UNIVERSIDADE NOVA DE LISBOA
INSTITUTO DE HIGIENE E MEDICINA TROPICAL

Lyme Borreliosis in Portugal

Study on Vectors(s), Agent (s) and Risk Factors

Lisboa 2006

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UNIVERSIDADE NOVA DE LISBOA

INSTITUTO DE HIGIENE E MEDICINA TROPICAL

LYME BORRELIOSIS IN PORTUGAL. STUDY ON VECTOR(S), AGENT(S) AND RISK FACTORS

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Dissertation of Candidature to a PhD degree
in Biomedical Sciences, Microbiology Speciality
by the New University of Lisbon, Institute of Hygiene and Tropical Medicine

(Dissertação de Candidatura ao Grau de Doutor
no Ramo de Ciências Biomédicas, Especialidade de Microbiologia
pela Universidade Nova de Lisboa, Instituto de Higiene e Medicina Tropical)

LISBOA

2006

This work was financed by a PhD grant from Fundação para a Ciência e Tecnologia (refª SFRH / BD / 996 / 2000)

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To my son Bruno

The present work should not be concluded before I pronounce my gratitude to those that, directly or indirectly, contributed to the achievement of my objectives.

To the Principal Researcher Doctor Margarida Collares Pereira, Director of Leptospirosis and Lyme Borreliosis Unit (ULBL) at Institute of Hygiene and Tropical Medicine, I leave here a special acknowledgement for these last years, where I was able to learn, to experience new things, to work with satisfaction, always having a feedback full of encouragement and trust. During the thesis work and in the revision of this manuscript, your pertinent suggestions and comments contributed, with no doubt, for the improvement of the final document.

To Doctor Matthew Nicholson, I am grateful for knowing you and I am honor to be able to share some of your experience in an area that also become a passion to me. Your suggestions in the beginning of this work and at the end were crucial for the achievement of the main objectives. Thank you for hosting me at your residence and allow me to be part of your family. A special thank to Erica and a big hug to your two beautiful girls, Terra and Splash.

To my Tutorial Committee, Professor Doctor Leonard Amaral from Micobacteria Unit and Professor Doctor Paulo Almeida from Medical Entomologic Unit, I present my gratitude for all the availability, suggestions and comments that both of you gave in the preliminary reports and in this final document.

To Professor Doctor Celso Cunha, I am grateful for the permission for use installations and equipment from Biologic Molecular Unit to develop part of my work.

Professor Doctor Isabel da Franca, an enthusiastic for Lyme Borreliosis, allowed me to “observe” the human side of this disease by contacting with suspected patients and understand some of the epidemiological factors related to these cases, crucial for development of this work, which I am gratefull.

To my colleagues from ULBL, I leave here a special reference. To Jacinta Gama I am grateful for all the help, availability, friendship, and experience of life that I was able to enjoy. To Maria do Céu Mateus, always there when needed, I thank also for her sincere friendship. To my younger colleagues, I may say here that I made friends for life with all of them. A special thank for João, Rita, Sofia, Lígia, Ana Tânia, Gisela, Isabel Castro, Ana Teresa and Liliana.

To Luisa Vieira, my friend and always my colleague, I leave my acknowledgments for all the trust, support, teachings, advises and availability that you always have. Thank you for always being there.

To all the Teachers from all Departments of this Institute, to all the colleagues that work in others UEI's, I leave a word of gratitude for all the encouragement gave during this thesis. I thank also those that brought some ticks from various places, contributing to enlarge my sampling in the country.

At last, but not the least, I dedicate this work to my son, Bruno, my future. I especially thank Milo for the support in the field work and the patience that he always demonstrated. To my sister Rita and my brother-in-law, Rui, a special thanks for the support in some difficult days. To my father I am gratefull for your trust. To my mother, I will always love you. I am here today because of you.

Abstract

The status of Lyme Borreliosis (LB) in Portugal was evaluated through identification of the main vectors (ticks), their distribution, infection rates with *Borrelia burgdorferi* sensu lato species and human disease cases.

Ixodes ricinus, the main vector of this disease, was studied extensively in a 5-year focal study in Tapada Nacional de Mafra, a protected area. An unimodal dynamic cycle was found for all developmental stages and a 1-1.5 year developmental cycle was observed. Climatic variables, including temperature, humidity and precipitation were significantly correlated with seasonal variation in *I. ricinus* abundance. Other tick species, namely *Dermacentor marginatus, Haemaphysalis punctata, Rhipicephalus sanguineus* and *Ixodes hexagonus*, were also collected. An overall infection rate of 11.8% for *I. ricinus* and 5.2% for the other tick species were detected. Several *Borrelia* species were identified in these ticks, probably due to the great variety of hosts present in this area.

In a nationwide study during a 4-years period, 55 sample sites were surveyed and 2801 ticks were collected, including *Rhipicephalus* spp, *D. marginatus, I. ricinus, Hyalomma marginatum, H. punctata* and *Ixodes* spp, with different collection efforts. All of these ticks were found infected with *B. lusitaniae*, the main strain of *Borrelia* found in Portugal. Confirmed pathogenic bacterial strains (*B. garinii)* were only registered in Mafra and near Coimbra (Soure).

Detection of human LB cases was achieved through routine diagnosis in Institute of Hygiene and Tropical Medicine, where several diagnostic techniques were applied. Positive cases were confirmed by immunoblotting (15.5%) and/or amplification of *B. burgdorferi* s.l. intergenic-spacer of rRNA 5S-23S (*rrf-rrl*) (28%), with identification of two pathogenic genospecies (*B. garinii* and *B. afzelii*), besides *B. lusitaniae*. Lisboa, Coimbra, Tomar, Viseu and Almada were the main geographic origins of LB positive patients.

The main environmental determinants of tick distribution and thus in the epidemiological cycle of Lyme Borreliosis in Portugal were related to climate (temperature, humidity and precipitation) and landscape composition (open areas, mixed and deciduous forests). Landscape structure (*e.g*

fragmentation) was also important in determining tick presence in an area. These environmental factors were used to build risk maps were created for the three main tick-species potentially implicated in the transmission of LB agents in Portugal (*I. ricinus, D. marginatus* and *Rhipicephalus* spp).

In conclusion, Lyme Borreliosis exists in Portugal and presents a complex epidemiology, as follows: i) besides the known European *I. ricinus*-vector, other potential tick species were found as vectors for LB spirochetes, being susceptibe to be associated with numerous reservoir hosts (still to investigate) and specific biotopes; ii) an higher diversity of genomic species belonging to *B. burgdorferi* s.l. complex, which resulted from this large amplitude of both vectors and reservoirs; and iii) a generalized distribution of LB patients, with important infection rates associated with the referred diversity of pathogenic agents, not only with the two more prevalent LB genomic species already recognized in Europe (*B. garinii* and *B. afzelii*), as with the recently isolated *B. lusitaniae*, which induces a clinical status apparently restricted to the Mediterranean basin.

Resumo

A situação da Borreliose de Lyme (BL) em Portugal foi avaliada com base na identificação dos principais vectores (carraças) e sua distribuição, taxas de infecção com os agentes do complexo *Borrelia burgdorferi* sensu lato (s.l.) e os casos humanos com confirmação laboratorial.

Ixodes ricinus, o principal vector desta doença, foi estudado durante um período de 5 anos na Tapada Nacional de Mafra (área protegida), durante o qual foi observado um ciclo unimodal para todos os estados de desenvolvimento, por um período de 1 a 1,5 anos. Confirmou-se uma correlação significativa entre a variação sazonal da abundância de carraças e algumas variáveis climáticas, nomeadamente, a temperatura, humidade e precipitação. Além de *I. ricinus*, foram colhidas outras espécies de carraças tais como *Dermacentor marginatus, Haemaphysalis punctata, Rhipicephalus sanguineus* e *Ixodes hexagonus*. As taxas de infecção atingiram valores globais de 11,8% para *I. ricinus* e de 5,2% para as restantes espécies, com identificação de vários agentes do complexo *B. burgdorferi* s.l., provavelmente relacionada com a acentuada diversidade de hospedeiros presentes na área investigada.

Num estudo a nível nacional, durante 4 anos, foram amostrados 55 pontos para colheita de vectores, tendo-se obtido um total de 2801 carraças distribuídas pelos seguintes géneros/espécies *Rhipicephalus* spp, *D. marginatus, I. ricinus, Hy. marginatum, H. punctata* e *Ixodes* spp, com diferentes taxas de colheita. Todos estes ixodídeos foram encontrados infectados por *B. lusitaniae*, a principal espécie genómica detectada no vector (até ao momento). Em Portugal, para além da Tapada Nacional de Mafra, foram apenas identificadas estirpes patogénicas de *B. garinii*, num local perto de Coimbra (Soure).

A confirmação laboratorial de casos humanos foi obtida com base no diagnóstico de rotina desta doença, realizado no Instituto de Higiene e Medicina Tropical, quer ao nível serológico por Western-Blot (15.5%), quer por amplificação do espaço intergénico de rRNA 5S-23S (*rrf-rrl*) de *B. burgdorferi* s.l. (28%). Neste último caso, foram identificadas duas espécies genómicas patogénicas (*B. garinii* e *B. afzelii*), além de *B. lusitaniae*. A principal proveniência dos doentes com Borreliose de Lyme foi Lisboa, Coimbra, Tomar, Viseu e Almada.

Os principais factores envolvidos na distribuição das carraças e consequentemente no ciclo epidemiológico da Borreliose de Lyme em Portugal encontram-se associados com o clima (temperatura, humidade e precpitação) e composição do habitat (áreas expostas, florestas mistas e de caducas). A estrutura da paisagem (ex. fragmentação) foi igualmente considerada como um factor essencial para a presença de carraças numa determinada área. Com base nestas variáveis, mapas de risco foram criados para os três ixodídeos (*I. ricinus, D. marginatus, Rhipicephalus* spp) potencialmente mais implicados na transmissão dos agentes de BL em Portugal

Em conclusão, a Borreliose de Lyme existe em Portugal e apresenta uma epidemiologia complexa, como a seguir se demonstra: i) além do vector Europeu, registaram-se outros potenciais vectores, susceptíveis de estarem associados a uma maior diversidade de hospedeiros reservatórios (ainda por investigar) e biótopos específicos, ii) uma elevada diversidade de espécies genómicas do complexo *B. burgdorferi* sensu lato, decorrente deste espectro alargado de vectores-reservatórios, iii) e uma distribuição generalizada de doentes de BL, com importantes taxas de infecção, resultante da referida diversidade de agentes patogénicos, não só das duas espécies genómicas mais reconhecidas na Europa (*B. garinii* e *B. afzelii*), como da recentemente isolada *B. lusitaniae*, indutora de um quadro clínico aparentemente diferente e restricto à zona do Mediterrâneo.

LIST OF ABBREVIATURES AND ABBREVIATIONS

ACA – Acrodermatitis chronica atrophicans

ACF – Autocorrelation plots

AM – Águas de Moura

ARIMA – AutoRegressive Integrated Moving Average

BSK – For Barbour, Stoenner and Kelly

CNS – Central Nervous System

CSF – Cerebrospinal fluid

DLA – Distributed Lag Analysis

DNA – Deoxyribonucleic acid

EM – Erythema migrans

GIS – Geographic Information System

HRA – Herdade Ribeiro Abaixo

IFA – Indirect ImmunoFuorescence Assay

IgG – IgG Imunoglobulin

IgM – IgM Imunoglobulin

IHMT – Instituto de Higiene e Medicina Tropical

LB – Lyme Borreliosis

LBC – Lymphadenosis benigna cutis

LD – Lyme Disease

MSF – Mediterranean Spotted Fever

NB – Neuroborreliosis

PACF – Partial Autocorrelation Plots

PCR – Polymerase Chain Reaction

rDNA – Ribossomic Deoxyribonucleic acid

RFLP – Restriction Fragment Lenght Polymorphism

RLB – Reverse Line Blot

RNA – Ribonucleic acid

rRNA – Ribossomic Ribonucleic acid

TPN – Tapada Nacional de Mafra

TSA – Time Series Analysis

ULBL – Unidade de Leptospirose e Borreliose de Lyme

WB – Western Blot

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INTRODUCTION

Introduction and general objectives

Lyme Borreliosis (LB) is a tick transmitted disease and is defined as a zoonosis involving a sylvatic cycle with several hosts and reservoirs, where humans are intervenients when in accidental contact with the ticks. The agents of this disease belong to the bacteria family of Treponemataceae and to the complex *Borrelia burgdorferi* sensu lato (s.l.) with 11 genomic species so far. The pathogenic strains of this complex are responsible for a multi-organ syndrome, with particular clinical characteristics. *Borrelia* and tick vectors develop a narrow relationship that is crucial for the success of this zoonosis around the world, including Portugal. This disease occurs in Europe, the United States and Asia, with cases in South Africa and Australia. About 50,000 cases are estimated annually in Europe, in some countries with up to 155 cases per 100 000 inhabitants (Gustafson, 1994; O'Connell *et al.*, 1998 *fide* Lebech, 2002; Stanek & Strle, 2003), and an overall incidence of six per 100,000 in the United States (CDC, 2001; Stanek & Strle, 2003; Brownstein *et al.*, 2003).

A considerable amount of studies were performed in this disease, especially on its agents and vectors. *B. burgdorferi* s.l. characterization was the aim of multiple researches on taxonomy, biology, genetics, immunology (in the host and the vectors) and pathogenicity. New molecular tools are being applied, allowing the discovery of new strains, new expressed genes and evading proteins. Chronicity is still a doubtful matter, but ability of *Borrelia* to rapidly alter the expression of surface antigens may allow the spirochetes to survive. Different clinical manifestations are also attributed to the three pathogenic bacterial strains, but the discovery of other potential genomic species linked to classic and non classic clinical cases has lead to several questions to resolve.

Several ticks and hosts have been implicated in LB cycles, with ticks from genus *Ixodes* appearing as the main vectors, associated worldwide with a list of several mammals and bird species. In endemic countries, as in Central and Eastern Europe, other vectors have been mentioned to carry LB spirochetes without, so far, sufficient proofs of successful transmission.

In Portugal, the status of Lyme Borreliosis is still undefined., Several clinical cases and species of *B. burgdorferi* s.l. have been already confirmed, with only one tick species implicated (*I. ricinus*). *B. lusitaniae* has been associated with a chronic clinical condition, but its pathogenicity is still unclear. The reservoirs of this particular bacterial species are unknown. Although the scarcity of information obtained so far, it seems that this zoonotic disease has more importance than previously accounted for.

In order to clarify some of the questions still rising from the latter studies, the **general objectives** of the present study were as follows:

- i) to search the role of Portuguese tick species as vectors of LB agents;
- ii) to ascertain the prevalence and distribution of *Borrelia* spirochetes;
- iii) to define which risk factors can highly influence or contribute to the epidemiological cycle of this zoonosis, based on environmental variables.

Dissertation Plan

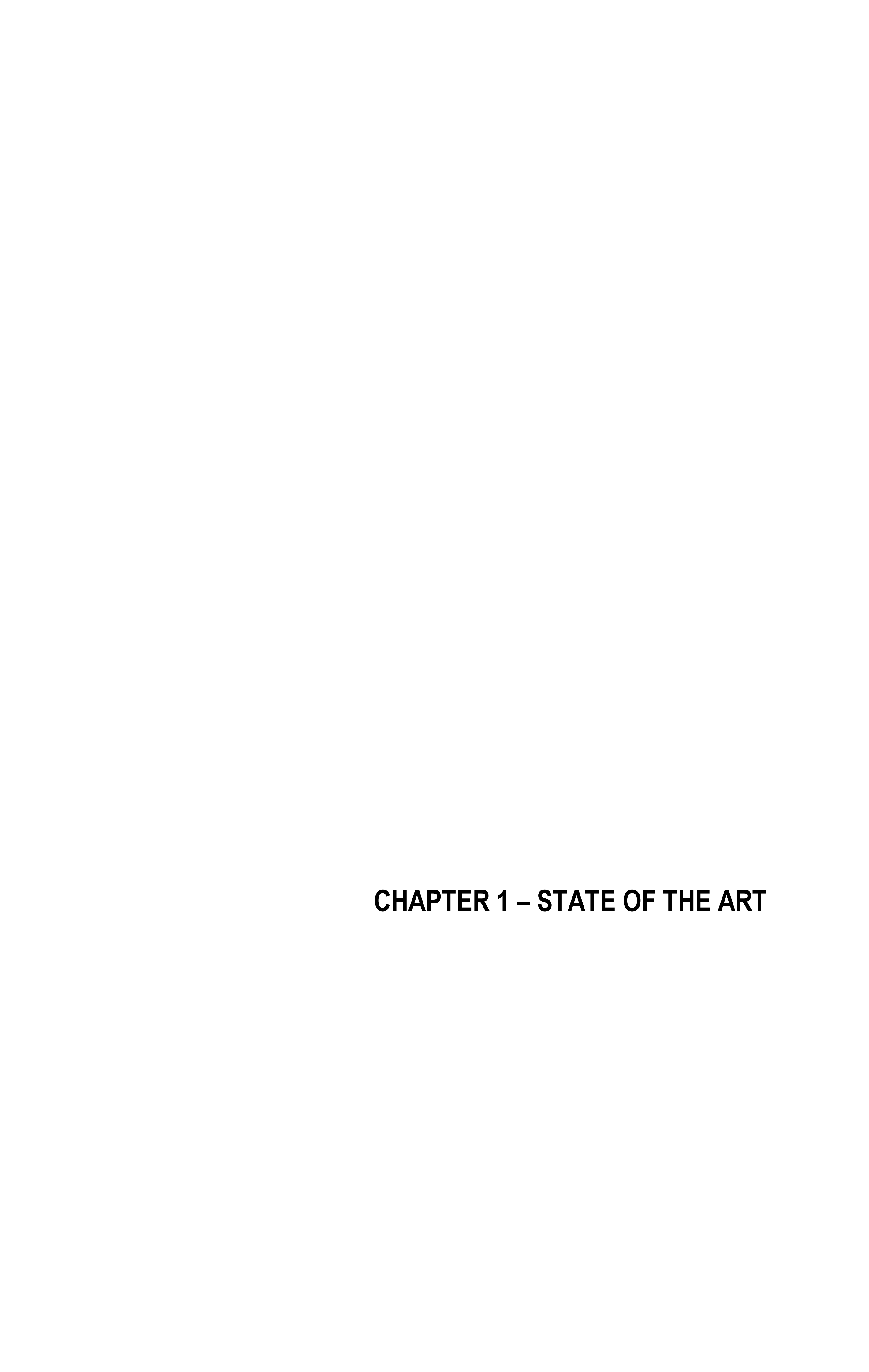
This thesis will be presented considering four specific chapters.

The first chapter named "State of the art" presents a broad and updated perspective about the knowledge on Lyme Borreliosis epidemiology and the bioecology of ticks.

The second chapter deals with the "Characterization of vectors" through two different approaches: i) a 5-year focal study in Mafra, to determine the seasonal dynamics of questing *I. ricinus* abundance (per month) together with *B. burgdorferi* s.l. tick-infection rates, with application of Time Series Analysis, a newly statistical approach in tick-studies; ii) the nationwide 4-year study, to map and characterize the distribution of ixodids and *Borrelia* tick infection rates by collection effort and presence/absence of ticks, in assigned sample sites, using a geostatistical approach.

In the third chapter “Lyme disease agents”, human and tick infection rates by *B. burgdorferi* s.l. agents are presented. Ticks were submitted to several lab techniques. Whenever possible, identification of tick *Borrelia* genotypes was ascertained to better understand the prevalence and distribution of *B. burgdorferi* s.l. agents in Portugal. Situation of LB in humans, based on the routine diagnosis performed at the Unit of Leptospirosis and Lyme Borreliosis (IHMT), is also analyzed in this chapter, with a global discussion of the prevalence of laboratory confirmed LB cases and their origin (distribution).

Finally, the fourth chapter “Lyme transmission risks” describes, with some detail, all the environmental variables that were obtained in the characterization of each sample site, associating them with presence / distribution of infected ticks and human data. These associations will be analyzed in multivariate models to determine the major risk factors involved in LB epidemiology in Portugal, followed by the creation of risk LB maps as a dynamic picture of this emergent disease allowing future improvements at a national level.



CHAPTER 1 – STATE OF THE ART

1 – LYME BORRELIOSIS, THE DISEASE

Lyme borreliosis (LB) is a multisystemic disorder caused by the tick-borne spirochete of the *Borrelia burgdorferi* sensu lato complex. Above all it is a zoonosis, mainly affecting a wide range of wild vertebrates, being only accidentally transmissible to man. Humans are dead-end hosts for the pathogens, along with a variety of incompetent hosts (Humair & Gern, 2000). Lyme borreliosis occurs with similar frequencies in men and women and affects people of all ages, especially children (Stanek *et al*., 1988; Hengge *et al*., 2003).

1.1 - In the World

1.1.1 - Historical Notes

Lyme disease was first identified in the United States in 1975, after a mysterious outbreak of arthritis among the residents of Lyme, Connecticut. A clinical sign, resembling an annular erythema, was obvious on each patients. In 1982, the causative spirochete that caused the disease was isolated by Burgdorfer and colleagues from *Ixodes dammini* (now called *Ixodes scapularis*) (Burgdorfer *et al*., 1982; Burgdorfer, 2001). In Europe this disease was probably referred to for the first time in 1883 in Germany, with the identification of the late cutaneous manifestation - achrodermatitis chronica atrophicans (ACA) - and was followed by the description, in 1909, of the first skin lesion - erythema migrans (EM) - by the dermatologist Arvid Afzelius, which would be observed years latter in Old Lyme (Afzelius, 1921 *cited* in Barbour & Fish, 1993, Gustafson, 1994; Weber, 2001). In 1983, *Borrelia* was detected for the first time in Europe in *Ixodes ricinus* collected in Switzerland (Gern *et al*., 1997). Since then, spirochete-infected ticks have been detected in many European countries.

Based on morphological studies, antigen profile and DNA structure, the first isolate was defined as a new species of the genus *Borrelia* and was named *Borrelia burgdorferi* in 1984 by Johnson and coworkers, in 1984. During recent years, the term "Lyme Borreliosis" (LB) has been more commonly used than Lyme Disease as an expression of all the various manifestations known to be caused by different *Borrelia burgdorferi* sensu lato agents (Gustafson, 1994).

1.1.2 – Medical importance

Lyme Borreliosis occurs throughout the northern hemisphere and is now the most common vectorborne disease in Europe, the United States and Asia, with few cases in South Africa (Berger *et al*., 1993) and Australia (**Fig. 1.1**). It is estimated that annually about 50,000 cases occur annually in Europe, with

some countries having up to 155 cases per 100,000 inhabitants (Gustafson, 1994; O'Connell *et al.*, 1998 *cited* in Lebech, 2002; Stanek & Strle, 2003). The incidence of the disease is not the same throughout Europe. The highest reported frequency occurs in central Europe and Scandinavia, particularly in Germany, Austria, Slovenia and Sweden (Gustafson, 1994, Huppertz *et al.*, 1999; Strle *et al.*, 1999; Mejlon, 2000; Hengge *et al.*, 2003), but other countries are reported, as follows: France (Jaulhac *et al.*, 2000), Denmark (Lebech *et al.*, 2000), Croatia (Situm *et al.*, 2002), Switzerland (Schwaiger *et al.*, 2001), Portugal (Morais *et al.*, 1989), Spain (Oteo *et al.*, 1992), Czech Republic (Stanek *et al.*, 1988), United Kingdom (Smith *et al.*, 2000), Hungary (Stanek *et al.*, 1988), Romania (Hristea *et al.*, 2001), Poland (Niscigorska *et al.*, 2003), Netherlands (Kuiper *et al.*, 1994), Italy (Ciceroni *et al.*, 2001), Finland (Juntilla *et al.*, 1994), Estonia (Kisand *et al.*, 2004) and Greece (Diza *et al.*, 2004). In the United States, Lyme borreliosis is currently the most prevalent vector-borne disease, with 16,273 cases of Lyme borreliosis reported in 1999 and has an overall incidence of six per 100 000 (CDC, 2001; Stanek & Strle, 2003; Brownstein *et al.*, 2003). As already mentioned, Portugal is also included in the long list of countries with reported cases of Lyme Borreliosis and more detailed considerations will be given bellow.

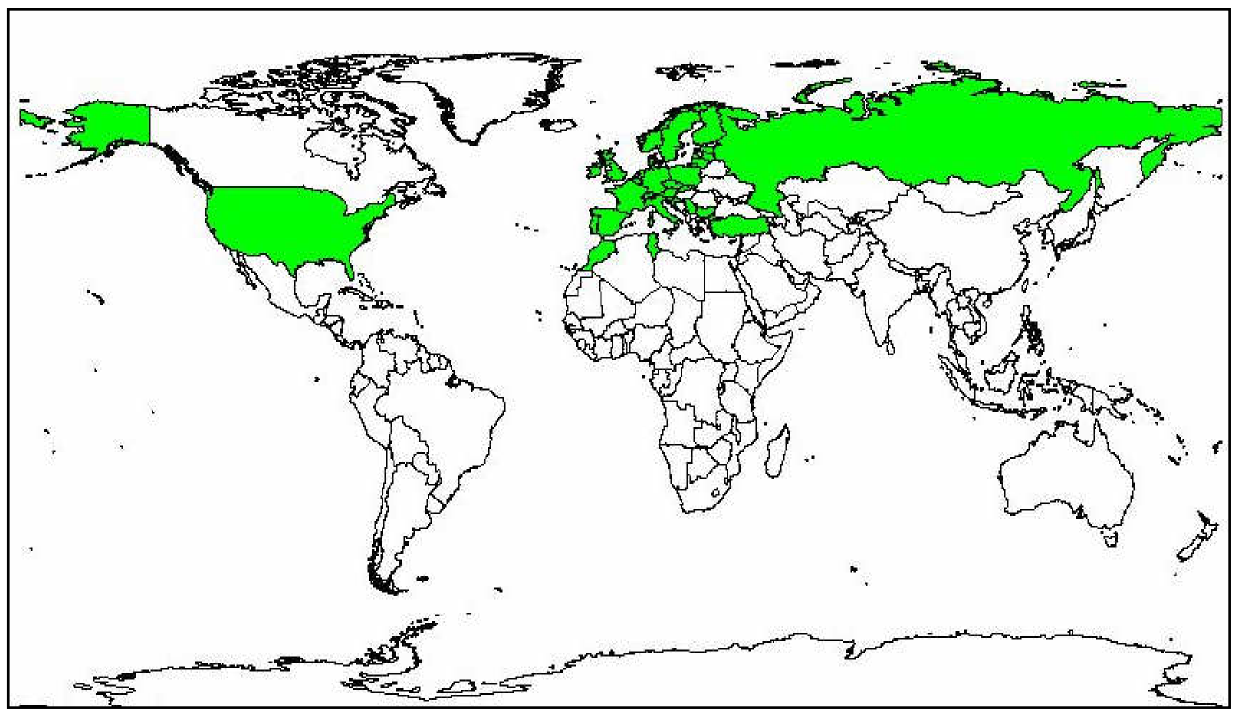


Figure 1. 1- World distribution of Lyme Borreliosis cases, according to the literature**.**

1.1.3 - Clinical Manifestations

As a result of its protean clinical manifestations, LB was described as the new “great imitator” of various human diseases (Wang *et al.*, 1999; Stanek & Stle, 2003). Lyme borreliosis is actually described in three stages, defined by time after tick bite, clinical manifestations (**Fig. 1.2**) and specific laboratory results (Brown *et al*, 1999; Wang *et al.*, 1999; Weber, 2001; Reed, 2002; Stanek & Strle, 2003; Hengge *et al.*, 2003; Hansmann *et al.*, 2004):

- Early localized disease (days to a month after tick bite) – Erithema cutaneos migrans (ECM), Lymphadenosis benigna cutis (LBC), and viral flulike symptoms (fever, malaise, headache, stiff neck and fatigue)

- Early disseminated disease (days to 10 months after tick bite) – multiple erythema, severe systemic symptoms, including progression to cardiac abnormalities, neurologic manifestations (*e.g.* Bell’s paralysis) and development of arthritis. In 25 to 50% of patients, recognized ECM does not precede Early disseminated disease.

- Late disease (months to years after tick bite) – migratory polyarthritis or chronic monoarthritis, central nervous system manifestations (*e.g.* psychiatric symptoms, mimic of multiple sclerosis) or cutaneous manifestations (*e.g.* ACA - acrodermatitis chronica atrophicans).

It is extremely important to recognize that LD has, above all, a clinical diagnosis. Any laboratory test used to supplement that evaluation should be ordered and interpreted in the context of careful investigation of the patient’s tick bite and physical examination, to assess if the patient actually has a borrelial infection (Reed, 2002):

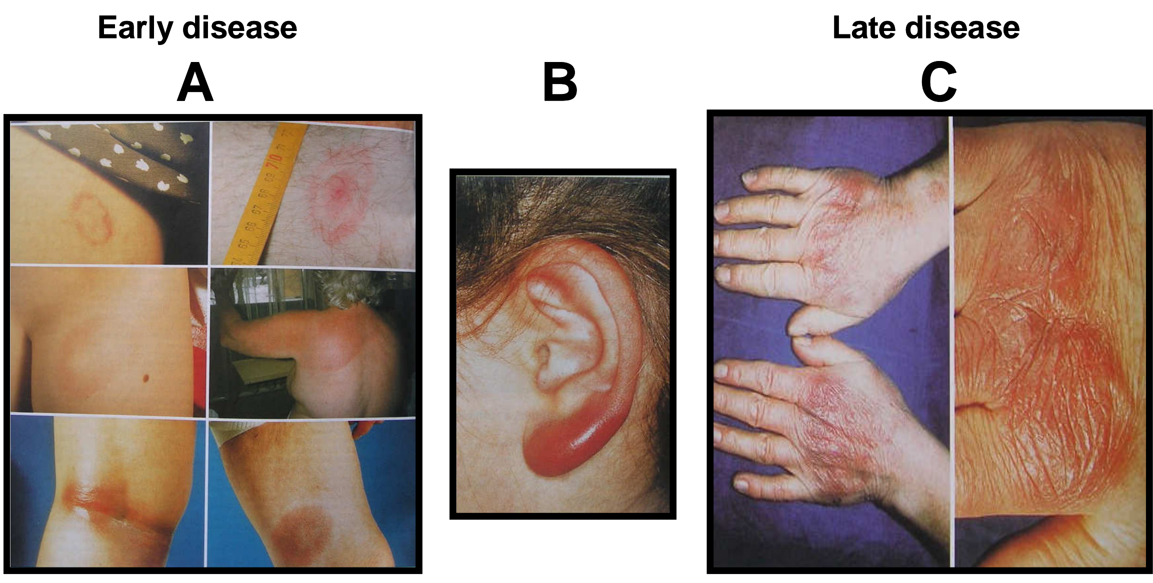


Figure 1. 2 – Clinic manifestations of early disease (A – Erythema chronicum migrans, B - Lymphadenosis benigna cutis) and late disease (C – Acrodermatitis chronica atrophicans) (Stanek *et al.*, 1997).

Differences in clinic manifestations can be observed between continents, in the northern hemisphere (**Table 1.1**). In the United States, the Centers for Disease Control and Prevention (CDC) defines the confirmed presence of LB whenever there is the evidence of an erythema migrans rash of 5 cm or greater in diameter, or laboratory confirmation of the infection by culture with at least, one manifestation of musculoskeletal, neurologic or cardiovascular disease.

In Europe, a panel of experts from 17 countries proposed guidelines that define features for the diagnosis of clinical forms in European patients, based on a previous risk of exposure to ticks (Stanek *et al.*, 1996; Dhote *et al.*, 2000; Stanek & Strle, 2003). As a whole, a clinical definition was proposed, based on the *major* manifestations of ECM, LCB and ACA, with no need of laboratory confirmation. For ACA, a high level of specific IgG antibodies should be present, and compatible histological findings and positive culture from skin biopsy are optional. Early neuroborreliosis can be diagnosed if lymphocytic pleiocytosis and cerebrospinal fluid (CSF) specific antibodies are associated with meningoradiculitis, facial palsy, or cranial neuritis. Lyme arthritis involves the concurrent findings of objective swelling of large joints and high levels of specific serum IgG antibodies. Other clinical manifestations are also defined.

Table 1. 1– Comparison of Lyme borreliosis in North America and Eurasia (Wang *et al.*, 1999; Strle *et al.*, 1999; Weber, 2001; Lebech, 2002, Hengge *et al.*, 2003).

CLINICAL FEATURE	EURASIA	NORTH AMERICA
Early localized disease		
Erythema migrans *	77% (Common)	60-90% (Common)
Systemic symptoms	38-51%	50-69%
Early disseminated disease		
Multiple EM	6% (Unusual)	>18% (Common)
Neuroborreliosis (NB)	16-80% (Common)	10-20% (Common)
Meningoradiculitis	37-61%	3-21%
Meningitis	4-27%	2-17%
Arthritis/arthralgia	7% (Unusual)	-
Carditis	0.5-4%	0.5-10%
Borrelial lymphocytoma *	3% (Well documented)	Rare
Late disease		
Lyme arthritis	7% (Unusual)	51-57% (Common)
Acrodermatitis chronica atrophicans *	3% (Well documented)	Rare
Peripheral neuropathy	40-63% ACA patients	30-70% late NB
Central Nervous System involvement	<9% NB	Well documented
Encephalomyelitis	4-6%	0.1% (Rare)
Meningoencephalitis	0.5-4%	9%

* Cutaneous *major* manifestation

Borrelia has been isolated and/or detected in various organs or tissues such as skin, blood, joints, internal organs (spleen, heart, liver, urinary blader, kidneys, nervous system) in various vertebrate hosts (Humair & Gern, 2000).

Currently, only *B. burgdorferi* sensu stricto (s.s.), *B. garinii*, and *B. afzelii* have been cultured frequently from patients with LB. Isolates recovered from LB patients in Slovenia and identified as *B. bissettii* (Strle *et al.*, 1997) could implicate this genospecies as the fourth *Borrelia* species that causes disease in humans. *B. valaisiana* might also be pathogenic for humans, since DNA specific for this species has been detected by PCR from patients with LB (Rijpkema *et al.*, 1997; Weber, 2001; Schaarschmidt *et al.*, 2001; Godfroid *et al*, 2003; Diza *et al.*, 2004), and positive immunoblots were obtained from patients with neuroborreliosis and EM (Ryffel *et al.*, 1999). An human isolate in a Portuguese chronic patient was identified as *B. lusitaniae* incriminating this species for the first time in humans (Collares-Pereira *et al.*, 2004). A new human isolate, *B. burgdorferi* A14S, was also described (Wang *et al.*, 1999b). The remaining five *B. burgdorferi* sensu lato species – *B. japonica, B. andersonii, B. turdi, B. tanukii, B. sinica* – are considered nonpathogenic for humans (Godfroid *et al.*, 2003).

The different species of *B. burgdorferi* sensu lato are particularly associated with distinct clinical manifestations of LB as follows: *B. burgdorferi* s.s. infection with Lyme arthritis, *B. garinii* with

neuroborreliosis and *B. afzelii* with ACA (Balmelli & Piffaretti, 1995; Wang *et al.*, 1999; Ryffel et al., 1999; Humair & Gern, 2000).

Infection with different strains or different antigens and/or plasmid profiles may cause distinct epidemiological and clinical characteristics of Lyme borreliosis (Escudero *et al.*, 2000; Ruzic-Sabljic *et al.*, 2001, 2001a; Logar *et al.*, 2004), but there seems to be a clear correlation between the geographic distribution of the tick *Borrelia* species and the local predominance of a given clinical presentation (Baranton *et al.*, 2001; Schaarschmidt *et al.*, 2001). For instance, a Spanish tick isolate *B. lusitaniae* was not able to disseminate through the skin of mice or to infect internal organs (Escudero *et al.*, 2000), but the same tick strain isolated in Portugal caused cardiac problems (Zeidner *et al.*, 2001) and was the cause of chronic disease in a human patient (Collares-Pereira *et al.*, 2004).

LB in domestic and sylvatic animals is also reported in many studies. Most of knowledge of Lyme disease in nonhuman vertebrates comes from veterinary research on domestic animals. Some clinical cases were observed in horses (Dzierzecka, 2002; Muller *et al.*, 2002) and in dogs and cats (Garcia *et al.*, 1991; Levy *et al.*, 1993; Simpson, 2002; Skotarczak & Wodecka, 2003; Korshus *et al.*, 2003). Seropositive zoo animals were described associated with presence of *Ixodes ricinus* (Stoebel *et al.*, 2003).

Lameness, loss of vigour, and listlessness behaviour are common in nonhuman LB, although these generalized symptoms are frequently encountered in domestic animals and can result from numerous physical conditions or disease agents. Furthermore, animals infected with Lyme spirochetes are frequently assymptomatic, even in species that often show symptoms. One potential influence of Lyme disease on conservation efforts is the effect of the disease on wild canids (Ginsberg, 1994).

1.2 - In Portugal

1.2.1 Medical importance, causative species and clinical manifestations

The occurrence of tick-borne diseases in Portugal has been reported by numerous authors (Bacellar *et al.*, 1991; Filipe *et al.*, 1991; Filipe *et al.*, 1992; Bacellar *et al.*, 1995; Santos *et al.*, 2004). Most of these diseases are notifiable to the Health Authorities, including Mediterranean Spotted Fever (MSF), Rickettsiosis, Q Fever and Lyme Borreliosis. LB is the most recent addition to this group.

The interest in LB, compared to other countries, is recent. The difficult laboratory diagnosis, along with a well known polymorphic clinical picture, may be factors that have contributed to the low number of cases reported so far (Collares-Pereira & Franca, 2000).
The first clinical case of LB, from Alentejo region, was described in 1989 by Morais and others. In this work the authors suggested, either new vectors implicated in the transmission of *B. burgdorferi* s.l., since *I. ricinus* was considered uncommon in that region, or the potential existence of a new *Borrelia* strain in the country.

Subsequent studies in the same Portuguese region confirmed the presence of more seropositives, some of them with confirmed clinical signs of Lyme borreliosis (Filipe *et al*., 1990, Núncio *et al*., 1991, 1992; Santos *et al*., 1995; Morais & Henriques, 1999; Morais *et al*., 1999).

Scientists of the National Institute of Health Dr. Ricardo Jorge reported a prevalence of 10% of seropositive cases in 1991 suggesting the growing importance of this disease in Portugal (Rocha & Caniça, 1991). Actual data from Direcção Geral de Saúde show a total of 22 cases of LB, among other tick-borne diseases (www.dgsaude.pt, 2005), namely MSF and Q Fever with 2,847 and 61 cases, respectively, between 2000 and 2004.

In 1993, Núncio and colleagues isolated a *B. burgdorferi* s.l. strain from *I. ricinus* adult ticks in the region of Águas de Moura (Setúbal District), Alentejo. This isolate was further identified as *Borrelia lusitaniae*, a new genospecies, by Fleche and co-workers (1997). One type strain, PoTiB2, was defined based on complete sequence of *rrs* genes. The humoral response and pathogenicity evaluation was monitored in C3H/HeN mice (Zeidner *et al*., 2001). The lesions were characterized as a severe, necrotising endarteritis of the aorta, with a minimal, mixed inflammatory infiltrate (neutrophils, macrophages and lymphoid cells) extending into the adjacent myocardium. Analysis of immunoblots revealed a reaction only to flagellin protein (41kDa) or to flagellin and OspC.

A study was conducted on the island of Madeira to understand the southern limit of Lyme disease spirochetes (Matuschka *et al*., 1998). Although a low infection rate in *I. ricinus* ticks was found on this island, the spirochetes were surprisingly diverse, including *B. afzelii, B. burgdorferi* s.s. and at least two serotypes of *B. garinii.* The authors suggested that, in this particular focus, the observed diversity may be caused by numerous and repeated introductions of spirochetes from migrating birds or from domestic animals transported to the island. Regardind serology, some local human infections have also been detected, as regards serology (Núncio *et al*., 2002; ULBL).

To understand the distribution and prevalence of LB in mainland Portugal, De Michelis and others (2000) analyzed the genetic diversity of *B. burgdorferi* s. l. in a sylvatic habitat in the south (Grandola District). *Ixodes ricinus* and *Hyallomma marginatum* ticks were found infected with *B. lusitaniae* type strains by PCR-RFLP. Phylogenetic analysis of *rrf-rrl* spacer revealed a population with multiple strains of *B. lusitaniae* harbouring 10 distinct sequence types in *I. ricinus* ticks

Diagnosis of Lyme Borreliosis in Portugal has been sustained by two reference institutions (Instituto Ricardo Jorge – CEVDI and Instituto de Higiene e Medicina Tropical - ULBL), whose geographic areas complement each other. Human samples come from the main hospitals, regional health centres and private medical offices. Although there is an increase in the use of commercial kits in some central hospitals, doubtful diagnoses coupled with the existing reliable protocols within the above reference institutions allow the maintenance of a circuit that improves the knowledge of this disease in Portugal.

Finally, an intensive study on the dermatological aspects of LB in Portugal performed by Isabel Franca (Franca, 2005) and the consequent increased number of samples arriving at the ULBL laboratory were very successful. This study, not only resulted in the description and characterization of the skin involvement of this disease, with DNA detection of new strains (*B. garinii* and *B. afzelii*) not yet identified in this part of Europe, but also contributed to the first human isolate (PoHL1) of *B. lusitaniae* in Portugal and in the world, as already reported (Collares-Pereira *et al.*, 2004), in a patient with chronic skin lesions (10 years of duration) and a weak serological response.

2 – *BORRELIA BURGDORFERI* SENSU LATO, THE AGENTS

2.1 - Taxonomy

Bacteria from the *B. burgdorferi* sensu lato complex are spirochetes belonging to the Order Spirochaetales, Family Treponemataceae and genus *Borrelia* (**Fig. 1.3**). This genus represents a tight phylogenetic cluster, which is differentiated from other spirochetal phylogenetic groups by base signature analysis of *rrs*. This genus currently contains 37 species of spirochetes, all transmitted by ticks, except *B. recurrentis* which is transmitted by the human body louse. Two major categories of *Borrelia*, the relapsing-fever *borreliae* and the LB *borreliae*, include more than 20 species, so far (Wang *et al.*, 1999; Schwan & Piesman, 2002).

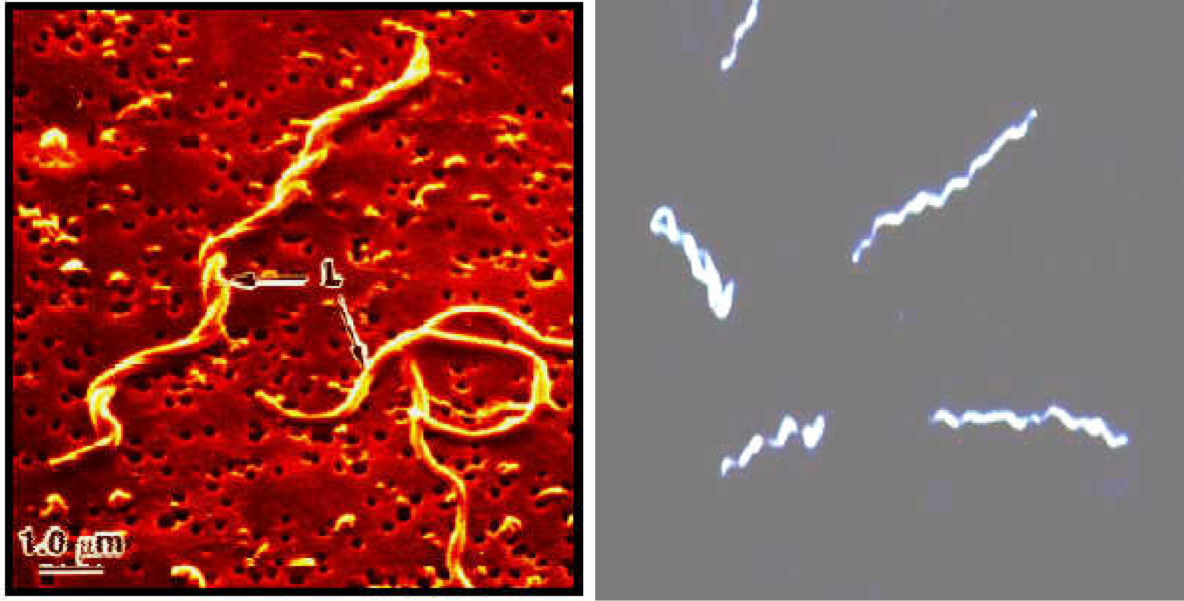


Figure 1. 3 – *Borrelia burgdorferi* sensu lato spirochetes (EUCALB), visualized by electron microscopy (picture on the left) and immunofluorescence (picture on the right).

Numerous LB strains have been isolated from patients, ticks and reservoir hosts and are genotypically and phenotypically diverse. At present the genus *Borrelia* is divided into 11 species or genomic groups, as specified in **Table 1.2** (Sparagano *et al.*, 1999; Wang *et al.*, 1999; Lebech, 2002; Stanek & Strle, 2003). The term "*B. burgdorferi* sensu lato" is now collectively used to refer to all *Borrelia* isolates within this cluster and within which is the species "*B. burgdorferi* sensu stricto" (Wang *et al.*, 1999; Lebech, 2002; Stanek & Strle, 2003). **Fig. 4 (A, B)** represents the global distribution of these species based on a review of the literature.

Table 1. 2 – Geographic distribution of the genospecies assigned to the *B. burgdorferi* sensu lato complex, according to the first or confirmed published reference(s)

***BORRELIA* SPECIES**	**HUMAN DISEASE**	**DISTRIBUTION**	**FIRST OR CONFIRMED REFERENCE(S) (VECTORS and HOSTS)**
***B.burgdorferi* s.s**	Yes	USA, Europe, Asia	Baranton *et al*., 1992;
B. garinii	Yes	Europe, Asia	Baranton *et al*., 1992; Yabuki *et al*., 1999; Masuzawa, 2004 Baptista *et al*., 2004
B. afzelii	Yes	Europe, Asia	Canica *et al*., 1993; Masuzawa, 2004; Baptista *et al*., 2004
B. valaisiana	Uncertain (Rijpkema *et al*., 1997)	Europe, Asia	Wang *et al*, 1997; ¸Masuzawa, 2000, 2004; Baptista *et al*., 2004
B. lusitaniae	Uncertain (Collares-Pereira *et al*., 2004)	Europe, Asia, North Africa	Le Fleche *et al*., 1997; Zhiuoa *et al*., 1999; Baptista *et al*., 2004
B. bissettii	Uncertain (Strle *et al*., 1997)	USA, Slovenia	Strle *et al*., 1997; Postic *et al*., 1998
B. japonica	No	Japan	Kawabata *et al*., 1993; Masuzawa *et al*., 2001; Masuzawa, 2004
B. andersonii	No	USA	Marconi *et al*, 1995
B. tanukii	No	Japan	Fukunaga *et al*., 1996, 1996a
B. turdae	No	Japan	Fukunaga *et al*., 1996, 1996a
B. sinica	No	China	Masuzawa *et al*., 2001; Masuzawa, 2004

B. burgdorferi s.s. was first isolated in *I. scapularis* ticks from USA (Burgdorfer *et al*., 1982) and was the first species assigned to the *B. burgdorferi* s.l. complex.

B. garinii was identified in 13 isolates from Europe and Japan associated with ticks and LB human patients, and was firstly identified by its specific rRNA gene restriction pattern by Baranton and colleagues (1992).

B. afzelii (type strain VS461) was firstly isolated from *Ixodes ricinus* in Switzerland (Canica *et al*., 1993) and first named in honor of Arvid Afzelius in a study by Baranton and coworkers (1992) that also documented the involvement of this new species as a specific aetiological agent of ACA.

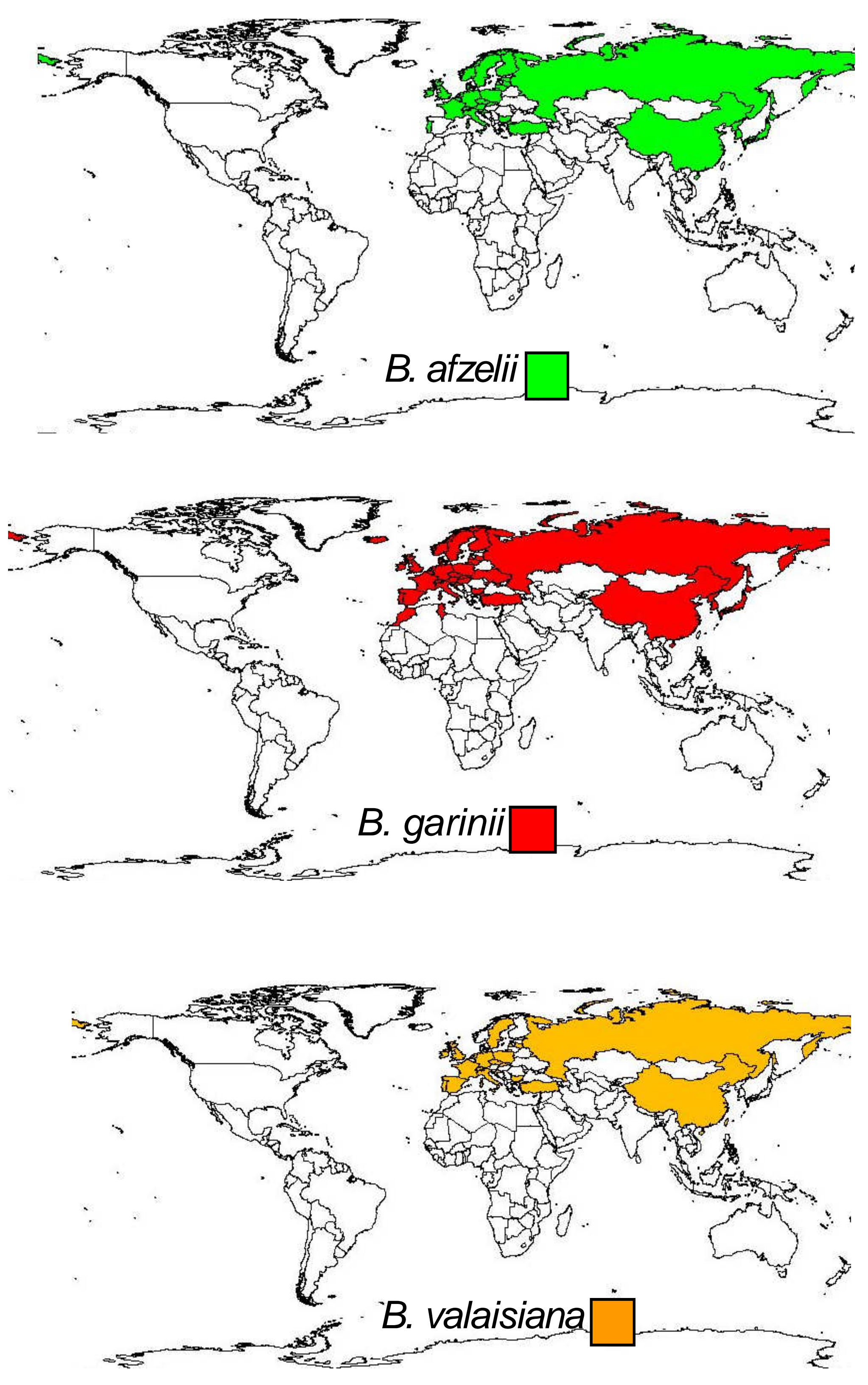


Figure 1. 4 (A) – Global geographic distribution of genospecies *B. afzelii, B. garinii* and *B. valaisiana*

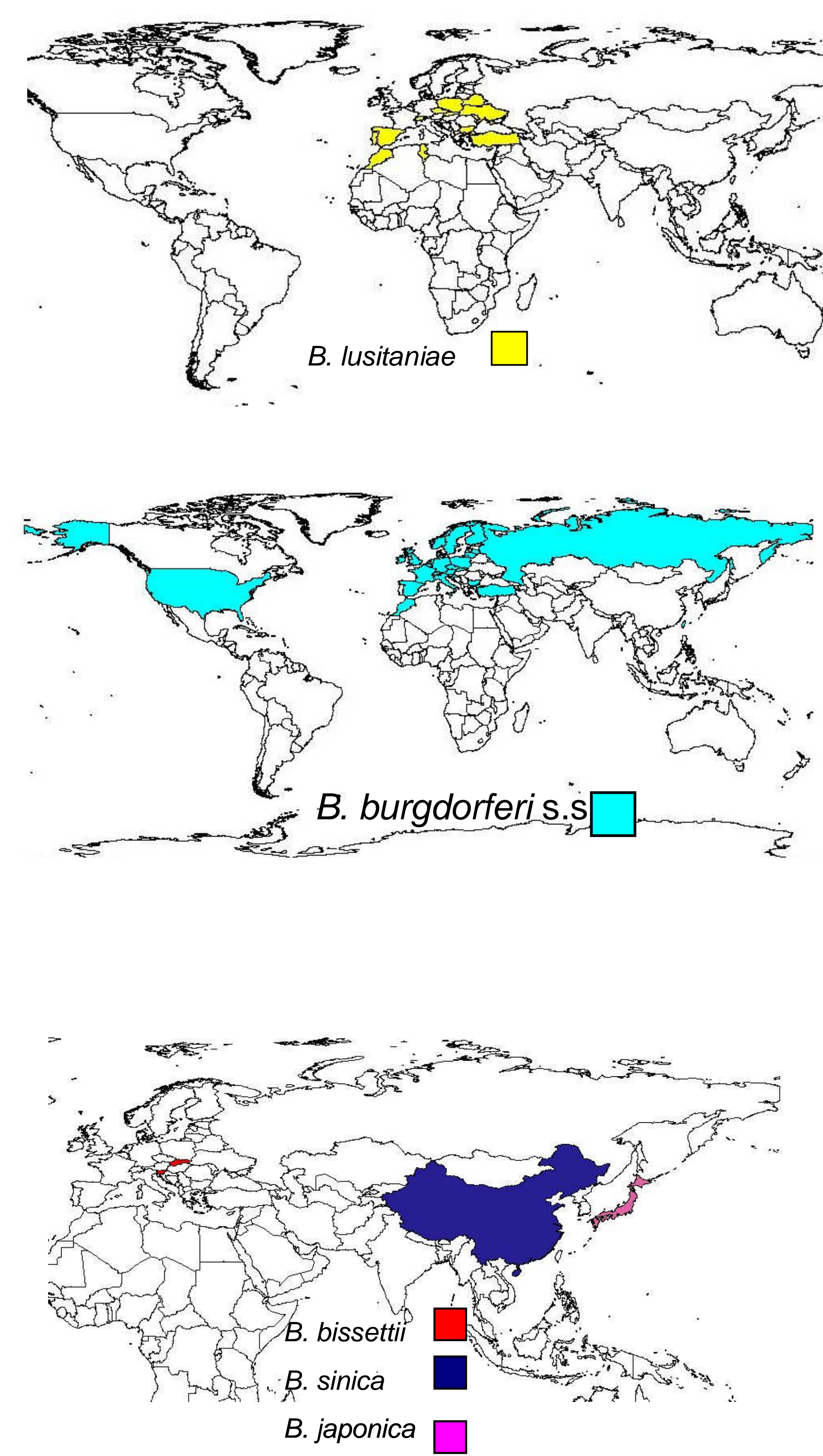


Figure 1.4 (B)– Global geographic distribution of genospecies *B. lusitaniae, B. burgdorferi s.s.*, *B. bissetti, B. sinica and B. japonica.*

B. valaisiana, former Groups VS116 and M19, was described based on DNA-DNA hybridization by Wang and others (1997). The few isolates of *B. valaisiana* obtained so far may be due to the difficult growth in selective medium or this strain is more prone to spirochetal decrease during tick moult, which makes isolation more laborious (Humair & Gern, 2000)

B. lusitaniae (PoTiB1, PoTiB2) was firstly isolated in Portugal in an *I. ricinus* tick (Núncio *et al*., 1993) and further described by Fleche and others (1997). The first human *B. lusitaniae* isolate (PoHu1) was described by Collares-Pereira *et al*. (2004), as already mentioned.

B. bissettii, formely Group DN127, was described by Postic *et al*., (1998) and is involved in several enzootic transmission cycles. *B. japonica* was first isolated from the tick *Ixodes ovatus* in Japan (Kawabata *et al*., 1993). *B. andersoni*, formerly group 21038, was described by Marconi and others (1995). *B. turdae* and *B. tanukii* were isolated from *Ixodes turdus* and *I. tanuki* respectively and were both identified by 16S RNA gene target (Fukunaga *et al*., 1996a). *B. sinica* (type strain CMN3T) was isolated from *I. ovatus* and *Niviventer confucianus* ticks and identified by Masuzawa *et al*. (2001).

It is assumed that speciation within the *B. burgdorferi* s.l. complex is a recent phenomenon. Diversity of *B. burgdorferi* sensu lato in Eurasia is much greater than in North America, suggesting a Eurasian origin for this complex. *B. garinii* shows the greatest genetic diversity and is the species which is closest to the common ancestor of *B. burgdorferi* sensu lato in this region (Rosa *et al*., 1991; Postic *et al*., 1994, 1999; Wang *et al*., 1999; Baranton, 1999; Olsen *et al*., 2000).

In North America isolates are somewhat heterogeneous, some of them genetically distinct from *B. burgdorferi* sensu stricto (Zingg *et al*., 1993; Liveris *et al*., 1995; Peavey & Lane, 1996; Liveris *et al*., 1999; Postic *et al*., 1999; Lin *et al*., 2001; Bunikis *et al*., 2004). Also, in a recent study, *B. afzelii* in Europe showed greater diversity than previously estimated (Bunikis *et al*., 2004). Geographic differences between isolates, even at local scales, have been observed (Masuzawa *et al*., 1996; Bunikis *et al*., 1996; Etti *et al*., 2003). This heterogeneous and habitat-specific distribution is maintained by strong ecological factors that are related to the structure of host communities (Etti *et al*., 2003).

2.2 – Morphology and growth

The agents of the *B. burgdorferi* s.l. complex are motile with 7 to 11 periplasmic flagella and helically shaped with dimensions of 0.2-0.3 μm by 20-30 μm (Gustafson, 1994; Stanek, 1997; Wang *et al*., 1999;

Lebech, 2002; Stanek & Strle, 2003; Hengge *et al*., 2003). They have an outer cell membrane that surrounds the protoplasmic cylinder which consists of cytoplasm, the inner cell membrane, and the peptidoglycan. Bipolar flagella are located in the periplasmic space between outer cell membrane and the protoplasmic cylinder. Conversion of *B. burgdorferi* to cystic forms (speroblast-L forms) in spinal fluid and the transformation to mobile spirochetes by incubation in BSK (for Barbour, Stoenner and Kelly) medium, but also *in vivo* (studies with mice) were observed. These microbial variants might contribute to subclinical or chronic infections or be a way to overcome or escape unfavorable conditions (exposure to the enzymes and tissue barriers in the tick midgut) (Burgdorfer, 2001; Gruntar *et al*., 2001). Flagella loss can also be observed in conjunction with decreased invasiveness (Sellek *et al*., 2002).

These spirochetes are microaerofilic gram-negative bacteria that can be grown in the BSK medium at 33ºC-35ºC (Barbour, 1984). The *in vitro* generation time is 12-24 hours. Utilization of carbohydrates as a source of energy by *B. burgdorferi* is well known, during transmission from host to naive feeding ticks, with ingested blood providing nutrients during tick colonization (Lackum & Stevenson, 2005).

2.3 - Genetic structure

The genome sequence of a Lyme disease spirochete was carried out by Fraser and coworkers, in 1997. The genome of the type strain *B. burgdorferi* sensu stricto B31 is composed of a linear chromosome of 1,521,419 bp with an average G+C content of 28.6% and 21 plasmids (9 circular and 12 linear) with a combined size of more than 613,000 bp (**Fig. 1.5**) (Fraser *et al*., 1997). Repetitive DNA sequences found throughout the genome of *B. burgdorferi* are specific and plasmid associated, with a probable role in physiology and pathogenicity (Simpson *et al*., 1990; Roberts *et al*., 2000; Steward *et al*., 2005). The level of DNA relatedness among different *B. burgdorferi* sensu lato species is about 48 to 70% (Baranton *et al*., 1992).

In most of the *B. burgdorferi* sensu lato strains, the *rrn* cluster of the linear chromosome contains a single copy of 16S rRNA (*rrs*) and tandem repeated 23S rRNA (*rrlA* and *rrlB*) and 5S rRNA (*rrfA* and *rrfB*). The rDNA gene cluster is located at the center of the chromosome and is arranged in the following order: *rrs-rrlA-rrfA-rrlB-rrfB.* These copies of rRNA genes are commonly used for molecular detection purposes, either in routine diagnosis (Couceiro *et al*, 2003) or in ticks to evaluate the *Borrelia* infection rate (Baptista *et al*., 2004).

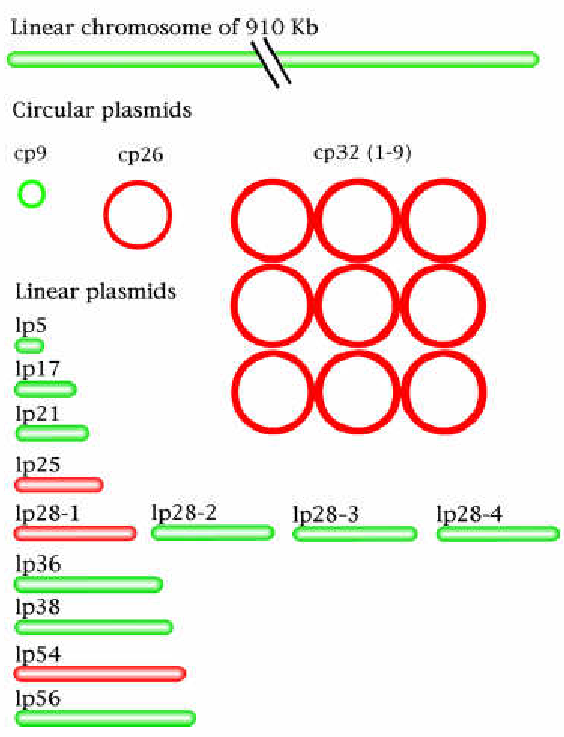


Figure 1. 5 - The segmented genome of *B. burgdorferi* s.l. (Steward *et al*., 2005)

Adaptation and gene expression may be required for *B. burgdorferi* to effectively colonize the host, evade humoral responses, and cause disease (Anguita *et al*., 2000; Stevenson & Babb, 2002). These spirochetes utilize a long list of genes to mediate and regulate protein expression, namely *ospC*, *bmp* (p39), *vlsE*, *ospA* and *B*. The proteins modulated can have a remarkable heterogeneity during tick transmission and mammalian infection. This diversity is generated not only by temporal changes in antigen expression but also by modulation of the surface lipoproteins during infection (**Fig. 1.6**). Regulation of the temporal and spatial expression patterns of lipoproteins during infection likely contributes to persistent infection of mammals by *B. burgdorferi* s.l. (Hefty *et al*., 2002):

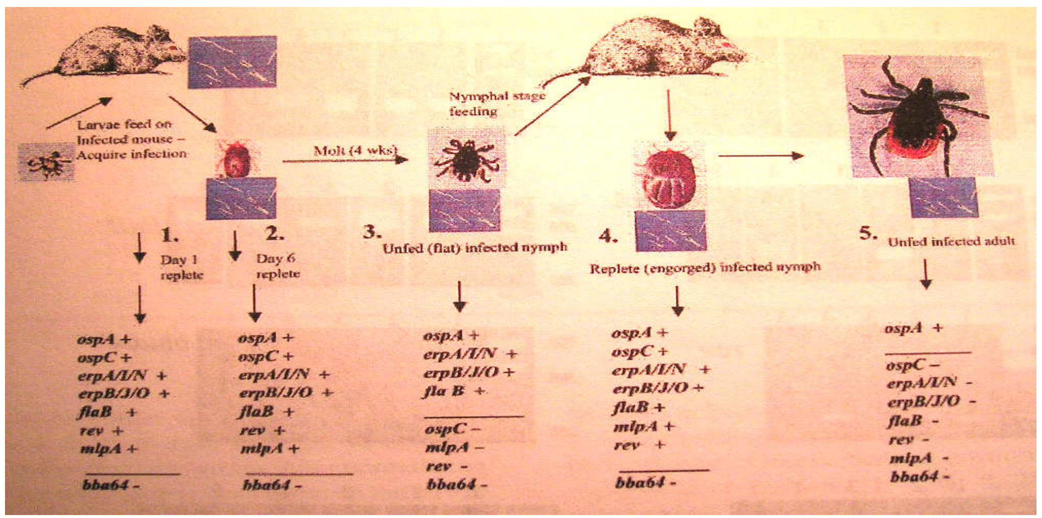


Figure 1. 6 – Flow chart illustrating the stages of the tick life cycle and the transcription of several *borrelia* genes (Gilmore *et al*., 2001; Hodzic *et al*., 2002).

2.4 - Antigenic characterization

More than 100 proteins have been described for *B. burgdorferi* s.l. (Cluss & Boothby, 1990), but the more important are the flagellin antigen and three outer surface proteins. The first one is a protein monomer of the flagellum, the motility organelle of *B. burgdorferi* s.l. Flagella are essential for the bacterium to invade host tissues and have been shown to be highly immunogenic, with a variable portion specific to *B. burgdorferi* s.l. and a conserved one, with homology to other bacteria.

The outer surface lipoproteins (OspA - OspF) (**Table 1.3**) seem to be involved in *B. burgdorferi* s.l. evasion of the host (human and tick) immune response (Munderloh & Kurtii, 1995; Gustafson, 1994; de Silva *et al*., 1998; Wang *et al*., 1999a; Beermann *et al*., 2000; Belperron & Bockenstedt, 2001; Lebech, 2002; Stanek & Strle, 2003; Hengge *et al*., 2003; Pal *et al*., 2004). When various *B. burgdorferi* s.l. isolates are compared, the Osp's show considerable heterogeneity within and among the different species (Wang *et al*., 1999).

Table 1. 3- Outer surface lipoproteins found in plasmids of *B. burgdorferi* sensu lato complex (Brandt *et al*., 1990; Stanek, 1997; Beermann *et al*., 2000)

Osp PROTEINS	MOLECULAR WEIGHT (kilodaltons)	GENE (in Linear and Circular plasmids)
OspA	31	49 kb (L)
OspB	34	49 kb (L)
OspC	21-25	25-27 kb (C)
OspD	28	38 kb (L)
OspE	19	45 kb (L)
OspF	26	45 kb (L)

The relationships among OspA and OspC serotypes and the delineated *Borrelia* species can be seen in **Fig. 1.7** for some strains (Wang *et al.*, 1999). For instance, *B. garinii* can harbour 5 OspA serotypes (Stanek, 1997; Wang et al., 1999; Marconi *et al.*, 1999; Hu *et al.*, 2001; Huegli *et al.*, 2002). OspA serotypes 1 and 2 correspond to *B. burgdorferi* s.s. and *B. afzelii*, respectively (Stanek, 1997; Huegli *et al.*, 2002). *B. valaisiana* also can harbour two distinct *ospA* genes (Wang *et al.*, 2000). Molecular polymorphism of the *OspC* gene has been reported in *B. burgdorferi* s.s., *B. garinii, B. afzelii* and *B. valaisiana* (Wang *et al*, 1999a, 1999c) with a probable occurrence of lateral gene transfer, not only between members of the same species but also between strains from different species (Wang *et al.*, 1999). *OspC* genes from strains of the same species appear to be more closely related to each other than to *ospC* genes from different species (Wang *et al.*, 1999, 1999a; Baranton, 1999). Only ten out of the 58 *OspC* genes known so far are invasive and cause Lyme disease: four of *B. burgdorferi* s.s., two of *B. afzelii* and four of *B. garinii* (Baranton *et al.*, 2001)

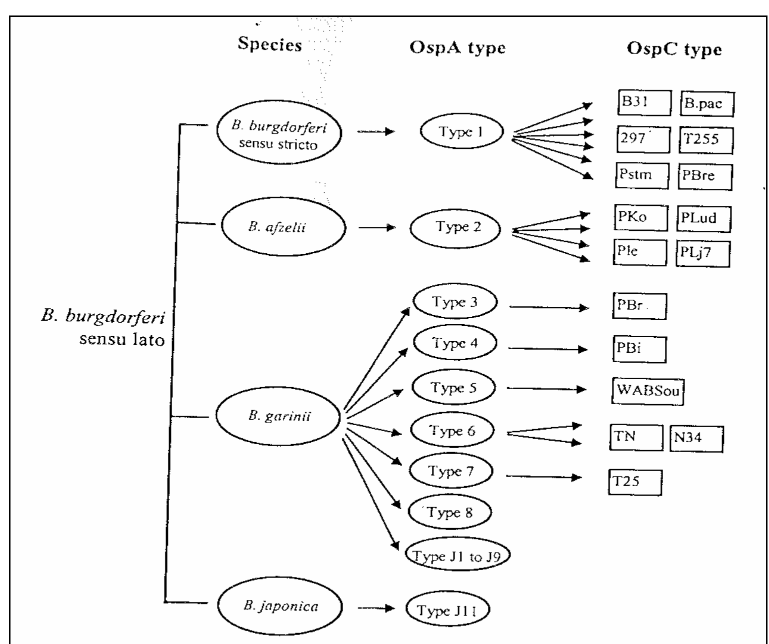


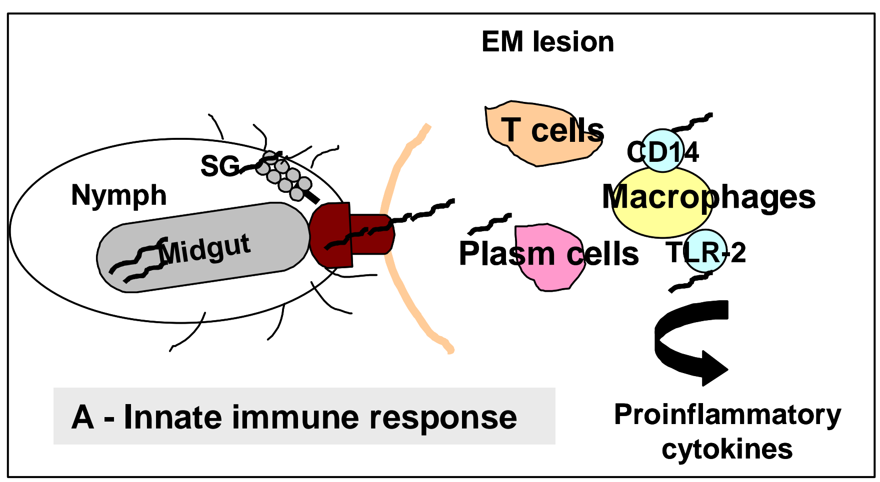
Figure 1. 7 – Relationship between species and OspA and OpsC serotypes of *B. burgdorferi* sensu lato (Wang *et al.*, 1999)

2.5 – General immune response

B. burgdorferi s.l. induces complex cellular and humoral immune responses to a number of spirochetal proteins in patients with Lyme disease (Vaz *et al.*, 2001). Spirochetes that have adapted to either the feeding tick or host are relatively invulnerable to the protective effects of immune sera, unlike

spirochetes grown *in vitro*, which are highly susceptible. Their ability to rapidly alter the expression of surface antigens may be the common factor that allows the spirochetes to survive (de Silva *et al*., 1998; Barbour & Restrepo, 2000)

Spirochetal proteins, which bind the complex CD14 molecule and Toll-like receptor 2 (TLR2) on macrophages, are potent activators of the innate immune response and the subsequent secretion of macrophage-derived inflammatory cytokines (Vaz *et al*., 2001; Hengge et al., 2003). Toll-like receptors are receptors for highly inflammatory lipoproteins and provide a mechanism by which localized bacteria could directly activate inflammation, namely, Lyme arthritis (Hirschfeld *et al*., 1999). T-helper 1 (Th1) cells are preeminent in the early phase of infection (**Fig. 1.8**) (Malovrh *et al*., 2000; Hengge *et al*., 2003). For instance, secretion of IFN-γ (Th1) alone is not sufficient to eliminate *Borrelia* spirochetes but still has a beneficial role acting in concert with other mechanisms (Ekerfelt *et al*., 1999).



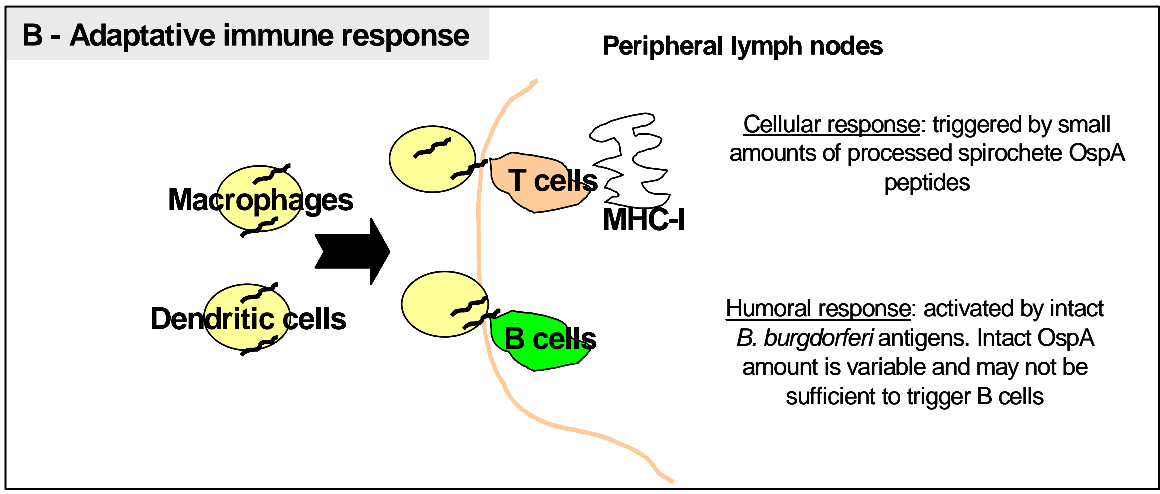


Figure 1. 8 – Immunity against *B. burgdorferi* sensu lato in early (A- Innate immune response) and disseminated disease (B – Adaptative immune response) (adapted from Vaz *et al*., 2001)

In disseminated infection, adaptative T-cell and B-cell responses in lymph nodes lead to the production of antibodies against different components of the organism (Sellati *et al.,* 1999; Vaz *et al*., 2001; Hengge et al., 2003). In both disease stages, MHC class-II molecules on Langerhan's cells are down-regulated which may impair the capacity of these cells to eliminate *B. burgdorferi* s.l. and thus, permit chronic infection (Beermann *et al*., 2000; Hengge et al., 2003).

Differences in immune responses were found between the major antigens present during infection. For OspA, OspC and FlaB, a cellular immune response is frequently observed, but a humoral response is more characteristic for the two latter proteins. However the strong cellular and humoral immune responses to OspA in some patients suggest that the spirochete may sometimes up-regulate the expression of this protein in inflamed joints, after prolonged exposure (Vaz *et al*., 2001; Crowley & Huber, 2003). The dissociation of the types of immune responses may occur because small amounts of processed peptides may trigger T cells, whereas intact antigen is required to activate B cells.

Neuroborreliosis pathogenesis is induced by secretion of IL-6 in the CNS by *B. burgdorferi* s.l.*,* causing symptoms associated with this interleukin, including fever and affective disorders of late-stage Lyme disease (Habicht *et al*., 1991).

Host serum complement activity may play a direct role in pathogen transmission, by selectively lysing spirochetes that are expressing different outer surface proteins (Schwan & Piesman, 2000). Different genospecies of the etiological agents of LB differ in their sensitivity to complement from different host species in a manner that reflects the ecological pattern observed (Kurtenbach *et al*., 1998, Lawrie *et al*., 2005). Complement-mediated selection of *B. burgdorferi* sensu lato appears to be a major factor in the evolution and ecology of Lyme borreliosis spirochetes (Kurtenbach *et al*., 2002, 2002a; Hanincová *et al*., 2003)

B. garinii strains are considered sensitive to serum complement-mediated bacteriolysis, *B. burgdorferi* s.s. are intermediate complement-sensitive and *B. afzelii* are resistant (Hellwage *et al*., 2001; Kraiczy *et al*., 2001; Alitalo *et al*., 2001). This resistance, that helps the bacteria to evade complement attack and phagocytes, occurs by a specific binding between *ospE* and the complement regulators factor H and FHL-1/reconectin (factor-H like protein 1), promoting inactivation of C3b (Hellwage *et al*., 2001; Kraiczy *et al*., 2001; Alitalo *et al*., 2001; Kraiczy *et al*., 2002, 2002a). *B. garinii* is more susceptible to complement

probably because of its preference for causing borreliosis in the central nervous system, where the amount of complement activity is much smaller (Alitalo *et al*., 2001)

B. burgdorferi s.l. are invasive bacterial pathogen that may also benefit by use of the host's plasminogen activation system (proteolytic activity), degrading components of human extracellular matrix (fibronectin, laminin and vitronectin) found in the connective tissue of the organs affected in LB. The resulting proinflammatory degradation products are also likely to contribute to the focal inflammation characteristic of the affected tissues (Coleman *et al*., 1999)

3 - VECTORS OF LYME BORRELIOSIS AGENTS

3.1 Definition

Ticks have evolved to become one of the most important groups of arthropod vectors of human and animal pathogens (Gustafson, 1994; Estrada-Pena & Jongejan, 1999; Mejlon, 2000; Parola & Raoult, 2001; Anderson, 2002; Gray, 2002), besides being a cause of severe paralysis caused by neurotoxic substances produced by the salivary glands, during prolonged attachment (5-7 days) (Sauer *et al*., 1995; Parola & Raoult, 2001). Parola and Raoult (2001) present an extensive review on the ticks commonly acting as vectors of human bacterial disease.

Ticks are a highly specialized group of obligate-bloodsucking, nonpermanent ectoparasitic arthropods that feed on mammals, birds, and reptiles. Their life cycles are often complex, and even though ticks are associated with their parasitic habits, ticks spend most of their life off hosts and in vegetation or soil. Maintenance of water balance during periods of overhydration while feeding and periods of dehydration while fasting is significant in the distribution, survival, activity and transmission of disease-causing pathogens to humans and animals.

Ticks are ideally suited as disease vectors for several reasons (Márquez-Jiménez *et al*., 2005):

1 - strong fixation and for long periods, along with hosts with certain mobility, which enhances the possibility of maintaining and spreading pathogenic agents,

2 – intracellular digestion of the blood ingested and persistent hematophagy,

3 - possibility of trans-ovaric and trans-stadial transmission of distinct agents,

4 – coincidence of feeding of different instars of the same species, favouring horizontal transmission of the pathogenic agents,

5 – almost no host specificity, allowing transmission of the agents between different hosts,

6 – great reproductive potential, with females capable of laying hundreds to thousands of eggs, which contribute to fast growing of tick populations,

7 – great resistance to hunger and draughtness,

8 – long longevity and sylvatic cycle,

9 – cuticula resistance and few natural enemies.

The maintenance cycle of *B. burgdorferi* s.l. in nature is composed of 4 steps: 1) *Borrelia* occurs in the tick vectors, 2) is transmitted from the tick to the host, 3) occurs in the hosts, and 4) is transmitted from the host to the tick (Humair & Gern, 2000). In this cycle, ticks are considered the main vectors of Lyme Borreliosis (**Table 1.4**), however numerous species have been implicated as potential or confirmed vectors, although with insignificant importance. Reports as early as 1904 describe the presence of spirochetes in the gut contents of numerous tabanids and mosquitoes that may suggest contaminative transmission by these insects (Magnarelli *et al*., 1986 *cited* in Ribeiro *et al*., 1987; Burgdorfer, 1998; Zeman, 1998; Lane *et al*., 1999; Zakovská *et al*., 2002; Halouzka *et al*., 2002). Dissemination to the salivary glands, as occurs in ticks, would seem unlikely, considering the brief feeding period of these insects, but studies are in progress to understand if systemic infections exist, including salivary glands tissues (Burgdorfer, 1998; Matuschka & Richter, 2002). The role of insects as LB vectors remains to be demonstrated (Ribeiro *et al*., 1987; Gustafson, 1994; Burgdorfer, 1998; Zeman, 1998; Zakovská *et al*., 2002; Kosik-Bogacka *et al*., 2002; Sun & Xu, 2003). So far, observations by some authors, show that mosquitoes could acquire spirochetes but they are unable to transmit them to a second level of hosts (Matuschka & Richter, 2002). Although naturally infected *Aedes* and *Culex* spp were found overwintering, the observed low values of the prevalence rates and infection intensity of female mosquitoes, as compared to ticks, suggest that the former do not pose a serious epidemiological threat in the spreading of Lyme disease (Hubalek *et al*., 1998; Halouzka *et al*., 2002; Kosik-Bogacka *et al*., 2002, 2004, 2004a; Janoukovcová *et al*., 2004). There has been only one published record of erythema migrans associated with mosquito bite (Hard, 1996 *cited* in Kosik-Bogacka *et al*., 2002).

Along with insects, mites (*Neotrombicula autumnalis*) have also been incriminated as potential vectors for *B. burgdorferi* s.l. in an extremely rare event with a possible transstadial and transovarial transmission (Kampen *et al*., 2004). Fleas (*Orchopeas leucopus*) can also be infected with *Borrelia*, but the very low number of spirochetes suggests that this insect is a poor vector (Lindsay *et al*., 1991).

Table 1. 4 – Ticks as potential and confirmed (#) vectors of Lyme Borreliosis agents (Sparagano *et al.*, 1999; Wang *et al.*, 1999; Baranton, 1999; Estrada-Pena & Jongejan, 1999; Sun & Xu, 2003).

SPECIES OF TICKS	SPECIES of *B. burgdorferi* sensu lato	SPECIFIC REFERENCES
Amblyomma americanum	*B. burgdorferi* sensu stricto	Feir *et al.*, 1994
Dermacentor reticulatus	*B. burgdorferi* s.l.	Hubbard *et al.*, 1998
Dermacentor variabilis	*B. burgdorferi* sensu stricto	Feir *et al.*, 1994
Haemaphysalis punctata (#)	*B. burgdorferi* s.l.	Marquez & Constan, 1990 *cited in* Kahl *et al.*, 1992; Hubbard *et al.*, 1998
Dermacentor marginatus (#)	*B. burgdorferi s.l.*	Baptista *et al.*, 2004
Ixodes columnae	*Borrelia* sp; *B. valaisiana*	Fukunaga *et al.*, 1996; Wang *et al.*, 1997; Masuzawa, 2004
Ixodes dammini (#)	*B. burgdorferi* sensu stricto	Persing *et al.*, 1990b *cited in* Sparagano *et al.*, 1999
Ixodes dentatus	*B. andersonii*	Fukunaga *et al.*, 1996; Lin *et al*, 2001
Ixodes hexagonus (#)	*B. burgdorferi* s.l.	Gern *et al.*, 1991; Hubbard *et al.*, 1998
Ixodes canisuga	*B. burgdorferi s.l.*	Estrada-Pena *et al.*, 1995
Ixodes frontalis	*B. burgdorferi s.l.*	Estrada-Pena *et al.*, 1995
Ixodes ovatus (#)	*B. japonica*	Kawabata *et al.*, 1993; Fukunaga *et al.*, 1996; Masuzawa, 2004
Ixodes pacificus	*B. burgdorferi* sensu stricto; *B. bissettii*	Baranton *et al.*, 1992; Postic *et al.*, 199; Fukunaga *et al.*, 1996
Ixodes persulcatus (#)	*B. garinii; B. afzelii; B. valaisiana*	Lesnyak *et al.*, 1998; Aleksheev *et al.*, 1998; Yabuki *et al*, 1999; Masuzawa, 2004
Ixodes ricinus (#)	*B. burgdorferi* s.l.	Kron *et al.*, 1991 *cited in* Sparagano *et al.*, 1999; Wittenbrink *et al.*, 1994; Livesley *et al.*, 1994; Gray *et al.*, 1994; Olsén *et al.*, 1995
	B. burgdorferi sensu stricto*; B. garinii; B. afzelii; B. valaisiana; B. lusitaniae; B. bissettii*	Núncio et al., 1993; Rijpkema *et al.*, 1995, 1996a,b; Cinco *et al.*, 1996 *cited in* Sparagano *et al.*, 1999; Kirstein *et al.*, 1997a; Strle *et al.*, 1997; Liebisch *et al.*, 1998; Misonne *et al.*, 1998; Michel *et al.*, 2003
Ixodes scapularis (#)	*B. burgdorferi* s.l.	Johnson *et al.*, 1992; Schwan *et al.*, 1993; Guttman *et al.*, 1996
	B. burgdorferi sensu stricto	Rosa & Schwan, 1989 *cited in* Sparagano *et al.*, 1999; Persing *et al.*, 1990a,b *cited in* Sparagano et al., 1999; Lin *et al.*, 2001
Ixodes trianguliceps	*B. burgdorferi* s.l.	Hubbard *et al.*, 1998
Ixodes columnae	*B. valaisiana*	Fukunaga *et al.*, 1996; Wang et al., 1997; Masuzawa, 2004
Ixodes uriae (#)	*B. burgdorferi* s.l.; *B. garinii*	Olsén *et al.*, 1993; Bunikis *et al.*, 1996
Ixodes takunii	*B. takunus*	Masuzawa *et al.*, 1996; Fukunaga *et al.*, 1996
Ixodes turdus (#)	*B. turdae*	Fukunaga *et al.*, 1996
Ixodes minor	*B. bissettii*	Lin *et al.*, 2001
Ixodes granulatus	*B. valaisiana*	Masuzawa, 2004
Ixodes jellisoni	*B. burgdorferi s.l.*	Lane *et al.*, 1999
Ixodes muris	*B. burgdorferi* sensu stricto	Dolan *et al.*, 2000
Rhipicephalus sanguineus	*B. burgdorferi* s.l.	Hubbard *et al.*, 1992b *cited in* Sparagano *et al.*, 1999

3.2 Taxonomy

The firsts attempts to classify ticks were made by Latreille (1775), Linnaeus (1794), Hermann (1804), Leach (1815), Heyden (1826), Sundeval (1833) and Dugés (1834) but all of them associated ticks with other kinds of mites (Santos Dias, 1994). Koch (1844) made the first distinction between these arthropods with the following families: Argasiden (gen. *Argas* and *Ornithodoros*); Ixodiden (gen. *Hyalomma*, *Haemalastor*, *Amblyomma* and *Ixodes*); Rhipistomiden (gen. *Dermacentor*, *Haemaphysalis*, *Rhipistoma* and *Rhipicephalus*). Since then, a number of authors, namely Santos Dias (1994) and Horak *et al*. (2002) gave new suggestions to discriminate the main taxa based on morphological and biological characteristics of ticks, presented as follows.

Phylum ARTHROPODA Siebold & Stannius, 1845
Subphylum CHELICERATA Heymons, 1901
Class ARACHNIDA Lamark, 1801
Subclass ACARIDA (Nitzsch, 1818)
Superorder ANACTINOTRICHIDA (Grandjean, 1935)
Order IXODIDA (Sundeval, 1833)
Suborder METASTIGMATA Canestrini, 1892
Superfamily IXODOIDEA Banks, 1894
Family IXODIDAE Murray, 1877

The group comprises 825 species divided into 3 families: Argasidae (soft ticks), Ixodidae (hard ticks) and Nutalliellidae. The phylogeny based on 18S rDNA (**Fig. 1.9**) resembles the phylogeny based on morphology and it is a good marker for supraspecific differentiation and eespecially for taxa grouping purposes (Black IV *et al*., 1997; Mangold *et al*., 1998).

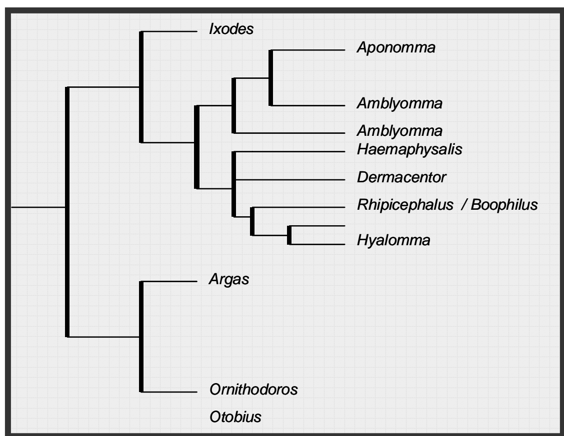


Figure 1. 9 - Phylogeny of ticks based on the 18S rDNA gene (Black IV *et al*., 1997; Mangold *et al*., 1998)

In Portugal, the monograph "Revisão sistemática dos Ixodídeos Portugueses" (Tendeiro, 1962 *cited* in Caeiro, 1999) was one of the most important revisions of ticks distribution and taxonomy, describing at that time 14 tick species. Since then, other species have been described by different authors and at present, 24 species are known to occur in Portugal and are classified as follows (Santos Dias, 1994a; Caeiro, 1999):

Superfamily Ixodoidea Banks, 1894

Family Ixodidae Murray, 1877; Subfamily Ixodinae Salmon and Stiles, 1901

I - **Genus *Ixodes*** Latreille, 1795

Subgenus *Ixodes* s.str. Latreille, 1795: *Ixodes acuminatus* Neumann, 1901; *Ixodes bivari* Travassos Dias, 1989; *Ixodes frontalis* (Panzer, 1798); *Ixodes ricinus* (Linnaeus, 1758); *Ixodes ventalloi* Gil Collado, 1936

Subgenus *Eschatocephalus* Frauenfeld, 1853: *Ixodes vespertilionis* Koch, 1844; *Ixodes simplex simplex* Neumann, 1906

Subgenus *Pholeoixodes* Schulze, 1942: *Ixodes canisuga* Johnston, 1849; *Ixodes hexagonus* Leach, 1815

Subfamily Rhipicephalinae Salmon and Stiles, 1901

II – **Genus *Rhipicephalus*** Koch, 1844

Subgenus *Rhipicephalus* s. str. Koch, 1844: *Rhipicephalus bursa* Canestrini and Fanzago, 1887; Rhipicephalus pusillus Gil Collado, 1936; *Rhipicephalus sanguineus* (Latreille, 1806); *Rhipicephalus turanicus* Pomerantzey, Matiskasvili and Lototski, 1940

III – **Genus *Boophilus*** Curtice, 1891: *Boophilus annulatus* (Say, 1821)

IV – **Genus *Hyalomma*** Koch, 1844

Subgenus *Hyalomma* s.str. Koch, 1844: *Hyalomma lusitanicum* Koch, 1844; *Hyalomma marginatum marginatum* Koch, 1844

Subfamily Amblyomminae Neveu-Lemaire, 1938

V – **Genus *Dermacentor*** Koch, 1844

Subgenus *Dermacentor* s.str. Koch, 1844: *Dermacentor marginatus* (Sulzer, 1776); *Dermacentor pictus* (Hermann, 1804)

VI – **Genus *Haemaphysalis*** Koch, 1844

Subgenus *Alloceraea* Schulze, 1918: *Haemaphysalis inermis inermis* Birula, 1895

Subgenus *Aboimisalis* Travassos Dias, 1963: *Haemaphysalis punctata* Canestrini and Fanzago, 1877

Subgenus *Rhipostoma* Koch, 1844: *Haemaphysalis hispanica* Gil Collado, 1936

Family Argasidae Canestrini, 1890

Subfamily Argasinae Pospelova-Shtrom, 1946

VII – **Genus *Argas*** Latreille, 1796

Subgenus *Carios* Latreille, 1796: *Argas vespertilionis* (Latreille, 1802)

Subfamily Ornithodorinae Pospelova-Shtrom, 1946

VIII – **Genus *Ornithodoros*** Koch, 1844

Subgenus *Alectorobius* Pocock, 1907: *Ornithodoros maritimus* Vermeil and Marguet, 1967

Subgenus *Pavlovsvskyella* Pospelova-Shtrom, 1946: *Ornithodoros erraticus* (Lucas, 1802)

Analysis of internal transcribed spacer (ITS2) indicated that *B. burgdorferi* sensu lato species associated with Lyme disease are found mainly in ticks of the *Ixodes ricinus* species complex (**Fig. 1.10**). There is a high degree of concordance in the phylogenetics of *Borrelia* taxa and the phylogenetic relationships among *Ixodes* ticks (Fukunaga *et al*., 2000).

Figure 1. 10 – Female of *Ixodes ricinus* (EUCALB)

I. ricinus complex consists of two Eurasian species (*I. persulcatus* Schulze and *I. ricinus* Linnaeus, 1758) and two Neartic species (*I. scapularis* Say and *I. pacificus* Cooley & Kohls) (Mejlon, 2000). All of these ticks are responsible for *B. burgdorferi* s.l. transmission.

The first detailed studies on *I. ricinus* took place in Britain between 1932 and 1955 mostly in sheeps reared on hill farms, resulting in the perhaps inappropriate common name of sheep tick for *I. ricinus* (Gray, 1991). In 1983, this tick aquired new significance when it was shown to be a vector of the newly described spirochaetal disease, Lyme Borreliosis (Burdorfer *et al*., 1983 *cited* in Gray, 1991).

In Europe, *I. ricinus* and *I. persulcatus* are the main vectors for Lyme Borreliosis agents. The importance of *I. ricinus* is due to its widespread distribution (**Fig. 1.11**), feeding habitats and willingness to bite humans (Estrada-Pena & Jongejan, 1999; Vassalo & Pérez-Eid, 2003).

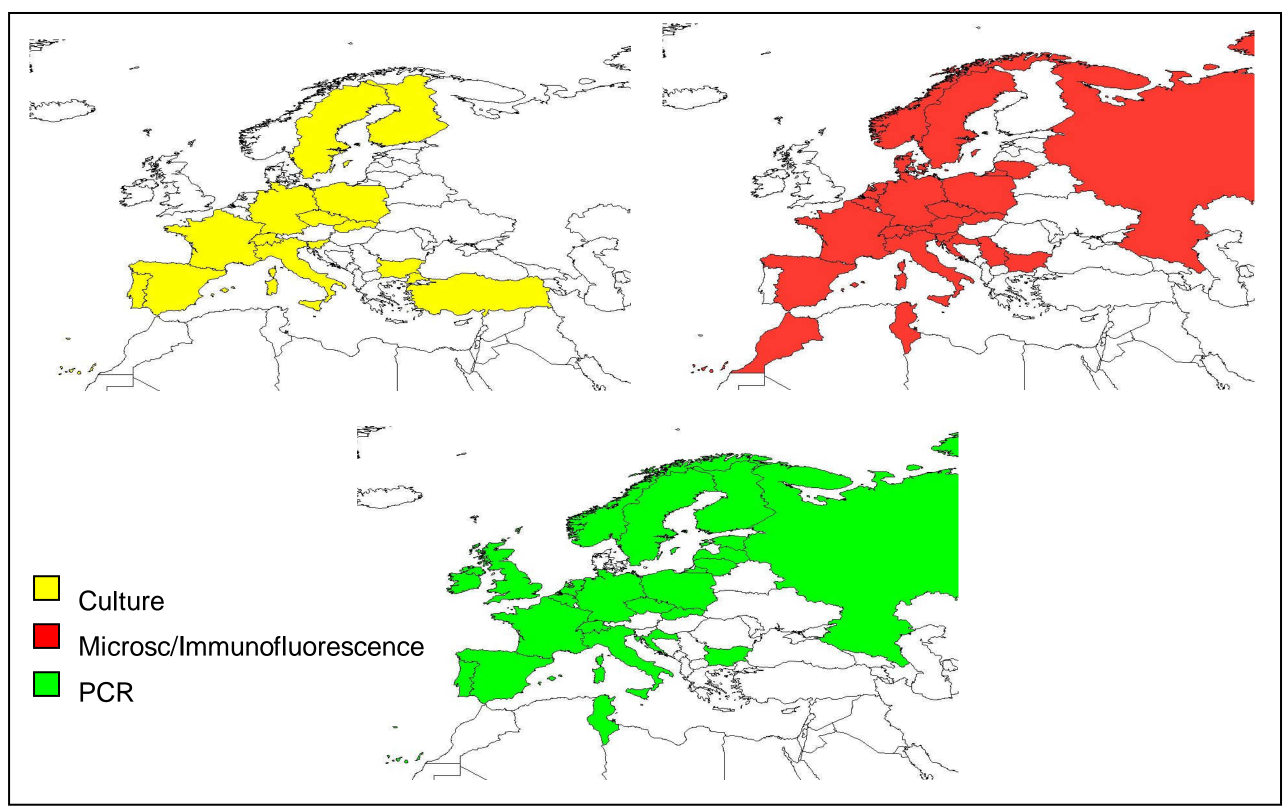


Figure 1. 11 – Distribution of *I. ricinus* ticks infected with *B. burgdorferi* sensu lato based on a review of the literature.

Besides LB causative agents, *I. ricinus* is implicated in the transmission of an extensive list of pathogenic agents, namely *Rickettsia* spp (Fernández-Soto *et al.*, 2004), *Borrelia* spp (Richter *et al.*, 2003), *Coxiella burneti* (Q Fever) (Tylewska-Wierbanowska *et al.*, 1996), Tick-borne encephalitis virus (Daniel *et al.*, 2004), Louping ill virus (Gaunt *et al.*, 1997 *cited* in Sparagano *et al.*, 1999), Human granulocytic ehrlichiosis (HGE) (*Anaplasma phagocytophila*) (Ambrasiene *et al.*, 2004), *Babesia* spp (Ambrasiene *et al.*, 2004*), Toxoplasma gondii* (Sroka *et al.*, 2003), Crimean-Congo Hemorrhagic fever virus (CCHF) (Estrada-Pena & Jongejan, 1999), *Bartonella* spp (Schouls *et al.*, 1999), *Francisella tularensis* (Wicki *et al.*, 2000), *Wolbachia* spp (Hartlet *et al.*, 2004), trypanosome and filarias (Aeschlimann *et al.*, 1979) and other Gram-negative bacteria (Stojek & Dutkiewicz, 2004). This diversity of pathogenic agents increases the importance of this tick in Europe and, more specifically, in Portugal, where some of these diseases are known to occur.

3.3 Morphology

Morphology of Ixodidade (hard-body ticks) ticks, the largest and most important family of ticks is presented in **Fig. 1.12.** The capitulum (gnatosoma) is the most anterior portion and includes the mouth and surrounding structures, including the bisegmented chelicerae, the hypostome and sensorial palps. The extremities of the chelicerae are flat, triangular, presenting a series of laterally orientated teeth. The bases of the palps are joined forming the basis capituli, the shape of which is specific to each tick genera. On the ventral side, this structure supports the hypostome characterized by a dense denticulation on the distal portion, which contacts the skin of hosts. On the dorsal side of females, the capitulum has several porous areas and glands that secrete pheromones (Gustafson, 1994; Estrada-Pena *et al.*, 2004**)**

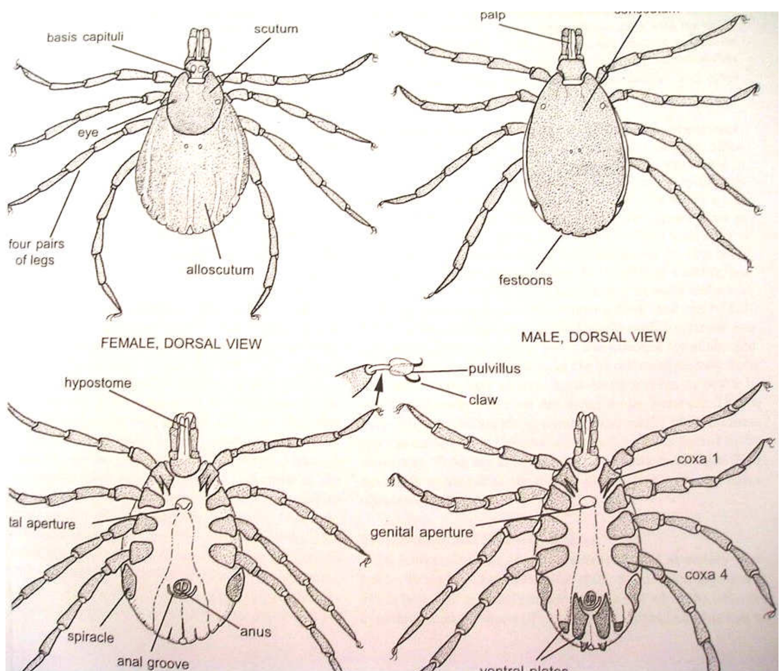


Figure 1. 12 – External morphology of ixodid ticks from ventral and dorsal side (Estrada-Pena *et al.*, 2004).

On the posterior part of the tick body (idiosoma), there is a sclerotized zone, the dorsal scutum. This structure can be ornamented wih colour spots (grey, white, blue or red, on dark base coulour). In males, this scutum covers the entire idiosoma (and is named the conscutum) while in females, nymphs and larvae it is incomplete, covering only the anterior region. Eyes, when present, are located on the lateral margin of the dorsal scutum.

On the anterior portion of the body, four pairs of legs are found on adults and nymphs and three pairs are found on larva. Each leg is joined to the body by a coxa, which can have one or two spurs (internal or external). Several segments, namely the trochanter, femur, knee, tibia and tarsus, terminating in small white pads, the pulvilli, compose the rest of the leg. In the tarsus of the first pair of legs, is located a sensorial structure named Haller's organ, with several sensorial setae involved in water or host localization. Behind the fourth pair of legs, in the lateral margin of the idiosoma, a pair of spiracular plates with various shapes (circular, oval or comma) contains openings for the breathing tubes (=tracheae).

Sexual differentiation is not seen in immature tick stages. However, males and females have a genital aperture, the gonoporum, on the ventral side of the idiosoma, between the coxae of the second pair of legs. A study of *I. scapularis* revealed that body weights of engorged nymphs that became females were significantly greater than those of nymphs that became males, probably because nymphs that become females imbibe more blood than those that become males (Hu & Rowley, 2000). In *I. ricinus* weight and body size are two characteristics that can be measured to determine the sex of nymphs (Kahl *et al*, 1990; Dusbabek, 1996). Specimens of greater body dimensions (e.g. 2.30-2.94 mm long) and with more numerous dorsal alloscutal setae moulted predominantly into females. Dusbabek (1996) found that about 98% of females moulted from nymphs weighting more than 3.42-3.60 mg. The author also found that characteristics of body weight, while important in differentiating between the sexes, can differ among tick populations and in ticks engorged on different host animals (Dusbabek, 1996).

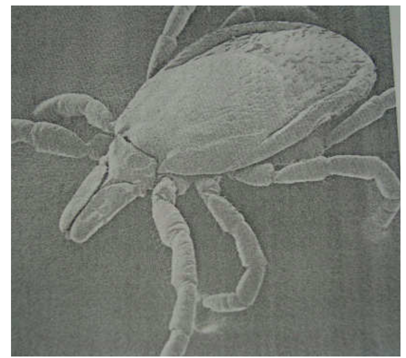
The anus is also located on the ventral side, near the posterior margin of the body, behind the fourth pair of legs, and is associated with being surrounded by the anal groove (**Fig. 1.13**). This groove may be absent or located anterior or posterior to the anus, depending on the genera (Estrada-Pena *et al*., 2004; Márquez-Jiménez *et al*., 2005).

Figure 1. 13 – Ventral side of *I. ricinus* showing the anterior anal groove (EUCALB).

3.4 Feeding Process

3.4.1 Physiological aspects

Ticks attach to the skin of the hosts by using their hypostome with numerous recurved teeth (**Fig. 1.14**) as an anchor and create a feeding lesion to ingest blood or tissue fluids, damaging the skin of the host. Soft-bodied ticks feed relatively rapidly (hours or less) and ingest only blood. Hard-bodied ticks take days to complete feeding (4 for immature stages to 14 for adult females) and feed on blood, lymph and lysed tissues from a pool that forms around their mouthparts. Host tissue fluids and tick saliva progress in alternate directions through a common buccal canal during tick attachment and feeding (Gustafson, 1994; Sauer *et al.*, 1995; Nuttall, 1999; Mejlon, 2000; Kuthejlová *et al.*, 2001; Parola & Raoult, 2001; Anderson, 2002; Gray, 2002; Estrada-Pena *et al.*, 2004). The tick diet consists of 90 to 95% of proteins, haemoglobin being the major constituent.

A 

B

Figure 1. 14 – Electron micrograph of an adult female *Ixodes ricinus* (A) with magnified view of mouthparts (B) (Gustafson, 1994)

Ixodid digestion displays three phases, two of which occur on the host and the last, after tick has droped off. The continuous digestion phase is initiated by feeding and corresponds with the slow-feeding period last for several days after attachment. During this time ticks utilize nutrients to synthesize new cuticle and for growth of internal organs. The second phase is characterized by reduced-digestion and a period of rapid engorgement period, which is generally initiated by mating, except for *I. ricinus* (Graf, 1978 *cited in* Brossard & Papatheodorou, 1990; Parola & Raoult, 2001). In this phase (12 to 24 hours before drop off) the tick weight increases by ca. 150 fold, with a marked increase in the alloscutal length and width, but no change in the dimensions of the hard scutal plate (Yeh *et al*., 1995; Parola & Raoult, 2001). In the final phase of continuous digestion, during the post-feeding period of preoviposition and oviposition, digestion of the blood meal takes place. The majority of this digested blood meal is used to produce the female specific protein vitellogenin and consequently the eggs (Brossard & Papatheodorou, 1990).

The constitution of the midgut epithelium of feeding nymphs and unfed females is derived from the digestive cells of larval and nymphal stages, respectively. Digestion is primarily of the main part of blood meal is performed by the generation of digestive cells of nymphal phase after detachment, during moult. Secretory vacuoles are formed in the digestive cells of the larval phase. All these functioning cells form a peritrophic membrane (PM) on their apical surface. (Munderloh & Kurtii, 1995; Grigor'eva, 2003, 2004).

PM can function as a selectively permeable physiological barrier or as a mechanical barrier protecting the midgut epithelium from damage afflicted by hard food particles during intracellular digestion, namely in the process of micropinocytosis (Grigor'eva, 2003). In *I. ricinus*, a PM is observed in all three tick stages at no later than 18 h after their placement on hosts and has been found to remain intact until at least 11, 30 and 10 days after repletion in larvae, nymphs and females, respectively. This membrane has a rapid secretion without well-defined boundaries at the onset of its appearance (Zhu *et al*., 1991; Munderloh & Kurtii, 1995). This temporary structure atrophies after repletion as the tick prepares to molt. In blood-sucking vector arthropods that exhibit a PM, some correlations have been found between this membrane and the infection process of a pathogenic agent. PM interfere with microbial adherence to and penetration of the midgut cells and their entry into the hemocoel, unless the microbes possess the means to penetrate the membrane (Zhu *et al*., 1991; Munderloh & Kurtii, 1995).

In the feeding process, salivary glands (SG), two branched alveolar (acinar) organs lying anterolaterally and extending posteriorly on both sides of the body, play an important role in water balance by eliminating excess water and ions and by taking up atmospheric water during nonfeeding periods, besides representing an important vehicle for transmission of tick-borne pathogens to the vertebrate hosts, namely Saliva Activated Transmission (SAT) (Munderloh & Kurtii, 1995; Sauer *et al*., 1995; Wang *et al*., 1999). Granular and agranular acini present in these organs have specific functions, including absorption of water and secretion of anticoagulants, cement, electrolytes and water (Munderloh & Kurtii, 1995; Sauer *et al*., 1995). A heterogeneity in the protein profile of salivary glands between individuals has been observed (Wang *et al*., 1999), namely between unfed an partially fed ticks (Slovak *et al*., 2000; Lawrie & Nuttall, 2001), life stages (Lawrie & Nuttall, 2001), gender and host species (Lawrie & Nuttall, 2001; Rolníková *et al*., 2003).

For a tick, maintaining the water balance with a minimum of energetic expense in the non-parasitic phases becomes a delicate physiological task crucial for survival. Quiescent ticks have a pattern of regular discontinuous ventilation, a state that is replaced by rapid ventilation and an increased metabolic rate during active uptake of water in dehydrated individuals placed at high humidity (Krober & Guerin, 1999; Perret *et al*, 2003). Besides unfed ticks, engorged and detached larvae and nymphs of some ixodid ticks consistently take up substantial amounts of atmospheric water vapour and with this, maintain water balance in subsaturated air when ambient relative humidity (RH) surpasses 75-90%, a threshold called the critical equilibrium humidity. Net uptake of vapour generally begins some days after detachment and persists until shortly after initiation of apolysis. This was shown for *I. ricinus, H. punctata* and the North American *I. dammini*. In *I. ricinus* both teneral nymphs and adults are capable of achieving net water gains on the first day following ecdysis (Hostis *et al*., 1995; Kahl, 1996; Kahl & Knulle, 1988; Kahl & Alidousti, 1997; Randolph & Storey, 1999; Gray, 2002). Agranular alveoli are responsible for producing the primary secretion involved in vapour uptake (Kahl *et al*., 1990). Type I SG acini secrete hygroscopic saliva that absorbs atmospheric humidity when exposed to the air and then is swallowed by the tick.

Ticks can also be in contact or close to drops of water (dew, raindrops and plant discharges), which can be used when the ambient RH is not sufficiently high to allow water vapour absorption. All life stages of *I. ricinus* ticks tend to react to an existing RH gradient when encountering or when already within the "catchment area" of a droplet, without making direct contact with the liquid. An increasing level of dehydration also increases positive reaction to water droplets. Ticks take a typical resting position with their forelegs folded and occasionally with their palps splayed, features strongly indicative of water

vapour adsorption (WVA) in non-feeding ixodid ticks. Sensory organs located outside Haller's organ (setae) and the papal organ are involved in the perception of liquid water (Krober & Guerin, 1999). Ticks close to a water body gain large amounts of net water within a relatively short period of time. Although *I. ricinus* can survive for several weeks when immersed in pure water, they do not insert their mouthparts into liquid water, which suggest that WVA is responsible for water gain. This could be a way for ticks to avoid contamination of the midgut lumen by pathogenic organisms (Kahl & Alidousti, 1997; Krober & Guerin, 1999). This capacity to use water droplets in nature could be of ecological relevance, particularly in habitats with a lack of a nearby stable humidity reservoir (*e.g.* permanent thick layer of leaf litter) or exposed areas with frequent wind but with a high annual precipitation (maritime and mountainous) (Kahl & Alidousti, 1997).

3.4.2 Immunological aspects

Besides the functions mentioned above, SG of ticks can also act as a pharmacy (**Table 1.5**) to overcome the host's haemostatic, inflammatory and immune responses (Sauer *et al.*, 1995; Bowman *et al.*, 1996; Brossard & Wikel, 1997; Kopecky & Kuthejlová, 1998; Kopecky *et al.*, 1999; Nuttall, 1999, 2004; Lawrie *et al.*, 1999; Wang *et al.*, 1999; Lacombe *et al.*, 1999; Gillespie *et al.*, 2000; Christe *et al.*, 2000; Kuthejlová *et al.*, 2001). Tick saliva stimulates host immune regulatory and effector responses involving antigen-presenting cells, cytokines, T lymphocytes, basophils, mast cells, eosinophils, complement and circulating antibodies, natural killer cells, interferon and nitric oxid (Bowman *et al.*, 1996; Wikel *et al.*, 1997; Brossard & Wikel, 1997; Kopecky & Kuthejlová, 1998; Christe *et al.*, 2000; Gillespie *et al.*, 2000; Kuthejlová *et al.*, 2001; Nuttall, 2004). Salivary gland extract (SGE) inhibits activation of the alternative pathway of complement, inactivates anaphylatoxins, and prevents phagocytosis and the production of superoxide by neutrophils (Pechová *et al.*, 2002).

The immunological interaction between ticks and hosts is very complex, involving on one hand the induction of the host response to tick antigens, and on the other the tick immune evasion mechanisms facilitating feeding on the host (**Fig. 1.15**). Tick feeding exerts a pronounced effect on the cytokine regulation of host immunity. Production of macrophage proinflammatory cytokines is usually suppressed, as is secretion of Th1 cytokines. For instance, tick saliva can upregulate IL-10 production at the tick feeding site, which consequently inhibits the elaboration of proinflammatory cytokines, for example IFN-γ or IL-12. Th2 cytokines are up-regulated, indicating the polarization of the immune response towards Th2 lymphocytes, *ie*, an humoral immunity (Brossard & Wikel, 1997; Christe *et al.*, 1999; Kopecky *et al.*, 1999; Willadsen & Jongejan, 1999; Schoeler *et al.*, 1999; Gillespie *et al.*, 2000; Kuthejlová *et al.*, 2001; Kovar *et al.*, 2001; Pechová *et al.*, 2002).

Table 1. 5 – The pharmacopeia of *Ixodes* ticks saliva (Nuttall, 1999; Gillespie *et al.*, 2000)

Activity	Target
Anti-haemostatic	Adenosin difosfat (ADP)
	Prostaglandin receptor
	Prostacyclin receptor
	Thrombin
Anti-inflammatory	Anaphylatoxins
	Bradykinin
	Histamine
Immunomodulatory	Alternative complement system (C3)
	Neutrophils
	Splenic T lymphocytes
	Interleukin 2
	Macrophages, nitric oxide
	Natural killer cells
	Type 1 interferon action
	Immunoglobulin G

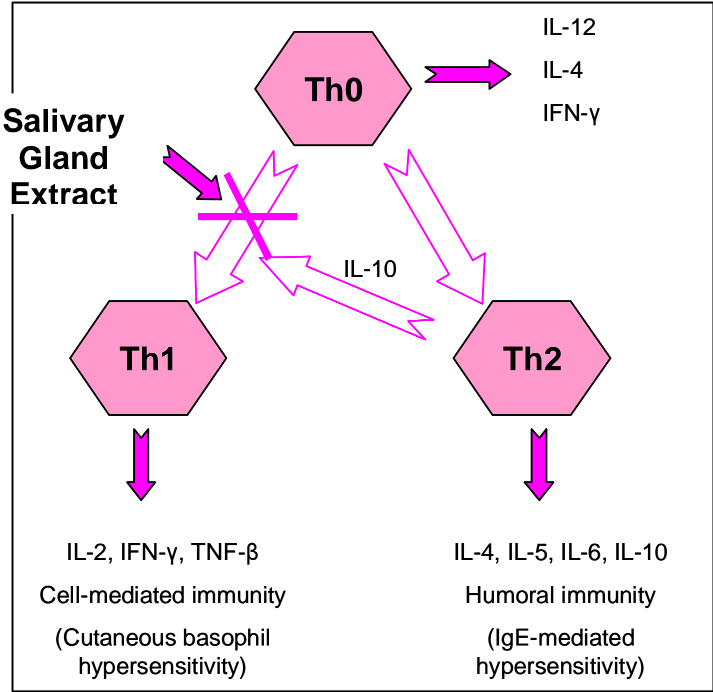


Figure 1. 15 – Effect of tick salivary gland extract on polarization of the host immune response (Kopecky, BDV, Prague)

The feeding success of a tick upon a host depends on its ability to suppress host anti-tick responses, which including activation of the complement system. For instance, in a study by Lawrie and others (1999), *I. ricinus* SGE inhibited alternative pathway activity in human, red deer, hedgehog and (weakly) pigeon sera. However, complement from pheasant or dog was not inhibited. The complement system

acts as a bridge between innate and adaptative immunity (Alitalo *et al.*, 2001). Activation of either results in the conversion of C3 from the inactive to the active form (C3b). In the presence of a suitable activating surface, bound C3b stimulates membrane attack complex formation and opsonization of foreign proteins. An anticomplement activity has been detected in *I. ricinus* SGE of unfed ticks as well as throughout the feeding process. *I. ricinus* SGE was shown to strongly inhibits the generation of the potent anaphylatoxin C3a. SGE inhibits the cleavage of C3 by the C3 convertase and appears to be at a stage before the formation of the C3b required to form the convertase (Alitalo *et al.*, 2001; Lawrie *et al.*, 1999; 2005).

Among inflammatory and immunological responses developed by hosts against ticks, skin sensitivity (immediate and delayed type) can affect tick nutrition and reproductive mechanisms (Brossard & Fivaz, 1982; Schorderet & Brossard, 1993; Mbow *et al.*, 1994; Wikel *et al.*, 1997; Willadsen & Jongejan, 1999; Odgen *et al.*, 2002), along with involvement of molecules that have a role in the physiological basis of pruritus, causing host grooming to limit tick infestation (Brossard & Wikel, 1997).

After successive infestations of rabbits with *I. ricinus* a skin sensitivity develops against the tick's salivary gland antigens. Female *I. ricinus* feed and lay fewer eggs after infestation on these immune rabbits. They also convert their blood meal less effectively into eggs (Brossard & Papatheodorou, 1990; Schorderet & Brossard, 1993; Wikel *et al.*, 1997; Brossard & Wikel, 1997; Nazario *et al.*, 1998; Willadsen & Jongejan, 1999; Lacombe *et al.*, 1999; Odgen *et al.*, 2002). On the other hand, no resistance to these ticks was acquired in BALB/c mice, although they developed cutaneous immediate- and delayed-type hypersensitivity reactions when reinfested (Mbow *et al.*, 1994; Christe *et al.*, 1998). Cases of immediate and/or delayed IgE-dependent hypersensitivity following bites by *I. ricinus* in humans were also reported (Beaudouin *et al.*, 1997). *I. muris* bites were associated with a severe reaction in domestic animals characterized by extreme pain and swelling at the site and, with more complete engorgement, lethargy, anorexia and high fever (Lacombe *et al.*, 1999). In sheeps, Odgen and others (2002) demonstrated that, although exposure to a particular density of ticks appeared to result in the development of sheep anti-*I. ricinus* resistance, resistance was inhibited or overcome when attachment rates of adult ticks were continuous and high during seasonal tick activity peaks. With increasing levels of infestations sheep simultaneously suffer increased doses of immunosuppressive tick saliva, which may act systematically or locally at the tick feeding lesion, and increasing blood loss, which may indirectly influence the host immune response (Odgen *et al.*, 2002).

Development of this tick immunity involves the interactions of tick antigens with host antibodies, T cells, B cells, mast cells and basophils. Langerhans cells in the skin process present tick antigens to lymphocytes that develop into sensitized lymphocytes and plasma cells secreting antibodies of various isotypes; among these are tissue-binding or homocytotropic antibodies. Antibodies bound to mast cells and basophils through their Fc receptors recognize tick antigens and induce degranulation resulting in development of microvesicles at the attachment site. Also contributing to the various manifestations of tick immunity are the multiple limphokines, monokines and chemokines released at the site of attachment. Basophil accumulation of tick attachment sites characterizes the immune reaction termed cutaneous basophil hypersensitivity (Brossard & Fivaz, 1982; Girardin & Brossard, 1989; Schorderet & Brossard, 1993; Mbow *et al*., 1994; Brossard & Wikel, 1997; Nazario *et al*., 1998; Kopecky & Kuthejlová, 1998; Odgen *et al*., 2002).

Glycoproteins found as soluble or membrane-bound receptors in tick tissues can play an important role in the parasite's interaction with the host organism or in the interaction with lectins of arthropod-borne infectious agents. Host antibody attack on tick gut antigens and other antigens of internal organs also can be involved in resistance to tick feeding (Uhlir *et al*., 1994). The most important antigens for resistance to tick feeding are those from salivary glands, midgut and 25kDa protein from *I. ricinus* (Uhlir *et al*., 1994). These are some common antigens found in larvae and nymphs and in different tissues of adult *I. ricinus*. Larvae are more efficient at inducing specific humoral responses.

3.5 Tick-*Borrelia* relations

3.5.1 Physiological aspects

B. burgdorferi s.l. are obligate parasites, persisting in nature only by infecting vertebrates and certain tick species. This requires adaptation strategies, for example, to escape different immune mechanisms or to survive the physiological changes between the tick and host tissue. Their main residence in guts of nonfed, infected ticks, progressively emerges into the hemolymph and appears in saliva when the tick is in contact with its host. A salivary route of spirochetal transmission from the vector enhance intradermal placement of spirochetal pathogens in vertebrates due to the already mentioned pharmacological properties of the vector's saliva (anti-inflammatory and immunosuppressive) that inhibit cellular immunity in these hosts (Ribeiro *et al*., 1987; Monin *et al*., 1989; Lebet & Gern, 1994; Piesman, 1995; Silva & Fikrig, 1995; Gern *et al*., 1996; Shih & Liu, 1996; Levin *et al*., 1997; De Silva *et al*, 1998; Nuttall, 1999; Kuthejlová *et al*., 2001; Schwan & Piesman, 2002; Crippa *et al*., 2002).

In their vector, *Borrelia* are primarily cell-surface parasites capable of occasional intracellular invasion (**Fig. 1.16**). Penetration of the tick gut occurs during active nutrient transport and extensive reorganization of the epithelial cell membranes. Attachment involves the alignment of the electron-dense, tapered end of the spirochete with a coated pit at the cell surface. Invagination of this area results in the entry of the organism into the cells. On the other side of the host cell, spirochetes emerge indicating that these pathogens utilize mechanisms of endocytosis and exocytosis, similar to those employed by *Rickettsiae* (Kurtii *et al*., 1993; Munderloh & Krutii, 1995). This intracellular residence is not common and they remain inside cells only for short periods. Residence within cells also occurs in the vertebrate host and it may be a strategy to help *Borrelia* survive when the extracellular environment becomes hostile because of the presence of antibiotics or borreliacidal antibodies, alternatively it could be a an efficient mechanism to spread through their vertebrate and invertebrate hosts (Stanek & Strle, 2003).

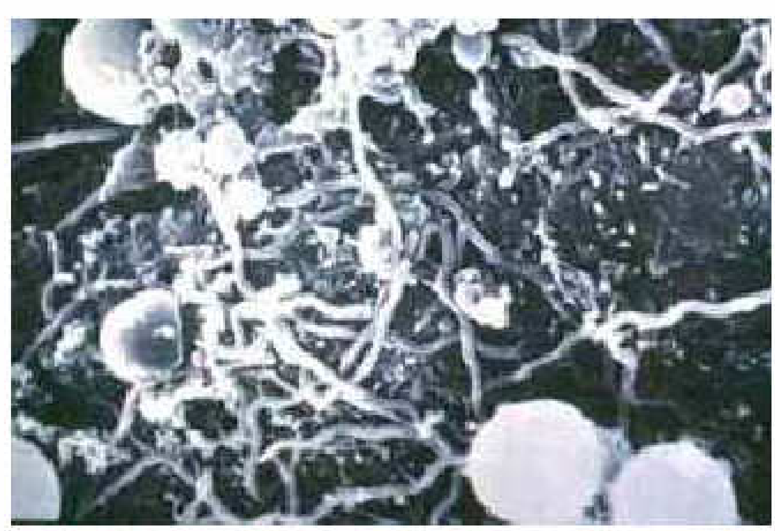


Figure 1. 16 – *Borrelia burgdorferi* attached to tick midgut (Labodia, 2003 *cited* in Aires, 2004)

Studies with *I. scapularis* and *I. ricinus* demontrate that these ticks deposit Lyme disease spirochetes in the skin of their hosts mainly after two days of attachment. The success of transmission is increased with duration of attachment. For *I. scapularis*, virtually no transmission occurs during the first day of nymphal feeding, inefficient transmission takes place during the second day of tick feeding, and transmission is extremely efficient during the third day of nymphal feeding. For *I. ricinus*, this situation may be more complex, with some risk of transmission during the first day of tick attachment. Successful transmission of spirochetes may occur even 17 or 29 h after *I. ricinus* attachment with differences for the various *Borrelia* species transmitted by this tick. (Obonyo *et al*., 1999; Gilmore & Piesman, 2000; Humair & Gern, 2000; Schwan & Piesman, 2002; Crippa *et al*., 2002; Fingerle *et al*., 2002).

Engorgement indices provide useful information on the duration of attachment *vs* risk of transmission of tick-vectored pathogenic agents (Yeh *et al.*, 1995; Falco *et al.*, 1996; Logar *et al.*, 2002). They can be computed, for instance, as the ratios between total body length and scutum length (index 1), total body length and scutum width (index 2), and alloscutum width and scutum width (index 3)

Larval ticks ingest spirochetes from infected reservoir hosts as early as 1 day after attachment, molt, and emerge as nymphs. When spirochetes are ingested by larvae, they rapidly multiply in the replete tick until the next molt, when a sudden drop in spirochete numbers occurs. When questing nymphs contact their potential hosts, spirochetes are at their lowest abundance and restricted to the lumen of the tick midgut, along the microvillar surfaces of the gut cells and between cells (Munderloh & Kurtii, 1995; Burgdorfer, 2001; Hodzic *et al.*, 2002). When nymphal feeding begins, a pronounced multiplication of the pathogen population in the tick takes place (Schwan & Piesman, 2002; Hodzic *et al.*, 2002).

During the blood meal the spirochetes start to migrate from the gut, invading the salivary glands (SG) and ultimately the mammalian host via the saliva (**Fig. 1.17**). The infectivity of spirochetes for the mammalian host increases during the tick's blood meal (Munderloh & Kurtii, 1995; Obonyo *et al.*, 1999; Gilmore & Piesman, 2000; Gillespie *et al.*, 2000; Humair & Gern, 2000; Crippa *et al.*, 2002; Hodzic *et al.*, 2002). This is linked to changes in the structural organization and function of midgut and salivary glands during feeding and salivation (Munderloh & Kurtii, 1995).

In one study by Gern and others (1996), spirochetes in tick midguts increased sixfold, from 998 per tick before attachment to 5,884 at 48h of attachment. Spirochetes in tick salivary glands increased >17-fold, from 1.2 per salivary gland pair before feeding to 20.8 at 72h post-attachment. The period of the most rapid increase in the number of spirochetes in the salivary glands occurred from 48 to 60h post-attachment; this time period coincides with the maximal increase in transmission risk during tick feeding. The presence of spirochetes in salivary glands may reduce the time delay for the transmission of *B. burgdorferi* s.l. During the first few hours of blood feeding, a drop in the number of spirochetes occurs followed by an increase at the end of the blood meal. This might be due to an elimination of spirochetes from the tick midgut via faeces. Once inoculated into the tick feeding lesion, spirochetes can be observed and remain at the inoculation site in the host skin for a period of one to several weeks. Soon after completion of feeding and detachment from the host, spirochetes decreased in number and are cleared from organs other than the midgut (Piesman, 1995; Silva & Fikrig, 1995; Gern *et al.*, 1996; Shih & Liu, 1996, 1998; de Silva *et al.*, 1999; Gilmore & Piesman, 2000; Fingerle *et al.*, 2000; 2002; Piesman *et al.*, 2001; Piesman & Schneider, 2002; Crippa *et al.*, 2002; Hodzic *et al.*, 2002).

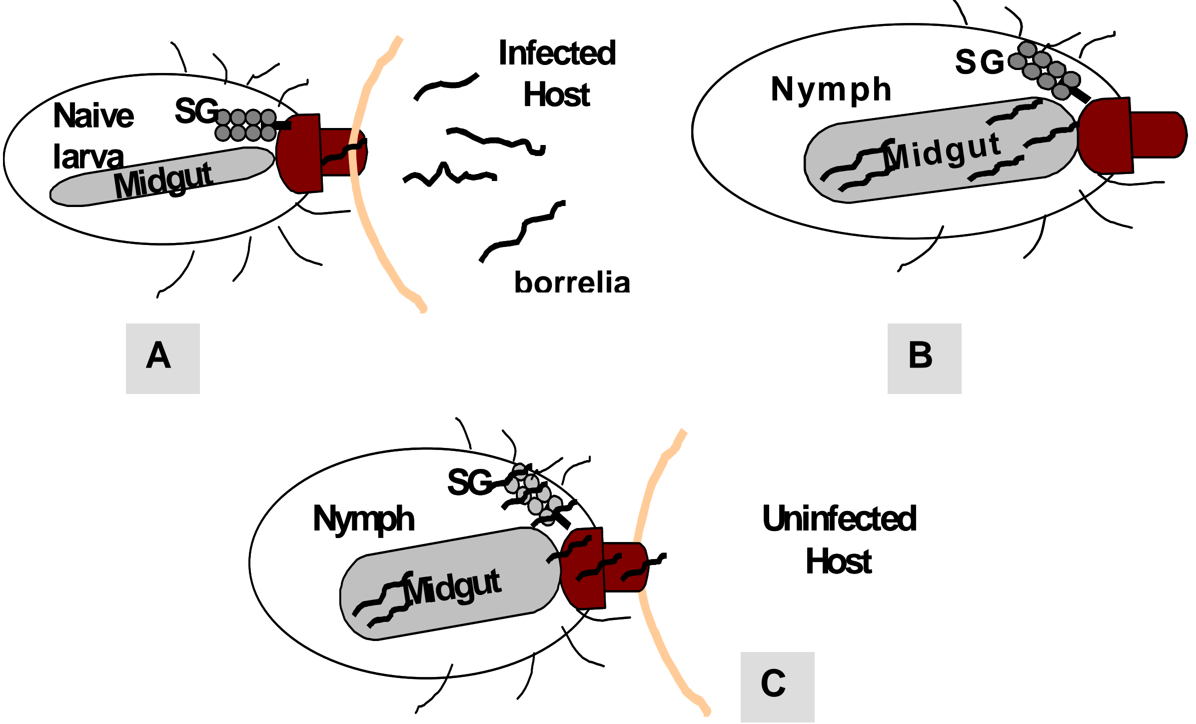


Figure 1. 17 – Transmission relations between *B. burgdorferi* s. l. and its' tick vector. A – tick feeds on an infected host and ingest *borrelia*; B – spirochetes migrate to the tick's midgut; C – the infected tick feeds on an uninfected host, *borrelia* migrates through the salivary glands (SG) and to the feeding site.

Although *Borrelia* is generally limited to the midgut tissues, there is a reasonable percentage of ticks (11-36%) that present a systemic infection involving various organs such as the midgut, salivary glands, central ganglion, muscles and hypodermis (Lebet & Gern, 1994; Leuba-Garcia *et al.*, 1994; Gern *et al.*, 1996; Humair & Gern, 2000; Piesman & Schneider, 2002). *B. afzelii* may be responsible for a more frequent systemic infection in unfed ticks (Crippa *et al.*, 2002).

3.5.2 Immunological aspects

One of the first immune components encountered by spirochetes within the midgut of ticks feeding on naïve hosts are natural antibodies present in the sera and interstitial fluids, the majority of which are IgM isotype and polyreactive. They facilitate uptake, processing and presentation of antigens by B cells and they may help localize pathogens and their antigens to lymphoid organs (Belperron & Bockenstedt, 2001). The effect of these antibodies on spirochetes is preferentially exerted within the tick midgut, with borrelicidal activity reducing spirochete numbers in feeding ticks (Belperron & Bockenstedt, 2001).

BALB/c mice repeatedly infested with pathogen-free *I. scapularis* nymphs were resistant to subsequent infection with tick-transmitted *B. burgdorferi* s.l.. The host immune response induced by repeated infestations could neutralize the tick immunosuppressant(s) introduced into the host during feeding and

thus enhance resistance to infection with *B. burgdorferi* (Wikel *et al*., 1997). Tick immunity also interferes with the capacity of *B. burgdorferi*-infected *I. scapularis* ticks to transmit borrelial infection to guinea pigs. The reduction of duration of tick attachment and size of blood meal could decrease the opportunity for spirochetes to multiply and reach the salivary glands before detachment takes place. Spirochetes could also be directly affected by an immune process and die before reaching the salivary glands (Nazario *et al*., 1998)

Saliva-activated transmission (SAT) of pathogen is possible because of the effects of tick saliva on the host, rather that direct action on the pathogen during transmission (Nuttall, 2004). The effects are local, occurring at the skin site of tick feeding, and occur over a period of several days during the prolonged feeding of ixodid ticks. A correlation between pathogen, vector competence and SAT has been observed (Nuttall, 2004). The type of antiectoparasite immune response (Th1 or Th2) could potentiate the transmission of some pathogens (Ganapamo *et al*., 1997). A polarization towards Th2, with high levels of IL-4, can strongly down-regulate the Th1 cell mechanism responsible of intracellular microorganism destruction, and with this, create an optimal microenvironment for the *borrelia* to develop and disseminate. IL-10 as well as some glycosylated components of tick saliva could decrease some macrophage activities and prevent the destruction of infectious agents in the vicinity of their entry site (Ganapamo *et al*., 1997; Kopecky *et al*., 1999; Schoeler *et al*., 1999; Gillespie *et al*., 2000) Tick anti-complement activity may be a key factor in the maintenance of particular *Borrelia* genospecies within the tick population.

Two studies showed that salivary gland extract (SGE) from *I. ricinus* inhibit the killing of *B. afzelli* spirochetes by murine macrophages, reducing also the production of superoxide and nitric oxid phagocytes molecules and proinflammatory cytokines (Kuthejlová *et al*., 2001; Pechová *et al*., 2002). This suppression facilitates survival or even reproduction of spirochetes at the tick feeding site and its dissemination into the body. Another study with *B. burgdorferi* sensu stricto and *B. lusitaniae* showed that SGE enhanced spirochetes load and that there was a tick specific interaction between the tick species (*I. scapularis* and *I. ricinus*) and *Borrelia* (Zeidner *et al*., 2002; Nuttall, 2004). In epidermal cells of Lyme disease susceptible mice (C3H/HeN) stimulated with *B. afzelii*, SGE was shown to down regulate production of tumour necrosis factor alpha (TNF-alpha) and up-regulate Th2 cytokine, IL-4 (Pechova *et al*., 2004)

Another possible mechanism for survival of *B. burgdorferi* between different hosts is the variation in expression of outer surface proteins, namely OspA and OspC. Under culture conditions, OspC

expression is detectable in about 40% of strains, and OspA expression is detectable in more than 90% of strains. In contrast, the primary human immune response against *B. burgdorferi* is directed mostly against OspC and only rarely against OspA (Hu *et al*., 1996; Obonyo *et al*., 1999; Pal *et al*., 2000; Schwan & Piesman, 2000; Belperron & Bockenstedt, 2001; Fingerle *et al*., 2002). This is because *OspA* is the main protein expressed by the spirochete in the unfed tick, confined mainly to the luminal surfaces of gut cells (Silva & Fikrig, 1995; Hu *et al*., 1996; de Silva *et al*., 1998, 1999; Obonyo *et al*., 1999; Gilmore & Piesman, 2000; Gilmore *et al*., 2001; Pal *et al*., 2001; Piesman & Schneider, 2002; Schwan & Piesman, 2002; Fingerle *et al*., 2002). OspA mediates spirochete attachment to the tick gut by binding to an *I. scapularis* protein. The binding domains reside in the central region and COOH-terminus of OspA (Li *et al*., 1997). It also binds to itself, suggesting that spirochete-spirochete interactions may further facilitate adherence in the gut. Downregulation during the bloodmeal may also facilitate dissemination to the tick salivary glands and the host's dermis by preventing the bacteria from clumping to one another during transmission (Fingerle *et al*., 2000; Schwan & Piesman, 2000; Belperron & Bockenstedt, 2001; Piesman & Schneider, 2002; Pal *et al*., 2004).

OspC plays an important part during dissemination in the tick and/or early colonization of the vertebrate host and may be a determinant of infectivity for the mammal (de Silva *et al*., 1998, 1999; Obonyo *et al*., 1999; Gilmore & Piesman, 2000; Schwan & Piesman, 2000; Gilmore *et al*., 2001; Pal *et al*., 2001; Schwan & Piesman, 2002; Fingerle *et al*., 2002; Pal *et al*., 2004). Expression of OspC by *B. burgdorferi* s.l. in salivary glands of *I. ricinus* is up-regulated during blood meal after the ticks reach a critical body weight and during the 24 to 72 h of feeding. Down-regulation of OspA may occur at the same time, but in most cases it can persist and may even be present in the absence of OspC expression by *borrelia* resident in the midgut and salivary glands of nearly fully engorged nymphal ticks. According to the above authors, OspA- and OspC-negative *Borreliae* can be detected in both organs. A strain-specific dynamics of Osp expression and strain-specific kinetics (namely the time for invasion of salivary glands) of systemic infection in vector tick *I. ricinus* was observed (Fingerle *et al*., 2002).

Exposure to a combination of increased temperatures (from ambient temperature to more than 34ºC) and blood affects gene expression, remodelling the outer surface of the spirochetes (Obonyo *et al*., 1999; Fingerle *et al*., 2000; Schwan & Piesman, 2002; Tokarz *et al*., 2004). For instance, the synthesis of OspC *in vitro* is influenced by a change in temperature, spirochete density and growth phase and changes in environmental pH (Schwan & Piesman, 2000; Gilmore *et al*., 2001; Schwan & Piesman, 2002; Fingerle *et al*., 2002). Environmental factors also may influence pathogenicity of *B. burgdorferi* s.l. A study by Hu and others (1996) showed variation of expression of a 22/23 kDa protein (OspC) of a *B. garinii* isolate from *I.*

ricinus in vitro subcultures and in ticks, with reexpression after reexposure to its former environment in the tick.

OspA antibodies reduce the number of spirochetes within feeding ticks and interfere with the ability of spirochetes to induce OspC and invade the salivary glands of the vector (Silva *et al*., 1999). Additionally, nonborreliacidal OspA antibodies can inhibit *B. burgdorferi* s.l. attachment to the tick gut, highlighting the importance of OspA in spirochete-arthropod interactions *in vivo* (Pal *et al*., 2001)

4. RESERVOIRS AND HOSTS OF LYME BORRELIOSIS AGENTS

4.1 Definition

Reservoirs of Lyme disease agents are defined as host species whose individuals have an inherent ability to become infected, to perpetuate and to subsequently transmit the disease agent. A number of conditions have to be met: the host population should (1) be relatively abundant; (2) serve as host to large numbers of vectors, and (3) infect the vectors (Brown & Lane, 1992; Jaenson & Talleklint, 1992; Gustafson, 1994; Humair & Gern, 2000; EUCALB; Rosà *et al*., 2003).

For any single host to support endemic cycles of parasites carried by such vectors that feed only once per life stage, it must feed more than one stage in order to be considered as an amplifying host (Humair *et al*., 1993; Randolph & Storey, 1999; Richter *et al*., 2002). Many animal species serve as hosts for potential Lyme disease vector ticks, but in both Europe and in North America, relatively few species of small mammals appear to exhibit the combination of characteristics that enhance their potential as reservoirs of *B. burgdorferi* s.l (Jaenson & Talleklint, 1992).

The absolute and relative contributions of each species towards infecting tick populations in individual foci is variable in time and space and appears to be influenced by various intrinsic and extrinsic factors, such as species composition of the vertebrate host cenosis, tick/host attachment ratio, climate and quality of the immune response, and even heterogeneity among isolates of *B. burgdorferi* s.l. (Kurtenbach *et al*., 1994). For example, a study showed that testosterone levels in sexually active males (most actively involved in the transmission of *B. burgdorferi* s.l.) reduced both innate and acquired resistance to tick feeding. These effects generate heterogeneities within the rodent population with respect to tick distribution and microparasite transmission (Hughes & Randolph, 2000) and it is an important density-dependent regulatory factor in the horizontal transmission of this agent in nature (Kurtenbach *et al*., 1994, 1995; Dizij & Kurtenbach, 1995, Humair *et al*., 1999; Pawelczyk & Sinski, 2000).

B. burgdorferi s.l. strains that are associated with a particular host species are always resistant to the alternative pathway of complement of this host. In contrast, sera from hosts that are reservoir incompetent for a particular strain often lyse that strain, normally in the gut of the feeding tick (Hanincová *et al*., 2002). Different genospecies of the complex *B. burgdorferi* s.l. differ in their sensitivity to complement from different host species in a manner that reflects the ecological pattern observed (Lawrie *et al*., 1999; Kuthejlová *et al*., 2001; Kurtenbach *et al*., 1998; Lawrie *et al*., 2005).

In every habitat, an assemblage of different host species contributes to the maintenance of microparasite cycles, each species contributing differentially according to its transmission competence and the relative numbers of each feeding ticks stage (Randolph & Storey, 1999). Tick xenodiagnosis is the best way to assess the reservoir status of a vertebrate species (Humair & Gern, 2000). Besides competent reservoirs, reservoir incompetent vertebrate hosts appear to constitute an important component affecting the force of transmission (Richter *et al*., 2002; Humair & Gern, 2000; Rosà *et al*., 2003). These hosts may have either a positive effect, by amplifying infection in tick populations, or a negative ("dilution") effect, by wasting tick bites. A non-viraemic transmission between ticks co-feeding on the same host can also occur (Gilot *et al*., 1996; Odgen *et al*., 1997; Rosà *et al*., 2003). With non-viraemic transmission, the "dilution" effect becomes less relevant. In this type of transmission the effects of tick aggregation on the hosts and correlation of tick stages have an important effect on infection persistence (Rosà *et al*., 2003).

Larvae cause 90 to 99% of infected tick bites in mice and insectivores, due to the greater abundance of larvae in the biotope compared to the other stages and the high infestation on these small mammals, even with the low importance of transovarial transmission (Humair *et al*., 1993; Talleklint & Jaenson, 1994; Randolph *et al*., 1995; Kurtenbach *et al*., 1995; Randolph & Storey, 1999; Humair & Gern, 2000). Transstadial transmission is dependent on the infestation of rodents by nymphal *I. ricinus*, and this appears to vary geographically. Under dry climatic conditions during spring and summer, nymphs quest lower in the vegetation to avoid desiccation and consequently come into contact with small hosts (Humair *et al*., 1993; Randolph & Storey, 1999; Humair & Gern, 2000). Once humidity increases, the previously quiescent larvae, begin searching for hosts, thus contributing to the seasonal and geographical variation in disease transmission dynamics. As a general rule, the smaller the host's body size, the lower the proportion of ticks of later life stages feed upon it, with rodents feeding more proportionally larvae than nymphs (Randolph & Storey, 1999)

The presence of certain *Borrelia* species in particular hosts could be interpreted in several ways. In some cases, there is a real association as with *B. garinii*, *B. valaisiana* and *B. burgdorferi* s.s and birds (Humair, 2002). Alternatively, an apparent association may be a result of less significant phenomena in *B. burgdorferi* s.l. transmission such as transovarial transmission (Monin *et al*., 1989; Zhioua *et al*., 1994), co-feeding transmission (Gern & Rais, 1996; Odgen *et al*., 1997; Humair & Gern, 2000), or interrupted feeding followed by attachment to another host (Shih & Spielman, 1993).

4.2 Reported reservoir hosts

As already mentioned, each parasitic stage (larva, nymph and adult female, males do not engorge) feeds on a different host from a wide range of species for a period of a few days. Adults prefer larger animals, most often deer and livestock. Immatures ticks can parasitize almost any warm-blooded animal, such as insectivores, rodents, hares, cervids and some reptiles (Gray, 1991; Talleklint *et al*., 1993; Talleklint & Jaenson, 1994, 1995, 1997; Gustafson, 1994; Jaenson & Talleklint, 1996).

In the table presented in **Appendix 1** a brief description of each animal group will be performed, indicating their specific importance in Lyme Borreliosis sylvatic cycle and transmission of Lyme spirochetes.

5. ECO-EPIDEMIOLOGY, THE TRANSMISSION CYCLES

5.1 Silvatic transmission cycles

Two specific enzootic transmission cycles (rodent-tick and bird-tick) involved in maintaining different *B. burgdorferi* sensu lato species in nature are recognized (Nakao *et al*., 1994; Humair *et al*., 1995; Olsen *et al*., 1995, Kurtenbach *et al*., 1998b, 2002; Gern & Humair, 2000; Humair & Gern, 2000; Gray *et al*., 2000; Hanincová *et al*., 2003). *B. afzelii* and *B. burgdorferi* s.s. have been associated with both Muridae and Sciuridae in various ecological situations. *B. garinii* and *B. valaisiana* have been associated with certain avian hosts and associated ticks (*e.g. I. uriae*-seabirds) in particular ecologic situations (Humair *et al*., 1995; Bunikis *et al*., 1996; Gern & Humair, 2000; Gray *et al*., 2000; Kurtenbach *et al*., 2002; Hanincová *et al*., 2003) (**Fig. 1.18**).

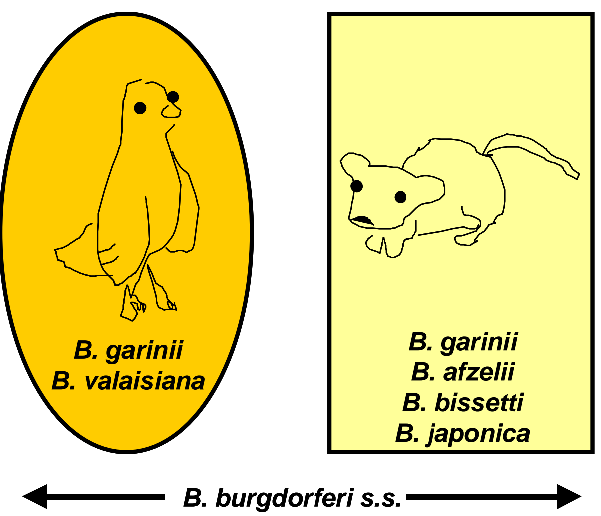


Figure 1. 18 – Schematic diagramme showing main host associations of some *B. burgdorferi* sensu lato genospecies (adapted from Kurtenbach *et al*., 2002)

As already mentioned, differences in serum complement sensitivity among *Borrelia* species could be a key factor in LB ecology, since there is evidence that the various host species do not transmit all *B. burgdorferi* sensu lato strains to ticks with equal efficiency (Kurtenbach *et al*., 1998, 1998b, 2002; Humair & Gern, 2000). With *B. afzelii*, transmission might not be sustained if a larval tick aquired these spirochetes from a mouse and attached subsequently as a nymph to a bird. If *B. garinii*, on the other hand, thrived mainly in birds, a corresponding diversion to a rodent would similarly result in transmission failure. Efficient perpetuation of rodent-borne *B. afzelii* as well as bird-borne *B. garinii* by the same subadult *I. ricinus* vector ticks, therefore, would seem paradoxical (Richter *et al*., 1999; Gern & Humair, 2000) However, in several studies different genospecies (*B. garinii, B. afzelii* and *B. burgdorferi* s.s.) share the same reservoir hosts (Richter *et al*., 2000a; Huegli *et al*., 2002). No confirmatory experimental proof yet demonstrates an eespecially intense association of *B. afzelii* with rodents and *B. garinii* or *B. valaisiana* with birds (Richter *et al*., 2000a). Because of the above studies, Hanincová and others (2003) suggested a third ecotype, besides the rodent-associated and bird-associated ecotypes: an ecotype that thrives in both rodent and avian hosts.

A trans-stadial transmission of *B. burgdorferi* sensu lato in *I. ricinus* has been confirmed (larvae to nymphs 21.25%; nymphs to adults 33.65%) although an important loss of spirochetes is observed

especially during the passage from the larval stage to the nymphal stage. Trans-ovarial transmission is another mode of transmission (eggs to larvae 3.2%) (Monin *et al.*, 1989; Humair & Gern, 2000) (**Fig. 1.19**).

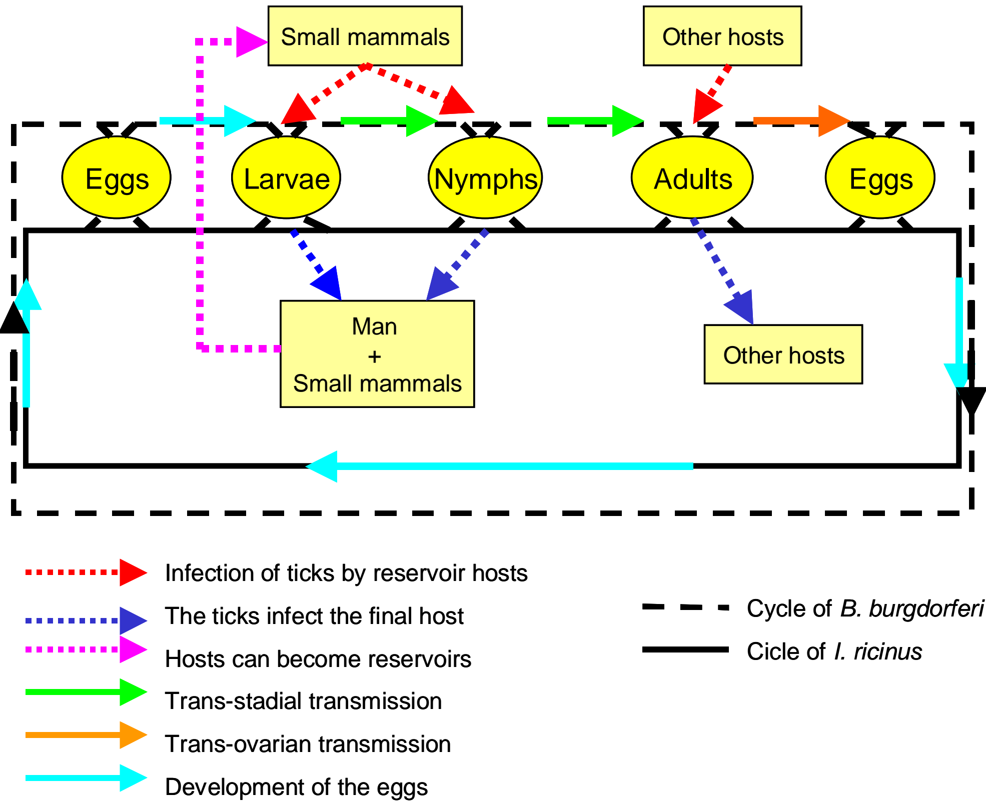


Figure 1. 19 — Transmission sylvatic cycles of *B. burgdorferi* s.l. among *I. ricinus* ticks and hosts (adapted from Monin *et al*, 1989)

In co-feeding transmission, ticks may acquire infection from a localized site on a host at which they feed simultaneously with infected ticks and may continue to do so after the infected ticks have dropped off (Randolph *et al.*, 1996). This co-feeding transmission increases with the duration of attachment of the infecting tick. Regarding larvae and nymphs, this co-feeding window is open briefly during the period of 2 days of attachment for larvae and 3 days for nymphs of *I. ricinus*. Co-feeding transmission becomes less efficient as distance from the infecting tick increases. The critical distance, that is, the distance which spirochetes can migrate between feeding ticks by way of host skin, is <1cm (Sato & Nakao, 1997; Richter *et al.*, 2002). The probability that a nymph acquires *Borrelia* infection from a co-feeding infected adult increases with the density of feeding ticks because the efficiency of co-feeding depends on the temporal and spatial proximity with which infected adult and uninfected immature ticks feed on hosts (Odgen *et al.*, 2002). The co-feeding method is also a very sensitive technique that can be used to detect very early infection in mice used in xenodiagnosis (Hua *et al.*, 2003).

In *B. burgdorferi* s.l. transmission, the non-systemic pathway (co-feeding) apparently contributes considerably less than does the systemic pathway, largely because of the much more limited duration of infectivity thought to arise from an original infected tick bite, unless sequestration and repeated transmission occur (Randolph *et al.*, 1996). On the other hand, addition of a range of new vertebrate species that do not develop systemic infection but can be a host for tick and a place for co-feeding transmission is important but, as Gray (2002) suggests, the fact that there are many animals in typical LB habitats that can be infected systemically by this pathogen, co-feeding probably has limited significance in its eco-epidemiology.

No penetration of intact skin has been observed for *B. burgdorferi* s.l., as it ocurs in relapsing fever spirochetes (Crippa *et al.*, 2002). Regurgitation in ticks, alone or with salivation is also a mechanism for transmission (Gustafson, 1994)

The risk of acquiring infection by the agents of Lyme disease following a bite by an infected tick has been associated with the duration of attachment of the infected tick (*e.g.* transmission of *B. burgdorferi* s..l from *Ixodes* spp to mice increases with duration of tick attachment) and the efficiency of transmission of the different strains of *B. burgdorferi* s.l. by vector ticks (Yeh *et al.*, 1995; Crippa *et al.*, 2002).

LB associated-spirochetes are rapidly acquired by nymphal ticks during the first 24 h of attachment, even before the tick ingests any significant amounts of blood (Schwan & Piesman, 2000; Hodzic *et al.*, 2002). Partially fed nymphal ticks (detached from their hosts by grooming or host-derived antitick immunity) transmit spirochetal infection more rapidly than do ticks that have never been attached to a host (as early as 8 h post attachment) (Shih & Spielman, 1993; Shih & Liu, 1998; Wang *et al.*, 1999). The span of spirochetal transmission may be accelerated by the active dispersal of spirochetes to the tick salivary glands during the previous attachment of infected nymphal ticks (Shih & Liu, 1998). In the case of *B. afzelii*, associated with dermatological manifestations, these spirochetes may disseminate rapidly from the site of tick attachment (Crippa *et al.*, 2002).

For an effective transmission, an estimated minimum of 300 organisms may be required in a host-seeking nymphal tick to be able to transmit infection to mice while feeding on mice (Wang *et al.*, 2003). A study with qPCR showed variation in the number of spirochetes in individual ticks from 25 to 197,200

with a mean of 1,964 spirochetes per nymphal tick and a mean of 5,351 spirochetes per adult tick (Wang *et al*., 2003).

Ticks feeding at higher density acquired spirochetes more efficiently than those feeding at lower density (Levin *et al*., 1997; Ogden *et al*., 1998). Modulation of host immune competence by the ticks facilitating the growth of the pathogen or attraction of spirochetes to the feeding site by factors in tick saliva can contribute to a higher proportion of ticks acquiring or transmiting the pathogen when at high density, *via* co-feeding (Levin *et al*., 1997; Sato & Nakao, 1997; Ogden *et al*., 1998, 2002).

5.2 Tick-developmental cycle

The length of the period defined as the developmental cycle has many definitions. When comparing data from different geographic regions it is necessary to consider which definition was used by the respective author (Cerny *et al*., 1974). For instance, it could be the period from adult to adult stage; from ovipositing female to developing eggs of the next generation, from the engorged female to the engorged female of the next generation, from the time when the engorged female leave the host to that when the unfed adults of the next generation quest for hosts or the period from unfed adult to unfed adult of the next generation (Alfeev, 1974; Pomerantsev, 1950; Serdyukova, 1960; Kheisin 1955; Uspenskaya, 1969 *cited in* Cerny *et al*., 1974).

The length the developmental cycle is determined not only by macroclimatic and biotic factors, but also microclimate. These factors also influence the duration of the pre-oviposition period, developmental period of eggs and pre-hatch period (Cerny *et al*., 1974). Predictions of the timing of emergence of unfed ticks from the previously engorged stage, and thereby their potential recruitment into the questing population, depend upon knowing the duration of interstadial periods under field conditions (Randolph *et al*., 2002).

The completion of the tick cycle requires not only optimal abiotic but also biotic conditions, which means a sufficient number of suitable hosts ensuring tick-host contact as early as possible. Microenvironment factors ensuring higher air temperatures near ground, while maintaining relatively high humidities play the most important role affecting tick development and survival. On the other hand increased heat accompanied by an acute decrease in humidity leads to desication of ticks. These facts are important for the prognosis of tick occurrence and of the degree of tick infestation in the biotopes. Seasonal and regional variations in microclimatic conditions can result in the life cycle spanning up to six years (Daniel *et al*., 1977; Gardiner & Gettinby, 1983; Gray, 1991; Gustafson, 1994; Vassalo *et al*., 2000a).

In Britain, tick activity in each developmental stage does not start until at least the month following completion of development. After activity is initiated, ticks can survive for approximately one month (in Britain) during which they alternate periods of activity (2 to 3 days) and inactivity (5 days). During activity (**Fig. 1.20**) it is assumed a tick has a ca. 50% probability of contacting a host provided daily temperature exceeds an activity threshold of 7ºC. Once attachment has occurred, the period of engorgement is assumed to be three, five and eight days for larvae, nymphs and adult females, respectively (MacLeod, 1932 *cited in* Gardiner & Gettinby, 1983). After the final blood meal (7-9 days), the adult female drops off her host and falls to the ground. In the subsequent month she deposits the eggs in one or several ovipositions concluding with her death. The eggs, coated with a waxy secretion, have an oval shape and are visible to the naked eye. The incubation period of the eggs varies considerably, depending on the ambient temperature (Gustafson, 1994; Mejlon, 2000; Gray, 2002). The total egg output is estimated as 2000 and 1000 for spring- and autumn-fed females (**Fig. 1.21**), respectively, though these figures may vary depending on the size of the blood meal consumed by the female and depending on the nature of tick habitat (Gray, 1981; Gustafson, 1994). Among exophilic ticks, those living in dense forests encounter more stable and favourable environmental conditions than those inhabiting more open areas. In order to compensate for the higher mortality, tick species living in open areas must produce more eggs as compared with forest ticks (> 20000 eggs/female) to ensure successful reproduction (Uspensky *et al*., 1999).

After hatching from the egg, the 6-legged larva, measuring 0.5 – 1.0 mm, takes its first blood-meal and then moults to an 8-legged nymph and after a second blood meal develops into an adult (imago). An adult female is 3 – 4 mm in size, while a male tick is 2.5 mm long. A male can fertilize several females but dies shortly afterwards (Gustafson, 1994; Mejlon, 2000; Gray, 2002). To complete its life cycle a tick must survive the overwintering, development, activity and engorgement periods. Survival is greatly affected by the timing of these events. For instance, the probabilities of larvae surviving development when engorgement takes place in the months of April, May and June are estimated to be 0.7, 0.81 and 0.93, respectively (Gardiner & Gettinby, 1983).

30d
Oviposition period
2-36w
Eggs hatch
2-6d
Larvae feed
Larvae moult
4-51w
7-22d
Preoviposition period
Nymphs feed
3-7d
Females engorge
5-14d
Nymphs moult
8-28w

Figure 1. 20 – Development times for *I. ricinus* (adapted from Gray, 1991, Parola & Raoult, 2001)

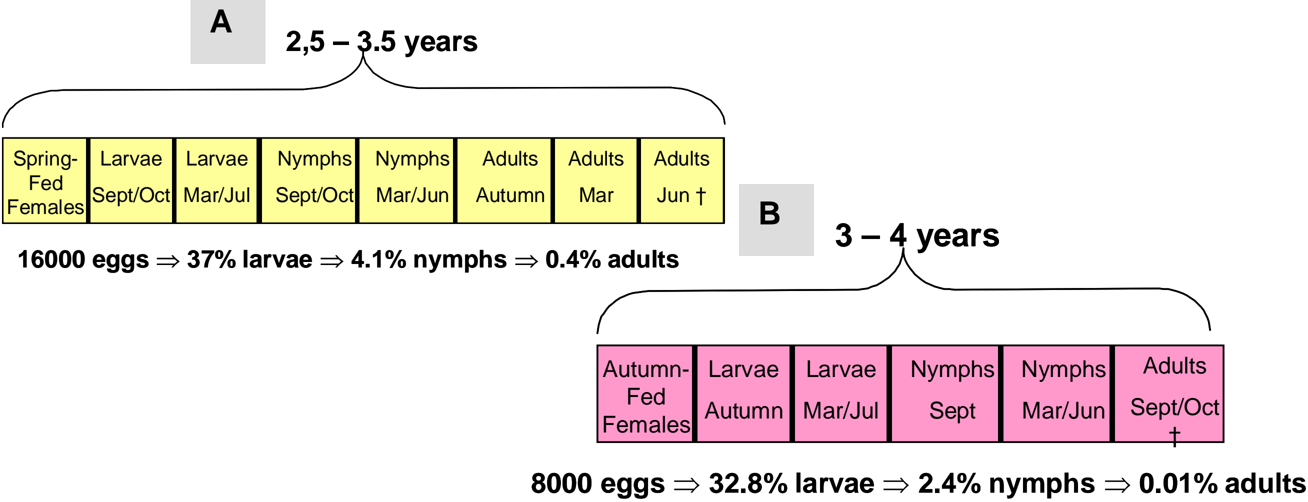


Figure 1. 21 —Predictions for timing of activity of ticks derived from spring (A)- and autumn-fed (B) females, with estimated survival rates at each stage (Gardiner & Gettinby, 1983).

Only a small proportion of eggs succeed in completing the whole lifecycle and reaching the adult stage (**Fig. 1.22**). In *I. ricinus*, a full year may separate the active feeding periods of successive instars (Gray, 2002).

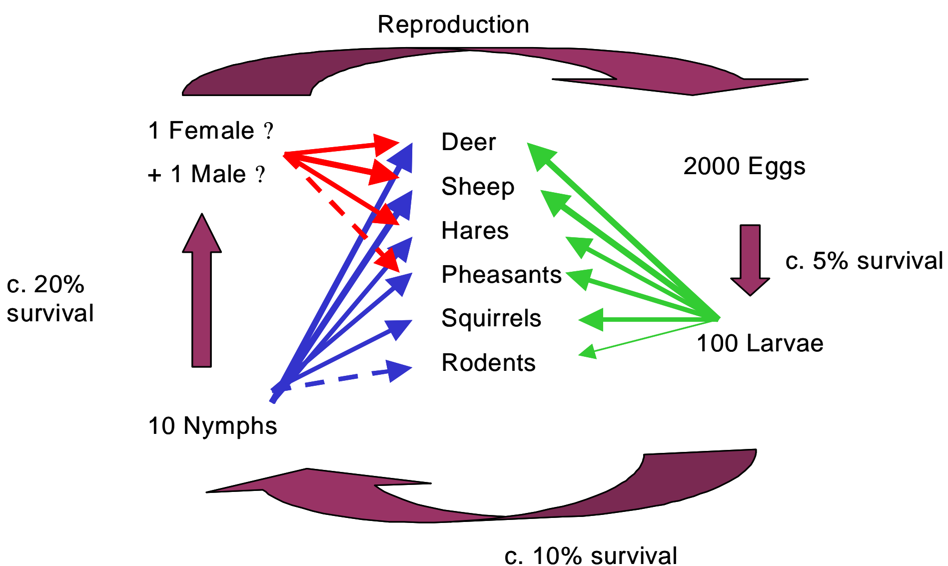


Figure 1. 22 - Life cycle of *I. ricinus* with host relations and approximate reproduction and mortality rates. Dashed or light coloured lines means light use of host (adapted from Randolph *et al.*, 1995, 1998)

5.3 Environmental determinants

Microclimate affects behavioural activity of various *Ixodes* species (Uspensky, 2002). For instance, a study with *I. scapularis* nymphs showed that temperature and humidity accounted for 51% of the variance in seasonally adjusted drag samples. According to the authors, other unmeasured variables such as solar radiation, soil temperature or other environmental characteristics may contribute to the unexplained variation (49%) (Vail & Smith, 1998). Another study, the mean daily survival rates of free *I. scapularis* were negatively related to air temperature, vapor pressure deficit and the coefficient of variation of relative humidity (Bertrand & Wilson, 1996)

Different geographic populations show different patterns of activity. Furthermore, adult females ticks are more active than other stages, nymphs being more active than larvae (Alekseev *et al.*, 2000). Temperature thresholds for the onset of activity varies with the size of the tick, with nymphs and adults appearing before larvae, and larger nymphs and adults appearing before smaller individuals. For temperate regions, it was shown that the larger the body size of a tick (several species), the lower the uncoordinated activity threshold (temperature below which a tick can no longer seek a host in a coordinated manner) and activity threshold temperatures (temperature at which all activity ceases) (Clark, 1995).

Once recruitment ceases, tick numbers fall, with the largest nymphs and adults disappearing first. This probably reflects not only their earlier arrival, but also their relatively higher energy levels permitting

them to quest more continuously, thus permiting them to find hosts more rapidly. Smaller ticks (larvae and small nymphs and adults) persist longer through the summer, partly because they become active later, and also because they are more likely to be forced into temporary inactivity by high moisture stress during the summer and when this occurs it takes them longer to find hosts (Randolph *et al.*, 2002).

Different stages of *Haemaphsalis* and eespecially *Ixodes* species (both forest ticks) develop better under high humidity conditions whereas species of *Dermacentor*, *Rhipicephalus* and *Hyalomma* genera (the first two have some features of forest ticks, but are close to the ticks of open country and the latter is a tick of open country) are more tolerant to low humidity (Uspensky, 2002). This toleration of dessication is eespecially important for egg survival since this is the only stage in tick metamorphosis that is unable to seek more favorable conditions.

5.3.1 Habitats

Tick populations are classically considered as large homogeneous local populations at equilibrium, however high variability in tick abundance is observed when comparing relatively close habitats (Gray *et al.*, 1998 *cited in* Estrada-Pena, 2003).

Tick relationships with landscape structure are influenced by the differential spatial scales at which hosts of ticks (rodents, birds, medium-sized mammals or wild ungulates) interact with the distribution of resources, even within the same landscape. Patterns of tick colonization are also be influenced by the spatial distribution of suitable habitat. Heterogeneity in habitat quality, differences in patch size and shape, and variation in the degree of isolation of patches lead to spatial variation in connectivity of tick landscapes. Recolonization of a local population of ticks depends on the number of potential immigrats and the degree of patch isolation (Estrada-Pena, 2003; Ginsberg *et al.*, 2004).

Ticks survived longer in edge and forest habitats than in open fields, which are characterized by greater extremes in air temperature, soil temperature, relative humidity, and vapor pressure deficit than the other two habitats. *I. ricinus* is known to be a hygrophilic tick (Daniel *et al.*, 1977; Gray, 1991; EUCALB). Plant communities with humid conditions throughout the year appear to provide favorable microhabitats for this tick species, supporting high survival rates and providing ideal sites for oviposition (**Fig. 1.23**).

Figure 1. 23 – Types of habitat occupied by *Ixodes ricinus* ticks (EUCALB, 2004)

Well-expressed litter layers and humus strata that buffer effects of variable weather conditions most likely serve as favorable microhabitats of *I. ricinus* (Kurtenbach *et al*., 1995, EUCALB). But these ticks are also able to complete their developmental cycle in open grassy areas, where development proceeds more quickly than in the forested habitats but with considerably higher losses, eespecially in the months of June and July (Daniel *et al.,* 1977; Gray, 1991; EUCALB). Warmer environmental conditions resulting from higher solar insolation in grasslands appear to foster the shorter developmental cycle (Cerny *et al*., 1974; Gray, 1982, 1991).

5.3.2 Questing behaviour

In order to explain the timing and size of the peaks of tick activity it is necessary to obtain data on the rates of development and subsequent activity of ticks deposited in the field at various times of years (Gray, 1982; Clark, 1995; Korenberg, 2000). For instance, during most of the active tick, the population is comprised of different ages that die at different rates, leading to a gradual decrease in the abundance of ticks and their natural disappearance from landscapes (Korenberg, 2000). Behaviourally, ticks avoid questing during unfavorable times of the year such as a midsummer, when temperatures are high and air humidity is low, and in winter when temperatures are too low (Gray, 1991).

Physiological age at which females start questing is dependent on the time of their activation. Ticks that start questing activity at the beginning of the season and possess considerable nutritional reserves, live longer than individuals that become active latter in the season when they are getting emaciated In an unfed tick, the maximal duration of the active life period before finding a hostdepends on its calendar age and the physiological age at the moment of activation (Uspensky, 1995; Korenberg, 2000; Walker, 2001). Feeding success and the level of sexual pheromones are not affected by female aging (Korenberg, 2000). On the other hand, older females require higher humidities to survive, have a weaker

induction of photoperiod and maintenance of diapause, and quest less (Uspensky, 1995; Van Es *et al*., 1999). Fat is a non-renewable source of energy derived from each blood meal and can be used as a marker of physiological ageing in the field. After emergence, fat is used to fuel the tick's locomotory and physiological activity. Its natural rate of usage varies with seasonal activity and climatic conditions, allowing a distinction between the calendar age and physiological age of ticks (Randolph & Storey, 1999; Randolph *et al*., 2002).

Behavioural factors that affect the manner in which ticks come in contact with a host largely determine host specificity. Indirectly, the time of day at which an engorged tick separates from a host would also contributes to host specificity. This time of detachment appears to depend more on properties of the host than on the periodicity of tick behaviour (Matuschka *et al*., 1990). *I. ricinus* on rodents tend to detach late in the afternoon; those on hedgehog detach around midnight and those on lizards and birds, during the morning. Ticks on carnivores (dog, cat) detach throughout the day light hours. On edible dormice, *I. ricinus* become replete and detach from edible dormice during late afternoon when dormice are at rest in their nests (Matuschka *et al*., 1994).

Nymphs present a random moderately aggregated distribution in the field. Since the lateral displacement of the ticks is highly limited in the field, nymphs and adults moult or oviposit in places where they land after dropping from the host (Nilsson & Lindquist, 1978; Nilsson, 1988; Vassalo *et al.* 2000). Therefore, the distribution of individuals in these lifestages largely mimics that of their hosts. Larvae present an aggregated distribution, probably caused by hatching of clumps of eggs from individual female ticks and scarce lateral movements of larvae (Humair *et al*., 1993; Mannelli *et al*., 2003) Distribution of these aggregates is dependent on the site of oviposition. The movements of the larvae are more or less vertical and there is a tendency for the larvae to gather (Nilsson & Lundquist, 1978; Nilsson, 1988). If a small mammal passes through such an aggregate, it is very likely to be infested, although the number of ticks climbing onto a host is species independent, but degree of infestation after 4 hours seems to be species dependent.

The vertical distribution of questing *I. ricinus* is influenced by many factors including the height and other physical properties of the vegetation, humidity and temperature. Desiccation tolerance increases with tick age (stage), therefore, larvae tend to quest lower in the vegetation than nymphs or adults, where each life stage contacts with specific hosts (Mejlon & Jaenson, 1997; Randolph & Storey, 1999). In a study by Mejlon and Jaenson (1997) significant correlations were found between the vertical distribution of immature ticks and the height of vegetation. Larval numbers were greatest close to the ground (0-29

cm) in both high and low vegetation, whereas in high vegetation, the greatest mean numbers of nymphal and adult ticks were observed at height intervals of 50-59 cm and 60-79 cm, respectively. These ranges are within the estimated height range (40-100 cm) for the main body surface of roe deer (*Cervus capreolus*), their "preferred" host (Kahl, 1996; Mejlon & Jaenson, 1997; Randolph & Storey, 1999; Mejlon, 2000).

Tick strategy of host-seeking can be passive (ambush) or active (hunter), depending on the presence of chemoreceptors or eyes (Uspensky, 2002), respectively, though a mixed strategy is utilized rather often. When a potential host approaches a tick, the parasite waves its forelegs (bearing clusters of sensilla and sensory setae, the Haller's organ) to facilitate contact and cling on to the animal (Kahl, 1996; Krober & Guerin, 1999; Osterkamp *et al*., 1999). *I. ricinus* is able to locate a vertebrate from a certain distance (at least 1 m) (Kahl, 1996). This species and other from the same genus have epithelial photosensitive cells (all stages) that equip them for efficient life in forest environments, eespecially for recognizing hosts at large distances (Uspensky, 2002; Perret *et al*., 2003).

Stimulii such as host odours (*e.g*. kairomones), radiant heat, vibrations caused by walking animals and shadows might facilitate location of hosts. Host-specificity of ticks may be achieved by specific sensory stimuli which are perceived before attachment (Kahl, 1996; Carroll, 1999; Krober & Guerin, 1999; Osterkamp *et al*., 1999). For instance, the use of kairomone cues to aid in acquiring suitable hosts is of adaptive value to ticks as it increases the likelihood of survival to reproduction successfull (Carroll, 1999; 2002).

For regulating attachment and mating behaviour, several types of pheromones are produced and released by ixodid ticks, such as assembly pheromones, aggregation-attachment pheromones and sexual pheromones (Rechav *et al*., 2000; Uspensky, 2002; Zemek *et al*., 2002). Feeding status of *I. ricinus* females affects their sexual attractiveness to males. Mated engorged females are highly attractive to males at a distance but males copulate more with unengorged or semiengorged females, probably because these females produce higher amounts of the genital sex pheromone (Zemek *et al*., 2002).

In many habitats, most *I. ricinus* females have mated by the time they locate a host, eespecially when these ticks are more numerous (Gray, 1987). Various *Ixodes* ticks mate preprandially, before they have attained host-contact, as well as perprandially, after they have begun to feed. Males (smaller than females and with shorter life span) complete spermatogenesis, while females become receptive to insemination within a few days after the nymphal-adult molt. Thereafter mating occurs as soon as appropriate contact is attained. Males, which either feed several times within a short period of time or

not feed at all, can mate only a few times and inseminate very few females, with the probability of male death increasing with each copulation (Kiszewski & Spielman, 1999; Uspensky, 2002). Open habitat ticks, such as *Dermacentor, Rhipicephalus* and *Hyalomma*, when unfed are unable to mate. Males, with bodies larger or similar in size to females and longer life, initiate contact with females after receiving small blood meals whereas females can be inseminated only after becoming partially engorged. After insemination, females gorge blood to complete repletion. The prolonged stay of males on the host makes possible insemination of a large number of females and may explain their presence on hosts in the temperate zone during wintertime (Uspensky, 2002). Frequency of intersexual encounters correlates linearly with tick density, and local tick abundance can be estimated simply from the number of days elapsed from the onset of adult activity and the prevalence of inseminated females (Kiszewski & Spielman, 1999).

Subadult *I. ricinus* probably concentrate in the host's nest when feeding on mammals but are scattered over the ground when feeding on lizard or avian hosts. The time of larval drop-off determines the place at which larvae detach from their hosts (with sufficient humidity) and, hence, the point of host contact for the resulting nymphs, affecting for instance, its capacity as a vector of this and other infections (Matuschka *et al*., 1990; 1991).

The orientation and movements of ticks in hosts differs according to tick species. On *A. sylvaticus, I. ricinus* ticks moves faster towards the head than on *C. glareolus*. Ticks move from the place of attachment on the rodent host to the areas where they settle. This movement is against the direction of the host's fur and results in an accumulation of ticks on the head. Rapid movement of ticks reduces the risk of their being scratched off by the host or brushed off by the vegetation (Nilsson & Lindquist, 1978). In deer, *I. ricinus* is found mainly at the lateral sides of the feet, but can also be found in other areas of the body (hears, perineum) (Gilot *et al*., 1994). *D. variabilis* favor the head and neck of human hosts. *A. americanum* favors the lower extremities, buttocks and groin. *I. scapularis* has widely distributed attachment sites with no apparent site preference (Felz & Durden, 1999)

5.3.2.1 - Behavioural aspects in infected ticks

In a study by Alekseev and Dubinina (2000), a significant relationship was found between the activity of *Borrelia*-infected *I. persulcatus* and the air temperature, soil temperature below the surface an at the soil surface, as well as relative humidity. Greater numbers of infected nymphs were collected when temperatures ranged from 10 to 14 ºC and lower numbers were collected between 15 and 20ºC. No

infected nymphs were collected at temperatures >26ºC. Infected adults appeared to be more tolerant of higher temperatures than nymphs (Alekseev & Dubinina, 2000).

The behavioural and ecological effects of infection by *B. burgdorferi* s.l. in nymphal and adult *I. scapularis* ticks were also studied by Lefcort & Durden (1996). The effects of infection were more pronounced in adults. Compared to uninfected adults, infected adults were less able to overcome physical obstacles, avoided vertical surfaces, were less active and quested at lower heights. These could result, on one hand, in a decrease of contact with potential hosts and with potential mates, but on the other hand, can help ticks to husband resources and decrease the tick's exposure to predators and dessication, ensuring longetivity of adult tick. Infected nymphs showed increased phototaxis and attraction to vertical surfaces and some tendency for increased questing height and overcoming of physical obstacles. These behaviours could result in dessication, but increases the rate of contact between the tick and hosts.

The bacteria in infected ticks need to ensure that the tick is able to survive and find an appropriate host to continue transmission. These altered behaviours may affect survival or pathogen transmission and may reflect kin selection in the bacterial pathogen (Lefcort & Durden, 1996). A longitudinal study showed no differences in either fecundity or length of survival between *B. burgdorferi* s.s.-infected and uninfected *I. scapularis* (Burkot *et al*., 2004).

Another study with *I. ricinus* showed that female ticks are more often infected by *B. burgdorferi* s.s. than males and thus transmit this agent more often. Dispersal capabilities, especially of males (the most vagile sex), are diminished by *B. afzelii* infection, modifiying tick population genetics and promoting specificity towards small rodents, the main reservoir of this spirochete, instead of the not so competent larger hosts, representing an adaptive host manipulation (Meeus *et al*., 2004). It is probable that infected and uninfected immature ticks show different behaviours and that the different genospecies of *B. burgdorferi* are vectored by *I. ricinus* in different ways, as a function of sex of the ticks and of the genospecies of the *Borrelia* (Meeus *et al*., 2004). Locomotor activity of both adult and immature *I. ricinus* that were infected with *B. burgdorferi* sensu lato was decreased compared with uninfected specimens (Alekseev *et al*., 2000). Interestingly, *I. persulcatus* females with exoskeleton anomalies infected by borreliae could crawl faster on humans and reach uncovered parts of the body vulnerable for attachment and feeding quicker than normal females (Alekseev *et al*., 2000)

5.4 Seasonal dynamics of *Ixodes ricinus*

This part of the chapter will be restricted to *I. ricinus*, the main vector of Lyme Borreliosis in Europe. *I. ricinus* is a tick of temperate regions in Europe (**Fig. 1.24**), being mainly found from approximately 10ºW (Ireland) to 45ºE (Ural mountains, Russia) and from 60ºN (Sweden) to 30ºS (Egypt). The geographic distribution of *I. ricinus* covers southern Scandinavia, the British Isles, central Europe, France, Spain, Portugal, Italy, the Balkans, eastern Europe and North Africa (Stanek *et al.*, 1988; Gray, 1991; Estrada-Pena, 2001; Randolph *et al.*, 2002)

The southern limit of its continuous distribution in Europe is a transition zone between the cold and wet western deciduous forests and the warm and dry eastern areas closer to the Mediterranean Sea. Because of the human pressure, the original habitat composed mainly of *Quercus* spp and *Pinus* spp has become deeply fragmented, creating a mosaic of patchy environments where habitat suitability changes drastically across small distances (Estrada-Pena, 2001).

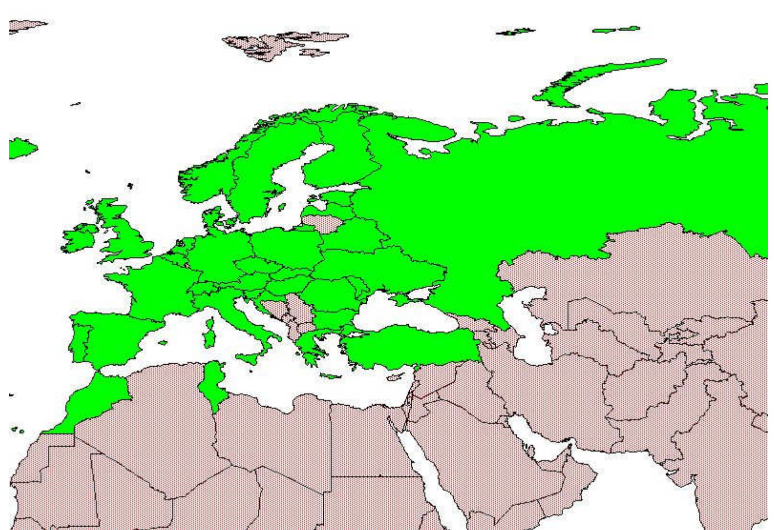


Figure 1. 24 – Distribution of *I. ricinus* in EuroAsia and Northern Africa (in green colour), based on the literature

The life cycles of *I. ricinus* complex ticks from across their range are generaly similar. Larvae and nymphs who feed in the season in which they emerge generally develop into nymphs and adults respectively in the next season. If they do not feed in their first season (summer), most die off but some (eespecially nymphs) can survive through winter and feed in the following year; they mate, with the male dying shortly after mating and the female remaining longer on the host. Afterwards, the female drops off the host and deposits about 3000 eggs. The hatching of larvae takes several weeks (5-19 weeks) starting in summer (Gosh & Pugliese, 2004)

As already reported *I. ricinus* activity is highly dependent on the mean and ground temperatures. Activity was recorded in a temperature range of 0-35ºC (Hostis *et al*., 1995). When the temperature reaches 24ºC, immature ticks demonstrate a positive geotropism (Perret *et al*., 2000). Questing behaviour of *I. ricinus* nymphs and density of questing ticks (about 4 months at its maximum) are dependent on temperature and relative humidity (saturation deficit). Abrupt declines in the density of questing ticks have been shown to coincide with abrupt increases in saturation deficit at field sites (Randolph & Storey, 1999; Perret *et al*., 2000; Randolph *et al*., 2002; Perret *et al*., 2003).

I. ricinus is freeze-susceptible as well as highly susceptible to inoculative freezing. Furthermore, engorged *I. ricinus* larvae acclimatized for 10 days at 3ºC did not reduce cold-related mortality (Dautel & Knulle, 1997). Because of this, ticks overwinter in leaf litter and the uppermost (≤5 cm) soil layer (Dautel & Knulle, 1997, Gustafson, 1994). Soil maximum and minimum temperatures measured at a depth of 5 cm explain much of the variation in development (Gardiner & Gettinby, 1983). In Russia, the gradient between soil at a depth of 1-1.5 cm and soil surface temperatures appeared to be the main physical parameter that initiated *I. persulcatus* migration out of the leaf litter. For instance, 60% of the ticks, eespecially nymphs, were collected when the soil surface and subsoil temperature difference ranged from 0 to 2.0 ºC. The gradient between soil temperature and relative humidity appeared to increase the importance of the above gradient at the time the maximum number of ticks were caught (Alekseev & Dubinina, 2000). For *I. ricinus*, influence of the depth in the soil during hibernation on survival of different stages showed that for larvae a depth of –30 cm was the most favourable and that of –20 cm was the least favourable. In engorged females of the highest overwintering survival was observed for ticks on the surface. No affect of hibernation depthon survival was observed for engorged nymphs (Daniel *et al*., 1977).

Probably the most critical periods for the survival of adults are both the summer months and those months with temperatures less than –10ºC when the highest tick mortality occurs (Daniel *et al*., 1977; Gray, 1981)

There are several patterns to the seasonal dynamics for *I. ricinus*, even within the same country (**Appendix 2**). This seasonality is defined as the capacity of the tick to regulate its activity to deal with the adverse effects of its environment. Behaviour of *I. ricinus* is complex and therefore studies based on limited numbers of seasonally distributed samples must be evaluated with caution (Jensen & Kaufmann, 2003). The questing tick population is the product of the balance between the rates of

recruitment and loss, either one of which may vary depending on seasonal variation in rates of tick development, survival, death and host-contact (host availability) (Craine *et al.*, 1995; Gray, 2002).

Classically *I. ricinus* are described as having a bimodal pattern, with each life cycle showing a major peak in the spring, followed by a distinct, but minor peak in the autumn (Gray, 1991; Randolph *et al.*, 1995, 2002; Gray, 2002). In mild, sheltered habitats, there is a single, prolonged period of tick activity throughout the year from early spring to late autumn, as long as temperatures are sufficient for seeking hosts in a coordinated manner (Clark, 1995; Craine *et al.*, 1995; Korenberg, 2000). On the other hand, in cold and more exposed conditions, this unique peak starts later (early summer) and finishes earlier (early autumn) (Gray, 1991; Randolph *et al.*, 1995; Korenberg, 2000) (**Fig. 1.25**). In the Mediterranean countries, virtually nothing is known about the seasonality pattern of this tick species, except for a recent study by Estrada-Pena and others (2004a).

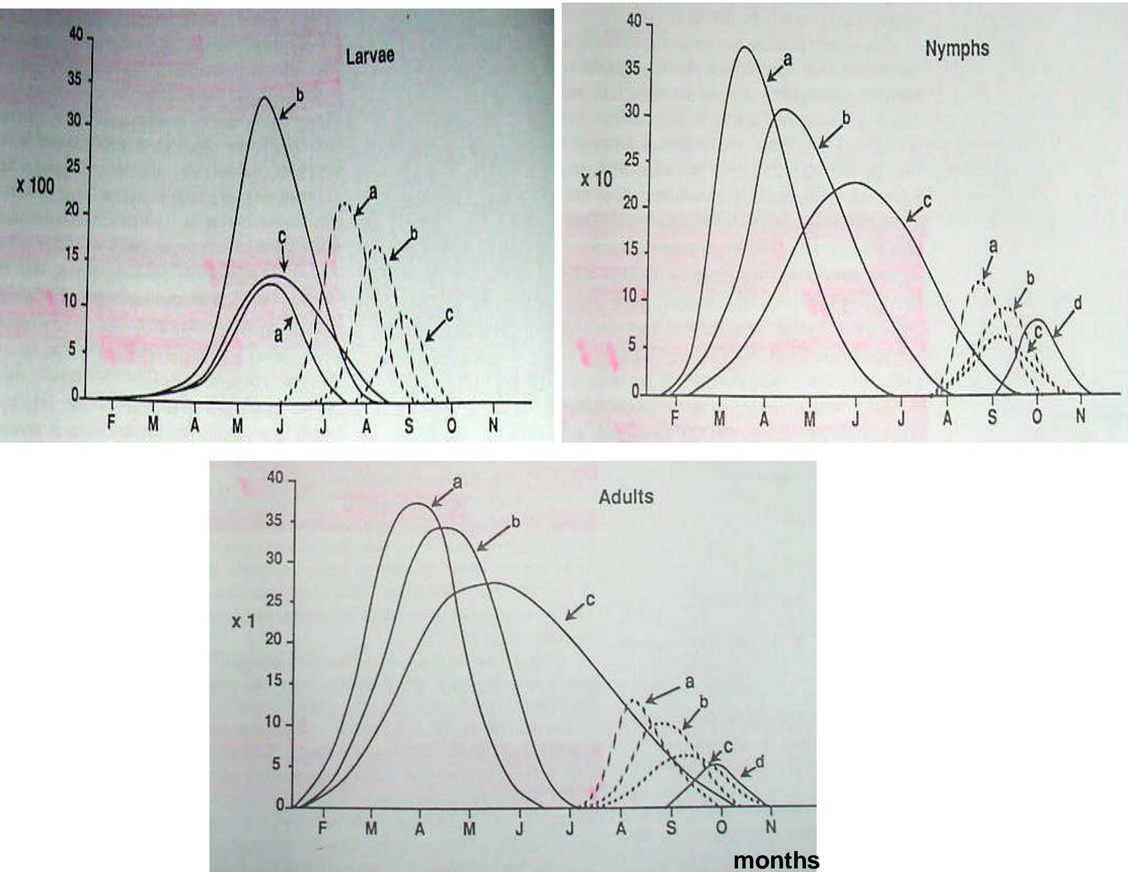


Figure 1. 25 – Different patterns of seasonal activity of *I. ricinus* developmetal stages in various habitats (adapted from Gray, 1991; EUCALB; Korenberg, 2000); (a) exposed meadow, (b) dense hill vegetation, secondary deciduous woodland, (c) highly sheltered, (d) spring-derived but autumn-feeding, (solid line) spring population, (dashed line) autumn population

Besides host densities, vegetation and climate, diapause phenomena are one of the strategies most important for regulating *I. ricinus* seasonal activity. This behaviour is defined as a neurohormonally mediated dynamic state of low metabolic activity, during unfavourable conditions, which can manifest as delayed morphogenesis, increased resistance to environmental extremes and altered or reduced behavioural activity (Gray, 1991, 2002; Walker, 2001; Belozerov *et al.*, 2002). Diapause can be responsible for delay, until the following spring, on development of eggs laid by autumn-engorged females and for the development of autumn-engorged larvae and nymphs (Gardiner & Gettinby, 1983; Vassalo *et al.*, 2000a).

Two types of diapause are recognized, a behavioural and a morphogenetic diapause (**Fig. 1.26**). The first applies to unfed ticks and is most commonly manifested as a reluctance to quest for a host, while the second applies to engorged ticks and eggs and is manifested in a delay in their development. Photoperiod (latitude) and temperature have a role in maintaining diapause as well as inducing it. Behavioural diapause can be interrupted by factors such as high density of ticks and morphogenetic diapause by high temperature causing some transfers between spring and autumn tick populations in some years (Gray, 1991, 2002; Walker, 2001; Belozerov *et al.*, 2002; Randolph *et al.*, 2002).

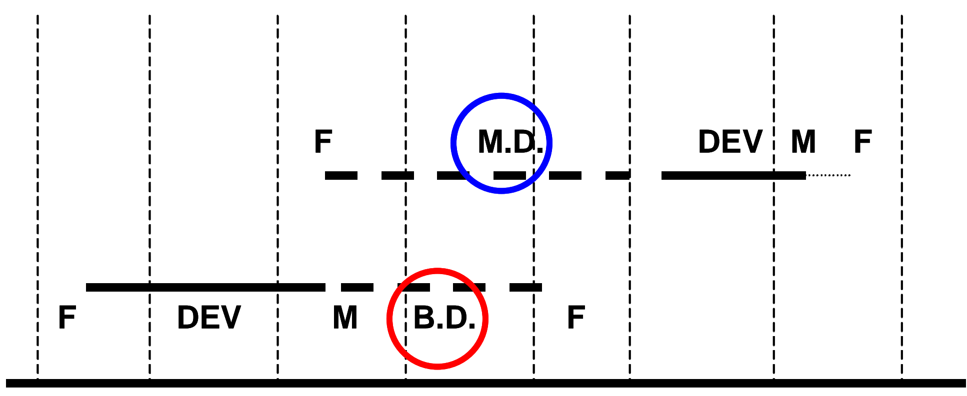


Figure 1. 26 – Example of the role of behavioural (B.D.) and morphogenetic (M.D.) diapause in developmental cycle of *I. ricinus* at a continental northern climate (F-feeding, M-moult, DEV development) (adapted from Gray, 1991).

These two types of diapause are most common in continental northern climates where winters are cold and summers hot (**Fig. 1.27**) (Gray, 1991; Walker, 2001; Randolph *et al.*, 2002). In mild oceanic climates, behavioural diapause may not be strongly developed in the tick population. This probably explains the occurrence of large numbers of autumn-feeding in such areas. For these mild areas, that only go through a morphogenetic diapause, the tick population remains smaller in autumn than in spring

because of higher mortality in engorged ticks and because a proportion of larvae derived from autumn-fed females can feed early in the next year, contributing to low or high level of recruitment of these larvae to the spring population.

In warmer countries (southern margin of *I. ricinus* range) morphogenetic diapause will probably not occur. Ticks here emerge earlier avoiding diapause induction, developing directly to the next stage within the same calendar year (*e.g.* feed July, moult September) and so complete a full life cycle in less than 3 years (Gray, 1991; Randolph *et al.*, 2002).

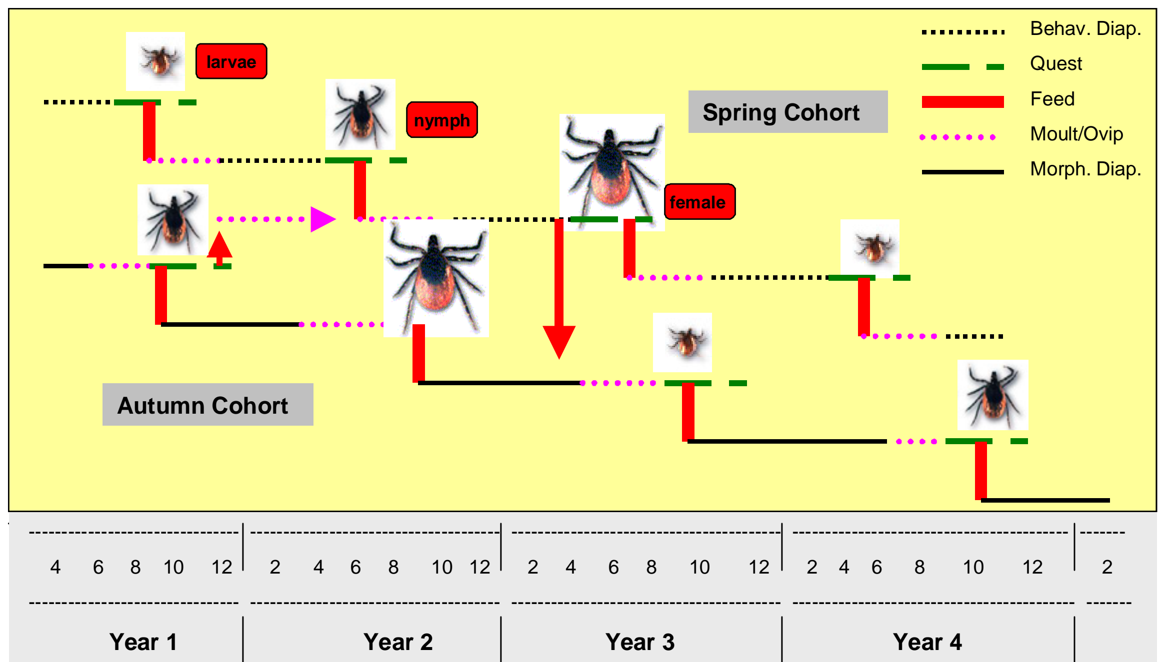


Figure 1. 27 – Descriptive model of the population dynamics of *I. ricinus* for a continental northern climate. The top horizontal line represents a cohort of ticks which feed in spring at all stages. The bottom horizontal line represents a cohort of ticks which feed in autumn at all instars. All moulting and oviposition periods occur in summer. Red arrowed lines represent potential transfers between cohorts, where late feeding nymphs from an autumn cohort join early feeding nymphs of the next spring cohort or where early feeding adults of the spring cohort produce eggs which develop into late feeding larvae of the autumn cohort in the same year (adapted from Walker, 2001)

I. ricinus larvae, nymphs and adult females (not males) can all exhibit behavioural diapause. Eggs, larvae and nymphs show morphogenetic diapause (Gray, 1991). Exposure to males abolish the behavioural diapause in females, which can contribute to transfer of ticks between spring and autumn populations, and since the mortality rate of the autumn population is higher could also contribute to density-dependent population regulation (Gray, 1987).

Few studies have dealt with the diel activity patterns of ticks and these show contrasting results. The endogenous rhythm of ticks and metereological factors, in particular the temperature, humidity and insolation are considered to be the main parameters governing tick diel activity (Mejlon, 1997; Van Es *et al*., 1999; Schulze & Jordan, 2003). The diel variation in activity of *I. ricinus* has also been associated with the physiological age of the ticks and a genetic makeup.

Regarding seasonality of the risk of exposure to *Borrelia*-infected *I. ricinus*, numerous studies were already performed (Mejlon & Jaenson, 1993; Wegner *et al*., 1994; Kovalevskii & Korenberg, 1995; Gray *et al*., 1995, 1999; Doby *et al*., 1995; Talleklint & Jaenson, 1996, 1996a; Hubalek *et al*., 1996a, 2003a; Dorn & Sunder, 1997; Stafford III *et al*., 1998; Talleklint-Eisen & Lane, 1999; Stepánová-Tresová *et al*., 2000; Jensen & Frandsen, 2000; Rizzoli *et al*., 2002; Barral *et al*., 2002; Quaresma, 2004), but further studies are needed on seasonal patterns of both tick density and prevalence of *Borrelia* infection as well as the factors influencing them, such as production of fed larvae by tick hosts and climatic conditions (Randolph, 1995; Craine *et al*., 1995; Talleklint & Jaenson, 1996a). At present, there is no evidence of a direct influence of any genospecies of *B. burgdorferi* s.l on the previously described seasonal pattern of ticks (Humair & Gern, 2000; Hanincová *et al*., 2003)

6. *BORRELIA* DETECTION (IN TICKS AND HOSTS)

The study of Lyme Borreliosis in natural communities has been favored by advances in biological research, eespecially in molecular biology. New techniques such as the polymerase chain reaction (PCR) provide ecologists with tools to identify with precision the presence or absence of *Borrelia* within hosts. A variety of immunological methods can provide sensitive serodiagnostic tools to identify whether a particular host has ever been infected by a specific infectious agent (Anderson, 1995). These methods do not require the destructive sampling of the host population and, increasingly, diagnosis of past infections can be based on secretions and excretions, in addition to samples of blood serum (**Fig. 1.28**).

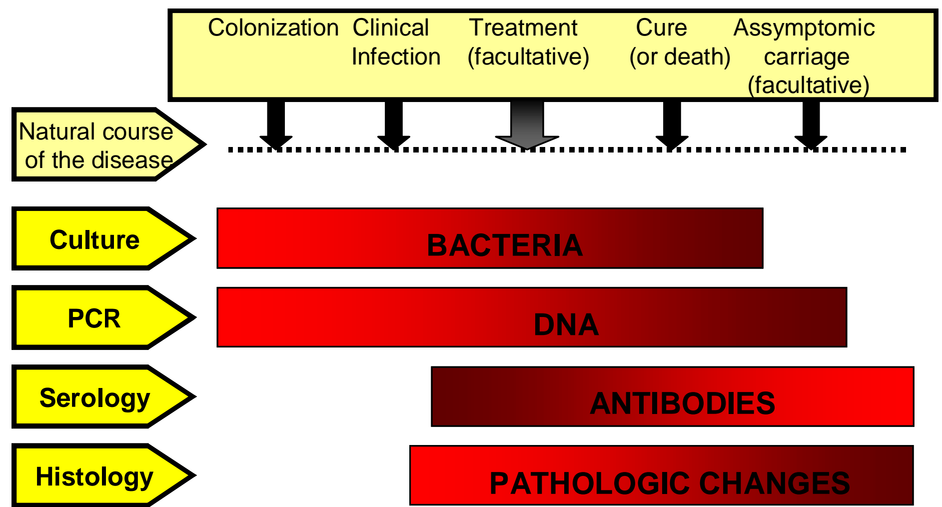


Figure 1. 28 – Laboratory techniques available for diagnosis of infectious diseases (adapted from Houpikian & Raoult, 2002)

Microbial (direct search) and/or serologic confirmation of human infection are needed for all manifestations of Lyme borreliosis except for typical early skin lesions. Culture and PCR can only be done satisfactorily in specialized laboratories, but antibody detection is currently most widely used in microbiological diagnosis of Lyme borreliosis (**Table 1.6**). An efficient diagnosis has to be based on a combination of various techniques such as serology, PCR and culture and not solely on serology (Tylewska-Wierzbanowska & Chmielewski, 2002). Differences in positivity rates between culture and PCR indicate unequal distribution of *Borrelia* within skin samples (Zore *et al*., 2002; Couceiro *et al*., 2003). It is important to inform clinicians about the limitations of the tests used (Lange & Seyyedi, 2002)

Table 1. 6 – Indications for microbiological testing related to some clinical manifestations of Lyme disease (Stanek *et al*., 1996; Wilske, 2002; Stanek & Strle, 2003; Hansmann *et al*., 2004)

Clinical signs	Serology	Tissue or fluid for culture, PCR, or both
Erythema chronicum migrans	No; If necessary a paired sample should be tested: IgM and IgG antibodies	Lesional skin
Borrelial lymphocytoma	Yes; IgM and IgG antibodies	Lesional skin
Acrodermatitis chronica atrophicans	Yes; IgG antibodies	Lesional skin
Early neuroborreliosis	Yes; Intrathecal IgG (rarely IgM) antibodies	Cerebrospinal fluid
Chronic neuroborreliosis	Yes: Intrathecal IgG antibodies	Cerebrospinal fluid
Lyme carditis	Yes; IgG antibodies	Endomyocardial biopsy
Lyme arthritis	Yes; IgG antibodies	Synovial fluid or synovia

6.1 Tick Collection techniques

Active populations of *Ixodes ricinus* and other ticks have been sampled by a variety of methods, namely:

a) **Sentinel animals** - collection of ticks over a period of days namely with sheep or cattle, or use of trapped rodents for sampling the immature stages. Constitutes a long-term methodology but it is very real (Gray, 1985; Kiszewski & Spielman, 1999; Van Es *et al.*, 1999);
b) **flags or blankets -** which are brought into contact with questing ticks - "flag" or "drag" techniques (Philip, 1937; Milne, 1943 *cited in* Gray, 1985; Daniel *et al.*, 1986; Hostis *et al.*, 1995; Schulze *et al.*, 1997; van Es *et al.*, 1999; Vassalo *et al.*, 2000; Jensen, 2000; Talleklint-Eisen & Lane, 2000). It is a method for use in short periods of time and the researcher has a great deal of control. Results between collections may vary with weather, vegetation structure and host abundance. This method is inefficient for adults (Sutherst *et al.*, 1978 *cited* in Gray, 1985; Ginsberg & Zhioua, 1999) and provides no information about the activity patterns of individual ticks, only on the overall patterns (Van Es *et al.*, 1999; Pichon *et al.*, 1999; Jensen, 2000);
c) **walking** surveys - which consists of collecting ticks seen directly on oneself after moving through the vegetation (Schulze *et al.*, 1997; Vassalo *et al.*, 2000);
d) **carbon dioxide traps** - this method overcomes problems associated with the nature of the vegetation and seems suitable for sampling adult ticks, when sufficiently vagile and numerous. It can operate from just a few hours to up to 7 days (Gray, 1985; Vassalo *et al.*, 2000),
e) observing **confined groups** - it can give additional insight into activity patterns and time of initiation of activity, but containment may influence results (Van Es *et al.*, 1999);
f) modified **Berlese-Tullgren funnel** – it can be used to extract live ticks (*I. ricinus* and *Ixodes uriae*) from small soil and litter samples (Mermod *et al.*, 1973; Barton, 1995);
g) **mark and recapture studies** can be done by capture by flagging, mark with cellulose paint, and recaptured again a few days later (Gray, 1985; Van Es *et al.*, 1999)

Several factors are important when selecting a sampling method: efficiency (best method to collect all stages) and purpose of collections. To evaluate tick populations over time, dragging and walking surveys seem adequate (after standardization by distance or duration). For distribution studies, hosts can be efficient collectors. Resources and logistics also play a role in selection of the collection method. Walking and dragging techniques require few resources and cost relatively little as compared to trapping of hosts or use of dry-ice-baited traps (Gilot *et al.*, 1975; Ginsberg, 1992; Schulze *et al.*, 1997).

Considering dragging/flagging method, several key parameters need to be accounted for: i) size of cloth lure (sufficient to cover all types of vegetation); ii) period of sampling (should be always equal between subsamples and permit enough captures); iii) minimum sample surface area required (minimal number of samples per collection required to give a representative sample of the area. To obtain a representative sample of tick population a minimum of two collections are required; iv) some form of randomization of sampling (sampling by transepts or random captures is indiferent) and nonselectivity. Types of cloth (toweling optimizes the collection) and speed of passage are also parameters to observe. A slow speed of passage (50 $cm.s^{-1}$) is better and position of the investigator doesn't have any influence (Vassalo *et al.*, 2000).

Wet vegetation (after rain) hampers collection of ticks by dragging or walking surveys. There is a decreased capacity of flannel strips to collect ticks when they are wet (Hostis *et al.*, 1995; Sheaves & Brown, 1995). However, high humidity seems to enhance questing tick. Collections seem highest after rainfall once vegetation is dry, while wind has a negative effect on *I. scapularis* collections in particular, presumably because high winds accelerate desiccation, making ticks less inclined to quest. Daily temperature extremes also affect collection efficiency. In early spring or late fall, collections should be performed during the warmest part of the day, while in summer, the collection of subadults is less productive during this period (Schulze *et al.*, 1997).

Besides collection of questing ticks, removing ticks when attached to humans or hosts has been the subject of various methodological recommendations in the popular literature and in information sheets available to the public. Attached ticks may be treated with oils, ether, gasoline, methylated spirit, fingernail polish, or other agents to induce detachment or to make it easier to remove the tick mechanically. The best method is to remove the tick without delay (*i.e.* with no previous treatments) by rotating without pulling. A mechanical device (forceps) to grab and rotate the tick that exerts a minimal pressure on the abdomen readily results in removal, leaving only the cement and the tip of the hyposthome, which consists mainly of chitin, unlikely to be contaminated with microbes. When the tick is pulled, large portions of tick tissue (possibly containing pathogens) often are left behind in the skin (De Boer *et al.*, 1993; Kahl, 1996; Parola & Raoult, 2001; Stanek & Strle, 2003)

6.2 Direct Detection of *Borrelia*

6.2.1 Microscopic examination

With a microscope, spirochetes can be directly observed in biological samples (blood, liquor, synovial fluid), using dark-field microscopy, coloration or imunohistochemical techniques. These disease agents are easily observed after coloration with aniline derivates and Romanovski pigments or using silver staining in tissue samples (Lebech, 2002). Due to difficult interpretation, this method should be limited to research and selected clinical situations where experienced observers are available to interpret the results (Reed, 2002). In the routine human diagnosis, the low concentration of bacteria in the biological samples and the limitations of microscopic observation make this direct technique not practical.

Detection of spirochetes in ticks can also be performed using dark-field microscopy (Hubalek *et al*., 1996; Kahl *et al*., 1998; Liebish *et al*., 1998). Along with histology of ticks embebbed in fresh paraffin (Lebet & Gern, 1994), fluorescence *in situ* hybridisation (FISH) with oligonucleotide probes permits simultaneous studies on presence, localization and identification of *Borrelia* inside the tick. This method has the advantage of preserving the architecture of tissues, with high specificity (discriminating several species), along with precise spirochete localization of *Borrelia* in tick tissues (Hammer *et al*., 2001)

6.2.2 Culture in selective media

The ability to isolate and culture the etiological agents of Lyme disease is essential in both research and clinical environments. Besides providing a mechanism for pathogen identification, culture is the basis of other supplemental tools that elucidate the causes of microbial disease and permits the study of the clinical and biological features of emerging bacterial diseases (Houpikian & Raoult, 2002). For instance, the ability to produce a viable culture of *B. burgdorferi* s.l. *in vitro* has facilitated production of spirochete antigens, which allow detection of Lyme pathogens in clinical specimens (Steere *et al*., 1983; Wang, 2002) In addition, isolation of the pathogen from infected hosts and ticks has been effective for defining endemic Lyme disease areas (Andersen *et al*., 1985,1989 *cited* in Polovinchik, 1999; Callister *et al*., 1990).

Cultivation in the laboratory is possible only with complex media that include undefined components, such as yeast extract. Elimination of any of those constituents results in significant inhibition of *B. burgdorferi* s.l. growth (Lackum & Stevenson, 2005). Attempts to culture *Borrelia* go back to the early 20th century, when Kligler and Robertson in 1992 defined the conditions for maintenance and growth of the relapsing fever pathogen in derivatives of Noguchi's medium. Today, variations of the complex Barbour-Stoener-Kelly (BSK) growth media are traditionally used with *in vitro* cultivation of *Borrelia* spirochetes

(Hofmann *et al.,* 1997; Barbour, 1984, Norris *et al.*, 1997, Picken *et al.*, 1997 *cited in* Polovinchik, 1999; Reed, 2002).

Two media are routinely used to grow *B. burgdorferi* in vitro, BSK II and BSK-H (Calllister et al.1990). The first has to be prepared in the individual laboratories from several components. Batch variations in media can influence growth kinetics, morphology, and antigenic characteristics of spirochetes (Callister *et al.*, 1990). BSK-H was developed in order to create a source of readily available commercial standardized medium (Reed, 2002). Gelatin and flavin adenine dinucleotide (FAD) are the two compounds that differ the most between BSK-II and BSK-H media. FAD acts as an electron carrier in the Krebs cycle and in oxidative phosphorylation steps of cellular respiration (Campbell, 1996 *cited* in Polovinchik, 1999) and this compound has no apparent role in the media. Conversely, according to Barbour *et al.* (1986 *cited* in Polovinchik, 1999), spirochetes generally prefer a viscous environment, thus, gelatin may be an important component of the medium. Also Bovine Serum Albumin (BSA - Fraction V) is an important factor for growing *Borrelia* and different batch of this component can affect the ability to detect low numbers of spirochetes and morphology of *Borrelia* (Callister *et al.*, 1990).

The reason for the peculiar nature of the above growth media becomes obvious upon sequence analysis of a *B. burgdorferi* s.s. genome, which reveals homologs of very few metabolic enzymes. It appears that this bacterium is unable to synthesize amino acids, nucleotides, fatty acids, or most other cellular building blocks. Fermentation of sugars to lactate, and the absence of both a citric acid cycle and oxidative phosphorylation are also observed. *B. burgdorferi* s.l. is capable of utilizing only a small number of diferent carbohydrates as energy sources, consistent with the general paucity of enzymes encoded by the spirochete (Lackum & Stevenson, 2005).

Glucose is a major energy source for *B. burgdorferi* s.l.. These spirochetes grow quite well on media containing maltose, confirming that this bacterium encodes an enzyme for breakdown of glucose polysaccharides. The only other hexose found to support *B. burgdorferi* s.l. growth is mannose, with mannose-modified host proteins as likely natural sources of this sugar. N-acetylglucosamine (GlcNAc) is an essential component of artificial media for cultivation of *B. burgdorferi* s.l. Genomic analysis suggests this is due to an inability of the organism to synthesize GlcNAc de novo. While this molecule is a building block of cell wall peptidoglycan, the present studies demonstrated that GlcNAc also also serve as an energy source. Glycerol is another source of energy can for *B. burgdorferi* s.l, presumably acquired from host blood and is more likely to be used for phospholipid and lipoprotein synthesis than as an energy source in nature (Lackum & Stevenson, 2005).

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The optimal temperature for *Borrelia* multiplication is 33-35ºC. The bacteria are slow growing, so weekly examination (during almost a year) of the culture medium by dark-field microscopy is usually recommended before stating a negative result of culture (Gustafson, 1994).

Other types of culture media have been used to detect *B. burgdorferi* s.l. Speck and others (2002) used BSK with other media, *e.g* BSK/EMEM (1:1, Eagle's minimum essential medium Biochrom) and BGM (buffalo green monkey cells ECACC 90092601), to take advantage of the interactions of *Borrelia* with eucaryotic cells *in vitro*, extra and intracellularly. This type of culture seems to shorten the log phase generation time from 12 hours in the normal medium to 4 hours in cultures of mammal cells. It increases growth of several species and is more sensitive for clinical samples. BSK/EMEM (1:1) is the best for cell line cultures, although it does not increase growth for adapted strains. Tick cell line media have also been used (Obonyo *et al*, 1999; Munderloh & Kurtii, 1995). Finally, inoculation in laboratory animals (C3H/He mouse) has proven faster than culture for recovery of *Borrelia* (Oteo *et al*., 1998).

6.2.3 DNA amplification and genotyping

Polymerase chain reaction (PCR) analysis, developed by Saiki *et al*. (1985) and mainly by Mullis *et al*. (1987) (Schmidt, 1997), is a widely used method for amplification of *Borrelia burgdorferi* s.l. DNA in biological specimens and ticks (Rosa *et al*., 1991, Schwartz *et al*. 1997; Schmidt, 1997; Kahl *et al*., 1998; Liebisch *et al*., 1998; Sparagano *et al*., 1999; Wang, 2002; Situm *et al*., 2002), allowing, among other features, a distinction between different species of *B. burgdorferi* sensu lato (Zore *et al*., 1999)

PCR tests to detect the organism directly in clinical samples are still too expensive for routine use and have yet to be fully evaluated (Davidson *et al*., 1999). But, even with these considerations, many clinical laboratories have turned to molecular assays in an attempt to increase sensitivity and specificity and reduce the time for LB laboratory results. Both single-stage and nested PCR assays have been developed, and detection methods vary from gel electrophoresis and Southern hybridisation to real-time PCR with quantification of product. Targets carried on plasmids, such as *ospA*, *ospC*, and *VlsE*, are present in multiple copies within each bacterium, and assays with these targets have greater sensitivity than those employing single-copy chromosomal targets such as *fla*, *recA*, *rpoB*, 16S and 23 ribosomal DNA (rDNA), and rDNA intergenic spacers (Reed, 2002) (**Table 1.7**).

Table 1. 7 – Several targets for DNA detection of *B. burgdorferi* s.l. by PCR (Schmidt, 1997; Sparagano *et al*., 1999; Lebech, 2002)

Original Reference	Target for DNA detection of *B. burgdorferi* s.l.
Rosa *et al*., 1989	Chromosomal gene, clone 2H1 (surface protein). Only American isolates
Persing *et al*., 1990	46-kb plasmid (outer surface proteins OspA and OspB)
Nielsen *et al*., 1990	145-bp DNA fragment of the OspA gene
Goodman *et al*., 1990	*Ly*1 gene, fragment of the RNA polymerase C gene
Wallich *et al*., 1990	Flagellin gene (inner part)
Rosa *et al*., 1991	Clone 2H1. Detection of European isolates with new primers.
Schwartz *et al*., 1992	Ribosomal 16S and 23S rRNA genes
Valsangiacomo *et al*., 1996	*hbb* gene, encoding a conserved histone-like protein

Attention also must be paid to genetic stability and specificity. No reaction with *Borrelia hermsii* and detection of all DNA subtypes of *B. burgdorferi* s.l. (pathogenic or not, including novel uncharacterised isolates) down to the limit of one organism, should be two major factors to be considered (Schmidt, 1997; Dumler, 2001). The intergenic spacer region between the 5S and 23S rRNA genes has been used as a target for a nested PCR as well as a single step PCR (Rijpkema *et al*., 1995; Alekseev *et al*., 1998). An increase in sensitivity and specificity can be accomplished with a nested-PCR procedure, using two rounds of amplifications and two sets of specifics primers (Rijpkema *et al*., 1995). With this PCR technique it is possible to detect 10 fg of *B. burgdorferi* s.l. DNA diluted in sample lysate, which corresponds to approximately two organisms per sample. A similar PCR is the single-tube nested PCR technique, with primers annealing at different temperatures in one reaction vessel, thus avoiding contaminations (Picken *et al*., 1996). In all PCR procedures, steps have to be taken to control contamination. Any contaminant, even the most minute airborne remnant carried over from previous PCR procedures, may be multiplied and can give false-positive results (Schmidt, 1997).

An optimal primer pair for detection of *B. burgdorferi* s.l. DNA should have the following characteristics (Schmidt, 1997; Sparagano *et al*., 1999):

- Amplify all strains of *B. burgdorferi* sensu lato
- Amplify all strains with the same sensitivity
- Be highly specific, not amplifying other borrelia, spirochetes, or other bacterial and viral pathogens
- Form stable duplexes with the template (all strains)
- Be 15 to 30 nucleotides long

- Have a high G+C content (the overall G+C content for *Borrelia* species is 28% (Hyde *et al*, 1984 *cited in* Schmidt, 1997)
- Not form 3'-end duplexes with themselves
- Not contain homooligomers or short repeated sequences
- Be stable at the 5'-termini but somewhat unstable at their 3' ends, as this promotes primer dimers
- Have melting temperatures that are very close to each other
- Have a difference in melting temperatures with respect to the target that does not exceed 20ºC, otherwise target self-annealing will predominate

In practice it is not possible to meet all of the above conditions. Studies must be conducted to test the best performing primers and to reduce the change of false negatives due to the changeable nature of borrelial DNA, with sequence differences in the target gene. A study on molecular detection of pathogens in ticks by Sparagano and others (1999) presents most of the available primer sequences to detect several agents, including *B. burgdorferi* sensu lato and species within the complex.

Depending on the specimen analysed (plasma, synovial fluid, CSF, serum, urine, skin biopsies and ticks), various procedures have been developed for optimising the amount of *B. burgdorferi* s.l. DNA available for amplification. For instance, in relation to ticks, because of the hard chitinous exoskeleton of ticks, **extraction of the nucleic acids** can take several steps (Schmidt, 1997; Priem *et al*., 1997; Kahl *et al*, 1998; Sparagano *et al*., 1999; Mannelli *et al*., 2003), as follows: disruption of cells, Inactivation of RNAs and/or DNAs with chaotropic salts, degradation of proteins with proteinase K, DNA extraction with phenol-chloroform, precipitation with alcohols (ethanol) or detergents (DNAzol), addition of carriers before centrifugation (glycogen and hyaluronidase), boiling, centrifugation and boiling, crushing, alkali lysis (with ammonia), adsorption to coated or uncoated silica in the presence of chaotropic salts, boiling and concentration by centrifugation and ultrafiltration or boiling in the presence of a cation exchanger (Chelex 100, BioRad).

A recently developed technology is Real-Time PCR for quantitative detection of *B. burgdorferi*. It is advantageous for i) the early efficient control of the therapeutic regimen, ii) the correlative analysis of the seriousness of symptoms and bacterial burden, and iii) the study of the influence of immune modulation on bacterial replication or elimination in animal models of Lyme disease (Pahl *et al*., 1999; Straubinger, 2000). This technology measures the release of fluorescent oligonucleotides during the PCR, which is proportional to the quantity of accumulated PCR product. For instance, a study with

Flagellin gene as target, showed a close correlation between clinical symptoms and bacterial burden of tissues (Pahl *et al*., 1999) and allowed distinction between *Borrelia* species (Schwaiger *et al*., 2001). Quantitative real-time PCR may also be used to assay spirochetes in feeding ticks and field-collected ticks, contributing to ecological and epidemiological surveillance of Lyme disease spirochetes (Piesman *et al*., 2001; Wang *et al*., 2003).

As in serology research, numerous other studies have led to the development of new targets, new primers and new molecular techniques, including DNA microarray assessment of putative *Borrelia burgdorferi* s.l. lipoprotein genes (Liang *et al*, 2002), One-step Reverse Transcriptase PCR (RT-PCR) for detecting flagellin mRNAa (Limbach *et al*., 1999) and several PCRs with primers for 66-kDa protein gene-p66, flagellin gene, *ospA* gene, *ospA/ospB* gene (Priem *et al*., 1997), for detecting the gene encoding the RNA polymerase beta subunit (*rpoB*) (Renesto *et al*., 2000), for detecting *lysK* sequences of *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* (Mejlhede *et al*., 2003) and to differentiate *B. burgdorferi* s.l. from non-Lyme *Borrelia* (Cyr *et al*., 2005)

Typing techniques can be divided into phenotyping and genotyping. Serotyping represents the most commonly used phenotypic method for *B. burgdorferi* s.l., along with whole cell protein pattern by polyacrylamide gel electrophoresis (PAGE) (Postic *et al*., 1994; Hubalek *et al*., 1996; Busch & Nitschko, 1999; Wang *et al*., 1999). Another technique is Multilocus Enzyme Electrophoresis (MLEE), which is a protein-based typing method whose results can be directly correlated with the genotype. It provides an estimate of the overall genetic relatedness and genetic diversity of *B. burgdorferi* sensu lato population. But because it is a labor-intensive method (needs large quantities of culture), MLEE is now used mainly to elucidate the population genetics of *B. burgdorferi* sensu lato (Postic *et al*., 1994; Balmelli & Piffaretti, 1995; Busch & Nitschko, 1999; Wang *et al*., 1999).

During the past several years, a number of genotyping methods have been used to assess the genetic relationships among *B. burgdorferi* s.l. species (**Table 1.8**).

Table 1. 8 – Examples of multiple techniques currently used to genotype *B. burgdorferi* s.l (Zingg *et al.*, 1993; Postic *et al.*, 1994; Ciceroni *et al.*, 1998; Busch & Nitschko, 1999; Wang *et al.*, 1999; Olsen *et al.*, 2000; Lee *et al.*, 2000; Mueller *et al.*, 2000; Situm *et al*, 2000; Iyer *et al.*, 2001; Dumler, 2001; Farlow *et al.*, 2002; Godfroid *et al.*, 2003 Derdáková *et al.*, 2003; Lagal *et al.*, 2003

Technique	Performance
DNA-DNA reassociation analysis	* *Borrelia* isolates showing less than 70% homology to each other do belong to different genospecies
rRNA Restriction Analysis (Ribotyping)	* *Borrelia* strains can be distinguished based on the profiles obtained by restriction fragment patterns of chromosomal DNA.
Pulse-Field Gel Electrophoresis (PFGE)	* Allows discrimination of species and strains within each *Borrelia* species
Plasmid Fingerprinting (linear plasmids)	* Strain and species identification of *B. burgdorferi* sensu lato (the number of plasmids in the isolates rang from 4 to 10, and the size of these plasmids range from 13.3 to 57.7 kb)
Randomly Amplified Polymorphic DNA (RAPD) and Arbitrarily Primed PCR (AP-PCR)	* Powerful tools to distinguish the different *Borrelia* species from each other as well as to recognize *Borrelia* strains within each of the species.
Species-specific PCR (typing PCR)	* Species identification with species-specific primers
PCR-Restriction Fragment Length Polymorphism (PCR-RFLP); PCR-Reverse Line Blott (PCR-RLB)	* Allows rapid discrimination of various *B. burgdorferi* s.l. species in large isolates collections and uncultured samples
DNA sequence analysis	* Useful for the study of bacterial genetics, evolution, taxonomy and epidemiology. DNA sequence analysis of some highly conserved gene loci can be used, for example, *rrs*, *fla* and *ospA*
Single-Stranded Conformation Polymorphism (SSCP)	* Suitable for rapid screening of sequence identity of isolates of *B. burgdorferi* sensu lato
Multiple-Locus Variable-Number Tandem Repeat Analysis (MLVA)	* Individual strains discrimination of *B. burgdorferi* sensu lato

In one of the most used genotyping techniques, PCR-RFLP, PCR amplicons are characterized by subsequent restriction with different enzymes (restriction endonucleases) to yield defined restriction patterns due to sequence variation within the amplified DNA fragment (Busch & Nitschko, 1999). Enzymes cut DNA at a defined position within (or close to) a specific recognition sequence and provide a reproducible pattern of DNA fragments whereby the number and size of fragments depends on composition of the DNA. It can result from a minor change of DNA such as single base substitution within the restriction enzyme recognition and/or cleavage site, or major changes like insertions, deletions or sequence rearrangements (Busch & Nitschko, 1999). *rrfA-rrlB* spacer of the 23S(*rrl*)-5S(*rrf*) rRNA gene is widely used as a template for amplification and for restriction polymorphism analysis of the amplified sequence to fingerprint genotypes and for preliminary assessment of genetic diversity (Postic *et al.*, 1994, 2000; Rijpkema *et al.*, 1995; Lee *et al.*, 2000; Olsen *et al.*, 2000; De Michelis *et al.*, 2000; Lin *et al.*, 2001; Lunemann *et al.*, 2001).

Digestion of amplicons (225-266 bp) with *Mse*I, isolated from *Micrococcus* species (**Fig. 1.29**) result in different restriction fragments with species-differentiating characteristics (**Table 1.9**) (Postic *et al*., 1994, 2000; Liveris *et al*., 1996; Wang *et al*., 1999; Lee *et al*., 2000; Lin *et al*., 2001). The limitations of this method include misleading novel patterns and the similarity of certain genospecies patterns (De Michelis *et al*., 2000).

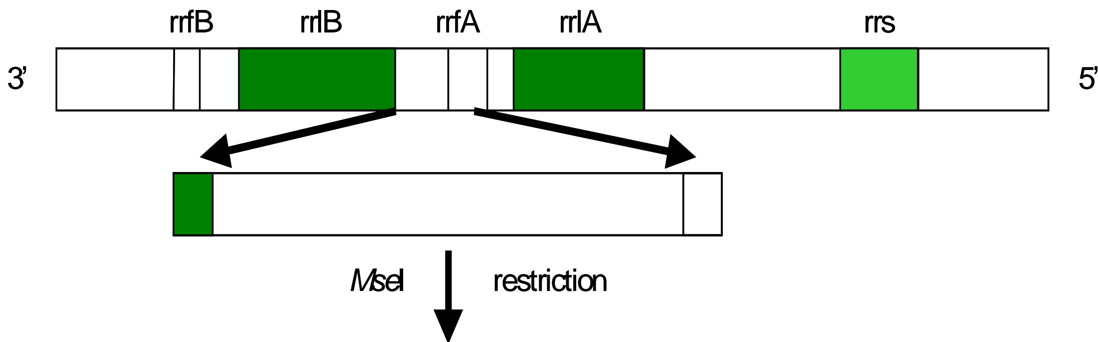


Figure 1. 29 – Digestion of *rrfA-rrlB* spacer of the 23S(*rrl*)-5S(*rrf*) rRNA gene of *B. burgdorferi* s.l. by a restriction enzyme (*MseI*) (adapted from Postic *et al*., 2000)

Table 1. 9 – Different restriction patterns obtained for most of the species of *B. burgdorferi* s.l. complex, after digestion with *MseI* (Postic *et al*., 1994; Liveris *et al*., 1996[a]; Wang *et al*., 1999; Postic *et al*., 2000; Lee *et al*., 2000; Lin *et al*., 2001[b])

Species	Strain	Amplicon size	RFLP pattern	MseI restriction fragment size (bp)
B. burgdorferi s.s.	B31	254	A	108, 51, 38, 29, 28
			M1	258, 149, 136, 128, 102 [(a)]
			M2	364, 258, 136, 102 [(a)]
	B31			105, 42, 38, 29, 28 [(b)]
B. garinii	20047	253	B	108,95,50
	NT29	253	C	108, 57, 50, 38
B. afzelii	VS461	246	D	108, 68, 50, 20
B. japonica	HO14	236	E	108, 78, 50
B. valaisiana	VS116	255	F	175, 50, 23, 7
	Am501	249	Q	169, 51, 23, 6
B. lusitaniae	PotiB2	257	G	108, 81, 39, 29
	PotiB3	255	H	108, 79, 52, 16
B. bissettii	DN127	257	I	108, 51, 38, 33, 27
	CA	226	J	108, 51, 38, 29
	25015	253	K	108, 51, 34, 27, 17, 12, 4
B. andersonii	19857	266	L	120, 67, 51, 28
	CA2	255	M	91, 50, 40, 28, 22, 17, 7
B. tanukii	Hk501	245	O	174, 51, 20
B. turdi	Ya501	248	P	107, 51, 38, 21, 16, 8, 7
Borrelia sp.	A14S	225	R	108, 66, 51
Haenam strains	HN-6 to HN-19 *(Korea)*	254	S	150, 58, 24, 22

Amplification of other regions has been attempted, namely, a region of the 16S-23S rDNA spacer with *Hinf*I digestion of the products (Liveris *et al*., 1995; Liveris *et al*., 1996), *ospC* gene with *Mbo*I *and Dra*I digestion of the products (Masuzawa *et al*., 1997) and *ospA* gene (Michel *et al*., 2003). In this last study, *B. garinii* showed 5 different RFLP patterns, corresponding to the OspA types 3-7 associated with this species. *B. burgdorferi* s.s., *B. afzelii, B. valaisiana* and *B. lusitaniae* showed 1 or 2 characteristic RFLP patterns.

Reverse Line Blot (RLB), another technique presently used, consists of hybridisation of the PCR product to multiple genomic-group specific oligonucleotide probes immobilized on a membrane in 45 lines perpendicular to each other. In one assay, the reactivity of 45 PCR products with up to 45 different probes can be determined (Rijpkema *et al*., 1995; Kurtenbach *et al*., 1998b; Gubbels *et al*., 1999; Heijden *et al*., 1999; Lunemann *et al*., 2001; Schnittger *et al*., 2004). This system has several advantages: DNA sequencing of the PCR product is not required, small amounts of PCR product can be typed, different genomic groups can be detected concurrently and coinfections of different genomic groups of *B. burgdorferi* sensu lato can be distinguished (if the ratio does not exceed 1:10) (Rijpkema *et al*., 1995; Heijden *et al*., 1999). Tick components do not appear to strongly inhibit the PCR results (Rijpkema *et al*., 1995). Again, the use of *rrf*A-*rrl*B spacer, in this PCR-RLB, is a reliable and rapid laborathory method for the distinct detection of *B. burgdorferi* sensu lato species in both bacterial cultures and clinical specimens from patients with Lyme borreliosis (Rijpkema *et al*., 1995; Heijden *et al*., 1999; Lunemann *et al*, 2001). Adaptations of this technique include studies like a Dot-Blot hybridisation with specific probes to different genomic groups, in a positively charged nylon membrane filters (Jaulhac *et al*., 2000), hybridization to ribosomal RNA of *B. burgdorferi* with probes specific for sequences in 23rRNA (Danishefsky *et al*., 2000) and a RLB with colorimetric detection for *ospA* gene (Godfroid *et al*., 2003).

6.3 Indirect detection of *Borrelia* (serology)

Serologic assays for the search of specific antibodies anti - *B. burgdorferi* s.l agents in USA were first used in 1983. In 1995, the CDC recommended a fundamental change in serologic procedures, a conditional 2-step serologic test, in which a serum specimen with a positive or equivocal first-step test (screening) result (e.g., ELISA) is further tested by an immunoblot assay, the reference test. A positive IgG immunoblot positivity for patients with illness lasting longer than 30 days is also required. This methodology has been adopted also in Europe (Wormser *et al*., 1999; Brown *et al*., 1999; Goossens *et al*., 1999; Davidson *et al*., 1999; Hauser *et al*., 1999; Guerra *et al*., 2000; Magnarelli *et al*., 2000; Dumler, 2001; Wilske, 2002, 2003).

Normally, the host immune response begins with the appearance of specific immunoglobulin M (IgM) antibodies against the 41 kDa flagellar protein, usually within the first several weeks after initial exposure. Studies suggest, however, that this protein is a natural antibody that confers protection against some strains and species (Ulvestad *et al.*, 2001). The IgM response (p41 and various outer surface proteins) may persist for many months or years despite effective antimicrobial therapy, or even, not may not even be detected at anytime. Thus, the presence of specific IgM antibodies cannot be used as the sole criterion to diagnose a recent infection. Most patients will have detectable IgG antibodies after 1 month of active infection. Like that of IgM, the IgG response can persist for years after LB symptoms have resolved. Both IgG and IgM responses can be greatly diminished or absent in patients receiving antimicrobial therapy early in the course of disease (Gustafson, 1994; Ulvestad *et al.*, 2001; Reed, 2002).

A negative test result indicates that there was no reliable serologic evidence of *B. burgdorferi* infection present at the time the specimen was collected. A positive second-step result indicates that there is either serologic evidence of past and current infection with *B.burgdorferi* or presence of cross-reacting antibodies.

False negative test results may occur during the first few weeks after infection before the production of a humoral response or when the level of antibodies is below the analytical sensitivity of the test. In first-step IgM assays, false-positive test results may be due to cross-reacting antibodies to *B. burgdorferi* s.l. in patients with other tickborne diseases such as relapsing fever (*Borrelia* spp) and an ehrlichiosis infection, autoimmune disease, or viral (Eipstein-Barr virus and others), musculoskeletal and neurological diseases (Gustafson, 1994; Brown *et al.*, 1999; Davidson *et al.*, 1999; Reed, 2002). False-positive test results for IgG may occur because of cross-reaction with syphilis, an ehrlichiosis infection, babesiosis, *Heliobacter pylori*, or musculoskeletal disease (e.g multiple sclerosis or lupus). Exclusion of infections with Epstein-Barr virus or cytomegaloviruses in a positive IgM EIA (enzyme immunosorbent assays) is more important than the confirmation with a Western blot (Goossens *et al.*, 1999)

For the majority of LD patients with objective findings (e.g. ECM and ACA), serologic testing is unnecessary. On the other hand, serologic testing is of little value in the absence of clinical findings suggestive of Lyme Disease. Tugwell *et al* (1997 *cited* in Brown *et al.*, 1999) recommended no serologic testing if the pretest probability of LD is below 0.20 or above 0.80. If a patient has nonspecific signs and symptoms of illness such as headache, fatigue, mylagia, or arthralgia, then their pretest probability will

be low (<0.20) and their symptoms should be treated, with common clinical presciptions. If the patient presents with a rash resembling EM, and a previous tick bite, then their pretest probability is high (>0.80) and antibiotic therapy is recommended. For patients not presenting EM or a history of EM but with other objective clinical signs (0.20 < pretest probability < 0.80), serologic testing may be useful. Serologic tests do not become positive until weeks after a tick bite. Later serologic testing may be negative if patients were treated with antibiotics. Without EM, positive 2-step serology indicates only exposure and does not indicate whether viable spirochetes are present (Brown *et al*., 1999)

Serological techniques can also be used in domestic animals. Guerra and others (2000) applied immunoblots for serodiagnosis of Lyme Disease in dogs. A logistical regression model was developed to distinguish infected from vaccinated dogs based on the band patterns of immunoblots.

Antibodies to tick salivary gland proteins (anti-tick saliva antibody – ATSA) in humans may be a biological marker of exposure to ticks (Schwartz *et al*., 1990, 1991; Lane *et al*., 1999; Mayoral *et al*., 2004). Ticks are excellent candidates to develop biological markers. Studies conducted with these ATSA suggest their role as biological markers of tick exposure associated with serologic status of Lyme Disease patients or spotted fever group (Schwartz *et al*., 1990, 1991; Mayoral *et al*., 2004). A marker of tick exposure without the need for collecting ticks could evaluate interventions to decrease tick exposure, to control differences between tick exposures (preventive activities, geographic areas) and have clinical utility identifying patients with recent tick bite and nonspecific symptoms.

6.3.1 Screening Tests (IFA, ELISA)

The earliest immunoserologic tests for LB were indirect fluorescence assay (IFA) and enzyme-linked immunosorbent assays (ELISAs). These methods ar suitable for detecting IgM and/or IgG antibodies, but many laboratories find ELISA more convenient for testing large numbers of specimens. Because these tests use whole-cell preparations, cross-reactions as described above can occur more frequently. Additionally they still lack sensitivity for early disease.

Improvement of sensitivity and specificity has been achieved using some modifications, as follows: i) recombinat antigens in ELISA (Magnarelli *et al*., 2000), like recombinants protein rFlaA (flagelinn A) (Panelius *et al*., 2001), BBK32 (Heikkila *et al*., 2002); ii) immune complexes (ICs) isolated in high levels from serum, liquor and synovial fluids in patients with LD as antigens for ELISA (Brunner *et al*., 2000); iii) a synthetic peptide (C_6) with the sequence of an immunodominant conserved region named IR_6 present at variable surface antigen of *B. burgdorferi* s.l. VlsE for ELISA (Liang *et al*., 1999; Wilske, 2003)

Another indirect imunoreaction technique easy to perform and readable by a simple routine light microscope is the immunoperoxidase slide test (IPT), incubated with H2O2 and 3,3-diaminobenzidine tetrahydrochloride as a chromogenic substracte and was developed by Kriuchechnikov (1998).

Direct (DFA) and indirect immunofluorescence assays (IFA) (Ribeiro *et al.*, 1987; Rijpkema *et al.*, 1995; Kahl *et al.*, 1998) are commonly used for detecting *Borrelia* in ticks. IFA is a less expensive technique, less sophisticated, it demands only one fluorescence microscopy but it needs experienced personnel because of its subjectivity. Immunofluorescence may not be as sensitive as PCR for detecting *B. burgdorferi* s.l. in tick material (Tylewska-Wierzbanowska *et al.*, 1996), but it is still a common technique in several laboratories.

6.3.2 Reference Test (Western Blot)

Immunoblotting allows detection of antibodies to individual antigens of B. burgdorferi s.l. and is more specific than IFA or ELISA. Antigens can be derived from whole-cell preparations or from expressed proteins taken from recombinant DNA.

In Europe, three species of *B. burgdorferi* that are pathogenic for humans and numerous surface protein A (OspA) serotypes are known, which complicates the comparability and standardization of assay systems (Hauser *et al.*, 1999; Wilske *et al.*, 1999; Reed, 2002). This is due to either the extensive range of blotting methodologies in use (antigens prepared from the different genospecies of *Borrelia burgdorferi* s.l., different polyacrylamide gel electrophoresis and immunoblotting protocols) or the interpretation of band patterns (Robertson *et al.*, 2000; Gutiérrez *et al.*, 2000). A previous study included identification of a series of key proteins with monoclonal antibodies and densitometric determination of the molecular masses of all visually distinguishable bands (more than 40 bands per strain) separately for each of the three strains used (**Fig. 1.30**). With a panel of European sera, a new interpretation criteria for WB was developed (EUCALB):

- WB IgG – at least two bands among p83/100, p58, p43, p39, p30, OspC, p21, p17 and p14 for *B. afzelii* (Pko) and at least one band among p83/100, p39, p30, OspC, p21, p17 for *B. garinii* (Pbi).
- WB IgM – at least one band among p39, OspC, p17 or a strong p41 for Pko or at least one band among p39 and OspC or a strong p41 band for Pbi

Pko strain gives a more sensitive WB and is recommended for Europe (Hauser *et al.*, 1999). For other authors, *B. garinii* 20047 is the best strain for the development of a generic WB for LB in Europe (Norman *et al.*, 1996).

European rules are not intended for the interpretation of any single immunoblot, but should be used by diagnostic laboratories as a guide for the formulation of working rules suited to their methodology and local populations (Robertson *et al.*, 2000). For instance, a study of six laboratories revealed the most discriminatory bands for reliable detection of antibodies. These bands were used to construct individual interpretation rules for the immunoblots used in those laboratories (Robertson *et al.*, 2000).

The growing use of recombinant immunoblots with recombinat proteins has the main advantage of allowing easier identification of diagnostic bands that could belong to different strains (homologous proteins) and be used on one blot. The identification of bands in the whole cell lysate immunoblot is difficult. The recombinant immunoblot is eespecially suitable where large series of sera need to be investigated (Wilske *et al.*, 1999; Lebech, 2002). Use of monoclonal antibodies to identify species of *Borrelia* also has been attempted (Canica *et al.*, 1993)

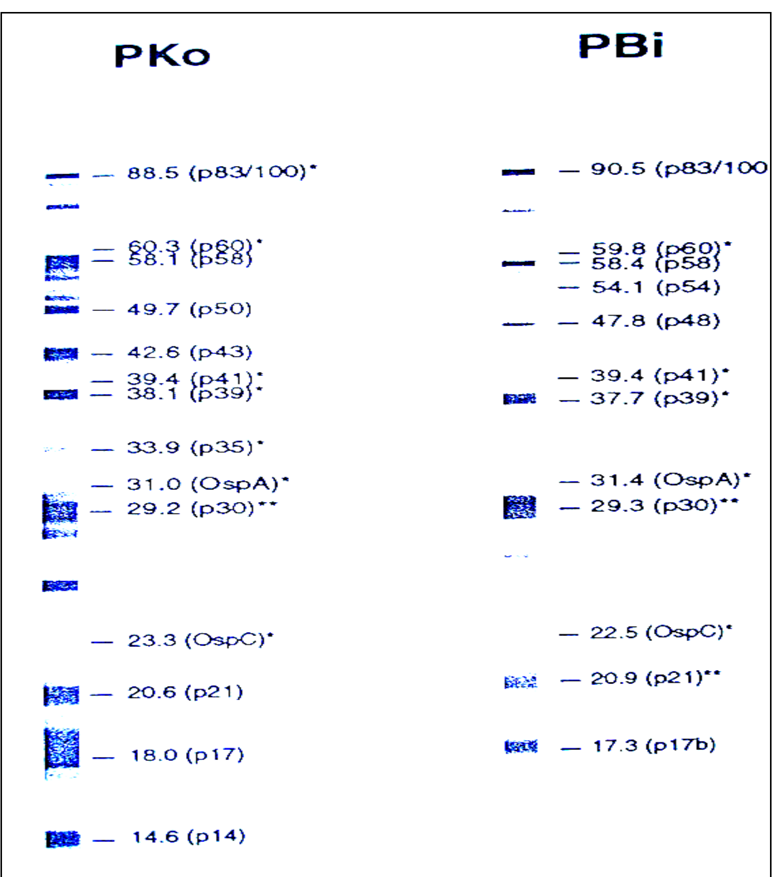


Figure 1. 30 – Molecular masses of all visual bands for *B. afzelii* strain(Pko) and *B. garinii* strain (Pbi), determined by immunoblotting (Hauser *et al.*, 1999)

6.4 Concluding Remarks on Diagnostics

As already mentioned, molecular methods may have specific adjunctive roles in the diagnosis of Lyme disease, but they are not likely to be useful as the primary diagnostic test and should only be used in patients with a high probability of infection based on clinical manifestations and an appropriate exposure history. Clinical diagnosis is mandatory and a negative PCR result does not exclude Lyme disease in any situation (**Fig. 1.31**). Generally *Borrelia* seems to be trophic for tissues avoiding body fluids. But PCR tests have been able to detect them emphasizing its diagnostic power. On the other hand, the presence of PCR-detectable sequences does not necessarily prove the presence of an active disease (Schmidt, 1997; Sparagano *et al.*, 1999; Dumler, 2001; Lebech, 2002; Reed, 2002; Stanek & Strle, 2003).

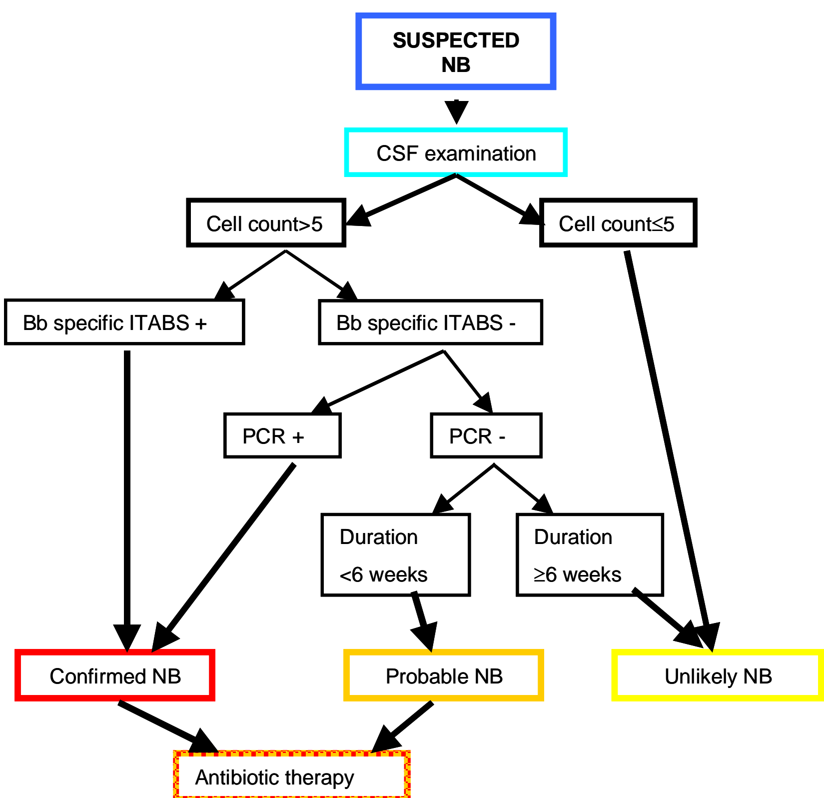


Figure 1. 31 – Laboratory steps for diagnosis of Lyme Neuroborreliosis (NB). Legend: CSF – Cerebrospinal fluid, ITABS – intrathecal antibodies (adapted from Lebech, 2002)

PCR results are dependent on the concentration of spirochetes in the specimens taken for analysis. A comparison of results for PCR and serological testst showed an inverse correlation. Culture has lower sensitivity than PCR and only detects viable spirochetes. This is in contrast to PCR, where reactivity can be due to dead organisms, portions of spirochetes that include DNA, or soluble DNA.

Interpretation of results from ticks removed from the skin or clothing of patients needs careful attention. A positive result for *B. burgdorferi* s.l. in ticks removed has a low predictive value for the subsequent development of LB in the exposed patient, and a negative result does not exclude transmission of infection from the submitted ticks or other ticks that the patient was not aware of. Coinfection with other tick-borne pathogens has to be also when choosing the testing method (Liebish *et al.*, 1998; Reed, 2002).

7 TREATMENT, CONTROL AND PREVENTION

Lyme Borreliosis surveillance and control is difficult and the effectiveness of any public health intervention is determined by its theoretical efficacy and by the level of engagement of the target population. This assessment of the various interventions should be presented to the community to be applied as a preventable option for any disease (Hayes *et al.*, 1999). Several steps are needed: description of the public health problem (*e.g.* Lyme disease), interventions to be used (*e.g.* acaricides, modification of tick habitat, personal protective measures), description of a hypothetical community (within risky habitats), definition of the target population (entire community or a subset), estimation of the levels of commitment (*e.g.* amount of acaricide, controlled burning, use of repellents) and estimation of effectiveness (number of cases prevented) (Hayes *et al.*, 1999). The main surveillance approaches for LB are summarized In **Table 1.10**,.

Table 1. 10 - Management options Lyme borreliosis surveillance and control and their potential environmental effects (Ginsberg, 1994).

Control Method	Site Applicability	Environmental Effects
Self-protection precautions	All tick-infested areas	Negligible, possible health effects of repellents on user
Habitat manipulation	Trails, campsites, residential yards	Powerful local effects (can be limited to heavy-use areas)
Manipulation of host populations	Potential applicability in managed areas (efficacy not established)	Powerful effects on host species and associates
Biological control	All tick-infested areas (efficacy not established)	Depends on species utilized
Broadcast pesticide applications	Heavily utilized, highly impacted areas, residential areas	Powerful local effects on nontarget species
Targeted pesticide applications	Residential areas, recreational areas	Main effects on nest associates of targeted host species

7.1 Antibiotics

Treatment with antibiotics is beneficial for all clinical manifestations of Lyme borreliosis. Such therapy is most effective in the course of the illness. Doxycyclin, Amoxiciclin and Ceftriaxone are some antibiotics used for treatment of LB (Gustafson, 1994; Weber, 2001; Kaiser, 2002; Hengge *et al.*, 2003; Stanek & Strle, 2003). *B. garinii* seems to be eespecially susceptible to azithromycin, *B. burgdorferi* s.s. to amoxicillin and *B. afzelii* to ceftriaxone (Sicklinger *et al.*, 2003).

7.2 Biological tick control

Numerous organisms have been very competent in controlling tick population, from entomopathogenic nematodes and fungi, bacteria *Bacillus thuringiensis*, protozoa, parasitoids to tick predators. The anti-tick activity of entomopathogenic nematodes (*Steinernema carpocapsae* and *S. glaseri*) (Zhioua *et al.*, 1999; Samish & Rehacek, 1999) is due to the susceptibility of engorged ticks to nematodes, co-occurrence in resting sites, easy application (irrigation or spraying) and long infectivity (Samish & Rehacek, 1999). Fungi belonging to Hyphomycetes were found in field *I. ricinus,* eespecially in the stretched integument of females when engorging near the skin of hosts, with temperatures that facilitate germination of spores (Kalsbeek *et al.*, 1995; Samish et al., 2001). These entomopathogenic fungi have significant impacts on the size of tick populations. Fungal strains seem to be of similar efficacy against different tick species and stages, so they may be used as a biological weapon to control ticks (Kalsbeek *et al.*, 1995; Samish & Rehacek, 1999; Samish et al., 2001; Gindin *et al.*, 2002).

As with other vectors of medical importance (*e.g.* mosquitoes), pathogenicity of the bacteria *Bacillus thuringiensis* has also been considered as a new strategy to control *I. scapularis* (Zhioua *et al.*, 1999; Samish & Rehacek, 1999). Rickettsial infection may lead to alterations in tick behaviour and development and even death, depending on the degree of infection. Latent bacterial flora may become acute and cause death when the ticks are under physiological stress (Samish & Rehacek, 1999).

Protozoa (e.g. *Babesia* spp and *Theileria* spp) can also affect development and survival of ticks (Samish & Rehacek, 1999). Parasitoid wasp *Ixodiphagus hookeri* was used for controlling tick populations with encouraging results (Samish & Rehacek, 1999; Knipling & Steelman, 2000). Ticks are preys of numerous predators: spiders (Araneida), mites (Acarina), bugs (Hemiptera), moths (Lepidoptera), flies (Diptera), ants (Hymenoptera), beetles (Coleoptera), amphibians (toads and tortoises), reptiles (lizards), birds (mainly oxpeckers, cattle egrets, domestic fowl, other birds), mammals (e.g. shrews, rodents) and other

vertebrates that groom. Tick cannibalism, mainly in argasid ticks, is also described (Samish & Rehacek, 1999).

7.3 Pesticides and repelents

Along with the above methods, use of organic and inorganic substances is another solution to decrease tick populations (**Table 1.11**), when the risk of contact between ticks and hosts is extremly high. Pesticides and repelents are commonly used and there is an effort to diminish their toxicity, but increase their effectiveness.

Table 1. 11 – Presentation of some studies regarding pesticides

Plant compounds	
Lemon eucalyptus extract (Citriodol) against tick bites	- Gardulf *et al.*, 2004
Alaska yellow cedar for *I. scapularis* nymphs and land eastern red cedar for larvae	- Panella *et al.*, 1997
African plants, acaricidal and repellent properties	- Kayaa, 2000
CO_2 extract of the seeds of the Mediterranean plant *Vitex agnus castus* (monk's pepper) as a spray against *Ixodes ricinus* and *Rhipicephalus sanguineus*	- Mehlhorn *et al.*, 2005
Organic compounds	
Inclusion of pheromone components in a permethrin-impregnated oily matrix increase the lethal activity for *I. scapularis*	- Sonenshine *et al.*, 2003
Inorganic compounds	
Anti-parasiticide chemical Ivermectin against *I. ricinus*	- Taylor & Kenny, 1990
Use of permethrin-treated cotton or apllicator given to reservoir hosts to reduce tick infestation	; Sonenshine *et al.*, 1996
Desiccant Drione (1% pyrethrin) and Safer's insecticidal soap (0.2% pyrethrin) to reduce substantially nymphs and adults of *I. scapularis*	- Patrican & Allan, 1995

Repellents are substances that induce a movement of the arthropod away from the host. They are used primarily on the skin, but also on materials. A variety of repellent assays for ticks are described in literature (**Table 1.12**).

Table 1. 12 – List of some tick repellents currently in use (Nentwig, 2003; McMahon *et al.*, 2003; Dautel, 2004)

Ticks Repellent	Effect	Characteristics
A13-35765	Equal to Deet	Results less satisfactory
A13-37220	Better than Deet	Results satisfactory
Bayrepel	Better than Deet	Replacing Deet in many countries
CIC-4	Weaker than Deet	Results less satisfactory
DEPA	Weaker or Equal to Deet	Efficacy, dermal toxicity and cosmetic properties similar to Deet
Ocimum suave	Equal to Deet	

7.4 Traditional prophylatic methods

The number of cases of Lyme borreliosis may be reduced by giving prophylactic information annually about ticks and ways to remove them, plus additional education of patients about the recognition of erythema migrans (den Boon *et al*., 2004). Also, prophylactic therapy could be reserved only for those patients presenting a tick (a situation not very common) whose engorgement index suggests a period of attachment longer than 36 hours for nymphs and 48 hours for adults, or that have a confirmed positivity (*e.g.* by PCR) (Yeh *et al*., 1995; Falco *et al*., 1996; Maiwald *et al*., 1998).

Repeated exposure to tick bites in sites that are endemic for Lyme disease has been associated with the development of cutaneous hypersensitivity, which results in persistent itch and local swelling at the site of tick attachment. Itching provides an early sign of tick bite and may facilitate removal of the attached tick before the pathogen can be transmitted. Additional inflammatory reaction to tick salivary proteins also may help prevent transmission. This acquired reaction may limit the incidence of Lyme disease by protecting persons who have been previously exposed to bites of vector ticks (Burke *et al*., 2005).

Before ticks initiate their feeding, the number of spirochetes, when present, is low. These spirochetes may not be infectious or may be too few to initiate an infection. Removing the tick at this time, along with the localized dermal tissue attached to the hypostome, could preempt infection because the spirochetes do not have time to disperse (Piesman & Schneider, 2002).

Minimizing the natural risk and human exposure and/or maximizing prophylatic measures against tick bites, it is theoretically possible to bring about a considerable reduction in the risk of people acquiring Lyme borreliosis (Kahl, 1996).

Those people frequenting forests in tick-infested areas should walk only on broad forest paths, keeping off path edges and not venturing away from the paths during the tick season. Immediately after tick exposure, one should thoroughly examine one's clothes and whole body for ticks. The wearing of light-coloured clothes makes it easier to detect crawling ticks. Long trouser legs tucked into socks do not protect per se against tick bites but make it harder for a tick to get to the skin and thus give one more time to detect a tick before it starts to bite. If a tick has already started to feed, a pair of forceps (or fingernails) should be used to pull the tick off, as already mentioned (Ginsberg, 1992; Ginsberg, 1994; Fish, 1995; Kahl, 1996).

Removal of leaf litter in wooded areas can significantly reduce abundance of questing larvae and nymphs of *I. scapularis* and *I. ricinus*. Direct effects include physical removal of ticks along with the leaf litter and indirect effects include mortality caused by exposure to extreme temperatures and desiccation. This method achieves the same results as with use of acaricides or burning and mowing of vegetation and can be used to create a hostile environmental barrier between wooded and residential areas (Schulze *et al*., 1995; Sheaves & Brown, 1995).

Burning vegetation for tick control has a limited and temporary impact, strongly influenced by the intensity and timing of the burn. It should be applied only on a large scale in areas with little or no human habitation (Mather *et al*., 1993; Stafford III *et al*., 1998).

7.5 Vaccines

Vaccines are the most complete prophylatic technique that can be used, but are also the most difficult to obtain. Vaccination can be performed on humans, ticks or on natural reservoirs blocking transmission of Lyme spirochetes.

Vaccination of human was attempted with an OspA vaccine with little success. *Bmp* gene products may be used as reagents in the preparation of vaccines due to their well conserved genetic structure across different species of *B. burgdorferi* (Gorbacheva *et al*, 2000).

Vaccinations for wild reservoirs have used OspA proteins (Kurtenbach *et al*., 1997; Tsao *et al*., 2004) with reasonable results. Immunization of natural reservoirs with *bbk32* antisera has a significant impact on the survival of spirochetes within ticks and in the ability of ticks to maintain spirochetes throughout the molt. It also reduces the larval acquisition of spirochetes and reduces the ability of engorged nymphs and adults to harbor the pathogen (Fikrig *et al*., 2000).

Vaccination of a variety of hosts using tick guts or internal organs (*e.g*. Bm86, a membrane-bound protein located on the surface of gut cells in *Boophilus microplus*) has also been suggested. This approach can affect survival and fecundity of ticks that feed on treated hosts, but demands booster vaccinations (Willadsen & Jongejan, 1999). Antibodies specific to a 25-KDa protein of *I. ricinus* involved in the process of material deposition in the cuticle during feeding of ixodid ticks, could interfere with cuticle formation (anti-tick vaccine) (Rutti & Brossard, 1989; Uhlir *et al*., 1994).

8 GEOGRAPHIC INFORMATION SYSTEMS AND MODELLING TOOLS FOR DISEASE SURVEILLANCE / MANAGEMENT

8.1 Geographic Information Systems (GIS)

To understand and ultimately predict the distribution and abundance of a vector, various aspects related of its biology, ecology and habitat asociations, as well as the nature of the data involved must be considered (Wilson, 1998):

- Spatial scale or resolution of the final analysis: *e.g.* countrywide, district. For instance, in the case of Lyme disease, tick habitat preference may operate on the microscale, mice and other intermediate host may operate on the mesoscale and establishment of new disease foci on the macroscale (Kitron, 1998; Wilson, 1998; Robinson, 2000; Cortinas *et al.*, 2002).
- Presence/Absence and Type of tick population measurements: presence/absence, abundance, density, prevalence and intensity of infestation
- Biasing factors that confound estimation: sampling method variation (systematic samples represent the sole source of information that is useful in determining abundance and density), temporal variation (seasonality, diel activity, duration of tick attachment), host-associated variation (observations from host species that are both numerous and heavily parasitized will probably provide the best relative estimates of tick abundance. Attention must be taken with stage-specific differences in host associations) and spatial variation (spatial heterogeneity of vegetation types, vertebrates or spatial units)
- Habitat associations of ticks: availability of suitable hosts, physical environment (vegetation and associated microclimate)
- Dispersal and tick distribution: tick movement and transport (hosts movements within homeranges, dispersal or migration)

The dynamics of ticks distributed over large areas could potentially be assessed using metapopulation dynamics (Wilson, 1998). According to this approach, the dynamics of a spatially structured population is best understood by analysing extinction, inter-patch movement and colonization. A metapopulation can be defined as a collection of subpopulations of the same species, each of which occupies a separate patch of a subdivided environemnt (Dobson, 2003). The patchy distribution of *I. scapularis*, for instance, corresponds to the typical pattern that characterizes a metapopulation. Subpopulations occur at different densities in various diverse, spatially heterogeneous habitats. The amount and distribution of suitable habitat (patchiness) have an impact on the rate and direction of expansion. Patchiness in host

diversity, abundance, vegetation and microclimate may combine to represent unequally suitable habitats that, in turn, can influence immigration, colonization, and population stability. This stability is dependent on successful invasion and establishment by ticks (Wilson, 1998). In general, distances among patches and extent of habitat fragmentation appear to be inversely related to the probability of successful invasion and establishment. In contrast, the density of ticks at a source for emigration and the proportion of suitable habitat within the region where migration can occur are positively associated with establishment (**Fig. 1.32**). This conceptual framework suggested by Wilson might help guide future studies that aim to understand the population ecology and changing spatial pattern of this vector.

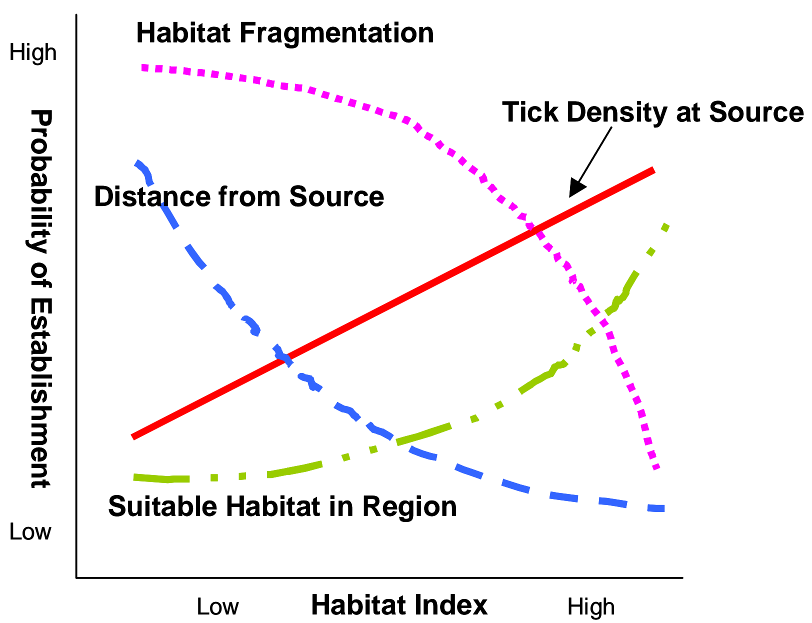


Figure 1. 32 – Hypothetical relationships between indices of environment and probability of tick establishment (Wilson, 1998)

Landscape ecology is the study of the dynamics of spatial heterogeneity, interactions among heterogeneous landscapes, effects of spatial heterogeneity on biotic processes, and the management of spatial heterogeneity. Spatial heterogeneity is the underlying mechanism that determines ecosystem structure and refers not only to the variables that describe the phenomenon being studied (*e.g.* vector density, disease cases, reservoir host presence, microhabitat conditions), but also to the nature of the spatial units themselves (e.g. administrative units, forested areas), their size, shape and configuration. Spatial dependency, on the other hand, deals with the relationship within spatially referenced data and is based on the notion that a stronger relationship exists when variables are located near each other

(spatial autocorrelation) (Turner & Gardner, 1990; Wilson, 1998; Kitron, 1998; Robinson, 2000; Westerberg & Wennergren, 2003).

Quantifying spatial heterogeneity and its dynamics has been facilitated by recent advances in computer processing and geographic information (GIS) technologies. Landscape pattern analysis involves four basic types of spatial data corresponding to different representation of landscape pattern: i) spatial point patterns, where geographic locations of sample sites are of primary interest (*e.g.*); ii) linear network patterns, like a map of streams, where collections of linear landscape elements intersect to form a network, with georeferenced display of nodes and corridors; iii) surface patterns, where quantitative measurements vary continuously across the landscape (*e.g.* digital elevation model, kriged surfaces); iv) categorical (or thematical) map patterns, where data is represented as a mosaic of discrete patches (*e.g.* map of land cover types) (McGarigal & Marks, 1995).

Geographic Information Systems (GIS) are computer-based tools that can rapidly combine, manipulate, display and analyse mapped information and data providing insight into complex spatial phenomena at multiple scales of resolution (Kitron *et al.*, 1992; Nicholson & Mather, 1996; Kitron & Kazmierczak, 1997; Kitron, 1998; Thomson & Connor, 2000). It includes spatial data (locations) in the form of geographic coverages (maps) and descriptive data (attributes) in the form of a relational database associated with the mapped features. Spatial data in a GIS include vector data (arc-based GIS) with points, lines and polygons (areas) and raster data (grid-based GIS) comprising regular arrays of pixels (picture elements), with characteristics of both point and area data (**Fig. 1.33**). Areas may consist of pixels (satellite images) or line vectors (traditional maps). The information required to geo-reference vector data includes the coordinate reference system (*e.g.* latitude and longitude), the reference units (*e.g.* meters) and either the coordinate position of the corners of the “bounding rectangle” (defining the boundary of the mapped area) or multiple reference control points. Grid-based images require geo-referencing by dx/dy coordinates for the centre of projection or the actual boundaries/edges of the image. This latter GIS is usually the most appropriate system for analysis of entomological and/or epidemiological data with environmental data (**Fig. 1.34**) (Thomson & Connor, 2000).

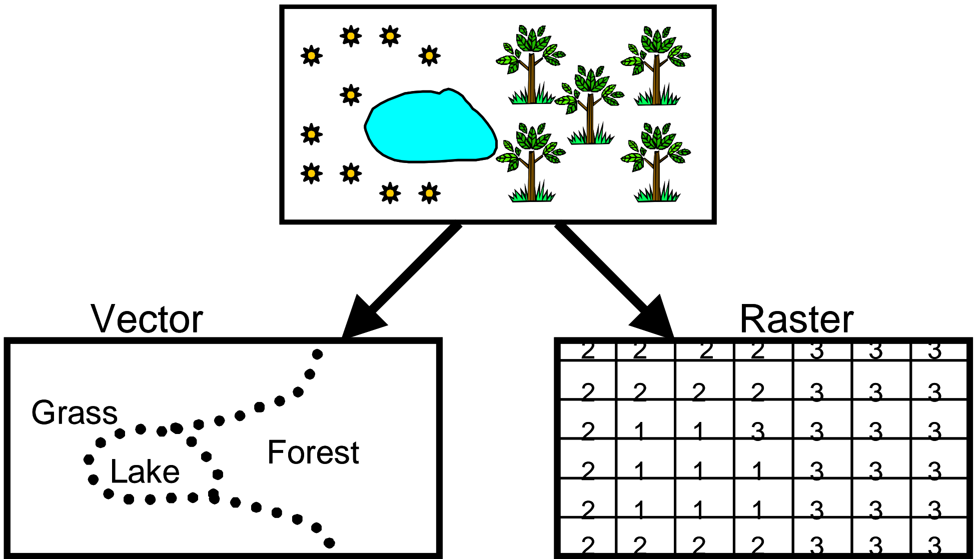


Figure 1. 33 – Illustration of the two principal methods for representing map information in a GIS: vector-based representation and raster (cell)-based representation (adapted from Coulson *et al.*, 1990)

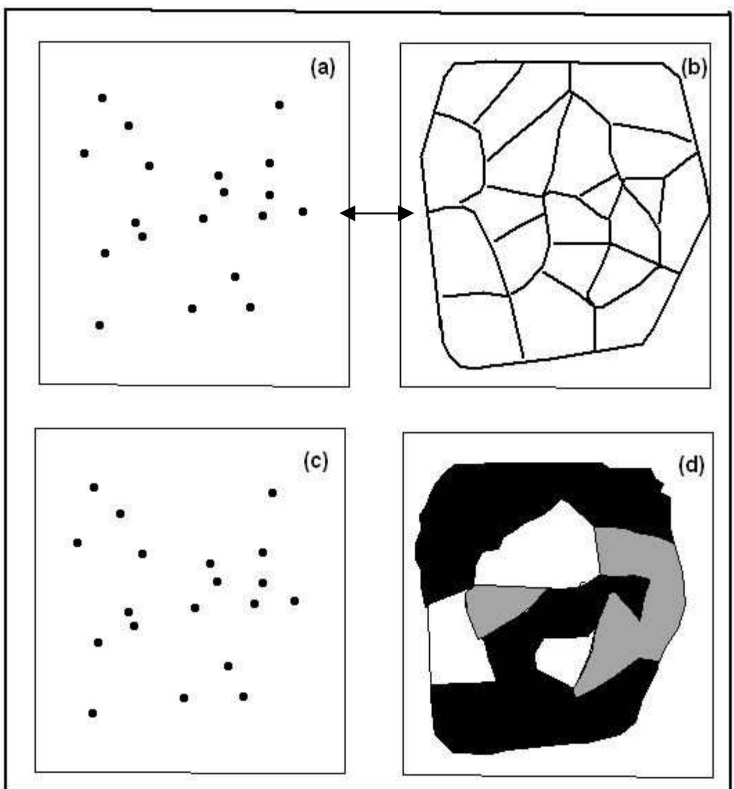


Figure 1. 34 - Different data types used in geographic information systems (GIS) and their inter-conversion: (a) point data, whose size is determined by the value of an attribute, (b) polygons (Thiessen polygon method can convert points to polygons), (c) and (d) raster format for points and polygons, respectively (Robinson, 2000)

With overlay analysis, two or more map layers (*e.g.* climate, vegetation type, soil pattern, population size) that are stored in a common planimetric projection and a similar geographic coordinate system are united to produce new geographic data that combines the attributes of the original information. The choice of a map projection depends on the size and shape of the area to be represented. Once the overlay process is complete, tabular data contained in the GIS can be exported to statistical packages where relationships between geographic data layers can be determined (Nicholson & Mather, 1996; Estrada-Pena, 1997; Kitron, 1998; Robinson, 2000; Thomson & Connor, 2000). Data transfer between different GIS software packages allows the performance of a variety of data manipulation and analysis tasks (Glass *et al*., 1992)

Analyses within GIS indicate that small- and large-scale ranges of hard tick species are determined more by climate and vegetation than by host-related factors. Spatial distributions of ticks can be analysed by statistical methods that seek correlations between known tick presence/absence and ground- or remotely-sensed (RS) environmental factors (Randolph, 2000; Brownstein *et al*., 2003). Sufficient ground observations are needed to derive predictive maps based on these correlations in order to understand the processes underlying the patterns of distribution, filling the gaps and updating the distributions as they shift with changing environmental conditions.

Habitat suitability models for tick distribution provides an essential first step toward a more precise estimate of the geographic distribution of Lyme disease and a stronger evidence base for determining human risk in specific endemic regions. By adding other related spatial data (infection rates, canine seroprevalence, host species composition, human case report data), these models can enable improved predictions of emerging risk, as well as aid in implementation of efficient control strategies and help target disease prevention efforts toward high-risk populations (Brownstein *et al*., 2003). Surveys, for testing habitat-relationship models require a minimum number of detections for a species and not a specified number of years. Given proper study design, it should be possible to obtain sufficient data to test habitat-relationship models for certain abundant species with one season of surveys. For other less abundant species, it may take many years of very specialized surveys to gather enough observations to make reliable estimates of model performance (Karl *et al*., 2000)

Mapping vector distributions on the basis of field collections can be extremely time consuming and therefore not likely to be performed over large areas. It is true that maps based solely on direct observation of tick density would provide little if any information on the underlying biotic and abiotic factors regulating tick populations; however these data are essential to gain a deeper understanding of

the spatial ecology of ticks and the diseases they vector. Analyses of tick abundance records can employ 3 fundamental approaches:

- standard statistical tests of habitat-specific differences in tick abundance (*e.g.* simple rank correlations, analysis of variance ANOVA) – they are helpful to establish associations, but depends on appropriate sampling designs to test specific hypothesis.
- Exploratory spatial data analysis (ESDA) coupled with geographical information systems (GIS) – permit application of conventional statistical approaches in a spatial environment.
- Statistical analysis of spatial patterns – address the spatial distribution of occurrences, amounts or rates, either at points or within areas (crowding indices, nearest neighbourhood analysis, Moran's I, kriging) (Wilson, 1998; Robinson, 2000)

By combining tick field observations with more ubiquous spatial data, in a GIS, statistical trends can be detected and predictive models developed. For instance, structure and composition of a landscapes can be related to the epidemiology of disease and therefore strong predictors of disease risk. Using the methods described above, even 3-dimensional surfaces can be generated to predict vector-borne disease risk beyond the spatial and temporal dimensions of field data, predicting potential risk for disease transmission (Glass *et al*., 1992, 1994; Washino & Wood, 1994; Hay *et al*., 1996; Nicholson & Mather, 1996; Vine *et al*., 1997; Kitron & Kazmierczak, 1997; Kitron, 1998; Estrada-Pena, 1998; Bavia *et al*., 1999; Tanser & Wilkinson, 1999). But one must remember that data used in prediction are often composed of a combination of continuous and categorical variables, so defining an index of potential risk may be difficult (Merler *et al*., 1996).

8.2 Modelling tools

Numerous statistical techniques have been used to predict species the potential distribution species by relating known species distributions to the spatial distribution of environmental variables. These techniques enable a probability of occurrence to be predicted in a location where no species information is known (Zaniewski *et al*., 2002)

Spatial statistics and geostatistics are tools that help to describe, explain, extrapolate, and predict the distribution of objects and processes in space. Geostatistics relate the spatial variation among population densities to the distance separating them. Incorporating geostatistical analysis into epidemiological studies, it is possible to model the spatial and temporal structure of

vector/host/pathogen systems where information is often autocorrelated (Nicholson & Mather, 1996; Kitron, 1998; Estrada-Pena, 1998; Thomson *et al.*, 1999; Robinson, 2000; Thomson & Connor, 2000). The choice of model is likely to be influenced by the aims of the study, the biology of the target organism, the level of knowledge the target organism's biology, and data quality (Robertson *et al.*, 2003)

Scale must be considered in the choice of spatial statistics. Accuracy of model predictions generally improved at coarse map scales because very large areas are considered and very fine local changes in presence and abundance are often ignored. Model predictions are sensitive to the resolution of the data used. For instance, a study on spatial autocorrelation in fine-scale distributions of *I. scapularis* demonstrated a lack of autocorrelation in *Ixodes* numbers because of higher variability of nymphal densities as compared with their distribution at both national and state scale, where strong spatial autocorrelation has been observed (Pardanani & Mather, 2004). Consequently, information needs and analytical approach for accurately predicting suitable habitat for a species differ with level of application.

Autocorrelation tests whether the observed value of a variable at one locality is significantly dependent on values of the variable at other localities. It assumes a Normal distribution of the data. Spatial autocorrelation is a method where repetitions of the sequence are found by computing a measure of self-similarity in the data. Strong similarities and dissimilarities between every point compared to every point will be evident. Comparisons are usually calculated for several lags (distance between any two points) and the resulting autocovariance (autocorrelation) is plotted in a correlogram (or semivariogram). This graphic shows the autocorrelation coefficients as a function of distance between pairs of localities and summarizes the patterns of geographic variability (Turner *et al.*, 1990).

Kriging, after Krige (1966 *cited in* Zhengquan *et al.*, 1997), is one common geostatistical technique that provides a means of accounting spatial relationships among data, interpolating values for unrecorded locations and calculating a measure of variance around estimated values. Estimates area using only the sample values of 1 variable, by a weighted linear combination of the known sample values around the point to be estimated. It also provides a means to understand and model the directional (e.g., north-south, east-west) trends of their data (anisotropy) (Nicholson & Mather, 1996; Zhengquan *et al.*, 1997; Estrada-Pena, 1997, 1998; Kitron, 1998; Robinson, 2000; Thomson & Connor, 2000

Finally, along with the rise of these new powerful statistical techniques and GIS tools, the development of predictive habitat distribution models has rapidly increased in ecology (Guisan & Zimmermann, 2000). Among some of the more commonly employed statistical techniques, are multiple regression models, generalized linear modeling (GLM) and generalized additive modeling (GAM) (Guisan & Zimmerman,

2000; Zaniewski *et al*., 2002; Hirzel & Guisan, 2002). The qualification of any model depends primarily on the goals of the study for which the model was and on the usability of the model, rather than on statistics alone (Guisan & Zimmerman, 2000).

Potential distribution maps can be defined in several ways, as cartographic representations of: 1. the *probability of occurrence* (*e.g*. from logistic GLMs), 2. the *most probable abundance* (e.g. from ordinal GLM); 3. the *predicted occurrence* based on non-probabilistic metrics (e.g. from CCA); 4. the *most probable entity* (e.g. from hierarchical considerations) (**Fig. 1.35**) (Guisan & Zimmerman, 2000).

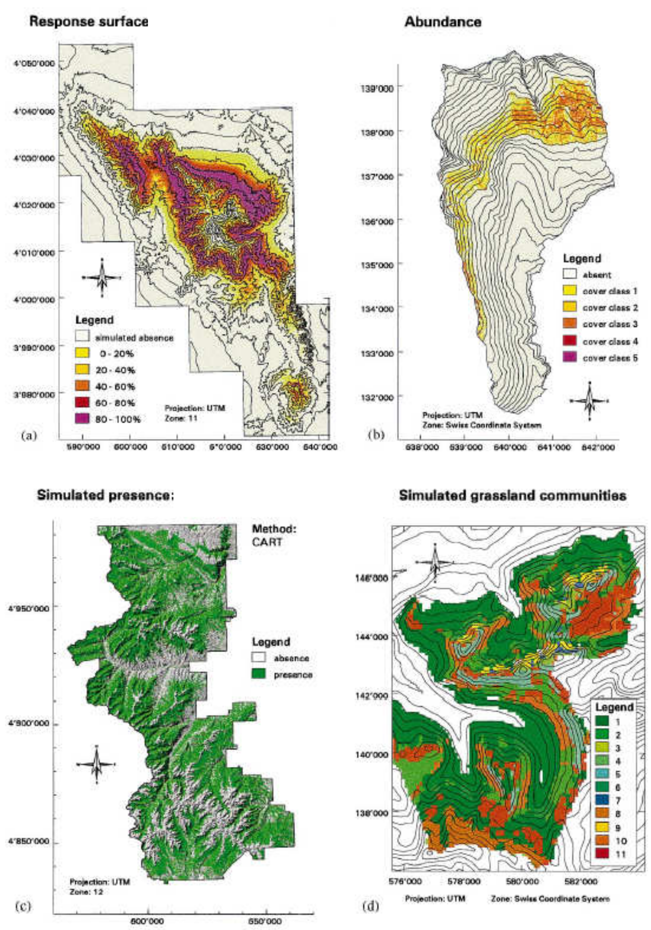
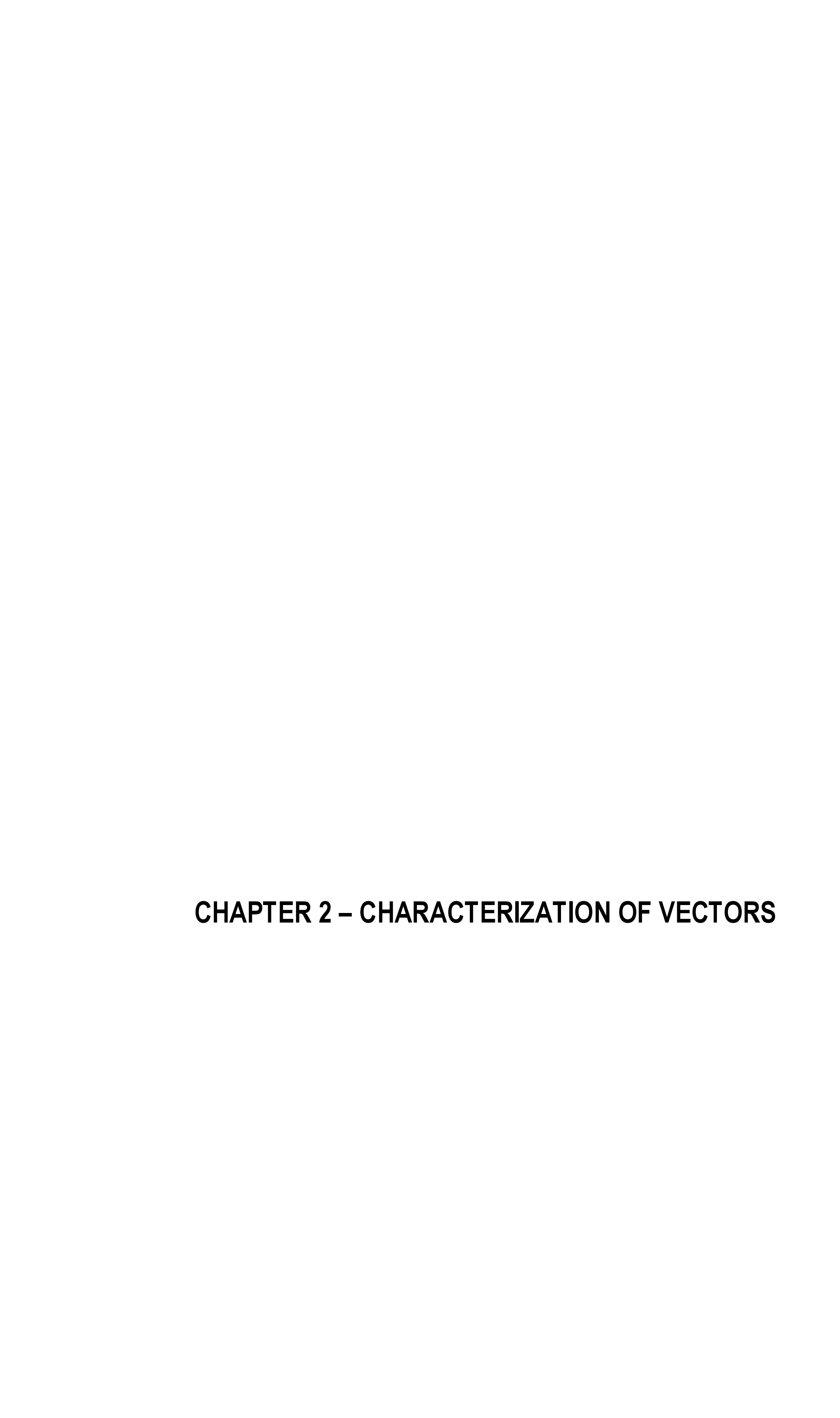


Figure 1. 35 – Examples of potential distribution maps (Guisan & Zimmerman, 2000)

All these computer tools will be used and tested against data collected to examine the spatial dynamics of Lyme Borreliosis occurrence in Portugal. Understanding the spatial distribution of vectors and disease is essential for management and will contribute to increase the knowledge of Portuguese epidemiologic situation.



CHAPTER 2 – CHARACTERIZATION OF VECTORS

CHAPTER 2 – CHARACTERIZATION AND DISTRIBUTION OF TICKS AS VECTORS

2.1 - Introduction

Transmission of *B. burgdorferi* sensu lato involves the presence of suitable tick vectors collected in habitats with appropriate environmental conditions. In Portugal, *I. ricinus* was found to be a competent vector for *B. burgdorferi* s.l. (Núncio *et al.*, 1993; Quaresma, 2003; Aires, 2004; Baptista *et al*, 2000, 2004), occupying different habitats, included in several climatic regions (Caeiro, 1999), based on the scarce studies on Portuguese ticks distribution (Caeiro, 1999; Estrada-Pena & Santos, 2004).

Within specific suitable habitats for the main vector *I. ricinus*, Tapada Nacional de Mafra (near Lisbon) was chosen for a **focal study** to evaluate the seasonal dynamics of this tick species, the main European vector of Lyme Borreliosis. At this site, besides monthly collection of ticks, climate variables were recorded and correlated with tick abundance for the first time in Portugal.

Based on the *I. ricinus* temporal pattern obtained from the focal study, a **nationwide approach** was initiated to understand the overall spatial distribution of questing *I. ricinus* in Portugal. The role of other collected ticks as potential vectors of LB causative agents was also surveyed.

2.2 - Objectives

a) (**Focal study**) To characterize the seasonal dynamics of the main *Borrelia burgdorferi* s.l. tick-vector *I. ricinus,* at Tapada Nacional de Mafra b) (**Nationwide study**) To quantify the overall spatial dynamics of this tick species and other potential vectors of LB agents nationally.

2.3 - Material and Methods

2.3.1 - Focal study (at Tapada Nacional de Mafra)

2.3.1.1. Location of study area

This study area, located 25 Km North of Lisbon, in Estremadura region (**Fig. 2.1**), is a Protected Area with dense forests of deciduous oaks (*Quercus faginea* Lam.), pinus (*Pinus pinaster* Act.), eucalyptus (*Eucalyptus globulus*, Labill.) and some chestnuts and planetrees. The herbaceous and arbustive extract is abundant and diverse (**Fig. 2.2**). There are numerous animal hosts, mainly *Gama gama* (fallow-deer), *Sus scrofa* (wild pig), some carnivorous and several species of small rodents. The climate is temperate and humid with moderate precipitation.

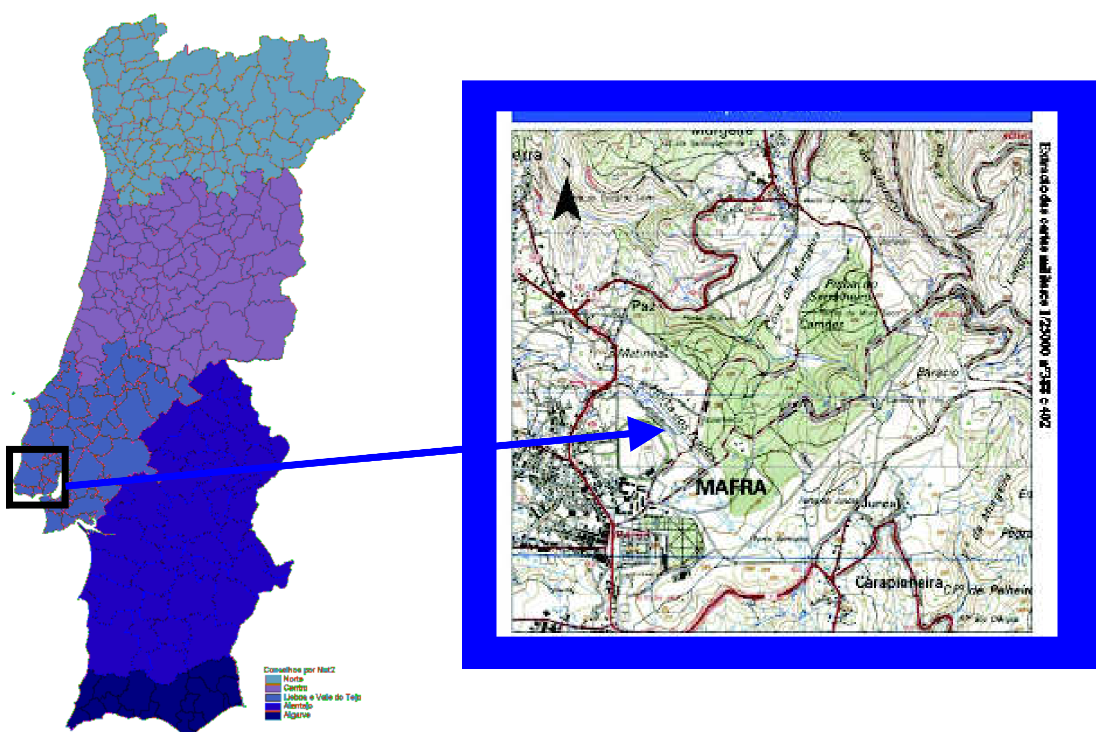


Figure 2. 1– Location of Tapada Nacional de Mafra, in Mafra county, Estremadura region, Portugal.

Figure 2. 2– Some of the habitats present at Tapada Nacional de Mafra (photos by the author)

2.3.1.2 Tick's collection and maintenance

From March 1999 to December 2004, monthly collections of questing ticks were made. Tick sampling was conducted using a one square meter white towel with a stick attached to the leading edge and pulled by string in transepts of 30 seconds for a total duration of one hour (dragging/flagging method)(**Fig. 2.3 A**). The cloth size allowed unrestricted passage across all types of vegetation and facilitated density calculations. Because of its weight, the stick permitted the entire cloth surface to be in contact with the vegetation (Vassalo *et al*., 2000). Using this approach, tick abundance was estimated as number of ticks collected per hour of sampling.

All ticks were collected between 2.00 and 4.00 p.m., stoked in small plastic tubes (**Fig. 2.3 B**) and taken to the Unit of Leptospirosis and Lyme Borreliosis (ULBL) at Institute of Hygiene and Tropical Medicine (IHMT). They were kept at 4ºC until further manipulation. In the laboratory, ticks were identified using both taxonomic keys (Núncio, 1988, Santos Dias, 1994, Estrada-Pena, 2000), and illustrations (Estrada-Pena, 2000; Habela, 2000; Estrada-Pena *et al*., 2004), prior to separation according to species, stage and gender with the help of a binocular stereomicroscope (Euromex Holland®) and finally counted (**Fig. 2.3 C**).

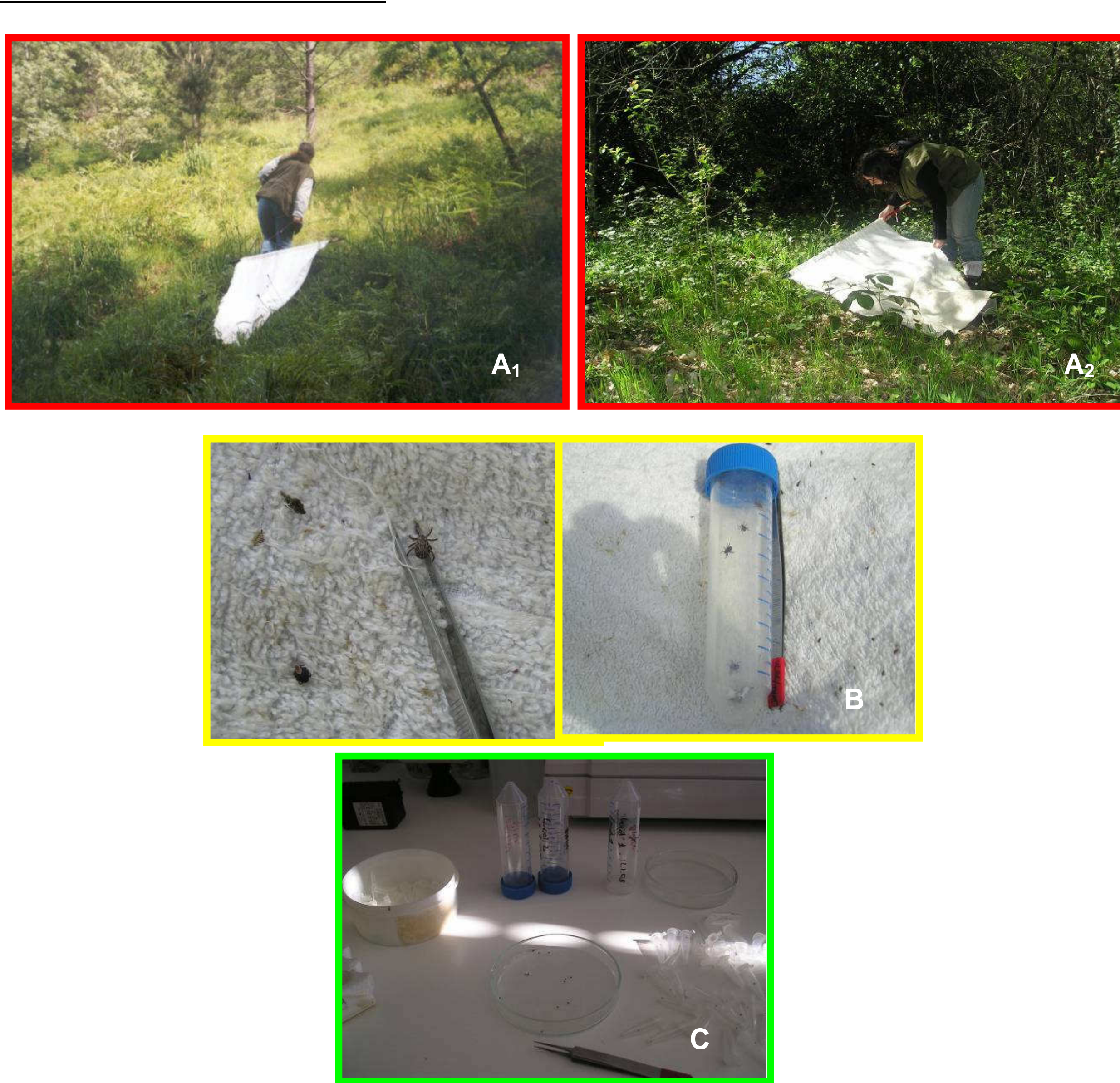


Figure 2. 3– Photographs illustrating collection (A_1 e A_2), preservation (B), counting and taxonomic separation of ticks (C) (photos of the author)

2.3.1.3 Data analysis

Monthly abundance was estimated for *I. ricinus* stages and for other tick species. This abundance was summarized as graphs of seasonal dynamics for each tick species and stage of interest. Statistical analysis of *I. ricinus* dynamics included univariate analysis ($\alpha = 0.05$) for comparison of frequencies (number of ticks/hour, according to Daniel *et al*, 1986; Vassalo *et al*., 2000) between stages and species (*STATISTICA* v. 6.0 or *SPSS* v. 11.0).

Seasonal data and forecasting of future patterns were studied with Time Series Analysis (TSA), using the Time series & Forecasting module of *STATISTICA* v. 6.0 package. A time series is a long sequence of observations that are ordered in equally spaced time intervals (Woolons & Norton, 1990). TSA analysis accounts for the fact that data points taken over time may have an internal structure (such as trend, seasonal variation, ciclicity and randomness) that should be accounted for. There are two main goals in TSA: a) obtain an understanding of the underlying forces and structure that produced the observed data and b) fit a model and proceed to forecasting (predicting future values of the time series variable. Both of these goals require that the pattern of observed time series is identified and more or less formally described. This methodology was used for quantify the developmental cycle of *I. ricinus* and forecast future trends. A detailed description of this analytical method and its basic structure are given in **Appendix 3**, for a better understanding of the analysis carried out.

Distributed Lag Analysis (DLA) is a specialized technique for examining the relationships between variables that involve some delay. There is an independent or explanatory variable (X) that affects the dependent variables (Y) with some lag. DLA allows the investigation of those lags, defining the period of time between two variables (STATSOFT, 1994; Sánchez-Lafuente *et al*., 2001). This methodology was used to estimate the lag time between developmental stages of *I. ricinus* thus allowing the estimation of the developmental cycle for this tick in Portugal.

Multiple Regression (*SPSS* v. 11.0 or *STATISTICA* v. 6.0) was applied to determine important climate variables (*e.g*. temperature, humidity and precipitation) influencing the seasonal dynamics (Gray, 1981). These climate variables were collected by a climate station located inside the limits of Tapada Nacional de Mafra (Eng. José Ferreira, *in litt*). Distributed Lag Analysis was also attempted to study time relations between climate variables and tick stages.

2.3.2 - *Nationwide* study

2.3.2.1 Climate (general overview)

The characterization of Portugal climate and landscapes has been the purpose of several studies. The climate of Portugal may be considered oceanic along the coast and in the Northern inlands, and Mediterranean in the Southern part of the country. The annual average temperature varies

between 16ºC and 26ºC in summertime, and 3ºC and 14ºC in the winter season. Mean precipitation varies from 350 mm in the South to 1,600 mm in the North. In the mountains precipitation can reach 3,000 mm. Atmospheric humidity is related to precipitation and shows mean values between 65% and 70% and sometimes in wintertime 85% (Caeiro, 1999).

According to Rivas-Martinez (1981), four bioclimatic stages can be found on the Iberian Peninsula (**Fig. 2.4**). The most widespread bioclimatic stage, Mesomediterranean stage (in yellow), is characterized by annual mean temperatures between 11 and 16ºC, with long winters (up to 5 months). In the northwest littoral, Eurossiberian or Oromediteranean stage (in red), communities of vegetation experience low temperatures (<11ºC) and high precipitation (between 650 to more than 1600mm). The Supramediterranean stage (in green) includes the highest mountain in Portugal, Serra da Estrela, and is characterized by temperatures from 8 to 12ºC and rigorous long winters (median temperatures between 0 and –3ºC). Finally, along the coast of south and southwest coast of Portugal, reaching Lisbon (in blue), the thermomediterranean stage is the bioclimatic stage with higher temperatures (more than 16ºC). In all of these bioclimatc divisions, vegetation distribution is conditioned by precipitation (ombroclimates), soil and topography.

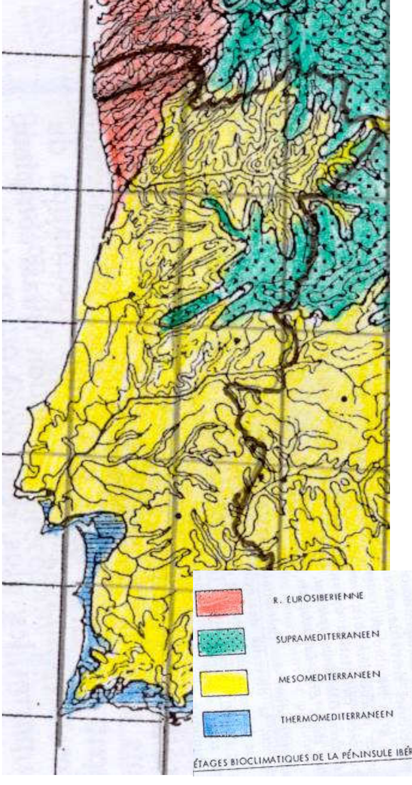


Figure 2. 4– Bioclimatic stages in Portugal (adapted from Rivas-Martinez, 1981)

On the other hand, Manique de Albuquerque (1957) described five types of climate – Atlantic, Mediterranic, Continental, Alpine and Tropical – that interchange in several ways, constituting a multiplicity of climates. This author created an Ecological Chart composed of 23 ecological zones characterized by different average values of temperature and precipitation. These zones are represented by symbols, depending on the influence of each type of climate mentioned above: OA – Oro Atlante, A – Atlantic, MA – Mediterranic-Atlantic, AM – Atlantic-Mediterranic, SM – Sub-Mediterranic, IM – Ibero-Mediterranic, I – Iberic, M – Mediterranic, A^M – Thermo Atlantic Mediterranic, SÂ – Thermo Sub-Atlantic (**Fig. 2.5**).

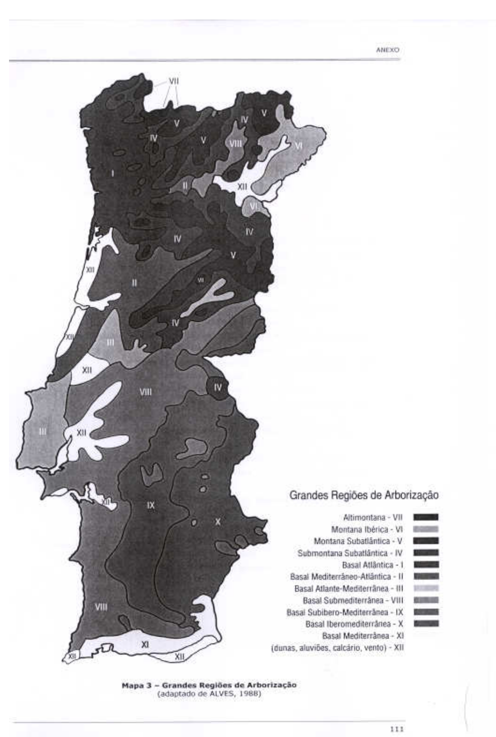


Figure 2. 5– Distribution of Ecological zones in Portugal country

These ecological zones have been used to sustain the forestation of the main trees species in Portugal. In 1999, one third of the territory was covered by forests, totaling 3 467 000 ha. The main forest species is *Pinus pinaster*, followed by evergreen oaks (*Quercus suber* and *Quercus ilex*) and eucalyptus. Deciduous oaks, including *Q. robur, Q. pyrenaica, Q. faginea* and recently introduced *Q. rubra*, occur mainly in the north, and in high mountain areas in the central part of the country. *Q. robur* occurs predominantly in north coastal areas, where the climate has a stronger Atlantic

influence, while *Q. pyrenaica* is found mainly in the interior, with a Mediterranean climate. *Q. faginea* is scattered throughout all the country, with subspecies *faginea* in the north and subspecies *broteroi* in the center and south (Chambel *et al.*, 1999). These species occur often in small areas and mixed stands, with *Pinus* spp., *Castanea sativa* and *Juglans regia*, although several large pure stands have also been reported. The main shrub species are *Ulex europaeus*, *Erica umbellata*, *Rosmarinus officinalis*, etc. (Caeiro, 1999).

2.3.2.2 Sample sites

Prior to the selection of the sampling sites, a search was made on literature and digital databases for information considered important in the bioecology of *Ixodes ricinus*. Printed information about distribution of Portuguese forests was obtained from Forests Agency and Hunting Agency (Engº Vitorino Lopes – *personal comm.*). A digital map depicting Corine Land Cover was obtained from Centro Nacional de Informação Geográfica (CNIG), now called Instituto Geográfico Português (www.igeo.pt). This map, at 1: 100000 scale and dated from 1985/87 presents information on land use cover including distribution of urban, agricultural, forested areas and wetlands. Because of its detailed information it was primarily used in the selection of sample points (**Fig. 2.6**).

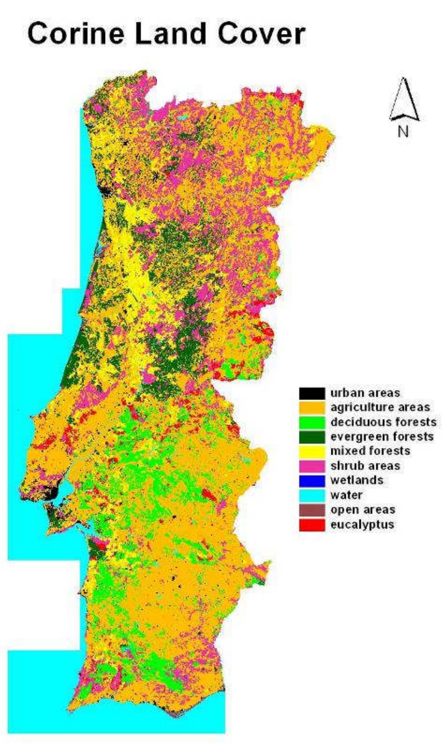


Figure 2. 6 – Map depicting the distribution of major land cover types in Portugal (source www.igeo.pt)

Along with Corine Land Cover, a globe map from DATASCAPE™, an application for *ArcView* 3.x (ESRI, 1999), was a source of general spatial information on political borders, road system (IPs and secondary roads), and localities from Portugal. All geographic data were transformed to Bonne projection.

Using *ArcInfo* or *ArcGis 8.0* software, twenty-two major population centers were identified around Portugal (**Fig. 2.7**). Using these centers, areas or polygons of interest (Thiessen or Voronoi Polygons) were calculated around each location. Each point selected within a given polygon is closer to the associated population center than to any other center (Robinson, 2000; Caeiro *et al.*, 2003). The major centers identified in analysis included **Braga** and **Venda Nova** (Minho region), **Bragança, Vila Real** and **Vila Nova de Foz Côa** (Trás-os-Montes region), **Porto** (Douro Litoral region), **Viseu** (Beira Alta region), **Coimbra** and **Leiria** (Beira Litoral region), **Guarda** and **Castelo Branco** (Beira Baixa region), **Rio Maior** and **Lisboa** (Estremadura region), **Abrantes** and **Coruche** (Ribatejo region), **Portalegre** (Alto Alentejo region), **Évora, Grândola, Aljustrel** and **Mértola** (Alentejo region), **Monchique** and **Loulé** (Algarve region).

Within each Thiessen polygon at least one site was chosen for collect of ticks and further environmental analysis. In this way, all geographic areas within Portugal were assured sampling, and observations at sample points could be summarized by Thiessen Polygon and associated population centers. Thus, with the estimation of tick abundance and infection with *Borrelia* at each site, populations at risk to LB could be identified.

According to the types of vegetation more important for *I. ricinus*, the attributes coniferous forests, deciduous forests and shrub landscape were defined from Corine Land Cover map (**Fig. 2.8**). All the roads were manually identified according to a 2000/01 edition of an ACP Road Map. Before beginning fieldwork every polygon was worked in *ArcView* 3.2 to layout a picture ready to use, with zoomed data on roads and vegetation (**Appendix 4**).

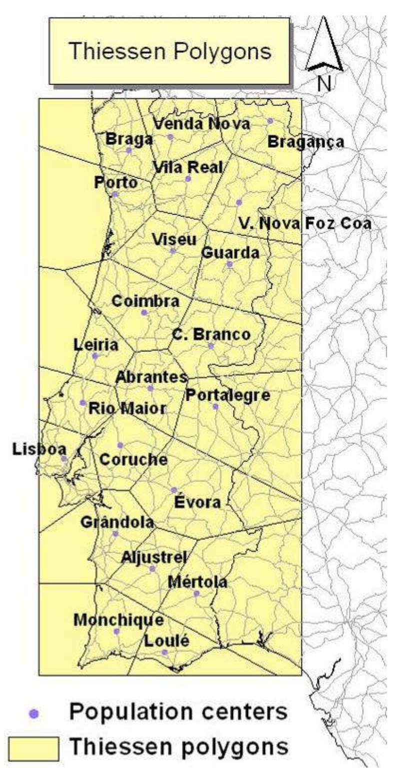


Figure 2. 7 – Thiessen polygons created around the 22 population centers

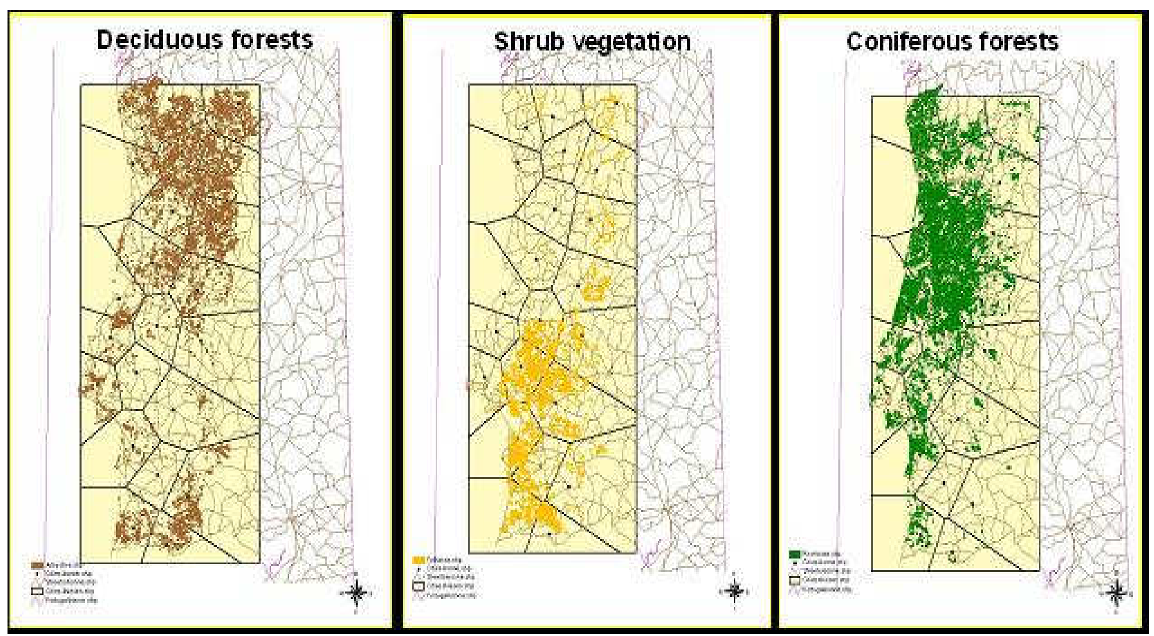


Figure 2. 8 – Vegetation types used for habitat assessment of *I. ricinus* ticks

All aspects of the above maps were visually confirmed in the field. The primary choices for sample points were type of vegetation (variety/density of trees and herbs), accessibility, exposure (forest cover), and/or presence of hosts in the neighbourhood. A field book was filled (**Appendix 4**) characterizing the habitat (Daniel *et al*., 1998; Gray *et al*., 1998 fide Estrada-Pena, 2001) according to some variables described in **Table 2.1** along with date of collections, period of time spent and geographic references of the sample site (GPS coordinates, number of road, nearest localities or nearest milesotones). In relation to soil drainage, this feature was visualized in the terrain according to three main types of soil of the litter layer, namely sandy soils, clay soils and rocky soils. The first is characterized by high to medium permeability and the last one by a low or none permeability (Fernandes, 1994). Although a suitable methodology was necessary to define correctly the type of soil and percentage of each constituent, a simple visual discrimination was performed.

For each sample point, the coordinates (latitude and longitude) were measured with a MAGELLAN® GPS device and the Temperature and Humidity measured in the air and near the ground with a Termohygrometer. Photos were taken for the majority of sites.

Table 2. 1– Ecological and entomological variables measured in each sample location.

Variable Name	Description
Vegetation (ordinal variable)	*Categories defined by EUCALB (2000)*
(1) Coniferous forests (eucalyptus and pinus) without secondary vegetation	A Yes or No variable, depending on its predominance
(2) Coniferous forests with secondary vegetation	A Yes or No variable, depending on its predominance
(3) Mixed forests with predominance of coniferous	A Yes or No variable, depending on its predominance
(4) Deciduous shrubs with short shrubs and young trees	A Yes or No variable, depending on its predominance
(5) Homogeneous deciduous trees (oaks and others)	A Yes or No variable, depending on its predominance
(6) Mixed forests dominated by deciduous trees and highly heterogeneous deciduous forests with several ecotones	A Yes or No variable, depending on its predominance
(7) Others (e.g. grassland)	A Yes or No variable, depending on its predominance
Soil (ordinal variable)	*Categories defined visually in the field by the author*
(1) Good drainage	A Yes or No variable, depending on its predominance
(2) Reasonable drainage	A Yes or No variable, depending on its predominance
(3) Bad drainage	A Yes or No variable, depending on its predominance
Type of soil	*Descriptive variable*
Exposition (Forest Cover) (ordinal variable)	*Categories defined visually in the field by the author*
(1) Wide exposition (open space)	A Yes or No variable, depending on its predominance
(2) Reasonable exposition (some shadow)	A Yes or No variable, depending on its predominance
(3) Weak exposition (lots of shadow)	A Yes or No variable, depending on its predominance
Temperature	*Measured in the field by the author*
Air Temperature	Average temperature for each sample site
Soil Temperature	Average temperature for each sample site
Humidity	*Measured in the field by the author*
Air Humidity	Average humidity for each sample site
Soil Humidity	Average humidity for each sample site
Human Use	*Categories defined visually in the field by the author*
(1) Abandoned Forest	A Yes or No variable, depending on its predominance
(2) Hunting Area	A Yes or No variable, depending on its predominance
(3) Agricultural zone edge	A Yes or No variable, depending on its predominance
(4) Habitation zone edge	A Yes or No variable, depending on its predominance
(5) Public garden / Picnic area	A Yes or No variable, depending on its predominance
(6) Others	A Yes or No variable, depending on its predominance
Ticks	*Collections in the field by the author*
Collection of ticks	A Yes (Presence) or No (Absence) variable
Ticks per hour (collection effort)	Estimated density of all tick stages for each sample site and for each tick genera
Hosts	*Categories defined visually in the field by the author*
Presence confirmed visually or through remains	A Yes or No variable with identification of host

2.3.2.3 Tick collection and maintenance

The spatial study at a national level took place from 2001 to 2004. In 2001, the nationwide survey was carried out from March to July, which coincided with the major peak of activity observed for *I. ricinus* in the focal-study. As already said, collections of ticks were attempted at least at one sample point per Thiessen polygon. Also, in most of the sites, there was more than one visit in subsequent years (2002 and 2003). In those sites with the presence of *I. ricinus* and *Borrelia* positive ticks, a fourth visit was carried out during 2004.

The already described (see 2.3.1.2) dragging/flagging methods were used for collecting questing ticks in vegetation, with one person in the years 2001, 2002 and 2004, and two persons (the author and a training student) in 2003 (= two white towels). At least a 15 to 30 minutes period was spent in each site. Collections were made at different periods of the day, depending on the time spent driving between each sample site (from 10 a.m to 18 p.m). Ticks were put in separated plastic tubes with moist plasticine or a piece of vegetation, in a refrigerated box, to keep them alive until arrival at ULBL lab (in Lisbon). Again, they were counted and separated by genus and/or species (whenever possible), stage and gender and kept at 4ºC until posterior manipulation for detection of *Borrelia* species, by culture and DNA amplification (see Chapter 3).

2.3.2.4 Data analysis

The number of ticks per site was calculated as a “collecting effort” defined by the number of ticks collected per hour (all 15 to 30 minutes were extrapolated to 60 minutes) per person (1 or 2, depending on the years). This was thus possible to compare all sites regardless the number of visits and periods of collection. The collections at Tapada Nacional de Mafra (TPN) were also included in this nationwide study as a specific sample site. For this and for two other long studies performed in Grândola and Coruche regions, namely Herdade da Ribeira Abaixo (HRA) and Águas de Moura (AM), respectively, the following methodology was adopted:

- Mafra - data from March 2002, 2003 and 2004, 1 person/3 hours
- HRA - data from 9 months (October 2002 to June 2003), 1 person/9 hours
- AM – data from 12 months (January 2003 to December 2003), 1 person/12 hours.

Comparison of results for every sampling site was done by Qui-Square analysis, for number of ticks and presence/absence of species per site.

Data exploration on soil occupation pretends to determine the influence of these variables in the tick population densities. Using tick presence as the independent variable, linear regression modelling for each studied variable will be performed in the chapter 4.

Prediction of unsampled values for numerical field variables of air and soil temperature and air and soil humidity were studied by geostatistical techniques (kriging). Semivariograms were constructed for each variable. These calculations were performed with GS^+ for Windows v. 5.0 (Gamma Design Software), which provides easy access to geostatistical computationally intense analysis.

Semivariance is evaluated by calculating γ (*h*) for all possible pairs of points in the data set (defined as variable Z) with or without transformation and assigning each pair to a lag or distance interval class h. The graph of all h's *vs* all semivariances for each interval class in the analysis constitutes the variogram (or semivariogram).

There are several functions used to model semivariance. The random model has a slope = 0 and demonstrates no spatial structure in the measured variable. The linear model has a constant slope and indicates a continuous gradient in the measured variable. The similar exponential and spherical models have a lag distance over which there is spatial structure in the variable (Range Ao) and no spatial structure beyond it (**Fig. 2.9**). These models have certain important characteristics: (i) it shows the nature of the geographic variation in the property of interest, and (ii) it is needed to provide kriged estimates at previously unrecorded points (Golaszewski, 2002).

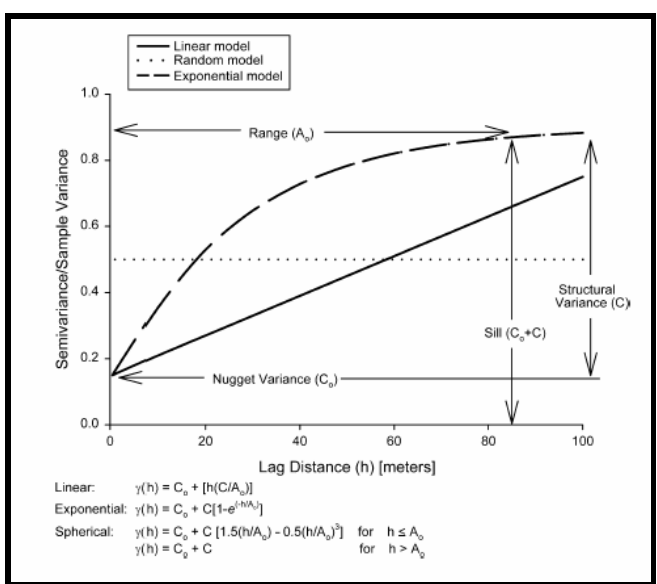


Figure 2. 9 – Example semivariogram showing functions used by geostatistical programs to model semivariance (γ) as a function of the lag distance (h).

Each model creates specific values for Nugget variance (Co), Sill (Co+C) and Range (Ao). *GS+*, for instance, also gives three statistics to aid the interpretation of model output:

- Residual Sum of Squares (RSS) – provides an exact measure of how well the model fits the variogram data; the lower the RSS, the better the model fits;
- r2 – provides an indication of how well the model fits the variogram data, although not as sensitive or robust as the RSS;
- Proportion C/(Co+C) – this statistic provides a measure of the proportion of sample variance (Co+C) that is explained by structural variance C. This value will be 1.0 for a variogram with no nugget variance (where the curve passes through the origin) indicating that spatial dependence is very low and spatial pattern occurs mainly at scales smaller than the average distance in the first lag interval. It will be 0 where spatial dependency is very high at the range specified (Zhengquan *et al.*, 1997)

In the larger "Interpolation Window" of *GS+* 5.0, kriging based on the underlying spatial relationships provided by semivariograms offers a mean of interpolating values for points not physically sampled.

Description of data, normality of data, and comparisons between categories within each variable (Qui-Square and T-Student Tests) were analysed with *SPSS* v.11.0 or *STATISTICA* v.6.0

2.4 - Results

2.4.1 - Focal tick-study (at Tapada Nacional de Mafra)

2.4.1.1 Collections

A total of 7139 ticks were collected during the 5-year period. Of these, 6800 (95.2%) belonged to *Ixodes ricinus* ticks distributed by 271 females (4.0%), 285 males (4.2%), 4452 nymphs (65.5%) and 1792 larvae (26.3%) (**Table 2.2**). These proportions varied through the five years. Years 2002 and 2003 were the most productive (18.3 and 40.1%, respectively), due to the high number of larvae and nymphs in each year (X^2=4021.56, g.l=15, $p<0.05$)

Table 2. 2– Total annual numbers of *I. ricinus* ticks collected during the 5-year study at Tapada Nacional de Mafra (legend: F – females, M – males, N – nymphs, L – larvae)

	F	M	N	L	TOTAL
1999	114	117	202	0	**433**
2000	61	62	577	235	**935**
2001	25	30	511	75	**641**
2002	10	16	333	888	**1247**
2003	47	42	2535	103	**2727**
2004	14	18	294	491	**817**
TOTAL	**271**	**285**	**4452**	**1792**	**6800**

Monthly numbers of ticks (**Fig. 2.10**) were different among stages and gender of *I. ricinus*, with highly and moderate significant differences (K-W, $p<0.01$ and $p<0.05$, respectively). Relatively to this, a sex ratio of 1:1 was found in this population of Mafra.

During the five-year period, other tick species were captured with the following frequencies per collected species: 138 *Haemaphysalis punctata*, 102 *Rhipicephalus sanguineus*, 88 *Dermacentor marginatus*, 10 *Ixodes hexagonus* and 1 *Hyalomma marginatum*, in a total of 339 ticks (**Table 2.3**)

Table 2.3- Total annual numbers of *Haemaphysalis* spp (Haem), *Rhipicephalus* spp (Rhip) and *Dermacentor* spp (Derm) collected during the 5-year study at Tapada Nacional de Mafra, according to gender and stages (legend: F – females, M – males, N – nymphs, L – larvae)

Haem	F	M	N	L	TOTAL
1999	5	2	0	0	**7**
2000	5	4	19	1	**29**
2001	1	5	9	0	**15**
2002	0	1	12	28	**41**
2003	2	5	32	0	**39**
2004	0	3	4	0	**7**
TOTAL	**13**	**20**	**76**	**29**	**138**

Rhip	F	M	N	L	TOTAL
1999	11	7	0	0	**18**
2000	5	14	2	0	**21**
2001	5	7	0	0	**12**
2002	0	7	5	20	**32**
2003	5	0	0	0	**5**
2004	9	5	0	0	**14**
TOTAL	**35**	**40**	**7**	**20**	**102**

Derm	F	M	N	L	TOTAL
1999	29	35	0	0	**64**
2000	6	3	0	0	**9**
2001	1	4	0	0	**5**
2002	0	3	0	0	**3**
2003	1	2	3	0	**6**
2004	1	0	0	0	**1**
TOTAL	**38**	**47**	**3**	**0**	**88**

Similar numbers between (adult) females and males were found for all the three species. Nymph collections were lower than adults for *Rhipicephalus* and *Dermacentor*, but only the former was significantly different (t♀=2.35 p=0.02; t♂=2,86, p=0.004). *Haemaphysalis* had more nymphs than adults (t♀ =-4.08, p=0.00008; t♂=-3.63, p=0.0004). The other two species had insufficient numbers to perform a proper statistical analysis.

2.4.1.2 Seasonal Dynamics

I. ricinus

Seasonal dynamics of *I. ricinus* ticks was studied during a period of 68 months, within the 5 year-study. Some months (November and December in 1999, May and September in 2001, August and December in 2002 and July in 2003) were not surveyed for several reasons (rainy months, trainings abroad, etc). Using Time Series Analysis, an interpolation of missing values was performed to give the total pattern of this tick species for the period studied (**Fig. 2.11**).

An apparent unimodal cycle was dominant for all stages. Except for larvae, this cycle extends for a large number of months, beginning at the end of autumn until the mid of summer, with a major peak in spring (March-April). Larvae have only one short single peak at the end of spring (May-July). In some years (2001 for females and males, 2002 for larvae and 2003 for nymphs and males), a subdivision of the major peak can be observed, with a small decrease of tick numbers to increase again during the following month.

In 2002, a great increase in the larvae population was seen, followed by a correspondent increase in nymphs in 2003. Although not so evident as in the first year (1999), a small increase in the average number of females was also observed and probably was responsible for the second highest peak of larvae in 2004.

When averaging values between all years (small graphs within **Figure 2.10**), it is possible to predict the human risk to bites of each *I. ricinus* developmental stage. Nymphs show a distinct unimodal pattern, beginning at September and ending before August. Females and males show a similar pattern and it can be described also as a unimodal cycle beginning at September, with a small decrease between December and January, increasing afterwards until April-May and decreasing before August. Larvae, as already referred, occur mainly during warmer months, preferring the late spring and mid summer.

Considering the total number of individuals (juveniles and adult stages), it is possible to define a risk period for contact with this tick species. *I. ricinus* ticks can occur all year reaching average values between 14 ticks/hour (in September) to as high as 200 ticks/hour in the Spring.

When analysing the correlation between groups (Spearman Rank Correlation), it is possible to draw some conclusions. Females correlate positively with males ($r=0.79$, $p=0.00$) and nymphs ($r=0.66$, $p=0.00$), but negatively with larva ($r=-0.35$, $p=0.03$); Males correlate with all, in the way as females. Nymphs correlate positively with females and males.

Time Series Analysis was applied to each stage to model seasonality and forecast future trends. Seasonal data plots, autocorrelation (ACF) and partial autocorrelation (PACF) graphs, ARIMA

models (p and q), normality of data and forecast plots were produced (**Fig. 2.11 and 2.12**). Data were analysed by their residual behaviour, with inspection of the respective correlograms to identify the most suitable ARIMA models. For the purpose of analysis, a lag length of 12 months was used. Some methodological criteria used in this analysis can be seen in **Appendix 3**.

Female and male seasonal data were smoothed (2-moving average) to reduce irregularities (residuals). Stationary (constant mean and variance and autocorrelation through time) was then assessed from an autocorrelation plot (ACF) or correlograms The ACF and PACF for females and males (**Figs. 2.11/2.12 A, B**) showed that only lags 1 and 2 were positively autocorrelated (cut-off), decaying exponentially afterwards. With this, p and q terms of ARIMA model were identified, suggesting that a first order autoregressive and moving average models would be appropriate – AR(1)+MA(1). Thus the model suggested was an ARIMA (1,0,1) (**Figs. 2.11/2.12 D**). The residuals were not autocorrelated (**Figs. 2.11/2.12 E**) and had a normal distribution (**Figs. 2.11/2.12 G**). It was also possible to forecast the evolution of these populations, where both females and males maintain low densities, but without a tendency to either increase or disappear (**Fig. 2.11/2.12-H**).

Nymphs seasonality was more pronounced and because of this, several procedures were attempted. Two transforming methodologies were tried - a smoothing (2-moving average) and a natural logarithm (**Fig. 2.13**). In both, the ACF had a sine-wave shape pattern and PACF plots spiked at lags 1 and 2, with no correlation for other lags (except a small one at lag 5). An ARIMA model with a AR(2) was proposed. Their residuals were not autocorrelated but they did not follow a normal distribution. Forecasting of data showed a similar pattern to the last months, with a small peak and a decrease afterwards

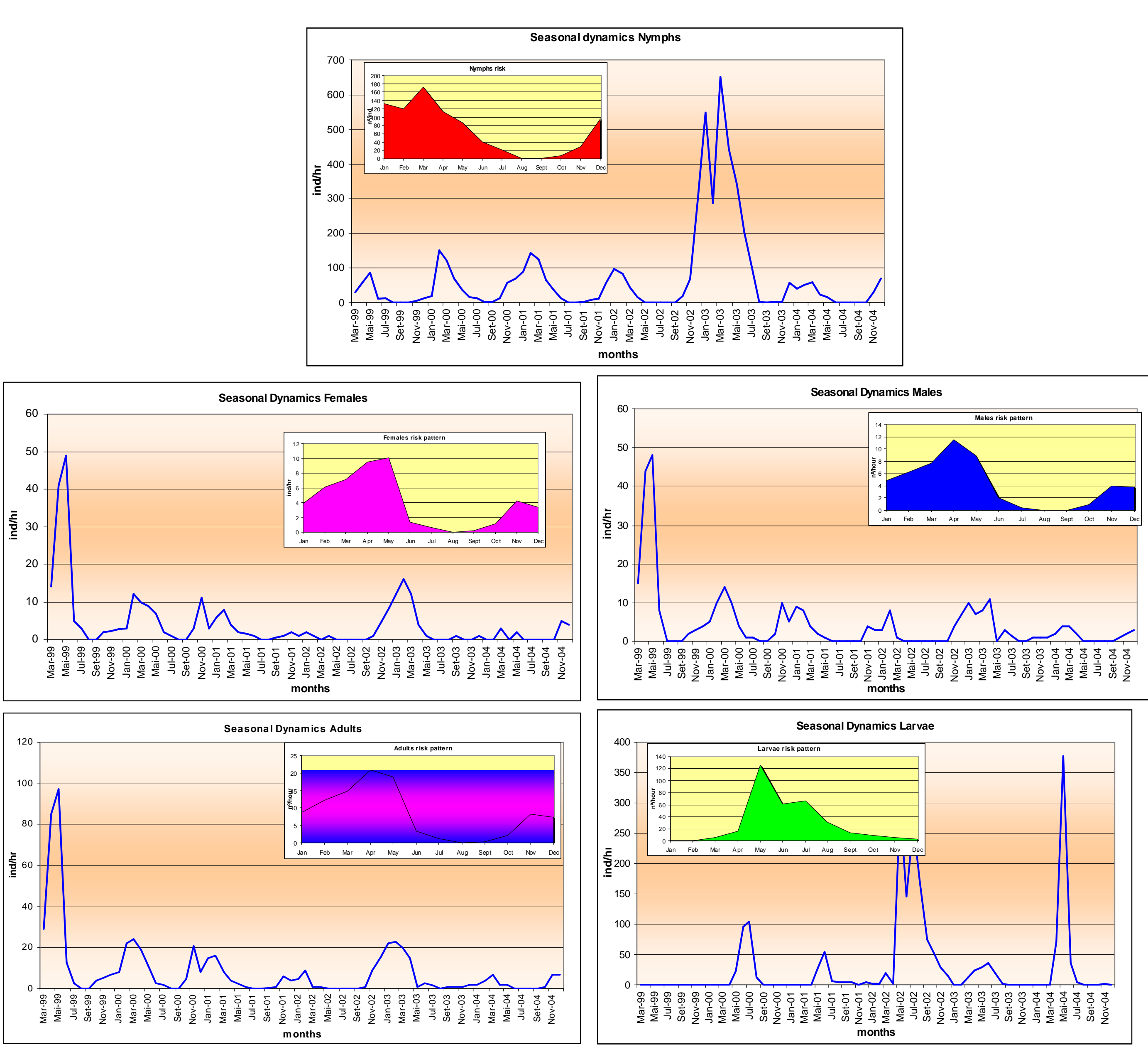
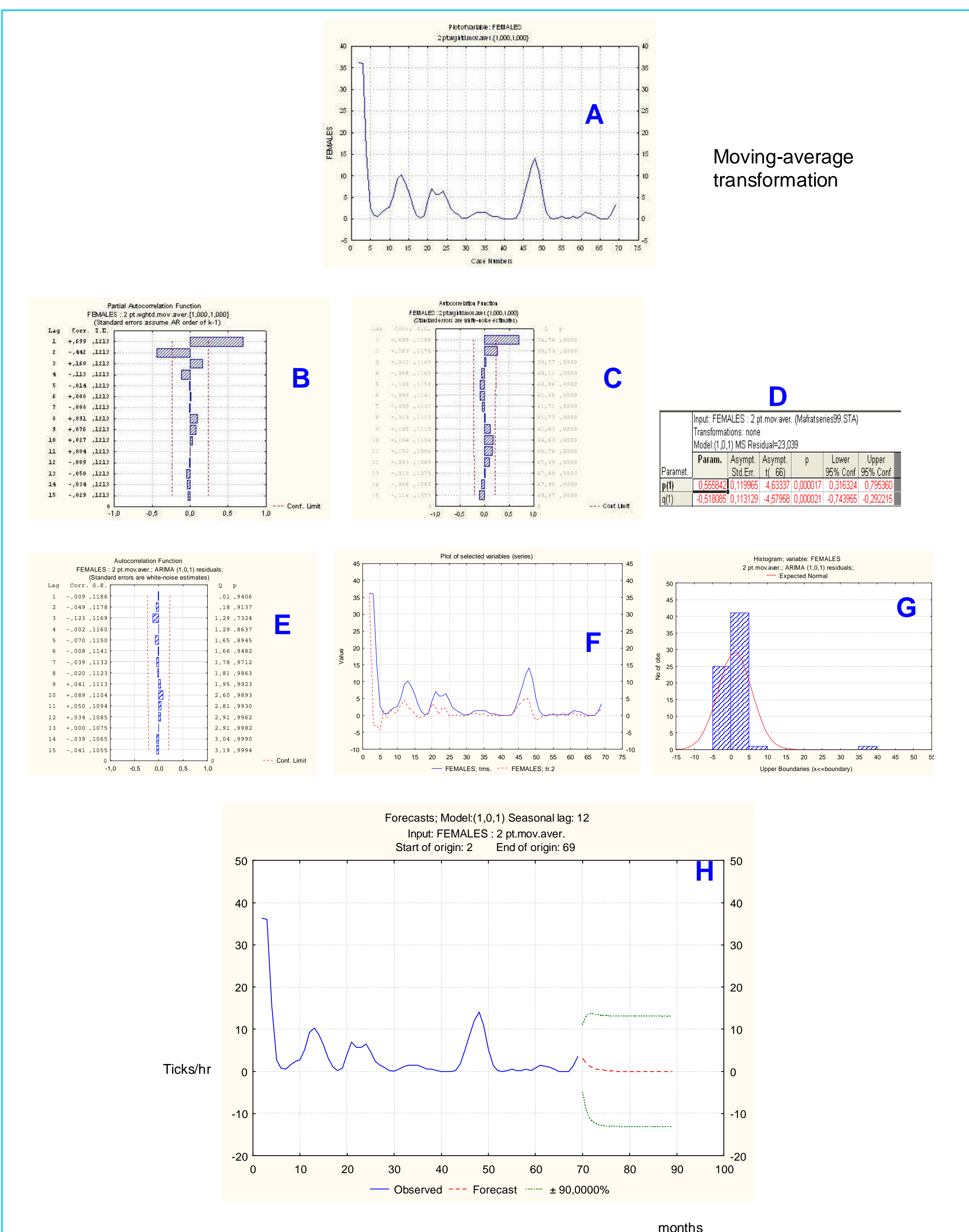


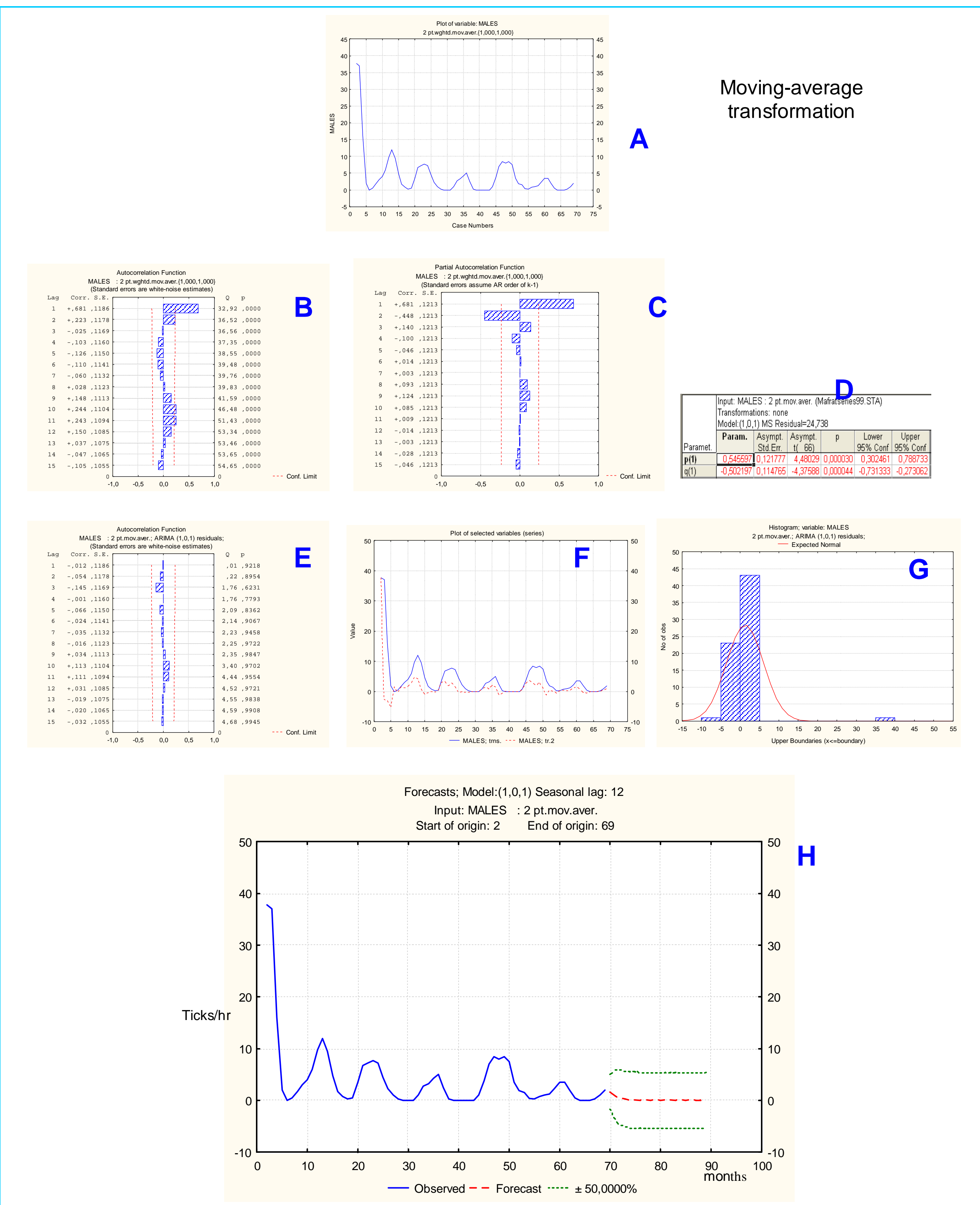
Figure 2. 10 – Seasonal dynamics of the different stages (larvae, nymphs, adults) and gender (females, males) of *I. ricinus* ticks at Tapada Nacional de Mafra from March 1999 to December 2004. Small graphs within each plot present the specific risk pattern for each developmental stage or gender.



Input: FEMALES : 2 pt.mov.aver. (Mafratseries99.STA)
Transformations: none
Model:(1,0,1) MS Residual=23,039

Paramet.	Param.	Asympt. Std.Err.	Asympt. t(66)	p	Lower 95% Conf	Upper 95% Conf
p(1)	0,555842	0,119965	4,63337	0,000017	0,316324	0,795360
q(1)	-0,518085	0,113129	-4,57958	0,000021	-0,743955	-0,292215

Figure 2. 11 – Time Series Analysis for female population at Tapada Nacional de Mafra (1999-04). A – seasonal plot (transformed data), B – ACF plot, C – PACF plot, D – ARIMA model (variables), E – residuals ACF plot, F – Original and residual data plots, G – Histogram (normality), H – Forecast plot



Input: MALES : 2 pt.mov.aver. (Mafratseries99.STA)
Transformations: none
Model:(1,0,1) MS Residual=24,738

Paramet.	Param.	Asympt. Std.Err.	Asympt. t(66)	p	Lower 95% Conf	Upper 95% Conf
p(1)	0,545597	0,121777	4,48029	0,000030	0,302461	0,788733
q(1)	-0,502197	0,114765	-4,37588	0,000044	-0,731333	-0,273062

Figure 2. 12 – Time Series Analysis for male population at Tapada Nacional de Mafra (1999-04). A – seasonal plot (transformed data), B – ACF plot, C – PACF plot, D – ARIMA model (variables), E – ACF plot (residuals not autocorrelated), F – Original and residual data plots, G – Histogram (normality), H – Forecast plot

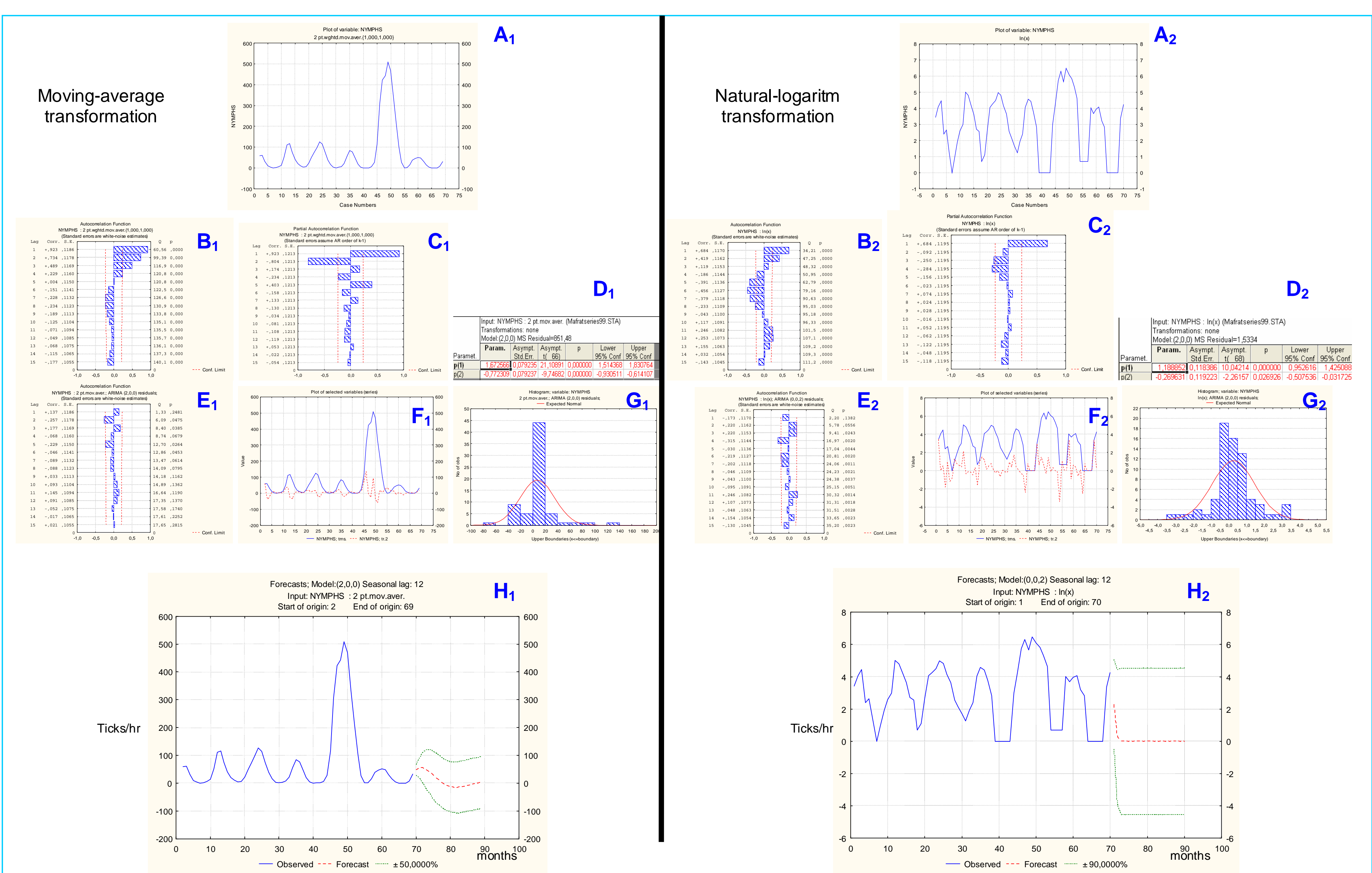


Figure 2. 13 – Time Series Analysis for nymph population at Tapada Nacional de Mafra (1999-04). $A_{1,2}$ – seasonal plot (transformed data), $B_{1,2}$ – ACF plot, $C_{1,2}$ – PACF plot, $D_{1,2}$ – ARIMA model (variables), $E_{1,2}$ – ACF plot (residuals not autocorrelated), $F_{1,2}$ – Original and residual data plots, $G_{1,2}$ – Histogram (normality), $H_{1,2}$ – Forecast plot.

Because of pronounced seasonality of nymphs, a seasonal (p=12) autoregressive term (meaning an ARIMA model with a AR12) was applied to both of these transformed variables. Seasonality is maintained but with a more pronounced effect, especially in log transformed data (**Fig. 2.14**).

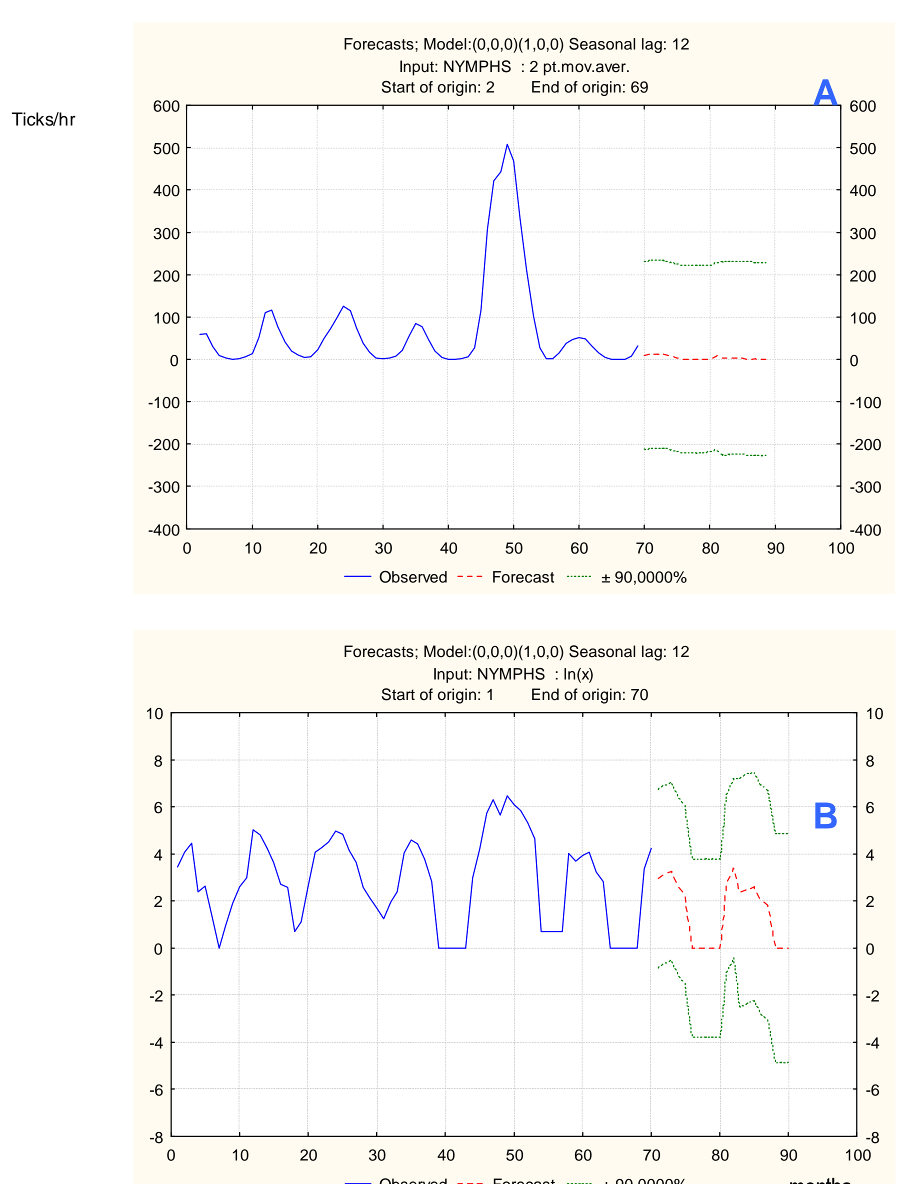
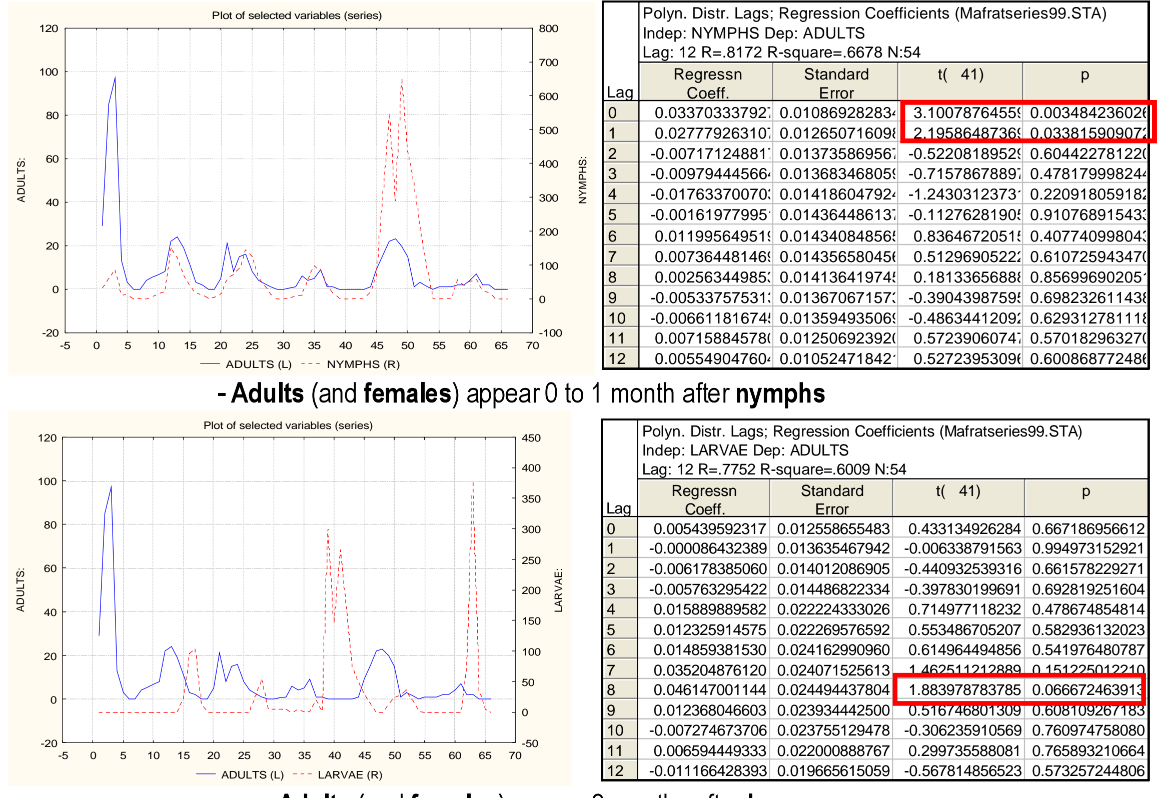


Figure 2. 14 – Forecast plots for nymph population using a 2-moving average smoothing (A) and a logarithmic transformation (B) including a seasonal autoregressive term (AR12).

Larvae population was also analyzed but no model was obtained. Residual data were always autocorrelated, thus not following the premises for time series analysis.

Distributed Lag Analysis (DLA) was performed between *I. ricinus* stages, defining independent and dependent variables (STATSOFT, 1994; Sánchez-Lafuente *et al.*, 2001). For instance, when nymphs are the independent variable and females the dependent one, DLA show when females appear in relation to the appearance of nymphs. This lag is obtained by observing the t value that is statistically significant (p>0.05) or at least the lowest p. Analysis was performed between each possible pair – nymphs/adults, nymphs/females, larvae/adults, larvae/females, larvae/nymphs. **Fig. 2.15** presents some examples of this technique.



Polyn. Distr. Lags; Regression Coefficients (Mafratseries99.STA)
Indep: NYMPHS Dep: ADULTS
Lag: 12 R=.8172 R-square=.6678 N:54

Lag	Regressn Coeff.	Standard Error	t(41)	p
0	0.033703337927	0.010869282834	3.100787645559	0.003484236026
1	0.027779263107	0.012650716098	2.195864873669	0.033815909072
2	-0.007171248817	0.013735869567	-0.522081895529	0.604422781220
3	-0.009794445664	0.013683468059	-0.715786788897	0.478179998244
4	-0.017633700703	0.014186047924	-1.24303123731	0.220918059182
5	-0.001619779951	0.014364486137	-0.112762819005	0.910768915433
6	0.011995649519	0.014340848565	0.836467205155	0.407740998043
7	0.007364481469	0.014356580456	0.512969052222	0.610725943470
8	0.002563449853	0.014136419745	0.181336568888	0.856996902051
9	-0.005337575313	0.013670671573	-0.390439875595	0.698232611438
10	-0.006611816745	0.013594935069	-0.486344120922	0.629312781118
11	0.007158845780	0.012506923920	0.572390607477	0.570182963270
12	0.005549047604	0.010524718421	0.527239530096	0.600868772486

- **Adults** (and **females**) appear 0 to 1 month after **nymphs**

Polyn. Distr. Lags; Regression Coefficients (Mafratseries99.STA)
Indep: LARVAE Dep: ADULTS
Lag: 12 R=.7752 R-square=.6009 N:54

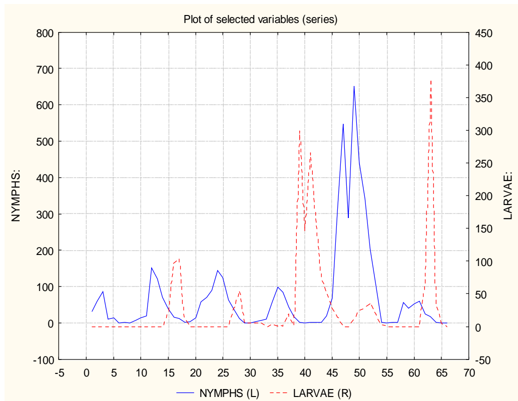
Lag	Regressn Coeff.	Standard Error	t(41)	p
0	0.005439592317	0.012558655483	0.433134926284	0.667186956612
1	-0.000086432389	0.013635467942	-0.006338791563	0.994973152921
2	-0.006178385060	0.014012086905	-0.440932539316	0.661578229271
3	-0.005763295422	0.014486822334	-0.397830199691	0.692819251604
4	0.015889889582	0.022224333026	0.714977118232	0.478674854814
5	0.012325914575	0.022269576592	0.553486705207	0.582936132023
6	0.014859381530	0.024162990960	0.614964494856	0.541976480787
7	0.035204876120	0.024071525613	1.462511212889	0.151225012210
8	0.046147001144	0.024494437804	1.883978783785	0.066672463913
9	0.012368046603	0.023934442500	0.516746801309	0.608109267183
10	-0.007274673706	0.023755129478	-0.306235910569	0.760974758080
11	0.006594449333	0.022000888767	0.299735588081	0.765893210664
12	-0.011166428393	0.019665615059	-0.567814856523	0.573257244806

- **Adults** (and **females**) appear 8 months after **larvae**

Polyn. Distr. Lags; Regression Coefficients (Mafratseries99.STA)
Indep: FEMALES Dep: LARVAE
Lag: 12 R=.2762 R-square=.0763 N:54

Lag	Regressn Coeff.	Standard Error	t(41)	p
0	1.86183781577	5.683430005948	0.327590524353	0.744888227106
1	-4.22265562066	7.566159469570	-0.558097623721	0.579812769220
2	4.82141335350	7.480236430717	0.644553604442	0.522806664458
3	-2.21209323596	7.515861871159	-0.294323295701	0.769995894426
4	4.78786451519	7.061649928064	0.678009326993	0.501574518487
5	-0.75655986654	6.762371072342	-0.111877898808	0.911466166839
6	-0.72694640703	6.876483570172	-0.105714846784	0.916324129308
7	0.14953966166	6.916228176086	0.021621562773	0.982854719495
8	2.57826148084	6.830558459001	0.377459836749	0.707778850757
9	-1.85954520357	5.590470629395	-0.332627667122	0.741110154091
10	0.98816123301	3.247144307238	0.304317005810	0.762425659995
11	-1.39118784592	3.095605010376	-0.449407415110	0.655504622486
12	0.84076775754	2.211444175584	0.380189455751	0.705767298744

- **Larvae** appear 4 months after **females** (not statistically significant)



Polyn. Distr. Lags; Regression Coefficients (Mafratseries99.STA)
Indep: LARVAE Dep: NYMPHS
Lag: 12 R=.9812 R-square=.9627 N:54

Lag	Regressn Coeff.	Standard Error	t(41)	p
0	-0.018308252491	0.068149587887	-0.26864802940	0.789547755339
1	0.015604751974	0.073992914456	0.21089522001	0.834013736208
2	-0.014018771724	0.076036638578	-0.18436864104	0.854633173184
3	-0.059090364905	0.078612792046	-0.75166348081	0.456546837242
4	0.027246469002	0.120600420865	0.22592349849	0.822383407111
5	0.195852788975	0.120845935236	1.62068164388	0.112752765499
6	0.113756197194	0.131120554923	0.86756952227	0.390679638952
7	0.669723770236	0.130624217896	5.12710262327	0.000007445089
8	1.198592778584	0.132919152380	9.01745728229	0.000000000028
9	-0.188843810734	0.129880335906	-1.45398307924	0.153563747874
10	0.789031581449	0.128907293163	6.12092273517	0.000000291141
11	0.210650431610	0.119387899814	1.76442027993	0.085110616867
12	-0.143206395858	0.106715528872	-1.34194523863	0.186998584111

- **Nymphs** appear 7-8 months and sometimes 10 months after **larvae**

Figure 2. 15 – Time lag differences between *I. ricinus* stages using Distributed Lag Analysis (DLA).

With these lag differences it was possible to hypothesize a developmental cycle for *I. ricinus* population at Tapada Nacional de Mafra, with a duration of 1 year to 1,5 year between the appearance of the first year larvae population to the second year larvae population (**Fig. 2.16**).

7-10months (L→N)+
0-1months (N→F)+
4months (F →L)
≈ 1-1,5 years

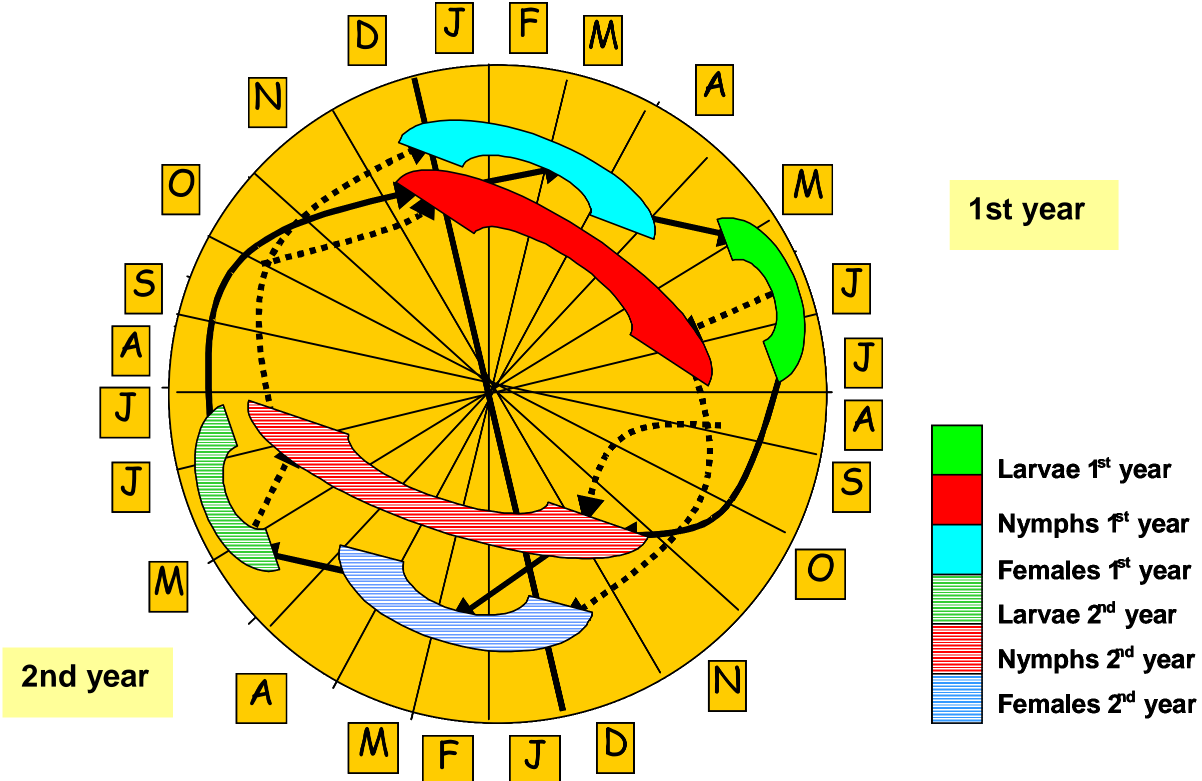


Figure 2. 16 – Schematic depicting the developmental cycle for *I. ricinus* at Tapada Nacional de Mafra during the 5-year study, from 1999 to 2004. Bold arrow is the more common cycle and split arrow is the parallel cycle.

As can be seen in the previous figure, females of the first year give rise to larvae in the same year with a lag time of 4 months. For instance, eggs from females appearing in cold season (December/January) hatch as soon as weather conditions permits it and the first larvae begin to quest at May. These larvae molt to nymphs 7 to 10 months latter (beginning at November until July), being responsible for the second year nymph population. Usually it is not possible that larva emerging in May be able to become an active nymph in the same year (Vassalo *et al.*, 2000a), but if temperatures are warm enough, this may occur (Randolph *et al.*, 2002).

Nymphs begin to appear in the autumn season, either from larvae of the preceding year (7-10 months earlier) or from the previous nymph population that may have suffered a diapause (probably behavioural) during the hotter months and began questing again at this colder season. A portion of nymphs develop to

females almost immediately (0 to 1 month latter) between the same year populations, but females can also derived from the previous year nymph population.

These cycles and periods between stages were confirmed by the actual dynamic cycle. As it can be seen by the extent of the coloured arrows in **Fig. 2.17**, emergence peaks for each stage are in agreement, in the majority of years, with those lags determined by Distributed Lags Analysis methodology.

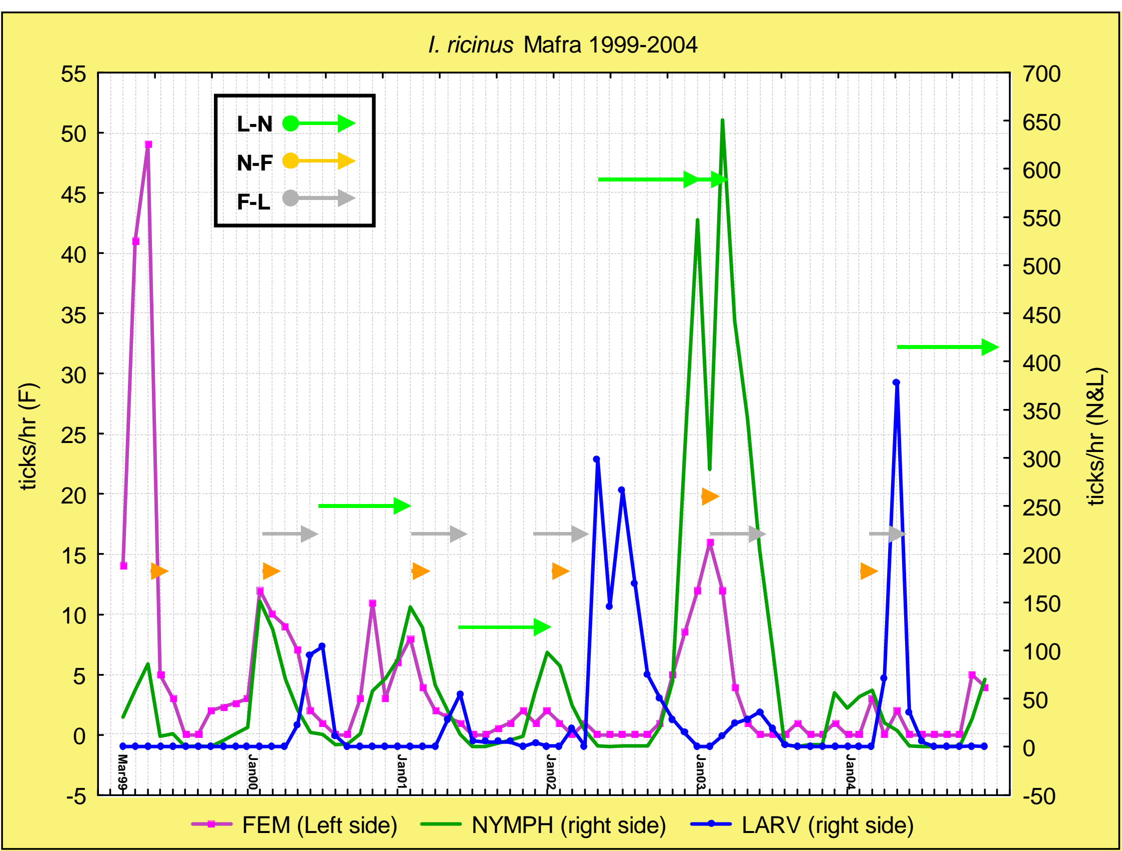


Figure 2. 17 – Grouped seasonal dynamics of females, nymphs and larvae with reference to time lags between each emergence peak stage (coloured arrows).

Climate variables were also analysed. **Fig. 2.18** shows Temperature, Humidity and Precipitation values between November 1999 and May 2004. Previous values were not possible due to the inexistence of the referred climate station and latter values were not obtained mainly because of logistic reasons. Temperature and Precipitation variables showed seasonality with annual peaks more or less pronounced. Humidity is a more stable variable with values ranging from 61 to 94%.

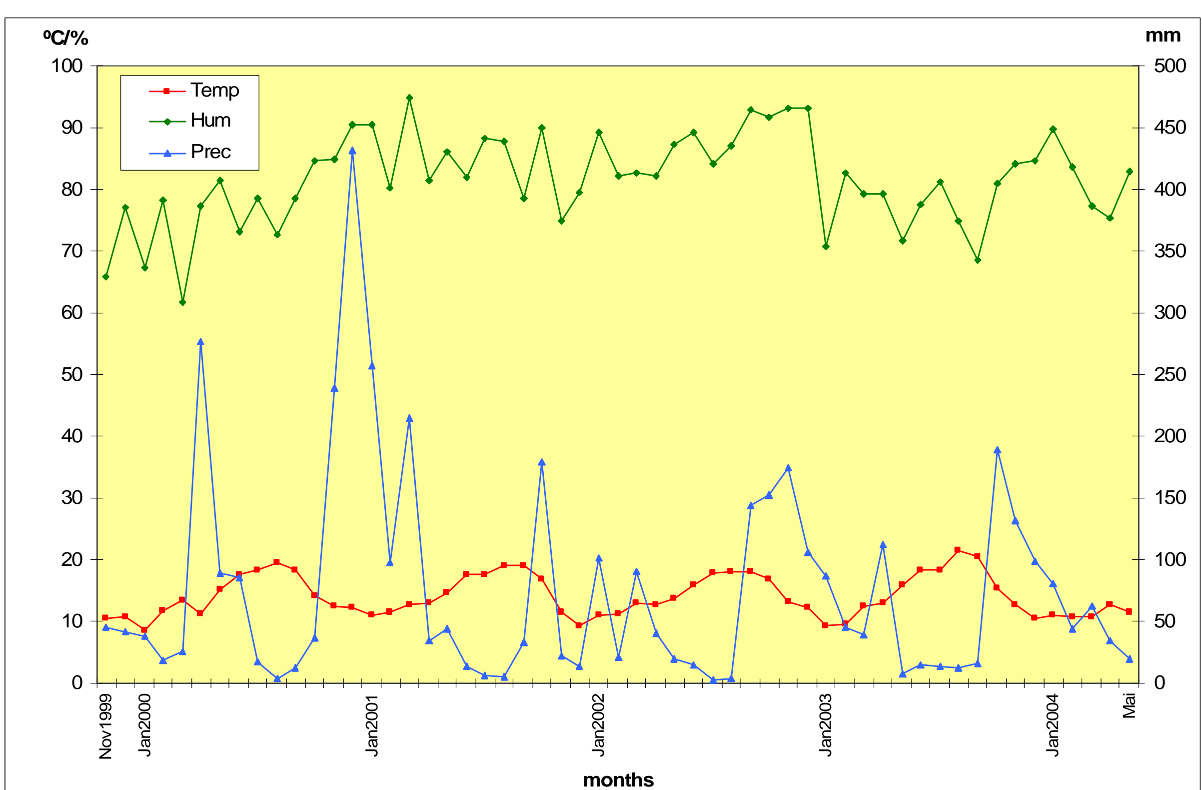


Figure 2. 18 – Temperature, Humidity and Precipitation values at Tapada Nacional de Mafra from November 1999 to May 2004.

Multiple regression was used to relate these variables with *I. ricinus* stages (**Tables 2.4**). Results for total ticks and each gender or developmental stage were as follows: (i) for the number of **total ticks**, there is no influence of these variables; (ii) Temperature and Humidity have a negative correlation for the number of **females**; (iii) Temperature and Humidity have a negative correlation with **males**. Precipitation has a positive correlation; (iv) Temperature has a negative correlation with **nymphs;** Precipitation has a negative correlation with **larva.**

Table 2. 4 – Multiple regression between climate variables and *I. ricinus* developmental stages (ticks/hour) from Tapada Nacional de Mafra between November 1999 and May 2004

N=48	Regression Summary for Dependent Variable: Totalticks (R= .32766579 R^2= .10736487 Adjusted R^2= .04650338 F(3,44)=1.7641 p<.16795 Std.Error of estimate: 152.52					
	Beta	Std.Err. of Beta	B	Std.Err. of B	t(44)	p-level
Intercept			551.8163	280.4155	1.96785	0.055407
Temp	-0.293607	0.147487	-13.8253	6.9448	-1.99073	0.052744
Hum	-0.117601	0.159029	-2.5272	3.4175	-0.73949	0.463535
Prec	-0.117403	0.163907	-0.2040	0.2848	-0.71628	0.477605

N=48	Regression Summary for Dependent Variable: FEMALES (R= .54889427 R^2= .30128492 Adjusted R^2= .25364525 F(3,44)=6.3242 p<.00116 Std.Error of estimate: 3.5769					
	Beta	Std.Err. of Beta	B	Std.Err. of B	t(44)	p-level
Intercept			23.94572	6.576225	3.64126	0.000711
Temp	-0.400905	0.130487	-0.50039	0.162869	-3.07237	0.003637
Hum	-0.318972	0.140699	-0.18169	0.080146	-2.26706	0.028353
Prec	0.258570	0.145014	0.01191	0.006679	1.78307	0.081474

N=48	Regression Summary for Dependent Variable: MALES (I R= .70753460 R^2= .50060521 Adjusted R^2= .46655557 F(3,44)=14.702 p<.00000 Std.Error of estimate: 2.7929					
	Beta	Std.Err. of Beta	B	Std.Err. of B	t(44)	p-level
Intercept			28.85173	5.134856	5.61880	0.000001
Temp	-0.485889	0.110316	-0.56013	0.127171	-4.40451	0.000067
Hum	-0.440488	0.118949	-0.23174	0.062579	-3.70316	0.000591
Prec	0.368735	0.122598	0.01569	0.005215	3.00768	0.004341

N=48	Regression Summary for Dependent Variable: NYMPHS (N R= .39356815 R^2= .15489589 Adjusted R^2= .09727515 F(3,44)=2.6882 p<.05789 Std.Error of estimate: 134.50					
	Beta	Std.Err. of Beta	B	Std.Err. of B	t(44)	p-level
Intercept			664.4792	247.2873	2.68707	0.010134
Temp	-0.313273	0.143507	-13.3694	6.1244	-2.18298	0.034416
Hum	-0.248407	0.154737	-4.8381	3.0137	-1.60534	0.115573
Prec	0.029065	0.159483	0.0458	0.2511	0.18225	0.856227

N=48	Regression Summary for Dependent Variable: LARVAE R= .35811008 R^2= .12824283 Adjusted R^2= .06880484 F(3,44)=2.1576 p<.10657 Std.Error of estimate: 77.317					
	Beta	Std.Err. of Beta	B	Std.Err. of B	t(44)	p-level
Intercept			-218.258	142.1506	-1.53540	0.131846
Temp	0.068937	0.145752	1.665	3.5205	0.47298	0.638569
Hum	0.284646	0.157159	3.138	1.7324	1.81120	0.076940
Prec	-0.342145	0.161979	-0.305	0.1444	-2.11228	0.040374

Distributed Lag Analysis was also used in this analysis to understand the relation between climate variation and tick emergence, confirming also the Multiple Regression results. To simplify this methodology, females and males were grouped as adult stage. Some conclusions were obtained, although not statistically significant.

- **Adults** emerge 6 months after the annual peak of Temperature, when values are lower; 4 months after the Humidity highest values, appearing when moisture values decrease abruptly, 0 to 2 months after annual peak of precipitation, with the same pattern or when Precipitation values decrease.
- **Nymphs** emerge 3 months after Temperature peaks; when Humidity reaches higher values (0 months lag); 2 to 4 months after Precipitation peaks.
- **Larvae** emerge at the same time as Temperatures reaches its highest values (0 months lag); when Humidity values are higher but also after 8 months of the latter peaks; 7 months after Precipitation peaks, when values are low.

The highest peak of larvae seen in 2002 and in 2004 coincided with the highest values of Humidity. On the other hand, the nymphal peak in 2003 followed the lowest peak in Temperature values.

Other tick species

Although there was a clear discrepancy between numbers of *I. ricinus* and other tick populations, it was possible to observe some seasonality for *R. sanguineus* and *D. marginatus*. The first species has two main emergences, one at the end of winter until spring and other during summer. On the other hand, *D. marginatus* is a characteristic spring tick. *H. punctata* has a more irregular pattern and is present all year (**Fig. 2.19**).

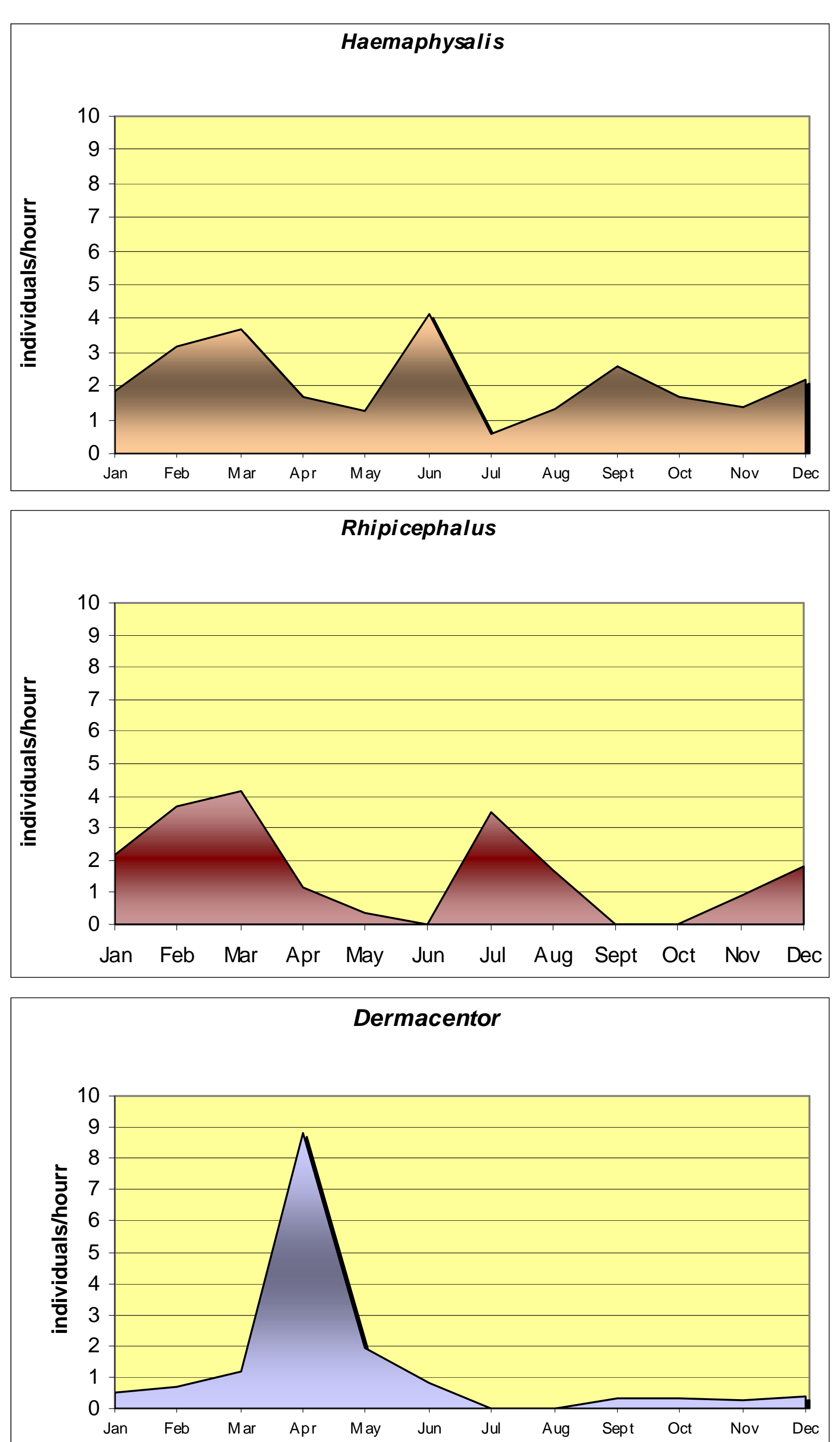


Figure 2. 19– Seasonal dynamics of other tick species (total number of individuals of *Haemaphysalis*, *Rhipicephalus* and *Dermacentor* spp) at Tapada Nacional de Mafra from 1999 to 2004.

2.4.2 - Nationwide study

2.4.2.1 Habitat assessment

Extensive field characterization of each sampling point, from a total of 55 (**Fig. 2.20)** is presented at **Appendix 4**, following the field categories described earlier. Records of each site with photographs and general descriptions for the majority of the sample points are also presented (**Appendix 5**). Sites that were not georeferenced with GPS device were subjected to a query on website *www.heavens-above.com* to obtain the respective latitude and longitude coordinates. These coordinates were all transformed to rectangular coordinates (X and Y coordinates).

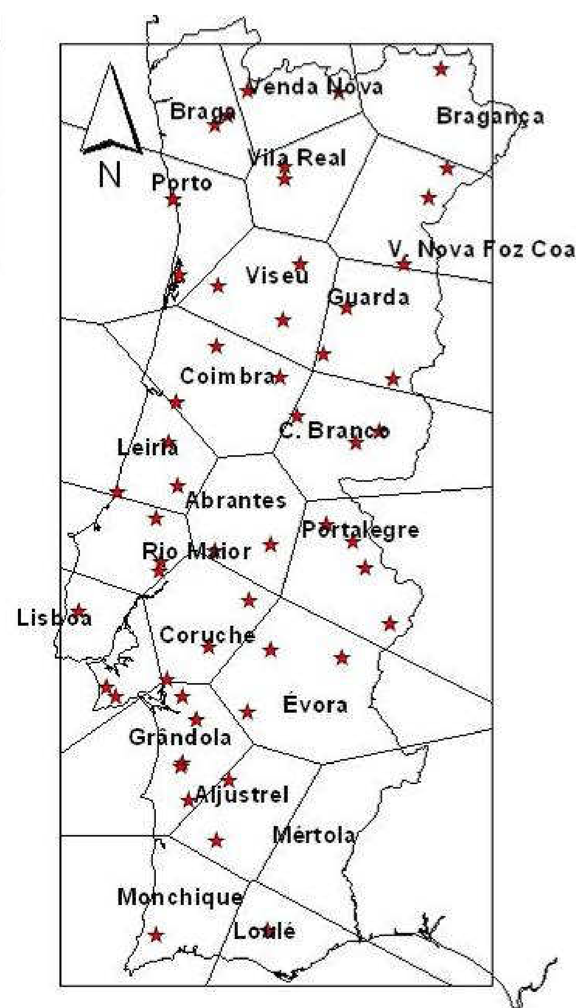


Figure 2. 20– Distribution of sample points per Thiessen polygon and local name of each site

Although there will be a more extensive description of environmental variables in Chapter 4 (with GIS-based variables), a brief characterization of sites is presented here, concerning mainly the variables observed in the field work.

Regarding the vegetation variable, some knowledge on general botanic was necessary to separate each category. Category 1 or coniferous forests without secondary vegetation was attributed to large areas of pinus (*P. pinea* or *P. pinaster*) or eucalyptus without the presence of any shrubs or herbaceous flora (**Fig. 2.21 A**). Areas with these species but with a dense secondary layer (*Ulex europaeus*, *Erica umbellata*, *Rosmarinus officinalis*, etc), suitable for maintenance of hosts and ticks were selected for Category 2 (**Fig. 2.21 B**). Mixture of pinus or eucalyptus with deciduous trees (oaks or chestnuts), with dominance of the first ones was included in category 3 (**Fig. 2.21 C**). Shrubs or arbustive areas (Category 4) were identified by large open areas with predominance of *Erica* spp, *Ulex* spp, *Cytisus* spp, *Genista* spp, *Cistus* spp (**Fig. 2.21 D**). Category 5 was characterized by the presence of forests of evergreen oaks (*Q. suber* or *Q. ilex*) or *Olea europeae* with a homogeneous structure (**Fig. 2.21 E**). Finally, the category 6 included dense forests of mixture of species, mainly deciduous trees (*Q. pyrenaica*, *Q. faginea*, *Castanea sativa*) with a secondary layer of shrubs and herbaceous species, with sufficient shadow to maintain some moisture and mild temperatures (**Fig. 2.21 F**). A seven category was added for only one site (local 10) with a large open area (grassland) that was selected only because the presence of suitable hosts (sheep) (**Fig. 2.21 G**).

Distribution of these habitats in mainland Portugal (**Fig. 2.22**) followed the distribution observed in the Corine Land Cover map at a broader scale. Some exceptions were identified, specifically a change in species of trees described for a certain area (areas of pinus converted in eucalyptus areas). Most of the polygons harboured more than one type of habitat and Category 5 and 6 were also the most widespread along the main continent (21 and 13 locals, respectively). Other habitats were also surveyed, namely coniferous forests with and without secondary vegetation (8 and 2 locals, respectively), mixed forests (6 locals), shrubs (4 locals) and 1 local occupied by grass

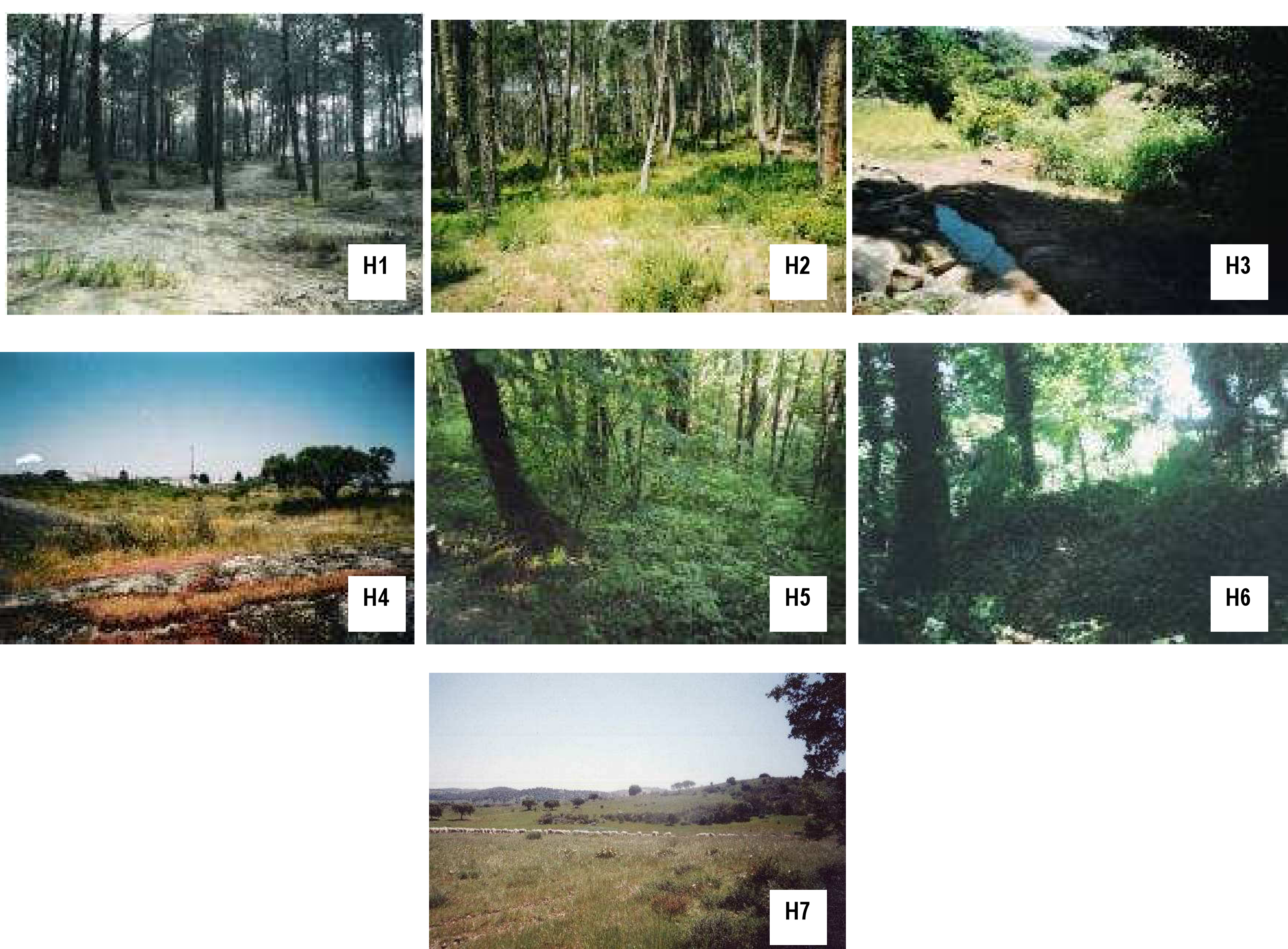


Figure 2. 21– Major habitats found at sites surveyed as part of the nationwide study. Legend: Habitat 1 - forests without secondary vegetation (Site 20 - Nazaré); Habitat 2 - forests with secondary vegetation (Site 21 – Soure); Habitat 3 – Mixed forests (Site ; Habitat 4 – shrubs (Site ; Habitat 5 – Homogeneous deciduous forests (Site 23 – Margaraça); Habitat 6 – Heterogeneous forests (site 34. Bom Jesus); Habitat 7 – grassland (Site 10, Arraiolos)

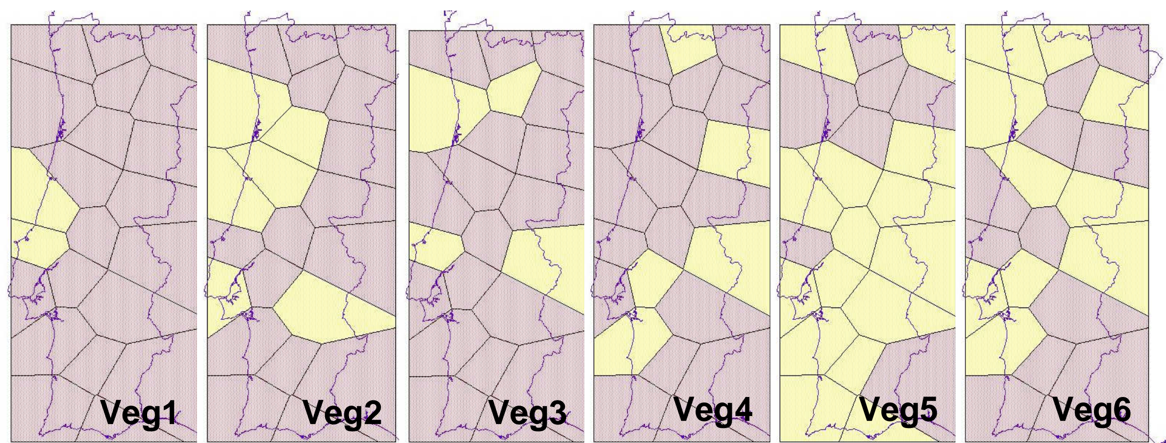


Figure 2. 22 – Distribution of vegetation categories in the different polygons: Legend: Veg1- Coniferous forests without secondary vegetation Veg2 – Coniferous forests with dense secondary layer; Veg3 – Mixture of pinus or eucalyptus with deciduous trees (oaks or chestnuts); Veg4 - Shrubs or arbustive areas; Veg5 - Forests of evergreen oaks; Veg6 – Mixed heterogeneous forests

The sites were mostly composed of soils with good (25 sites) and reasonable drainage (24 sites), with 4 sites with bad drainage, mainly sandy soils covered with herbaceous species (*e.g.* graminae, umbeliferae, grass). These two categories were found in the majority of the polygons. For exposition feature or, more precisely, the forest cover, again a visual discrimination was used. The amount of shadow was directly correlated with amount of tree coverage. A medium exposition was prevalent in the majority of the sites (41 sites against 6 locals with open areas and 7 locals with high forest cover.

The values of temperature and humidity (air and soil) correspond to the measurements of both parameters in the first visit, or, rarely, in the second when there was not possible to measure it in the first visit (logistic reasons). Some statistical values for the total sites that were surveyed can be seen in **Table 2.5**. All variables have a normal distribution and significant differences were obtained in each variable between sample points (T-Student Test).

Table 2. 5 – Statistical summary of temperature and humidity measured at sample locations.

	Air Temperature (ºC)	**Soil Temperature (ºC)**	**Air Humidity (%)**	**Soil Humidity (%)**
Medium	25.15	26.25	36.56	46.19
St. Dev.	5.76	5.63	13.98	14.31
Maximum	38.7	37.2	73.0	72.2
Minimum	11.8	11.6	9.4	9.0
Normality (K-S Test)	0.487; p=0.972	0.628; p=0.825	0.825; p=0.504	0.578; p=0.892
	TEMPAIR	TEMPSOIL	HUMAIR	HUMSOIL
Differences between sites (Student test)	30.57; p=0.000	32.33; p=0.000	18.30; p=0.000	22.36; p=0.000

Analysis with *GS+* programme gave the variogram characteristics for each variable (**Fig. 2.23**) for the default lag distance (40000 m - mean distance between points for the aggregated data set, 10% of sample sites range) (**Table 2.6**). Air related variables followed the exponential model and soil variables followed the spherical model. Total Sill was higher in humidity values indicating a higher correlation with distance between sampling values. The nugget to sill ratio was generally below 0.5 (49%), indicating that a 51% (or more) degree of spatial structure was present. Anisotropic variograms showed no variations in any direction for all variables (C/C0+C≈1).

Table 2. 6 – Summary of variogram characteristics for each climate variables measured in the nationwide study (lag interval of 40000).

Variable	**Model**	**Nugget (Co)**	**Total Sill (Co+C)**	**Range (Ao)/effective range[1] (m)**	**Nugget / Total Sill**	R^2
AirTemp	Exponential	26.82	53.65	636000	0.49	0.62
SoilTemp	Spherical	24.62	49.28	911000	0.49	0.62
AirHum	Exponential	59.60	218.20	63400	0.27	0.83
SoilHum	Spherical	135.30	384.00	911000	0.35	0.76

[1]the **effective range** is the separation distance at which spatial dependence is apparent

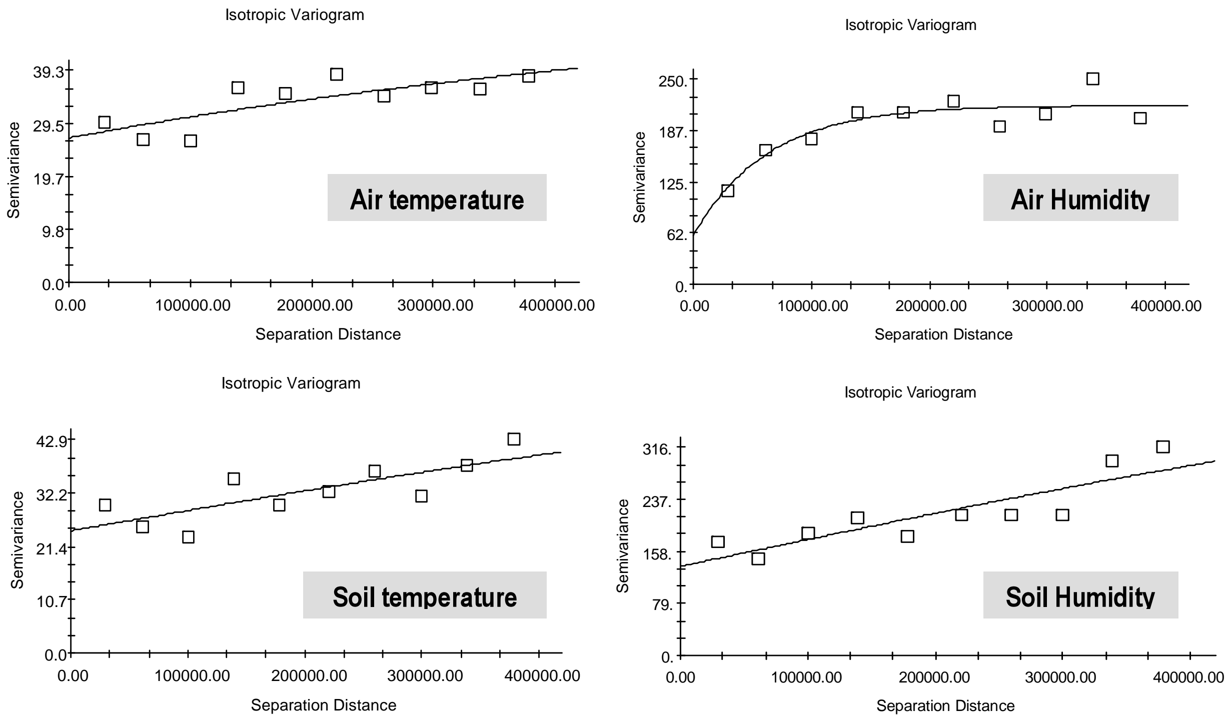


Figure 2. 23 – Semivariograms for the different climate variables (Air temperature, Soil Temperature, Air Humidity and Soil Humidity) measured in the nationwide study for a lag distance of 40000

Simulation of gridded surfaces of estimated values (interpolation) performed by kriging techniques, was also created for these variables (**Fig. 2.24**).

As it would be expected lower temperatures and higher humidity appear in the littoral regions, influenced by the Atlantic Ocean.

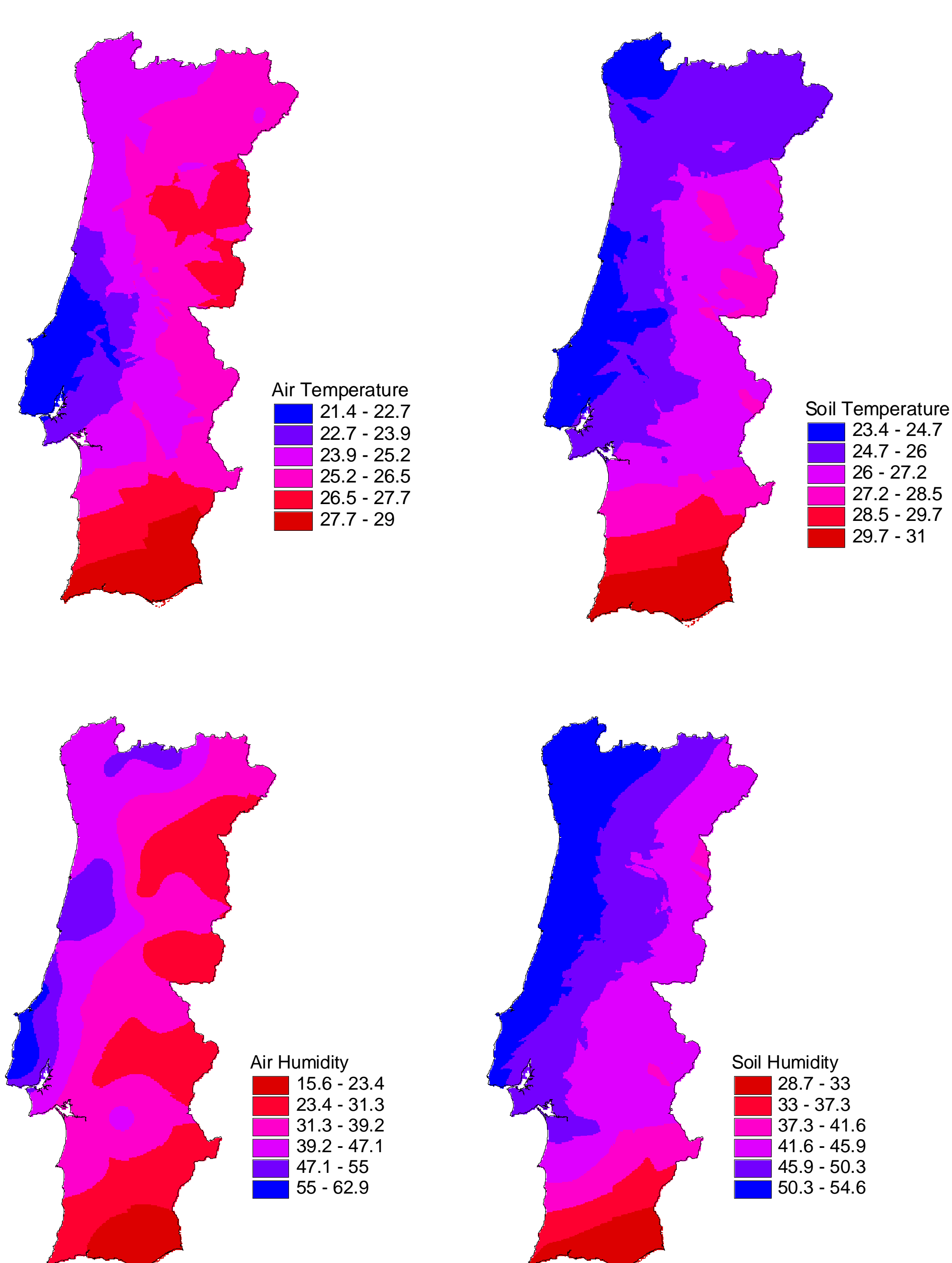


Figure 2. 24 – Grid surface of estimated values (Z variates) for field climate variables (Air and Soil Temperature, Air and Soil Hunidity)

2.4.2.2 Tick collection and maintenance

As already mentioned, a total of 55 sampling sites distributed all over the country were prospected. In the majority (64%, 35 sites), it was possible to collect tick specimens. There were 20 locals where collections were negative. **Table 2.7** presents all the collected tick species during this study and the number of sites where each species was present. Taxonomic identification lead to the following species: *Ixodes ricinus* (Linnaeus, 1758), *Rhipicephalus* spp Koch, 1844, *Dermacentor marginatus* (Sulzer, 1776), *Haemaphysalis punctata* Canestrini and Fanzago, 187, *Hyalomma marginatum marginatum* Koch, 1844, *I. hexagonus* Leach, 1815 and *I. bivari* Travassos Dias, 1989, these two latter species included in the *Ixodes* spp group.

Table 2. 7 – Number of sites with positive and negative collections for each tick species

Tick species	Nº positive sites (%)	Nº negative sites (%)
Ixodes ricinus	9 (16.4)	46 (83.6)
***Rhipicephalus* spp**	29 (52.7)	26 (47.3)
***Dermacentor* spp**	13 (23.6)	42 (76.4)
***Haemaphysalis* spp**	2 (3.6)	53 (96.4)
***Hyalomma* spp**	3 (5.4)	52 (94.5)
***Ixodes* spp**	2 (3.6)	53 (96.4)

There were sites where only one species was collected and others where more than one species were confirmed. **Table 2.8** show the distribution of all ticks species / genera within sites and **Fig. 2.25** presents distribution of the most important ticks among Thiessen polygons (*Rhipicephalus* spp, *I. ricinus* and *D. marginatus*)

As it can be seen, *Rhipicephalus* spp has the widest distribution appearing in 16 of the 22 polygons created. Most of these polygons are distributed in the north, centre and south of Portugal, mainly in the coastal areas. On the other hand, *I. ricinus* has a more restrict distribution with only 6 polygons positive for this tick (Braga, Coimbra, Guarda, Lisboa, Coruche and Grândola). *D. marginatus* was found in 9 polygons distributed mainly in the coastal regions, from north to south of Portugal.

Table 2. 8 – Distribution of tick species at sample sites (presence/absence)

Species	Site number (total)
IR	L38 (**1**)
R	L2, L3, L7, L8, L9, L30, L31, L33, L36, L39, L40, L41, L44, L45, L46, L47, L51 (**17**)
D	L4, L25, L50 (**3**)
IR + R	L1, L21 (**2**)
IR + D	L23 (**1**)
R + D	L5, L13, L19, L43 (**4**)
R + HY	L10, L18 (**2**)
IR + R + D	L16, L55 (**2**)
IR + D + Ispp	L22 (**1**)
IR + R + D + HY + HA	L54 (**1**)
IR + R + D + HA + Ispp	L53 (**1**)

(Legend: IR – *Ixodes ricinus*, R – *Rhipicephalus*, D – *Dermacentor*, HY – *Hyalomma*, HA – *Haemaphysalis*, Ispp – *Ixodes* spp)

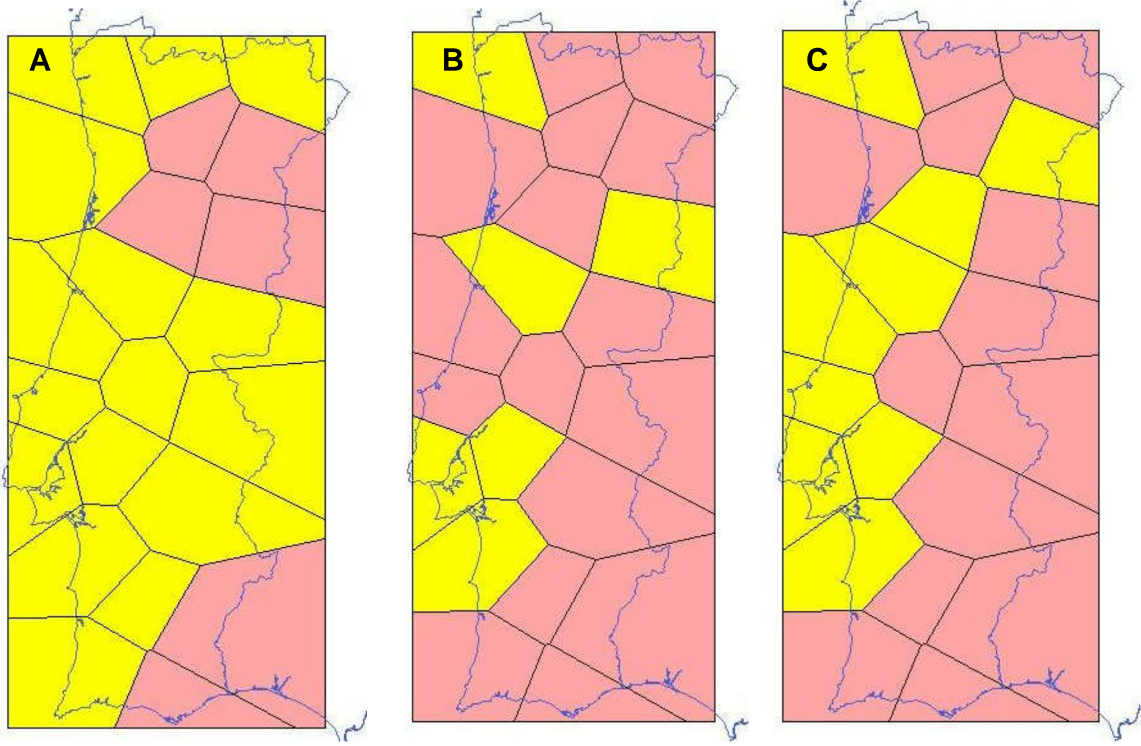


Figure 2. 25 – Distribution of *Rhipicephalus* spp ticks (A), *I. ricinus* ticks (B) and *Dermacentor* spp ticks (C), the three more important species collected during nationwide study, among Thiessen polygons.

The other three species were found in three or less polygons, namely Rio Maior, Portalegre and Grândola for *H. marginatum*, Lisboa and Grândola for *Ha. punctata* and Lisboa for other species of *Ixodes*.

Regarding the number of ticks collected, a total collect effort of 682 ticks/person/hour (corresponding to 2801 ticks collected) was obtained distributed by the previous species: *I. ricinus* (287 ticks/hour), *Rhipicephalus* spp (232 ticks/hour), *D. marginatus* (137 ticks/hour), *Ha. punctata* (15 ticks/hour), *H. marginatum* (13 ticks/hour), *Ixodes* spp (2 ticks/hour) (**Table 2.9**). A 1♀:1♂ sex ratio for *Rhipicephalus* and *Ix. ricinus* was found, along with a 1.5♀:1♂ for *Dermacentor*.

Sites 53 and 54 (Tapada Nacional de Mafra and Herdade da Ribeira Abaixo) correspond to the more important collection sites with 270 and 191 ticks/hour, respectively, compared to the other positive sites where the average number is about 7 ticks/hour (range: 1-20). As it was seen in the previous table 2.10 these sites were also the sites which had more species collected together.

Geostatistical anaysis with GS+ was performed for the most important species (*I. ricinus*, *Rhipicephalus* spp and *Dermacentor* spp) to extrapolate tick abundance at non-sampled locations. Following the same methodology as in the previous chapter (see analysis of field temperature and humidity variables), the semivariance graphs of collection effort data were created for default lag (40000m) (**Fig. 2.26**). Variables of semivariograms are presented in **Table 2.10**.

Table 2. 9 – Summary of variogram characteristics for the total numbers (collect effort) of each tick species measured in the nationwide study (lag interval of 40000)

Variable	Model	Nugget (Co)	Total Sill (Co+C)	Range (Ao)	Nugget / Total Sill	R^2
Total Ticks	Spherical	0.810	4.312	911000	0.19	0.95
Rhip Ticks	Spherical	0.497	3.004	905300	0.16	0.83
Derm Ticks	Spherical	0.435	1.335	911000	0.32	0.57
Ixric Ticks	Exponential	0.001	0.855	43400	0.001	0.52

Table 2. 10 – Total Collect Effort within the 55 sample sites

LOCAL	POLYGON	LOCAL_NAME	ColeffTot	ColRhip	ColDerm	ColIxric	ColHyal	ColHaem	ColIxspp
Site01	Lisboa	Alfarim	17	16	0	1	0	0	0
Site02	Lisboa	V Nog Azeitão	3	3	0	0	0	0	0
Site03	Grândola	Pinheiro (E Arq)	12	12	0	0	0	0	0
Site04	Grândola	Albergue	6	0	6	0	0	0	0
Site05	Grândola	Santiago Cacém	12	7	5	0	0	0	0
Site06	Grândola	S Domingos	0	0	0	0	0	0	0
Site07	Aljustrel	Canhestros	18	18	0	0	0	0	0
Site08	Aljustrel	Gavão	14	14	0	0	0	0	0
Site09	Évora	Alcaçovas	8	8	0	0	0	0	0
Site10	Évora	Arraiolos	6	4	0	0	2	0	0
Site11	Portalegre	Alpalhão	0	0	0	0	0	0	0
Site12	Coruche	Mora	0	0	0	0	0	0	0
Site13	Coruche	Lavre	20	10	10	0	0	0	0
Site14	Porto	Fund Serralves	0	0	0	0	0	0	0
Site15	Porto	Pq Cidade	0	0	0	0	0	0	0
Site16	Braga	Póvoa Lanhoso	8	1	6	1	0	0	0
Site17	Bragança	Mougadouro	0	0	0	0	0	0	0
Site18	Rio Maior	Vale Santarém	11	5	0	0	6	0	0
Site19	Rio Maior	Azambuja	13	5	8	0	0	0	0
Site20	Leiria	Nazaré	0	0	0	0	0	0	0
Site21	Coimbra	Soure	7	1	0	7	0	0	0
Site22	Coimbra	Buçaco	2	0	1	1	0	0	1
Site23	Coimbra	Mata Margaraça	2	0	1	1	0	0	0
Site24	Viseu	Paradela	0	0	0	0	0	0	0
Site25	Viseu	V Nova Paiva	1	0	1	0	0	0	0
Site26	Viseu	Canas Senhorim	0	0	0	0	0	0	0
Site27	Guarda	Celorico Beira	0	0	0	0	0	0	0
Site28	Guarda	Penamacor	0	0	0	0	0	0	0
Site29	C. Branco	Oledo	0	0	0	0	0	0	0
Site30	Abrantes	Ulme	8	8	0	0	0	0	0
Site31	Monchique	S Espinhaço Cão	8	8	0	0	0	0	0
Site32	Loulé	Besteiros	0	0	0	0	0	0	0
Site33	Porto	Murtosa	2	2	0	0	0	0	0
Site34	Braga	Bom Jesus	0	0	0	0	0	0	0
Site35	Venda Nova	Gerês	0	0	0	0	0	0	0
Site36	Venda Nova	Chaves	12	12	0	0	0	0	0
Site37	Vila Real	S. Marão	0	0	0	0	0	0	0
Site38	C. Branco	Loriga	1	0	0	1	0	0	0
Site39	Portalegre	Elvas	4	4	0	0	0	0	0
Site40	Rio Maior	Amiais Cima	2	2	0	0	0	0	0
Site41	Abrantes	Rosmaninhal	6	6	0	0	0	0	0
Site42	Leiria	Meirinha	0	0	0	0	0	0	0
Site43	Leiria	Fatima	7	5	2	0	0	0	0
Site44	Portalegre	Rasa	2	2	0	0	0	0	0
Site45	Portalegre	Arronches	1	1	0	0	0	0	0
Site46	Évora	Bencatel	1	1	0	0	0	0	0
Site47	C. Branco	Escalos Baixo	1	1	0	0	0	0	0
Site48	C. Branco	Orvalho	0	0	0	0	0	0	0
Site49	V.N.FozCoa	FCastRodrigo	0	0	0	0	0	0	0
Site50	V.N.FozCoa	Freixo Esp Cinta	1	0	1	0	0	0	0
Site51	Bragança	Pq Montesinho	1	1	0	0	0	0	0
Site52	Vila Real	Pq Alvão	0	0	0	0	0	0	0
Site53	Lisboa	TapNacMafra	270	1	2	262	0	4	1
Site54	Grandola	HRA	191	71	93	12	5	11	0
Site55	Coruche	Aguas Moura	4	3	1	1	0	0	0

All variables followed the spherical model, except *I. ricinus*. Total Sill was higher in *Rhipicephalus* ticks indicating a higher correlation with distance between sampling values. The nugget to sill ratio was generally below 0.5, indicating that spatial structure was present in all tick species.

Fig. 2.27 shows the spatial distribution of the above species, by kriging analysis. *Rhipicephalus* spp presents a crescent probability of occurring from north to south of Portugal, with no distinction between inland and coastal regions. *D. marginatus* increases its distribution towards NW and SW, with a preference for more southern regions. On the other hand, *I. ricinus* ticks have a more restricted distribution although it is present throughout the littoral region.

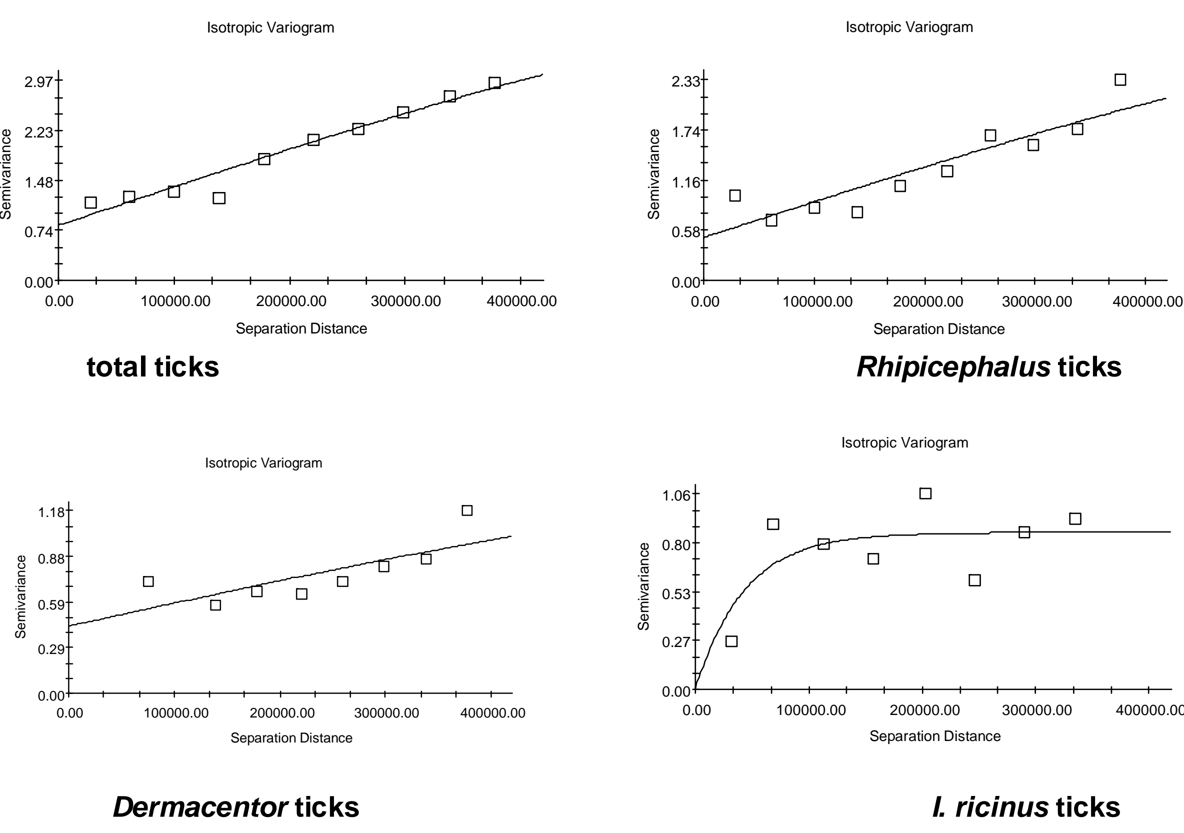


Figure 2. 26– Semivariograms for the total numbers (collect effort) of different tick species measured in the nationwide study (lag interval of 40000)

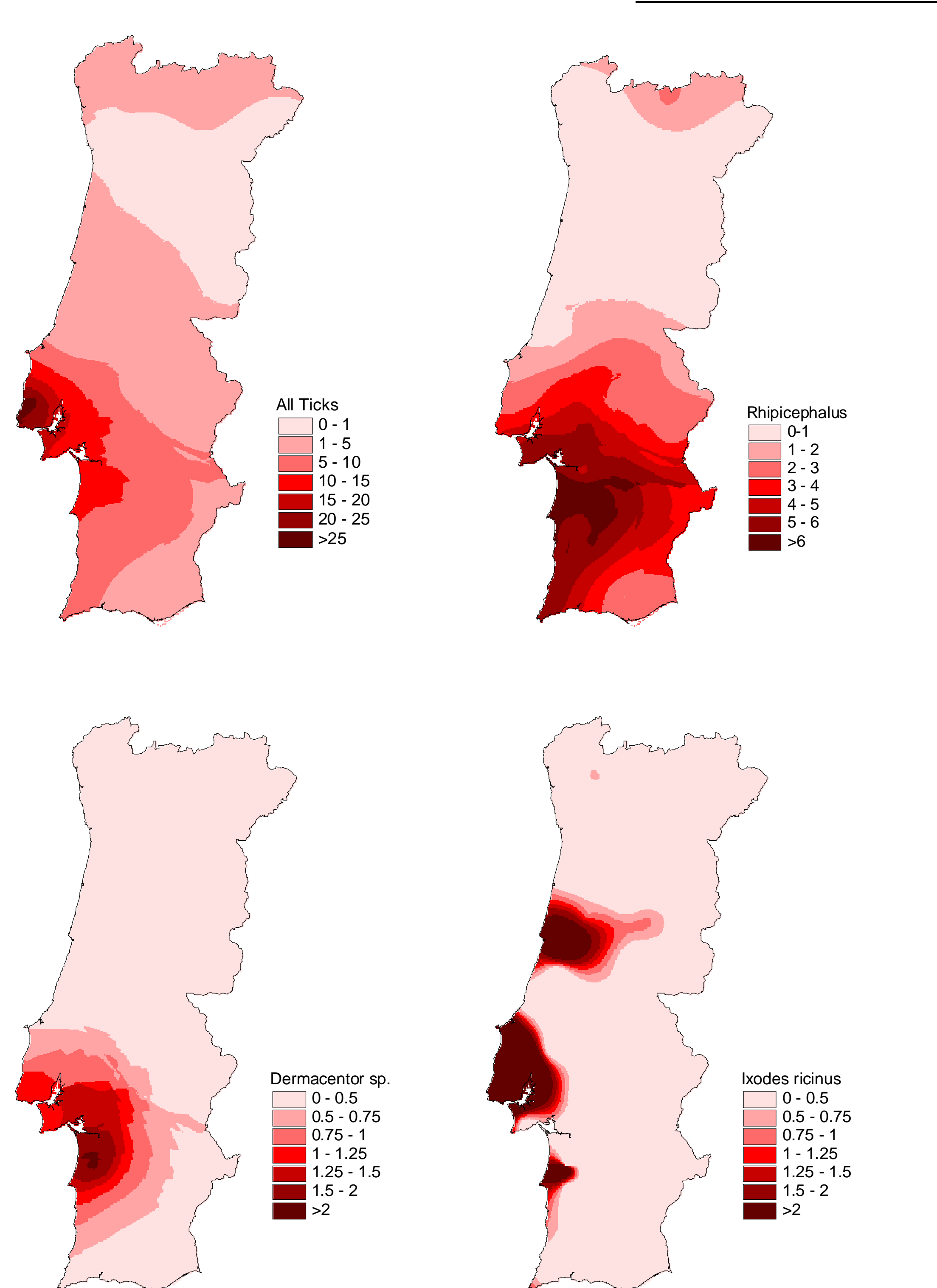


Figure 2. 27 – Grid surface of estimated values of collect effort (Z variates) for the more important collected species in Portugal (Total ticks, *Rhipicephalus*, *Dermacentor*, *I. ricinus*)

An indicator kriging was also attempted using an indicator variable for the presence (1) or absence (0) of total ticks (**Fig. 2.28**). The use of indicator variables in geostatistics avoids problems of outlier observations or skewed distributions and allows the estimation of the probability that a given sample has a higher value than the cutoff point (in this case a non-zero count) (Crist, 1998).

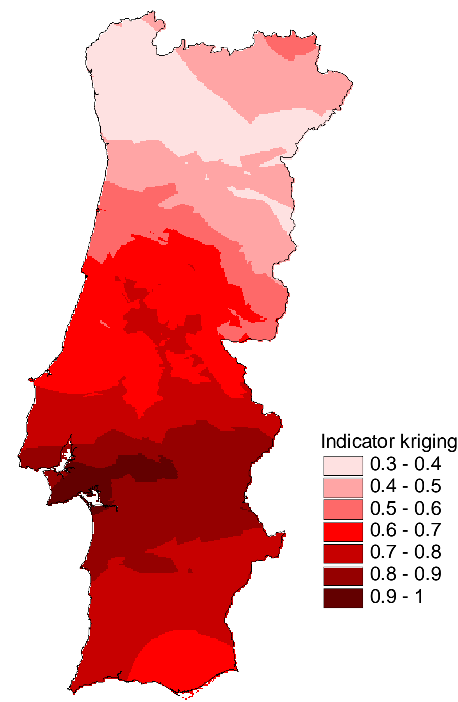


Figure 2. 28 – Grid surface depicting likelihood of occurrence for all tick species collected in this study based on indicator kriging of point estimates.

A total of 155 ticks were brought to the laboratory from different sources and different capture methods. **Table 2.11** presents the distribution of those ticks regarding the polygon where they were picked up and species of ticks collected.

Table 2. 11– Source of additional ticks contributed by other collectors

Local name	Polygon	Species	Source	Total Number
Mougadoro	V.N. Foz Côa	R	dog	6
Montalegre	Venda Nova	D	wolf	18
Cinfães	Porto	R	dog	1
Tomar	Abrantes	R	human	23
		H	human	1
		Ispp	human	1
Coruche	Coruche	R	vegetation	25
Pegões	Coruche	R	dog	5
Sesimbra	Lisboa	Iric	vegetation	1
Caparica	Lisboa	R	dog	18
Oeiras	Lisboa	R	dog	2
Lisboa	Lisboa	R	vegetation	1
Sintra	Lisboa	R	dog	2
Mafra	Lisboa	R	dog	28
Estremoz	Évora	R	vegetation	4
Alcaçovas	Évora	H	vegetation	1
Alqueva	Évora	R	vegetation	2
Melides	Grândola	R	vegetation	1
Faro	Loulé	R	dog	15
Total	9 polygons	5 species	4 sources	155

Legend: R – *Rhipicephalus* spp, D – *Dermacentor* spp, H – *Hyalomma* spp, Iric – *I. ricinus*, Ispp – other *Ixodes*.

2.5 Discussion

2.5.1 Methodological considerations (sampling and collections)

When collecting ticks, it should be remembered the main characteristics of each sampling technique and its efficiency to collect each developmental stage or gender (biases), so that the resulting data is not misinterpreted and does not lead to inappropriate intervention strategies. These factors, along with the experience of the collector(s), will influence the final numbers of collected ticks that depend primarily on the type of habitat, environmental conditions and presence of suitable hosts.

Sampling effort design is also an essential step in ecological research. In this study, a nationwide study was performed creating Thiessen polygons around 22 localities distributed along Portugal, allowing the creation of a grid with a minimum number of samplings (one to four sample sites) in a total of 55 sampling sites. The extension of Portugal did not permit a sampling grid defined by equal size squares (e.g 10x10km) as Nicholson & Mather (1996) did for Rhode Island County, in their study on distribution of *I. scapularis*. Also, since only one person (rarely two) was involved in the collections and characterizations, there were no logistic conditions to increase the sampling strategy and effort.

Prior to the beginning of the field survey, there was an intention of performing an equal-stratified sampling, with at least three sample points per polygon at suitable habitats. But due to unequal distribution of forested areas, especially in the south of Portugal, some places had less sample points and others had more sites. All these 55 sites surveyed had common biases. All of them were chosen according to prior preferences of the collectors (specific habitat or known presence of a given tick species), near transportation pathways and easily accessible. A similar study on Rhode Island selected the sample sites based on their location in the state, the type (deciduous, coniferous, mixed) and amount of forested habitats available, and their accessibility by road networks (Nicholson & Mather, 1996).

In future studies a more precise grid based on these 55 sample sites and their semivariograms can be defined and be the basis of other ecological approaches contributing to the knowledge of vector-borne diseases in Portugal.

Nevertheless, even with the mentioned biases, the study on distribution of vectors of LB in Portugal was performed and it was possible to detect spatial heterogeneities and interpolate and create contour maps for specific variables, namely temperature, humidity and tick collections.

In the collection methodologies several sources for sampling bias are expected: methodological differences (for instance, vertical targets in walking surveys and horizontal target in dragging method), habitat effects, weather (humidity, wind) and behavioural influences (host acquisition) (Gilot *et al*., 1994; Schulze *et al*, 1997; Kiszewski & Spielman, 1999; Ginsberg & Zhioua, 1999).

Using the same person in both techniques eliminates the biases attributable to the investigator sampling techniques and attractiveness to ticks (Ginsberg, 1992; Schulze *et al*, 1997; Vassalo *et al*., 2000). This aspect was followed in almost of the collections (Mafra and nationwide study), except in those made in the nationwide study, during 2003, where two persons (the author and a training student) performed the dragging technique with two different towels. In these cases, the biases associated were diminished by creating an average number of ticks between the two collections as the measure for the "collecting effort" (Daniel *et al*, 1986; Vassalo *et al*., 2000). Several expressions used in the different chapters of this thesis, have been related to the quantity of ticks (*i.e.* density, activity, abundance, availability, number of) that essentially represent equally the fraction of unfed, host-seeking ticks present in a particular area and moment, that will attach to the sampling device (Mejlon, 2000). These expressions do not imply the total number of ticks present in an area.

As regards the correct use of drags, it was possible in the Mafra focal study, to strictly follow the well known recommendations, that is, the same texture and dimensions all through the experiment a constant dragging distance and at the same hours, under similar weather conditions (Hostis *et al*., 1995). In the nationwide study, the distance between sample sites influenced the arrival time and consequently the beginning of collections.

Considering the period spent in each of the sample sites (1 hour for Mafra and 15-30 minutes for nationwide studies), with transepts of 30 seconds each, more than 5 subsamples were executed which gave a good representation of the local populations, as confirmed by Vassalo and others (2000). These authors showed that the number of subsamples could be small (*e.g.* 5 subsamples) to be representative of

a homogeneous area, if regularly checked because of variations on host populations and consequently on distribution of ticks. Besides, impact of intensive sampling on the tick population could be negligible since the flag was dragged over a surface inferior than the actual total area and due to the transport of ticks by hosts over the whole forest (Perret *et al*., 2000).

In addition, assuming that the efficiency of a single drag-sampling occasion to estimate the total population size of *I. pacificus* nymphs in leaf-litter habitats was estimated to be 5.9% (Talleklint-Eisen & Lane, 2000) or 10% (Mermod *et al*., 1973), and that a third day of flagging, representing the number of ticks ascending into the vegetation, is closely related to the size of the free-living tick population (Jensen, 2000), the low values of collected ticks in the nationwide study can be explained. Most of the sites had only one visit and even those that had more surveys (with exception of sites 53, 54 and 55) did not necessarily had a great increase in tick numbers. Besides, it is worthy to point out that in high fragmented landscapes, the presence of a species at a particular site and time does not mean that the species has a persistent population at that site and vice-versa (Estrada-Pena, 2003). Non-establishment may be caused by low hatchability, innate increment mortality of each instar, difficulty in finding a mate and low density of medium to large mammals hosts for adults (Lindsay *et al*., 1995)

The reduced number of collected ticks in the nationwide study could also be the result of a reduced tick's activity at the time of collection (daytime), which was not the most appropriate, as shown by Quaresma (2004), in a study at a southern Portuguese region where all tick species were more active immediately after dawn and before night fall. Sampling in open areas and/or exclusively during the daytime (not taking in account the diel activity pattern) could be more seriously biased and underestimate tick abundance than if carried out in woodland areas and/or during extended diel periods (Mejlon, 1997; Randolph & Storey, 1999; Schulze & Jordan, 2003). However, another study in Sweden showed that *I. ricinus* is present during all 24 hours, meaning that tick's activity has a regional pattern which can differ per specific habitat and stage (Mejlon, 1997, 2000). In the meadow habitat, the availability peaked between 11 p.m. and 3 a.m. for all stages but in the forest, the patterns of nymphs and adults diel activity were similar, while that of the larvae was more irregular; no differences between behavior of male and female *I. ricinus* were found. In Scotland, the mean proportion of time spent questing for *I. ricinus* was 13.8%, but activity for the majority of ticks fell after four hours of questing, probably due to increased desiccation or unsuitable climatic conditions, of limited duration (namely temperature and humidity) (Van Es *et al*., 1999). Other species, like *I. scapularis*

and *A. americanum* in USA, demonstrated responses to diel changes in local metereological conditions. Numbers of ticks were correlated with ambient temperature, humidity and with conditions recorded in the leaf litter that appeared to play a significant role in mediating questing behavior of both species (Schulze & Jordan, 2003).

With exception of Mafra site and a nymph from Site 1 (Alfarim, Lisboa Polygon), only adults were collected. Some authors referred that use of flags or blankets which are brought into contact with questing ticks is not a good method for sampling adults, because of their failure to attach and their higher capacity to release from flag due to larger size (Gray, 1985; Gilot *et al*., 1994; Hostis *et al*., 1995). A study showed that walking surveys are more efficient for collecting *Ixodes scapularis* and *Amblyomma americanum* adults compared to dragging (2:1) (Schulze *et al*., 1997). The most efficient method for this stage consists on the increasing of dragging duration (10 minutes) with inspection of the entire flag and removal of ticks after this period. This was not performed in our study (30 seconds for each transect) but was sufficiently valuable to obtain reasonable collections. This ten-minute sampling is not suitable for immature stages, along with time consumed to remove them at each sweep (Gray, 1985).

The explanation for collections of higher number of adults in the nationwide study may be due to two main reasons: the period of year and time of day when collections were made, where variables like temperature and humidity are important and the use of the stick in the towel that allowed the entire cloth surface to be in contact with the vegetation (Vassalo *et al*., 2000) increasing the probability of attachment.

For defining the sex ratio of tick population, the flagging method could involve the possibility of some distortion of the actual situation in case of different behaviour between genders (activity and attachment to host) (Cerny, 1977). A study in Czech Republic showed that the male predominance in *I. ricinus* populations occurred primarily in the years characterized by low numbers of adults, while the predominance of females was observed mainly in the years with high numbers of adults. A ratio of 1♂:1.24♀ was registered. This sex ratio may differ in various parts of the species range (Cerny, 1977). In nationwide and Mafra studies, a 1:1 ratio was found for *I. ricinus* population.

The blanket method is no doubt, the best method for nymphs and larva (the latter, when on uniform ground) (Gray, 1985). Also, immature stages are rarely collected in the walking technique (Schulze *et al*., 1997). The

leaf litter may affect the number of nymphs that are picked or brushed off while a person walks, since nymphs tend to quest within the litter layer, being more difficult to contact with the person or the towel (Talleklint-Eisen & Lane, 2000; Carroll & Kramer, 2001). This aspect can explain both the absence of nymphs in the nationwide study and the high presence of nymphs in Mafra study, where conditions of leaf litter were more propitious and developed.

There were great variations on larval activity between our two studies. Only in Mafra it was possible to collect larvae, mainly in more open areas where it was possible to confirm the aggregated distribution of this developmental stage. The presence of larvae on the vegetation is a good indication of the presence in the previous year of suitable hosts that feed adult ticks (Gray *et al*., 1995), as is the case of fallow deer and wild pig hosts in Mafra. Other explanations can also rely on one or more of the following factors: a) larvae are not easy to sample on blankets in some habitats because a high proportion of them does not reach the vegetation surface and they also tend to be easier to blanket-sample in open areas where they respond vigorously to sunshine, and die off rapidly which tends to separate peaks from each other, b) counts from rodents may not always represent the total availability of questing larvae in the habitat; c) in some areas with long winters and short summers, spring larval activity may be so late that a second larval peak may not be discernible and d) the autumn feeding adult population may be too small or unsuccessful to produce a noticeable rise in larval activity in late summer of the following year (Gray, 1991; Vassalo et al., 2000a; Walker, 2001).

2.5.2 Focal tick study

In Mafra study, a cycle of 5 years was studied for the population of *I. ricinus*, the first one made in Portugal and one of the few studies made in Iberian Peninsula.

As already referred in Chapter 1, transition into a new stage and completion of post-moulting development (defined as activation) (Korenberg, 2000) is the basis of the life cycle of ticks. Periods of activity are periods during which the ticks occur in the active stage and are capable of attaching to their animal hosts and humans (Korenberg, 2000). As already mentioned, ticks have the capacity to regulate their activity to deal with the adverse effects of environment (Jensen & Kaufmann, 2003). This activity is defined as seasonality and depends on host abundance, climate, vegetation and diapause phenomena (Gray, 1991; Walker, 2001; Belozerov *et al*., 2002; Gray, 2002).

Ixodes ricinus portuguese population presented a unimodal cycle with particular characteristics, that can be included in the warmer climates seasonal dynamics pattern, already explained in Chapter 1 (Figure 1.25). Except for larvae, which was restricted to late spring and mid summer, nymphs and adults were present during most or even the whole year, with an extended questing period, confirmed by correlation results. The beginning of higher temperatures and lower precipitation was the trigger for the increase of ticks.

As already reported (see **Appendix 2**), the literature on this subject is immense and for the same country authors have found different patterns of seasonal dynamics for *I. ricinus*. This diversity of seasonal patterns may be related to duration of vegetation period, density of hosts and host seeking periods, open *vs* woodland areas, climate and photoperiod (Mejlon, 2000; Jensen, 2000; Jensen & Kaufmann, 2003). The presence of ticks during all year, as a result of the present study and of another from Caeiro (1999), was only observed in southern European countries, like Italy (Frusteri *et al.*, 1994; Manelii *et al.*, 1999, 2003), Spain (Revuelta, 1991; Guerrero, 2001) and exceptionally in United Kingdom (Randolph *et al.*, 2002). In the majority of the patterns presented, maximal activity begins usually in March-April, extends until early or late summer where activity begins to decrease. In early autumn, a smaller peak is observed decreasing again in the winter months, where ticks take refuge in the soil (Mermod *et al.*, 1973, 1974). In Tunisia, a warmer country, *I. ricinus* prefers these cold months to initiate and increase its activity (Younsi *et al.*, 2001).

Differences in seasonal pattern between stages, as shown in this focal study, are also common in other countries. For instance, in Italy, Mannelii and others (1999, 2003) observed that larvae showed a summer peak of activity in July, like in our study, but maintained low densities during autumn and winter and peaked again in December (probably due to mild climate). Nymphs were also collected during most of the year, with a significant spring peak (mainly April). Adult *I. ricinus* were active in spring and, more markedly, in autumn. In a nine year study in north-central Spain, larvae had a unimodal activity pattern, beginning its activity in May with a maximum observed around July–August, whereas nymphs displayed a bimodal pattern (May–June and August–September) with strong dominance of spring activity. An inversion of this pattern, with larger autumn peak, was observed in years with wet summers. Adults showed a small spring peak and a large autumn one. In the later years of the study, a small increase in the adult spring peak of activity was noticed, correlated with mild winters (Estrada-Pena *et al.*, 2004a).

Studies in Spain also show this diversity of patterns depending on the geographic region. The different stages of I. ricinus appear in autumn and winter periods in the more southern populations and in autumn and spring periods in northern populations (Márquez-Jiménez *et al.*, 2005). In Italy, seasonal patterns of nymphs and adults did not differ between inland and coastal sites (Mannelli *et al.*, 2003).

Unimodal patterns were observed for numerous countries (France, United Kingdom, Spain, etc), with just a small peak or with an extended spring/summer peak, especially for nymphs and larva stage. For example, a long study made in France by Vassalo and others (2000a) demonstrate the existence of only one peak during the year for nymphal activity. The lower the peak, the longer the nymph activity season. A stable annual nymph population density between years was apparent, explained by a stability and variety of vertebrate hosts for non-discriminating nymphs in their choice of hosts. In opposite, Mafra presented some years with higher nymph densities compared to others. This may be explained by an enhanced survival of the 7-10 months earlier moulting larvae (by e.g. presence of suitable and numerous hosts in those particular years) that produce these higher nymphal peaks (Estrada-Pena *et al.*, 2004). An increase in adult abundance has also been detected in the years following these high nymphal peaks and it seems plausible that the high nymphal peak had produced this accumulation of adults in the same year or in the following years.

Like in other studies, larvae from Mafra had simple peaks, always occurring in mid-May, suggesting that these larvae were laid in the previous year, have passed the winter and emergence is relatively synchronous, when temperature is sufficiently high.

Adding to these observations, the unimodal cycle of Mafra represented by a very long activity period (almost all year) and thus a higher risk period of contact, may be regulated by a developmental diapause in such a manner that all ticks (especially nymphs and adults) initiate activity in the autumn and they then die out the following summer before a new cohort commence activity (Randolph *et al.*, 2002; Jensen & Kaufmann, 2003).

Mathematical population models can show which factors are capable of generating cyclic dynamics (Kendall *et al.*, 1999). Times-series analysis is an area of statistics which departs a little from the mainstream of statistical theory and practice (Woollons & Norton, 1990; Kendall *et al.*, 1999). These techniques are well

developed, the sampling properties of the statistics are well understood and the approach makes relatively few assumptions about the data. But some caution has to be present since they tend to be biologically naïve, ignoring any qualitative or quantitative information about the ecological system that generates the time series and not providing any information about the underlying ecological mechanisms.

Although Times series analysis was already performed in other biologic communities (Woollons & Norton, 1990; Sánchez-Lafuente *et al*., 2001), it was used here for the first time in tick's populations. Application of Box-Jenkins equations on seasonal dynamics of *I. ricinus* population of Mafra allowed a reasonable prediction of future seasonal behavior, which maintains its actual tendency.

With Distributed lags analysis it was possible to hypothesize a developmental cycle for *I. ricinus* population at Tapada Nacional de Mafra, with a duration of 1 year to 1,5 year between the appearance of first year larvae population to the second year larvae population.

Although the biological cycle of *I. ricinus* in Mafra can be completed in one year, according to climate conditions in its geographic distribution, it may extend up to 3 years (Cerny *et al*., 1974; Daniel *et al*., 1977; Guerrero, 2001; Márquez-Jiménez *et al*., 2005).

Climatic variables are responsible for developmental cycle length, being important to understand that in studies of 1 year or more, weather conditions are not obviously the same (Hostis *et al*., 1995).

In Mafra, females, males and nymphs correlate negatively with temperature, females and males correlate negatively with humidity, males correlate positively with precipitation and larvae correlate negatively with this variable. In another study, in Scotland, no correlation was observed with temperature, but humidity correlate negatively with nymphs and adults questing. Questing of larvae was positively correlated with temperature and negatively correlated with relative humidity (Walker, 2001). In Sweden, in meadow ticks, a negative correlation with temperature was found for all stages and the relative humidity was correlated with the adult tick numbers. In the forested site only the larval tick numbers were associated with the temperature and humidity (Mejlon, 1997, 2000). Relative humidity and also solar radiation are dominant variables and are modified with vegetation, leading to differences in host seeking periods between habitats (Jensen, 2000).

Nymphs were present in Mafra all through the year, mainly because humidity was always higher than 70% and temperatures within the range of 0-35ºC were always observed. (Hostis *et al*., 1995). In Scotland, nymphs and adults were found questing at all months of the year and at temperatures ranging from 3.5ºC to 29ºC and humidies from 53% to 100% (Walker, 2001).

According to Vassalo and others (2000a) the unimodal annual cohorts (nymphal stock) reflect the temperature-dependent temporal distribution. The higher the temperature during the initial activation period, the faster the stock depletion and nymph activity does not spread into the year. On the other hand, cooler temperatures early on lead to an extended activity pattern with no or a reduced peak. The reduction of the proportion of questing nymphs in late summer months may be either related to a peak in saturation deficit or to a drop in maximal relative humidity (Perret *et al*., 2000; 2004). This maximal humidity was responsible for the highest peaks of larvae in Mafra. Adults, compared with nymphs, have a greater resistance to low relative humidity (Mejlon & Jaenson, 1993). Also, accelerated developmental rates of all three stages leading to 1-1.5 year cycle like the one in Mafra may be correlated with seasonal changes of soil temperatures (not recorded) and the time of placement in the field (Padget & Lane, 2001; Randolph *et al*., 2002; Jensen & Kaufmann, 2003).

Another aspect that was not studied in this thesis but has a crucial importance in this developmental and seasonal dynamic cycle of *I. ricinus* at Tapada Nacional de Mafra is the presence of a wide range of suitable hosts that allows the maintenance of a permanent population.

Fallow deer and wild pig permit the success of adult engorgement and subsequent reproduction (Chemini *et al*., 1997; Ginsberg & Zhioua, 1999). There is a greater facility with which the female ticks can feed, hence the more frequent production of larger batches of eggs (Pichon *et al*., 1999), that contributes to the renewal of the population.

The population of small and medium mammals, along with birds, sustain larva and nymphs which tends to follow their dynamic cycle with differences according to each tick stage ((Matuschka *et al*., 1990; Humair *et al*., 1993; Craine *et al*., 1995; L'Hostis *et al*., 1996a). In Mafra, these mammals are, with certainty, an important factor defining seasonal dynamics and questing peaks of larva and nymphs and must be carefully studied.

2.5.3 Nationwide tick study

Tick species collected in the nationwide study were in accordance with collections performed by other authors, mainly from captures on hosts and only a few from dragging technique (Bacellar *et al*., 1995; Caeiro, 1999; Estrada-Pena & Santos-Silva, 2005). *Rhipicephalus* spp and *Dermacentor marginatus* adults ticks were the main species collected, despite the fact that dragging is not the recommended technique for collecting these stages (as already mentioned) and collection on hosts is normally more successful (Casado *et al*., 2004). Bacellar and others (1995) collected, on hosts, about 68% of *Rhipicephalus* spp, 16.4% of *D. marginatus*, 6.2% of *H. marginatum*, 4.3% of *Ha. punctata* and 1% of *I. ricinus*, indicating a higher prevalence of these two genera.

These two species had also the widest distribution in the nationwide study. *Rhipicephalus* spp had the widest distribution appearing in 16 of the 22 polygons. *D. marginatus* was found in 9 polygons distributed mainly in the littoral regions, from north to south of Portugal Most of these polygons are distributed in the centre and south of Portugal. On the other hand, *I. ricinus* had a more restricted distribution with only 6 polygons positive for this tick (Braga, Coimbra, Guarda, Lisboa, Coruche and Grândola)..

The other three species were found in three or less polygons, *H. marginatum* in Rio Maior, Portalegre and Grândola, *Ha. punctata* in Lisboa and Grândola and other species of *Ixodes* in Lisboa, although other distributions were already confirmed in the studies above.

With kriging analysis, it was possible to predict the distribution of the three more important species. *Rhipicephalus* spp presents a crescent probability of occurring from north to south of Portugal, with no distinction between inland and coastal regions. *D. marginatus* increases its distribution towards NW and SW, with a preference for more southern regions. On the other hand, *I. ricinus* have a more restricted distribution although it is present throughout the littoral region.

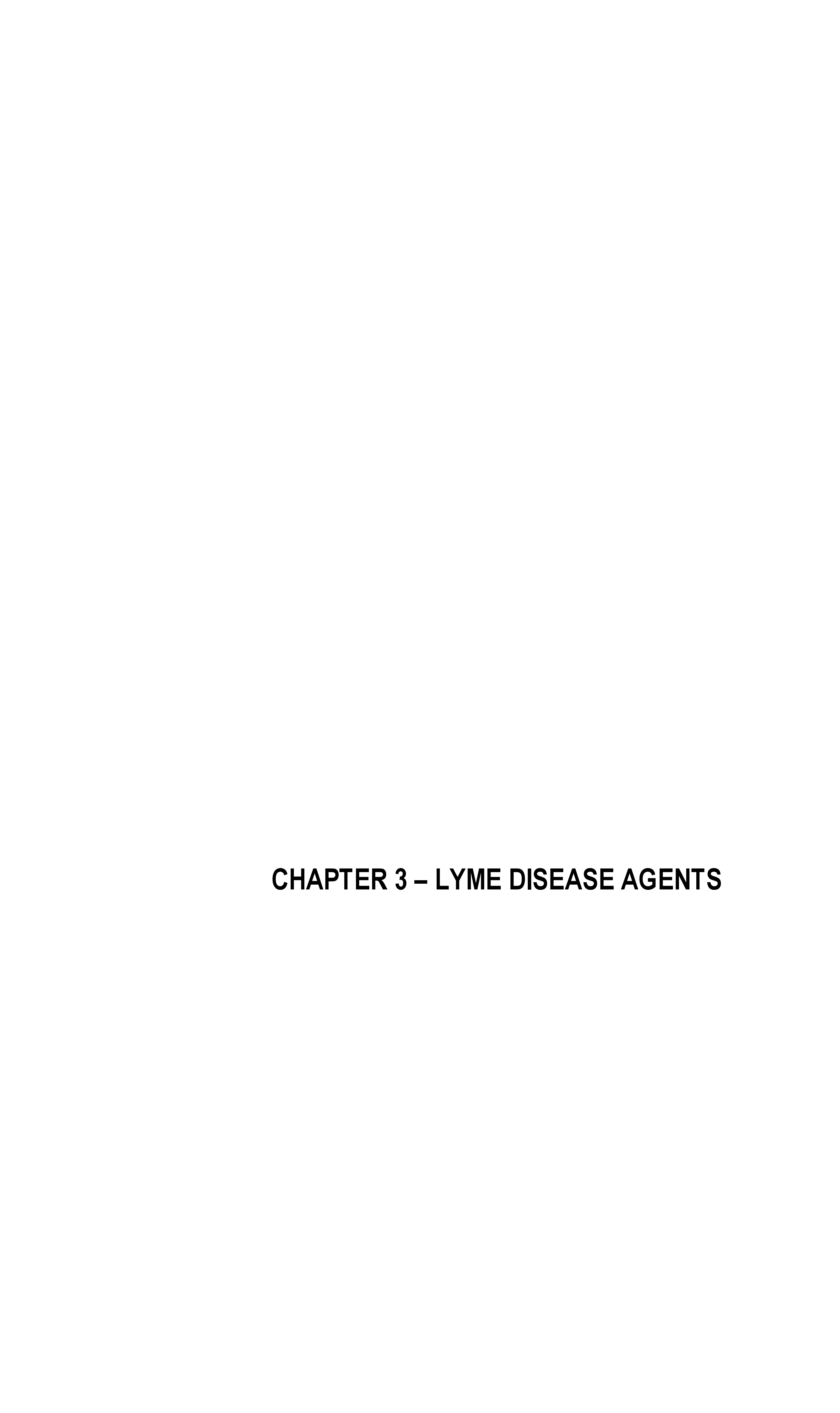
Although kriging will produce results that honour the data, the estimated values at locations between sample sites are nonunique. In many, if not most cases, there is not enough data available to clearly and absolutely define the semivariogram, but by incorporating the modeler's knowledge or expert opinion about the site, uncertainty may be reduced, possibilities limited, and reasonable results may be identifiable. One

must keep in mind that this statistical method has limitations and it is not correct to assume the presence of trends nor assume a simple pattern without sufficient observation (William & Poeter, 1993). Since data collection is time-consuming and expensive, a future improvement can be performed by examining data as it is collected, preparing experimental semivariograms, plotting information errors and using the results to select subsequent data types and locations. This can be performed in any time and allows the improvement of our distribution maps obtained in the present study.

Nevertheless, in a recent study, Estrada-Pena & Santos-Silva (2005) compiled data from collections carried out in 1988-1998 based on the smallest administrative divisions (AD) available in Portugal (the *freguesia*), that confirmed reasonably the results obtained in this thesis. These collections were made from hosts (about 97%) and standard dragging (3%). *Rhipicephalus* group was the genus collected in the majority of AD, followed by *D. marginatus*, *I. ricinus* and *H. marginatum*, among others. *Ha. punctata* has also been collected in some points of the country. *Rhipicephalus* spp, *D. marginatus* e *H. marginatum* are mostly restricted to south-eastern parts of the country. However, both *D. marginatus* and *Rhipicephalus* spp are also found in some points of central and northern parts of the country in colder areas at higher altitude. *I. ricinus* has a very patchy distribution and has been found in southern zones of the country, but also in the humid western part, the western coast and in northern parts.

Host availability is one of the possible limiting factors for tick distribution, along with vegetation type and structure, climate and elevation. These variables have been the basis of tick distribution studies in several countries. For instance, in Spain, Estrada-Pena (2001, 2003) divided a study area into 18 categories as defined by Spanish Land Use Inventory Office (SLUIO), slightly modified, to follow the classification already used in previous studies about habitat suitability for *I. ricinus* (Daniel *et al.*, 1998; Gray *et al.*, 1998 fide Estrada-Pena, 2001). Habitat categories were determined according to the prevalent tree species, the fraction of area covered by that dominant species, together with the age of the forest (old, young, reforested) and the structure of the site (homogeneous or heterogeneous). Other authors studying *I. ricinus* habitat preferences cited plant communities using syntaxonomic botanical units (Gilot *et al.*, 1979 *fide* Estrada-Pena, 2001), but this technique is highly time-consuming and difficult to apply in large areas. Definition of homo-ecologic zones based on a solid floristic analysis and a profound study on environmental conditions (climate, soil, hydric systems, animal biocenosis) was also attempted. Vegetation is not considered alone but integrated within the different elements of environment. Cartography of these zones allowed a delimitation of

favourable or non-favourable territories for installation of animal populations (Gilot *et al*., 1975, 1975a). Geographic data used as GIS layers, like altitude (numerical), exposure, geological substratum, vegetation type and dominant plant species, wood age, wood density, ground cover, roe deer density (numeric), sky cover, road networks, land use and hidrography, can also be considered as possible long-term predictors of the presence/absence of ticks in the modelling (Glass *et al*., 1995; Merler *et al*., 1996; Nicholson & Mather, 1996; Rizzoli *et al*., 2002)



CHAPTER 3 – LYME DISEASE AGENTS

Chapter 3 – Distribution and Diversity of *Borrelia burgdorferi* sensu lato complex

3.1 - Introduction

The epidemiology of Lyme Borreliosis (LB) in the temperate Old World presents a greater diversity within Lyme disease systems resulting in a spatially variable risk of transmission. Infection prevalence in unfed nymphal ticks varies widely, depending on the resulting pattern of vector distribution, abundance and seasonality (Randolph, 1998; Vassalo *et al*., 2000a). Other factors related to this biotic diversity are the genetic diversity of *B. burgdorferi* sensu lato and the wide range of hosts available to ticks (Randolph, 2000).

An active LB foci is dependent on the fulfilment of three conditions: tick survival, pathogen survival and opportunities for human exposure. Tick survival depends on the availability of habitats that provide food sources, protection from environmental extremes and ultimately afford the opportunity for successful reproduction. Pathogen survival is dependent on sufficient densities of ticks and suitable reservoir hosts, and opportunities for transmission between them in order to maintain infection. Human exposure occurs when there are sufficient encounters between infected ticks and humans (Cortinas *et al*., 2002).

To identify LB foci, it is therefore necessary to determinate *B. burgdorferi* s.l. infection rates in ticks as a crucial first step to understanding the spatial distribution of LB in Portugal. Ultimately, the confirmation of LB in the human Portuguese population will allow the preliminary identification of areas with the greatest potential risk areas which will be refined in the last chapter through exploration of environmental mechanisms driving the patterns observed here. Furthermore, the distribution of human cases will help our understanding of LB epidemiology in Portugal (Zeman, 1997).

3.2 - Objectives

a) (**Ticks**) To quantify the *B. burgdorferi* s. l. infection rates in *I. ricinus* and other tick species by ascertaining data about: i) seasonality of locally transmitted *Borrelia* genotypes (Focal study at Tapada Nacional de Mafra), ii) distribution of infected ticks and diversity of transmitted genotypes at a national level (Nationwide study)

b) (**Humans**) To determine the status of this disease in suspected LB patients, contributing to our knowledge of LB in the human population of Portugal.

3.3 – Material and Methods

3.3.1 Ticks (*Borrelia* detection)

Detection of *B. burgdorferi* s.l. agents was carried out in questing ticks (ixodids) collected during the focal study and the nationwide study, as described earlier (Chapter 2). Almost all adults, at least 30 nymphs per month or site (or less when the number collected was lower), and several pools of larvae (10 larvae per pool) were used in this approach.

3.3.1.1 Culture in selective medium

Initially, ticks were disinfected with sodium hipochlorite, alcohol and distilled water, during a five minutes washing step (Sparagano *et al*., 1999; Speck *et al*, 2002; Couceiro *et al*, 2003). Each adult and nymph was divided longitudinally into two halves with sterile forceps and surgical blade One half was inoculated (**Fig. 3.1**) into sterile 5 ml Nunk® plastic tubes containing BSK-II or BSK-H semi-solid (with agar) medium supplemented with antibiotics (Riphampicine and Phosphomicine) and a fungicide (Amphotericine B) (Kurtenbach *et al*, 1998; Polovinchik, 1999; Couceiro *et al*, 2003). Those tubes were filled with media to 70% capacity and sealed tightly to maintain the gas balance for growth of *B. burgdorferi* s.l agents (Polovinchik, 1999).

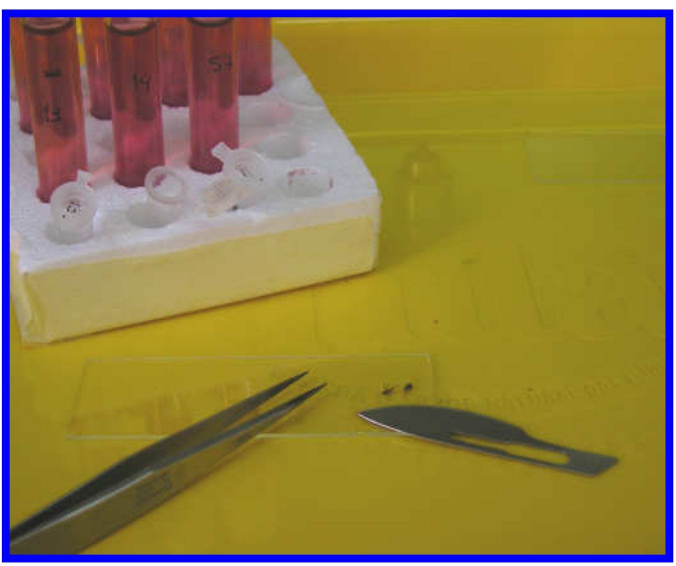


Figure 3. 1- Culture of ticks in selective medium BSK, after bisection (photo by the author).

Cultivated ticks were maintained at 34ºC in an incubator (MemMert). Weekly, the presence of *borrelia* was confirmed after observation at 200X in an Olympus BH-2® dark-field microscope, for up to 4 months. A change in colour (from red to yellow) and some turvation of the medium caused by changes in pH were signs of bacterial growth (not necessarily *B. burgdorferi* s.l.)

Positive cultures were passed to new tubes with clean liquid medium, without antibiotics (4 to 12 droplet, depending on the observed concentration; 1 droplet ≈ 33 μl), in order to adapt and maintain the bacteria *in vitro*. For every new tube, a glass tube with a nutritive broth (Difco, Quilaban, Portugal) was prepared to check the occurrence of contaminations with other organisms (cocci, bacillus, fungi). Whenever needed, small sterile filters (0.45 and 0.22 μm, NUCLEPORE Corporation®) attached to a 5 ml syringe were used to filtrate each contaminated medium to a new Nunk® tube. Passages of the positive culture were stored in 1.5 ml cryotubes and kept in a –80ºC freezer for further DNA analysis.

3.3.1.2 DNA amplification

The other half of each sample of *I. ricinus* was stored in a sterile tube at -20ºC and along with other tick-species, these specimens were directly processed for DNA purposes (**Fig. 3.2**). All genomic DNA was extracted with ammonium hydroxide solution at 20% using the methodologies described by Guy *et al*. (1991), Kirstein *et al*. (1996), Rijpkema *et al*. (1996a), Kahl *et al*. (1998), De Michelis *et al*. (2000) and Couceiro *et al*. (2003). Each tick was homogenized in a 100µl aliquot (nymphs and larvae) and 500µl (adults) of freshly prepared solution of 1:20 ammonium hydroxide in a 1.5 ml safe-lock eppendorf (Eppendorf®) with a sterile pipette tip. The tubes were sealed, and the contents were denatured at 100ºC for 20-25 minutes in a thermobloc (TEMBLOC, Selecta). Subsequently, the lids of the tubes were opened and the tubes were boiled until the solution was reduced to half of the initial volume (50µl and 250µl, respectively). The lysates (template) were stored at 4ºC or –20ºC until ready for use. For controlling contamination in this step, four 1.5 ml safe-lock tubes ($1n_1$ to $1n_4$) were prepared with only 100µl of ammonia, and the former procedures were also done.

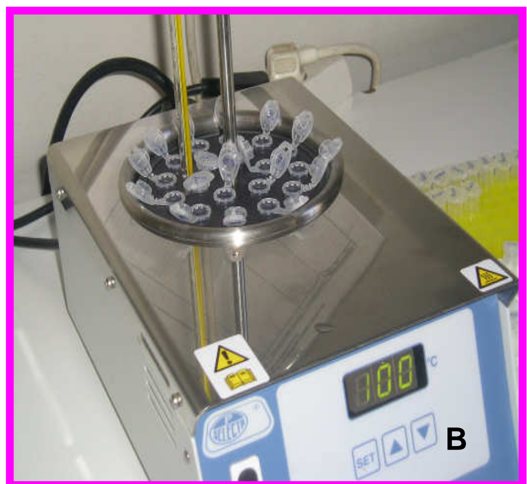


Figure 3. 2 – DNA extraction procedure. Use of sterile safe-lock eppendorfs to homogenize ticks in a 20% ammonium hydroxide solution (A) and boiling in a thermobloc (B) (photos by the author).

All templates, including negative controls of extraction ($1n_1$-$1n_4$), were subsequently analyzed by a *nested*-PCR (Polymerase Chain Reaction) for the intergenic spacer *rrf*(5S)-*rrl*(23S) of *B. burgdorferi* s.l. (Rijpkema *et al.*, 1995, 1996a; Kurtenbach *et al.*, 1998a, 1998b; Gray *et al.*, 2000; De Michelis *et al.*, 2000; Couceiro *et al.*, 2003). This region allows the assignment of any given strain to genospecies according to the current taxonomic criteria (**Table 3.1**), as already described in Chapter 1.

Table 3. 1 - Oligonucleotide sequences of primers used in PCR for detection of *B. burgdorferi* sensu lato genomic groups (Schwartz *et al.*, 1992 *fide* Rijpkema *et al.*, 1995)

Designation	**Nucleotide sequence**	**Position on 5S-23S intergenic spacer region**
Primers for first PCR		
23SN1	5'- ACCATAGACTCTTATTACTTTGAC	469-446
23SC1	5' - TAAGCTGACTAATACTAATTACCC	92-115
Primers for second PCR		
23SN2	5' - ACCATAGACTCTTATTACTTTGACCA	469-444
5SCB	5'- biotin- GAGAGTAGGTTATTGCCAGGG	243-263

After the first standard PCR, 20 µl of the first reaction plus template mix in a 0.2 µl eppendorf (Sarsted) was removed and a second master mix containing primers specific for an inner part of the generated amplicon was applied (**Table 3.2**). All the amplifications were done in a thermal cycler (MyCycler™ Thermal Cycler BioRad).

Table 3. 2 – Protocol for *nested*-PCR with two rounds of amplification.

	First PCR	
Mix 1 (20 µl)	**Concentrations**	**Amplification conditions**
dH_2O $MgCl_2$ (1; 2) NH_4 buffer (1; 2) dNTP (3) Primer 1 (23SN1) (1; 4) Primer 2 (23SC1) (1; 4) Taq-DNA polymerase (1; 2) **Template**	----- 25 mM / 50 mM 10x 10mM (each) 10pmol/µl 9.7pmol/µl 5U/µl 5 µl	1 cycle: Initial denaturating – 1 min, 94.5ºC 25 cycles: Denaturating – 30 s, 94.0ºC Annealing – 30 s, 52.0ºC Extension – 1 min, 72.0ºC 1 cycle: Final extension – 5 m, 72.0ºC
	Second PCR	
Mix 2 (20 µl)	**Concentrations**	**Amplification conditions**
dH_2O $MgCl_2$ NH_4 buffer dNTP Primer 3 (23SN2) Primer 4 (5SCB) Taq-DNA polymerase	----- 25 mM / 50 mM 10x 10mM (each) 10pmol/µl 10pmol/µl 5U/µl	1 cycle: Initial denaturating – 1 min, 94.5ºC 40 cycles: Denaturating – 30 s, 94.0ºC Annealing – 30 s, 55.0ºC Extension – 1 min, 72.0ºC 1 cycle: Final extension – 5 m, 72.0ºC

(1) P.Elmer, (2) Bioline; (3) Amersham; (4) MWG

To avoid contaminations during *nested*-PCR technique, several procedures were performed: a) all manipulations were made in different sterile chambers, with filtered pipette tips; b) *B. japonica* was used as a positive control, since this genospecies was for now only described in Japan (Wang *et al.*, 1999); c) three negative controls with sterilized distilled water were prepared for first and second rounds each ($2n_1$-$2n_3$ and $3n_1$-$3n_3$, respectively) to avoid false positives (Rosa & Schwan, 1989; Rijpkema *et al.*, 1995; De Michelis *et al.*, 2000).

PCR products were analysed in ethidium bromide (BIORAD) stained 2% agarose gels (SeaKem LE Agarose), in TBE 1x (BIORAD) and visualized under UV lights (Eagle Eye® Still Video System (Stratagene). Two DNA fragments of ~380 bp (first product) and ~ 225 bp (second product) were

expected in PCR using samples with a considerable number of spirochetes (x10^3 bacteria/ml). Low copies of DNA template can give origin just to the second product. All PCR products were kept at 4ºC or –20ºC until further use.

3.3.1.3 Genotyping

To provide a genospecies identification, only a small fraction of the intergenic spacer amplicons from positive ticks was subjected to Restriction Fragment Lenght Polimorphisms (RFLP), besides all tick-isolates, either due to logistic reasons or to small amounts of amplified *Borrelia* DNA.

First, positive ticks and reference strains went through a second reamplification of ITS region with primers 3 and 4 to increase DNA concentration (Postic *et al*., 1994; 2000; Masuzawa et al, 2000; De Michelis *et al*., 2000; Huegli *et al*., 2002). For each sample, 10 µl of DNA was digested for 2 hours at 37ºC in a solution with 1.25 U of *Mse*I, 500 µg of BSA and 10xNEBuffer 2 (50 mM NaCl, 10 nM Tris-HCl, 10 mM MgCl2, 1 mM DTT) (New England Biolabs®), in a final volume of 25 µl.

A 16% acrylamide gel (19:1 acrylamide-Bis acrylamide 40%) was used to analyze the restriction profiles of digested PCR products (**Table 3.3**). After 2 hours of polymerization, another 5% acrylamide gel (concentration gel) was poured on top and a comb was inserted. Fourty-five minutes latter, the comb was removed and 22.5 µl of each sample and positive reference strains with loading buffer (3 µl) were pipetted into each well. Electrophoresis was run for 2 h at 100 V in a Mini-Protean II Cell (BIO-RAD®) filled with TBE 1x.

Finally, the gel was stained with ethidium bromide (12.5 µl in 250 ml TBE 1x) and visualized in UV light (EagleEye). Molecular weight of each band was compared with those already known for reference strains (Postic *et al*., 1994; 2000; Wang *et al*., 1999; Lee *et al*., 2000; Collares-Pereira *et al*., 2004). (**Table 3.4**).

Table 3. 3 – Reagents for preparation of acrylamide gels

16% acrylamide gels (10x8 cm), n=2	5% acrylamide gels, n=2
Acrylamide-Bisacrylamide 19:1 (40%) – 8 ml (BIO-RAD®)	Acrylamide-Bisacrylamide 19:1 (40%) – 650 µl (BIO-RAD®)
MiliQ H_2O – 9.9 ml	MiliQ H_2O – 3.8 ml
TBE 10x – 2 ml (BIO-RAD®)	TBE 10x – 500 µl (BIO-RAD®)
10% Ammonium Persulfate - 150 µl (BIO-RAD®)	10% Ammonium Persulfate - 40 µl (BIO-RAD®)
TEMED – 17 µl (BIO-RAD®)	TEMED – 12 µl (BIO-RAD®)

Table 3. 4 – Source and RFLP patterns of reference strains used in this work

Strain	Source	Country	Restriction fragments with *MseI*
B. afzelii			
Pgau	Human (skin)	Germany	108, 68, 50, 20
Pk0	Human (skin)	Germany	108, 68, 50, 20
B. lusitaniae			
PoHL1	Human (skin)	Portugal	108, 81, 39, 29
PoTiBL37	*I. ricinus*	Portugal	108, 81, 39, 29
B. burgdorferi s.s.			
IP3	Human (LCR)	France	108, 51, 38, 29, 28
Vs219	*I. ricinus*	Switzerland	105, 55, 41, 33
B. garinii			
Pbi	Human (LCR)	Germany	108, 95, 50
PoTiBG163	*I. ricinus*	Portugal	108, 50
B. valaisiana	*I. ricinus*	Portugal	175, 50, 23, 7
PoTiBV6			
B. japonica			
H014	*I. ovatus*	Japan	108, 78, 50

Another technique used to genotype PCR products was Reverse Line Blot (RLB), a DNA-DNA hybridisation performed with a line blotter with two plastic blocks (Miniblotter 45; Immunetics). One block contained 45 small channels where the 5 probes specific to *B. burgdorferi* s.l. (BBSL), *B. garinii* (BG), *B. afzelii* (BA), *B. valaisiana* (BV) and *B. burgdorferi* s.s. (BBSS) (**Table 3.5**) were covalently bound to an activated membrane (Byodine C) by the C6-5' aminolink (a) group (1 minute, room temperature). After rotating the membrane 90°, the biotin-labelled products amplified

in the *nested*-PCR hybridized to the oligo's during an incubation period (1h30m, 42ºC). PCR amplicons of DNA templates derived from cloned cultures of reference strains were included as positive controls for the RLB. After a series of steps including incubations with 2xSSPE/0.1% and 2xSSPE/0.5% solutions in a rotative oven, membrane was incubated (30 minutes, 42ºC) with streptadivin-peroxidase conjugate (500U/ml - Sigma) and hybrids were visualized with an enhanced chemiluminescence system (type ECL: Amersham Life Sciences) (**Fig. 3.3**) (Rijpkema *et al.*, 1995, 1996a; Kurtenbach *et al.*, 1998b; De Michelis *et al.*, 2000).

Table 3. 5 – Probe sequences of some reference strains used in Reverse Line Blot (legend: BBSL – *B. burgdorferi* sensu lato, BG – *B. garinii*, BA – *B. afzelii*, BV – *B. valaisiana*, BBSS – *B. burgdorferi* sensu stricto)

Designation	Nucleotide Sequence	Position in ITS 5S-23S
BBSL	5'-a-CTTTGACCATATTTTTATCTTCCA-3'	453-430
BG	5'-a-AACATGAACATCTAAAAACATAAA-3'	322-298
BA	5'-a-AACATTTAAAAAATAAATTCAAGG-3'	305-278
BV	5'-a-CATTAAAAAAATATAAAAAATAAATTTAAGG-3'	303-278
BBSS	5'-a-AACACCAATATTTAAAAAACATAA-3'	322-298

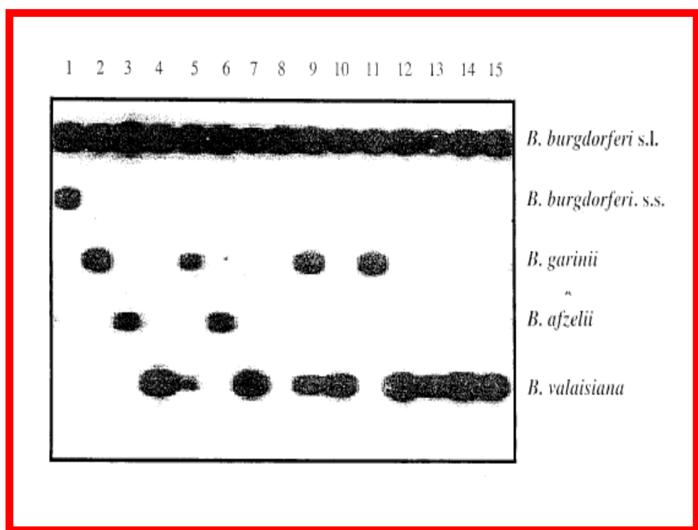


Figure 3. 3 – Visualization of positive dots in the activated Byodine-C membrane

Common univariate statistics were used for comparisons and analysis of data, namely Kolmogorov-Smirnov, X^2, Anova or Kruskal-Wallis and Correlation using *Statistica* 6.0 and *SPSS* 11.0 programs.

3.3.2 Patients (*Borrelia* detection)

The study of human LB was conducted, from 2000 to 2004, in Lyme patients suspected of having infection, as part of the routine diagnosis of Lyme Borreliosis at Unidade de Leptospirose e Borreliose de Lyme (ULBL/IHMT). A total of 469 requests for routine LB diagnosis of 369 suspected patients from different origins were studied, of which 91 (24.7%) individuals entered in 2001, 113 (30.6%) in 2002, 83 (22.5%) in 2003 and 82 (22.2%) in 2004 (**Fig. 3.4**). In the year 2002, the number of patients and samples was higher, due to existing protocols with hospitals from distinct regions.

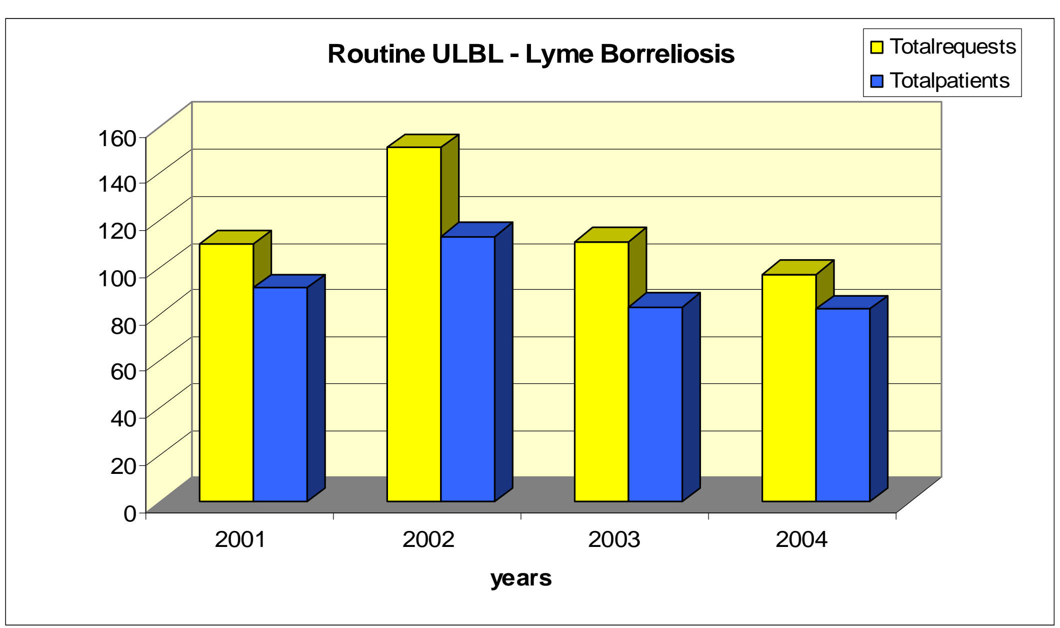


Figure 3. 4 – Annual distribution of requests and of Lyme suspected patients analyzed at the ULBL/IHMT (2001 to 2004).

A total of 582 clinical specimens were received for routine Lyme diagnosis, distributed as follows: 420 (72.2%) sera, 73 (12.5%) cerebro spinal fluid (CSF), 76 (12.9%) skin biopsies, 4 (0.7%) were

synovial fluids and 10 (1.7%) blood samples (**Fig. 3.5**). These different clinical samples were submitted to different techniques as summarized in **Table 3.6.**

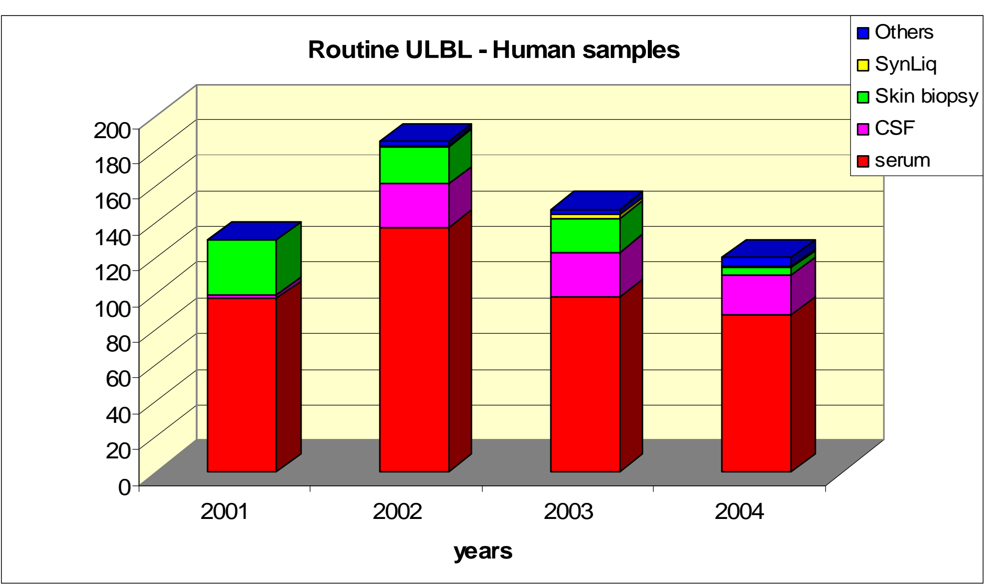


Figure 3. 5 – Annual distribution of analyzed human samples at ULBL/IHMT (2001 to 2004), per tested material.

Table 3. 6 – Lyme Borreliosis diagnostic techniques performed at ULBL/IHMT for each type of clinical specimen

Clinical specimen	Direct search		Indirect search	
	Culture	PCR-RFLP	IFA	WB
CSF	✓	✓	✓	✓
serum		✓	✓	✓
skin/synovial tissues	✓	✓		
synovial fluid	✓	✓		
whole blood	✓	✓		

3.3.2.1 Culture in selective medium

Skin/synovial tissues were divided into two halves in a sterile chamber and one piece was inoculated in BSK-H medium. Approximately 3 to 4 droplets of synovial fluid, CSF and blood were

pipetted directly into the 5 ml tubes with BSK medium and the remaining volume was frozen (-20ºC) in 1.5 ml cryotubes for further use. All culture tubes were checked for spirochetes every week and if no growth was observed, a monthly visualization was done until one year after the first culture.

3.3.2.2 DNA amplification and genotyping

Molecular diagnosis was conducted using the same technique already presented for ticks (i.e. *nested*-PCR, see 3.3.1.2). For DNA extraction, commercial kits were used –from Quiagen® and Gentra® -, both based on proteinase K extraction. All biological samples were subjected to these kits and followed the manufacturer's instructions. Finally, genotyping of PCR derived amplicons was performed with the RFPL technique also described earlier (3.3.1.3).

3.3.2.3 Serological diagnosis

Human serological diagnosis followed the recommended two-step protocol, *i.e.*, screening of specific *B. burgdorferi* sensu lato antibodies by a genus-specific test, indirect immunofluorescence assay (IFA), followed by reference Western blotting (WB), whenever a doubtful or positive reaction was detected. Herewith, only IFA will be described, since WB was not performed by the author.

A home-made polyvalent IFA was performed according to Collares-Pereira *et al.* (2000a). An human isolate of *B. garinii* (reference strain *Pbi*, kindly provided by Dr. Bettina Wilske) was used to prepare an antigenic suspension due to its higher sensitivity and specificity (Collares-Pereira *et al.*, 2000a). This antigen was prepared by centrifugating 50 ml of culture at 30,000 rpm x 20'. The supernatant was discarded and the tube was filled (washed) with PBS buffer solution, pH 7.2 (0.01M) and centrifuged again (2x20'). The pellet was finally transferred to a cryotube with ≈ 500 μl of PBS. To prepare IFA slides, a best dilution (1:20, 1:40, 1:60, 1:80) was chosen according to the concentration of *Borrelia* in the diluted pellet, visualized in a dark-field microscope. An adequate amount of spirochetes in all microscope fields was preferred. Normally a dilution of 1/20 was chosen to prepare several slides to use in routine diagnosis (40 to 70 slides, in packs of 4, wrapped in aluminum foil).

IFA procedure was initiated with dilution of serum samples and controls (positive and negative) in a microtiter plate (**Fig. 3.6**). Serum dilutions were prepared with PBS, as follows: 1:32, 1:64, 1:128 and 1:256. For CSF the starting dilution was 1:4, followed by three serial double dilutions (1:8, 1:16 and 1:32). The positive control had the same serum dilutions, whereas for the negative only one dilution (1:32) was needed. A volume of 4 μl of serum or CSF was applied to the slide (per each well) and incubated in a moist chamber for 30 min at 37ºC, washed with buffer and left to dry. In the meantime, the fluorescent polyconjugate, *i.e*, a rabbit anti-human IgA/IgG/IgM/kappa/Lambda FICT (Dako) was prepared at a 1:40 dilution with PBS. Again 4 μl of conjugate was applied per well on each slide, reincubated for 30 minutes at 37ºC in a moist chamber, washed with buffer and distilled water, and allowed to dry.

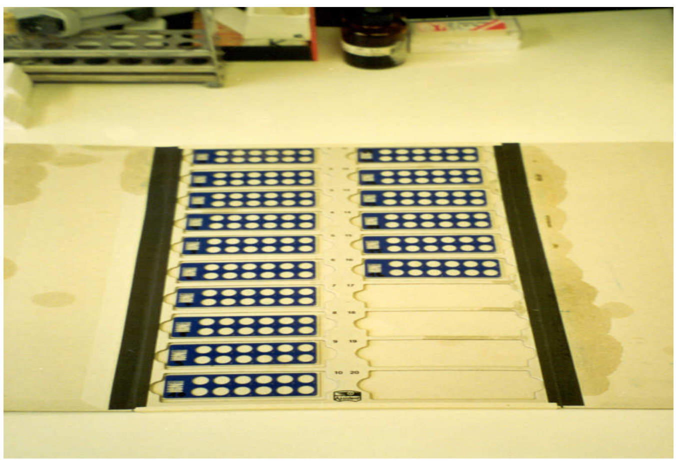


Figure 3. 6 – Microtiter plates used in immunofluorescence

IFA results were read under a fluorescence microscope (Olympus), between 450 and 490 nm. If the serum or CSF contained specific antibodies, the coated slide *Borrelia* fluoresced a yellow-green light (**Fig. 3.7**); if there were no antibodies, the *Borrelia* did not fluoresce at all. The test was considered not conclusive if the *Borrelia* fluoresced at a serum dilution ≤ 1:64 (1:8 for CSF), doubtful at a serum dilution ≤ 1:128 (1:16 for CSF)) and positive at a serum dilution ≥ 1:256 (≥1:32 for CSF). The positive control was a serum with a high titer of antibodies (≥ 1:256) from a patient with confirmed Lyme disease and the negative control was a serum without specific antibodies.

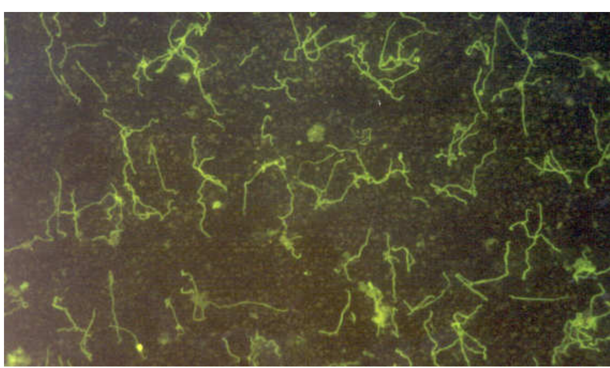


Figure 3. 7 – Green-fluorescence of *Borrelia garinii* antigen in reactive samples

As already mentioned, a doubtful and a positive result were confirmed by the WB reference technique (a home-made test or using a diagnostic kit), but negative or inconclusive results were also re-examined, depending on results from other techniques (culture, DNA amplification), clinical diagnosis and epidemiological aspects (e.g. trips abroad, contact with ticks, residence in risk areas). WB results followed the same scheme as IFA (negative, not conclusive, doubtful and positive) according to the strength and amount of specific diagnostic bands visualized in the immunoblot strips as recommended by Hauser *et al*. (1999).

(Data analysis)

A confirmed laboratory diagnosis for Lyme Borreliosis was always based on at least one of the following criteria: a positive immunobloting, a DNA specific amplification or a positive culture.

Univariate analysis was performed to compare the number of positive samples *versus* patients and their geographic origin.

3.4 - Results

3.4.1 Ticks (*Borrelia* infection rates)

3.4.1.1 Focal study

From a total of 6,800 questing *I. ricinus* ticks collected during monthly surveys, 2,842 (41.8%) were tested for *B. burgdorferi* s.l. infection using direct search. Most adults were tested including 76.7% females and 72% males, while 35.9% of nymphs and 46.4% larvae were examined.

Borrelia DNA amplification was attempted in all adults, 208 females and 205 males, in 1,598 nymphs and in 831 larvae. All adults and 744 out of 1,598 nymphs (46.6%) were also processed for *Borrelia* isolation. Seven isolates were obtained, from six females and one male, yielding an infection rate for adults of 1.7% (7+/413 adults)

When analyzed via *nested*-PCR test (**Fig. 3.8**), a larger proportion of ticks were found infected, with a total infectivity rate of 11.8% (335+/2,842). Infection rates differed significantly between all stages and gender (X^2=262.33, $p<0.05$) with 18.7% in females (39+/208), 9.3% in males (19+/205), 17.3% in nymphs (276+/1598) and 0.12% in larvae (1+/831).

Seasonal dynamics of infection rates based on *Borrelia burgdorferi* s.l. DNA detection were analysed for adults and nymphs (**Fig. 3.9**). A positive correlation (r_{fem}=0.66, r_{mal}=0.39; r_{nym}=0.76; p=0.000; Spearman rank test) was found between seasonal variation of number of ticks and number of infected ticks during the 5-year period, indicating a higher risk of being infected during the higher peaks of ticks and a lower risk when density of ticks is lower.

When analyzed specifically the group of nymphs, some short periods occurring in 2000, 2001 and 2002, presented a negative correlation described by a positive peak of infected ticks when the number of questing nymphs was decreasing or at lower densities. This situation was not observed for the other stages.

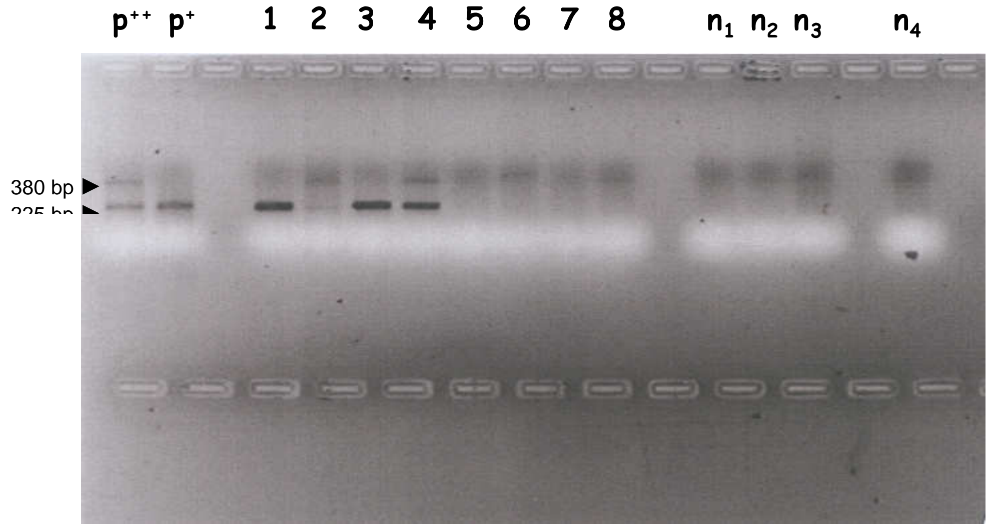


Figure 3. 8 – Example of a nested PCR targeting the *rrf* (5S) - *rrl* (23S) intergenic spacer of *B.burgdorferi* .s.l. in *I. ricinus* ticks from Tapada Nacional de Mafra, where is possible to detect specific DNA in ticks from lanes 1, 3 and 4 (legend: p - positive controls; 1-8 – *I. ricinus* ticks; n - negative controls)

A total of 328 questing ticksof species other than *I. ricinus* were examined, of which 102 (31.1%) were *Rhipicephalus* spp, 88 (26.8%) were *Dermacentor* spp and 138 (42.1%) were *Haemaphysalis* spp (**Table 3.7**). *B. burgdorferi* s.l. DNA was found in 5.2% of these ticks. *Dermacentor marginatus* was the most infected tick species, reaching almost 8%, followed by *Haemaphysalis punctata* and *Rhipicephalus* spp, but no statistical differences were found (F=0.17, p=0.84).

Table 3. 7 – Distribution of *B. burgdorferi* sensu lato infected tick species, *Rhipicephalus* (R), *Dermacentor* (D) and *Haemaphysalis* (Ha) (Focal study, TNMafra, 1999-2004)

	Rhip		Derm		Haem	
	n	**pos**	**n**	**pos**	**n**	**pos**
1999	18	0	64	0	7	0
2000	21	1	9	3	29	1
2001	12	2	5	3	15	0
2002	32	0	3	0	41	0
2003	5	0	6	1	39	5
2004	14	1	1	0	7	0
total	**102**	**4**	**88**	**7**	**138**	**6**
		3.9%		7.9%		4.3%

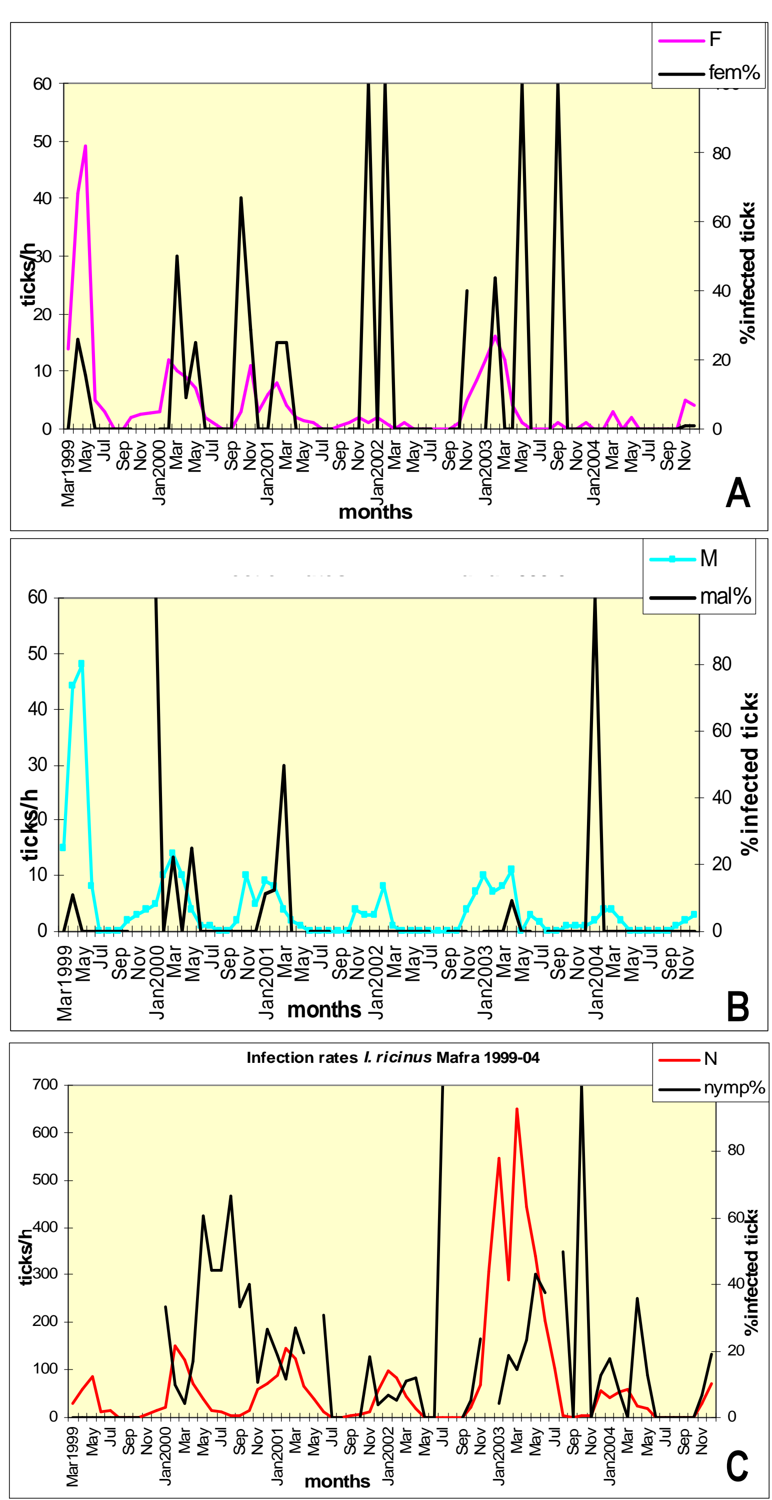


Figure 3. 9 –Seasonal dynamics of *Ixodes ricinus* and *Borrelia* infection rates by stage and gender - number of collected ticks (colored line) and rate of infected ticks (black line) for females (A - pink), males (B - blue) and nymphs (C - red) (Focal study, TNMafra, 1999-04)

3.4.1.2 Nationwide study (ticks)

A total of 788 ticks collected at 55 sampling sites were examined for the presence of *B. burgdorferi* s.l. spirochetes by amplification of specific DNA. Culturing was only attempted for ticks collected at sites 53 and 54 (Mafra and Grândola sites, respectively), where the presence of *B. burgdorferi* s.l. was already known (Baptista *et al*., 2000; De Michelis *et al*., 2000)

Table 3.8 presents the number of analyzed and *B. burgdorferi* s.l. infected ticks for the three main tick species collected in the nationwide study (*Rhipicephalus, Dermacentor* and *I. ricinus*). In addition, *H. marginatum* and *Ha. punctata* were found to be positive for *B. burgdorferi* s.l. infection, at sites 53 and 54 (**Table 3.9**).

Table 3. 9– Distribution of ticks (N) examined using PCR and of *B. burgdorferi* s.l. positive ticks per sampling site from 2001 to 2004 in the nationwide study (Hy – *Hyalomma*; Ib – *Ixodes bivari*; Ha – *Haemaphysalis*)

LOCAL	POLYGON	LOCAL_NAME	N	+ (%)
Site 10	Évora	Arraiolos	1 Hy	0
Site 22	Coimbra	Buçaco	2 Ib	0
Site 53	Lisboa	TapNacMafra	12Ha; 1 Ih	3 (25) Ha
Site 54	Grândola	HRA	44 Hy; 60 Ha	5 (11.4) Hy 11 (18.3) Ha

An overall *B. burgdorferi* s.l. infection rate of 21.2% (167^{+} out of 789) for all species in this nationwide study was found. Of these, *D. marginatus* was the most infected (34.5%), followed by *I. ricinus* (24.5%), *Ha. punctata* (19.4%), *H. marginatum* (11.1%) and *Rhipicephalus* spp (3.7%), but these differences were not significant (K-W=5.10, p=0.28).

No positive cultures were obtained from ticks collected at Mafra site in the months studied, but in the HRA site (Grândola polygon), 15 out of 88 (17.0%) *Borrelia* tick-isolates were obtained: 3 from males (3+/46, 6.5%) and 12 from females (12+/42, 28.6%). These differences between sexes were significantly different ($p<0.01$; Quaresma, 2003). Ticks with a positive culture for *B. burgdorferi* s.l., also showed the presence of specific *Borrelia* DNA by *nested*-PCR. Distribution of infected ticks within sites and polygons can be observed in **Fig. 3.10** and the spatial distribution resulting from kriging analysis is shown in **Fig. 3.11**.

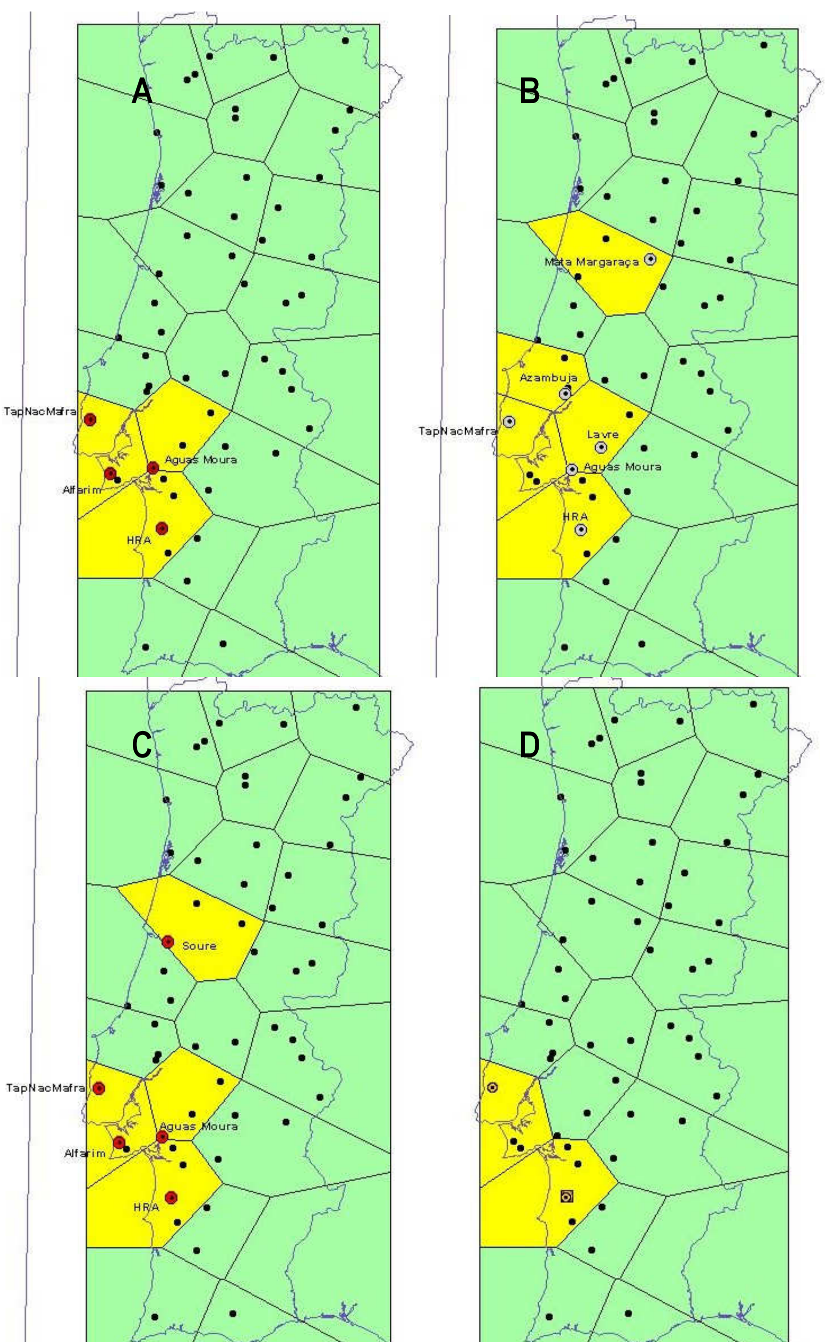


Figure 3. 10 – Distribution of infected tick species within sample sites and Thiessen polygons: A – *Rhipicephalus*, B – *Dermacentor*, C – *I. ricinus*, D – *Hyalomma* (square) and *Haemaphysalis* (circle).

Table 3. 8 – Total number of worked and positive ticks (by detection of specific DNA) of the main species (*Rhipicephalus*, *Dermacentor* and *I. ricinus*) collected in the nationwide study.

LOCAL	POLYGON	LOCALNAME	total worked	*Rhip* (N)	+ (%)	*Derm* (N)	+ (%)	*I.ric* (N)	+ (%)
Site 01	Lisboa	Alfarim	23	22	1 (4.5)	0	0	1	1 (100)
Site 02	Lisboa	V Nog Azeitão	2	2	0				
Site 03	Grândola	Pinheiro (E Arq)	8	8	0				
Site 04	Grândola	Albergue	1			1	0		
Site 05	Grândola	Santiago Cacém	9	5	0	4	0		
Site 06	Grândola	S Domingos	0						
Site 07	Aljustrel	Canhestros	9	9	0				
Site 08	Aljustrel	Garvão	7	7	0				
Site 09	Évora	Alcaçovas	5	5	0				
Site 10	Évora	Arraiolos	3	2	0				
Site 11	Portalegre	Alpalhao	0						
Site 12	Coruche	Mora	0						
Site 13	Coruche	Lavre	52	26	0	26	6 (23.1)		
Site 14	Porto	Fund Serralves	0						
Site 15	Porto	Pq Cidade	0						
Site 16	Braga	Póvoa Lanhoso	13	1	0	10	0	2	0
Site 17	Bragança	Mougadouro	0						
Site 18	Rio Maior	Vale Santarém	11	5	0	6	0		
Site 19	Rio Maior	Azambuja	23	9	0	14	9 (64.3)		
Site 20	Leiria	Nazaré	0						
Site 21	Coimbra	Soure	11	1	0			10	3 (30)
Site 22	Coimbra	Buçaco	5			1	0	2	0
Site 23	Coimbra	Mata Margaraça	3			2	1 (50)	1	0
Site 24	Viseu	Paradela	0						
Site 25	Viseu	V Nova Paiva	1			1	0		
Site 26	Viseu	Canas Senhorim	0						
Site 27	Guarda	Celorico Beira	0						
Site 28	Guarda	Penamacor	0						
Site 29	C. Branco	Oledo	0						
Site 30	Abrantes	Ulme	2	2	0				
Site 31	Monchique	S Espinhaço Cão	2	2	0				
Site 32	Loulé	Besteiros	0						
Site 33	Porto	Murtosa	2	2	0				
Site 34	Braga	Bom Jesus	0						
Site 35	Venda Nova	Gerês	0						
Site 36	Venda Nova	Chaves	7	7	0				
Site 37	Vila Real	S. Marão	0						
Site 38	C. Branco	Loriga	1					1	0
Site 39	Portalegre	Elvas	1	1	0				
Site 40	Rio Maior	Amiais Cima	1	1	0				
Site 41	Abrantes	Rosmaninhal	3	3	0				
Site 42	Leiria	Meirinha	0						
Site 43	Leiria	Fatima	7	5	0	2	0		
Site 44	Portalegre	Rasa	1	1	0				
Site 45	Portalegre	Arronches	1	1	0				
Site 46	Évora	Bencatel	1	1	0				
Site 47	C. Branco	Escalos Baixo	1	1	0				
Site 48	C. Branco	Orvalho	0						
Site 49	V.N.FozCoa	FCastRodrigo	0						
Site 50	V.N.FozCoa	Freixo Esp Cinta	1			1	0		
Site 51	Bragança	Pq Montesinho	1	1	0				
Site 52	Vila Real	Pq Alvão	0						
Site 53	Lisboa	TapNacMafra	166	4	1 (25)	5	1 (20)	144	14 (9.7)
Site 54	Grandola	HRA	357	20	4 (20)	150	59 (39.3)	83	39 (47)
Site 55	Coruche	Aguas Moura	48	34	1 (2.9)	9	4 (44.4)	5	4 (80)
total			**789**	**188**	**7 (3.7)**	**232**	**80 (34.5))**	**249**	**61 (24.5)**

Figure 3. 11 (A, B) – Kriging distribution of infected ticks (indicator variable): A – *Rhipicephalus; B -Dermacentor*

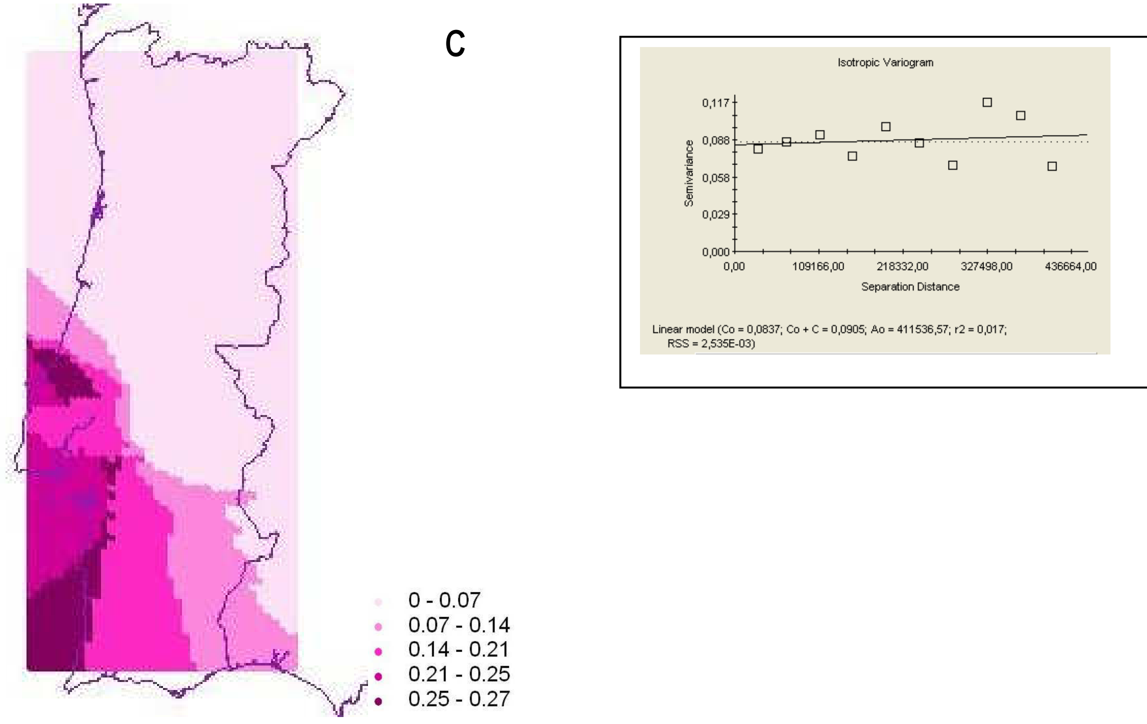


Figure 3.11 (C) – Kriging distribution of infected ticks (indicator variable): C – *I. ricinus*

Total Sill obtained in the semivariograms was higher in *I. ricinus* infected ticks values indicating a higher correlation with distance between sampling values. The nugget to sill ratio was below 0.5 in both *Rhipicephalus* and *I. ricinus* species indicating that some spatial structure was present, but not in *Dermacentor* where the nugget to sill ratio was 1. Infection with *B. burgdorferi* s.l. was found to be more prevalent in the coastal region (Lisboa, Grândola, Coruche and Coimbra polygons), regardless of tick species, with a decreasing probability of occurrence from outer to interior regions of Portugal.

3.4.1.3 Other collections (ticks)

A total of 86 (55.5%) ticks out of 155 that were obtained from other sources were examined for *B. burgdorferi* s.l infection (**Table 3.10**). Only one *Rhipicephalus* tick, from Tomar, was infected.

Table 3.10 – Distribution of *B. burgdorferi* s.l. infected ticks per species obtained from other collection sites

POLYGON	Local Name	Examined (n)	*Rhipicephalus* n/ +	*Dermacentor* n/+	*I. ricinus* n/+
Abrantes	Tomar	25	23/1+		
Bragança	Mogadouro	6	6/0+		
Coruche	Pegões	5	5/0+		
Évora	Estremoz	4	4/0+		
	Alcaçovas	1			
	Alqueva	2	2/0+		
Grândola	Melides	1	1/0+		
Loulé	Faro	15	15/0+		
Lisboa	Caparica	18	18/0+		
	Sesimbra	1			1/0+
	Oeiras	2	2/0+		
	Lisboa	1	1/0+		
	Sintra	2	2/0+		
	Mafra	28	28/0+		
Porto	Cinfães	1	1/0+		
V. Nova	Montalegre	18		18/0+	

3.4.2. Patients (*Borrelia* infection rates)

As described above, use of laboratory techniques was based on the type of clinical sample and its available quantity and condition of storage previous to the arrival in the lab, upon medical request. Thus, in a total of 940 diagnostic tests, different numbers of analyses per method were performed, with distinct diagnostic values (**Table 3.11**). All serum samples were subjected to immunofluorescence technique (with four repetitions) and c.a 62.8% were submitted to both IFA and WB. Of these, 15.5% were found positive for the presence of antibodies anti-*B. burgdorferi* s.l. These antibodies were not found in any of the CSF samples, but the PCR technique allowed the detection of 19 samples with specific *B. burgdorferi* s.l. DNA (28%). Twenty-two percent of synovial fluid and blood samples were found positive for *B. burgdorferi* s.l. using PCR. Only one positive culture was obtained from a skin chronic biopsy.

Table 3.11– Laboratory diagnosis of Lyme Borreliosis suspected patients according to the test, clinical specimen and respective result (N - negative, NC - not conclusive, D - doubtful, P - positive) at ULBL/IHMT (2000-2004)

	Lab technique	N	NC	D	P
serum (n=420)	IFA (n=424)	255 (60%)	88 (20.7%)	39 (9.2%)	42 (9.9%)
	WB (n=264)	91 (34.5%)	89 (33.7%)	43 (16.3%)	41 (15.5%)
CSF (n=73)	IFA (n=73)	73 (100%)			
	PCR (n=68)	49 (72%)			19 (28%)
biopsy (n=75)	Culture (n=76)	75 (98.7%)			1 (1.3%)
	PCR (n=76)	39 (51.3%)			37 (48.7%)
synfluid (n=4)	PCR (n=3)	2 (66.7%)			1 (33.3%)
blood (n=10)	PCR (n=6)	5 (83.3%)			1 (16.7%)

Origin of samples was known for 365 (99%) of the patients studied. A total of 22 sites from different geographic regions, including Madeira and Azores islands, were registered (**Table 3.12**). Lisboa was the main origin of the studied population (mainly admitted at Egas Moniz Hospital or consulted in some private medical offices), followed by Coimbra (at University Hospital), Tomar and Viseu (at City Hospital) and Almada (at Garcia de Orta Hospital). From these Lyme suspected patients, 32% had confirmed laboratorial diagnosis for Lyme Borreliosis. The regional distribution of these positive patients is shown in **Table 3.12**, with infection rates ranging from 18% to 71.4%. Within Thiessen polygons created for the nationwide study (**Fig. 3.12**), human cases of Lyme Borreliosis are observed primarily in coastal regions, following the same distribution as tick vectors, presented in **Fig. 3.10**.

Table 3.12 – Origin of suspected Lyme Borreliosis patients with a confirmed *Borrelia* infection at ULBL, by either serology, DNA amplification and/or culture (2000-2004)

Provenience	Polygon	Nº individuals	Positive cases	%	Cases per 10000 pop
Alhandra	Lisboa	1	0	0,0	
Almada	Lisboa	10	6	60,0	**0.36**
AngraHeroísmo		4	0	0,0	
Arganil	Coimbra	1	0	0,0	
Avis	Portalegre/Évora	1	0	0,0	
Barreiro	Lisboa	7	5	71,4	**0.63**
Benedita	Rio Maior	1	1	100,0	*
Braga	Braga	2	1	50,0	**0.06**
CaldasRainha	Rio Maior	1	1	100,0	**0.20**
Coimbra	Coimbra	40	11	27,5	**0.76**
Covilhã	Guarda	1	0	0,0	
FigueiraFoz	Coimbra	9	0	0,0	
Funchal		2	1	50,0	
Grândola	Grândola	3	0	0,0	**
Lisboa	Lisboa	248	77	31,0	**1.42**
PontaDelgada		3	1	33,3	**
Porto	Porto	2	1	50,0	**0.04**
Santarém	Rio Maior	2	1	50,0	**0.15**
Setúbal	Lisboa	2	2	100,0	**0.17**
Tomar	Leiria	11	2	18,2	**0.46**
VFXira	Lisboa	3	1	33,3	**0.07**
Viseu	Viseu	11	6	54,5	**0.62**
TOTAL		**365**	**117**	**32,1**	

* - without official data; ** - not assessed for the present study

Figure 3. 12– Distribution of human confirmed cases of Lyme Borreliosis according to the Thiessen Polygons created for the nationwide study.

3.4.3 Genotyping

3.4.3.1 Tick isolates and PCR amplicons

Focal study

All tick isolates were sequenced and registered in GeneBank with the following accession numbers: 5 isolates of *B. garinii* - PoTiP4 (♀) PoTiP20 (♂), PoTiP82 (♀), PoTiP86 (♀), PoTiP163 (♀); 1 isolate of *B. valaisiana* - PoTiP6 (♀); 1 isolate of *B. lusitaniae* - PoTiP37 (♀) (Baptista *et al.*, 2004).

Within *I. ricinus* species, a total of 12 (15%) ITS amplicons were genotyped as *B. garinii*, 42 (52.5%) as *B. lusitaniae*, 16 (20%) as *B. valaisiana*, 5 (6.3%) as *B. afzelii* and 5 (6.3%) as mixed infections of *B. garinii*+*B. valaisiana* (**Fig. 3.13**).

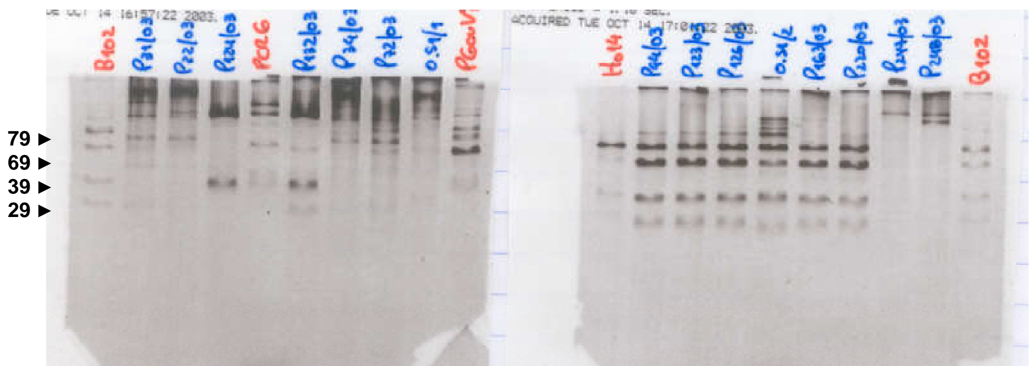


Figure 3. 13 - Restriction Fragments of positive *I. ricinus* (P_n) and other tick-species ($o.s_n$) ticks from Tapada de Mafra. Positive controls are indicated in red color (B102 – human isolate *B. lusitaniae*; PCR6 – tick isolate *B. valaisiana*; Ho14 – tick isolate *B. japonica*; PGauVi – human isolate *B. afzelii*).

B. lusitaniae detection was more prevalent in nymphs (n=35). *B. garinii*, on the other hand was more prevalent in females (n=7). *B. valaisiana* was found in all stages, except larvae and *B. afzelii* was only found in nymphs and larvae. Mixed infections were only found in nymphs (**Fig. 3.14**). These differences were not statistically significant, however these results suggest specific associations between genomic species and tick developmental stage ($p>0.05$).

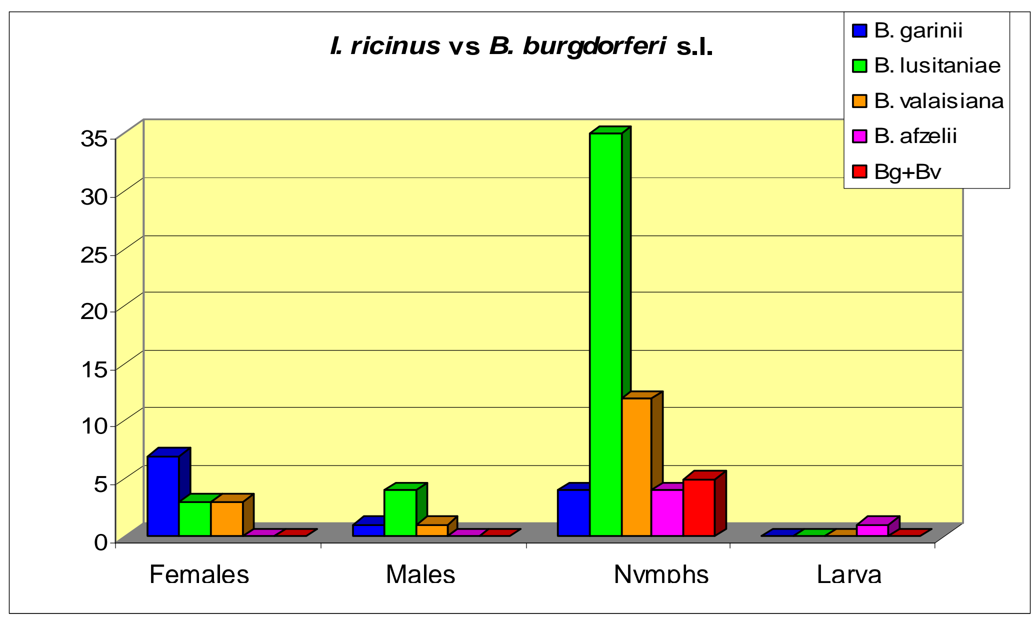


Figure 3. 14 – Distribution of *B. burgdorferi* sensu lato strains per developmental stages of *I. ricinus* at Tapada Nacional de Mafra (Focal study).

Three *Borrelia* genospecies were detected in the other tick species (*B. lusitaniae, B. valaisiana* and *B. afzelii*) (**Table 3.13**).

Table 3.13 – *Borrelia* genotyping of PCR derived amplicons of positive *Haemaphsyalis*, *Dermacentor* and *Rhipicephalus* tick species collected at Tapada Nacional de Mafra (Focal study)

Species and developmental stage	Capture date	*Borrelia* spp
H. punctata - nymph	November 2000	*B. lusitaniae*
- female	February 2003	*B. lusitaniae*
D. marginatus – 2 females	March 2000	*B. afzelii*
- male	February 2001	*B. lusitaniae*
- 2 males	April 2001	*B. lusitaniae*
R. sanguineus - male	December 2000	*B. lusitaniae*
- male	February 2001	*B. valaisiana*
- male	March 2001	*B. lusitaniae*
- female	May 2004	*B. lusitaniae*

Nationwide study (ticks)

Twenty-eight percent (47 out of 167) of the positive ticks collected during this study were subjected to RFLP technique and *Borrelia* DNA was identified as belonging to several members of the *B. burgdorferi* s.l. complex, including *B. lusitaniae*, *B. garinii* and *B. valaisiana*. **Table 3.14** presents the distribution of these genotypes by tick species and sampling sites.

Table 3.14 – Distribution of *Borrelia* genotypes within tick species and sample sites (Ln) in the nationwide study

	B. lusitaniae	***B. garinii***	***B. valaisiana***
***Rhipicephalus* spp**	**L54 (1), L55 (1) (total=2)**		
D. marginatus	**L13 (6), L19 (9), L23 (1), L54 (3), L55 (3) (total=22)**		
I. ricinus	**L21 (1), L53 (4), L54 (14), L55 (4) (total=23)**	**L21 (1)**	**L53 (1)**

B. lusitaniae was the most widespread species in this study (considering the positive polygons presented earlier in this chapter), identified for the first time in several regions (Coimbra, Rio Maior and Coruche) and in two tick species (*Dermacentor* and *Rhipicephalus* spp), besides *I. ricinus*. *B. garinii* was restricted to *I. ricinus* and appeared only at site 21 (Soure, Coimbra polygon), in addition to Mafra site. This was the first identification of *B. garinii* (**Fig. 3.15**) outside the restricted habitat of Tapada Nacional de Mafra. *B. valaisiana* was only found in Mafra.

All culture isolates obtained at Grândola (site 54) were identified, also by RFLP, as *B. lusitaniae*.

Other collections (ticks)

The only positive tick obtained from other collected ticks (*Rhipicephalus* tick from Tomar) was submitted to RFLP but due to insufficient DNA, it was not possible to obtain a consistent result.

3.4.3.2 Human isolate and PCR amplicons

Genotyping was performed for the unique human strain, obtained by which was described with great precision by Collares-Pereira *et al.*, (2004) and for PCR derived amplicons of *B. burgdorferi* s.l. from human samples. Three species were detected, namely *B. lusitaniae*, *B. garinii* and *B. afzelii*. Although interesting, no description of the association between *Borrelia* strains and type of specimen was performed.

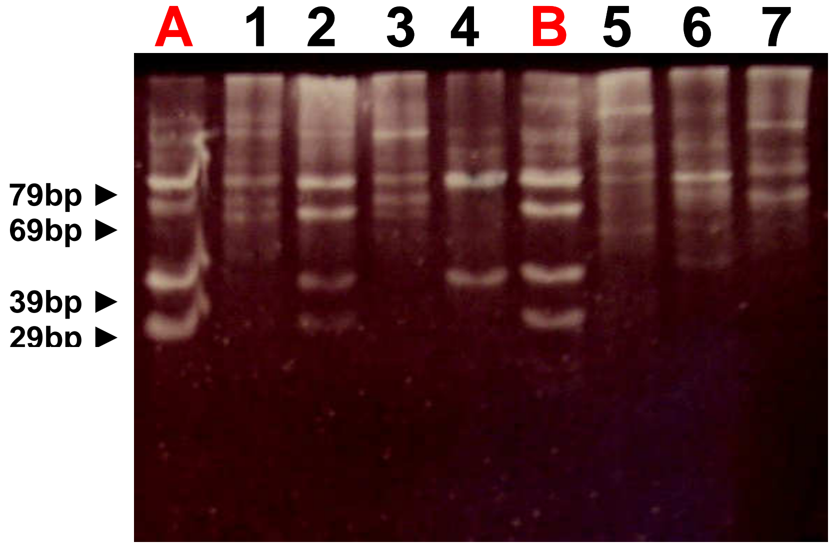


Figure 3. 15 - Restriction Fragments of PCR positive ticks from Portugal: 1 – *D. marginatus*/ Azambuja; 2,3 - *D. marginatus*/Lavre; 4,5,6 – *I. ricinus*/Soure; 7 – *D. marginatus*/Mata Margaraça. Positive controls are indicated in red colour: A - Vs219 (tick isolate *B. burgdorferi sensu stricto*); B - B102 (human isolate *B. lusitaniae*).

3.5 – Discussion

(Ticks)

The main goal of the present study was to gain a greater understanding of the epidemiology of Lyme Borreliosis in Portugal. Both DNA detection and isolation of specimens of *Borrelia burgdorferi* sensu lato complex in its primary vector *I. ricinus* constituted, without doubt, one of the most important achievements. The results from the nationwide study, although requiring a larger sampling in future studies, were also important in their contribution to our understanding of the sylvatic cycle of *B. burgdorferi* s.l. agents in the country.

Besides *I. ricinus*, a tick already incriminated as a vector of *B. burgdorferi* in Portugal (Núncio *et al*., 1993; De Michelis *et al*., 2000; Baptista *et al*., 2000, 2004), other ticks were found to be infected. Furthermore, the distribution of *Borrelia* was not limited to the southern regions, as previously observed (Santos *et al*., 1995; Morais & Henriques, 1999; Morais *et al*., 1999) Infection rates were significant in comparison with other countries. Finally a great diversity of spirochetes was found between different regions in Portugal.

The different *B. burgdorferi* s.l. infection tick-rates found in ticks by culture, as illustrated by Mafra (1.7%) and Grândola (17.0%), although important, point out the recognized difficulty of this diagnostic technique, due to the slow growth of these spirochetes. Indeed, culturing *Borrelia* strains can be time-consuming, due to different responses of the various genospecies to a given environmental factors (*e.g.* media's viscosity). This also represents a challenge when attempting to detect simultaneous infection with several *Borrelia* species or to detect strains from different geographic regions, as observed differences can be due to characteristics of individual *Borrelia* species (Livesley *et al*., 1994; Zore *et al*., 1999; Norris *et al*., 1997 *fide* Derdáková *et al*., 2003; Dumler, 2001; Ruzic-Sabljic & Strle, 2004). Besides, other culture conditions, such as, osmotic strength, dissolved oxygen and carbon dioxide concentration, and temperature of the growth media can influence retention and/or expression of the infective phenotype (Elias *et al*., 1998; Austin, 1993 *fide* Polovinchik, 1999; Houpikian & Raoult, 2002). Also, BSK-H has superior growth supporting capabilities. For instance, in this medium, *B. burgdorferi* s.s. usually overgrow either to *B. afzelii* or

B. garinii, whereas in a mixture of *B. afzelii* and *B. garinii* the latter is the predominant species (Ruzic-Sabljic & Strle, 2004).

Assuming that it was not only a question of medium used (commercial BSK), the above aspects could explain the low number of positive cultures obtained and the predominance of one *Borrelia* species (e.g. *B. garinii* predominance in Mafra and only *B. lusitaniae* in Grândola sites).

Another major aspect to consider is the reduced sensitivity of *Borrelia* selective culturing from ticks and clinical specimens, mainly because of suboptimal inoculation of the growth medium, which depends on the number of spirochetes present (Gustafson, 1994; Livesley *et al.*, 1994; Juntilla *et al.*, 1999; Dumler, 2001;Hu et al., 2001; Houpikian & Raoult, 2002),

Moreover, bisection of ticks to perform two distinct techniques (culture and PCR) limited the chances for spirochetes to adapt to BSK medium by reducing the number of spirochetes which were introduced in BSK medium, especially if the tick had a lower grade of infection (Gern *et al.*, 1999; Juntilla *et al.*, 1999). Mafra and HRA (Sites 53 and 54 repectively) were the only sites with positive cultures, which may be due to a particularly higher infection rate in *I. ricinus* ticks from these regions. This fact was not confirmed by quantitative techniques (e.g. DFA, IFA, real-time PCR), however.

Viability of ticks during preparation can also affect results on *Borrelia* detection. Living ticks give more positive cultures than dead ticks (Juntilla *et al.*, 1999). This fact occurred several times, especially in warmer months, increasing also the probability of higher contamination by other microorganisms that were not successfully cleaned by the pre-disinfection.

Mafra had a lower isolation success compared to some of the European countries with published references (**Appendix 6**). An average of 18.1% (range: 4.0% in Turkey – 48.0% in France) of isolations was observed, similar to what was obtained for Grândola. Isolation rates from the southern Europe (Italy and Spain) achieved a 10.5% value, confirming the increased importance of *B. burgdorferi* s.l. in these regions.

Specific DNA was detected using a nested-PCR technique, already optimised at ULBL laboratory and PCR derived amplicons were visualized in the common, simple and inexpensive agarose gel electrophoresis with ethidium bromide staining. Schmidt (1997) reminded that this latter method suffers from a lack of sensitivity and specificity which may affect the final results with false negatives. Labelled probes with specific sequences can overcome this problem.

On the other hand, substances present in mammalian blood (residual heme molecules) or the presence of some inhibitors in the ticks can inhibit PCR amplification because of its chelatory mode of action on available magnesium. This would limit the utility of PCR for determination of *B. burgdorferi* infection in engorged ticks that have taken a blood meal from a human or other animal host. Steps have to be taken to remove such inhibitors, for instance using extraction kits with procedures to diminish these inhibitors (*e.g.* Isoquik DNA extraction kit – ORGA Research) (Kirstein *et al.*, 1996, Schwartz *et al.*, 1997; Schmidt, 1997; Wilson, 1997; Sparagano *et al.*, 1999; Basta *et al.*, 1999).

As already mentioned, the effect of detection level can also affect the good resolution of a PCR, especially concentration of *Borrelia* DNA near the detection limit of the PCR (Priem *et al.*, 1997; Wilson, 1997; Liebisch *et al.*, 1998). The lower number of spirochetes in samples may likely to become a contributing factor in failure of conventional PCR. Also the positive results in PCR but negative in culture may be due to the presence of nonviable spirochetes (Liveris *et al.*, 2002).

A considerable percentage (11.8%) of *I. ricinus* ticks collected during the Mafra study was found infected with a mean value of 14% for adults (18.7% females and 9.3% males), 17.3% for nymphs and 0.12% for larvae, along with a global infection rate of 24.5% in the nationwide surveyed ticks. In the **Appendix 6** a review of published literature on infection rates detected by PCR methodology is presented, with 79 articles belonging to 23 countries. As can be seen, even in the same country, there is a great variation in the percentages of overall tick infection or per each developmental stage, as was evidence in Mafra (focal study) and other nationwide sites. **Table 3.15** resumes the values obtained for the total number of ticks and separate stages in these publications (average numbers) and **Fig. 3.16** presents the distribution of these prevalences among European countries.

Values obtained for Mafra focal study and nationwide studies are within the range of values observed for these countries where differences between stages are also observed.

Table 3.15– Average values of global infection rates (%) of *B. burgdorferi* s.l. in *I. ricinus* ticks and per developmental stage as reported in the reviewed literature (79 articles).

Country	Total	Adults	Males	Females	Nymphs	Larvae
Belgium	23.0					
Bulgaria	40.0	34.0	8.0	42.0	7.5	
Croatia	45.0	47.0			41.0	
Czech Rep.	13.6	20.5	10.3	6.9	3.8	1.6
Estonia	15.0					
Finland	18.5	9.0			4.0	
France	8.3		12.0	17.6	11.4	
Ireland	16.7	17.9	19.5	20.7	15.6	
Italy	27.3	20.7			7.1	
Germany	22.3	27.0	15.8	22.6	18.1	
Latvia	28.0	20.0			20.0	
Lithuania	17.8					
Netherlands	19.5	11.0			23.0	5.3
Norway	16.5					
Poland	8.4	20.0	6.0	11.9	5.5	14.8
Portugal	7.5	40.1			1.1	
Russia	92.9					
Slovakia	30.6	41.2	27.2	35.0	26.1	
Spain	1.2	5.5			0.8	
Sweden	9.0		17.6	38.5	7.0	
Switzerland	13.7	49.0	21.1	20.2	25.9	
Tunisia	34.0	34.0	40.0	56.5	33.3	2.6
U.K.	34.2	18.0	13.8	10.8	25.9	8.8

The differences observed between infection rates in females and males could be because of different feeding behavior. Male-destined subadults ticks may take a relatively small meal and small meals, taken from marginally infected hosts, are less likely to infect a tick than is the inoculum in a larger meal (Matuschka *et al*., 1992; Gupta *et al*., 1995). Through their life stages, males may switch more easily than females from one type of host to another (for instance, a male may

feed on a bird as a larva and on a rodent as a nymph. A spirochete acquired during the first blood meal may be cleared from the tick during the second blood meal by activation of the alternative pathway of host complement (Derdáková *et al.*, 2003). Also, because female larvae and nymphs may feed preferentially on small mammals and because *Borrelia* species are host specific, it is possible that females and males are not identically infected (De Meeus *et al.*, 2004). Another hypothesis is the venereal transmission between sexual partners (Alekseev & Dubinina, 1996). Females can receive the pathogen from their partner and from an epidemiological standpoint this has to be considered in field-collected ticks kept in the same tubes. An increase in the estimated *Borrelia* spp. infection prevalence level can be observed, which stresses the importance of isolation of collected ticks according to gender (Alekseev & Dubinina, 1996, Mejlon, 2000).

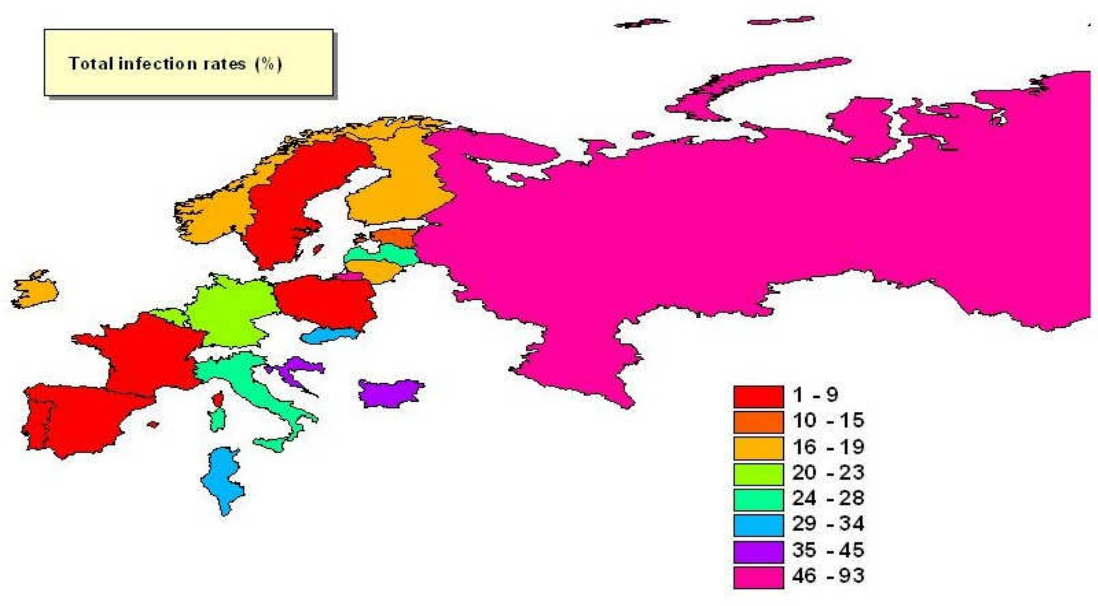


Figure 3. 16 – Distribution of infection prevalence rates of *B. burgdorferi* s.l. in *I. ricinus* ticks in European countries based on the published values for total ticks.

Infection rate of nymphs was found to be higher than adults. Infection in adults is mainly related to: i) specificity of adults life-cycle in nature that can increase the possibility of being infected with *B. burgdorferi* s.l. compared to nymphs or larvae (Ruzic-Sablijic *et al.*, 1993) and ii) to the number of bloodmeals ingested and to efficient transstadial transmission of the spirochetes (Mejlon & Jaenson,

1993; Gilot *et al*., 1996) if most larvae and a significant proportion of the nymphs feed on reservoir-competent small mammals (Gustafson *et al*., 1995). Higher *Borrelia* detection in nymphs may be derived from the local absence of reservoir competent hosts for adults, in contrast to their own hosts (Matuschka *et al*., 1992a; Gupta *et al*., 1995). Also, nymphs are more competent *B. burgdorferi* s.l. carriers than either adults or their hosts and adult infections results mostly from trans-stadial transmission or, less probable, higher levels of inhibitors in the larger adult samples were able to inhibit PCR for the small amount of *B. burgdorferi* s.l. DNA present but not for the tick DNA (Hubbard *et al*., 1998)

Larval *Borrelia* infection is known to be very low compared to the other stages and values obtained in Mafra followed that premise. Because of this low spirochete infection prevalences found in unfed larval populations, larvae are commonly thought to have a low or no vector potential.

The scarcity of spirochetal infection in larval field-collected ticks (about 1%), which generally is taken as evidence of transovarial transmission, suggests that vector ticks may only infrequently inherit infection (Zhioua *et al*., 1994; Matuschka *et al*., 1998; Humair & Gern, 2000; Nefedova *et al*., 2004). This may happen if their parents had been exposed to infection repeatedly or during some particular developmental stage. Low spirochete prevalence in larval ticks could result from low frequency or rate of transovarial transmission, low passage from eggs to the resulting filial ticks, or inefficient transovarial transmission in most (but not all) females (Zhioua *et al*., 1994). But, according to some authors (Zhioua *et al*., 1994; Matuschka *et al*., 1998), previously reported natural infection in larval ticks may reflect instead interrupted feeding, with partial engorgement on a spirochete infected host and premature detachment and that Lyme disease spirochetes rarely, if ever, are inherited by vector ticks.

Larval infection rates normally observed (between 1 and 3%) are, nevertheless, sufficiently high to contribute to spirochetes infections in susceptible host populations. Although transovarial infection is very low, its efficiency is very high. A rodent host passing through an aggregate containing transovarially infected questing larvae could, therefore, simultaneously pick up numerous infected ticks along with uninfected ones. Simultaneous transmission by several larvae, perhaps facilitated

by the salivation of non-infected co-feeding larvae, could establish an infection (Kurtenbach *et al*., 1995).

Seasonal dynamics of infection was studied and a positive correlation was found between infection rates and abundance of ticks, with some exceptions for nymphs where this correlation had an inverse behaviour.

It is known that in the bimodal pattern, each peak may be composed of cohorts of mixed developmental (therefore infection) history, and the time between the vector acquiring and transmitting the infectious agents may vary between ≈ 4 and >12 months. In unimodal seasonality, like the one observed for Mafra site, in the 5-year study, there may be an increase of transmission potential in sheltered habitats and a decrease of transmission potential in exposed habitats (Randolph, 1995; Craine *et al*., 1995).

Similarly to the *Borrelia* tick infection prevalence rates, seasonal dynamics of infection of *I. ricinus* with *B. burgdorferi* s.l. is not the same among European countries. In studies from Slovakia and Sweden, no correlation was found between the relative density and the prevalence of infected ticks with *B. burgdorferi* s.l. (Mejlon & Jaenson, 1993; Stepánová-Tresová *et al*., 2000). The positive correlation that was found in Mafra population was also observed in countries like Sweden, Russia, France, USA (for *I. pacificus*) and Spain (Doby *et al*., 1995; Kovalevskii & Korenberg, 1995;Talleklint & Jaenson, 1996, 1996a; Talleklint-Eisen & Lane, 1999; Barral *et al*., 2002).

A peak infection prevalence in spring or early summer, followed by lower infection prevalences in late summer was observed. With low numbers of ticks, the potential reservoirs harbour no or too few nymphal or adult ticks to become exposed to spirochetes. Larvae that feed on these hosts will emerge as questing nymphs free of spirochete infection. When the number of ticks increases, the result is higher larval loads on the few reservoir-competent mammals that will lead to an increase in infection prevalence in nymphs that fed on mammals as larvae (Talleklint-Eisen & Lane, 1999).

The negative correlation observed for nymphs in the beginning of the Mafra study may be related to the presence of non-incompetent hosts. This fact was confirmed in Ireland, Germany and Italy

(Gray *et al*., 1995, 1999; Dorn & Sunder, 1997; Rizzoli *et al*., 2002). A strong negative correlation between nymphal and adult abundance and percentage infection rate with *B. burgdorferi* s.l. were observed in places where large hosts are prominent (Gray *et al*., 1995, 1999). Large animals such as deer (namely the fallow deer, a common species at Tapada Nacional de Mafra) may not be greatly involved in the circulation of the spirochete between ticks and hosts (incompetent reservoirs-borrelicidial activity) and this makes little or no contribution to the population of infected ticks (Gray *et al*, 1995; Rizzoli *et al*., 2002).

The annual differences detected during the focal study in this nymph population may be due to fluctuations of larval abundance that may affect the prevalence of infection in nymphal ticks in the next season (Levin *et al*., 1997). Ticks feeding at higher density acquired spirochetes more efficiently than did those feeding at lower density (Levin *et al*., 1997; Ogden *et al*., 1998), which may explain the positive correlation after the larva peak of 2002 and subsequent peak of nymphs in 2003.

Another important aspect found in the present study was the discovery of other tick species, besides *I. ricinus*, infected with *B. burgdorferi* s.l.. Specimens from practically all the main Ixodidae genus found in Portugal were incriminated as potential vectors of these bacteria, sometimes with *Borrelia* infection rates higher than *I. ricinus*, distributed in different regions, along the littoral. Infection rates of other vectors, besides the European tick vector (*I. ricinus*), have been found in many countries, using a variety of laboratory techniques. For instance, *D. marginatus* was only found infected in Bulgaria (Angelov *et al*., 1996), *Ha. punctata* was found in Bulgaria (Angelov *et al*., 1996), Spain (Estrada-Pena & Revuelta, 1991; Barral *et al*., 2002) and United Kingdom (Hubbard *et al*., 1998), *R. sanguineus* was found in United Kingdom (Hubbard *et al*., 1998), and *Hyalomma* spp was only found infected in Portugal, so far (De Michelis *et al*., 2000, Baptista *et al*., 2004).

The extensive prevalence of spirochete infection in female adults of the above mentioned species indicated that they were strong candidate vectors of the pathogens to humans. However, the presence of the spirochetes in ticks does not necessarily mean that they are capable of transmitting them to new hosts. Tick species without the ability of transmission of the pathogen should not be recognized as vector competent. They should be called “carrier” species. Examples of these “carriers” are *I. hexagonus, I. muris*, *I. trianguliceps*, *I. acuminatus*, *I. uriae*, all involved in the maintenance of spirochetes among reservoirs hosts, and also *D. reticulates, D. andersoni, D.*

variabilis, D. occidentalis, H. concinna and *H. punctata*. All of these ticks were not recognized as vectors of LB for their inability of transstadiall transmission in natural conditions, but they might be only secondary vectors (Gern *et al*., 1991; Marquez & Constan, 1990 *fide* Kahl *et al*., 1992; Barbour & Fish, 1993; Angelov *et al*., 1996; Dolan *et al*., 2000; Sun & Xu, 2003).

D. marginatus was suspected as a secondary vector (Angelov *et al*., 1996), but without the necessary transmission evidence, its vector status was still doubted. *I. hexagonus* also was proved to be an efficient vector of *B. burgdorferi* s.l. in the laboratory, with transovarial and transstadial survival and transmission to laboratory mice (Gern *et al*., 1991; Estrada-Pena *et al*., 1995). A specific enzootic cycle may be involved for this species with a different niche of hosts and reservoirs and a different geographical distribution compared to *I. ricinus. I. frontalis* were found to be carriers of the spirochete only on those zones where *I. ricinus* is present, suggesting evidence for reservoir competence in a tick-bird cycle (Estrada-Pena *et al*., 1995). More recently, Gylfe and others (1999) demonstrated that *I. uriae* may be implicated in transfer of *B. garinii* to the human population of the Faeroe Islands.

Although these carrier species do not transmit Lyme spirochetes, they can acquire spirochetes when co-feeding with confirmed vectors in the same host. Spirochetes can survive the succeeding molt, but not be transmitted by that instar to a vertebrate host. Since most of these carriers share at least some hosts, co-feeding may explain high prevalence of infection in a number of tick species (Sun & Xu, 2003).

Genotyping through ribosomal spacer DNA-RFLP analysis of *B. burgdorferi* s.l. directly amplified from ticks or from isolated strains, depends on the concentration of positive amplicons that may not allow the visualization of their specific pattern and differences in electrophoresis which may cause different patterns (Lin *et al*., 2001). Regardless of this, and besides the low number of PCR products that were identified, a notorious diversity of *B. burgdorferi* s.l. species was observed in Mafra, including *B. lusitaniae, B. garinii, B. afzelii, B. valaisiana* and mixed infections of *B. garinii* and *B. valaisiana*. *B. lusitaniae* was the main spirochete found in Portugal, along with one single isolation of *B. garinii* in Coimbra district.

Developmental stages were found to be associated with different genospecies in Mafra, although these differences were not statistically significant. As Strle and others (1995) referred, there may be an association between *Borrelia* species and the stage of development. For instance, *B. afzelii* tends to be more frequent in questing nymphs, whereas *B. garinii* and *B. burgdorferi* s.s. are more frequently found on questing adults (Humair & Gern, 2000). Having in mind the reduced number of isolates, no association has been found with gender (Strle *et al*., 1995), but females from Mafra harboured more strains of *B. garinii* than males.

Although single genospecies become established in a tick far more frequently than do multiple genospecies (Richter *et al*., 1999), mixed infections of multiple *B. burgdorferi* sensu lato species have been found in ticks, reservoir hosts, and in patients with LB. The prevalence of mixed infections in ticks varies from 5 to 40% in different European geographic regions. Practically all possible variants of mixed infection by two or several *Borrelia* species circulating in a certain area occur in both unfed and fully or partially engorged ticks at all phases of their development, (Korenberg, 2004). Such mixed infections may result directly from feeding on a host infected with multiple *Borrelia* species or through cofeeding transmission (Gern & Rais, 1996; Randolph *et al*., 1996; Kirstein *et al*., 1997a; Kurtenbach *et al*., 1998b, 2001; Humair & Gern, 2000; Piesman & Happ, 2001; Richter *et al*., 2002; Odgen *et al*., 2002; Korenberg, 2004), by which various spirochetes may be exchanged among clustered ticks infesting a single host, infected for a long time (several months at least) (Randolph *et al*., 1996). This host could be a competent or a non-competent one (Randolph *et al*., 1996; Ritcher *et al*., 2002).

Ticks may also acquire different *Borrelia* species from different individual hosts during their three-stage life cycle, since the spirochetes can survive through molts and are present in all subsequent stages of the vectors (Wang *et al*., 1999). The co-occurrence of *Borrelia* genospecies within individual ticks might come from the existence of resistant and susceptible individuals in tick populations. Because the different genospecies display different specificities, there might be a potential conflict of interest for transmission to the vertebrate host (Kurtenbach *et al*., 1998b; De Meeus *et al*., 2004).

B. lusitaniae, the main genospecies found in Portugal during this study, has been found in several countries, with different prevalences. For instance, the prevalences of *B. burgdorferi* s.s., *B. bissettii* and *B. lusitaniae* in questing ticks were very low, a finding that appears to be typical of Central Europe (Hubalek & Halouzka, 1997). In the western Mediterranean Basin, however, high prevalences of *B. lusitaniae* have been observed in some localities, suggesting that this genospecies has a narrow spectrum of reservoir hosts, restricting its geographical range (De Michelis *et al*., 2000; Hanincová *et al*., 2003).

Reservoir hosts that may harbor each of these genospecies are an important issue in the epidemiological cycle of Lyme Borreliosis in Portugal. Unfortunately, little is known about the competence of the numerous hosts available for ticks. Some unpublished data detected positive DNA in mongoose (*H. ichneumon*) and badger (*M. meles*) of Herdade da Ribeira Abaixo (Sewell, pers. comm.) and in the same region Quaresma (2004) detected DNA in all small mammal species captured in her study: *Mus spretus, Apodemus sylvaticus, Rattus norvegicus* and *Crocidura russula*. These data mean that, at least in Herdade da Ribeira Abaixo (site 54), there is a sylvatic cycle specific to *B. lusitaniae* that is maintained by a variety of hosts and reservoirs and numerous tick species, other than *I. ricinus.*

In Mafra, a larger diversity of genospecies suggests the presence of the two sylvatic cycles known for *B. burgdorferi* s.l. transmission, a bird-tick cycle and a rodent-tick cycle. No data are available for detection of *Borrelia* in these hosts.

It is expected that similar cycles occur throughout Portugal, where presence of those small and medium reservoirs is common. The importance of each reservoir needs to be confirmed. For instance, in a Lyme disease endemic region in northern Spain, small mammals do not seem to play an important role as reservoirs for *B. burgdorferi* sensu lato (Gil *et al*., 2005), but in Czech Republic, a mosaic distribution of *Borrelia* infection in a more or less uniformly distributed tick population was caused by different home ranges of small rodents (*Apodemus flavicollis* and *C. glareolus*) inhabiting the forest (Zeman & Daniel, 1999).

Deer, one of the most important hosts for adult ticks in several European countries, have a restricted distribution in Portugal, being limited to some hunting areas and a few natural parks, and their importance may be confined to these sites, in contrast with other larger and medium sized mammals that have wider distributions.

As already mentioned, the two major elements of biotic diversity are the genetic diversity of *B. burgdorferi* s.l. and the tick's wide range of hosts (Randolph, 2000). Identification of environmental markers for the key elements of this biotic diversity is necessary to produce reliable risk maps for this complex zoonosis.

(Patients)

As in other European countries and only in the last few years; Portugal has increased interest in Lyme Borreliosis, an illness with diverse clinical, epidemiological and laboratory characteristics. However, the difficulties of specific immunological diagnosis and culture of the agent, on conjunction with an enhanced clinical polymorphism in the Portuguese population (Franca, 2005) have produced numerous constraints to scientific and medical research of this disease in Portugal.

The present work also provides a contribution to human research, through a transverse analysis of suspected Lyme patients who were analyzed at ULBL lab. However, it was not a goal to understand the clinical factors allied to this population that may influence final results, because this was carefully studied by Franca (2005), in her PhD manuscript. Thus, a comparison with other countries based on the results obtained with the different laboratory techniques will be presented to give an idea of the epidemiological importance of Portugal in the European context.

As already mentioned (3.3.1.1), culture of *Borrelia* from ticks and clinical specimens has little sensitivity, dependent on the number of spirochetes present. In the case of ECM, a study demonstrated (by quantitative PCR, qPCR) that the skin culture positivity was significantly associated with skin lesions containing larger numbers of spirochetes (Liveris *et al*., 2002).

The percentage of recovery from other clinical specimens besides skin is distinct and dependent on the sample itself. In total blood of untreated patients this recovery is ≤ 5%, but using plasma,

serum and higher quantities of blood increases this percentage to 25-50%. Culture of CSF has less than 10% efficacy and there has been no success so far with synovial liquid (Schmidt, 1997; Speck *et al*., 2002; Davidson *et al*., 1999; Dumler, 2001; Lebech, 2002; Reed, 2002). The number of organisms in these clinical specimens appears extremely low in Lyme Borreliosis. Although there can be up to 4,500 spirochetes in infected ticks, this number decreases in the urine or plasma of infected patients to less than 50 per ml and rarely exceeds 5,000 per ml. In CSF, the number of organisms might even be lower (Schmidt, 1997) The most sensitive human body fluids are plasma, synovial fluid and CSF (10 spirochetes per ml), followed by serum samples (20 spirochetes per ml), urine (50 spirochetes per ml), and whole blood (100 spirochetes per ml).

In most instances, it is not practical to attempt isolation of *B. burgdorferi* s.l. from CSF or synovial fluid on a routine basis because immunoserologic or molecular-based tests offer higher sensitivity (Reed, 2002). Another inconvenience is that these results are not timely, requiring up to 12 weeks (Dumler, 2001), which demands more rapid and sensitive assays to detect *B. burgdorferi* s.l. However, if cultures are examined for the presence of spirochetes at frequent intervals, especially during the first two weeks of incubation, it is possible to see an increase of positive cultures (Reed, 2002).

The only positive culture obtained in this work (1.3%) and so far in Portugal was obtained from a chronic patient with a small lesion in the thigh (erythematous macules associated with a local diffuse infiltration of the subcutaneous tissues). A 5-mm cutaneous biopsy was taken and all the described procedures were performed. A *Borrelia lusitaniae* strain was identified by genotyping techniques, this being the first ever identified in Portugal or in the rest of the World (Collares-Pereira *et al*., 2004). Probably the higher content of spirochetes in this piece of skin lesion was the main reason for a successful isolation and further maintenance.

It is known that *B. burgdorferi* s.l. can convert from cystic forms (speroblast-L forms) to mobile spirochetes (and vice-versa) to overcome or escape unfavorable conditions (Burgdorfer, 2001; Gruntar *et al*., 2001). These cystic forms are difficult to distinguish from other round-shape microrganisms under dark microscopy and may have been missed and ignored.

The conditions of storage before arriving at ULBL lab had a great influence on the number of viable spirochetes. For skin specimens, fresh frozen samples are known to give the best results (Schmidt, 1997), which was not always the case.

As already referred, the positive culture was obtained from an unspecific dermatological lesion. Isolation of *B. burgdorferi* from EM, one of the most typical skin lesions, lead to recovery percentages of 29% in Denmark (Lebech *et al.*, 2000), 38% in United Kingdom (Robertson *et al.*, 1999), 46% in Italy (Ciceroni *et al.*, 2001), 50% in Slovenia (Zore *et al.*, 2002) and 54% in U.S.A (Liveris *et al.*, 2002). These higher values reinforce the importance of EM compared to other skin lesions, but the atypical skin lesion in this Portuguese patient strengthens the above idea of an enhanced clinical polymorphism of this genospecies.

Culturing isolates in BSK-H medium reduces the number and diversity of *ospA* and *fla* alleles, which have to be accounted for when studying population genetics and phylogeny. Loss of pathogenic potential between *in vitro* and *in vivo* studies should also be envisaged (Norris *et al.*, 1997, 1999; Siebers *et al.*, 1999; Sellek *et al.*, 2002). Due to this evidence, use of different species and strains of *B. burgdorferi* s.l. as antigens or as controls to type and classify other strains (Busch & Nitschko, 1999; Wang *et al.*, 1999; Kaiser, 2000; Reed, 2002), may lead to confounding results because of variations in the expression of immunogenical proteins. Further serological difficulties result from i) subjectivity of interpreting band strength, ii) problems with band resolution and identification, and iii) differences in the immune response to the various clinical presentations (Robertson *et al.*, 2000).

Regarding the obtained serological results, the observed rates for IFA (9.9%) and WB (15.5%) suggest the important role of immunobloting on the laboratorial confirmation of Lyme reactivities in Portuguese patients, even when facing an IFA negative result. On the other hand, unusually low WB seroreactivity in Portuguese Lyme patients with a confirmed *B. burgdorferi* s.l. DNA amplification by PCR, has already been noted by Collares-Pereira and coworkers (2004). Thus, seronegative or doubtful IFA and WB results, but PCR positive (especially in cases of late disease), mean that specific antibodies to *B. burgdorferi* s.l. need a more specific test like WB to reach a detectable level in the patient's serum.

It is also important to note that prevalences of positive serologies in European patients present a wide range of values, according to the reviewed literature (Ruesca *et al*., 1991, 1991a; Núncio *et al*., 1991; Rocha & Caniça, 1991; Oteo *et al*., 1992; Santos *et al*., 1995; Golubic *et al*., 1998; Lebech *et al*., 2000; Situm *et al*., 2002; Hamlet *et al*., 1989 *fide* Situm *et al*., 2002; Burek *et al*., 1992 *fide* Situm *et al*., 2002; Núncio *et al*., 2002). Infection rates under 10% were observed in southern European countries, (i.e. Portugal and Spain), as compared to higher values such as 87% in Denmark or 40% in Croatia.

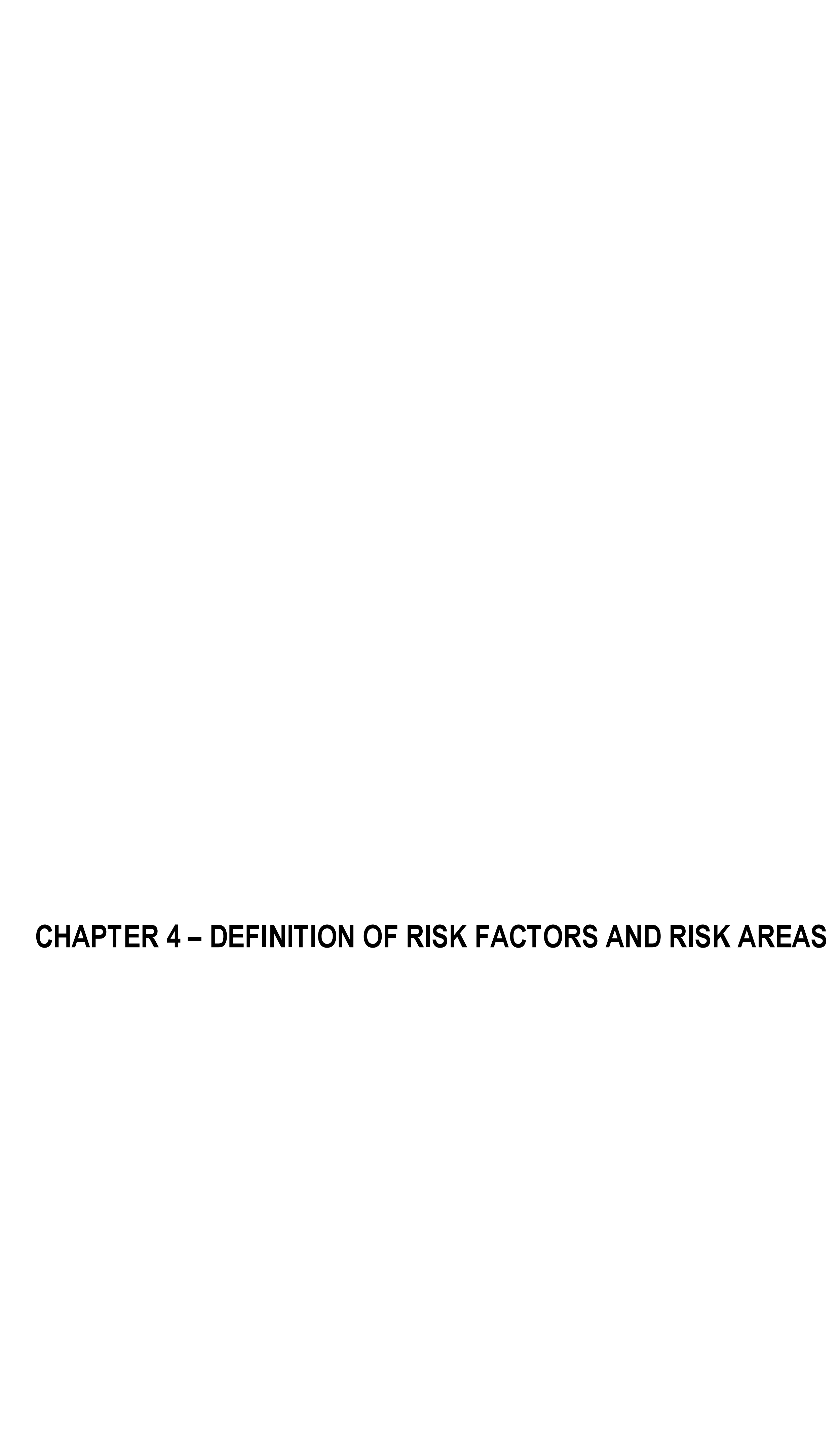
DNA detection by PCR was found to present higher sensitivity than culture for the direct search of pathogenic spirochetes, as also shown by Couceiro and others (2003). As already mentioned, results from the *nested*-PCR (27.9% for CSF, 48.7% for biopsies, 33.3% for synovial fluid and 16.7% for other samples) have increased the importance of this technique on the diagnosis of Lyme Borreliosis at ULBL. But even so, it is important to not forget that, whenever possible, this procedure should not be used as the primary diagnostic test.

For the knowledge of LB epidemiology in Portugal, this molecular approach may provide a reasonable advance given the characteristics of the Portuguese population where clinical manifestations are atypical and polymorphic and exposition to a tick bite is practically never mentioned. Clinical diagnosis is always mandatory and a negative PCR result does not exclude Lyme disease in any situation. On the other hand, the presence of PCR-detectable sequences does not necessarily prove the presence of an active disease. Reactivity can be due to dead organisms, portions of spirochetes that include DNA, or soluble DNA (Schmidt, 1997; Sparagano *et al*., 1999; Dumler, 2001; Lebech, 2002; Reed, 2002; Stanek & Strle, 2003).

Global DNA infection rates obtained herewith per surveyed specimen apparently fit in the wide range of values observed in a variety of European countries. CSF infection rates from 17 to 79% were observed in Germany and Denmark (Priem *et al*., 1997; Lebech *et al*., 2000; Schaarschmidt *et al*., 2001). Skin biopsies belonged all to EM, except one study with ACA lesions (Rijpkema *et al*., 1997). Values ranged from 20% in Germany (Schaarschmidt *et al*., 2001), 40% in France (Jaulhac *et al*., 2000), 56% in United Kingdom (Robertson *et al*., 1999), 71% in Denmark (Lebech *et al*., 2000) and 75% in Netherlands (Rijpkema *et al*., 1997). Synovial fluid was another human sample intensively

studied (mainly in Lyme arthritis patients), with positive PCR values of 17.9% in Switzerland (Schwaiger *et al*., 2001), 83% in France (Jaulhac *et al*., 2000) and 85% in Germany (Priem *et al*., 1997). The very reduced number of some surveyed samples did not allow further conclusions.

Distribution of positive cases with a confirmed laboratory diagnosis followed the distribution of ticks observed in Chapter 2. However, the origin of human samples that arrived at the ULBL lab influenced the final distribution: Thus, a more generalized study of suspected Lyme patients at a national level is needed, to improve the future prevalence map, like the one presented by Saz and others (1995), where they show the districts with at least one reported case of Lyme disease, and thus confirming the wide occurrence of this disease in Spain.



CHAPTER 4 – DEFINITION OF RISK FACTORS AND RISK AREAS

CHAPTER 4 – Definition of risk factors and risk areas

4.1 Introduction

An understanding of the determinants of Lyme Borreliosis risk is essential to evaluate human disease potential and to recommend strategies disease prevention (Fish, 1995). These determinants include some of the parameters presented in earlier chapters, such as:

- The geographic distribution and abundance of ticks as vectors
- Presence of various tick life stages
- Temporal coincidence between peak tick feeding activity and human outdoor activity
- Role of hosts in establishment of tick populations
- Presence of competent disease reservoirs and prevalence of infection in hosts and vectors through time and space
- Environmental characteristics that impact all of the preceding parameters.

Ultimately, all of the above factors combine to regulate human risk to Lyme Borreliosis. Risk, can most directly be measured by prevalence of LB cases in an area and often is indirectly estimated as the distribution and prevalence of tick vectors (Zeman, 1997; Jensen *et al*., 2000). Unfortunately, absent a complete census of the human population for disease, or a complete enumeration of tick populations and their infection with *Borrelia*, we are left to quantify more easily measured correlates to disease risk, especially those associated with tick habitat.

The study of ecology relies heavily on the description of associations between particular species and the habitats in which they occur. These preferred associations may be apparent at a variety of different scales. While some environmental variables cause heterogeneity in the distribution of organisms on a scale of meters or kilometres, others act over much larger areas; Broad-scale habitat requirements frequently take precedence over those that are relevant at smaller scales (Cumming, 2002).

To quantify human risk to Lyme Borreliosis, measures of environmental condition were evaluated for their ability to predict potential vector tick occurrence including the three main ixodids observed during this study, *I. ricinus, Rhipicephalus* spp and *D. marginatus*.

Combining these predictors into multivariate models, maps were generated to depict relative risk of LB across Portugal.

4.2 Objectives

a) To study the associations between tick presence / *B. burgdorferi* s.l. infection (accessed in chapters 2 and 3) and environmental variables;

b) To extrapolate a risk map based on the previous results and on a multivariate analysis (logistic regression) defining the most important areas for the occurrence of LB in Portugal, so far.

4.3 Material and Methods

(Population)

This chapter have the purpose of attempting to define risk areas for Lyme Borreliosis in Portugal based on the distribution of vector ticks, *Borrelia* strains, human cases and the biotic and abiotic variables. Since all of these tick species were infected, at various rates, with *B. burgdorferi* s.l. agents, analyses will be based solely on presence/absence of total ticks and the three main ixodid species / genus (*I. ricinus*, *Rhipicephalus* spp. and *D. marginatus*).

(Study sites)

To better characterize each sampling site, besides field variables already described, other GIS-based maps of Continental Portugal were used. Information namely on mean temperature and humidity, insolation, precipitation, soil type and pH, lithology, altitude, ecological areas and landscapes was obtained from "Atlas do Ambiente", a product from Instituto do Ambiente (www.ia.pt). These maps created as late as 1974, are the most complete digitised information to be used in a GIS system, although not updated and with a large scale (original map - 1: 1 000 000).

(Data analysis)

With a newly attribute table of sample sites (**Apendix**) it was possible to describe them more precisely and including other environmental variables that were not surveyed during this study. To also characterize the landscape of each sample site and thus the landscape for each tick population, Corine map variables of soil occupation were redefined (**Apendix**). Corine map was also used to ascertain landscape within circles of different radii (500m, 1km, 2 km and 5km) created around each sample points (**Figure 4.1**).

An attempt to analyze each circle was tried with *Patch Analyst* 3.3, a free extension of *ArcView* 3x, developed as a tool for quantifying spatial structure within *Arcview* (Rainis, 2003). using a simpler front page of the most used *FRAGSTATS* (see **Appendix** fore more details) to obtain some landscape classes and patch metrics. These variables were determined for each circle, as follows:

- **type1** number of 30m x 30m cells of type 1 (human development)
- **type2** number of 30m x 30m cells of type 2 (Open areas). Include agriculture (2), shrub/bush habitats (6), barren/unvegetated areas (9)
- **type3** number of 30m x 30m cells of type 3 (deciduous forest)

- **type4** number of 30m x 30m cells of type 4 (evergreen forest)
- **type5** number of 30m x 30m cells of type 5 (mixed forest)
- **NP** Number of Patches
- **MPS** Mean Patch Size
- **IJI** Interspersion/Juxtaposition Index – measure of patch adjacency (0 - without adjacency; 100 - all patches adjacent)
- **SDI** Shannon Diversity Index - measure of relative patch diversity

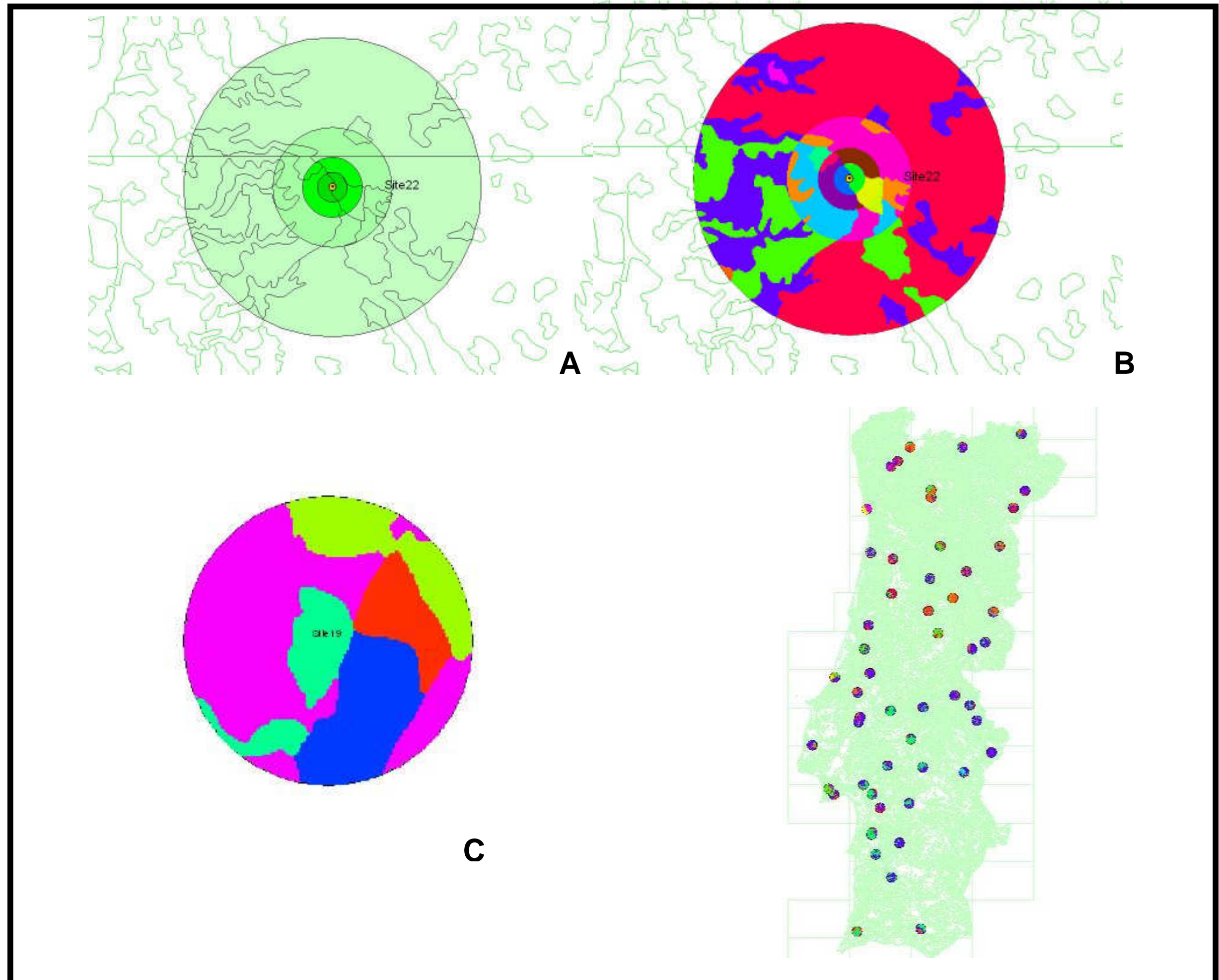


Figure 4. 1– Circles of several diameters were created around each of the 55 sample points (A), and then were used to intersect with CORINE LAND COVER map (B). Spatial Analyst® was used to create grids themes of each circle (C).

Presence/absence tick data were compared with all environmental variables by construction of contingency tables and Qui-Square statistics ($p<0.15$). Continuous values were, for the purpose of this analysis, modified to categorical variables by a simple Sturges rull (**Table 4.1**).

Table 4. 1– Redefinition of the multiple variables assembled in the nationwide study for association analysis with tick presence

Variable	Type of variable	Definition
Ticks	Nominal	**1** – Presence; **0** - Absence
***Borrelia* strains**	Nominal	**1** – Presence; **0** - Absence
Human cases	Nominal	**1** – Presence; **0** - Absence
Vegetation (field)	Categorical	**1** -Coniferous forests (eucalyptus and pinus) without secondary vegetation; **2** -Coniferous forests with secondary vegetation; **3** -Mixed forests with predominance of coniferous; **4** -Deciduous shrubs with short shrubs and young trees; **5** -Homogeneous deciduous trees (oaks and others); **6** -Mixed forests dominated by deciduous trees and highly heterogeneous deciduous forests with several ecotones
Soil (field)	Categorical	**1** -Good drainage; **2** -Reasonable drainage; **3** -Bad drainage
Forest cover (field)	Categorical	**1** -Wide exposition (open space); **2** -Reasonable exposition (some shadow); **3** -Weak exposition (lots of shadow)
Human use (field)	Categorical	**1** -Abandoned Forest; **2** -Hunting Area; **3** -Agricultural zone edge; **4** -Habitation zone edge; **5** -Public garden / Picnic area; **6** -Others
Air Temperature (field)	Numeric recoded as Categorical*	**1** -[11.8 – 16.28[; **2** -[16,28-20.76[; **3** -[20,76-25.24[; **4** -[25.24-29.72[; **5** -[29.72-34.2[; **6** -[34.2-38.70[(ºC)
Soil Temperature (field)	Numeric recoded as Categorical*	**1** -[11.55-15.77[; **2** -[15.77-20.04[; **3** –[20.04-24.31[; **4** -[24.31-28.58[; **5** -[28.58-32.85[; **6** -[32.85-37.20[(ºC)
Air Humidity (field)	Numeric recoded as Categorical*	**1** –[9.40-20[; **2** -[20-30.6[; **3** -[30.6-41.2[; **4** -[41.2-51.8[; **5** -[51.8-62.4[; **6** -[62.4-73[(%)
Soil Humidity (field)	Numeric recoded as Categorical*	**1** -[9-19.54[¸**2** -[19.54-30.08[; **3** -[30.08-40.62[; **4** -[40.62-51.16[; **5** -[51.16-61.7[; **6** -[61.7-72.24[(%)
Landscape use (GIS)	Categorical	**1** -Urban areas; **2** -Agricultural areas/ Shrubs-bush areas; **3** -Deciduous forests; **4** -Evergreen forests; **5** -Mixed forests; **6** –Wetlands; **7** -Eucalyptus forest
Average Annual Temperature (GIS)	Categorical	**1** -Less than 7.5ºC; **2** -Between 7.5 to 12.5ºC; **3** -Between 12.5 to 15.0ºC; **4** -Between 15.0 to 17.5ºC; **5** -More than 17.5ºC
Average Annual Humidity (GIS)	Categorical	**1** -Very dry (less than 55%); **2** -Dry (between 55 to 75%); **3** -Humid (between 75 to 90%); **4** -Very humid (>90%)
Average Annual Precipitation (GIS)	Categorical	**1** -Desertic (less than 125 mm); **2** -Arid (between 125 to 250 mm); **3** -Semiarid (between 250 to 500 mm); **4** -Moderately rainy (between 500 to 1000 mm); **5** -Rainy (between 1000 to 2000 mm); **6** -Excessively rainy (more than 2000 mm)
Average Annual Insolation (GIS)	Categorical	**1** -Less than 1800 h; **2** -Between 1800 to 2000 h; **3** -Between 2000 to 2200 h; **4** -Between 2200 to 2400 h; **5** -Between 2400 to 2600 h; **6** -Between 2600 to 2800 h; **7** -Between 2800 to 3000 h; **8** -More than 3000 h
Soil type (GIS)	Categorical	**1** –Podzois – sands and sandstones and rarely clayish; **2** -Cambissoils – sands and sandstones; thin soils and well drained; **3** -Litossoils – schists and grauvaques; soil above the rock; **4** -Luvissoils – clay, sandstones, gravelbed, quartzidorites; **5** -Fluvissoils – soils of marine or fluvial sediments; **6** -Regossoils – sands and sandstones; **7** -Rankers
Soil pH (GIS)	Categorical	**1** -Extremely acid (less or equal to 4.5); **2** -Very acid (between 4.6 to 5.5); **3** -Moderately acid (between 5.6 to 6.5); **4** -Neuter (between 6.6 to 7.3); **5** -Moderately alkaline (between 7.4 to 8.5)
Litology (GIS)	Categorical	**1** -Sedimentary formations; **2** -Sedimentary and metamorphic formations; **3** -Vulcanic/Plutonic eruptive rocks
Altitude (GIS)	Categorical	**1 - <300; 2 – 300-599; 3 – 600-899; 4 – 900-1199; 5 – 1200-1499; 6 – 1500-1799; 7 - >1800**

Besides these associations, predictive models were built based on logistic regression techniques (see **Appendix 3** for more details). Firstly, univariate analysis was performed using likelihood ratio test for the significance of the coefficient and the univariate Wald statistic (Hosmer & Lemeshow, 2000) to assess the most significant environmental variables for tick presence. Only continuous variables including field variables (air/soil temperature and air/soil humidity), GIS variables (land use, temperature, humidity, precipitation and insolation) and *Patch Analyst* variables for each 500, 1000, 2000 and 5000 m circle were included. All variables that had a p value equal or less than 0.15 were included in a stepwise logistic regression with a p value to enter of 0.25 and a p value to exit of 0.25, according to Hosmer & Lemeshow (2000) and Guerra *et al*. (2002).

To develop a risk map for Lyme Borreliosis in the area studied, the logistic equation was then used to generate the probability of the presence of tick species. The map was generated with probabilities divided into quartiles and deciles.

4.4 Results

As already presented in the previous chapters, 55 sites were surveyed in the nationwide study. Ticks were collected in 35 sites, being *Rhipicephalus* spp the most collected tick. All tick species were found infected with *B. burgdorferi* s.l (except other species of genus *Ixodes*) presenting distinct infection rates and distributions. Field observation of these sites was characterized by deciduous or heterogeneous forests, mostly composed of soils with good and reasonable drainage and with medium exposition. By GIS-variables, the majority of sites have a mean annual temperature from 15 to 17.5ºC, moderately rainy (500 to 1000 mm), dry to humid (55 to 90%) with about 2400 to 3000 h of sun. Main soil types include cambissols and luvissols, with several lithologic formations and an acid pH (between 4.6 to 6.5). Altitude of sample points is diverse but the majority is located at low altitudes (50 to 300 m). Landscape occupation confirms the field observation with forests of deciduous and evergreen species along with open areas (**Appendix 4**).

Distribution of landscape types within circles is more or less similar, with type 2 (open areas) being the most prevalent, followed by deciduous and evergreen forests. Urbanized areas (increased human contact) increases with increased circle radius, as well as mixed forests.

For all the sites in the nationwide study where it was possible to collect ticks, several associations were found with tick presence (**Table 4.2**). Ticks, in general, were found associated with a range of values from 12ºC to 29ºC for Temperature and about 30-52% of Humidity, living above very or moderately acid Soils. *Rhipicephalus* spp was influenced by a variety of environmental variables, namely average annual temperature (15-17ºC) and average annual precipitation of 500 to 1000mm. Sedimentary soils, including cambissoils and luvissoils, with a very or moderately acid pH were characteristics of soils occupied by this tick species. Abandoned forests and hunting areas were the most frequented by *Rhipicephalus* spp, mainly at low altitudes (less than 300m). Air humidity was the only variable responsible for *Dermacentor* presence among nationwide study, with a variety of values ranging from 20% to 62%. *I. ricinus* was found preferably in open habitats, abandoned or transformed in hunting areas, associated with warm temperatures (15ºC to 25ºC) and dry habitats (52-62%), also in low altitudes.

Table 4. 2 – Association between all Environmental variables and Presence of ticks in 55 sites (Total ticks, *Rhipicephalus*, *Dermacentor* and *I. ricinus*) with a Qui-Square analysis. Significant results ($p<0.15$) are marked in yellow colour.

Variable	Subject	X^2 ; p	Categories most frequent	Significant variables ($p<0.15$)
Vegetation (field)	Total ticks	2.116 ; 0.909	5	No
	Rhipicephalus	2.727; 0.842	5	No
	Dermacentor	3.227; 0.780	5, 6	No
	I. ricinus	2.909; 0.820	5, 6	No
Soil drainage (field)	Total ticks	2.091 ; 0.554	1, 2	No
	Rhipicephalus	3.373; 0.338	1, 2	No
	Dermacentor	1.684; 0.641	1	No
	I. ricinus	2.440; 0.486	1, 2	No
Forest cover (field)	Total ticks	0.724 ; 0.868	2	No
	Rhipicephalus	3.017; 0.389	2	No
	Dermacentor	2.493; 0.477	2	No
	I. ricinus	5.049; 0.168	2	Yes
Human use (field)	Total ticks	3.774 ; 0.582	1, 2	No
	Rhipicephalus	9.398; 0.094	1, 2	Yes
	Dermacentor	5.421; 0.367	1	No
	I. ricinus	9.426; 0.093	1, 2	Yes
Air Temperature (field)	Total ticks	9.282 ; 0.098	3, 4	Yes
	Rhipicephalus	6.282; 0.280	3, 4	No
	Dermacentor	6.639; 0.249	3, 4	Yes
	I. ricinus	9.190; 0.102	3	Yes
Soil Temperature (field)	Total ticks	2.820; 0.728	4	No
	Rhipicephalus	5.301; 0.380	4, 5	No
	Dermacentor	4.979; 0.418	4, 5	No
	I. ricinus	4.963; 0.420	3	No
Air Humidity (field)	Total ticks	9.108; 0.105	2, 3	Yes

	Rhipicephalus	3.055; 0.692	2, 3	No
	Dermacentor	8.261; 0.142	2, 5	Yes
	I. ricinus	13.447; 0.020	5	Yes
Soil Humidity (field)	Total ticks	6.173; 0.290	4	No
	Rhipicephalus	7.789; 0.168	4	Yes
	Dermacentor	1.818; 0.874	4, 6	No
	I. ricinus	5.702; 0.336	4, 6	No
Land use (GIS)	Total ticks	5.530; 0.355	2, 3	No
	Rhipicephalus	2.460; 0.782	2, 3	No
	Dermacentor	6.140; 0.293	2	No
	I. ricinus	12.014; 0.035	2	Yes
Temperature (GIS)	Total ticks	15.236; 0.002	4	Yes
	Rhipicephalus	15.458; 0.001	4	Yes
	Dermacentor	3.280; 0.350	4	No
	I. ricinus	5.854; 0.119	4	Yes
Humidity (GIS)	Total ticks	0.065; 0.799	2, 3	No
	Rhipicephalus	0.147; 0.701	3	No
	Dermacentor	0.009; 0.926	2, 3	No
	I. ricinus	0.035; 0.853	2, 3	No
Precipitation (GIS)	Total ticks	4.613; 0.202	4	Yes
	Rhipicephalus	10.869; 0.012	4	Yes
	Dermacentor	1.482; 0.686	4	No
	I. ricinus	2.077; 0.557	4	No
Insolation (GIS)	Total ticks	6.233; 0.284	6, 7	No
	Rhipicephalus	8.057; 0.153	6,7	Yes
	Dermacentor	6.848; 0.232	4, 7	Yes
	I. ricinus	5.077; 0.407	4, 7	No
Soil type (GIS)	Total ticks	7.620; 0.267	2	No
	Rhipicephalus	9.843; 0.131	2, 4	Yes
	Dermacentor	3.106; 0.795	2	No
	I. ricinus	5.314; 0.504	2	No
Litology (GIS)	Total ticks	3.224; 0.200	1, 2	No
	Rhipicephalus	5.929; 0.052	1	Yes
	Dermacentor	1.575; 0.455	1, 2	No
	I. ricinus	0.248; 0.883	1	No
Soil pH (GIS)	Total ticks	6.960; 0.138	2, 3	Yes
	Rhipicephalus	10.452; 0.033	2, 3	Yes
	Dermacentor	4.647; 0.325	2, 3	No
	I. ricinus	2.017; 0.733	2, 3	No
Altitude (GIS)	Total ticks	5.328; 0.255	2	No
	Rhipicephalus	7.414; 0.116	1	Yes
	Dermacentor	3.106; 0.540	1	No
	I. ricinus	7.547; 0.110	1	Yes

Among the broader areas of Thiessen polygons (**Table 4.3**), only four variables were found to be associated with tick presence, namely Type of Vegetation (evergreen forests with secondary vegetation, mixed forests with predominance of evergreen species and homogeneous deciduous forests for *I. ricinus*),

Human Use (hunting areas and edge of habitations for *Dermacentor* spp. and abandoned forests for *I. ricinus*), Soil Temperature (ranging from 24 to 29°C for *I. ricinus*) and very acid Soil pH (*Dermacentor* spp., *I. ricinus*).

Table 4. 3– Association between all Environmental variables and Presence of ticks within the 22 Thiessen polygons (Total ticks, *Rhipicephalus*, *Dermacentor* and *I. ricinus*) with statistical analysis performed with Qui-Square analysis. Significant results ($p<0.15$) are marked in yellow colour.

Variable	Subject	X^2 ; p	Categories most frequent	Significant variables ($p<0.15$)
Vegetation (field)	Total ticks	6.125; 0.190	5	No
	Rhipicephalus	5.099; 0.277	5	No
	Dermacentor	0.481; 0.975	5	No
	I. ricinus	7.616; 0.107	2, 3, 5	Yes
Soil drainage (field)	Total ticks	2.386; 0.303	2	No
	Rhipicephalus	1.147; 0.563	2	No
	Dermacentor	0.704; 0.703	2	No
	I. ricinus	0.491; 0.782	2	No
Forest cover (field)	Total ticks	2.118; 0.347	2	No
	Rhipicephalus	3.207; 0.201	2	No
	Dermacentor	0.463; 0.793	2	No
	I. ricinus	0.824; 0.662	2	No
Human use (field)	Total ticks	6.713; 0.243	1, 2	No
	Rhipicephalus	5.841; 0.322	1	No
	Dermacentor	10.710; 0.057	2, 4	Yes
	I. ricinus	8.75; 0.119	1	Yes
Air Temperature	Total ticks	4.063; 0.255	4	No
	Rhipicephalus	2.389; 0.496	4	No
	Dermacentor	4.156; 0.245	4	No
	I. ricinus	1.491; 0.684	4	No
Soil Temperature	Total ticks	1.821; 0.610	4, 5	No
	Rhipicephalus	0.808; 0.848	4, 5	No
	Dermacentor	1.575; 0.665	5	No
	I. ricinus	7.00; 0.072	4	Yes
Air Humidity	Total ticks	5.063; 0.281	3	No
	Rhipicephalus	3.332; 0.504	3	No
	Dermacentor	3.773; 0.438	3	No
	I. ricinus	3.306; 0.508	4	No
Soil Humidity	Total ticks	3.300; 0.654	4	No
	Rhipicephalus	2.201; 0.821	4	No
	Dermacentor	5.749; 0.331	3	No
	I. ricinus	3.85; 0.571	4	No
Land use (GIS)	Total ticks	2.250; 0.522	2, 3	No
	Rhipicephalus	1.742; 0.628	2, 3	No
	Dermacentor	2.089; 0.554	2, 3	No
	I. ricinus	1.150; 0.765	2, 3, 4	No
Temperature (GIS)	Total ticks	1.768; 0.413	3, 4	No
	Rhipicephalus	0.454; 0.797	4	No

	Dermacentor	1.181; 0.554	3, 4	No
	I. ricinus	1.847; 0.397	3	No
Humidity (GIS)	Total ticks	0.875; 0.350	2, 3	No
	Rhipicephalus	0.151; 0.697	2, 3	No
	Dermacentor	0.788; 0.375	2, 3	No
	I. ricinus	0.130; 0.719	3	No
Precipitation (GIS)	Total ticks	1,154; 0.764	4	No
	Rhipicephalus	1,592; 0.661	4	No
	Dermacentor	8.562; 0.036	5	No
	I. ricinus	2.573; 0.462	5	No
Insolation (GIS)	Total ticks	3.493; 0.479	7	No
	Rhipicephalus	3.796; 0.434	7	No
	Dermacentor	5.775; 0.217	6, 7	No
	I. ricinus	3.578; 0.466	6	No
Soil type (GIS)	Total ticks	2.196; 0.533	4	No
	Rhipicephalus	2.221; 0.528	4	No
	Dermacentor	0.772; 0.868	2, 4	No
	I. ricinus	4.667; 0.198	2	No
Litology (GIS)	Total ticks	1.714; 0.424	3	No
	Rhipicephalus	2.827; 0.243	3	No
	Dermacentor	2.10; 0.350	3	No
	I. ricinus	2.333; 0.311	1	No
Soil pH (GIS)	Total ticks	1.727; 0.422	2, 3	No
	Rhipicephalus	2.839; 0.242	2, 3	No
	Dermacentor	4.964; 0.084	2	Yes
	I. ricinus	4.667; 0.097	2	Yes
Altitude (GIS)	Total ticks	0.750; 0.687	2	No
	Rhipicephalus	1.918; 0.383	2	No
	Dermacentor	3.360; 0.186	1, 2	No
	I. ricinus	0.583; 0.747	1	No

Table 4. 4– Significant associations (p<0.15) between Patch Analyst variables (NP, MPS, IJI, SDI) and Presence of ticks in the 55 sites (Total ticks, *Rhipicephalus*, *Dermacentor* and *I. ricinus*) performed with Qui-Square analysis.

	NP	MPS	IJI	SDI
Total ticks	NP1000 (p=0.15),			
Rhipicephalus				
Dermacentor		MPS1000 (p=0.06);		
I. ricinus	NP500 (p=0.08); NP5000 (p=0.07)	MPS500 (p=0.04); MPS5000 (p=0.05)		

Legend: NP – number of patches; MPS – mean patch size; IJI - interspersion/juxtaposition index; SDI – Shannon's diversity index

Regarding Patch Analyst variables (**Table 4.4**), different associations were found. For Total ticks, only Number of Patches at 1000 m from the sample site influenced tick presence. MPS at 1000 m influenced *Dermacentor*'s distribution. For *I. ricinus*, the NP and MPS for the smallest and the major buffers (500 and 5000m) influenced tick presence.

With some of the original continuous environmental variables, logistic regression using Wald statistics was performed with tick presence/absence as dependent variable. **Tables 4.5** (A-D) show the respective results for total number of ticks and the three main ixodid vectors. Significant results correspond to p values < 0.15.

Based on the above results, the following variables were submitted to stepwise logistic regression (p<0.25): Total Ticks – northern coordinate of the site (NORTH), soil humidity (HUMSOIL), precipitation (PRECIP), insolation (INSOL), open lands 1000 (POP1000), deciduous forest 1000 (PDE1000);
Rhipicephalus – easting coordinate of the site (EAST), NORTH, annual temperature (ANTEMP), PRECIP, INSOL, POP5000, PDE1000, PDE2000, PEV5000, mixed forest 5000 (PMI5000);
Dermacentor – PMI5000, interspersion/juxtaposition index 1000 (IJI1000), mean patch size 500 and 5000 (MPS500, MPS 5000), number of patches 500 and 2000 (NP500, NP2000), Shannon's diversity index 2000 (SDI2000);
I. ricinus – EAST, air temperature (TEMPAIR), air humidity (HUMAIR), PRECIP, PMI2000, IJI1000, MPS5000.

Based in these variables the final prediction surfaces were created with ArcView® for Total Ticks, *Rhipicephalus* spp, *D. marginatus* and *I. ricinus*.

Table 4. 5A – Results of univariate analysis (Wald statistics) between presence/absence of ticks (Total ticks) and environmental variables.

	TICKS PRESENT		TICKS NOT PRESENT			
Variable	N	Mean ± Std Error	N	Mean ± Std Error	Wald X^2	P
EAST	34	271845,45 ± 22073,19	20	364476,2 ± 30587,67	0,95	0,33
NORTH	34	196401,55 ± 9320,77	20	211534,76 ± 12775,33	5,33	0,02
Air Temperature (C)	32	25,05 ± 0,91	17	25,33 ± 1,69	0,03	0,87
Soil Temperature (C)	32	27,98 ± 1,68	17	25,76 ± 1,68	0,69	0,40
Relative Humidity of Air (%)	31	36,00 ± 2,18	17	35,44 ± 3,64	0,02	0,89
Relative Humidity of Soil (%)	32	43,03 ± 2,48	17	49,79 ± 4,11	2,12	0,15
Average Annual Humidity (%)	35	74,79 ± 0,71	20	74,66 ± 1,20	0,01	0,92
Average Annual Temperature (C)	35	14,56 ± 0,40	20	14,22 ± 0,40	0,32	0,57
Total Annual Precipitation (mm)	35	852,45 ± 62,64	20	1100,85 ± 106,92	3,71	0,05
Total Annual Insulation (hours)	35	2722,96 ± 35,05	20	2540,52 ± 51,31	6,81	0,01

	TICKS PRESENT		TICKS NOT PRESENT			
Variable	N	Mean ± Std Error	N	Mean ± Std Error	Wald X^2	P
Open Lands (%)						
500m	35	44,73 ± 6,37	19	54,24 ± 8,71	0,79	0,37
1000m	35	50,42 ± 5,68	19	66,30 ± 6,47	2,89	0,09
2000m	35	56,00 ± 5,06	19	60,12 ± 6,44	0,25	0,62
5000m	35	66,19 ± 3,95	19	58,25 ± 5,64	1,36	0,24
Human Dominated Lands (%)						
500m	35	6,64 ± 3,06	19	4,40 ± 2,94	0,23	0,63
1000m	35	4,42 ± 1,62	19	5,69 ± 3,67	0,14	0,71
2000m	35	3,09 ± 1,04	19	5,27 ± 3,66	0,48	0,49
5000m	35	1,63 ± 0,41	19	3,70 ± 2,54	0,90	0,34
Deciduous Forest (%)						
500m	35	27,61 ± 6,41	19	18,52 ± 8,69	0,71	0,40
1000m	35	25,73 ± 5,81	19	5,31 ± 3,85	3,99	0,05
2000m	35	21,85 ± 5,06	19	11,03 ± 5,75	1,67	0,20
5000m	35	13,93 ± 3,33	19	10,84 ± 4,88	0,29	0,59
Evergreen Forest (%)						
500m	35	11,34 ± 4,48	19	15,77 ± 6,51	0,34	0,56
1000m	35	9,95 ± 3,90	19	15,54 ± 5,90	0,67	0,41
2000m	35	8,90 ± 3,44	19	13,57 ± 5,04	0,61	0,43
5000m	35	6,42 ± 2,45	19	13,11 ± 4,67	1,81	0,18
Mixed Forest (%)						
500m	35	6,49 ± 2,91	19	7,06 ± 4,78	0,01	0,91
1000m	35	6,69 ± 2,22	19	6,86 ± 3,57	0,00	0,97
2000m	35	7,81 ± 2,29	19	8,04 ± 2,90	0,00	0,95
5000m	35	8,58 ± 2,25	19	8,58 ± 2,75	0,00	1,00
Interspersion Juxtaposition Index (%)						
500m	35	16,23 ± 5,09	19	8,83 ± 5,81	0,01	0,37
1000m	35	27,45 ± 6,20	19	25,05 ± 7,14	0,06	0,81
2000m	35	34,62 ± 5,54	19	34,50 ± 7,47	0,00	0,99
5000m	35	44,96 ± 3,40	19	45,95 ± 4,57	0,03	0,86
Mean Patch Shape (%)						
500m	35	45,19 ± 4,02	19	47,20 ± 5,25	0,09	0,76
1000m	35	123,71 ± 15,11	19	130,80 ± 23,44	0,07	0,79
2000m	35	277,51 ± 40,79	19	286,04 ± 37,01	0,02	0,89
5000m	35	520,82 ± 70,60	19	603,70 ± 63,02	0,58	0,45
Number of Patches (%)						
500m	35	2,26 ± 0,19	19	2,11 ± 0,25	0,24	0,63
1000m	35	3,51 ± 0,32	19	3,63 ± 0,49	0,05	0,83
2000m	35	6,89 ± 0,65	19	6,05 ± 0,84	0,60	0,44
5000m	35	16,74 ± 1,07	19	16,32 ± 2,11	0,04	0,84
Shannon Diversity Index (%)						
500m	35	0,42 ± 0,06	19	0,35 ± 0,08	0,54	0,46
1000m	35	0,55 ± 0,07	19	0,50 ± 0,08	0,28	0,60
2000m	35	0,66 ± 0,07	19	0,62 ± 0,08	0,14	0,71
5000m	35	0,65 ± 0,06	19	0,57 ± 0,09	0,59	0,44

Table 4.5B – Results of univariate analysis (Wald statistics) between presence/absence of ticks (*Rhipicephalus* spp ticks) and environmental variables.

Rhipicephalus	TICKS PRESENT				TICKS NOT PRESENT					
Variable	N	Mean	±	Std Error	N	Mean	±	Std Error	Wald X^2	*P*
EAST	28	190923,45	±	10447,72	26	213941,97	±	10576,81	2,297	0,130
NORTH	28	254124,57	±	24271,14	26	362183,90	±	25037,69	7,482	0,006
Air Temperature (C)	26	24,98	±	1,05	23	25,35	±	1,32	0,050	0,823
Soil Temperature (C)	26	28,51	±	2,02	23	25,74	±	1,33	1,116	0,262
Relative Humidity of Air (%)	25	36,02	±	2,25	23	35,56	±	3,14	0,015	0,901
Relative Humidity of Soil (%)	26	42,69	±	2,61	23	48,42	±	3,55	1,690	0,194
Average Annual Humidity (%)	29	74,90	±	0,85	26	74,57	±	0,93	0,073	0,787
Average Annual Temperature (C)	29	15,05	±	0,34	26	13,75	±	0,45	4,507	0,034
Total Annual Precipitation (mm)	29	765,39	±	43,66	26	1140,64	±	98,60	8,529	0,004
Total Annual Insulation (hours)	29	2751,52	±	35,93	26	2550,76	±	44,47	9,097	0,003

Rhipicephalus	TICKS PRESENT				TICKS NOT PRESENT					
Variable	N	Mean	±	Std Error	N	Mean	±	Std Error	Wald X^2	*P*
Open Lands (%)										
500m	29	44,50	±	6,73	25	52,22	±	7,93	0,570	0,450
1000m	29	50,09	±	5,90	25	62,87	±	6,49	2,071	0,150
2000m	29	56,49	±	5,34	25	58,57	±	5,99	0,070	0,791
5000m	29	68,01	±	4,24	25	58,05	±	4,87	2,298	0,130
Human Dominated Lands (%)										
500m	29	6,93	±	3,56	25	4,60	±	2,51	0,272	0,602
1000m	29	4,68	±	1,91	25	5,09	±	2,83	0,016	0,901
2000m	29	3,38	±	1,24	25	4,41	±	2,79	0,126	0,723
5000m	29	1,86	±	0,48	25	2,94	±	1,94	0,317	0,574
Deciduous Forest (%)										
500m	29	28,98	±	7,27	25	19,12	±	7,25	0,916	0,339
1000m	29	28,64	±	6,74	25	6,84	±	3,42	5,245	0,022
2000m	29	25,39	±	5,89	25	9,51	±	4,40	3,592	0,058
5000m	29	16,48	±	3,86	25	8,62	±	3,77	1,956	0,162
Evergreen Forest (%)										
500m	29	8,94	±	4,42	25	17,50	±	6,02	1,315	0,251
1000m	29	7,75	±	3,79	25	16,76	±	5,43	1,792	0,181
2000m	29	6,08	±	3,11	25	15,71	±	4,82	2,553	0,110
5000m	29	4,49	±	2,50	25	13,74	±	3,86	3,326	0,068
Mixed Forest (%)										
500m	29	7,00	±	3,44	25	6,33	±	3,72	0,019	0,892
1000m	29	5,83	±	2,31	25	7,82	±	3,12	0,279	0,598
2000m	29	5,94	±	2,01	25	10,15	±	3,06	1,353	0,245
5000m	29	5,40	±	1,18	25	12,26	±	3,37	3,350	0,067
Interspersion Juxtaposition Index (%)										
500m	29	15,79	±	5,73	25	11,12	±	5,18	0,363	0,547
1000m	29	25,53	±	6,74	25	27,84	±	6,61	0,061	0,805
2000m	29	33,90	±	5,83	25	35,37	±	6,83	0,028	0,867
5000m	29	43,63	±	4,01	25	47,25	±	3,57	0,451	0,502
Mean Patch Size (%)										
500m	29	45,31	±	4,38	25	46,57	±	4,66	0,040	0,841
1000m	29	118,38	±	15,08	25	135,29	±	21,27	0,447	0,504
2000m	29	279,99	±	46,34	25	281,12	±	34,30	0,000	0,984
5000m	29	517,94	±	81,63	25	587,15	±	56,27	0,447	0,504
Number of Patches (%)										
500m	29	2,24	±	0,21	25	2,16	±	0,22	0,074	0,786
1000m	29	3,48	±	0,34	25	3,64	±	0,43	0,088	0,767
2000m	29	6,62	±	0,64	25	6,56	±	0,85	0,004	0,953
5000m	29	16,41	±	1,13	25	16,80	±	1,75	0,037	0,847
Shannon Diversity Index (%)										
500m	29	0,43	±	0,06	25	0,37	±	0,07	0,410	0,522
1000m	29	0,54	±	0,08	25	0,52	±	0,08	0,055	0,815
2000m	29	0,64	±	0,07	25	0,65	±	0,08	0,003	0,959
5000m	29	0,61	±	0,07	25	0,63	±	0,08	0,014	0,906

Table 4.5C – Results of univariate analysis (Wald statistics) between presence/absence of ticks (*Dermacentor* spp ticks) and environmental variables.

Dermacentor	TICKS PRESENT				TICKS NOT PRESENT					
Variable	N	Mean ± Std Error			N	Mean ± Std Error			Wald X^2	*P*
EAST	12	187353,70	±	15014,13	42	206192,94	±	8665,63	1,077	0,299
NORTH	12	293213,12	±	36272,69	42	309850,28	±	22007,16	0,138	0,711
Air Temperature (C)	11	22,78	±	1,80	38	25,84	±	0,91	2,328	0,127
Soil Temperature (C)	11	28,61	±	4,72	38	26,80	±	0,90	0,361	0,548
Relative Humidity of Air (%)	10	38,52	±	4,86	38	35,09	±	2,03	0,549	0,459
Relative Humidity of Soil (%)	11	43,62	±	5,94	38	45,89	±	2,28	0,191	0,662
Average Annual Humidity (%)	13	75,67	±	0,96	42	74,46	±	0,76	0,692	0,405
Average Annual Temperature (C)	13	14,30	±	0,59	42	14,47	±	0,34	0,061	0,805
Total Annual Precipitation (mm)	13	946,19	±	118,95	42	941,72	±	66,39	0,001	0,973
Total Annual Insulation (hours)	13	2654,76	±	65,06	42	2657,19	±	35,95	0,001	0,973

Dermacentor	TICKS PRESENT				TICKS NOT PRESENT					
Variable	N	Mean ± Std Error			N	Mean ± Std Error			Wald X^2	*P*
Open Lands (%)										
500m	13	44,04	±	10,17	41	49,36	±	5,99	0,200	0,655
1000m	13	49,41	±	8,68	41	58,10	±	5,14	0,713	0,399
2000m	13	54,39	±	8,57	41	58,42	±	4,50	0,193	0,661
5000m	13	65,83	±	6,58	41	62,63	±	3,77	0,181	0,671
Human Dominated Lands (%)										
500m	13	8,82	±	6,62	41	4,91	±	2,09	0,549	0,459
1000m	13	4,62	±	2,52	41	4,95	±	2,03	0,008	0,931
2000m	13	2,50	±	1,11	41	4,29	±	1,86	0,264	0,607
5000m	13	1,32	±	0,43	41	2,69	±	1,21	0,336	0,563
Deciduous Forest (%)										
500m	13	19,98	±	8,07	41	25,82	±	6,31	0,238	0,626
1000m	13	18,01	±	6,66	41	18,72	±	5,14	0,005	0,942
2000m	13	14,23	±	6,63	41	19,25	±	4,69	0,307	0,580
5000m	13	6,38	±	3,34	41	14,89	±	3,41	1,609	0,205
Evergreen Forest (%)										
500m	13	16,36	±	8,07	41	11,80	±	4,15	0,283	0,595
1000m	13	14,81	±	6,80	41	11,00	±	3,75	0,252	0,616
2000m	13	13,29	±	6,26	41	9,67	±	3,20	0,299	0,585
5000m	13	8,34	±	3,68	41	8,91	±	2,82	0,012	0,914
Mixed Forest (%)										
500m	13	10,36	±	5,07	41	5,53	±	2,89	0,658	0,417
1000m	13	12,39	±	4,56	41	4,96	±	1,98	2,513	0,113
2000m	13	15,19	±	4,73	41	5,58	±	1,70	4,663	0,031
5000m	13	16,56	±	5,06	41	6,05	±	1,46	5,262	0,022
Interspersion Juxtaposition Index (%)										
500m	13	27,61	±	9,70	41	9,20	±	3,91	3,713	0,054
1000m	13	41,06	±	9,67	41	22,02	±	5,24	2,910	0,088
2000m	13	41,43	±	10,22	41	32,41	±	4,85	0,769	0,381
5000m	13	49,77	±	4,94	41	43,89	±	3,19	0,864	0,353
Mean Patch Size (%)										
500m	13	35,33	±	5,69	41	49,25	±	3,64	3,245	0,072
1000m	13	111,21	±	26,12	41	130,96	±	14,60	0,445	0,505
2000m	13	268,69	±	90,52	41	284,26	±	26,70	0,053	0,819
5000m	13	422,84	±	78,73	41	590,29	±	61,14	2,266	0,132
Number of Patches (%)										
500m	13	2,77	±	0,32	41	2,02	±	0,16	4,135	0,042
1000m	13	4,23	±	0,58	41	3,34	±	0,30	1,993	0,158
2000m	13	8,08	±	1,27	41	6,12	±	0,54	2,530	0,112
5000m	13	17,08	±	2,10	41	16,44	±	1,16	0,075	0,784
Shannon Diversity Index (%)										
500m	13	0,56	±	0,08	41	0,35	±	0,05	3,671	0,055
1000m	13	0,77	±	0,12	41	0,46	±	0,06	5,498	0,019
2000m	13	0,79	±	0,14	41	0,60	±	0,05	2,568	0,109
5000m	13	0,57	±	0,13	41	0,63	±	0,05	0,247	0,619

Table 4.5D – Results of univariate analysis (Wald statistics) between presence/absence of ticks (*I. ricinus* ticks) and environmental variables.

Ixodes ricinus	TICKS PRESENT				TICKS NOT PRESENT					
Variable	N	Mean ± Std Error			N	Mean ± Std Error			Wald X^2	*P*
EAST	8	168175,76	±	17419,53	46	207890,04	±	8064,89	3,203	0,074
NORTH	8	319226,25	±	41806,31	46	303879,55	±	20975,80	0,086	0,770
Air Temperature (C)	7	19,86	±	2,26	42	26,03	±	0,82	5,806	0,016
Soil Temperature (C)	7	29,11	±	7,64	42	26,89	±	0,82	0,381	0,537
Relative Humidity of Air (%)	6	49,63	±	4,15	42	33,83	±	1,90	6,118	0,013
Relative Humidity of Soil (%)	7	50,21	±	8,18	42	44,57	±	2,18	0,819	0,366
Average Annual Humidity (%)	9	76,23	±	1,14	46	74,45	±	0,70	1,116	0,291
Average Annual Temperature (C)	9	13,58	±	1,05	46	14,60	±	0,28	1,629	0,202
Total Annual Precipitation (mm)	9	1140,36	±	181,09	46	904,12	±	58,22	2,068	0,151
Total Annual Insulation (hours)	9	2602,50	±	84,35	46	2667,20	±	33,64	0,589	0,443

Ixodes ricinus	TICKS PRESENT				TICKS NOT PRESENT					
Variable	N	Mean ± Std Error			N	Mean ± Std Error			Wald X^2	*P*
Open Lands (%)										
500m	9	47,85	±	14,15	45	48,12	±	5,55	0,000	0,984
1000m	9	48,13	±	12,02	45	57,58	±	4,75	0,639	0,424
2000m	9	46,40	±	10,25	45	59,66	±	4,26	1,536	0,215
5000m	9	61,77	±	9,83	45	63,72	±	3,42	0,051	0,821
Human Dominated Lands (%)										
500m	9	0,08	±	0,05	45	7,01	±	2,64	0,267	0,605
1000m	9	1,96	±	0,81	45	5,45	±	1,96	0,559	0,455
2000m	9	2,19	±	1,03	45	4,19	±	1,71	0,249	0,618
5000m	9	1,77	±	1,02	45	2,48	±	1,10	0,078	0,780
Deciduous Forest (%)										
500m	9	18,79	±	9,87	45	25,54	±	5,88	0,241	0,624
1000m	9	13,55	±	6,92	45	19,55	±	4,85	0,284	0,594
2000m	9	13,73	±	9,31	45	18,90	±	4,31	0,247	0,620
5000m	9	4,43	±	3,81	45	14,52	±	3,15	1,548	0,213
Evergreen Forest (%)										
500m	9	24,72	±	12,03	45	10,53	±	3,67	1,943	0,163
1000m	9	22,45	±	11,18	45	9,81	±	3,20	1,929	0,165
2000m	9	17,85	±	9,18	45	9,08	±	2,88	1,262	0,261
5000m	9	13,76	±	7,46	45	7,77	±	2,35	0,909	0,341
Mixed Forest (%)										
500m	9	8,56	±	4,43	45	6,32	±	2,88	0,112	0,738
1000m	9	13,91	±	4,46	45	5,32	±	2,04	2,515	0,113
2000m	9	19,64	±	6,58	45	5,54	±	1,51	6,833	0,009
5000m	9	17,03	±	6,65	45	6,89	±	1,53	3,937	0,047
Interspersion Juxtaposition Index (%)										
500m	9	19,01	±	11,51	45	12,55	±	4,10	0,385	0,535
1000m	9	45,20	±	12,04	45	22,88	±	4,98	2,953	0,086
2000m	9	45,26	±	10,32	45	32,44	±	4,86	1,161	0,281
5000m	9	47,38	±	9,11	45	44,90	±	2,74	0,119	0,730
Mean Patch Size (%)										
500m	9	42,05	±	7,39	45	46,67	±	3,52	0,298	0,585
1000m	9	105,03	±	28,50	45	130,44	±	14,15	0,553	0,457
2000m	9	234,94	±	68,16	45	289,63	±	32,51	0,485	0,486
5000m	9	364,66	±	64,92	45	587,04	±	58,17	3,596	0,058
Number of Patches (%)										
500m	9	2,33	±	0,37	45	2,18	±	0,17	0,151	0,698
1000m	9	4,22	±	0,64	45	3,42	±	0,29	1,236	0,266
2000m	9	8,67	±	1,58	45	6,18	±	0,52	3,016	0,082
5000m	9	19,44	±	2,62	45	16,02	±	1,08	1,547	0,214
Shannon Diversity Index (%)										
500m	9	0,45	±	0,12	45	0,39	±	0,05	0,287	0,593
1000m	9	0,70	±	0,15	45	0,50	±	0,06	1,962	0,161
2000m	9	0,80	±	0,14	45	0,62	±	0,05	1,746	0,186
5000m	9	0,69	±	0,15	45	0,61	±	0,05	0,395	0,530

Complete steps of logistic regression may be observed in **Appendix 4**. The following table (**Table 4.6**) presents the variables that remained in the final model with the respective level of concordance (correction of the model). As it can be seen, the presence of the different tick species can be predicted by various variables, mostly related to climate and type of habitat. *I. ricinus* was, with no doubt, the best model, with a 97.2% of concordance, followed by *Rhipicephalus* ticks.

Table 4.6 – Final variables of the stepwise logistic regression model for total ticks, *Rhipicephalus* spp., *Dermacentor* spp. and *I. ricinus* and respective percent of concordance.

TICKS	FINAL MODEL (variables)	Level of concordance (%)
Total ticks	HUMSOIL, PDE1000	73.5
Rhipicephalus	PRECIP, PDE1000, POP5000	83.6
Dermacentor	PMI2000	57.4
I. ricinus	EAST, PRECIP, PMI2000, IJI1000	97.2

Legend: HUMSOIL (soil humiity), PDE (proportion of deciduous forests), PRECIP (precipitation), POP (proportion of open areas), PMI (proportion of mixed forests), EAST (easting coordinate of the site), IJI (interspersion/juxtaposition index)

Based on these variables, predictive maps (**Fig. 4.2**) were built to define risk areas for each species, all infected with *B. burgdorferi* sensu lato agents. As it is possible to observe, the environmental conditions assessed in the logistic model offers suitable conditions for the presence of ticks in practically all Portugal. According to these prediction maps, the risk of contacting with ticks is higher in *Rhipicephalus* genera, manly in the inner regions and the south of Portugal. For *I. ricinus* and *D. marginatus* ticks, so far the most infected by *B. burgdorferi* s.l., the areas of higher risk are found in the coastal region, with increasing areas from South to North.

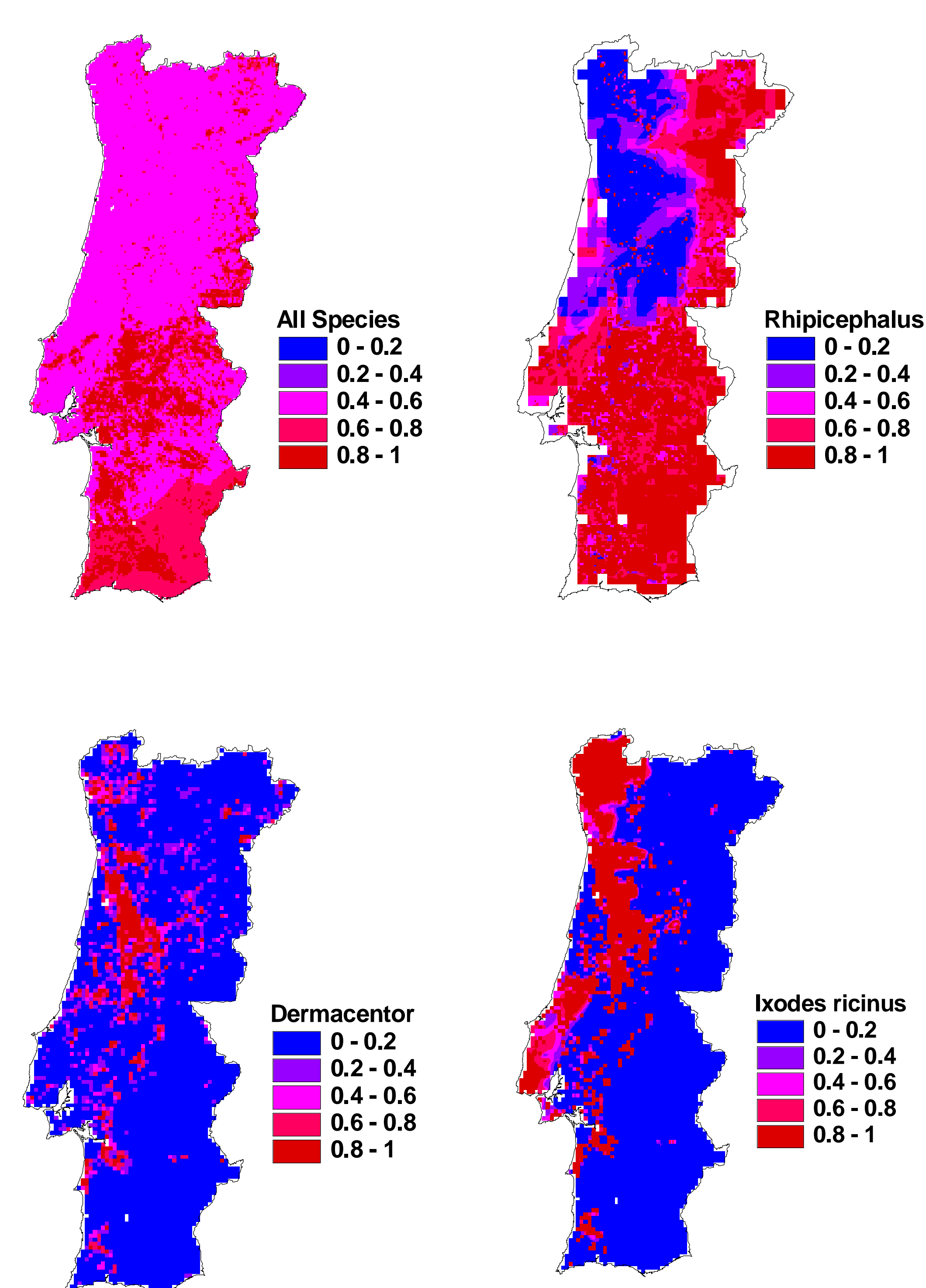


Figure 4.2 – Prediction surfaces of tick presence (Total Ticks, *Rhipicephalus* spp, *D. marginatus* and *I. ricinus*) based on the logistic models with the environmental variables.

4.5 Discussion

High risk areas for Lyme Borreliosis are generally considered to be heterogeneous deciduous woodland, with a recreational function and supporting a diverse fauna usually including deer (Kahl, 1996; Gray *et al*., 1998 *cited in* Estrada-Pena & Jongejan, 1999; Lindstrom & Jaenson, 2003).

Although important, locations cannot be classified as posing a high risk for LB simply on the basis of tick density. It is necessary to take several integrated abiotic and biotic factors that will influence the distribution and density of ticks. Vegetation, for instance, will have a strong influence on the variety and abundance of hosts present (Talleklint & Jaenson, 1996). The climate sets the limit for the possible geographic distribution of ticks and also may influence population density both directly and indirectly. Bioclimatic threshold temperatures influence the dynamics of transmission of LB agents (Lindgren *et al*., 2000). Patterns of tick natality and mortality at different sites and movement patterns of ticks or of their vertebrate hosts also influence the spatial distribution of ticks (Lindstrom & Jaenson, 2003; Ginsberg *et al*., 2004). All variables are interrelated, and extremes of each factor may adversely affect the tick's ability to survive (Guerra *et al*., 2002; Lindstrom & Jaenson, 2003).

In this chapter there was an attempt to describe with some accuracy all sample sites in the nationwide study. Field characterization and GIS environmental variables were the basis for this description. Although the GIS variables (Corine Map and IA maps) were based on old information (with 20 to 30 years), the local description fitted most of the broad-scale maps.

Coniferous and deciduous forests were the main habitats, along with open areas occupied by agricultural and pasture fields and shrub areas. Since sample points were chosen according to what was known about *I. ricinus* preferable habitats, and since these habitats are restricted to non-urbanized areas, habitats of type 1 (human development) were more frequent at higher distances from each sample site. Sample sites had a mean air temperature above 20ºC, indicating a warm climate, which is also in accordance with the specific temperatures for the seasons (Spring and Summer) when collections were performed, being also true for recorded values of humidity.

Mean temperature and humidity were found to be associated with tick presence collected in the nationwide study (Hostis *et al*., 1995). As mentioned before, temperatures were reasonably elevated, within the range of 12ºC to 29ºC. Maximum temperature may play a significant role in determining the range of ticks, since it may augment both the development and hatching rates while hindering overall survival and oviposition success (Brownstein *et al*., 2003). Local humidity related to the type of vegetation is another factor that contributes to lower or higher tick infestations. Moisture during summer and severe temperatures during winter are both factors that affect the activities of free-living ticks (Hostis *et al*., 1995; Kahl, 1996). Precipitation, which is known to increase tick activity and survival rate during wet conditions (McGabe & Bunnell, 2004), was important for *Rhipicephalus* and *I. ricinus* ticks and was able to predict their distribution.

Other variable important for ticks is the type of soil (Glass *et al*., 1992, 1994, 1995; Kurtenbach *et al*., 1995; Merler *et al*., 1996; Guerra *et al*., 2002; Cortinas *et al*., 2002; Bunnell *et al*., 2003). Soil orders are influenced by the type of underlying bedrock and by quaternary deposits. The soils, in turn, influence the type of vegetation overlying them. Soil texture is the component of soil that influences the extent of drainage. The soil texture classes are independent of soil order and are usually a function of the degree of soil weathering and the parent material (bedrock or quaternary deposit). Soil texture determines the extent of drainage, and the level of moisture of the ground layer, regardless of the amount of precipitation (Guerra *et al*., 2002). Geological characteristics of soils may indirectly influence their infestation by ticks owing to their draining capacity and thereby the selection of vegetation (Mémeteau *et al*., 1998).

Ticks were collected mainly in very or moderately acid soils, as in other studies (Kurtenbach *et al*., 1995). Although the pH of the soil is unlikely to affect ticks directly, this abiotic factor may have an indirect effect. The rate of decomposition of the leaf litter is known to be lower on acid soils than on alkaline ones (Kurtenbach *et al*., 1995)

The main soils found in the nationwide study were cambisols and luvisols. Cambisols can be described as weakly to moderately developed soils, characterized by soil structure, absence of rock structure, stronger chroma, redder hue or high clay content. Cambisols are medium textured and have good structural stability, high porosity, good water holding capacity and good internal drainage. In most cases Cambisols have a neutral to weakly acid soil reaction, a satisfactory chemical fertility and an active soil fauna. In general, Cambisols make good agricultural land and are intensively used. On the other hand, Luvisols are

characterized by the presence of a diagnostic argic horizon (textural differentiation), usually well drained. In a compacted argic horizon, internal permeability may be low so that water stagnates in the upper horizons. It has high silt content, a medium pH and a good level of fertility (Cardoso *et al.*, 1973; Fonseca & Marado, 1991; ISSS, 1998).

Increased seropositivity of LB in dogs (Guerra *et al.*, 2001) was associated with fertile, moist, calcareous soils, such as alfisoils and mollisols, that underlie prairie grasslands and deciduous forests, respectively, with sandy soils characterized by increased particle size. There was no significant association with soil drainage. These soils, which contain greater amounts of calcareous material and organic matter, are more suited for supporting biological activity. *I. scapularis* (the American tick) and ticks found in our study may survive better in soils containing higher amounts of organic matter that is associated with increased amounts of leaf litter. Particle size is related to the water-retaining properties of the soil. Sandy soils have better drainage and have been associated to tick endemicity. Soils of medium to fine particle size are usually saturated at the time of spring defrost, when adults begin questing, and tick survival may be adversely affected in soils with greater proportions of silt and clay (Guerra *et al.*, 2001).

Glass and colleagues (1994) found, based on 41 environmental variables, that *I. scapularis* abundance was negatively correlated with the amount of privately owned land, which harbour soils that tended to be saturated with water and one drainage system (*e.g.* sands with high content of silt and clay which tend to remain saturated or extended periods of time, reducing tick survival). Ticks abundance was positively correlated with well-drained, acidic sandy soils having low water tables.

Type of habitat and their diversity, adjacency and size were factors that contributed to presence of ticks. Several habitats were used by ticks such as open areas and deciduous and coniferous forests. Type of forest (deciduous and coniferous) by itself do not account for tick distribution patterns. The distinction between deciduous and coniferous woods influences tick distribution primarily because of the influence of habitat type on the movements of tick host animals and not because of differences in the physical environments of the different forest types (Lindstrom & Jaenson, 2003; Ginsberg *et al.*, 2004).

Fragmentation of the landscape has been proposed to play an important role in defining local scale heterogeneity in Lyme disease risk through influence on mammalian host density and species composition

(Brownstein *et al.*, 2005). For instance, edge habitat, indirectly defined by the Interspersion/Juxtaposition Index (IJI), is ideal as it provides preferred forage in abundant ecotonal vegetation. This type of landscape mosaic should experience higher tick densities than homogeneous forested landscapes because of the extremely high host numbers for adult tick-feeding and reproduction. Second, landscape structure will have an influence on abundance and composition of small- and medium-sized mammals, which serve as hosts for the immature ticks as well as maintain the enzootic cycle of *B. burgdorferi* s.l.. Finally landscape fragmentation may also influence human exposure to infected ticks, as the density of infected ticks on a residential property is positively correlated with the proportion of vegetation cover on the property. Increased fragmentation in the landscape through decreasing patch size may serve to increase tick-infection prevalence, while increasing interpatch distance serve to increase tick density (Brownstein *et al.*, 2005).

Environmental variables that were associated with tick presence, regardless the species, but with a special focus on *I. ricinus* ticks, was similar to what is obtained for other places. For instance, in Italy, *I. ricinus* has been recorded especially in thermomesophilous woods and shrubby habitats, according to what was obtained in our study (Rizzoli *et al.*, 2004). Altitude and geological substratum were the most important variables affecting tick occurrence, with a decrease of tick presence above 1,100 m and on volcanic rocks (Merler *et al.*, 1996). In another place, altitude, vegetation cover and roe deer density were the variables with the greatest effect on tick occurrence and infection prevalence (Rizzolli *et al.*, 2003). In Spain, local populations of *I. ricinus* in some mountain areas can be seen, except in those with higher altitudes because of the low temperatures. The tick is predicted to be associated to vegetation areas of termophilous oakwoods or Atlantic vegetation of *Quercus* spp (Degeilh *et al.*, 1994 *fide* Estrada-Pena, 1997). Barral and others (2002) observed that *I. ricinus* infected with *B. burgdorferi* s.l. were mainly collected in pine woodlands, located below 800 m above sea level with Atlantic climate and a predominant undergrowth mainly composed of heather, as well as bracken, furze and grass. Roe deer abundance and soil water capacity, but not *Borrelia* prevalence, were the main factors that influence tick density in Denmark (Jensen *et al.*, 2000).

Infection of ticks with *B. burgdorferi* s.l. agents is also associated with several biotic and abiotic aspects of the environment. First, diversity and prevalence of *B. burgdorferi* s.l. genomospecies is associated with woodland characteristics. Spatial distributions vegetation composition and host cenosis of the habitats were

identified as factors which may affect the distribution and prevalence of *B. burgorderi* s.l. genomospecies (Kirstein *et al*., 1997). Infection rates are also related to type of vegetation and host composition. For instance, in Ireland, Gray and others (1995) observed that highest infection prevalence in nymphs and adults occurred in deciduous amenity woodland from which livestock were excluded, but they were more abundant at sites with livestock or larger mammals. In Croatia, tick infection was at lowest degree in parks, little bit higher in woods and highest in mixed park-wood areas (Stajkovic *et al*., 1993). In France, hill vegetation was associated with presence of *I. ricinus* and *B. burgdorferi* s.l, mountain vegetation with presence of *I. ricinus* until 1200m and *B. burgdorferi* s.l. from 200 to 1200m, and supramediterranean vegetation associated with absence/presence of *I. ricinus* and presence of *B. burgdorferi* s.l. (Pichot *et al*., 1994).

Definition of risk areas for LB in Portugal was based on an extensive work of collections of ticks, laboratory analyses of infection rates on ticks and humans and genotyping of *Borrelia* species. The gathering of all these data and definition of significant associations was based on univariate and multivariate statistical analyses that allowed a comprehension of environmental variables that could contribute to enhance the risk of any person to contract LB, when on outdoor activities.

Choice of these models should depend on the nature of the data, whether assumptions for each method are satisfied, and on the needs of any particular analysis (Thomson & Connor, 2000). For instance, presence only data sets suffer three fundamental drawbacks that limit both their use and validity in models. The most obvious of these is the intrinsic lack of accurate absence data, which is a necessary component in most modeling methods. Second, is the unknown sampling bias associated with ad hoc or non-systematic data samples where the sample is often dependent upon factors such as distance to cities, accessibility and type of environment, rather than on a stratified or systematic strategy, an aspect already referred in chapter 2. A third drawback of these types of data sets lies in the unknown sampling bias of rare *versus* common species (Zaniewski *et al*., 2002), as seems to be the case of *I. ricinus* compared to *Rhipicephalus* spp.

Considering these aspects, risk factors for these tick populations were defined based on the results presented earlier on this thesis. Precipitation was the most important variable predicting presence of ticks, along with type of habitats (open areas, mixed forests and deciduous forests) in ranges of 1000 to 5000 m

around sample points, indicating that macroclimate impact was rather important for determining tick distribution and confirms the importance of fragmented landscape.

Risk maps created with these variables predict a wider distribution of ticks throughout the national territory, especially for *Rhipicephalus* genera, the less demanding species (Santos Dias, 1994). For *I. ricinus* and *D. marginatus* ticks, the areas of higher risk are restricted to the coastal region, dependent on the climate variables, more suitable in this region as already demonstrated (Chapter 2)

Again, these variables are similar to what was obtained for other countries and species (namely *I. scapularis*), with similar methods of multivariate analysis (logistc regression). Glass *et al*. (2002) used a total of 127 environmental variables linked to LB epidemiology extracted with the help of a GIS, most of them recorded as dichotomous (presence-absence) variables were analysed by logistic regression. Residence within two watersheds, on loamy soils (large percentages of sand with lesser amounts of clay and silt and may favour tick survival, and within forested areas served to increase risk, while residence in highly developed areas decreased risk of disease.

In Netherlands, a study from 1997 indicated that the ecological risk factors for both *I. ricinus* tick bites and erythema migrans were the area covered by woods, sandy soil, dry uncultivated land, the number of tourist--nights per inhabitant and sheep population density. The cattle population density was a risk factor for erythema migrans (de Mik *et al*., 1997). In a more recent research, some authors of the previous study found out that, at the municipal level, tick bites and erythema migrans were positively associated with the area covered by forest, sandy soil, the number of roe deer and tourism. There was a negative association with the degree of urbanization. Increases in tourism in areas with many ticks, new forests in urban regions and an increased number of horses were positively associated with the increase in tick bites and erythema migrans since 1994 (den Boon *et al*., 2004)

I. ricinus is expected to be prevalent in the northern portion of Spain. Highest risk is presumed for areas above the parallel 41ºN, where adequate combinations of temperatures, rainfall and soil saturation deficit exist. Lower risk is expected in areas of Supramediterranean oaks, forests of deciduous oaks, and Iberic Oromediterranean vegetation. *I. ricinus* is predicted to be absent in a rather small area at the west, close to the Spain-Portugal border. High temperatures and low rainfall in *Q. ilex* areas make it unsuitable for the

permanence of constant populations, although it has been recorded in this habitat (Degeilh *et al*., 1994 *cited in* Estrada-Pena, 1997). The small patches of relative risk in the south of Spain correspond to mountain areas with deciduous oaks (Aceri-Quercion Faginae). Small or near zero risk is predicted for wide areas of Spain, corresponding to steppe or semidesert areas. Presence of small sites with semihumid forest within these areas can harbour local populations (Estrada-Pena, 1997).

Importance of fragmentation between habitats was also observed by Mémeteau and others (1998) in France. Pastures located in the close vicinity of a wood containing deciduous or conifer trees, whatever their soil type or vegetation are at a six- to seven-fold increased risk than pastures without a wood in the close vicinity and consisting of seeded grass species. An intermediate risk level is related to the presence of large hedges and of naturally growing grass species.

In conclusion, determination of risk factors with prediction of tick distribution and consequently the potential areas where LB may prevail is important, not only to understand the epidemiology of this disease in Portugal but also to establish priorities for intervention strategies (Schulze *et al*., 1991). Steps like characterization of a site to respect to potential risk of transmission in the absence of tick activity by evaluating the suitability, amount, and accessibility of tick habitat, corresponding to a Phase I and use this information, in conjunction with the information on habitat, data on the presence and size of tick population, and rate of *B. burgdorferi* s.l. infection (Phase II) to categorize actual risk, needs to become a well understood and well used tool for the study of Lyme Borreliosis in Portugal.

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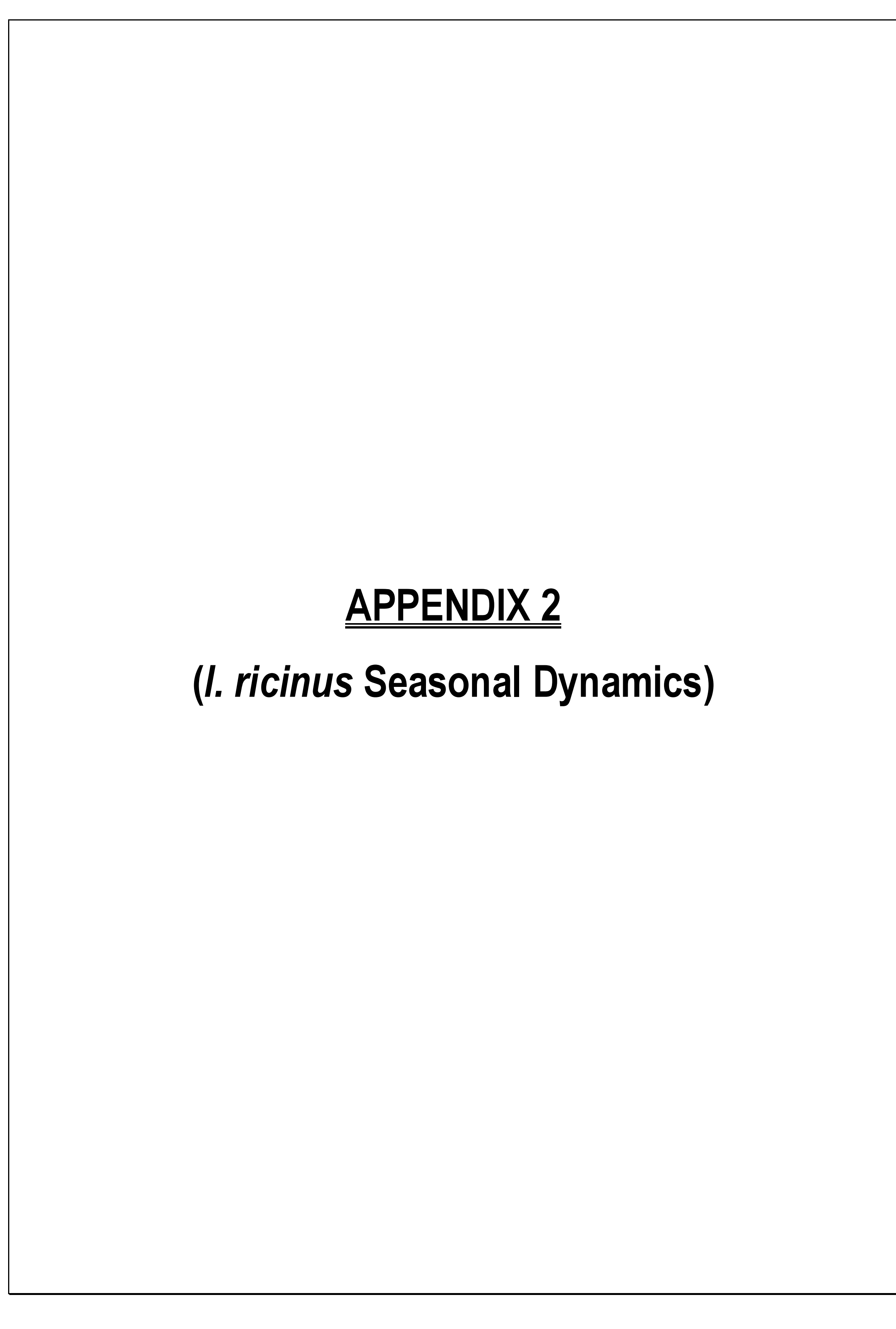
APPENDIX 1

(Hosts and *B. burgdorferi* s.l.)

Table A1.1 – List of various host species related with Lyme Borreliosis epidemiology

Small mammals	**Role/Characteristics**	**References**
Peromyscus leucopus (**white-footed mice)**	Suitable sentinel for *B. burgdorferi* s.l.	Oliver Jr *et al*., 1999¸ Hofmeister *et al*., 1999
*Apodemus agrarius (***black-striped mice)**	Correlation between mice presence and transmission of *B. burgdorferi* s.l.	Matuschka *et al*., 1992
A. flavicollis *A. sylvaticus*	Real reservoirs hosts for *B. burgdorferi;* the agent can persist and overwinter in *Apodemus* while *I. ricinus* is not active	Gern *et al*, 1994; Humair *et al*., 1999
C glareolus	May acquire resistance to higher densities of ticks; In some countries is the primary overwintering reservoir of *B. burgdorferi*	Kurtenbach *et al*., 1994, 1995; Dizij & Kurtenbach, 1995; Talleklint & Jaenson, 1997; Humair *et al*., 1999; Talleklint & Jaenson, 1995; Humair *et al*., 1999; Pawelczyk & Sinski, 2000
Glis glis (**edible dormice)**	Amplifies transmission	Matuschka *et al*., 1994
Elyomis quercinus (**Garden dormice)**	Recognized reservoir host of Lyme disease spirochetes	Matuschka *et al*., 1999
Rattus norvegicus, Rattus rattus (**Rats)**	Appear to perpetuate the Lyme disease spirochete; may establish urban enzootic foci	Matuschka *et al*., 1994a; 1996, 1997
Medium mammals		
Squirrels	Reservoir hosts of *B. burgdorferi* s.l; association with *B. afzelii* and *B. burgdorferi* s.s. genotypes	Craine *et al*., 1997; Humair & Gern, 1998
Erinaceus europaeus (**Hedgehogs)**	Enzootic transmission cycle in an urban area involving hedgehogs and *I. hexagonus*	Gern *et al*., 1997
Lepus timidus (**varying hares)** *Lepus europaeus* (**European hares)**	Some *I. ricinus* vector ticks acquire spirochetal infection in island sites where biodiversity is restricted	Jaenson & Talleklint, 1996, Talleklint & Jaenson, 1993 *cited in* Matuschka *et al*., 2000
Oryctolagus cuniculus (**European rabbits**)	Do not perpetuate Lyme disease spirochetes; zooprophylactic	Matuschka *et al*., 2000

Large mammals		
Deer	Feed several stages of *I. ricinus* but it is not a competent host for *B. burgdorferi*¸ It is only above a relatively high threshold of deer density that such species' inability leads to a reduction of infection levels in questing ticks	Telford III *et al*., 1988; Jaenson & Talleklint, 1992; Matuschka *et al*., 1993; Talleklint & Jaenson, 1994;1996; Gustafson, 1994; Randolph *et al*., 1995; Pichon *et al*., 1999, 2000; Mannelli *et al*., 2003
Sheep	May actually not become infected with *B. burgdorferi* but allow co-feeding infection to take place, since their complement is only partially borreliacidal	Odgen *et al*., 1997; Kurtenbach *et al*., 1998
Reptiles		
Lizards	Negative correlation between lizard presence and transmission of *B. burgdorferi* s.l.	Matuschka *et al*., 1992
Birds		
Pheasants	Adequate reservoirs for *B. garinii*, *B. burgdorferi* s.s and *B. valaisiana; B. afzelii* is killed by avian complement in the gut of the feeding tick	Craine *et al*., 1997; Kurtenbach *et al*., 1998a, 1998b, 2002
Turdus merula (**European blackbirds)**	Play no role in the introduction of the agent of Lyme Disease into new foci; the reservoir competence of this species has been cleary demonstrated by tick xenodiagnosis, transmitting *B.garinii*	Matuschka & Spielman, 1992; Humair *et al*., 1993 *cited in* Gern & Humair, 2000
Turdus spp	May transmit *B. burgdorferi* to *I. ricinus* and *I. persulcatus* ticks and are considered as amplifier hosts¸ *B. garinii* was isolated from these birds; latent infection can be reactivated and passed on to ticks as a result of migratory restleness (increased nocturnal activity)	Humair *et al*., 1993a; Olsen *et al*., 1995; Hubalek *et al*., 1996; Miyamoto *et al*., 1997; Gylfe *et al*., 2000; Richter *et al*., 2000; Scott *et al*., 2001; Humair, 2002; Hanincová *et al*., 2003
Blackbirds	Serve as an important vehicle for transporting various *Ixodes* ticks into new foci of infestation	Matuschka & Spielman, 1992; Humair *et al*., 1993a; Olsen *et al*., 1995; Humair & Gern, 2000; Scott *et al*., 2001
Seabirds	Competent reservoirs and amplifying hosts of *B. garinii;* specific cycle with *I. uriae* without the intervention of mammal hosts	Olsen *et al*., 1993, 1995; Gylfe *et al*., 1999; Humair, 2002
Songbirds	Important reservoir hosts for *B. garinii* and *B. valaisiana*, but are not transmission competent for *B. afzelii*	Hanincová *et al*., 2003



APPENDIX 2

(*I. ricinus* Seasonal Dynamics)

Table A2.1 – Patterns of seasonal dynamics of *I. ricinus* populations for a wide range of European countries and one from North Africa, based on the reviewed literature. (1) Zacovska & Martiniková, 2004; (2) Gilot *et al.*, 1975a; (3) Matuschka *et al.*, 1999; (4) Vassalo *et al.*, 2000a; (5) Hostis *et al.*, 1995; (6) Dorn & Sunder, 1997; (7) Kurtenbach *et al.*, 1995; (8) Frusteri *et al.*, 1994; (9) Cinco *et al.*, 1998; (10) Manelii *et al.*, 1999, 2003; (11) Bormane *et al.*, 2004; (12) Vascilo *et al.*, 2004; (13) Michalik *et al.*, 2003; (14) Wegner *et al.*, 1994; (15) Caeiro, 1999; (16) Walker, 2001; (17) Revuelta, 1991; Guerrero, 2001; (18) Barandika *et al.*, 2004; (19) Estrada-Pena *et al.*, 2004; (20) Mejlon & Jaenson, 1993; (21) Gustafson, 1994; (22) Nilsson, 1988; (23) Mejlon, 2000; (24) Mermod *et al.*, 1973, 1974; (25) Younsi *et al.*, 2001; (26) Odgen *et al.*, 1997; (27) Craine *et al.*, 1995; (28) Randolph *et al.*, 2002; (29) Gardiner & Gettinby, 1983

	jan	feb	mar	apr	may	jun	jul	aug	sep	oct	nov	dec
Czech Rep. (1)												
France (2)												
France (3)												
France (4)												
France (5)												
Germany (6)												
Germany (7)												
Italy (8)												
Italy (9)												
Italy (10)												
Latvia (11)												
Lithuania (12)												
Poland (13)												
Poland (14)												
Portugal (15)												
Scotland (16)												
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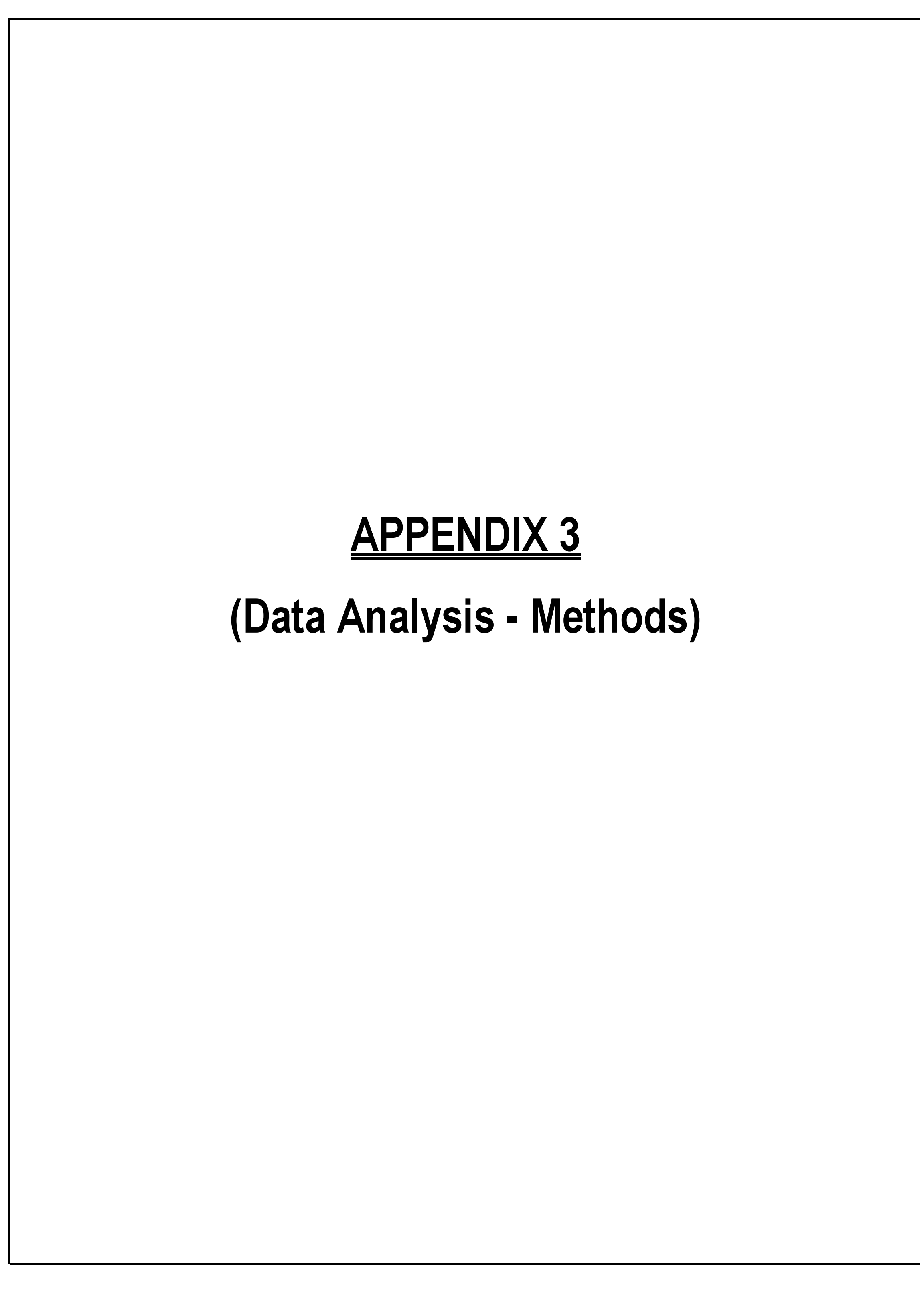
Sweden (23)												
Switzerland (24)												
Tunisia (25)												
U.K. (26)												
U.K. (27)												
U.K. (28)												
U.K. (29)												
	jan	feb	mar	apr	may	jun	jul	aug	sep	oct	nov	dec

Legend:
------ presence
_____ max. activity
_____ total ticks
_____ adults
_____ nymphs
_____ larva



APPENDIX 3

(Data Analysis - Methods)

A3.1 TIMES SERIES ANALYSIS

Module of Time Series Analysis in STATISTICA software "provides all major techniques for analysing time series data in a fully integrated environment".

There are several types of internal structure, like trend, seasonal variation and ciclicity, that can be extrapolated to prepare more accurate forecast, while the randomness cannot be predicted. Randomness is also call residuals or "white noise" and indicates the extent of variation between the actual and predicted results and can help to determine the extent of uncertainly in our future predictions. The general formula for the time series model is ACTUAL = PATTERN+RANDOMNESS. The common goal is to minimize these deviations or errors in the forecast (Turner *et al.*, 1990; Woolons & Norton, 1990; STATSOFT, 1994).

There are several steps in Time series analysis (**Figure A3.1**). Transformations and smoothing procedures (exponential smoothing, moving average smoothing and running medians smoothing) are used to reduce irregularities (residuals) in time series data. These techniques reveal more clearly the underlying trend, seasonal and cyclic components. It involves some form of local averaging of date such that the nonsystematic components of individual observations cancel each other out.

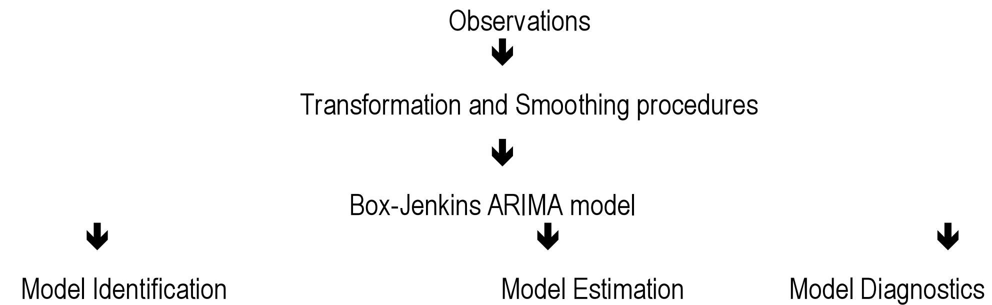


Fig. A1.1 – Steps to perform in Time Series analysis

Following these primary analyses, it is necessary to test whether and how current observations are influenced by prior observations or specific discrete events. The Box-Jenkins ARIMA model (1976) tries to describe those fluctuations about the equilibrium or perhaps "average" value and constitutes a combination of the Autoregressive Models (AR) and Moving Average Models (AM). An autoregressive model is simply a linear regression of the current value of the series against one or more prior values of the series. The value of **p** is called the order of the AR. The moving average model is a linear regression of the current value of the series against the residuals of one or more prior values of the series. These residuals or random shocks are propagated to future values of the time series. The value of **q** is called the order of the AM model.

The first step in ARIMA model (identification) is to determine if the series is stationary (constant mean and variance and autocorrelation through time) and if there is any significant seasonality that needs to be modelled. Stationary can be assessed from an autocorrelation plot (ACF) or correlograms. An autocorrelation plot with very slow decay often indicates non-stationary. ACFs are commonly used tools for checking randomness in a data set at varying time lags (Turner *et al.*, 1990; Woolons & Norton, 1990; ENGINEERING STATISTICS HANDBOOK; STATSOFT, 1994; Sánchez-Lafuente *et al.*, 2001). If random, such autocorrelations should be near zero for any and all time-lag separations. If non-random, then one or more of the autocorrelations will be significantly non-zero. For detecting seasonality, it is necessary to identify the order for the seasonal autoregressive and seasonal moving average terms (*e.g.* AR12 or AM12 for monthly data). After this, the p and q terms of ARIMA model have to be identified with the help of ACF and the PACF (partial autocorrelation plots) (**Table A3.1**)

After fitting a model to a given set of data the goodness of fit of the model is usually examined to see if it is indeed an appropriate model. If the model is not satisfactory, modifications are made to the model and the whole process of model selection, parameter estimation, and diagnostic checking must be repeated until a satisfactory model is found. The models are fitted with non-linear least squares and maximum likelihood estimation and should follow the assumptions for a stationary univariate process (model diagnostics). The residuals must be independent (not autocorrelated), and have a normal distribution, that is if the fitted model is the "true" model, the residuals should behave like a white noise process with zero mean and constant variance.

Table A3.1 - Identification of Box-Jenkins ARIMA model based on the shape of autocorrelation and parcial-autocorrelation plots (ACF and PACF).

SHAPE	***INDICATED MODEL***
ACF exponential decay; PACF spike at lag 1, no correlation for other lags	AR (p=1)
ACF sine-wave shape pattern or a set of exponential decays; PACF spikes at lags 1 and 2, no correlation for other lags	AR (p=2)
ACF spike at lag 1; no correlation for other lags; PACF damps out exponentially	AM (q=1)
ACF spikes at lags 1 and 2, no correlation for other lags; PACF a sine-wave shape pattern or a set of exponential decays	AM (q=2)
ACF exponential decay starting at lag 1; PACF exponential decay start at lag 1	AR (p=1) + AM (q=1)
ACF all zero or close to zero	Data is essentially random
High values at fixed intervals	Include seasonal autoregressive term (AR12)
No decay to zero	Series is not stationary

A3.2 GEOSTATISTICS (kriging and other methodologies)

The first step in kriging is to construct a semivariogram, a plot of the semivariance for a set of distance intervals or lags, and to choose the mathematical method which best approximates the shape of the variogram. The semivariogram is a model of the average degree of similarity between values as a function of their separation distance, i.e., expresses the way in which variance of a property changes over the land surface, based on the distance and direction separating two separate locations. Semivariance is calculated as

$$\gamma(h) = \frac{1}{2N(h)} \sum_{i=1}^{N(h)} [z(x_i) - z(x_i + h)]^2,$$

($\gamma(h)$ – semivariance statistic, calculated for each specific distance interval h in a data set; $z(x_i)$ – sample value at point x_i ; $z(x_i + h)$ – sample value at point $x_i + h$; $N(h)$ – number of pairs of points for the interval in question)

Semivariogram is a plot of semivariances [$\gamma(h)$] against all lags (h's) and the shape of the plot is a representation of the autocorrelation present (**Figure A3.2**). Small values for semivariance correspond to data that are more similar, whereas large values reflect data that are more dissimilar. Data that exhibit spatial autocorrelation produce a variogram with small values for short lags and increasing semivariance with increasing distance. These increases continue until the points are so far apart that they are not related to each other and their squared difference becomes equal to the average variance of all samples. The line becomes flat and is called a Sill. The area or neighbourhood where locations are related to one another is called the Range (Ao). This is the greatest distance over which the value at a point on the surface is related to the value at another point. For distances less than the Range, the estimate of $\gamma(h)$ is less than the Sill. Beyond the Sill the samples can be considered to be spatially independent. For instance, at the sill there may be a transition from forest to grassland. The Sill is the total amount of variance over the sampled lag distance and is composed of two components, the "nugget variance" (Co) and the "structural variance" (C). The nugget effect shows the pure random variation in population density (white noise) in distances shorter than the sampling interval or it may be associated with sampling error. Is the expected variance when two samples are separated by a zero distance (the smallest measurement). The remainder of the Sill, not defined by the Nugget, defined by C describes the amount of variance explained by the model (Turner *et al.*, 1990; Leduc *et al.*, 1994; Crist, 1998; Robinson, 2000; Golaszewski, 2002; Gs+ Notebook, 2004; Anderson *et al.*, 2004)

A

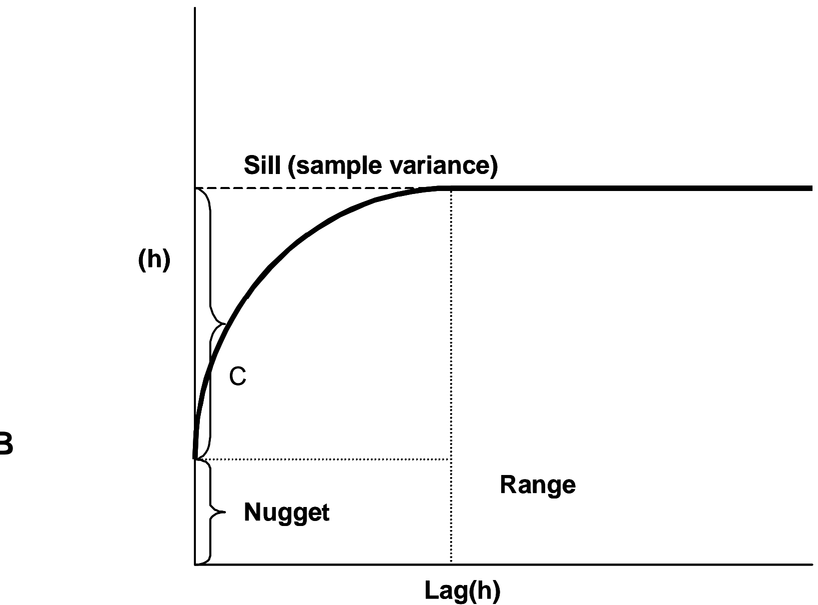


Figure A3.2— Generalized semivariogram. The *Nugget* is the value of the semivariance for which h=0; the maximum value of the semivariance is called the *Sill*; and the *Range* is the distance, Lag(h), at which the *sill* is reached, C is the Sill minus Nugget and *Lag interval* is distance class between neighbors (Robinson, 2000)

With the Nuggett, C, Range, and model equation type defined, the spatial statistics of the site are defined and the data can be kriged in a geostatistical program like GS+ (**Figure A3.3 and A3.4**)

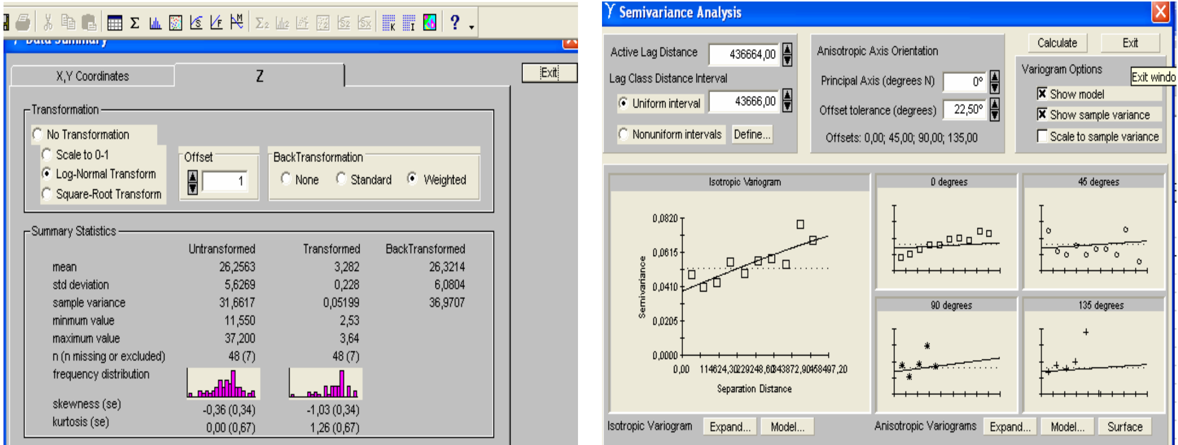


Figure A3.3 – Semivariance analysis obtained with GS+ 5.0 program; A – Variable transformation, B – Semivariance Analysis

Anysotropy is a characteristic of a random process that shows higher autocorrelation in one direction than another. An isotropic model reaches the level at which the semivariogram levels off (sill) at the same distance all distances. In an anysotropic model, some directions reach the sill more rapidly than others.

One important limitation of kriging is that it does not model variation at a scale smaller than the lag interval, often determined by the average spacing between neighbouring samples. Important local variation can be masked by this technique (Nicholson & Mather, 1996). But since kriging is a robust interpolator (Zhengquan *et al.,* 1997), even a naïve selection of parameters will provide an estimate comparable to many other grid estimation procedures.

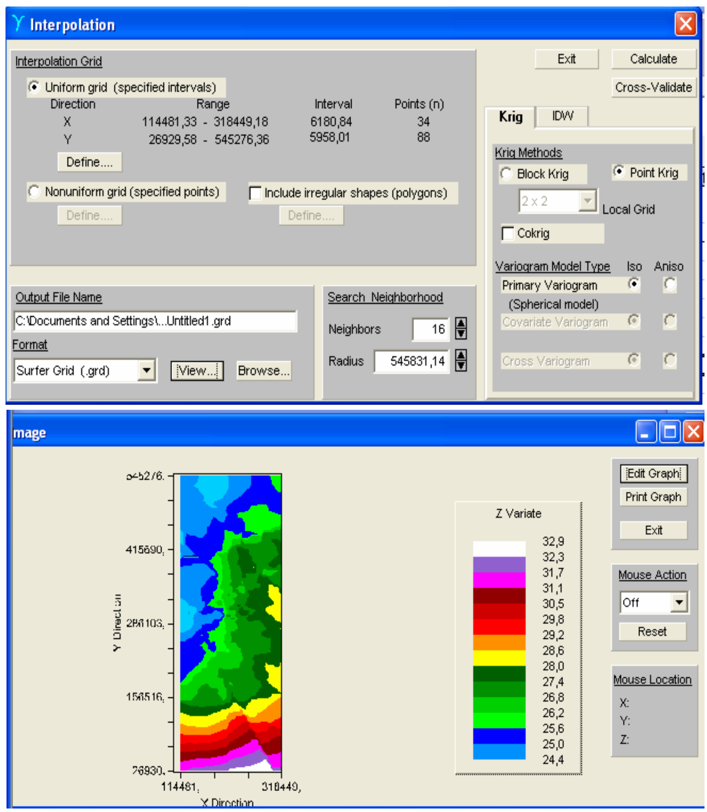


Figure A3.4 - Interpolation Window for semivariance analysis, with an example of a surface (grid) originated by kriging.

When this only variable is underestimated, more variables can be used to minimize the estimation error. For instance, variables as temperature and NDVI value (normalized derived vegetation index) are more frequently sampled than variable tick presence/absence (Estrada-Pena, 1998). For obtaining information about the "tick variable", cross-correlation with the "variable climate" can be achieved with a cokriging system. Cokriging provides the best predictive model in detecting missing values of a response variable when there are several explanatory variables, in opposite with kriging that only uses the idea that locations close together seem to be more similar (Cressie, 1991 *cited in* Estrada-Pena, 1998). This methodology was used by Estrada-Pena (1998) to estimate the distribution of *I. scapularis* based on two vegetation (standard NVDI) and four temperatures variables remotely sensed.

In cokriging, models for cross-covariance have to be developed. Cross-variance is the statistical tendency of variables of different types to vary in ways that are related to each other. Positive cross-

variance occurs when both variables tend to be above their respective means together, and negative cross-variance occurs if one variable tends to be above its mean when the other variable is below its mean (ESRI®).

A simple measure of spatial autocorrelation relies on standard significance tests for the null hypothesis that data are randomly distributed. One such test is Moran's *I*, expressed as follows:

$$I = \frac{n}{\Sigma \Sigma W_{ij}} \times \frac{\Sigma \Sigma W_{ij}(X_i - X)(X_j - X)}{\Sigma (X_i - X)^2}$$

(n - number of regions (*e.g.* counties), X_i and X_j – values of the variable of interest (*e.g.* number of human cases, tick distribution, degree of vegetation cover) at points *i* and *j* (centroids of counties), W_{ij} – weight based on the distance between county centroids)

This coefficient measures the degree of similarity between each areal unit and its contiguous neighbours, *i.e.*, it measures spatial autocorrelation as a function of distance between the centroids and simultaneously considers all locations a distance *d* apart. The inverse of the distances between county centroids to assign highest weight to pairs of neighbouring counties, and decreasing weights to associations of counties further apart can be used (Kitron & Kazmierczak, 1997; Robinson, 2000; Thomson & Connor, 2000; Glavanakov *et al*., 2001).

Spatial correlograms are here, a series of estimates of Moran's I, evaluated at increasing distances. Moran's I ranges from –1 to +1, and equal 0 when there is no spatial autocorrelation (no effect of distance on the distribution of a variable). The further the points are from value 0 (no spatial autocorrelation), the more the estimates of Moran's I differ from the null hypothesis of no spatial autocorrelation. Increased positive or negative values indicate a stronger spatial component, *i.e.*, positive or negative effect of distance on the variable distribution (Kitron & Kazmierczak, 1997; Robinson, 2000; Glavanakov *et al*., 2001).

Other authors used a classification tree methods in a GIS-generated database to determine on a mesoscale the habitat determinants of risk of exposure to Lyme disease. This approach was used to analyze the structure of the relationships among tick density, tick prevalence of infection, and a series of critical ecological variables (Merler *et al*., 1996; Rizzoli *et al*., 2003). The model is appropriate for summarizing large multivariate data sets described by a mix of continuous and categorical, ordered and unordered variables. It can predict the presence/absence of *I. ricinus* allowing the ranking of variables in terms of their potential effect on the classification (Merler *et al*., 1996).

A3.3 LANDSCAPE ANALYSIS

FRAGSTATS 3.3 software (**Figure A3.5**) is a free spatial pattern analysis program for categorical maps that quantifies the areal extent and spatial configuration of patches (building blocks for categorical maps) within a landscape (landscape metrics) (FRAGSTATS). Landscape metrics describe the spatial structure of a landscape at a set point in time and provide information about the contents of the mosaic or the shape of the component landscape elements (Rainis, 2003). They may be defined at three levels: a) patch-level metrics (characterize the spatial character and context of individual patches, *e.g.* size, edges); b) class-level metrics (integrate all the patches of a given type representing the amount and spatial distribution of a single patch type and may be interpreted as fragmentations indices); c) landscape-level metrics (integrates all patch types or classes over the full extent of the data, *i.e.*, the entire landscape and may be interpreted as landscapes heterogeneity indices).

Each run of *FRAGSTATS* produced some indexes like Number of patches (NP), Landscape contagion index (LC) and Patch richness (PR) (**Figure A3.6**).

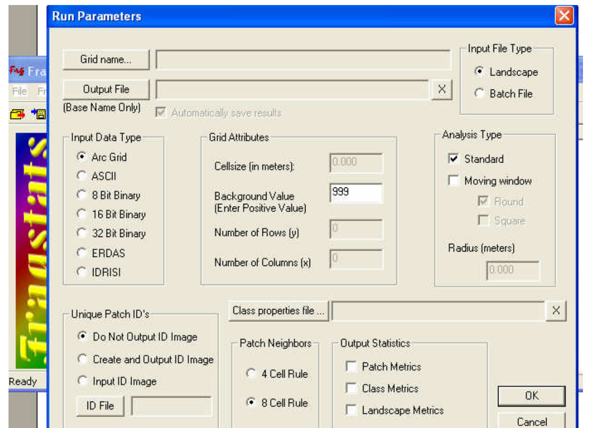


Figure A3.5– Preview of a Run Parameters file from FRAGSTATS 3.3

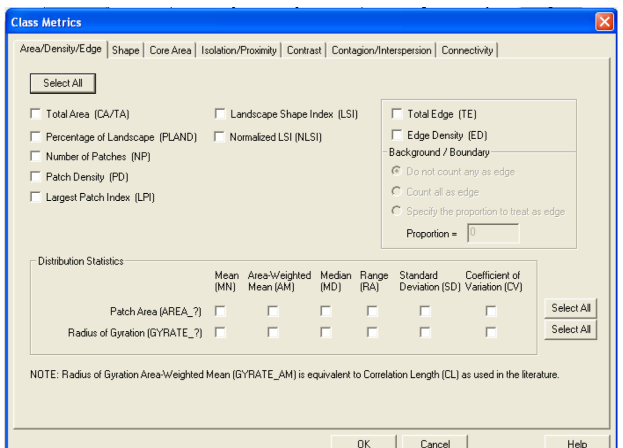


Figure A3.6 – Preview of available class metrics present in *FRAGSTATS* 3.3 program

PATCH ANALYST is a free ArcView extension that uses a simple front page of FRAGSTATS (**Figure A3.7**)

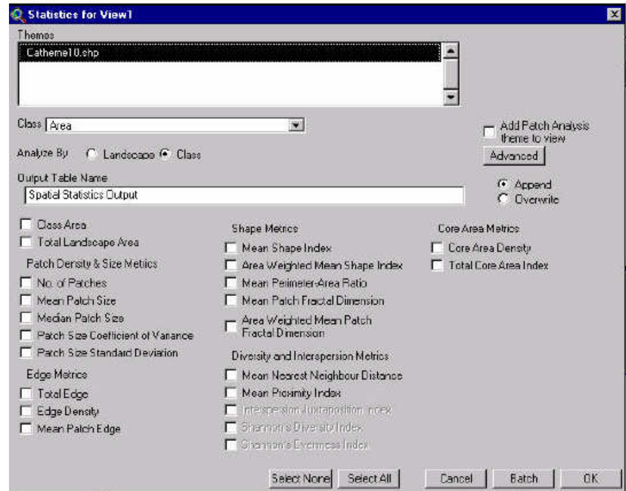


Figure A3.7– Preview of available class metrics present in PATCH ANALYST 3.3 program

Table A3.2 – *Patch Analyst* metrics definitions

Metrics	Metric Definition	Vector Theme	Grid Theme
AREA METRICS			
Class Area (CA)	Sum of areas of all patches belonging to a given class	X	X
Total Landscape area (TLA)	Sum of areas of all patches in the landscape	X	X
PATCH DENSITY & SIZE METRICS			
No. of Patches (NumP)	Total number of patches in the landscape if 'Analyze by Landscape' is selected, or Number of Patches for each individual class, if 'Analyze by Class' is selected	X	X
Mean Size Patch (MPS)	Average patch size. Indicator for the grain of landscape	X	X
EDGE METRICS			
Total Edge (TE)	Perimeter of patches	X	X
Edge Density (ED)	Amount of edge relative to the landscape area	X	X
SHAPE METRICS			
Mean Shape Index (MSI)	Shape Complexity. Value 1 when all patches are circular (polygons) or square (grids); <1 shape become simpler	X	X
Mean Patch Fractal Dimension (MPFD)	Shape Complexity. ≈1 for shapes with simple perimeters and ≈2 for shapes more complex	X	
DIVERSITY & INTERSPERSION METRICS			
Mean Nearest Neighbour Distance (MNN)	Measure of patch isolation. Distance between an individual patch to the nearest neighbouring patch of similar type (edge-to-edge). Smaller values indicate that patches of similar type are close or clustered together, and larger otherwise		
Mean Proximity Index (MPI)	Measure of the degree of isolation and fragmentation. =0 if all patches of the corresponding land use type have no neighbours of the same type with specified search radius. Increases as polygons becomes less isolated and the land use type becomes less fragmented in distribution		
Interspersion/Juxtaposition Index (IJI)	Measure of patch adjacency. Higher values (≈100) result where patch types are well interspersed (equally adjacent to each other); Lower values (≈0) characterise the opposite		
Shannons Diversity Index (SDI)	Measure of relative patch diversity. The index will equal zero when there is only one patch in the landscape and increases as the number of patch types or proportional distribution of patch types increases	X	X
Shannons Eveness Index (SEI)	Measure of patch distribution and abundance. equal to zero when the observed patch distribution is low and approaches one when the distribution of patch types becomes more even.	X	X

A3.4 LOGISTIC REGRESSION

General regression models are used to predict one variable from one or more other variables. In multiple regression, the dependent variable is predicted based on linear combinations of interval, dichotomous, or dummy independent variables. Multiple regression can establish that a set of independent variables explains a proportion of the variance in a dependent variable at a significant variable at a significant level (significance test of R^2), and can establish the relative predictive importance of the independent variables (comparing beta weights).

The multiple regression equation takes the form:

$$y = b_1x_1 + b_2x_2 + \ldots + b_nx_n + c.$$

The b's are the regression coefficients, representing the amount the dependent variable y changes when the independent changes 1 unit. The *c* is the constant, where the regression line intercepts the *y* axis, representing the amount the dependent y will be when all the independent variables are 0. The standardized version of the *b* coefficients are the beta weights, and the ratio of the beta coefficients is the ratio of the relative predictive power of the independent variables. Associated with multiple regression is R^2, multiple correlation, which is the percent of variance in the dependent variable explained collectively by all of the independent variables.

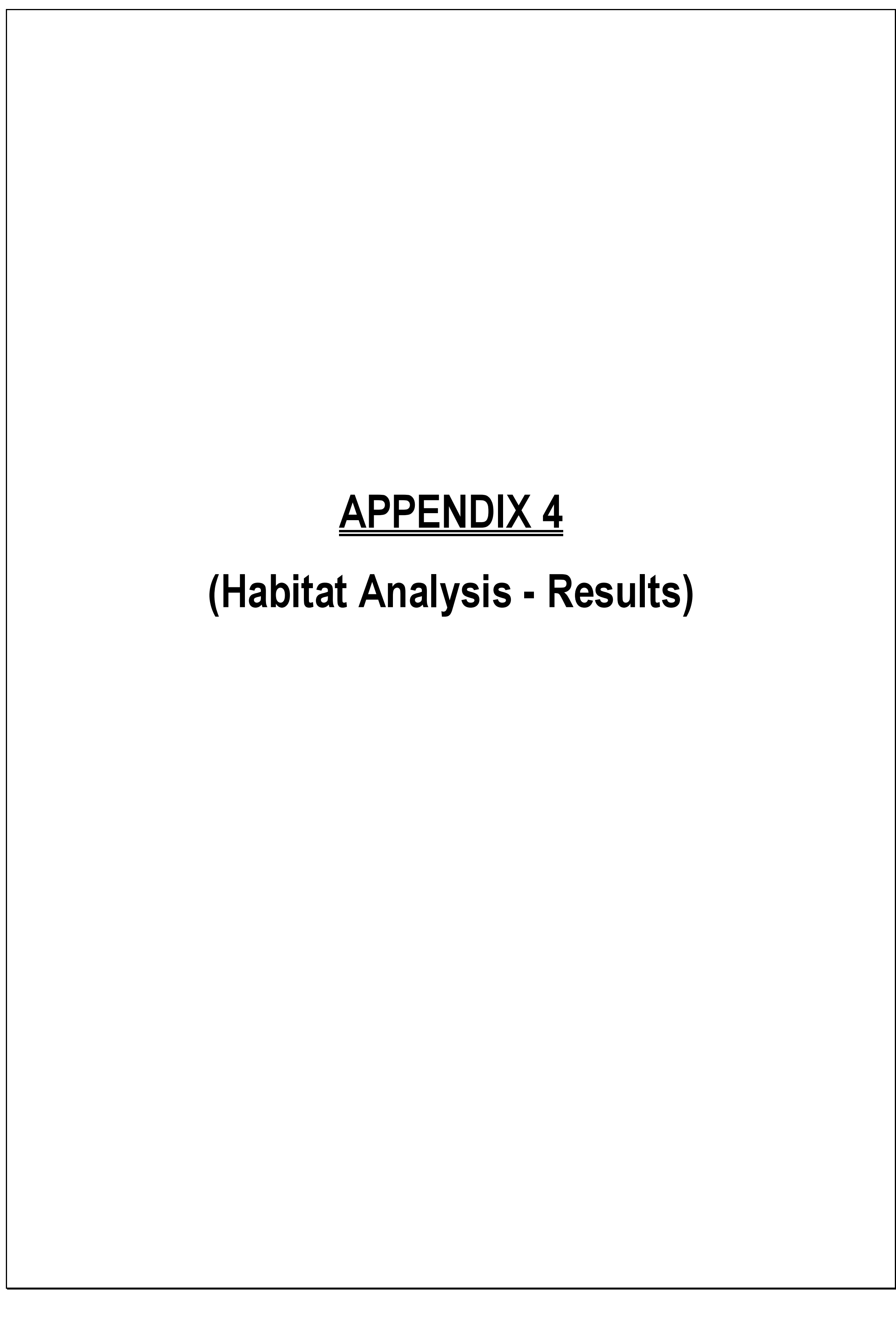
Generalized linear models (GLM) are used to model data with non-normally distributed errors. One example of a GLM is the logistic regression model where the dependent variable is dichotomous (0 or 1, presence/absence) as a function of a series of independent variables that can be either continuous or categorical (Thomson & Connor, 2000). Multiple regression models used to predict the spatial distribution of species are commonly limited to binary data regimes (e.g. presence/absence data sets) that have a specific and consistent sampling strategy, as they give the most interpretable and meaningful results (Guisan & Zimmerman, 2000; Zaniewski *et al*., 2002; Hirzel & Guisan, 2002)

The inclusion of categorical predictor variables gives logistic regression an advantage over standard multiple regression and discriminant analysis, though the other methods are more efficient for continuous data (Robinson, 2000). In instances where the independent variables are a categorical, or a mix of continuous and categorical, logistic regression is preferred.

Just like linear regression, logistic regression gives each regressor a coefficient b_1 which measures the regressor's independent contribution to variations in the dependent variable. It measures the probability

(*p*) that it is 1 rather than 0, by making a logistic tranformation of p, also called taking the logit of *p*. Logit(*p*) is the log (to base *e*) of the odds or likelihood ratio that the dependent variable 1. Logistic regression involves fitting to the data an equation of the form:

$$Logit(p) = a + b_1x_1 + b_2x_2 + b_3x_3 + \ldots$$



APPENDIX 4

(Habitat Analysis - Results)

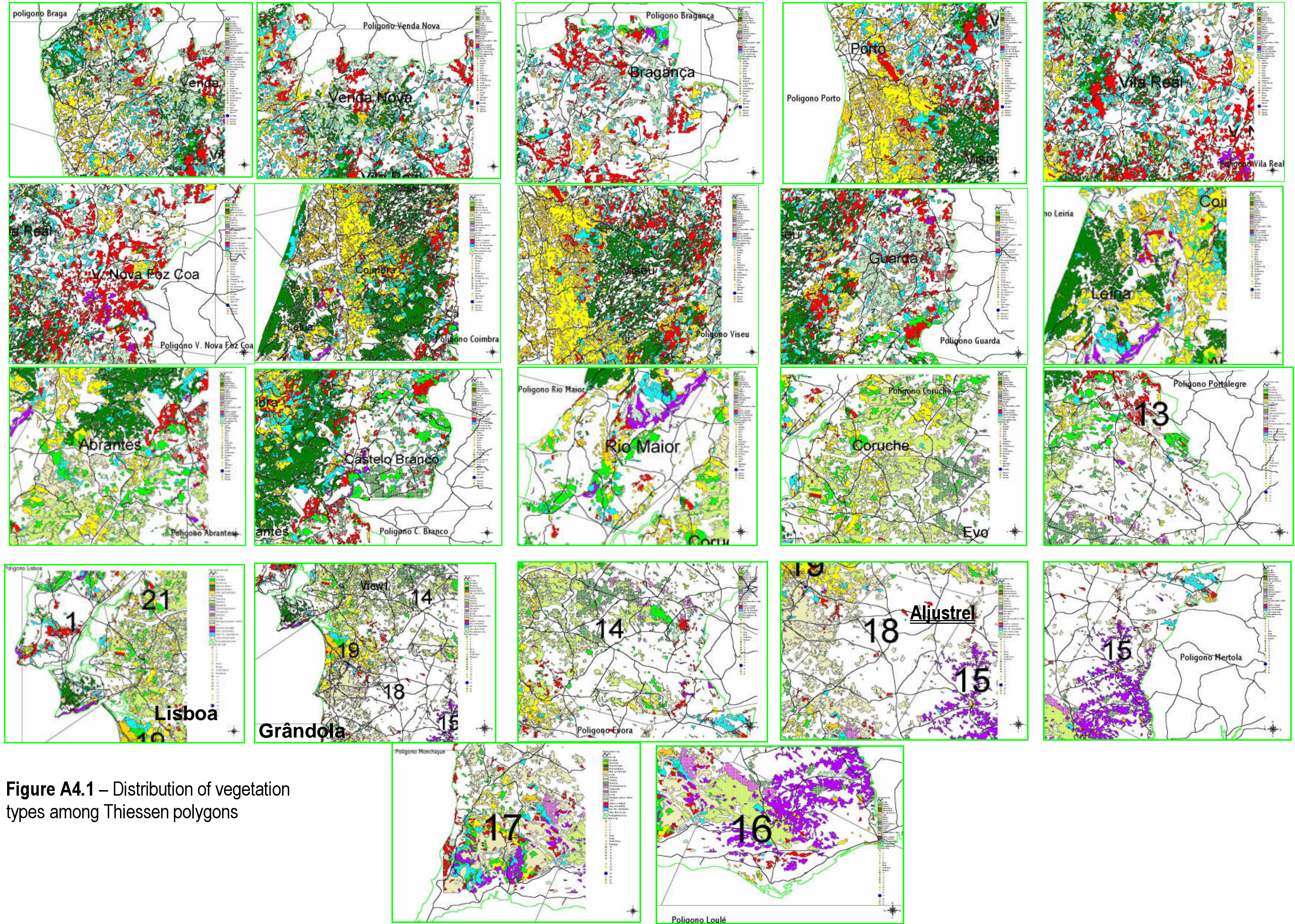


Figure A4.1 – Distribution of vegetation types among Thiessen polygons

Figure A4.2 – Field book

Utilização humana
1 - Floresta abandonada. ☐
2 - Zona caça. ☐
3 - Limite de zona agrícola. ☐
4 - Limite habitação. ☐
5 - Jardim público/Parque merendas ☐
6 - Outra ________ ☐

Carraça

Espécies________

Densidade *Ix. ricinus* (por cada 30")
larvas________
ninfas________
fêmeas________
machos________

Hospedeiros

Espécies________

ASSIN: ________

LOCAIS DE AMOSTRAGEM *IXODES RICINUS* (DOUTORAMENTO)

Data________ **Hora** início ________ fim ________

Local (descrição pormenorizada: Km, nº estrada, pontos cardeais, pontos de referência (edifícios, árvores), população mais próxima)
____

Vegetação
1 - Coníferas com estrutura homogénea/heterogénea. Com pouca ou ausência de vegetação herbácea. ☐
2 - Coníferas com alguma vegetação secundária e vegetação herbácea substancial. ☐
3 - Florestas mistas com prevalência de coníferas sobre caducas. ☐
4 - Arbustos caducos com mosaico de arbustos baixos e árvores novas. ☐
5 - Florestas de caducas com estrutura homogénea. ☐
6 - Florestas mistas (dominada pelas caducas) e florestas de caducas, ambas caracterizadas por estrutura altamente heterogénea e muitos ecótonos. ☐

Solo
1 - boa drenagem. ☐
2 - razoável drenagem. ☐ Tipo________
3 - má drenagem. ☐

Exposição
1 - muita (espaço aberto). ☐
2 - razoável (alguma sombra). ☐
3 - fraca (muita sombra). ☐

Temperatura Ar________ Solo________
Humidade Ar________ Solo________

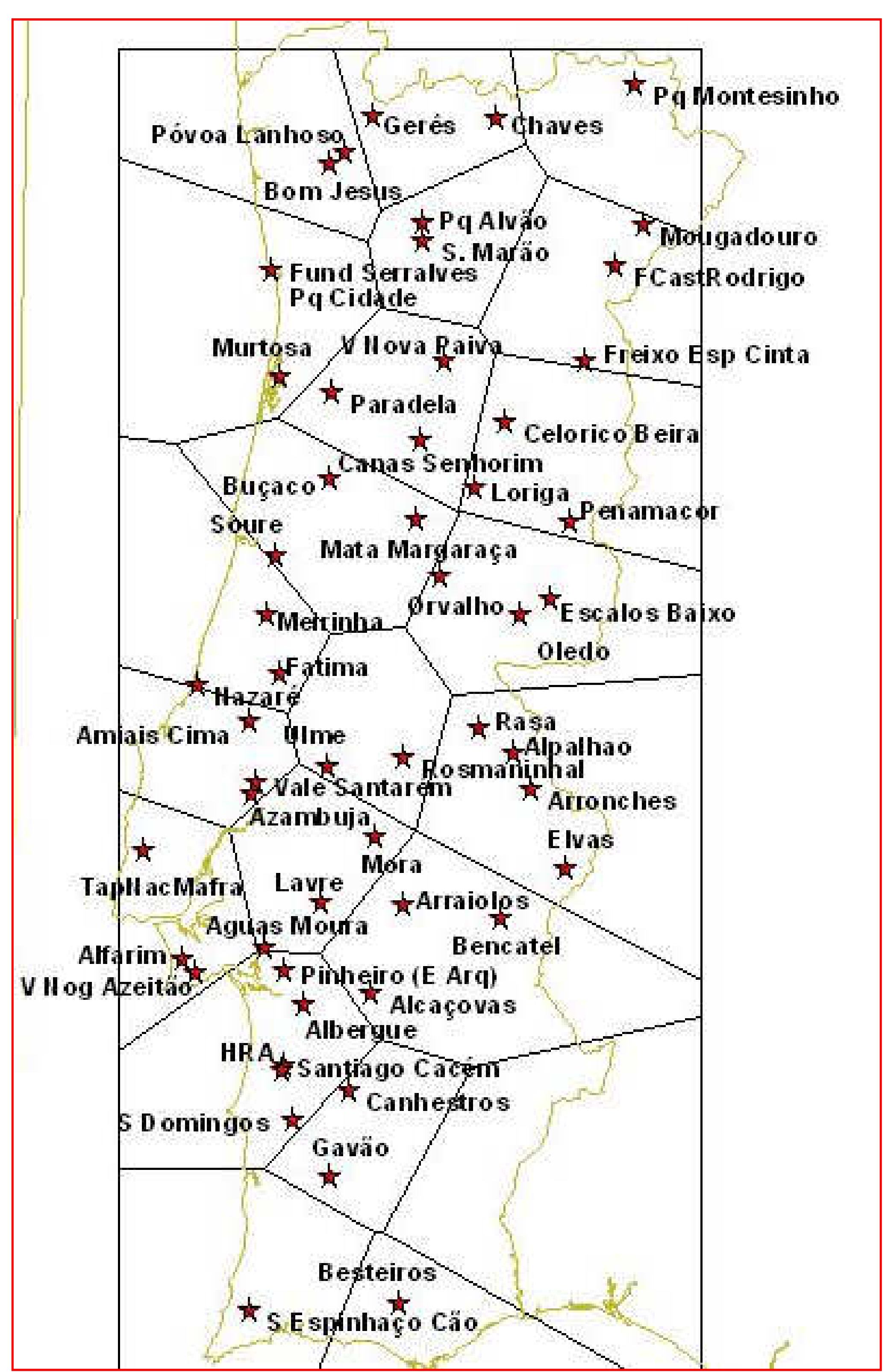


Figure A4.3– Distribution of the 55 sample points in the nationwide study.

Table A4.1 – Characterization of nationwide sample points (legend: TEMPAIR – air temperature, TEMPSOIL – soil temp., HUMAIR – air humidity, HUMSOIL – soil hum., VEG – vegetation types, SOILDR – soil drainage, EXPOS – exposition, HUMUSE – human use, TICKS – presence(1)/absence(0))

LOCAL	LOCAL_NAME	POLYGON	LATITUDE	LONGITUDE	NORTHING	EASTING	Nº VISITS
Site01	Alfarim	Lisboa	38,54116	-9,11276	175501,70	114481,33	3
Site02	V Nog Azeitão	Lisboa	38,54087	-9,11148	169780,43	119736,67	2
Site03	Pinheiro (E Arq)	Grândola	38,54961	-8,65398	170212,37	159582,97	2
Site04	Albergue	Grândola	38,36700	-8,50000	155776,55	167829,69	1
Site05	Santiago Cacém	Grândola	38,13375	-8,59257	129920,94	159610,43	2
Site06	S Domingos	Grândola	37,93142	-8,54890	107444,03	163338,59	1
Site07	Canhestros	Aljustrel	38,04012	-8,27000	119436,64	187876,93	2
Site08	Gavão	Aljustrel	37,71510	-8,35982	83375,79	179903,65	2
Site09	Alcaçovas	Évora	38,41100	-8,15398	160596,94	198071,97	2
Site10	Arraiolos	Évora	38,74831	-7,99046	198051,17	212296,80	2
Site11	Alpalhão	Portalegre	39,32928	-7,42263	262780,48	261159,30	2
Site12	Mora	Coruche	39,01386	-8,13492	227522,05	199738,95	2
Site13	Lavre	Coruche	38,76418	-8,41290	199840,96	175576,79	4
Site14	Fund Serralves	Porto	41,17038	-8,67957	467125,75	154038,86	1
Site15	Pq Cidade	Porto	41,17038	-8,67957	467125,75	154038,86	2
Site16	Póvoa Lanhoso	Braga	41,60080	-8,28160	516918,93	186067,71	3
Site17	Mougadouro	Bragança	41,33300	-6,71700	486008,07	318449,18	2
Site18	Vale Santarém	Rio Maior	39,21627	-8,74547	250173,15	147008,75	2
Site19	Azambuja	Rio Maior	39,20908	-8,74339	245175,06	145413,59	3
Site20	Nazaré	Leiria	39,59430	-9,04709	291734,11	120753,08	2
Site21	Soure	Coimbra	40,08013	-8,65041	346037,86	155772,64	3
Site22	Buçaco	Coimbra	40,37758	-8,37021	378965,78	179761,90	3
Site23	Mata Margaraça	Coimbra	40,21649	-7,91913	361072,23	218113,08	3
Site24	Paradela	Viseu	40,70284	-8,35661	415082,35	181008,96	2
Site25	V Nova Paiva	Viseu	40,82421	-7,77108	428599,18	230440,09	2
Site26	Canas Senhorim	Viseu	40,51824	-7,89969	394584,22	219680,00	2
Site27	Celorico Beira	Guarda	40,58141	-7,45237	402071,52	257580,34	2
Site28	Penamacor	Guarda	40,20241	-7,12493	359973,41	285739,76	2
Site29	Oledo	C. Branco	39,59433	-9,04711	276181,67	144232,59	2
Site30	Ulme	Abrantes	39,27958	-8,37881	257051,71	178695,05	1
Site31	S Espinhaço Cão	Monchique	37,20505	-8,76591	26929,58	143715,33	1
Site32	Besteiros	Loulé	37,23300	-8,01700	29849,49	210197,14	1
Site33	Murtosa	Porto	40,76141	-8,63035	421681,91	157910,47	2
Site34	Bom Jesus	Braga	41,55666	-8,37774	511587,89	179155,63	2
Site35	Gerês	Venda Nova	41,75249	-8,14454	531633,92	198949,17	1
Site36	Chaves	Venda Nova	41,70459	-7,49887	531419,52	253393,68	2
Site37	S. Marão	Vila Real	41,28073	-7,88180	479266,96	220954,14	1
Site38	Loriga	C. Branco	40,28605	-7,70010	374369,80	243450,15	2
Site39	Elvas	Portalegre	38,88300	-7,16700	213436,88	283728,43	1
Site40	Amiais Cima	Rio Maior	39,85787	-8,70055	321384,64	151338,43	1
Site41	Rosmaninhal	Abrantes	39,45263	-8,75622	260643,77	212528,71	1
Site42	Meirinha	Leiria	39,62997	-8,63025	296044,18	157213,64	1
Site43	Fatima	Leiria	39,42112	-7,60388	272870,21	245470,79	1
Site44	Rasa	Portalegre	39,32926	-7,42261	246828,62	268477,27	1
Site45	Arronches	Portalegre	38,70058	-7,49905	192933,12	255055,06	1
Site46	Bencatel	Évora	39,87235	-7,42848	321380,35	263571,29	1
Site47	Escalos Baixo	C. Branco	40,25555	-7,71827	337685,56	228076,09	1
Site48	Orvalho	C. Branco	38,76448	-8,41272	328172,41	277125,86	1
Site49	FCastRodrigo	V.N.FozCoa	40,58195	-7,45231	468375,67	306509,61	1
Site50	Freixo Esp Cinta	V. N.FozCoa	40,81847	-7,03909	428473,86	292201,09	1
Site51	Pq Montesinho	Bragança	41,86700	-6,75000	545276,36	314736,54	1
Site52	Pq Alvão	Vila Real	41,35000	-7,88300	486960,04	220831,54	1
Site53	TapNacMafra	Lisboa	38,95000	-9,31700	221100,41	97261,18	3
Site54	HRA	Grandola	38,11700	-8,60000	128064,90	158949,61	2
Site55	Aguas Moura	Coruche	38,58300	-8,70000	179843,30	150498,11	2

Table A4.1 – cont.

LOCAL	TEMPAIR	TEMPSOIL	HUMAIR	HUMSOIL	VEG	SOILDR	EXPOS	HUMUSE	TICKS
Site01	22,15	23,6	43,7	46,15	2	1	2	2	1
Site02	24,9	24,3	37,85	50,05	5	1	2	4	1
Site03	28,65	28,1	34,15	46	5	1	2	2	1
Site04	28,45	29	30,25	38,5	5	1	2	1	1
Site05	25,8	26,15	32	51,5	6	3	2	1	1
Site06	23,8	24,5	43,9	62,15	5	1	2	3	0
Site07	27,65	28,9	28	41,6	5	2	2	1	1
Site08	26,95	28,1	27,15	42,2	5	2	2	1	1
Site09	17,2	19,55	53,15	55,1	5	2	2	2	1
Site10	26,25	28,55	34,25	38,15	7	1	1	2	1
Site11	14,4	15,6	40,4	70,1	4	3	1	2	0
Site12	23,3	24,35	23,3	43,4	5	2	2	1	0
Site13	25,7	26,85	23,65	45,95	6	1	2	1	1
Site14	n.d	n.d	n.d	n.d	6	1	2	5	0
Site15	n.d	n.d	n.d	n.d	3	1	2	5	0
Site16	11,8	11,55	58,75	71,15	5	1	2	1	1
Site17	16,4	16,1	31,1	49,15	5	2	3	1	0
Site18	24,65	26,1	42,8	51,55	1	2	2	2	1
Site19	23,95	25,15	46,15	47,4	3	1	2	1	1
Site20	17,05	16,8	67,5	68,45	1	1	2	1	0
Site21	22,9	25,95	50,6	48,8	2	2	3	1	1
Site22	18,05	17,8	59,55	64,3	6	1	3	6	1
Site23	22,7	22	52,6	64,3	5	1	3	6	1
Site24	17,85	18,45	64,4	66,5	2	2	2	1	0
Site25	25	25,45	21,4	28,45	2	2	2	1	1
Site26	38,7	37,2	17	36,1	2	2	2	1	0
Site27	21,2	21,55	42,55	72,25	5	1	2	2	0
Site28	23,65	23,75	34,95	47,4	4	2	1	6	0
Site29	21,6	21,4	34,5	63,4	6	1	2	4	0
Site30	23,3	29	36,6	32	5	1	2	1	1
Site31	33,2	36,5	22,9	22,8	5	3	1	2	1
Site32	34,3	36,55	9,4	9	5	2	2	4	0
Site33	28,5	28,4	40,9	42,85	2	2	3	1	1
Site34	32,3	32,4	35,65	37,3	6	1	3	1	0
Site35	n.d	n.d	n.d	n.d	6	1	2	1	0
Site36	21,7	21,95	58,25	66,55	4	1	1	3	1
Site37	29,5	28,6	35,1	55,9	3	1	2	1	0
Site38	28,55	29,9	32,6	50,75	6	2	2	1	1
Site39	n.d	n.d	n.d	n.d	5	0	0	3	1
Site40	20,95	24,05	34	54,35	3	2	1	1	1
Site41	21,5	22,15	28,8	40,7	5	2	2	1	1
Site42	25	26,95	42,75	43,25	2	1	3	1	0
Site43	26,7	27,85	41,75	41,3	5	1	2	4	1
Site44	33,35	33,8	18,95	25,75	3	1	2	3	1
Site45	28,15	29,55	28,35	37,55	6	2	2	1	1
Site46	29,35	29,95	25,15	28,85	2	3	2	1	1
Site47	32,25	33,05	20,9	34,3	6	2	2	2	1
Site48	31,3	32,1	26,7	49,85	5	2	2	3	0
Site49	31,95	32,15	18,9	26,2	6	2	2	4	0
Site50	29,55	29,95	19,05	20,95	6	2	2	4	1
Site51	29,05	29,1	31,85	41,25	5	2	2	2	1
Site52	28,35	29,55	34,35	46	3	1	2	1	0
Site53	12,9		73		6	2	2	5	1
Site54	n.d	n.d	n.d	n.d	4	0	2	2	1
Site55	n.d	n.d	n.d	n.d	5	2	2	2	1

Table A4.2 – Recoding of Corine Land Cover map (www.igeo.pt) variables

CODE CORINE	Description	NEW CODE
1 - 11	***Zones dominated by human residences***	
1110	Continuous urban area	**1**
1120	Discontinuous urban area	**1**
12	Zones with artificial cover	**1**
1210	Industries, Business and General Equipments	**1**
1220	Highways and Train network	**1**
1230	Harbours	**1**
1240	Airports	**1**
13	Zones artificially altered, without vegetation	**1**
1310	Quarry, Sand extraction, open mines	**1**
1320	Industrial and Trash disposal areas	**1**
1330	Shipyards	**1**
14	Ordered Green areas	**1**
1410	Urban green spaces	**1**
1420	Sports equipment and leisure areas	**1**
2	***Agricultural Territory***	
21	Annual cultures	**2**
2110	Agricultural areas outside forest edge	**2**
2120	Irrigation areas	**2**
2130	Rice cultures	**2**
22	Permanent cultures	**2**
2210	Wineyard	**2**
	2211 – Wineyard + orchard	**2**
	2212 – Wineyard + olive-grove	**2**
2220	Orchards	**2**
	2221 – Orchard + wineyard	**2**
	2222 – Orchard + olive-grove	**2**
2230	Olive-grove	**2**
	2231 – Olive-grove + wineyard	**2**
	2232 – Olive-grove + orchard	**2**
23	Grasslands	**2**
2310	Grasslands	**2**
24	Heterogeneous agricultural areas	**2**
2410	Annual cultures with permanent cultures	**2**

2420	Complex partial and cultural systems	**2**
2430	Agricultural areas with important natural areas	**2**
2440	Agro forested areas	**2**
3	***Forests and Semi-Natural Territory***	
31	Forests	**3**
3110	Deciduous	**3**
	3111 – Cork-oak	**3**
	3112 – Holm-oak	**3**
	3113 – Cork/holm oaks	**3**
	3114 – Chestnut tree	**3**
	3115 - Oak	**3**
	3116 - Eucalyptus	**36**
3120	Evergreen	**4**
	3121 – *Pinus pinus* (wild pinus)	**4**
	3122 – *Pinus pinaster* (“gentle” pinus)	**4**
3130	Forests with mixture of several species	**5**
32	Shrub/bush areas	**6**
3210	Poor grassland, trails	**6**
3220	Acorn and thicket	**6**
3230	Sclerofitic vegetation	**6**
3240	Corrupted forest spaces	**6**
33	Open areas without or with few vegetation	**6**
3310	Beach, sand dunes, sandy areas and soils without vegetation	**9**
3320	Rocks	**9**
3330	Sub-deserted areas	**9**
3340	Recent burned areas	**9**
3350	Eternal snow areas	**9**
4	***Aquatic Territory***	
41	Continental wetlands	**7**
4110	Swamped areas	**7**
4120	Peat areas	**7**
42	Sea wetlands	**7**
4210	Marsh	**9**
4220	Salt production areas	**9**
4230	Coastal areas	**9**
5	***Water cover***	**8**

51	Sweat water	**8**
5110	Streams	**8**
5120	Lakes	**8**
52	Salt water	**8**
5210	Coastal lakes	**8**
5220	Estuary	**8**
5230	Sea and Ocean	**8**

Table A4.3 – Reclassification (coding) of the GIS-based variables obtained for nationwide sample sites.

Variable	Description	Code (numeric)
Temperature	Less than 7.5ºC	1
	Between 7.5 to 12.5ºC	2
	Between 12.5 to 15.0ºC	3
	Between 15.0 to 17.5ºC	4
	More than 17.5ºC	5
Humidity	Very dry (less than 55%)	1
	Dry (between 55 to 75%)	2
	Humid (between 75 to 90%)	3
	Very humid (>90%)	4
Precipitation	Desertic (less than 125 mm)	1
	Arid (between 125 to 250 mm)	2
	Semiarid (between 250 to 500 mm)	3
	Moderately rainy (between 500 to 1000 mm)	4
	Rainy (between 1000 to 2000 mm)	5
	Excessively rainy (more than 2000 mm)	6
Insolation	Less than 1800 h	1
	Between 1800 to 2000 h	2
	Between 2000 to 2200 h	3
	Between 2200 to 2400 h	4
	Between 2400 to 2600 h	5
	Between 2600 to 2800 h	6
	Between 2800 to 3000 h	7
	More than 3000 h	8
Landscapes	Urban areas	1
	Agricultural areas/ Shrubs-bush areas	2
	Deciduous forests	3
	Evergreen forests	4
	Mixed forests	5
	Wetlands	6
	Eucalyptus forest	7
Soil type	Podzois – sands and sandstones and rarely clayish	1
	Cambissoils – sands and sandstones; thin soils and well drained	2
	Litossoils – schists and grauvaques; soil above the rock	3
	Luvissoils – clay, sandstones, gravelbed,	4

	quartzidorites	
	Fluvissoils – soils of marine or fluvial sediments	5
	Regossoils – sands and sandstones	6
	Rankers	7
Soil pH	Extremely acid (less or equal to 4.5)	1
	Very acid (between 4.6 to 5.5)	2
	Moderately acid (between 5.6 to 6.5)	3
	Neuter (between 6.6 to 7.3)	4
	Moderately alkaline (between 7.4 to 8.5)	5
Litology	Sedimentary formations	1
	Sedimentary and metamorphic formations	2
	Vulcanic/Plutonic eruptive rocks	3

Table A4.4– Classification of nationwide sites with GIS-based variables.

LOCAL	LOCAL_NAME	POLYGON	TEMPERATURA	CODTEMP	PRECIPITAC	CODPREC	HUMIDADE	CODHUM	COUNTY
Site01	Alfarim	Lisboa	Between 15.0 and 16.0 C	4	Between 600 and 700 mm	4	Between 75 and 80%	3	Sesimbra
Site02	V Nog Azeitão	Lisboa	Between 12.5 and 15.0 C	3	Between 700 and 800 mm	4	Between 75 and 80%	3	Sesimbra
Site03	Pinheiro (E Arq)	Grândola	Between 16.0 and 17.5 C	4	Between 600 and 700 mm	4	Between 70 and 75%	2	Álcacer do Sal
Site04	Albergue	Grândola	Between 16.0 and 17.5 C	4	Between 500 and 600 mm	4	Between 75 and 80%	3	Álcacer do Sal
Site05	Santiago Cacém	Grândola	Between 16.0 and 17.5 C	4	Between 700 and 800 mm	4	Between 80 and 85%	3	Grândola
Site06	S Domingos	Grândola	Between 15.0 and 16.0 C	4	Between 500 and 600 mm	4	Between 80 and 85%	3	Santiago do Cacém
Site07	Canhestros	Aljustrel	Between 15.0 and 16.0 C	4	Between 400 and 500 mm	3	Between 75 and 80%	3	Ferreira do Alentejo
Site08	Gavão	Aljustrel	Between 15.0 and 16.0 C	4	Between 500 and 600 mm	4	Between 75 and 80%	3	Ourique
Site09	Alcaçovas	Évora	Between 15.0 and 16.0 C	4	Between 700 and 800 mm	4	Between 70 and 75%	2	Viana do Alentejo
Site10	Arraiolos	Évora	Between 15.0 and 16.0 C	4	Between 700 and 800 mm	4	Between 65 and 70%	2	Arraiolos
Site11	Alpalhão	Portalegre	Between 12.5 and 15.0 C	3	Between 700 and 800 mm	4	Between 75 and 80%	3	Nisa
Site12	Mora	Coruche	Between 15.0 and 16.0 C	4	Between 600 and 700 mm	4	Between 65 and 70%	2	Mora
Site13	Lavre	Coruche	Between 15.0 and 16.0 C	4	Between 600 and 700 mm	4	Between 65 and 70%	2	Montemor-o-Novo
Site14	Fund Serralves	Porto	Between 12.5 and 15.0 C	3	Between 1000 and 1200 mm	5	Between 80 and 85%	3	Porto
Site15	Pq Cidade	Porto	Between 12.5 and 15.0 C	3	Between 1000 and 1200 mm	5	Between 80 and 85%	3	Porto
Site16	Póvoa Lanhoso	Braga	Between 10.0 and 12.5 C	2	Between 1600 and 2000 mm	5	Between 80 and 85%	3	Póvoa de Lanhoso
Site17	Mougadouro	Bragança	Between 12.5 and 15.0 C	3	Between 800 and 1000 mm	4	Between 65 and 70%	2	Mogadouro
Site18	Vale Santarém	Rio Maior	Between 16.0 and 17.5 C	4	Between 700 and 800 mm	4	Between 75 and 80%	3	Santarém
Site19	Azambuja	Rio Maior	Between 16.0 and 17.5 C	4	Between 600 and 700 mm	4	Between 75 and 80%	3	Cartaxo
Site20	Nazaré	Leiria	Between 12.5 and 15.0 C	3	Between 700 and 800 mm	4	Between 80 and 85%	3	Nazaré
Site21	Soure	Coimbra	Between 15.0 and 16.0 C	4	Between 700 and 800 mm	4	Between 75 and 80%	3	Soure
Site22	Buçaco	Coimbra	Between 15.0 ãnd 16.0 C	4	Between 1400 and 1600 mm	5	Between 70 and 75%	2	Mealhada
Site23	Mata Margaraça	Coimbra	Between 7.5 and 10.0 C	2	Between 1400 and 1600 mm	5	Between 70 and 75%	2	Arganil
Site24	Paradela	Viseu	Between 12.5 and 15.0 C	3	Between 1000 and 1200 mm	5	Between 75 and 80%	3	Sever do Vouga
Site25	V Nova Paiva	Viseu	Between 10.0 and 12.5 C	2	Between 1400 and 1600 mm	5	Between 70 and 75%	2	Vila Nova de Paiva
Site26	Canas Senhorim	Viseu	Between 12.5 and 15.0 C	3	Between 1000 and 1200 mm	5	Between 70 and 75%	2	Nelas
Site27	Celorico Beira	Guarda	Between 10.0 and 12.5 C	2	Between 1000 and 1200 mm	5	Between 70 and 75%	2	Celorico da Beira
Site28	Penamacor	Guarda	Between 12.5 and 15.0 C	3	Between 800 and 1000 mm	4	Between 65 and 70%	2	Penamacor
Site29	Oledo	C. Branco	Between 15.0 and 16.0 C	4	Between 600 and 700 mm	4	Between 65 and 70%	2	Idanha-a-Nova
Site30	Ulme	Abrantes	Between 16.0 and 17.5 C	4	Between 800 and 1000 mm	4	Between 75 and 80%	3	Chamusca
Site31	S Espinhaço Cão	Monchique	Between 15.0 and 16.0 C	4	Between 500 and 600 mm	4	Between 80 and 85%	3	Lagos
Site32	Besteiros	Loulé	Between 16.0 and 17.5 C	4	Between 700 and 800 mm	4	Between 65 and 70%	2	Loulé
Site33	Murtosa	Porto	Between 15.0 and 16.0 C	4	Between 800 and 1000 mm	4	Between 80 and 85%	3	Murtosa
Site34	Bom Jesus	Braga	Between 12.5 and 15.0 C	3	Between 1600 and 2000 mm	5	Between 80 and 85%	3	Braga
Site35	Gerês	Venda Nova	Between 10.0 and 12.5 C	2	More than 2800 mm	6	Between 75 and 80%	3	Terras de Bouro
Site36	Chaves	Venda Nova	Between 10.0 and 12.5 C	2	Between 600 and 700 mm	4	Between 70 and 75%	2	Chaves
Site37	S. Marão	Vila Real	Between 10.0 and 12.5 C	2	Between 1200 and 1400 mm	5	Between 75 and 80%	3	Vila Real
Site38	Loriga	C. Branco	Less than 7.5 C	1	Between 2000 and 2400 mm	6	Between 70 and 75%	2	Seia
Site39	Elvas	Portalegre	Between 15.0 and 16.0 C	4	Between 600 and 700 mm	4	Between 65 and 70%	2	Elvas
Site40	Amiais Cima	Rio Maior	Between 16.0 and 17.5 C	4	Between 1000 and 1200 mm	5	Between 75 and 80%	3	Santarém
Site41	Rosmaninhal	Abrantes	Between 15.0 and 16.0 C	4	Between 600 and 700 mm	4	Between 75 and 80%	3	Ponte de Sôr
Site42	Meirinha	Leiria	Between 15.0 and 16.0 C	4	Between 800 and 1000 mm	4	Between 75 and 80%	3	Pombal
Site43	Fatima	Leiria	Between 16.0 and 17.5 C	4	Between 1200 and 1400 mm	5	Between 70 and 75%	2	Vila Nova de Ourém
Site44	Rasa	Portalegre	Between 12.5 and 15.0 C	3	Between 800 and 1000 mm	4	Between 70 and 75%	2	Portalegre
Site45	Arronches	Portalegre	Between 16.0 and 17.5 C	4	Between 700 and 800 mm	4	Between 70 and 75%	2	Arronches
Site46	Bencatel	Évora	Between 15.0 and 16.0 C	4	Between 600 and 700 mm	4	Between 70 and 75%	2	Redondo
Site47	Escalos Baixo	C. Branco	Between 15.0 and 16.0 C	4	Between 700 and 800 mm	4	Between 65 and 70%	2	Castelo Branco
Site48	Orvalho	C. Branco	Between 7.5 and 10.0 C	2	Between 1200 and 1400 mm	5	Less than 65%	2	Oleiros
Site49	FCastRodrigo	V.N.FozCoa	Between 12.5 and 15.0 C	3	Between 800 and 1000 mm	4	Between 65 and 70%	2	Torre de Moncorvo
Site50	Freixo Esp Cinta	V. N.FozCoa	Between 10.0 and 12.5 C	2	Between 500 and 600 mm	4	Between 75 and 80%	3	Figueira de Castelo Rodrigo
Site51	Pq Montesinho	Bragança	Between 10.0 and 12.5 C	2	Between 1000 and 1200 mm	5	Between 70 and 75%	2	Bragança
Site52	Pq Alvão	Vila Real	Between 10.0 and 12.5 C	2	Between 1400 and 1600 mm	5	Between 80 and 85%	3	Mondim de Basto
Site53	TapNacMafra	Lisboa	Between 12.5 and 15.0 C	3	Between 700 and 800 mm	4	Between 75 and 80%	3	Mafra
Site54	HRA	Grandola	Between 16.0 and 17.5 C	4	Between 700 and 800 mm	4	Between 80 and 85%	3	Grândola
Site55	Aguas Moura	Coruche	Between 16.0 and 17.5 C	4	Between 600 and 700 mm	4	Between 70 and 75%	2	Palmela

Table A4.4 - cont.

LOCAL	LOCAL_NAME	INSOLACAO	CODINSOL	LANDSCAPES	CODELANDS	C. CURVE	SOIL NAME	CODNAME	COMP_LIT	CODCOMPLIT	SOIL Ph	CODPh
Site01	Alfarim	Between 2900 and 3000 h	7	Wild Pinus (P. pinaster)	4	50	PODZOIS	1	SEDIMENTARY FORM	1	between 5.6 and 6.5 (+4.6 to 5.5	3
Site02	V Nog Azeitão	Between 2800 and 2900 h	7	Complex sistems of cultures and parcels	2	100	CAMBISSOIS	2	SEDIMENTARY FORM	1	between 6.6 and 7.3 (+7.4 to 8.5	4
Site03	Pinheiro (E Arq)	Between 2900 and 3000 h	7	Oak trees	3	50	PODZOIS	1	SEDIMENTARY FORM	1	between 5.6 and 6.5 (+4.6 to 5.5	3
Site04	Albergue	Between 2900 and 3000 h	7	Discontinuous urban area	1	50	PODZOIS	1	SEDIMENTARY FORM	1	between 5.6 and 6.5 (+4.6 to 5.5	3
Site05	Santiago Cacém	Between 2900 and 3000 h	7	Deciduous trees	3	200	LITOSSOILS	3	SEDIMENTARY and METAMORPHIC FORM	2	between 5.6 and 6.5	3
Site06	S Domingos	Between 2900 and 3000 h	7	Deciduous trees	3	100	LUVISSOILS	4	SEDIMENTARY and METAMORPHIC FORM	2	between 5.6 and 6.5	3
Site07	Canhestros	Between 2900 and 3000 h	7	Deciduous trees	3	50	LUVISSOILS	4	SEDIMENTARY FORM	1	between 5.6 and 6.5 (+6.6 to 7.3	3
Site08	Gavião	Between 2900 and 3000 h	7	Annual cultures associated with permanent cultures	2	100	LUVISSOILS	4	SEDIMENTARY FORM	1	between 5.6 and 6.5	3
Site09	Alcaçovas	Between 2900 and 3000 h	7	Oak trees	3	200	LUVISSOILS	4	VULCANIC ERUPTIV ROCKS	3	between 5.6 and 6.5	3
Site10	Arraiolos	Between 2900 and 3000 h	7	Oak trees	3	300	LUVISSOILS	4	SEDIMENTARY and METAMORPHIC FORM	2	between 6.6 and 7.3 (+5.6 to 6.5	4
Site11	Alpalhão	Between 2500 and 2600 h	5	Complex sistems of cultures and parcels	2	300	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site12	Mora	Between 2800 and 2900 h	7	Oak trees	3	100	PODZOIS	1	SEDIMENTARY FORM	1	between 4.6 and 5.5 (+5.6 to 6.5	2
Site13	Lavre	Between 2700 and 2800 h	6	Forest with mixture of tree species	5	100	PODZOIS	1	SEDIMENTARY FORM	1	between 4.6 and 5.5 (+5.6 to 6.5	2
Site14	Fund Serralves	Between 2600 and 2700 h	6	Wasteland and brushwood	2	0	CAMBISSOILS	2	SEDIMENTARY FORM	1	between 4.6 and 5.5	2
Site15	Pq Cidade	Between 2600 and 2700 h	6	Wasteland and brushwood	2	0	CAMBISSOILS	2	SEDIMENTARY FORM	1	between 4.6 and 5.5	2
Site16	Póvoa Lanhoso	Between 2300 and 2400 h	4	Annual cultures associated with permanent cultures	2	200	CAMBISSOILS	2	VULCANIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site17	Mougadouro	Between 2600 and 2700 h	6	Agricultural areas outside forest perimeters	2	700	LITOSSOILS	3	SEDIMENTARY and METAMORPHIC FORM	2	between 5.6 and 6.5	3
Site18	Vale Santarém	Between 2700 and 2800 h	6	Discontinuous urban area	1	50	CAMBISSOILS	2	SEDIMENTARY FORM	1	between 5.6 and 6.5	3
Site19	Azambuja	Between 2700 and 2800 h	6	Continuous urban area	1	50	CAMBISSOILS	2	SEDIMENTARY FORM	1	between 5.6 and 6.5	3
Site20	Nazaré	Between 2400 and 2500 h	5	Wild Pinus (P. pinaster)	4	50	FLUVISSOILS	5	VULCANIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site21	Soure	Between 2600 and 2700 h	6	No data	0	50	PODZOIS	1	SEDIMENTARY FORM	1	between 4.6 and 5.5	2
Site22	Buçaco	Between 2500 and 2600 h	5	No data	0	400	CAMBISSOILS	2	SEDIMENTARY and METAMORPHIC FORM	2	between 4.6 and 5.5	2
Site23	Mata Margaraça	Between 2200 and 2300 h	4	Deciduous trees	3	700	CAMBISSOILS	2	SEDIMENTARY and METAMORPHIC FORM	2	between 4.6 and 5.5	2
Site24	Paradela	Between 2400 and 2500 h	5	Agricultural areas with important natural areas	2	100	CAMBISSOILS	2	SEDIMENTARY and METAMORPHIC FORM	2	between 4.6 and 5.5	2
Site25	V Nova Paiva	Between 2400 and 2500 h	5	Wild Pinus (P. pinaster)	4	700	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site26	Canas Senhorim	Between 2600 and 2700 h	6	Wild Pinus (P. pinaster)	4	400	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site27	Celorico Beira	Between 2400 and 2500 h	5	Agricultural areas outside forest perimeters	2	500	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site28	Penamacor	Between 2500 and 2600 h	5	Agricultural areas outside forest perimeters	2	500	LITOSSOILS	3	SEDIMENTARY FORM	1	between 5.6 and 6.5	3
Site29	Oledo	Between 2700 and 2800 h	6	Deciduous trees	3	200	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site30	Ulme	Between 2700 and 2800 h	6	Deciduous trees	3	100	PODZOIS	1	SEDIMENTARY FORM	1	between 4.6 and 5.5 (+5.6 to 6.5	2
Site31	S Espinhaço Cão	Between 2700 and 2800 h	6	Deciduous trees	3	100	LUVISSOILS	4	SEDIMENTARY and METAMORPHIC FORM	2	between 5.6 and 6.5	3
Site32	Besteiros	Between 2800 and 2900 h	7	Annual cultures associated with permanent cultures	2	200	LUVISSOILS	4	VULCANIC ERUPTIV ROCKS	3	between 6.6 and 7.3 (+7.4 to 8.5	4
Site33	Murtosa	Between 2500 and 2600 h	5	Evergreen trees	4	0	REGOSSOILS	6	SEDIMENTARY FORM	1	between 4.6 and 5.5	2
Site34	Bom Jesus	Between 2300 and 2400 h	4	Agricultural areas with important natural areas	2	300	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site35	Gerês	Between 1800 and 1900 h	2	Forest with mixture of tree species	5	1000	RANKERS	7	PLUTONIC ERUPTIV ROCKS	3	less or equal to 4.5	1
Site36	Chaves	Between 2400 and 2500 h	5	Agricultural areas with important natural areas	2	400	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site37	S. Marão	Between 2300 and 2400 h	4	Degradated forest spaces	2	900	RANKERS	7	SEDIMENTARY and METAMORPHIC FORM	2	less or equal to 4.5	1
Site38	Loriga	Between 2500 and 2600 h	5	Wasteland and brushwood	2	1800	RANKERS	7	PLUTONIC ERUPTIV ROCKS	3	less or equal to 4.5	1
Site39	Elvas	Between 3000 and 3100 h	8	Agricultural areas with important natural areas	2	300	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 7.4 and 8.5	5
Site40	Arriais Cima	Between 2600 and 2700 h	6	Sclerofitic vegetation	2	200	LUVISSOILS	4	SEDIMENTARY FORM	1	between 6.6 and 7.3 (+7.4 to 8.5	4
Site41	Rosmaninhal	Between 2700 and 2800 h	6	Annual cultures associated with permanent cultures	2	200	PODZOIS	1	SEDIMENTARY FORM	1	between 4.6 and 5.5 (+5.6 to 6.5	2
Site42	Meirinha	Between 2500 and 2600 h	5	Wild Pinus (P. pinaster)	4	100	CAMBISSOILS	2	SEDIMENTARY FORM	1	between 5.6 and 6.5	3
Site43	Fatima	Between 2300 and 2400 h	4	Wild Pinus (P. pinaster)	4	300	LUVISSOILS	4	SEDIMENTARY FORM	1	between 7.4 and 8.5	5
Site44	Rasa	Between 2500 and 2600 h	5	Deciduous trees	3	500	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site45	Arronches	Between 2500 and 2600 h	5	Degradated forest spaces	2	300	LUVISSOILS	4	SEDIMENTARY and METAMORPHIC FORM	2	between 5.6 and 6.5	3
Site46	Bencatel	Between 2900 and 3000 h	7	Eucalyptus	4	300	LITOSSOILS	3	SEDIMENTARY and METAMORPHIC FORM	2	between 5.6 and 6.5	3
Site47	Escalos Baixo	Between 2900 and 3000 h	7	Oak/Holm-oak trees	3	300	CAMBISSOILS	2	PLUTONIC ERUPTIV ROCKS	3	between 4.6 and 5.5	2
Site48	Orvalho	Between 2500 and 2600 h	5	Degradated forest spaces	2	500	CAMBISSOILS	2	SEDIMENTARY and METAMORPHIC FORM	2	between 4.6 and 5.5	2
Site49	FCastRodrigo	Between 2600 and 2700 h	6	Agricultural areas with important natural areas	2	500	LUVISSOILS	4	SEDIMENTARY and METAMORPHIC FORM	2	between 4.6 and 5.5 (+5.6 to 6.5	2
Site50	Freixo Esp Cinta	Between 2800 and 2900 h	7	Orchards	2	400	LUVISSOILS	4	SEDIMENTARY and METAMORPHIC FORM	2	between 5.6 and 6.5	3
Site51	Pq Montesinho	Between 2700 and 2800 h	6	Agricultural areas with important natural areas	2	600	CAMBISSOILS	2	SEDIMENTARY and METAMORPHIC FORM	2	between 4.6 and 5.5	2
Site52	Pq Alvão	Between 2300 and 2400 h	4	Wild Pinus (P. pinaster)	4	500	CAMBISSOILS	2	SEDIMENTARY and METAMORPHIC FORM	2	between 4.6 and 5.5	2
Site53	TapNacMafra	Between 2300 and 2400 h	4	Agricultural areas with important natural areas	2	200	CAMBISSOILS	2	SEDIMENTARY FORM	1	between 5.6 and 6.5 (+7.4 to 8.5	3
Site54	HRA	Between 2900 and 3000 h	7	Annual cultures associated with permanent cultures	2	200	LITOSSOILS	3	SEDIMENTARY and METAMORPHIC FORM	2	between 5.6 and 6.5	3
Site55	Aguas Moura	Between 2900 and 3000 h	7	Evergreen trees	4	0	PODZOIS	1	SEDIMENTARY FORM	1	between 5.6 and 6.6 (+4.6 to 5.5	3

Table A4.5 – Values of Landscape Analysis of 55 sites obtained by Patch Analyst , for each circle of 500, 1000, 2000 and 5000m

ID500	type1	type 2	type3	type4	type5	type6	type7	NP	MPS	IJI	SDI	SEI
S1				870				1	78,30			
S2		602		156			112	3	26,10	93,38	0,83	0,75
S3			870					1	78,30			
S4	273	546				51		3	26,10	61,98	0,82	0,75
S5		259	611					3	26,10		0,61	0,88
S6		32	838					2	39,15	67,59		
S7		468	402					2	39,15		0,69	1,00
S8		349	521					3	26,10		0,67	0,97
S9			870					1	78,30			
S10		307	562					2	39,15		0,65	0,94
S11		869						1	78,30			
S12			869					1	78,30			
S13		314	54		501			3	26,10	58,27	0,86	0,78
S14	423	446						2	39,15		0,69	1,00
S16	4	562			303			3	26,10	23,23	0,68	0,62
S17	39	830						2	39,15		0,18	0,25
S18	521	214			134			3	26,10	62,84	0,95	0,86
S19	720	6	123	20				5	15,66	67,56	0,56	0,41
S20		191		678				2	39,15		0,53	0,77
S21	2	867						2	39,15		0,01	0,01
S22			426	434	9			4	19,57	48,22	0,74	0,67
S23			669		200			2	39,15		0,54	0,78
S24		810		59				2	39,15		0,23	0,34
S25		106		763				3	26,10		0,37	0,54
S26		360		509				4	19,58	8,65	0,69	0,63
S27		869						1	78,30			
S28		869						1	78,30			
S29		385	482					2	39,15		0,69	0,99
S30			867					1	78,30			
S31			867					1	78,30			
S32			867					1	78,30			
S33	86	206		575				4	19,58	52,98	0,84	0,77
S34	264	310			293			5	15,66	91,58	1,10	1,00
S35		120			747			2	39,44		0,41	0,59
S36	187	680						3	26,10		0,52	0,75
S37		867						1	78,30			
S38		867						1	78,30			
S39	226	641						2	39,15		0,57	0,82
S40		867						1	78,30			
S41		478	390					4	19,57		0,69	0,99
S42		195		673				3	26,10		0,53	0,77
S43		868						1	78,30			
S44		79	789					2	39,15		0,31	0,44
S45		868						1	78,30			
S46		61					807	2	39,15		0,25	0,36
S47		199			669			2	39,15		0,54	0,78
S48		667		201				3	26,10		0,54	0,77
S49		745			123			2	39,15		0,41	0,59
S50		868						1	78,30			
S51		868						1	78,30			
S52		385		483				3	26,10		0,69	0,99
S53		726		142				2	39,15		0,45	0,64
S54		494	374					2	39,15		0,68	0,99
S55		223		488	157			4	19,57	99,64	0,98	0,89

Table A4.5 – cont (1000m)

ID1000	type1	type2	type3	type4	type5	type6	type7	NP	MPS	IJI	SDI	SEI
S1		18		3459				2	156,47		0,03	78,30
S2		1905	356	831			385	5	62,59	83,76	1,15	26,10
S3		38	3439					2	156,47		0,06	78,30
S4	472	2604			60	341		6	52,15	68,55	0,79	26,10
S5		873	2301					2	142,83		0,59	26,10
S6	58	1069	2350					4	39,07	39,86	0,70	39,15
S7		2750	727					2	156,47		0,51	39,15
S8		2225	1252					4	78,23		0,65	26,10
S9		168	3309					3	104,31		0,19	78,30
S10		1131	2346					5	62,59		0,63	39,15
S11		3477						1	312,93			78,30
S12		3477						1	312,93			78,30
S13		1180	653		1644			7	44,70	72,82	1,03	26,10
S14	2387	894				196		5	62,59	62,12	0,77	39,15
S16	185	2543			749			7	44,70	45,45	0,72	26,10
S17	297	3149			31			4	78,23	46,37	0,34	39,15
S18	1136	1620	52		669			6	52,16	64,48	1,10	26,10
S19	1105	1281	729	362				4	78,23	72,87	1,30	15,66
S20		1317	3	2157				8	39,12	11,27	0,67	39,15
S21	102	2919			456			4	78,23	87,24	0,52	39,15
S22	191		923	1507	856			5	62,59	81,11	1,22	19,57
S23		586	1511	27	1353			6	52,16	70,42	1,07	39,15
S24		2431		730	316			3	104,31	63,08	0,80	39,15
S25		710		2767				3	104,31		0,51	26,10
S26	153	2151		1173				6	52,16	55,72	0,80	19,58
S27		3290			187			2	156,47		0,21	78,30
S28		3477						1	312,93			78,30
S29	179	2181	1117					4	78,23	68,55	0,81	39,15
S30			3477					1	312,93			78,30
S31		15	3462					2	156,47		0,03	78,30
S32		3437	40					2	156,47		0,06	78,30
S33	419	1931		1127				5	62,59	91,82	0,95	19,58
S34	685	1939			853			8	39,12	92,06	0,99	15,66
S35		1302			2175			4	78,23		0,66	39,44
S36	463	3014						2	156,47		0,39	26,10
S37		3477						1	312,93			78,30
S38		3477						1	312,93			78,30
S39	1176	2301						2	156,47		0,64	39,15
S40		3477						1	312,93			78,30
S41		1842	1632				3	6	52,15	8,64	0,70	19,57
S42		825		2652				3	104,31		0,55	26,10
S43		3477						1	312,93			78,30
S44		1111	2366					2				39,15
S45		3222	255					5	62,59		0,26	78,30
S46		489	333				2655	4	78,23	90,83	0,71	39,15
S47		2060			1417			2	156,47		0,68	39,15
S48		1692		1686	99			4	78,23	36,83	0,80	26,10
S49		2607			870			3	104,31		0,56	39,15
S50		3477						1	312,93			78,30
S51		3311	166					2	156,47		0,19	78,30
S52		1606		1871				5	62,59		0,69	26,10
S53	134	2195		852	296			5	62,59	54,62	0,97	39,15
S54		1883	1885					3	113,04		0,69	39,15
S55		1587	66	1181	643			5	62,59	67,98	1,11	19,57

Table A4.5 – cont. (2000m)

ID2000	type1	type2	type3	type4	type5	type6	type7	NP	MPS	IJI	SDI	SEI
S1	577	1547		11773				5	250,13	56,31	0,52	0,47
S2		6354	2764	3917	454		408	9	138,96	79,85	1,25	0,78
S3		1676	12221					5	250,13		0,37	0,53
S4	851	9130	1000	577	1858	481		11	113,69	66,02	1,16	0,65
S5		4147	68					4	94,97		0,08	0,12
S6	197	6331	7369					8	156,33	34,18	0,75	0,69
S7	107	10876	2714		195	5		12	104,22	27,68	0,61	0,38
S8	9	9002	4886					11	113,69	7,53	0,65	0,60
S9	74	3872	9951					5	250,13	20,24	0,62	0,57
S10		6047	7088			252	510	10	125,06	43,36	0,90	0,65
S11	256	13027	614					4	312,66	62,77	0,27	0,25
S12			13814		83			2	625,32		0,04	0,05
S13		5181	4724		3992			11	113,69	81,08	1,09	0,99
S14	9615	1027				3255		4	312,66	90,85	0,79	0,72
S16	240	9009			4648			12	104,22	28,16	0,72	0,65
S17	671	10788			2438			5	250,13	58,27	0,65	0,59
S18	1325	10147	266		2159			7	178,66	76,86	0,82	0,59
S19	1611	6306	2581	1334	2065			9	138,96	78,93	1,43	0,89
S20	210	7745	397	4931		614		9	138,96	47,03	0,99	0,62
S21	103	8154		603	5037			10	125,06	38,54	0,85	0,62
S22	557	1833	1240	4969	5298			17	73,57	82,66	1,35	0,84
S23		4260	1689	1052	6896			11	113,69	79,99	1,16	0,84
S24		7460		2891	2935		611	7	178,66	60,73	1,13	0,81
S25		2672		11225				5	250,13		0,49	0,71
S26	252	8562		5083				10	125,06	39,64	0,74	0,67
S27		13179			718			2	625,32		0,20	0,29
S28		12611	555				731	6	208,44	61,04	0,37	0,34
S29	218	10581	3098					4	312,66	60,75	0,61	0,55
S30		357	13360		180			7	178,66	64,72	0,19	0,17
S31		2232	11528				137	5	250,13	34,20	0,49	0,45
S32		11270	2627					4	312,66		0,49	0,70
S33	2105	8819		2972		1		12	104,22	48,08	0,90	0,65
S34	2331	8644			2922			15	83,38	80,05	0,92	0,84
S35		8790			5107			3	416,88		0,66	0,95
S36	3717	10180						3	416,88		0,58	0,84
S37		13863		34				3	416,88		0,02	0,03
S38		13883		14				2	625,32		0,01	0,01
S39	2388	11509						2	625,32		0,46	0,66
S40	114	13783						2	625,32		0,05	0,07
S41		8894	4173				830	7	178,66	47,91	0,81	0,74
S42		5130		8767				12	104,22		0,66	0,95
S43		13897						1	1250,64			
S44		6481	7416					4	312,66		0,69	1,00
S45		12199	1698					5	250,13		0,37	0,54
S46		4133	1228				8536	7	178,66	93,28	0,87	0,80
S47		11201	153		2543			4	312,66	34,69	0,53	0,49
S48	162	3996	647	7162	1930			10	125,06	60,23	1,17	0,73
S49		8802			5095			3	416,88		0,66	0,95
S50		12952		934				3	416,88		0,25	0,36
S51		12778	1108					2	625,32		0,28	0,40
S52		6939		6947				4	312,66		0,69	1,00
S53	1264	8388		2128	2030		76	8	156,33	57,13	1,12	0,70
S54		2463	15576					3	541,11		0,40	0,58
S55		9045	2243	1787	651	160		10	125,06	64,56	1,03	0,64

Table A4.5 – cont (5000m)

ID5000	type1	type2	type3	type4	type5	type6	type7	NP	MPS	IJI	SDI	SEI
S1	9734	17794	199	71987	368	613		20	453,13		0,84	0,47
S2	1541	45340	5751	415	9016		404	23	244,44	51,98	0,89	0,50
S3		20244	52509	163	10702	372	2834	17	459,66	54,06	1,05	0,59
S4	860	44276	1165	2570	34580	2187	1186	20	390,71	42,66	1,07	0,55
S5	113	11319	142		548			10	109,10	38,59	0,30	0,22
S6	303	39334	47187					19	411,27	383,35	0,71	0,65
S7	243	70067	15820	72	518	104		20	390,71	18,48	0,54	0,30
S8	133	57692	28999					18	434,12		0,66	0,60
S9	392	51847	31789	394			2402	13	601,09		0,86	0,53
S10	438	45336	38058			257	2735	17	459,66	37,65	0,82	0,51
S11	257	77567	4389				4611	8	976,77	16,94		
S12		15607	62069		8182	966		9	868,24	80,54		
S13	178	36796	27447		16309		6094	22	355,19	54,14		
S14	40567	9344			1255	35658		15	520,94	48,72		
S16	747	36035			6850	227		17	232,19	67,12		
S17	806	75933	395		9690			10	781,42	46,54		
S18	2038	52236	267		3318		348	12	436,55	51,30		
S19	4488	62976	4983	3352	8173			10	755,75	54,56		
S20	517	38578	398	26155	4535	16641		13	601,09	42,66		
S21	990	59596		8153	18085			27	289,41	53,16	0,86	0,62
S22	832	18440	1242	16600	49710			21	372,10	58,65	1,07	0,66
S23		42534	3008	5211	36071			28	279,08	55,12	1,00	0,72
S24	541	36617		11924	35845		1897	26	300,54	47,90	1,12	0,69
S25	1013	41643	596	42215	1357			23	339,75	38,20	0,85	0,53
S26	1574	47710		37540				24	325,59	26,67	0,76	0,70
S27		81573		1737	3514			8	976,77	70,81	0,27	0,24
S28		68262	1952	35	2418		14157	12	651,18	41,67	0,67	0,42
S29	220	69880	11718			1223	3783	13	601,09	58,59	0,66	0,41
S30		14234	63790	494	5391		2915	25	312,57	43,70	0,84	0,52
S31		37343	43729			80	5672	13	601,09	47,47	0,89	0,64
S32	2	47355	38873				594	7	1116,31	19,00	0,72	0,52
S33	7167	55975		11449		12233		18	434,12	69,08	1,03	0,74
S34	14211	48951			18891	790	1726	43	177,00	56,19	1,07	0,67
S35		57595	8207	1076	17111	2835		19	411,27	57,16	0,98	0,61
S36	5862	67669		11518	1151	624		18	434,12	50,39	0,74	0,46
S37	200	77545		7692				9	854,37	18,95	0,32	0,29
S38		77835		5223	2938	828		11	710,38	59,75	0,43	0,31
S39	2391	84232				201		3	2604,72	42,21	0,14	0,13
S40	1422	77196		270	3370		4566	18	434,12	65,07	0,47	0,29
S41	86	56022	10133		9545		11038	24	325,59	60,05	1,05	0,65
S42	940	36465		47780	1639			28	279,08	25,45	0,82	0,59
S43	2913	69842		3495	10574			13	601,09	51,41	0,67	0,49
S44	1363	60547	23059		1248		607	14	558,15	28,47	0,76	0,47
S45		71153	11087				4584	14	558,15	56,50	0,58	0,53
S46		51147	10771	562		323	24021	19	411,27	54,51	0,98	0,61
S47	168	80452	802		3016		2386	9	868,24	58,14	0,34	0,21
S48	492	24733	1940	48736	10923			23	339,75	56,91	1,06	0,66
S49	70	57974	1749		27031			10	781,42	29,12	0,72	0,52
S50		74403	2156	10265				7	1116,31	54,00	0,48	0,43
S51	426	73531	12142				725	11	710,38	24,76	0,48	0,35
S52		42109		29727			5352	14	496,21	45,79	0,88	0,80
S53	1717	69134		6060	5902		4011	13	601,09	66,87	0,77	0,48
S54		7471						8	84,05			
S55	441	41661	30176	4217	6483	2121	1725	30	260,47	65,72	1,26	0,65

Table A4.6 – Results from logistic regression model
Multi_all2 – Total ticks; Multi_RH2 – *Riphicephalus;* Multi_DE2 – *Dermacentor*; Multi_IR2 – *I. ricinus*

The LOGISTIC Procedure

Model Information

Data Set	WORK.ALL
Response Variable	YESTICK
Number of Response Levels	2
Model	binary logit
Optimization Technique	Fisher's scoring

Number of Observations Read	55
Number of Observations Used	49

Response Profile

Ordered Value	YESTICK	Total Frequency
1	1	32
2	0	17

Probability modeled is YESTICK=1.

NOTE: 6 observations were deleted due to missing values for the response or explanatory variables.

Stepwise Selection Procedure

Step 0. Intercept entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

multi_all2.lst
-2 Log L = 63.262

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
8.7539	6	0.1879

Step 1. Effect PDE1000 entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	65.262	61.196
SC	67.154	64.980
-2 Log L	63.262	57.196

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	6.0658	1	0.0138
Score	4.8028	1	0.0284
Wald	3.5560	1	0.0593

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
3.9081	5	0.5627

NOTE: No effects for the model in Step 1 are removed.

Step 2. Effect HUMSOIL entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	65.262	60.951
SC	67.154	66.627
-2 Log L	63.262	54.951

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	8.3109	2	0.0157
Score	6.6167	2	0.0366
Wald	5.0915	2	0.0784

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
1.8042	4	0.7717

NOTE: No effects for the model in Step 2 are removed.

NOTE: No (additional) effects met the 0.25 significance level for entry into the model.

Summary of Stepwise Selection

Step	Effect Entered	Effect Removed	DF	Number In	Score Chi-Square	Wald Chi-Square	Pr > ChiSq
1	PDE1000		1	1	4.8028		0.0284
2	HUMSOIL		1	2	2.1682		0.1409

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	1.6715	1.1068	2.2807	0.1310
HUMSOIL	1	-0.0323	0.0226	2.0498	0.1522
PDE1000	1	0.0348	0.0185	3.5231	0.0605

The SAS System

3

The LOGISTIC Procedure

Odds Ratio Estimates

Effect	Point Estimate	95% Wald Confidence Limits	
HUMSOIL	0.968	0.926	1.012
PDE1000	1.035	0.998	1.074

Association of Predicted Probabilities and Observed Responses

Percent Concordant	73.5	Somers' D	0.476
Percent Discordant	25.9	Gamma	0.479
Percent Tied	0.6	Tau-a	0.220
Pairs	544	c	0.738

Table A4.6 – Results from logistic regression model (cont.)

multi_RH2.lst

The SAS System

1

The LOGISTIC Procedure

Model Information

Data Set	WORK.ALL
Response Variable	YESRH
Number of Response Levels	2
Model	binary logit
Optimization Technique	Fisher's scoring

Number of Observations Read	55
Number of Observations Used	53

Response Profile

Ordered Value	YESRH	Total Frequency
1	1	28
2	0	25

Probability modeled is YESRH=1.

NOTE: 2 observations were deleted due to missing values for the response or explanatory variables.

Stepwise Selection Procedure

Step 0. Intercept entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

-2 Log L = 73.304

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
18.3290	8	0.0189

Step 1. Effect PRECIP entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	75.304	64.621
SC	77.274	68.561
-2 Log L	73.304	60.621

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	12.6831	1	0.0004
Score	10.1495	1	0.0014
Wald	7.9612	1	0.0048

□

The SAS System

2

The LOGISTIC Procedure

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
9.0870	7	0.2465

NOTE: No effects for the model in Step 1 are removed.

Step 2. Effect PDE1000 entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	75.304	62.914
SC	77.274	68.825
-2 Log L	73.304	56.914

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	16.3897	2	0.0003
Score	13.2472	2	0.0013
Wald	9.5908	2	0.0083

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
5.9679	6	0.4268

NOTE: No effects for the model in Step 2 are removed.

Step 3. Effect POP5000 entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	75.304	59.753
SC	77.274	67.635
-2 Log L	73.304	51.753

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	21.5502	3	<.0001
Score	17.6484	3	0.0005
Wald	12.0911	3	0.0071

The LOGISTIC Procedure

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
1.1334	5	0.9511

multi_RH2.lst

NOTE: No effects for the model in Step 3 are removed.

NOTE: No (additional) effects met the 0.25 significance level for entry into the model.

Summary of Stepwise Selection

Step	Effect Entered	Effect Removed	DF	Number In	Score Chi-Square	Wald Chi-Square	Pr > ChiSq
1	PRECIP		1	1	10.1495		0.0014
2	PDE1000		1	2	3.1769		0.0747
3	POP5000		1	3	4.9093		0.0267

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	0.1001	1.4877	0.0045	0.9464
PRECIP	1	-0.00293	0.00121	5.8590	0.0155
POP5000	1	0.0345	0.0164	4.4175	0.0356
PDE1000	1	0.0315	0.0152	4.3190	0.0377

Odds Ratio Estimates

Effect	Point Estimate	95% Wald Confidence Limits	
PRECIP	0.997	0.995	0.999
POP5000	1.035	1.002	1.069
PDE1000	1.032	1.002	1.063

multi_RH2.lst

Association of Predicted Probabilities and Observed Responses

Percent Concordant	83.6	Somers' D	0.677
Percent Discordant	15.9	Gamma	0.681
Percent Tied	0.6	Tau-a	0.344
Pairs	700	c	0.839

Table A4.6 – Results from logistic regression model (cont.)

multi_DE2.lst

The SAS System

1

The LOGISTIC Procedure

Model Information

Data Set	WORK.ALL
Response Variable	YESDE
Number of Response Levels	2
Model	binary logit
Optimization Technique	Fisher's scoring

Number of Observations Read	55
Number of Observations Used	49

Response Profile

Ordered Value	YESDE	Total Frequency
1	1	11
2	0	38

Probability modeled is YESDE=1.

NOTE: 6 observations were deleted due to missing values for the response or explanatory variables.

Stepwise Selection Procedure

Step 0. Intercept entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

-2 Log L = 52.188

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
11.0418	5	0.0506

Step 1. Effect PMI2000 entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	54.188	49.118
SC	56.080	52.902
-2 Log L	52.188	45.118

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	7.0697	1	0.0078
Score	8.0101	1	0.0047
Wald	6.3219	1	0.0119

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
3.2931	4	0.5100

NOTE: No effects for the model in Step 1 are removed.

Step 2. Effect NP500 entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	54.188	49.779
SC	56.080	55.454
-2 Log L	52.188	43.779

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	8.4091	2	0.0149
Score	9.0461	2	0.0109
Wald	7.2746	2	0.0263

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
1.9229	3	0.5886

Step 3. Effect NP500 is removed:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	54.188	49.118
SC	56.080	52.902
-2 Log L	52.188	45.118

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	7.0697	1	0.0078
Score	8.0101	1	0.0047
Wald	6.3219	1	0.0119

The SAS System

3

The LOGISTIC Procedure

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
3.2931	4	0.5100

NOTE: No effects for the model in Step 3 are removed.

NOTE: Model building terminates because the last effect entered is removed by the Wald statistic criterion.

Summary of Stepwise Selection

Step	Effect Entered	Effect Removed	DF	Number In	Score Chi-Square	Wald Chi-Square	Pr > ChiSq
1	PMI2000		1	1	8.0101		0.0047
2	NP500		1	2	1.3828		0.2396
3		NP500	1	1		1.3215	0.2503

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	-1.9190	0.4849	15.6642	<.0001
PMI2000	1	0.0665	0.0264	6.3219	0.0119

Odds Ratio Estimates

Effect	Point Estimate	95% Wald Confidence Limits	
PMI2000	1.069	1.015	1.126

Association of Predicted Probabilities and Observed Responses

Percent Concordant	57.4	Somers' D	0.388
Percent Discordant	18.7	Gamma	0.509
Percent Tied	23.9	Tau-a	0.138

Pairs	418	c	0.694

Table A4.6 – Results from logistic regression model (cont.)

The LOGISTIC Procedure

Model Information

Data Set	WORK.ALL
Response Variable	YESIR
Number of Response Levels	2
Model	binary logit
Optimization Technique	Fisher's scoring

Number of Observations Read	55
Number of Observations Used	48

Response Profile

Ordered Value	YESIR	Total Frequency
1	1	6
2	0	42

Probability modeled is YESIR=1.

NOTE: 7 observations were deleted due to missing values for the response or explanatory variables.

Stepwise Selection Procedure

Step 0. Intercept entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

multi_IR2.lst
-2 Log L = 36.170

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
22.2754	7	0.0023

Step 1. Effect PMI2000 entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	38.170	29.283
SC	40.041	33.026
-2 Log L	36.170	25.283

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	10.8868	1	0.0010
Score	14.0030	1	0.0002
Wald	8.4506	1	0.0036

The SAS System

2

The LOGISTIC Procedure

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
8.6543	6	0.1940

NOTE: No effects for the model in Step 1 are removed.

Step 2. Effect PRECIP entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	38.170	26.675
SC	40.041	32.289
-2 Log L	36.170	20.675

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	15.4946	2	0.0004
Score	18.5270	2	<.0001
Wald	8.7834	2	0.0124

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
6.1985	5	0.2874

NOTE: No effects for the model in Step 2 are removed.

Step 3. Effect EAST entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	38.170	25.020
SC	40.041	32.505
-2 Log L	36.170	17.020

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	19.1500	3	0.0003
Score	20.2945	3	0.0001
Wald	7.6675	3	0.0534

The LOGISTIC Procedure

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
5.0053	4	0.2868

NOTE: No effects for the model in Step 3 are removed.

Step 4. Effect IJI1000 entered:

Model Convergence Status

Convergence criterion (GCONV=1E-8) satisfied.

Model Fit Statistics

Criterion	Intercept Only	Intercept and Covariates
AIC	38.170	21.548
SC	40.041	30.904
-2 Log L	36.170	11.548

Testing Global Null Hypothesis: BETA=0

Test	Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	24.6220	4	<.0001
Score	21.0355	4	0.0003
Wald	2.5547	4	0.6349

Residual Chi-Square Test

Chi-Square	DF	Pr > ChiSq
1.9285	3	0.5874

NOTE: No effects for the model in Step 4 are removed.

NOTE: No (additional) effects met the 0.25 significance level for entry into the model.

Summary of Stepwise Selection

Step	Effect Entered	Effect Removed	DF	Number In	Score Chi-Square	Wald Chi-Square	Pr > ChiSq
1	PMI2000		1	1	14.0030		0.0002
2	PRECIP		1	2	5.3102		0.0212
3	EAST		1	3	2.6166		0.1058
4	IJI1000		1	4	4.1888		0.0407

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	5.8707	5.3564	1.2012	0.2731
EAST	1	-0.00011	0.000073	2.2505	0.1336
PRECIP	1	0.00954	0.00638	2.2353	0.1349
PMI2000	1	0.3470	0.2206	2.4743	0.1157
IJI1000	1	-0.1037	0.0700	2.1928	0.1387

The SAS System

4

The LOGISTIC Procedure

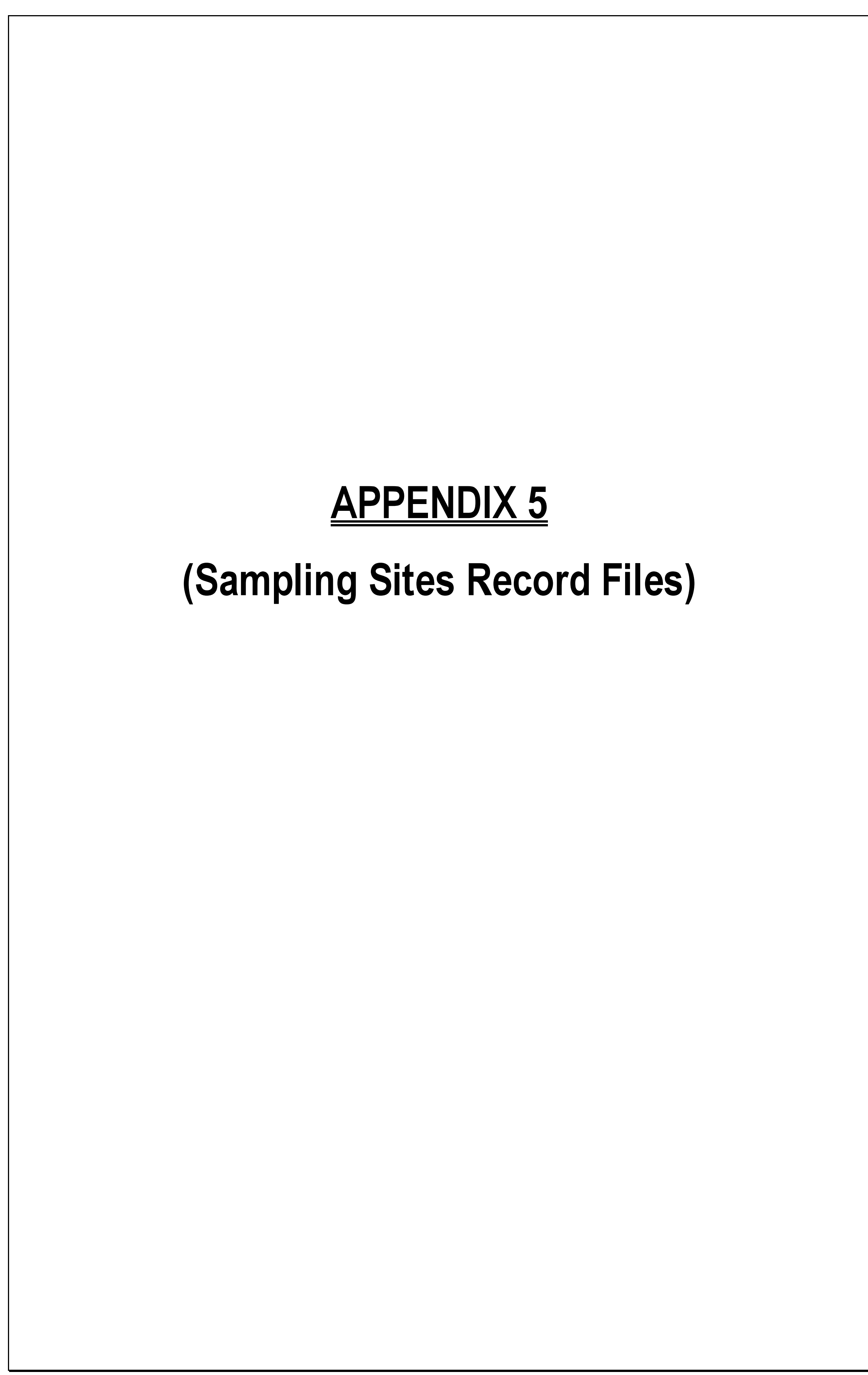
Odds Ratio Estimates

Effect	Point Estimate	95% Wald Confidence Limits	
EAST	1.000	1.000	1.000
PRECIP	1.010	0.997	1.022
PMI2000	1.415	0.918	2.180

IJI1000	0.902	0.786	1.034

Association of Predicted Probabilities and Observed Responses

Percent Concordant	97.2	Somers' D	0.944
Percent Discordant	2.8	Gamma	0.944
Percent Tied	0.0	Tau-a	0.211
Pairs	252	c	0.972



APPENDIX 5

(Sampling Sites Record Files)

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 1

REGION:	ESTREMADURA	*MILITARY CHART:*	*464*
COUNTY:	SESIMBRA		
POLYGON:	LISBOA	*NAME:*	ALFARIM

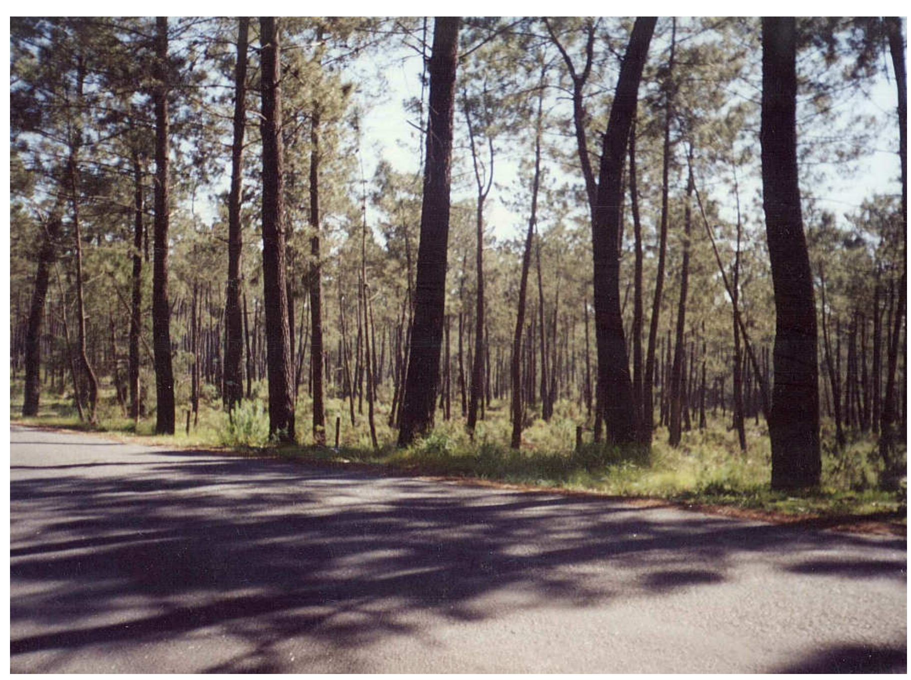
1. ACCESSIBILITY

EN 377 to Alfarim. Left side of the road. Hunting area symbol.

2. SITE CHARACTERIZATION

GPS coordinates	38.54116/-9.11276 (WGS84)	**Temperature Air/Soil**	22.15/23.6
No visits	3	**Humidity Air/Soil**	43.7/46.15
Vegetation	2	**Human use**	2
Soil / Type	1. Sandy	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS



RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 2

REGION:	ESTREMADURA	*MILITARY CHART:*	*464*
COUNTY	SESIMBRA		
POLYGON:	LISBON	*NAME:*	VILA NOGUEIRA DE AZEITÃO

1. ACCESSIBILITY

EN 379 after V. Nogueira de Azeitão. Right side of the road. Area with some olives. Some houses dispersed.

2. SITE CHARACTERIZATION

GPS coordinates	38.54087/-9.11148 (WGS84)	**Temperature Air/Soil**	24.9/24.3
No visits	2	**Humidity Air/Soil**	37.85/50.05
Vegetation	5	**Human use**	4
Soil / Type	1. Sandy and Clayish	**Ticks**	Presence
Forest cover	2	**Hosts**	Rabbits (excrements)

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 3

REGION:	ALENTEJO	*MILITARY CHART:*	*476*
COUNTY	ALCÁCER SAL		
POLYGON:	GRÂNDOLA	*NAME:*	PINHEIRO

1. ACCESSIBILITY

IC1 to Alcaçer do Sal. Right side of the road. Secondary road to Pinheiro (Estação Arqueológica).

2. SITE CHARACTERIZATION

GPS coordinates	38.54961/-8.65398 (WGS84)	**Temperature Air/Soil**	28.65/28.1
No visits	2	**Humidity Air/Soil**	34.15/46
Vegetation	5	**Human use**	2
Soil / Type	1. Sandy and Clayish	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d.

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 4

REGION:	ALENTEJO	*MILITARY CHART:*	*476*
COUNTY	ALCACER DO SAL		
POLYGON:	GRÂNDOLA	*NAME:*	ALBERGUE

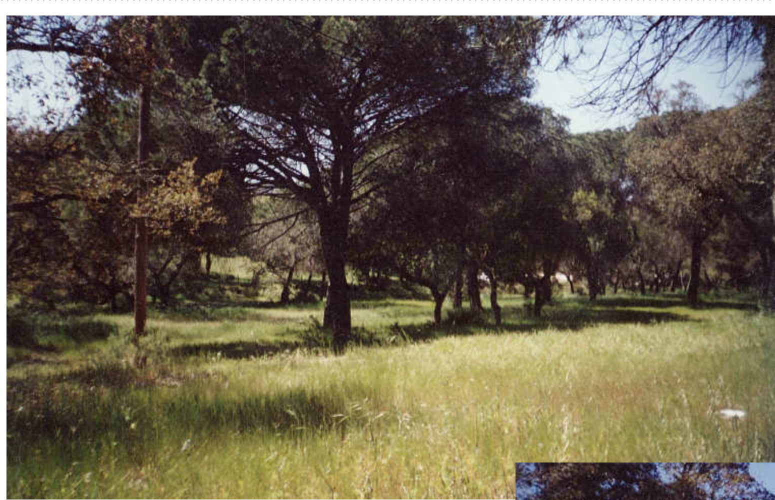
1. ACCESSIBILITY

Next to the entrance to Albergue. The road opens in two roadways and closes in just one roadway. Just before the traffic sign.

2. SITE CHARACTERIZATION

GPS coordinates	38.36700/-8.50000 (WGS84)	**Temperature Air/Soil**	28.45/29
No visits	1	**Humidity Air/Soil**	30.25/38.5
Vegetation	5	**Human use**	1
Soil / Type	1. Sandy	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d.

3. PHOTOS



RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 5

REGION:	**ALENTEJO**	*MILITARY CHART:*	*506*
COUNTY	**GRÂNDOLA**		
POLYGON:	**GRÂNDOLA**	*NAME:*	**SANTIAGO DE CACÉM**

1. ACCESSIBILITY

EN120 (bad condition). Road Grândola - Santiago de Cacém. Dense oakland in both sides. Right side of the road, near a steep slope.

2. SITE CHARACTERIZATION

GPS coordinates	38.13375/-8.59257 (WGS84)	**Temperature Air/Soil**	25.8/26.15
No visits	2	**Humidity Air/Soil**	32/51.5
Vegetation	6	**Human use**	1
Soil / Type	3. Clayish	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d.

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 6

REGION:	**ALENTEJO**	*MILITARY CHART:*	***517***
COUNTY	**SANTIAGO DE CACÉM**		
POLYGON:	**GRÂNDOLA**	*NAME:*	**S. DOMINGOS**

1. ACCESSIBILITY

EN261. 1 km after the intersection with the road that comes from S. Domingos (EN390). Right side near two oaks (*Q. suber*) with numbers 7 and 8

2. SITE CHARACTERIZATION

GPS coordinates	37.93142/-8.54890 (WGS84)	**Temperature Air/Soil**	23.8/24.5
No visits	1	**Humidity Air/Soil**	43.9/62.15
Vegetation	5	**Human use**	3
Soil / Type	1. Sandy and Clayish	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 7

REGION:	ALENTEJO	*MILITARY CHART:*	*508*
COUNTY	FERREIRA DO ALENTEJO		
POLYGON:	ALJUSTREL	*NAME:*	CANHESTROS

1. ACCESSIBILITY

EN121, Ferreira do Alentejo to Canhestros. Left side of the road (1,3 km of Canhestros). Oakland with dense shrubs after an intersection of two secondary roads.

2. SITE CHARACTERIZATION

GPS coordinates	38.04012/-8.27000 (WGS84)	**Temperature Air/Soil**	27.65/28.9
No visits	2	**Humidity Air/Soil**	28/41.6
Vegetation	5	**Human use**	1
Soil / Type	2. Clayish	**Ticks**	Presence
Forest cover	2	**Hosts**	Rabbits (excrements)

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 8

REGION:	ALENTEJO	*MILITARY CHART:*	*547*
COUNTY	OURIQUE		
POLYGON:	ALJUSTREL	*NAME:*	GAVÃO

1. ACCESSIBILITY

EN123, 15km to Ourique, just after Gavão. There are two secondary roads in both sides of the main road.

2. SITE CHARACTERIZATION

GPS coordinates	37.71510/-8.35982 (WGS84)	**Temperature Air/Soil**	26.95/28.1
N0 visits	2	**Humidity Air/Soil**	27.15/42.2
Vegetation	5	**Human use**	1
Soil / Type	2. Clayish?	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 9

REGION:	ALENTEJO	*MILITARY CHART:*	*470*
COUNTY	VIANA DO ALENTEJO		
POLYGON:	ÉVORA	*NAME:*	ALCAÇOVAS

1. ACCESSIBILITY

EN2, 2,5 km after Alcaçovas to Montemor-o-Novo. Left side of the road near an iron door (entrance of an hunting area)

2. SITE CHARACTERIZATION

GPS coordinates	38.41100/-8.15398 (WGS84)	**Temperature Air/Soil**	17.2/19.55
No visits	2	**Humidity Air/Soil**	53.15/55.1
Vegetation	5	**Human use**	2
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d.

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 10

REGION:	ALENTEJO	*MILITARY CHART:*	*437*
COUNTY	ARRAIOLOS		
POLYGON:	ÉVORA	*NAME:*	ARRAIOLOS

1. ACCESSIBILITY

Km 93 after Alcaçovas, EN370 Arraiolos-Pavia after the intersection to Santana do Campo. Right side of the road, near a drinking fountain.

2. SITE CHARACTERIZATION

GPS coordinates	38.74831/-7.99046 (WGS84)	**Temperature Air/Soil**	26.25/28.55
No visits	2	**Humidity Air/Soil**	34.25/38.15
Vegetation	7	**Human use**	2
Soil / Type	1. Clayish	**Ticks**	Presence
Forest cover	1	**Hosts**	Sheeps, Rabbits

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 11

REGION:	ALENTEJO	*MILITARY CHART:*	*324*
COUNTY	NISA		
POLYGON:	PORTALEGRE	*NAME:*	ALPALHÃO

1. ACCESSIBILITY

EN246 to Castelo de Vide, 1.5Km after Alpalhão. Hunting area with some garbage at the entrance.

2. SITE CHARACTERIZATION

GPS coordinates	39.32928/-7.42263 (WGS84)	**Temperature Air/Soil**	14.4/15.6
No visits	2	**Humidity Air/Soil**	40.4/70.1
Vegetation	4	**Human use**	2
Soil / Type	3. Rocks	**Ticks**	Absence
Forest cover	1	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 12

REGION:	RIBATEJO	*MILITARY CHART:*	*408*
COUNTY	MORA		
POLYGON:	CORUCHE	*NAME:*	MORA

1. ACCESSIBILITY

EN2 Montargil-Mora, after the dam and the intersection to Foros de Mocho. Oakland near a sign from "Direcção de Estradas do Distrito de Évora". In the right side of this road there is a sign from an hunting zone (Proc. 420-DGF)

2. SITE CHARACTERIZATION

GPS coordinates	39.01386/-8.13492 (WGS84)	**Temperature Air/Soil**	23.3/24.35
No visits	2	**Humidity Air/Soil**	23.3/43.4
Vegetation	5	**Human use**	1
Soil / Type	2. Sandy	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d.

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 13

REGION:	RIBATEJO	*MILITARY CHART:*	*446*
COUNTY	MONTEMOR-O-NOVO		
POLYGON:	CORUCHE	*NAME:*	LAVRE

1. ACCESSIBILITY

EN380, 3.8 km after Lavre to Vendas Novas. Left side of the road with a small berm.

2. SITE CHARACTERIZATION

GPS coordinates	38.76418/-8.41290 (WGS84)	**Temperature Air/Soil**	25.7/26.85
No visits	4	**Humidity Air/Soil**	23.65/45.95
Vegetation	6	**Human use**	1
Soil / Type	1. Sandy	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 14

REGION:	DOURO LITORAL	*MILITARY CHART:*	*122*
COUNTY	PORTO		
POLYGON:	PORTO	*NAME:*	FUNDAÇÃO SERRALVES

1. ACCESSIBILITY

In the center of Porto

2. SITE CHARACTERIZATION

GPS coordinates	41.17038/-8.67957 (WGS84)	**Temperature Air/Soil**	
No visits	1	**Humidity Air/Soil**	
Vegetation	6	**Human use**	5
Soil / Type	1	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 15

REGION:	DOURO LITORAL	*MILITARY CHART:*	122
COUNTY	PORTO		
POLYGON:	PORTO	*NAME:*	PARQUE DA CIDADE

1. ACCESSIBILITY

In the center of Porto

2. SITE CHARACTERIZATION

GPS coordinates	41.17038/-8.67957 (WGS84)	**Temperature Air/Soil**	
No visits	2	**Humidity Air/Soil**	
Vegetation	3	**Human use**	5
Soil / Type	1	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 16

REGION:	MINHO	MILITARY CHART:	57
COUNTY	PÓVOA DE LANHOSO		
POLYGON:	BRAGA	NAME:	PÓVOA DE LANHOSO

1. ACCESSIBILITY

19 km from Bouro (St. Maria). Road Amares-Póvoa Lanhoso, in a curve to the right. Left side of this curve.

2. SITE CHARACTERIZATION

GPS coordinates	41.60080/-8.28160 (WGS84)	**Temperature Air/Soil**	11.8/11.55
No visits	3	**Humidity Air/Soil**	58.75/71.15
Vegetation	5	**Human use**	1
Soil / Type	1. Sandy	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 17

REGION:	TRAS-OS-MONTES	*MILITARY CHART:*	*107*
COUNTY	MOGADOURO		
POLYGON:	BRAGANÇA	*NAME:*	MOGADOURO

1. ACCESSIBILITY

EN216, Mogadouro-Peneda (Alfândega da Fé), next to a traffic sign indicating directions to Alfândega da Fé and Macedo de Cavaleiros.

Both sides of the road.

2. SITE CHARACTERIZATION

GPS coordinates	41.33300/-6.71700 (WGS84)	**Temperature Air/Soil**	16.4/16.1
No visits	2	**Humidity Air/Soil**	31.1/49.15
Vegetation	5	**Human use**	1
Soil / Type	2	**Ticks**	Absence
Forest cover	3	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 18

REGION:	**ESTREMADURA**	*MILITARY CHART:*	*377*
COUNTY	**SANTARÉM**		
POLYGON:	**RIO MAIOR**	*NAME:*	**VALE DE SANTARÉM**

1. ACCESSIBILITY

EN25, Vale de Santarém to Póvoa de Isenta, after Zootecnic Station. Near a small sign "Santuário". Hunting area. Right side of the road.

2. SITE CHARACTERIZATION

GPS coordinates	39.21627/-8.74547 (WGS84)	**Temperature Air/Soil**	24.65/26.1
No visits	2	**Humidity Air/Soil**	42.8/51.55
Vegetation	1	**Human use**	2
Soil / Type	2. Sandy	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SITE 19

SAMPLE SITES (NATIONWIDE STUDY)

REGION:	ESTREMADURA	*MILITARY CHART:*	*377*
COUNTY	CARTAXO		
POLYGON:	RIO MAIOR	*NAME:*	AZAMBUJA

1. ACCESSIBILITY

EN3, Chã de Ourique-Azambuja. Left side of the road. Near a bus stop.

2. SITE CHARACTERIZATION

GPS coordinates	39.20908/-8.74339 (WGS84)	**Temperature Air/Soil**	17.05/16.8
No visits	2	**Humidity Air/Soil**	67.5/68.45
Vegetation	1	**Human use**	1
Soil / Type	1. Sandy	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 20

REGION:	BEIRA LITORAL	*MILITARY CHART:*	*307*
COUNTY	NAZARÉ		
POLYGON:	LEIRIA	*NAME:*	NAZARÉ

1. ACCESSIBILITY

EN8 to Nazaré. Pinus forest at the right side of the road, with a wide entrance full of sand.

2. SITE CHARACTERIZATION

GPS coordinates	39.59430/-9.04709 (WGS84)	**Temperature Air/Soil**	17.05/16.8
No visits	2	**Humidity Air/Soil**	67.5/68.45
Vegetation	1	**Human use**	1
Soil / Type	1. Sandy	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 21

REGION:	BEIRA LITORAL	MILITARY CHART:	250
COUNTY	SOURE		
POLYGON:	COIMBRA	NAME:	SOURE

1. ACCESSIBILITY

Road Soure-Louriçal, intersection to Louriçal and Figueira da Foz, turn to Samuel/Gesteira. Pinus forest at the left side of the road.

2. SITE CHARACTERIZATION

GPS coordinates	40.08013/-8.65041 (WGS84)	**Temperature Air/Soil**	22.9/25.95
No visits	3	**Humidity Air/Soil**	50.6/48.8
Vegetation	2	**Human use**	1
Soil / Type	2. Sandy	**Ticks**	Presence
Forest cover	3	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 22

REGION:	BEIRA LITORAL	*MILITARY CHART:*	*219*
COUNTY	MEALHADA		
POLYGON:	COIMBRA	*NAME:*	BUÇACO

1. ACCESSIBILITY

Mata Nacional do Buçaco, at the curve with the sign "Hotel Buçaco". Small green signs "Vale dos Fetos" and "Porto das Lapas"

2. SITE CHARACTERIZATION

GPS coordinates	40.37758/-8.37021 (WGS84)	**Temperature Air/Soil**	18.05/17.8
No visits	3	**Humidity Air/Soil**	59.55/64.3
Vegetation	6	**Human use**	6
Soil / Type	1	**Ticks**	Presence
Forest cover	3	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 23

REGION:	BEIRA LITORAL	*MILITARY CHART:*	*232*
COUNTY	ARGANIL		
POLYGON:	COIMBRA	*NAME:*	MATA DE MARGARAÇA

1. ACCESSIBILITY

End of the road from Coja and Fraga da Pena. Next to the shelter house.

2. SITE CHARACTERIZATION

GPS coordinates	40.21649/-7.91913 (WGS84)	**Temperature Air/Soil**	22.7/22
No visits	3	**Humidity Air/Soil**	52.6/64.3
Vegetation	5	**Human use**	6
Soil / Type	1	**Ticks**	Presence
Forest cover	3	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 24

REGION:	BEIRA ALTA	*MILITARY CHART:*	*175*
COUNTY	SEVER DO VOUGA		
POLYGON:	VISEU	*NAME:*	PARADELA

1. ACCESSIBILITY

Next to Sever do Vouga, to Paradela. Left side of the road there is steep road, before a sign "Paradela"

2. SITE CHARACTERIZATION

GPS coordinates	40.70284/-8.35661 (WGS84)	**Temperature Air/Soil**	17.85/18.45
No visits	2	**Humidity Air/Soil**	64.4/66.5
Vegetation	2	**Human use**	1
Soil / Type	2	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 25

REGION:	BEIRA ALTA	*MILITARY CHART:*	*158*
COUNTY	VILA NOVA DE PAIVA		
POLYGON:	VISEU	*NAME:*	VILA NOVA DE PAIVA

1. ACCESSIBILITY

Road Vila Nova de Paiva to Viseu. Pinus forest with dense shrubs. Entrances at both sides of the road.

2. SITE CHARACTERIZATION

GPS coordinates	40.82421/-7.77108 (WGS84)	**Temperature Air/Soil**	25/25.45
No visits	2	**Humidity Air/Soil**	21.4/28.45
Vegetation	2	**Human use**	1
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 26

REGION:	BEIRA ALTA	*MILITARY CHART:*	*200*
COUNTY	NELAS		
POLYGON:	VISEU	*NAME:*	CANAS DE SENHORIM

1. ACCESSIBILITY

After the exit from Canas de Senhorim, there is a pinus forest at the right side of the road, near an olive field.

2. SITE CHARACTERIZATION

GPS coordinates	40.51824/-7.89969 (WGS84)	**Temperature Air/Soil**	38.7/37.2
No visits	2	**Humidity Air/Soil**	17/36.1
Vegetation	2	**Human use**	1
Soil / Type	2	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 27

REGION:	BEIRA INTERIOR	*MILITARY CHART:*	*191*
COUNTY	CELORICO DA BEIRA		
POLYGON:	GUARDA	*NAME:*	CELORICO DA BEIRA

1. ACCESSIBILITY

Road Seia-Celorico da Beira, before Cortiço da Serra. Right side of the road.

2. SITE CHARACTERIZATION

GPS coordinates	40.58141/-7.45237 (WGS84)	**Temperature Air/Soil**	21.2/21.55
No visits	2	**Humidity Air/Soil**	42.55/72.25
Vegetation	5	**Human use**	2
Soil / Type	1	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 28

REGION:	BEIRA ALTA	*MILITARY CHART:*	*248*
COUNTY	PENAMACOR		
POLYGON:	GUARDA	*NAME:*	PENAMACOR

1. ACCESSIBILITY

Serra Malcata Natural Park. Entrance by the road from Sabugal to Penamacor, after Meimoa. Secondary road from the Shooting Area.

2. SITE CHARACTERIZATION

GPS coordinates	40.20241/-7.12493 (WGS84)	**Temperature Air/Soil**	23.65/23.75
No visits	2	**Humidity Air/Soil**	34.95/47.4
Vegetation	4	**Human use**	6
Soil / Type	2	**Ticks**	Absence
Forest cover	1	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 29

REGION:	BEIRA BAIXA	*MILITARY CHART:*	*281*
COUNTY	IDANHA-A-NOVA		
POLYGON:	CASTELO BRANCO	*NAME:*	OLEDO

1. ACCESSIBILITY

Road S. Miguel Anchor-Idanha-a-Nova. Before Oledo, in a private garden "Casa de Stanto António de Alvarinheiro"

2. SITE CHARACTERIZATION

GPS coordinates	39.59433/-9.04711 (WGS84)	**Temperature Air/Soil**	21.6/21.4
No visits	2	**Humidity Air/Soil**	34.5/63.4
Vegetation	6	**Human use**	4
Soil / Type	1	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SITE 30

SAMPLE SITES (NATIONWIDE STUDY)

REGION:	RIBATEJO	*MILITARY CHART:*	*342*
COUNTY	CHAMUSCA		
POLYGON:	ABRANTES	*NAME:*	ULME

1. ACCESSIBILITY

Road Ulme-Abrantes. Right side of the road. Oak land with a sand road.

2. SITE CHARACTERIZATION

GPS coordinates	39.27958/-8.37881 (WGS84)	**Temperature Air/Soil**	23.3/29
No visits	1	**Humidity Air/Soil**	36.6/32
Vegetation	5	**Human use**	1
Soil / Type	1. Sandy	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 31

REGION:	**ALGARVE**	*MILITARY CHART:*	***585***
COUNTY	**LAGOS**		
POLYGON:	**MONCHIQUE**	*NAME:*	**SERRA ESPINHAÇO DE CÃO**

1. ACCESSIBILITY

Road Aljezur-Lagos. Left side of the road. A small entrance after a curve. Near a hunting area symbol (Procº 1608).

2. SITE CHARACTERIZATION

GPS coordinates	37.20505/-8.76591 (WGS84)	**Temperature Air/Soil**	33.2/36.5
No visits	1	**Humidity Air/Soil**	22.9/22.8
Vegetation	5	**Human use**	2
Soil / Type	3. Rocks	**Ticks**	Presence
Forest cover	1	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 32

REGION:	ALGARVE	*MILITARY CHART:*	*597*
COUNTY	LOULÉ		
POLYGON:	LOULÉ	*NAME:*	BESTEIROS

1. ACCESSIBILITY

EN124 Silves-B. Velho. Just after Salir there is a small village named Besteiros. Oak land at the right side of the road, between to white houses

2. SITE CHARACTERIZATION

GPS coordinates	37.23300/-8.01700 (WGS84)	**Temperature Air/Soil**	34.3/36.55
No visits	1	**Humidity Air/Soil**	9.4/9
Vegetation	5	**Human use**	4
Soil / Type	2	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SITE 33

SAMPLE SITES (NATIONWIDE STUDY)

REGION:	DOURO LITORAL	*MILITARY CHART:*	*174*
COUNTY	MURTOSA		
POLYGON:	PORTO	*NAME:*	MURTOSA

1. ACCESSIBILITY

Road to Murtosa, before the entrance to the Industrial Area.

2. SITE CHARACTERIZATION

GPS coordinates	40.76141/-8.63035 (WGS84)	**Temperature Air/Soil**	28.5/28.4
No visits	2	**Humidity Air/Soil**	40.9/42.85
Vegetation	2	**Human use**	1
Soil / Type	2. Sandy	**Ticks**	1
Forest cover	3	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 34

REGION:	MINHO	*MILITARY CHART:*	*70*
COUNTY	BRAGA		
POLYGON:	BRAGA	*NAME:*	BOM JESUS

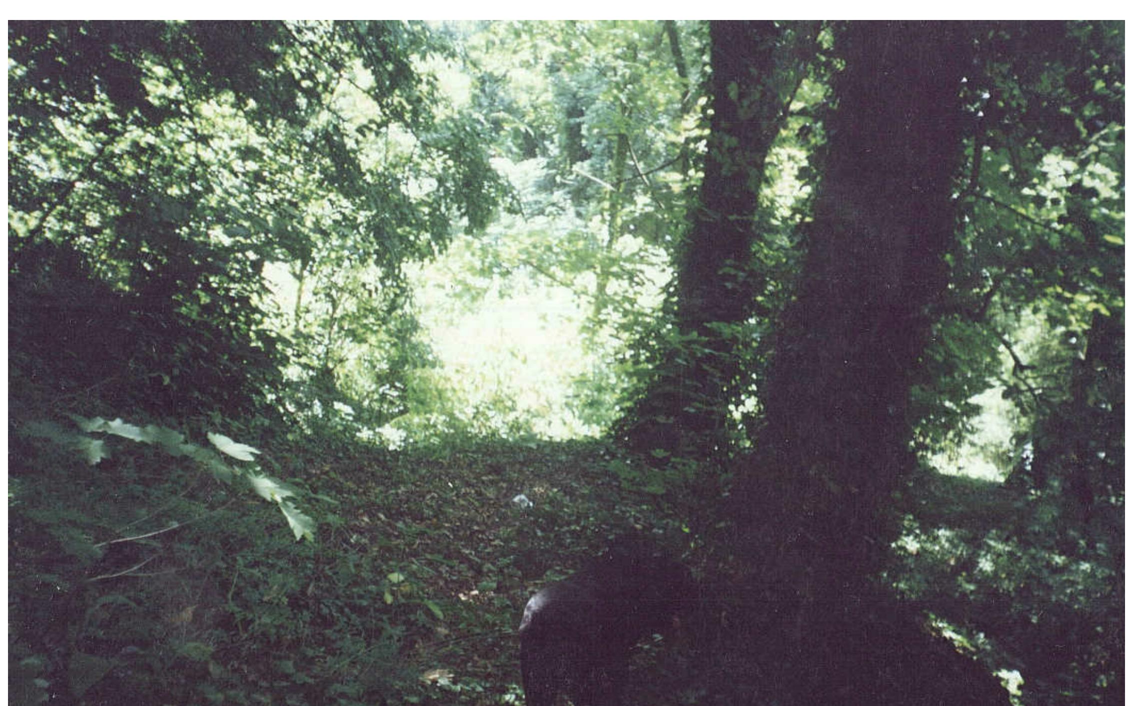
1. ACCESSIBILITY

Near Bom Jesus Church. Curve to the left, after an house that resembles a castle. Forest in the right side.

2. SITE CHARACTERIZATION

GPS coordinates	41.55666/-8.37774 (WGS84)	**Temperature Air/Soil**	32.3/32.4
No visits	2	**Humidity Air/Soil**	35.65/37.3
Vegetation	6	**Human use**	1
Soil / Type	1	**Ticks**	Absence
Forest cover	3	**Hosts**	n.d.

3. PHOTOS



RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 35

REGION:	MINHO	*MILITARY CHART:*	*30*
COUNTY	TERRAS DE BOURO		
POLYGON:	VENDA NOVA	*NAME:*	GERÊS

1. ACCESSIBILITY

National Park of Gerês, after Caldas do Gerês. Curve to the left with a small river. Steep area at the right side of the road.

2. SITE CHARACTERIZATION

GPS coordinates	41.75249/-8.14454 (WGS84)	**Temperature Air/Soil**	
No visits	1	**Humidity Air/Soil**	
Vegetation	6	**Human use**	1
Soil / Type	1	**Ticks**	Absence
Forest cover	2	**Hosts**	Several dejects

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 36

REGION:	**TRÁS-OS-MONTES**	*MILITARY CHART:*	***34***
COUNTY	**CHAVES**		
POLYGON:	**VENDA NOVA**	*NAME:*	**CHAVES**

1. ACCESSIBILITY

Road to "Chaves Camping Area". Grassland at the right side of the road, with 2 houses in the left side.

2. SITE CHARACTERIZATION

GPS coordinates	41.70459/-7.49887 (WGS84)	**Temperature Air/Soil**	21.7/21.95
No visits	2	**Humidity Air/Soil**	58.25/66.55
Vegetation	4	**Human use**	3
Soil / Type	1	**Ticks**	Presence
Forest cover	1	**Hosts**	Sheeps

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 37

REGION:	TRÁS-OS-MONTES	*MILITARY CHART:*	*102*
COUNTY	VILA REAL		
POLYGON:	VILA REAL	*NAME:*	SERRA MARÃO

1. ACCESSIBILITY

N15, Road Amarante-Vila Real. Serra do Marão. Steep side with *Pinus* and *Ulmus* spp, before a sign with number 9 (at the berm).

2. SITE CHARACTERIZATION

GPS coordinates	41.28073/-7.88180 (WGS84)	**Temperature Air/Soil**	29.5/28.6
No visits	1	**Humidity Air/Soil**	35.1/55.9
Vegetation	3	**Human use**	1
Soil / Type	1	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SITE 38

SAMPLE SITES (NATIONWIDE STUDY)

REGION:	BEIRA BAIXA	*MILITARY CHART:*	*212*
COUNTY	SEIA		
POLYGON:	CASTELO BRANCO	*NAME:*	LORIGA

1. ACCESSIBILITY

2. SITE CHARACTERIZATION

GPS coordinates	40.28605/-7.70010 (WGS84)	**Temperature Air/Soil**	28.55/29.9
No visits	2	**Humidity Air/Soil**	32.6/50.75
Vegetation	6	**Human use**	1
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 39

REGION:	**ALTO ALENTEJO**	***MILITARY CHART:***	***414***
COUNTY	**ELVAS**		
POLYGON:	**PORTALEGRE**	***NAME:***	**ELVAS**

1. ACCESSIBILITY

Elvas village

2. SITE CHARACTERIZATION

GPS coordinates	38.88300/-7.16700 (WGS84)	**Temperature Air/Soil**	
No visits	1	**Humidity Air/Soil**	
Vegetation	5	**Human use**	3
Soil / Type	0	**Ticks**	Presence
Forest cover	0	**Hosts**	Sheeps

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 40

REGION:	ESTREMADURA	*MILITARY CHART:*	*352*
COUNTY	SANTARÉM		
POLYGON:	RIO MAIOR	*NAME:*	AMIAIS DE CIMA

1. ACCESSIBILITY

N114, left side of the road Rio Maior-Alcanena. Village of Amiais de Cima, just before the end of the village (near the sign)

2. SITE CHARACTERIZATION

GPS coordinates	39.85787/-8.70055 (WGS84)	**Temperature Air/Soil**	20.95/24.05
No visits	1	**Humidity Air/Soil**	34/54.35
Vegetation	3	**Human use**	1
Soil / Type	2. Sandy	**Ticks**	Presence
Forest cover	1	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 41

REGION:	RIBATEJO	*MILITARY CHART:*	*356*
COUNTY	PONTE DE SOR		
POLYGON:	ABRANTES	*NAME:*	ROSMANINHAL

1. ACCESSIBILITY

N244 Ponte de Sôr-Gavião. Left side of the road, after the village Rosmaninhal. Steep side with oaks and pinus, with a small entrance before a brick wall at the right side

2. SITE CHARACTERIZATION

GPS coordinates	39.45263/-8.75622 (WGS84)	**Temperature Air/Soil**	21.5/22.15
No visits	1	**Humidity Air/Soil**	28.8/40.7
Vegetation	5	**Human use**	1
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 42

REGION:	BEIRA LITORAL	*MILITARY CHART:*	*285*
COUNTY	POMBAL		
POLYGON:	LEIRIA	*NAME:*	MEIRINHA

1. ACCESSIBILITY

EN1, Leiria-Pombal. After a Meirinha there is a gas bomb at the right side of the road. It's just after this bomb.

2. SITE CHARACTERIZATION

GPS coordinates	39.62997/-8.63025 (WGS84)	**Temperature Air/Soil**	25/26.95
No visits	1	**Humidity Air/Soil**	42.75/43.25
Vegetation	2	**Human use**	1
Soil / Type	1	**Ticks**	Absence
Forest cover	3	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 43

REGION:	BEIRA LITORAL	*MILITARY CHART:*	*309*
COUNTY	VILA NOVA DE OURÉM		
POLYGON:	LEIRIA	*NAME:*	FÁTIMA

1. ACCESSIBILITY

EN356, Ourém-Fátima. Right side of the road, curve with a secondary road going down the hill

2. SITE CHARACTERIZATION

GPS coordinates	39.42112/-7.60388 (WGS84)	**Temperature Air/Soil**	26.7/27.85
No visits	1	**Humidity Air/Soil**	41.75/41.3
Vegetation	5	**Human use**	4
Soil / Type	1	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 44

REGION:	ALTO ALENTEJO	*MILITARY CHART:*	*347*
COUNTY	PORTALEGRE		
POLYGON:	PORTALEGRE	*NAME:*	RASA

1. ACCESSIBILITY

Next to the intersection Marvão-Portalegre, after Castelo de Vide. Curve to the right side, near a small river (intersection with Rasa)

2. SITE CHARACTERIZATION

GPS coordinates	39.32926/-7.42261 (WGS84)	**Temperature Air/Soil**	33.35/33.8
No visits	1	**Humidity Air/Soil**	18.95/25.75
Vegetation	3	**Human use**	3
Soil / Type	1. Clayish	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 45

REGION:	ALTO ALENTEJO	*MILITARY CHART:*	*385*
COUNTY	ARRONCHES		
POLYGON:	PORTALEGRE	*NAME:*	ARRONCHES

1. ACCESSIBILITY

Road to Arronches. Curve to the left. There is a road at the left side that goes up.

2. SITE CHARACTERIZATION

GPS coordinates	38.70058/-7.49905	**Temperature Air/Soil**	28.15/29.55
No visits	1	**Humidity Air/Soil**	28.35/37.55
Vegetation	6	**Human use**	1
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 46

REGION:	ALENTEJO	*MILITARY CHART:*	*451*
COUNTY	REDONDO		
POLYGON:	EVORA	*NAME:*	BENCATEL

1. ACCESSIBILITY

Road Bencatel-Redondo. Eucalyptus forest at the right side. Left side has a secondary road.

2. SITE CHARACTERIZATION

GPS coordinates	39.87235/-7.42848 (WGS84)	**Temperature Air/Soil**	29.35/29.95
No visits	1	**Humidity Air/Soil**	25.15/28.85
Vegetation	2	**Human use**	1
Soil / Type	3. Sandy	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 47

REGION:	BEIRA BAIXA	*MILITARY CHART:*	292
COUNTY	CASTELO BRANCO		
POLYGON:	CASTELO BRANCO	*NAME:*	ESCALOS DE BAIXO

1. ACCESSIBILITY

EN240, Castelo Branco-Monfortinho. Right side of the road, a secondary road with some oaks.

2. SITE CHARACTERIZATION

GPS coordinates	40.25555/-7.71827 (WGS84)	**Temperature Air/Soil**	32.25/33.05
No visits	1	**Humidity Air/Soil**	20.9/34.3
Vegetation	6	**Human use**	2
Soil / Type	2. Sandy	**Ticks**	Presence
Forest cover	2	**Hosts**	Rabbits

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 48

REGION:	BEIRA BAIXA	*MILITARY CHART:*	*278*
COUNTY	OLEIROS		
POLYGON:	CASTELO BRANCO	*NAME:*	CARVALHO

1. ACCESSIBILITY

EN122, road to Oledos (direction Coimbra). Curve to the left with a pic-nic area. Secondary road, a few meters ahed that goes to some vegetable gardens

2. SITE CHARACTERIZATION

GPS coordinates	38.76448/-8.41272 (WGS84)	**Temperature Air/Soil**	31.3/32.1
No visits	1	**Humidity Air/Soil**	26.7/49.85
Vegetation	5	**Human use**	3
Soil / Type	2	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 49

REGION:	TRÁS-OS-MONTES	*MILITARY CHART:*	*152*
COUNTY	FIG. CASTELO RODRIGO		
POLYGON:	VILA NOVA FOZ COA	*NAME:*	FIGUEIRA CASTELO RODRIGO

1. ACCESSIBILITY

Bridge at the road from Pinhel to Figueira de Castelo Rodrigo. Right side of the road, a secondary road next to an hunting area symbol

2. SITE CHARACTERIZATION

GPS coordinates	40.58195/-7.45231 (WGS84)	**Temperature Air/Soil**	31.95/32.15
No visits	1	**Humidity Air/Soil**	18.9/26.2
Vegetation	6	**Human use**	4
Soil / Type	2	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 50

REGION:	TRÁS-OS-MONTES	*MILITARY CHART:*	*131*
COUNTY	TORRE DE MONCORVO		
POLYGON:	VILA NOVA FOZ COA	*NAME:*	FREIXO ESPADA CINTA

1. ACCESSIBILITY

Road Freixo Espada Cinta to Moncorvo. House at the right side of the road, with a sign "Parapente"

2. SITE CHARACTERIZATION

GPS coordinates	40.81847/-7.03909 (WGS84)	**Temperature Air/Soil**	29.55/29.95
No visits	1	**Humidity Air/Soil**	19.05/20.95
Vegetation	6	**Human use**	4
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 51

REGION:	**TRÁS-OS-MONTES**	*MILITARY CHART:*	***25***
COUNTY	**BRAGANÇA**		
POLYGON:	**BRAGANÇA**	*NAME:*	**PARQUE MONTESINHO**

1. ACCESSIBILITY

EN103-7, just next to the exit from IP4, at Portela. Secondary road at the left side going slightly up, next to Rabal, just before França

2. SITE CHARACTERIZATION

GPS coordinates	41.86700/-6.75000 (WGS84)	**Temperature Air/Soil**	29.05/29.1
No visits	1	**Humidity Air/Soil**	31.85/41.25
Vegetation	5	**Human use**	2
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 52

REGION:	**TRÁS-OS-MONTES**	*MILITARY CHART:*	***87***
COUNTY	**MONDIM DE BASTO**		
POLYGON:	**VILA REAL**	*NAME:*	**PARQUE NATURAL DE ALVÃO**

1. ACCESSIBILITY

EN304, Vila Real-Mondim de Basto. Large curve to the right with some trees and lots of shadow. Stone wall at the left side

2. SITE CHARACTERIZATION

GPS coordinates	41.35000/-7.88300 (WGS84)	**Temperature Air/Soil**	28.35/29.55
No visits	1	**Humidity Air/Soil**	34.35/46
Vegetation	3	**Human use**	1
Soil / Type	1	**Ticks**	Absence
Forest cover	2	**Hosts**	n.d

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 53

REGION:	**ESTREMADURA**	*MILITARY CHART:*	***402***
COUNTY	**MAFRA**		
POLYGON:	**LISBOA**	*NAME:*	**TAPADA NACIONAL DE MAFRA**

1. ACCESSIBILITY

Road to Mafra, turn to Gradil. Left side of the road.

2. SITE CHARACTERIZATION

GPS coordinates	38.95000/-9.31700 (WGS84)	**Temperature Air/Soil**	12.9
No visits	3	**Humidity Air/Soil**	73
Vegetation	6	**Human use**	5
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	Several species

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 54

REGION:	ALENTEJO	*MILITARY CHART:*	*506*
COUNTY	GRÂNDOLA		
POLYGON:	GRÂNDOLA	*NAME:*	HERDADE RIBEIRA ABAIXO

1. ACCESSIBILITY

Road Grândola- Santiago do Cacém. First intersection to the left. Secondary road at the right side, until reaching a white house.

2. SITE CHARACTERIZATION

GPS coordinates	38.11700/-8.70000 (WGS84)	**Temperature Air/Soil**	
No visits	2	**Humidity Air/Soil**	
Vegetation	4	**Human use**	2
Soil / Type	0	**Ticks**	Presence
Forest cover	2	**Hosts**	Several species

3. PHOTOS

RECORD FILES

SAMPLE SITES (NATIONWIDE STUDY)

SITE 55

REGION:	**RIBATEJO**	*MILITARY CHART:*	***444***
COUNTY	**PALMELA**		
POLYGON:	**CORUCHE**	*NAME:*	**ÁGUAS DE MOURA**

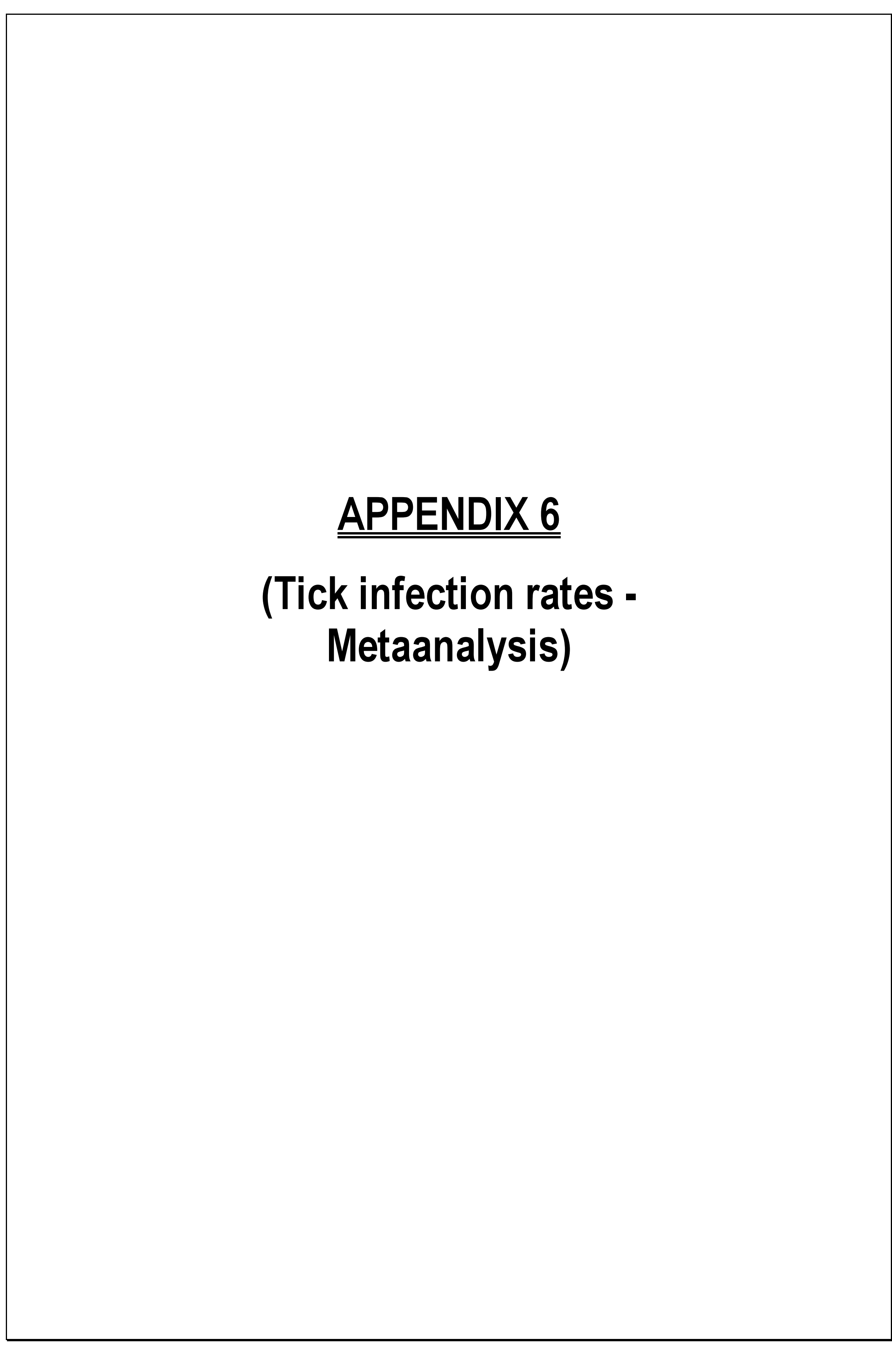
1. ACCESSIBILITY

EN5. Secondary road to Pinheiro. 1 km ahead the road opens in two roads. House in front.

2. SITE CHARACTERIZATION

GPS coordinates	38.58300/-8.70000 (WGS84)	**Temperature Air/Soil**	
No visits	2	**Humidity Air/Soil**	
Vegetation	5	**Human use**	2
Soil / Type	2	**Ticks**	Presence
Forest cover	2	**Hosts**	Several species

3. PHOTOS



APPENDIX 6

(Tick infection rates - Metaanalysis)

Table A6.1 – *I. ricinus* infection rates by culture technique

Country	Stage	Techniques	Infection rate (%)	Reference
Bulgaria	adult	Culture	17.0	Christova *et al.*, 1998
Czech Rep.	larvae	Culture	5.1	Hubalek *et al.*, 1996
	nymphs		11.7	Hubalek *et al.*, 1996
Finland	total	Culture	5.5	Juntilla *et al.*, 1994
	nymphs		3.8	
	females		7.5	
	males		5.3	
France	total	Culture	48.0	Pichot *et al.*, 1994
Germany	total	Culture	22.8	Gupta *et al.*, 1995
	nymphs		25.0	
	females		19.7	
	males		21.1	
	adults	Culture	16.3	Ackermann *et al.*, 1984 *fide* Hubalek & Halouska, 1998
Italy	adults	Culture	10.0	Solari *et al.*, 1996 *fide* Hubalek & Halouska, 1998
Poland	total	Culture	20.0	Dabrowski *et al.*, 1994
Portugal	total	Culture	17.0	Quaresma, 2004
	females		28.6	
	males		6.5	
	adults	Culture	1.2-14.0	Baptista *et al.*, 2004
	males	Culture	37.5	Núncio *et al.*, 1993
Slovakia	total	Culture	33.0	Gern *et al.*, 1999
	females		32.0	
	males		33.0	
Slovenia	total	Culture	19.0	Strle *et al.*, 1995
	nymphs		13.0	
	females		35.0	
	males		22.0	
Spain	total	Culture	11.0	Barral *et al.*, 2002
	nymphs		13.3	
	adults		4.4	
Sweden	total	Culture	26.0	Gustafson, 1994
Switzerland	larvae	Culture	14.0	Humair *et al.*, 1993
(on rodents)	nymphs		50.0	
(on birds)	larvae	Culture	16.0	Humair *et al.*, 1993a
	nymphs		22.0	
	nymphs	Culture	23.3	Humair & Gern, 1998
	females		41.2	
	males		9.1	
	adults	Culture	8.9	Miserez *et al.*, 1990
	total	Culture	5.0	Péter *et al.*, 1995
	total	Culture	13.7	Humair *et al.*, 1995
	nymphs		5.0	
	females		27.3	
	males		43.0	
	adults	Culture	16.5	Leuba-Garcia *et al.*, 1994
Turkey	total	Culture	4.0	Guner *et al.*, 2003

Table A6.2 – *I. ricinus* infection rates by microscopic techniques

Country	Stage	Techniques	Infection rate (%)	Reference
Austria	Total		20.0	Stanek *et al.*, 1988
	adults	DF	21.2	Kaaserer *et al.*, 1994 *fide* Hubalek & Halouska, 1998
	nymphs	DF	19.6	Stunzner, 1997 *fide* Hubalek & Halouska, 1998
	adults		24.1	
Belgium	nymphs	IFA	9.6	Martin *et al.*, 1990 *fide* Hubalek & Halouska, 1998
	adults		15.2	
Bulgaria	larvae	IFA	0.6	Angelov, 1995 *fide* Hubalek & Halouska, 1998
	nymphs		3.4	
	adults		26.9	
Croatia	total		27-31.7	Stajkovic *et al.*, 1993
Czech Rep.	larvae	DF	0	Hubalek *et al.*, 2004
	nymphs		9.0	
	females		20.0	
	total		8(3-20.0)	Stanek *et al.*, 1988
	larvae	DF	6.3	Hubalek *et al.*, 1998
	nymphs		17.2	
	adults		23.2	
	females		22.6	
	males		23.7	
	nymphs	DFA	6.0	Basta *et al.*, 1999
	adults		8.1	
	nymphs	DF	16.2	Hubalek *et al.*, 2003
	females		28.6	
	males		29	
	nymphs	DF	16.8	Hubalek *et al.*, 2003a
	females		24.9	
	males		26.1	
	total	DF	6.5	Janouskovcova *et al.*, 2004
	larvae		1.3	
	nymphs		6.0	
	females		8.3	
	males		12.2	
	nymphs	DFA	14.7	Zeman, 1998
	adults		19.1	
	adults	DF	12.8	Kmetv *et al.*, 1986 *fide* Hubalek & Halouska, 1998
	nymphs	DF	7.9	Svatková *et al.*, 1987 *fide* Hubalek & Halouska, 1998
	adults		9.1	
	adults	DF	42.9	Markvart *et al.*, 1987 *fide* Hubalek & Halouska, 1998
	nymphs	IFA	8.2	Pokornv, 1990 *fide* Hubalek & Halouska, 1998
	adults		15.9	
	nymphs	IFA	3.8	Pokornv & Zahradková, 1990 *fide* Hubalek & Halouska, 1998

	adults		14.5	
	nymphs	DF	7.0	Chmela, 1994 *fide* Hubalek & Halouska, 1998
	adults		11.8	
	larvae	DF	6.3	Halouzka *et al.*, 1995 *fide* Hubalek & Halouska, 1998
Denmark	total	IFA	7-22.0	Landbo & Flong, 1992
	nymphs		20.3	
	adults		15.7	
	nymphs	DFA	5.0	Jensen *et al.*, 2000
	adults		6.0	
France	nymphs	DF	27.0	Matuschka *et al.*, 1999
	nymphs	IFA	10-14.0	Doby *et al.*, 1991
	nymphs	IFA	7.14-9.1	Doby & Bigaignon, 1992
	total	IFA	9.56	Doby *et al.*, 1995
	total	IFA	7.06	Gilot *et al.*, 1996
	nymphs		5.0	
	females		12.5	
	males		11.2	
	nymphs	DFA	12.4	Zhioua *et al.*, 1996
	females		2.9	
	males		2.8	
	nymphs	DFA	21.1	Anderson *et al.*, 1986 *fide* Hubalek & Halouska, 1998
	adults		22.2	
	larvae	IFA	6.0	Perez-Eid, 1995 *fide* Hubalek & Halouska, 1998
	nymphs		10.0	
	adults		8.6	
Italy	total	IFA	0.55	Ciceroni *et al.*, 1998
Germany	nymphs	IFA	12.1	Fingerle *et al.*, 1995
	females		20.2	
	males		25.2	
(from human	total	IFA	36.6	Fingerle *et al.*, 1998
	nymphs		27.8	
	females		49.0	
	males		100	
	nymphs	IFA	19.0	Hu *et al.*, 2001
	nymphs	DF	25.0	Matuschka *et al.*, 1996
	total	IFA	13.6	Stanek *et al.*, 1988
	larvae		0-4.5	
	nymphs		3.1-25.7	
	adults		11.4-33.8	
	larvae	DFA	1.1	Wilske *et al.*, 1987 *fide* Hubalek & Halouzka, 1998
	nymphs		10.6	
	adults		20.9	
	larvae	IFA	1.0	Matuschka *et al.*, 1992
	nymphs		20.0	
	females		18.4	
	males		11.9	
	total	DF	16.7	Gupta *et al.*, 1995
	nymphs		19.4	

	females		10.9	
	males		10.8	
	total	IFA	23.9	
	nymphs		22.4	
	females		33.3	
	males		15.0	
	total	IFA	14.4	Kampen *et al.*, 2004
	nymphs	IFA	31.0	Dorn & Sunder, 1997
	females		35.0	
	males		34.0	
	nymphs	DF	6.7	Pelz *et al.*, 1989 *fide* Hubalek & Halouzka, 1998
	adults		28.7	
	nymphs	IFA	18.4	Kahl *et al.*, 1992 *fide* Hubalek & Halouzka, 1998
	larvae	DFA	1.1	Bergmann *et al.*, 1992 *fide* Hubalek & Halouzka, 1998
	nymphs		4.1	
	adults		12.5	
	nymphs	DFA	5.6	Oheim & Herrmann, 1994 *fide* Hubalek & Halouzka, 1998
	adults		11.6	
	larvae	IFA	1.0	Kurtenbach *et al.*, 1995
	nymphs		6.3	
	adults		10-20	
Lithuania	adults	DF	8.1-16.2	Rockiene, 1992 *fide* Hubalek & Halouska, 1998
Morocco	total	DF	47.8	Sarih *et al.*, 2003
Netherlands	nymphs	DF	11.6	De Boer *et al.*, 1993 *fide* Hubalek & Halouska, 1998
	adults		21.7	
	larvae	IFA	10.8	Rijpkema *et al.*, 1994 *fide* Hubalek & Halouska, 1998
	nymphs		24.6	
	adults		24.6	
Norway	nymphs	IFA	4.0	Mehl *et al.*, 1987 *fide* Hubalek & Halouska, 1998
	adults		24.1	
Poland	total	IFA	17.7	Kosik-Bogacka *et al.*, 2004
	total	IFA	10.8	Kosic-Bogacka *et al.*, 2004a
	nymphs		9.9	
	females		21.3	
	males		20.8	
	total	IFA	11.5	Wegner *et al.*, 1994
	nymphs		7.5	
	adults		18.8	
	females		18.7	
	males		18.9	
	larvae	IFA	2.7	Sinski *et al.*, 1994
	females		22.2	
	males		23.0	
	total	IFA	9.6-11.6	Bukowska, 2002

	total	DF	8.8	Cisak *et al.*, 2002
	nymphs		4.0	
	females		16.7	
	males		7.1	
	total	IFA	16.2	Michalik *et al.*, 2003
	nymphs		15.5	
	females		30.8	
	males		15.9	
	total	IFA	9.6-11.6	Bukowska *et al.*, 2003
	nymphs		8.6-10.8	
	females		19.3-25.9	
	males		19.7-22.8	
	total	IFA	10.3	Wegner *et al.*, 1997
	nymphs		8.2	
	females		14.9	
	males		18.9	
Russia	total	DF	11.2	Alekseev *et al.*, 1998
	total	DF	11.5	Alekseev *et al.*, 2001a
	adults	IFA	16.8	Korenberg *et al.*, 1987 *fide* Hubalek & Halouska, 1998
	adults	DF	34.4	Kovalevskii *et al.*, 1988 *fide* Hubalek & Halouska, 1998
	adults	DF	18.5	Alekseev *et al.*, 1993 *fide* Hubalek & Halouska, 1998
Serbia	adults	DF	24.0	Stajkovic *et al.*, 1996 *fide* Hubalek & Halouska, 1998
	adults	DF	30.6	Dmitrovio, 1995 *fide* Hubalek & Halouska, 1998
Slovakia	total	DF	8.8	Schwarzova & Ciznar, 2004
	total	DFA	49.0	Gern *et al.*, 1999
	females		42.0	
	males		56.0	
	total	DF	4.8-17.2	Stepanova-Tresová *et al.*, 2000
	nymphs		4-11.6	
	adults		5-19.3	
	adults	DF	9.1	Svatkova *et al.*, 1987 *fide* Hubalek & Halouska, 1998
	adults	DF	9.2	Kmety *et al.*, 1990 *fide* Hubalek & Halouska, 1998
	adults	DF	17.9	Rehacek, 1992 *fide* Hubalek & Halouska, 1998
	adults	IFA	5.2	Prokopcakova *et al.*, 1992 *fide* Hubalek & Halouska, 1998
Slovenia	nymphs	IFA	4.4	Ruzic-Sablijic *et al.*, 1993
	adults		23.5	
Spain	total	IFA	12.2	Revuelta, 1991
	females		13.0	
	males		5.6	
	total	IFA	28.3	Estrada-Pena & Revuelta, 1991
	total	IFA	11.0	Oteo *et al.*, 1991 fide Saz *et al.*, 1995
(on cows)	adults	IFA	14.0	Estrada-Pena *et al.*, 1995
	females		15.4	

	males		8.9	
(on birds)	nymphs	IFA	51.0	
	larvae	IFA	10.5	Marquez & Constan, 1990 *fide* Hubalek & Halouska, 1998
	nymphs		13.8	
Sweden	nymphs	IFA	3.8-21.1	Talleklint & Jaenson, 1996
	larvae	IFA	0.7	Matuschka *et al*., 1993
	nymphs		19.0	
	adults		15.0	
	total	IFA	30.0	Gustafson, 1994
	total	DF	7.0	
	nymphs	DF	2.0	Bergstrom *et al*., 1992
	adults		19.0	
	nymphs	IFA	7.0	Mejlon & Jaenson, 1993
	females		36.0	
	males		12.0	
	nymphs	IFA	10.0	Gustafson *et al*., 1995
	adults		15.0	
	nymphs	DF	9.0	Mejlon, 2000
	adults		20.0	
	females		26-29.0	
	males		14-15.0	
	nymphs	IFA	17.4	Berglund & Eitrem, 1993 *fide* Hubalek & Halouska, 1998
	adults		26.3	
Switzerland	nymphs	M	27.0	Lebet & Gern, 1994
	total	-	20(5-34)	Stanek *et al*., 1988
	nymphs	-	18.3	Aeschlimann *et al*., 1986 *fide* Gustafson, 1994
	larvae	DFA	3.2	Miserez *et al*., 1990
	nymphs		36.2	
	adults		25.0	
	larvae		3.1	Zhioua *et al*., 1994
	nymphs		12.8	
	adults		14.5	
	adults	IFA	23.4	Péter *et al*., 1995
	adults	DF	15.2	
	nymphs	DF	20.0	Jouda *et al*., 2004
	adults		35.0	
	nymphs	DFA	17.0	Burgdorfer, 1984 *fide* Hubalek & Halouska, 1998
	adults		36.3	
	nymphs	DFA	27.3	Caflisch *et al*., 1984 *fide* Hubalek & Halouska, 1998
	adults		18.6	
Tunisia	total	DFA	30.5	Zhioua *et al*., 1999
	females		36.6	
	males		22.6	
U.K.	larvae	IFA	1.7	Randolph *et al*., 1995
	nymphs		6.8	
	larvae	IFA	1.0	Kurtenbach *et al*., 1995
	nymphs		1-15.0	
	females		10-20.0	

	nymphs	DFA	7.4	Livesley *et al.*, 1994
	adults		18.7	
	total	IFA	5.2-17.0	Robertson *et al.*, 2000

Table A6.3 – *I. ricinus* infection rates by PCR technique

Country	Stage	Technique	Infection rate (%)	Reference
Belgium	total	PCR	23.0	Misonne *et al.*, 1998
Bulgaria	nymphs	PCR	10.0	Christova *et al.*, 2001
	adults		32.0	
	total	PCR	40.0	Christova *et al.*, 2003
	nymphs		0-10.0	
	adults		31-41.0	
	females		42.0	
	males		8.0	
Croatia	total	PCR	45.0	Rijpkema *et al.*, 1996a
	nymphs		41.0	
	adults		47.0	
	total	PCR	45.0	Golubic *et al.*, 1998
Czech Rep.	total	PCR	8-35.0	Danielova *et al.*, 2004
	total	PCR	5.6	Zakovská & Martiníková, 2004
	larvae		1.6	
	nymphs		5.6	
	females		6.9	
	males		10.3	
	nymphs	PCR	0.9-2.9	Basta *et al.*, 1999
	adults		7.7-33.3	
Estonia	total	PCR	15.0	Makinen *et al.*, 2003
Finland	total	PCR	32.0	Juntilla *et al.*, 1999
	total	PCR	5.0	Makinen *et al.*, 2003
	nymphs		4.0	
	adults		9.0	
France	total	PCR	3.3	Halos *et al.*, 2005
	nymphs	PCR	8.2	Pichon *et al.*, 1999
	total	PCR	13.2	Quessada *et al.*, 2003
	nymphs		14.1	
	females		17.6	
	males		12.0	
	nymphs	PCR	12.0	Pichon *et al.*, 1995 *fide* Hubalek & Halouska, 1998
Ireland	nymps	PCR	11.6	Gray *et al.*, 2000
	adults		15.8	
	total	PCR	18.4	Kirstein *et al.*, 1997
	nymphs		18.5	
	adults		17.8	
	total	PCR	14.9	Kirstein *et al.*, 1997a
	nymphs		13.1	
	adults		20.1	
	females		20.7	
	males		19.5	
	nymphs	PCR	10-28.0	Gray *et al.*, 1999
Italy	nymphs	PCR	8.7	Mannelli *et al.*, 2003

	adults		38.2	
	nymphs	PCR	1.32	Rizzoli *et al.*, 2004
	nymphs	PCR	0.8	Ciceroni *et al.*, 2001
	adults		3.1	
	nymphs	PCR	17.5	Rizzoli *et al.*, 2002
	total	PCR	0-70(40)	Cinco *et al.*, 1998
	total	PCR	4.4	Cinco *et al.*, 1998a
	total(pool	PCR	45.0	Favia *et al.*, 2001
	total	PCR	19.8	Cinco *et al.*, 1997
Germany	total	PCR	42.0	Liebisch *et al.*, 1998
	total	PCR	35.0	Rauter *et al.*, 2002
	nymphs		30.0	
	adults		40.0	
	total	PCR	17.9	Maetzel *et al*, 2005
	nymphs		17.3	
	females		26.6	
	males		12.5	
	nymphs	PCR	9.0	Richter *et al.*, 1999
	adults	PCR	22.0	Beichel *et al.*, 1996
	total	PCR	11.3	Maiwald *et al.*, 1998
	total	PCR	36.2	Fingerle *et al.*, 1999
	nymphs		30.8	
	adults		37.4	
	total	PCR	21.8	Baumgarten *et al.*, 1999
	total	PCR	15.0	Schaarschmidt *et al.*, 2001
	adults	PCR	18.1	Kurtenbach *et al.*, 2001
	total	PCR	11.1	Hildebrandt *et al.*, 2003
	nymphs		8.6	
	adults		21.0	
	females		20.6	
	males		21.4	
	total	PCR	14.0(11.9/16.5)	Kampen *et al.*, 2004
	nymphs		12.9	
	adults		21.1	
	females		20.6	
	males		13.6	
	total	PCR	14-24.0	Oehme *et al.*, 2002
	nymphs	PCR	18.3	Eiffert *et al.*, 1995 *fide* Hubalek & Halouska, 1998
	adults		34.7	
	adults	PCR	22.0	Wittenbrink *et al.*, 1994 *fide* Hubalek & Halouska, 1998
Latvia	adults	PCR	31.3	Kurtenbach *et al.*, 2001
	total	PCR	28.0	Etti *et al.*, 2003
	nymphs		20.0	
	adults		8.7	
	total	PCR	18-38.0	Bormane *et al.*, 2004
Lithuania	total	PCR	16.0	Ambrasiene *et al.*, 2004
	total	PCR	19.6	Vascilo *et al.*, 2004
Netherlands	nymphs	PCR	23.0	Rijpkema *et al.*, 1995
	adults		11.0	
(on deer)	total	PCR	26.0	Rijpkema *et al.*, 1996
	larvae	PCR	5.3	Rijpkema *et al.*, 1996b

	total	PCR	13.0	Schouls *et al.*, 1999
Norway	total	PCR	16-17.0	Jenkins *et al.*, 2001
Poland	larvae	PCR	5-10.0	Pawelczyk *et al.*, 2004
	total	PCR	12.4	Stanczak *et al.*, 2004
	total	PCR	0.77	Tylewska-Wierzbanowska *et al.*, 1996
	total	PCR	10.9-12.8	Bukowska, 2002
	total	PCR	5.3	Cisak *et al.*, 2002
	nymphs		1.7	
	females		6.9	
	males		11.2	
	total	PCR	7.4	Skotarczak *et al.*, 2002
	larvae		5.5	
	nymphs		5.8	
	females		19.5	
	males		4.2	
	total	PCR	4.4	Wodecka, 2003
	larvae		4.1	
	nymphs		3.8	
	females		9.4	
	males		2.5	
	total	PCR	16.7	Skotarczak *et al.*, 2003
	larvae		42.1	
	nymphs		10.7	
	adults		20.0	
Portugal	total	PCR	48.1	Quaresma, 2004
	females		42.9	
	males		26.1	
	total	PCR	14.2(10.5-63.0	Baptista *et al.*, 2004
	adults	PCR	75.0	De Michelis *et al.*, 2000
(pools)	total	PCR	0.7	Matuschka *et al.*, 1998
(pools)	nymphs		0.5-1.2	
(pools)	adults		0.6	
	adults	PCR	14.7-74.5	Kurtenbach *et al.*, 2001
	nymphs	PCR	1.3	Matuschka *et al.*, 1994a
Russia	total	PCR	92.9	Alekseev *et al.*, 2001
Slovakia	total	PCR	20.5	Derdáková *et al.*, 2003
	nymphs		18.0	
	females		30.0	
	males		19.5	
	total	PCR	33.0	Hanincová *et al.*, 2003
	nymphs		20.3	
	adults		42.0	
	adults	PCR	40.5	Kurtenbach *et al.*, 2001
	total	PCR	38.3	Derdáková *et al.*, 2003
	nymphs		40.0	
	females		40.0	
	males		35.0	
Spain	nymphs	PCR	1.5	Guerrero, 2001
	adults		9.4	
	total	PCR	1.2	Fernandez-Soto *et al.*, 2004
	nymphs	PCR	0.05; 1.5(p)	Barral *et al.*, 2002
	adults		1.5; 9.3(p)	

Sweden	nymphs	PCR	7.0	Bergstrom *et al.*, 1992
	females		38.5	
	males		17.6	
	total	PCR	7.0	Nilsson *et al.*, 1997
	total	PCR	11.0	Fraenkel *et al*, 2002
Switzerland	total	PCR	1.0	Bernasconi *et al.*, 1997
	adults	PCR	49.0	Leutenegger *et al.*, 1999
	total	PCR	26.5	Wicki *et al.*, 2000
	nymphs		25.9	
	females		20.2	
	males		21.1	
Tunisia	total	PCR	34.0	Younsi *et al.*, 2001
	larvae		2.6	
	nymphs		33.3	
	adults		34.0	
	females		56.5	
	males		40.0	
U. K.	total	PCR	75.0	Livesley *et al.*, 1994
	larvae	PCR	1.0	Kurtenbach *et al.*, 1998b
	nymphs		2.6	
	adults		16.0	
	total	PCR	20.0	Hubbard *et al.*, 1998
	larvae		20.0	
	nymphs		37.9	
	adults		11.6	
	females		10.8	
	males		13.8	
	total	PCR	6-9.5	Robertson *et al.*, 2000
	adults	PCR	16.0	Kurtenbach *et al.*, 2001
	adults	PCR	7.7	Guy & Farquhar, 1991 *fide* Hubalek & Halouska, 1998
	larvae	PCR	5.4	Nuttall *et al.*, 1994 *fide* Hubalek & Halouska, 1998
	nymphs		37.2	
	adults		38.6	