THE EPIDEMIOLOGY AND CONTROL OF CANINE VISCERAL LEISHMANIASIS IN AMAZON BRAZIL

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by
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ABSTRACT

This thesis describes a study of the dog and fox reservoir populations of *Leishmania infantum* in an endemic focus in Amazon Brazil. Following a brief review of the relevant literature on canine visceral leishmaniasis in **Chapter 1,** the aims of the study are described. Broadly they were to **(1)** quantify the courses (and their inter-relationships) of infection, disease and infectiousness in dogs, **(2)** investigate the implications for culling strategies, **(3)** assess the relative role of dogs and foxes in transmission. **Chapter 2** describes the overall study design. A cohort of 126 previously unexposed native dogs was established in 24 endemic study communities and monitored at bimonthly intervals over a period of 24 months. A total of 756 sera were tested by ELISA, and 514 bone marrow samples were examined either as smears, or following inoculation into hamsters or culture medium. Clinical examinations were performed on 116 dogs on 562 independent occasions, and 50 dogs were experimentally exposed to colony bred *Lu. longipalpis* (the vector) in 173 xenodiagnostic trials. Longitudinal demographic and serological data were also collected on the resident dogs of 15 communities and on a sympatric free-ranging population of crab-eating foxes, *Cerdocyon thous*. These data were added to those for the same populations collected during a previous study (by the author) giving 5 years of data, all of which is described in this thesis. Using comparable methods as for dogs, 37 foxes were clinically, serologically and parasitologically sampled on 74 occasions, 26 of which were also examined by xenodiagnosis in 44 trials. The principal results of this thesis are presented in Chapters 3–6. **Chapter 3** describes the demographic parameters relevant for transmission dynamics. These showed that the resident dog population had a high turnover rate (0.42 per year) characterised by a mortality rate of 0.40 per year. Dog abundance was sustained by immigration (owner-mediated) rather than by birth. The mortality rate was positively associated with seroprevalence, incidence, and sandfly abundance in houses and animal sheds. The seroconversion rate between villages was positively associated with sandfly abundance in houses and animal sheds, and negatively associated with the mean number of dogs per household. The fox population replacement and mortality rates were similar to dogs, but with no evidence of mortality due to *Leishmania*. **Chapter 4** describes the courses of infection and disease in the sentinel population. By the end of the study 80 dogs were identified as infected, representing 93% of the 86 dogs which remained in the study for > 3 sample rounds. The seroconversion and serological recovery rates were 0.269 and

0.006 per month, respectively; the average time to infection was 115 days, and time from infection to seroconversion was 94 days. All dogs developed one or more clinical signs of CVL by the end of 24 months, with an average time from infection to clinical onset of 66 days. Only 9% of dogs classified as symptomatic by their longitudinal clinical profile fully recovered from the disease. Clinical severity outcome was positively associated with antibody titre, parasite isolation success, and mortality. Forty-nine percent of dogs with confirmed *Leishmania* infections died by the end of the study. The risk of mortality was positively associated with the severity of infection and disease. In **Chapter 5**, the results from serial xenodiagnosis of the sentinel population revealed that the onset of infectiousness occurred a median 128 days after seroconversion with a latent period (time from infection to infectiousness) of 222 days. The heterogeneity of infectiousness was extensive both between and within dogs: 45% of seropositive dogs were observed to become infectious, 20% of the infectious dogs were responsible for 80% of all sandfly infections. Infectiousness was positively associated with high antibody titres, and severe clinical signs. However, neither antibody titre, clinical signs, biochemical parameters, nor any combinations of these, proved to be reliable surrogate markers of infectiousness. **Chapter 6** provides comparable longitudinal serological and parasitological data for the fox population. Fox infection rates were similar to those for dogs, though there was an absence of clinical signs. No foxes were observed to be infectious to *Lu. longipalpis* by xenodiagnosis. **Chapter 7** concludes that **(1)** the dynamics of canine *L. infantum* infection and disease in Marajó is similar to that in Europe, **(2)** the detection of potential clinically severe cases may be possible in early infection (e.g. for treatment by the veterinarians), however **(3)** selection of infectious dogs for targeted control (treatment, elimination or other) is not possible using the immunological and clinical parameters as surrogate markers, **(4)** foxes are not important for human transmission in the presence of infected (infectious) dogs.

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1. GENERAL INTRODUCTION

1.1 The Leishmaniases

The genus *Leishmania* (Kinetoplastida: Trypanosomatidae) is a heterogeneous group of protozoan parasites which cause a spectrum of clinical diseases in humans, broadly categorised as visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). Recognised as one of the re-emerging infectious diseases of significance to public health (PAHO, 1995; WHO, 1995), the leishmaniases occur in 88 countries throughout Africa, Asia, Latin America and Europe, with an estimated prevalence of 12 million cases among 350 million people at risk, representing annual incidences of 1–1.5 million cases of CL and 500,000 cases of VL (Desjeux, 1996). In endemic Latin America (Mexico to Argentina), the minimum number of cases is 16,000 per annum (Ashford *et al.*, 1992; Desjeux, 1996), of which 90% occur in Brazil (Grimaldi *et al.*, 1989).

The parasites which usually cause VL are members of the *L. donovani* complex, including *L. chagasi* in Latin America, *L. infantum* in the circum-Mediterranean region, eastern Europe, Central Asia, and China, *L. donovani sensu stricto* in the Indian sub-continent, and *L. donovani sensu lato* in East Africa (Lainson and Shaw, 1987). VL can also result from infection with *L. amazonensis* (Barral *et al.*, 1991), and dermatrophic *L. infantum* zymodemes have been isolated from patients in the Mediterranean Basin (Gradoni and Gramiccia, 1994; Rioux *et al.*, 1986; Bettini *et al.*, 1990; Giudice *et al.*, 1998), central Asia (Tagi-zad *et al.*, 1989) and in Latin America (Oliveira *et al.*, 1986; Noyes *et al.*, 1997; Ponce *et al.*, 1991). Similarities in the DNA fragment patterns, isoenzymes, and monoclonal reactivity patterns of *L. chagasi* and *L. infantum* have led many to regard these two species as synonymous (Beverley *et al.*, 1987; Rioux *et al.*, 1990; Thomaz-Soccol *et al.*, 1993; Mauricio *et al.*, 1998b). This is the view adopted in this thesis; hereafter both species are referred to as *L. infantum*.

Traditionally, VL due to *L. infantum* is a disease of children in rural communities. More recently, VL has become an increasing problem of suburban areas, including the Brazilian cities of Rio de Janeiro, Natal, Fortaleza, Teresina, and Sao Luis (Marzochi *et*

al., 1993; Costa, 1993; Jeronimo *et al*., 1994; Arias *et al*., 1996), as well as among immuno-compromised patients (Górgolas and Miles, 1994; Alvar *et al*., 1997). In southern Europe, for example, the advent of HIV and AIDS has contributed to a shift in the age-distribution of VL cases from 70% in children less than 15 years old, to 75% among adults, of which 50-60% are VL/HIV co-infections (Alvar *et al*., 1997). Alterations in the geographical distribution of the disease in Latin America are thought to be associated predominantly with environmental alteration and human migration behaviour resulting in increased vector/human contact (Lainson, 1988; Marzochi *et al*., 1993).

1.2 Invertebrate and vertebrate hosts

Leishmania parasites are biphasal, occurring as obligatory intracellular amastigotes in the vertebrate host, and as flagellated promastigotes in the gut of Phlebotomine sandflies (Diptera: Psychodidae). All the known vectors of the human leishmaniases belong to the genus *Lutzomyia* (New World) and *Phlebotomus* (Old World) (Lewis and Ward, 1987; Killick-Kendrick, 1990). Members of the species complex *Lu. longipalpis* are the vectors of *L. infantum* throughout Latin America, except in Córdoba, Colombia, where *Lu. evansi* has been incriminated (Travi *et al*., 1990). The Old World vectors of *L. infantum* comprise a number of geographically distinct species, including *P. ariasi*, *P. perniciosus*, and *P. chinensis* (Killick-Kendrick, 1990).

Most of the leishmaniases with the exception of *L. donovani s.s.* and *L. tropica* are classified as, or suspected to be, zoonoses, with one or more non-human vertebrate host (Lainson and Shaw, 1979; Shaw and Lainson, 1987, Ashford and Bettini, 1987; Ashford, 1996). The principal zoonotic maintenance host for VL due to *L. infantum* (hereafter referred to as ZVL) is the domestic dog (*Canis familiaris*) throughout the range of the parasite. Evidence for this followed the first identified canine infection in Tunisia in the early 1900s (Nicolle and Comte, 1908), with subsequent records in the Mediterranean region (Sergent and Sergent, 1910; Giraud and Cabassu, 1933), China (Andrews, 1933), central Asia (Chodukin, 1943), and Latin America (Ponde *et al*., 1942; Deane, 1956). The dog's role as an important reservoir was further indicated by **(1)** the high prevalence of parasite positives, **(2)** the co-prevalence and/or geographical

association of dog and human infection (e.g. Deane, 1956; Marty *et al.*, 1992; Mancianti *et al.*, 1986; Coutinho *et al.*, 1985), **(3)** the fulminating nature of the disease in dogs **(4)** its ability to infect the sandfly vector(s) (Parrot *et al.*, 1930; Adler and Theodor, 1932; Feng and Chung, 1939; Chagas, 1939, cited in Deane, 1956), and, more recently, **(5)** the similarity in zymodemes isolated from dogs, humans and the sandfly vector(s) (Gramiccia *et al.*, 1982; Bettini and Gradoni, 1986; Gradoni *et al.*, 1991; Jimenez *et al.*, 1995), **(6)** significant risk of human infection and/or disease associated dog abundance (Gavgani, 1998; Kotkat *et al.*, 1986). Dogs thus fulfil the criteria of a true reservoir host, in part defined by Killick-Kendrick and Ward (1981).

With few exceptions (see below), xenodiagnosis has not been performed on any of the proposed wildlife reservoirs of *L. infantum* to quantify their role in human transmission. Rather, their incrimination is inferred from conditions **(1)** and **(2)** above. Natural wildlife infections with *L. infantum* are documented for the Carnivora (predominantly foxes and jackals), Marsupialia (New World opossums), and Rodentia, all from endemic regions of ZVL (**Table 1.1**). Although differences in sampling and isolation techniques complicate inter-specific comparisons, noticeably high parasite prevalences recorded for individual populations include 18% in red foxes, *Vulpes vulpes* (Italy, Mancianti *et al.*, 1994), 32% in common opossums, *Didelphis marsupialis* (Colombia, Corredor *et al.*, 1989a; 1989b), and 42% in crab-eating foxes, *Cerdocyon thous* (Marajó, Brazil, Silveira *et al.*, 1982; Lainson *et al.*, 1990). Only *C. thous* and *Didelphis* have been experimentally exposed to sandflies to demonstrate their ability to infect the vector (Sherlock, 1996; Travi *et al.*, 1998; Deane and Deane, 1954b; Lainson *et al.*, 1990: see note on fox nomenclature in **Table 1.1**). However, population data are not available to quantify their precise roles in human transmission relative to that of domestic dogs.

Table 1.1. Wildlife species with natural infections of suspected or confirmed *L. infantum*.

number parasite positive /n (seropositive/n)	number of symptomatics / positives	country / region	source
Carnivora			
Golden jackal *Canis aureus*			
1/20	1/1	Iran	Nadim *et al.* 1978
4/161 (6/48)	1/4 (1/6)	Iran	Hamidi *et al.* 1982
2/30 (5/30)	0/2 (0/5)	Iran	Edrissian *et al.* 1993
1[a]	1/1	Spain	Hervas *et al.* 1996
≥5/nd	nd	C. Asia	Latyshev *et al.* 1961[†], Lubova. 1973[†] Dursunova *et al.* 1965[†]
Wolf *C. lupus*			
1/nd	0/1	C. Asia	Petrisceva. 1961[†]
Red fox *Vulpes vulpes*			
25/507 (29/82)[e, g]	2/25 (0/29)	Europe	see **Table 6.1** for detail
2/19	0/2	C. Asia	Maruashvili & Bardzhadze. 1966[†]
1/36	0/1	C. Asia	Maruashvili & Bardzhadze. 1966[†]
1/10	0/1	Iran	Nadim *et al.* 1978
1/10 (2/10)	0/1 (0/2)	Iran	Edrissian *et al.* 1993
Corsac fox *V. corsak*			
≥1/nd	nd	USSR	Sergiev, 1979
Sand fox *V. pallida*			
1/4	0/1	Sudan	Kirk, 1956
Fennec fox *V. zerda*			
1/2[‡‡]	1/1	N. Africa	Conroy *et al.* 1970
Racoon dog *Nyctereutes procyonoides*			
1/5[c]	0/1	Beijing, China	Zhi-Biao *et al.* 1982, 1984
2/nd	0/2	Beijing, China	Zhi-Biao *et al.* 1982
Crab-eating fox *Cerdocyon thous*			
26/266 (13/25)[e, g, h]	3/39 (0/13)	Brazil	see **Table 6.1** for detail
Serval cat *Felis serval*			
1/2[‡]	0/1	Sudan	Hoogstraal and Heyneman, 1969
Common genet *Genetta genetta*			
1/2[‡]	0/1	Sudan	Hoogstraal and Heyneman, 1969
≥1/nd	nd	Kenya	WHO, 1990
Eurasian badger *Meles meles*			
≥1/nd	nd	USSR	Petrisceva, 1971
Dwarf mongoose *Helogale parvula*			
≥1/nd	nd	Kenya	WHO, 1990
Marsupialia			
White-eared opossum *Didelphis albiventris*			
1/57	0/1	Brazil	Sherlock *et al.* 1984
1/62	0/1	Brazil	Sherlock, 1996
Common opossum *D. marsupialis*			
12/37	0/12	Colombia	Corredor *et al.* 1989a; 1989b
5/22	0/5	Colombia	Travi *et al.* 1994

Table 1.1. *continued*.

number parasite positive /n (seropositive/n)	number of symptomatic / positive	country / region	source
Rodentia			
Nile grass rat *Arvicanthis niloticus*			
4/117[‡]	0/4	Sudan	Hoogstraal and Heyneman, 1969
Spiny mouse *Acomys capirinus*			
1/29[‡]	0/1	Sudan	Hoogstraal and Heyneman, 1969
1/114[‡]	0/1	Sudan	Hoogstraal and Heyneman, 1969
Black rat *Rattus rattus*			
1/nd[‡]	0/1	Sudan	Hoogstraal and Heyneman, 1969
1/39[‡c]	0/1	Sudan	Hoogstraal and Heyneman, 1969
2/20	0/2	Yugoslavia	Petrovic *et al.* 1975[†††]
2/68	0/2	Yugoslavia	Petrovic *et al.* 1975[†††]
3/143[d, e]	1/3[f]	Italy	Bettini *et al.* 1978; 1980
1/94[d, e]	0/1[f]	Italy	Pozio *et al.* 1981b
1/3[d]	0/1	Spain	Marquez *et al.* 1985
Brown rat *R. norvegicus*			
4/nd[††]	0/4	Yugoslavia	Petrovic *et al.* 1975[†††]
Grass gerbil *Tatera robusta*			
3/58[‡]	0/3	Kenya	Hoogstraal and Heyneman, 1969
Brazilian porcupine *Coendu prehensilis*			
1/1[b]	1/1	Bolivia	Le Pont *et al.* 1989b
Indian porcupine *Hystrix indica*			
≥1/nd	nd	USSR	Petrisceva, 1971

a captive animals
b sentinel animal
c denominator include *Mastomys natalensis*
d nominator refers to number of isolates from hamsters or culture inoculated with homogenates of more than one animal.
e 4 *R. rattus* isolates typed by isoenzymes as *L. infantum* zymodeme MON–1) (Gramiccia *et al.*, 1982) and 1 *N. procyonoides* isolate as *L. infantum* (MON-1) (Zhi-Biao *et al.*, 1984). For fox isolates see **Table 6.1**.
f denominator refers to number of isolates as for d. above.
g totals for all studies in which at least one positive animal was recorded. For further detail see **Table 6.1**.
h includes samples originally cited as *Dusicyon vetulus* (= *Lycalopex vetulus*), since these are now considered to have been *C. thous* (= *Dusicyon thous*) (Courtenay *et al.*, 1996). The Canidae nomenclature here follows that described and discussed by Ginsberg and Macdonald (1990).
† cited in Abranches (1989).
†† cited in Bettini and Gradoni (1986).
††† cited in Ashford and Bettini (1987).
‡‡ referred to in source as *L. donovani*.
‡ specific identity of isolates recently redefined (see Ashford, 1996).
nd, not reported in source.

1.3 The disease in canids

Canine visceral leishmaniasis (CVL) is a systemic disease showing similarities to that described in humans. Following the bite of an infected sandfly, the parasites multiply in the macrophages at the site of inoculation, then spread to mononuclear phagocytes of the reticuloendothelial system, including the liver, spleen, and bone marrow. This can result in chronic or symptomatic disease, the latter manifested in a broad spectrum of signs including dermatitis, fur depilation (alopecia), nail hypertrophy (onychogryphosis), skin lesions (chancres), body emaciation and weight loss, general lymphadenopathy (lymph node enlargement), hepatospenamegaly (spleen and liver enlargement), conjunctivitis, anaemia, apathy and chronic renal insufficiency (**Table 1.2**; and references therein).

In advanced stages of the disease, additional signs may include paralysis of the hind limbs (paraplegia), vomiting, diarrhoea, and intestinal bleeding. Urinary and serum biochemical alterations associated with CVL include proteinuria, elevated protein/creatinine ratio, serum proteins and globulins, inversion of the albumin/globulin ratio, decreased packed cell volume (PCV), dysproteinaemia, and increased blood urea nitrogen (BUN) (Kenan *et al.*, 1984; Abranches *et al.*, 1991; Carrera *et al.*, 1996; Bourdoiseau *et al.*, 1997; Moreno *et al.*, 1998). Progressive CVL is usually fatal in the absence of a cell-mediated response (see Immunity below); death is usually caused by severe renal failure due to an immune-mediated glomerulonephritis (Slappendel, 1988; Palacio *et al.*, 1995).

The prevalence of symptomatic infections among seropositive dogs is approximately 50% (e.g. Gradoni *et al.*, 1988; Mancianti *et al.*, 1988; Saladrigas, 1992). The clinical presentation of CVL in dogs appears consistent across the parasite's geographical range (**Table 1.2**), and is not dissimilar to the disease presented by naturally and experimentally infected foxes (Lanotte, 1975; Rioux *et al.*, 1971; and references in **Table 1.1**). More usually, wildlife infections with *Leishmania* are asymptomatic with little evidence of pathology (**Table 1.1**; see also Lainson and Shaw, 1979; Ashford, 1996). The prevalence of clinical signs among parasitologically confirmed infections include 4% (3/26) *C. thous*, 8% (2/25) *V. vulpes*, and 38% (3/8) *C. aureus* (**Table 1.1**).

Table 1.2. Prevalence of clinical and clinicopathologic signs among serologically and/or parasitologically confirmed infected dogs from endemic regions. Values represent the percentage of dogs with the condition; "common", "rare" are as quoted in the original source; nd = not reported.

location	S. Brazil	Greece	Italy	Europe	France	Portugal	Iran
source[c]	1	2	3	4	5	6	7
clinical sign							
lymphadenopathy	86	96	89	90	100	66	60
alopecia	86	nd	14-18	89[a]	84	38[b]	71
dermatitis	43	75	56	89[a]	nd	38[b]	nd
chancres	43	nd	40	9	49	34	57
onychogryphosis	43	40	24	20	73	61	54
conjunctivitis	14	50	11	33	20	"rare"	50
weight loss	nd	31	32	64	nd	"rare"	61
anorexia	100	37	17	33	nd	"rare"	nd
hepatosplenomegaly	nd	"rare"	53	33	nd	nd	76
renal failure	nd	19	16	33-45	nd	nd	nd
hyperthermia	nd	nd	6-35	35	nd	nd	nd
biochemical sign							
anemia	nd	94	60	"common"	nd	nd	nd
proteinuria	nd	68	4	85	nd	nd	nd
azotemia	nd	19	4	45	nd	nd	nd
leukopenia	nd	6	0	22	nd	nd	nd
hypoalbuminaemia	nd	nd	68	94	nd	nd	nd
hyperglobulinaemia	nd	nd	71	100	nd	nd	nd
albumin/globulin	nd	nd	76	nd	nd	nd	nd
N cases	7	52	150	80	45	61	24[d]

a dermatitis and alopecia not differentiated in source, but referred to as "skin involvement".
b stated to include dermatitis and/or depilation.
c 1 Marzochi *et al.* (1985); 2 Kontos and Koutinas (1993); 3 Ciaramella *et al.* (1997); 4 Slappendel. (1988); 5 Lanotte *et al.* (1979); 6 Abranches *et al.* (1991); and 7 Gavgani (1998).
d confirmed parasitological cases only.

1.4 Diagnosis of canine infection with *L. infantum*

1.4.1 Diagnostic techniques

Diagnosis of *Leishmania* infection in the vertebrate host is based on **(1)** direct demonstration of amastigotes by microscopical examination of Giemsa-stained lymph node, bone marrow, or spleen aspirate smears, **(2)** indirect demonstration by *in vivo* or *in vitro* cultured aspirates inoculated into susceptible animals, e.g. Syrian hamsters, or growth media (reviewed by Schnur and Jacobson, 1987), **(3)** indirect detection of amastigotes in biopsies, aspirates, or blood, using parasite DNA-based techniques, including the polymerase chain reaction (PCR) (Smyth *et al.*, 1992; Ravel *et al.*, 1995; Mathis and Deplazes, 1995) and *Leishmania* specific DNA probes (Howard *et al.*, 1991; Wilson *et al.*, 1992). Other indirect methods include **(4)** detection of lymphocyte proliferation *in vitro*, or by a delayed-type hypersensitivity reaction *in vivo* using a Montenegro Skin Test (MST), following exposure to *Leishmania* antigen (Montenegro, 1926; Cabral *et al.*, 1992; Pinelli *et al.*, 1994), and **(5)** detection of serum anti-*Leishmania* antibodies, notably IgG and IgM fractions, by immunological test. The serological tests commonly used in both canine and human population studies include the Immunofluorescence Antibody Test (IFAT) (Shaw and Voller, 1964), Enzyme Linked Immunosorbent Assay (ELISA) (Hommel *et al.*, 1978), Dot ELISA (Pappas *et al.*, 1983), Falcon Assay Screening Test (fast ELISA) (Ashford *et al.*, 1993), and Direct Agglutination Test (DAT) (Harith *et al.*, 1986; 1989).

1.4.2 Defining infection

Serological test are relatively cheap and suited to large scale surveillance, the most commonly used for dogs is IFAT. The documented sensitivities and specificities of these tests vary between laboratories, study populations, and according to the threshold titre chosen to define infection. Cut-off titres are not standardised for *Leishmania*; only in exceptional cases is the appropriate cut-off titre indicated intrinsically by a bimodal frequency distribution of endemic titres (Lanotte *et al.*, 1979; Abranches *et al.*, 1991). In this case, true positive and negative infections are differentiated by the trough between the two peaks, taken to represent the lowest titre of true infection. More commonly, titres are unimodally or multimodally distributed, and the cut-off titre

selected according to the behaviour of the test sera relative to endemic or non-endemic positive and negative controls (e.g. Evans *et al.*, 1990; Harith *et al.*, 1986).

In canine leishmaniasis, the median period between natural infection and seroconversion (the prepatent period) is estimated to be 3 months (Quinnell *et al.*, 1997). During this period, parasitological methods are useful to detect early, otherwise cryptic, infection (Berrahal *et al.*, 1996; Quinnell *et al.*, 1997). In humans, MST and T cell proliferation assays are negative during active infection but become positive after cure (i.e. post treatment, or, for asymptomatic cases, after serorecovery), and thus identify historical infection (Badaro *et al.*, 1986a; Marty *et al.*, 1992). The limited information for dogs suggest that they also mount *Leishmania* specific cellular responses only after spontaneous (clinical) recovery (Pinelli *et al.*, 1994; Martinez *et al.*, 1993; 1995). There is uncertainty, however, about the definition of "cure", and therefore the timing of T cell responsiveness (see Immunology below), since dogs may show a cellular response in the presence of anti-*Leishmania* antibody and/or *Leishmania* DNA detected by PCR (Cabral *et al.*, 1993; 1998). The performance of MST to diagnose canine *L. brasiliensis* infection has also yielded some ambiguous results (Genaro *et al.*, 1992; Pirmez *et al.*, 1988; Marzochi and Barbosa-Santos, 1988).

Anti-*Leishmania* antibody persistence following clinical recovery or apparent parasitological clearance is not well defined in dogs either, though it appears to be similar (3–12 months) to that in humans (Abranches *et al.*, 1991; Deplazes *et al.*, 1995; Badaro *et al.*, 1996a). Despite these uncertainties, a seropositive titre is generally taken to represent current infection in both humans and dogs.

1.5 Immunology

1.5.1 Cellular immunity

The importance of T cell and cytokine production in immunity against *Leishmania* infection has been demonstrated in murine models (Scott *et al.*, 1989; Liew, 1990; McSorley *et al.*, 1996). The only published studies of cytokine production in dogs -the natural host- infected with *L. infantum* are those by Pinelli *et al.* (1994; 1995). These show that in both experimentally and naturally infected dogs, resistance is generally

associated with a Th1 type CD4+ T helper cell response and secretion of interferon-γ (IFN-γ), interleukin-2 (IL2), and tumor necrosis factor-α (TNFα), which activate macrophages to kill intracellular amastigotes. In contrast, susceptibility and disease progression is related to down regulation of the Th1 response in the absence of IFN-γ and IL2. Since Pinelli and co-workers recorded this apparent dichotomous response 3 years post experimental infection, it is still unclear how soon after infection dogs mount distinct responses.

1.5.2 Differentiating resistant and susceptible dogs

Self-resolving infections or "resistant" dogs are thus indicated by a positive lymphocyte proliferation or MST reaction (Pinelli *et al*., 1994). In contrast, specific antibody is not thought to play any role in protective immunity. Consequently there are no proven serological techniques to differentiate the "resistant" and "susceptible" classes of dog. Pinelli *et al*. (1994) (see also Killick-Kendrick *et al*., 1994) were able to distinguish clinically classified susceptible and resistant dogs by the presence and absence respectively of specific antibody 3–5 years post infection. In the shorter term, antibody is probably not a useful tool since both experimentally and naturally infected dogs of mixed ages may mount simultaneous cellular and humoral responses (Cabral *et al*., 1992; 1993; 1998; Abranches *et al*., 1991). It may prove possible to differentiate the two types of dog by a subclass of antibody (such as IgG1), since its persistence in chronically ill dogs which do not respond to treatment is substantially longer than in dogs which resolve their clinical condition (Deplazes *et al*., 1995).

1.6 Control strategies for ZVL

The principal methods of ZVL control include one or combination of **(1)** human case-detection and treatment, **(2)** treatment of symptomatic infected dogs, **(3)** elimination of seropositive dogs, and **(4)** spraying domestic or peridomestic vectors with residual insecticide (Desjeux, 1996). Control measures **(2)**–**(4)** are briefly described below.

1.6.1 Treatment

The anti-leishmanial drugs used for human chemotherapy (reviewed by Olliaro and Bryceson, 1993; Gradoni *et al*., 1995) are also used, or are being evaluated, to combat

the disease in dogs. Traditionally, the first line drugs are the pentavalent antimonials in the form of sodium stiboglutconate (Pentostam®, Wellcome Foundation, UK) and meglumine antimoniate (Glucantime®, Rhone Poulenc, France). The second line drugs include Pentamidine, Amphotericin B, Liposomal Amphotericin B (AmBisome®, Vestar, USA), Aminosidine (paromomycin), and Allopurinol (Slappendel and Teske, 1997; Ginel *et al.*, 1998; Oliva *et al.*, 1995; 1998, Vexanat *et al.*, 1998; Ferrer *et al.*, 1995; Poli *et al.*, 1997; Alvar *et al.*, 1994). Despite varying degrees of clinical remission, and in some cases reductions in antibody titre, treatment against CVL does not generally lead to parasitological cure, thus prognosis is usually poor, relapses may occur in up to 75% of treated cases, and as few as 10% of advanced viscero-clinical infections show spontaneous long-term improvement (Lanotte *et al.*, 1979; Saladrigas, 1992; Gradoni *et al.*, 1987; Mancianti *et al.*, 1988). The probability of remission following treatment is inversely related to the severity of signs (Mancianti *et al.*, 1988; and references cited). Reasons for the poor efficacy of drugs against CVL, in contrast to against the human disease, is thought to be due to non-specific drug targetting (e.g. against dermal infection) and fast excretion rates of the active compounds (Valladares *et al.*, 1996; 1997).

1.6.2 Insecticides

Sandflies are generally highly susceptible to residual insecticides (reviewed by Lane, 1991), thus reductions in peridomicilary sandfly populations as well as disease transmission have been achieved in both the New and Old World by spraying houses with DDT, often as part of an anti-malarial campaign (Davies *et al.*, 1994; Vioukov, 1987). Similar success has been observed in trials using pyrethroid deltamethrin (Falcao *et al.*, 1991; Alexander *et al.*, 1995b). There is little evidence, however, of parasite eradication after the cessation of spraying (Mukhopadhyay *et al.*, 1987; Davies *et al.*, 1994). In Latin America, efforts to control peridomicilary *Lu. longipalpis* with residual pyrethroids resulted in only partial or temporary reductions in sandfly abundance (Le Pont, 1989a; Marcondes and Nascimento, 1993; Kelly *et al.*, 1997), as did unsustained spraying of exophilic sandfly sylvatic resting sites (Ready *et al.*, 1985). In Brazil, some 100,000–200,000 houses are reportedly sprayed with organophosphorates or residual insecticides (DDT or deltamethrin) per annum (Lacerda, 1994).

1.6.3 Dog culling

Anecdotal reports that eliminating seropositive dogs reduces canine and/or human incidence exist for a number of countries including China (Guan, 1991; Zhi-Biao, 1989), Brazil (Alencar, 1961; Nunes *et al.*, 1991), and Italy (Gradoni *et al.*, 1988). In each case, however, culling formed only part of an integrated control effort which included spraying insecticides, or, in the latter case, treatment of mild to asymptomatic dogs with Glucantime®. Consequently, these studies do not provide conclusive evidence that culling on its own reduces transmission.

Recent work in Brazil suggests that, in fact, it does not. In Bahia, Ashford *et al.* (1998) measured the effect of culling seropositive dogs at 12 month intervals in the absence of other forms of intervention. They reported temporary reductions in dog seroconversion rates (from 36% to 6% in one year only), but with no change in cumulative incidence by the end of 5 years. An overall 25% reduction in the crude number of new human cases was observed, but again transmission was not fully interrupted. Unfortunately since the migration of seropositive dogs into the intervention area was not prevented, and only 42%-73% of the seropositive population was eliminated in any cull year, it is impossible to conclude from these results whether a blanket cull of all seropositive dogs would be more effective. One such study in Espirito Santo, Brazil which did remove all seropositive dogs (at 2 x 6 month culling intervals), reported no reduction in seroconversion relative to controls in either humans (33-54% *vs.* 36-54%) or dogs (36-14% *vs.* 52-11%) (Dietze *et al.*, 1997). These results corroborate the conclusions of Evans *et al.* (1992), working in Ceará, Brazil, who detected no change before and after seropositive dog elimination in either the human seroconversion rate, or the number of new or retrospective ZVL cases.

1.6.4 Wildlife reservoir control

Past attempts to control ZVL have not generally been directed at wildlife hosts. The single possible exception, albeit anecdotal, is a report from Iraq suggesting that the disposal of carcasses around chicken farms and abattoirs led to a decline in infantile ZVL due to a reduction in contact between canid reservoirs (dogs and jackals) (WHO, 1988;

1990). By contrast, direct action against the wildlife reservoir, *Rhombomys opimus* (the great gerbil) of *L. major*, has been successful in reducing human cases for up to 7 years post intervention in the central Asian steppes (Sergiev, 1978). It seems that in some control foci at least, reduction in vector abundance played a major role in interrupting transmission. Successful control was associated with mechanical destruction of the rodent burrows located in sandy soils, in which the exit holes were more likely to deteriorate and close, thus blocking access to predominant breeding and resting sites of the vectors. By contrast, exit holes in non-sandy soils were more resilient with no consequential change in sandfly abundance (see review by Vioukov, 1987).

1.6.5 Anti-*Leishmania* vaccines

Neither a canine nor a human anti-*Leishmania* vaccine is presently available. Evaluation of the safety (phase I) and immunogenicity (phase II) of a "first generation" vaccine consisting of a killed *L. braziliensis* strain gave promising results by providing protection and a positive lymphocyte proliferative response in 9/10 dogs, 26 months after experimental challenge with *L. infantum* promastigotes (Mayrink *et al.*, 1996). However, preliminary results after 18 months of the clinical phase III trial in Minas Gerais, Brazil, indicate that the vaccine does not in fact provide the protection expected relative to unvaccinated controls (Genaro *et al.*, 1996b). Previous experimental canine vaccines incorporating *L. infantum* antigen resulted in an increase, rather than decrease, in susceptibility to *L. infantum* infection (Ogunkolade *et al.*, 1988; Dunan *et al.*, 1989), whereas "second generation" vaccines (e.g. live vaccines, recombinant molecules) are still in preclinical development (Grimaldi, 1995; Modabber, 1995).

1.7 Study rationale

Until an effective vaccine becomes available, ZVL control has to rely on a more traditional integrated approach. In Europe, CVL is primarily a concern of veterinarians, so clinically suspicious canine cases are confirmed by the presence of anti-*Leishmania* antibodies, and treated by chemotherapy. Treating symptomatic dogs, however, may not have the desired effect if cryptic infections (e.g. prepatent and asymptomatics) prove to be as infectious as clinical cases (Poli *et al.*, 1997; Alvar *et al.*, 1994; Molina *et al.*, 1994a); prolonged treatment also runs the risk of selecting drug resistant parasite strains

(Gramiccia *et al.*, 1992), and may lead to chronic potentially infectious conditions which are unresponsive to treatment (Deplazes *et al.*, 1992, cited in Deplazes *et al.*, 1995). The size and expense of a potential mass treatment campaign is indicated from the number of seropositive dogs recorded in Italy between 1995 and 1997, which amounted to some 50,000 out of a total 183,000 examined by IFAT (Gradoni, pers. com.). Approximately half of these are expected to be symptomatic infections.

In Brazil, where *L. infantum* infection is more of a public health problem, ZVL control includes elimination of seropositive dogs following serological screening by governmental health laboratories, in conjunction with residual insecticide (DDT or deltamethrin) application (Lacerda, 1994). Despite the lack of conclusive efficacy trials, this approach accounted for the elimination of more than 80,000 dogs among 4.5 million serologically screened between the years 1991 and 1994. The incidence of reported human disease during this period increased by almost 100% (Monteiro *et al.*, 1994; Dietze *et al.*, 1997).

In search of more quantitative approaches to the implementation of present or future ZVL control strategies, a more thorough understanding of the infection and transmission dynamics of the parasite in the reservoir populations is required. Indeed, despite the role of dogs in an estimated 56 human zoonotic diseases (Hubbert *et al.*, 1975), including rabies (e.g. Eng *et al.*, 1993; Perry, 1993), Chagas disease (e.g. Gurtler *et al.*, 1990), hydatidosis (e.g. Wachira *et al.*, 1990; Baronet *et al.*, 1994), toxocariasis (Lloyd, 1998), Paget's disease (Khan *et al.*, 1996; Fraser, 1997), and both cutaneous and visceral leishmaniasis (Aguilar *et al.*, 1990; references herein), surprisingly little is known generally about the dynamics of infection and disease in domestic dog populations. Even less is known about these processes in wildlife reservoirs. Interest in additional ZVL reservoirs arises not least due to reports of human infections in regions with no known dog infection, and, indeed, in regions void of dogs (Ahmed and Burney, 1962, cited in Rab *et al.*, 1995).

1.8 Study aims

To quantify the dynamics of infection, infectiousness and disease in domestic and wildlife reservoir populations of *L. infantum*, a study was conducted in an endemic region in Brazil: using a novel study approach, a previously unexposed cohort of sentinel dogs was established in an area of high transmission and subsequently monitored over a period of 24 months (as described in **Chapter 2**). These data were related to longitudinal epidemiological and demographic information obtained on the local resident dogs, in addition to a sympatric population of crab-eating foxes, *C. thous*.

The broad aims of this thesis, defined by chapter, are:

(1) to describe the population dynamics of the resident dogs and foxes, and to measure demographic and transmission parameters which quantify the control problem **(Chapter 3)**

(2) to describe the serological and parasitological courses of infection in the sentinel population, and relate these measures to the long-term clinical outcome **(Chapter 4)**

(3) to quantify the dynamics of population infectiousness, in addition to testing the sensitivity and specificity of surrogate markers to identify infectiousness **(Chapter 5, Part I)**

(4) to develop a compartmental model of infectiousness, and with the empirically derived demographic and epidemiological estimates to predict the outcome of present and potential strategies of dog culling on transmission **(Chapter 5, Part II)**

(5) to describe the course of infection, infectiousness and clinical development in the fox population, and to assess the epidemiological significance of foxes relative to that of domestic dogs **(Chapter 6)**.

An overview of the collective results is presented and discussed in relation to possible ZVL control strategies in the final chapter **(Chapter 7)**.

2. GENERAL METHODS

2.1 Study site and study populations

2.1.1 Location

Fieldwork was conducted in the municipality of Salvaterra, Marajó island, Pará, Brazil (48°03'W, 00°46'S) (**Figure 2.1**). Marajó is approximately 50,000 km^2 in area, and situated in the Amazon delta. The vegetation in the study region is broadly characterised as savannah ('campo') (Prance, 1987; Pires and Prance, 1985), comprising seasonally flooded grassland interspersed with woodland, regenerating scrub, cultivation (pineapples and manioc), remnant *terra firme* forest, and some *varzeá* forest along river courses. More detailed accounts of the vegetation in the study site are given by Bastos (1984), and Macdonald and Courtenay (1996).

2.1.2 Climate

The mean daily precipitation ranged from 0.4mm (*S.D.* 0.26) in the peak dry season (August - November), to 21.0mm (*S.D.* 14.62) in the peak wet season (January - March), with corresponding mean temperatures of 27.9°C (*S.D.* 0.27) and 26.8°C (*S.D.* 1.244), and relative humidity levels of 79.1% (*S.D.* 2.79) and 85.2% (*S.D.* 6.79), respectively (data for 1988-91 & 1993-4: MAARA, National Institute of Meteorology, Belém).

2.1.3 Study populations

The study focused on 25 rural communities within an 18 km radius of the main town of Salvaterra (15,000 inhabitants), which were known to harbour *Lu. longipalpis* (Lainson *et al.*, 1983; Quinnell and Dye, 1994a; 1994b; Kelly *et al.*, 1997), and, in nine villages, where human VL cases had been previously confirmed (Shaw and Lainson, 1987; Instituto Evandro Chagas, unpublished data). The resident dogs of 15 of these villages had also been shown positive for anti-*Leishmania* antibodies (IFAT) by a cross-sectional survey conducted in 1988 (O. Courtenay and Instituto Evandro Chagas, unpublished data). Observations were made on three discrete populations of canids, including (1) a cohort of sentinel dogs (2) the resident dogs of 15 of the villages, and (3) a free-ranging population of crab-eating foxes, *C. thous*, living in the vicinity of 15 of

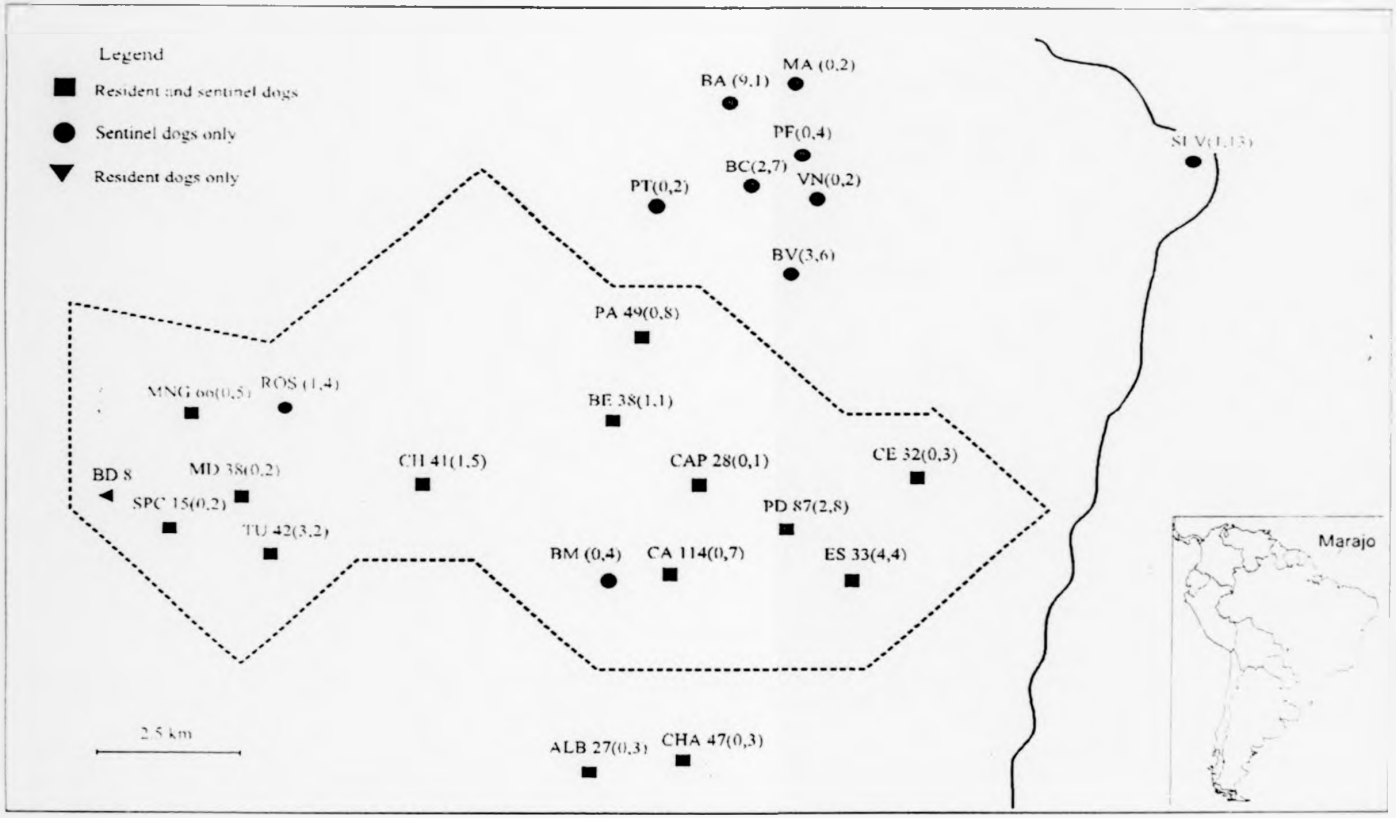


Figure 2.1 The distribution of study villages (symbols) in Marajó, Brazil (inset) showing the number of resident dogs per village. The numbers of local and Belem sentinel dogs, respectively, are shown in brackets. (-----) demarks the area in which study foxes were trapped

the villages. The spatial distributions of the three populations are shown in **Figure 2.1**. The study methods for each are described separately below.

2.2 Study design and procedures

2.2.1 Longitudinal study of the sentinel population

A sentinel population of 126 dogs previously unexposed to *L. infantum* was established in the 24 endemic Marajó villages.

a) Recruitment and instalment

Dogs were recruited from two sources: 99 dogs were acquired from the nearby city of Belém (1.5 million inhabitants), where *Leishmania* infection is known not to occur, and 27 were recruited locally ("local dogs") from 10 of the study villages in Marajó. (The majority of dogs were obtained from Belém due to a scarcity of young dogs in the study area: the reasons for this are discussed in Chapter 3). The criterion for local dog recruitment was a negative IFAT anti-*Leishmania* antibody response, as defined below (see Serology). Belém recruits were acquired for a small fee from owners and transported to Marajó by ferry.

In Marajó, local recruits remained with their original owners, and Belém dogs were given (without payment) to willing householders. Their distribution among the study villages is illustrated in **Figure 2.1**. During the course of the study, 12 dogs had to be relocated to different houses to avoid them being neglected, and 3 of the locally recruited dogs were discontinued due to withdrawal of their owners' permission. None of the sentinel dogs belonged to a recognisable breed. Due to the practical constraints of transporting large quantities of animals to the island, dogs were recruited and installed gradually during the course of the study, as shown in **Table 2.1.**

b) Sampling regime

Dog instalment dates corresponded with the sampling schedule (**Table 2.2**). The study ran from April 1993 to July 1995 during which period samples were taken at approximately bimonthly intervals (mean 67.3 days, *SE* 0.854, range: 58-80 days), in a

Table 2.1. Numbers of dogs originating from Belém and Marajó ("local") which were recruited into the sentinel study population between April 1993 and July 1995.

sampling round	date	day	Belém dogs	Local dogs	Total dogs
1	11 Apr 93	0	19	11	30
2	30 Jun 93	80	20	1	21
3	28 Aug 93	139	30	7	37
4	05 Nov 93	208	10	6	16
5	13 Jan 94	277	6	0	6
6	23 Mar 94	346	2	0	2
7	30 May 94	414	7	2	9
8	06 Aug 94	482	0	0	0
9	12 Oct 94	549	0	0	0
10	10 Dec 94	608	5	0	5
11	19 Feb 95	683	0	0	0
12	24 Apr 95	746	0	0	0
13	06 Jul 95	818	0	0	0
Total			99	27	126

Table 2.2. Numbers of Belém and local sentinel dogs sampled from the time of their instalment into the study villages.

sampling round	Belém dogs	Local dogs	Total dogs sampled
instalment 1	99	27	126
2	97	27	124
3	76	22	98
4	62	16	78
5	59	12	71
6	48	11	59
7	40	11	51
8	30	9	39
9	23	10	33
10	22	6	28
11	17	6	23
12	12	5	17
13	6	4	10

total of 13 sample rounds. The total number of dogs sampled in each round aligned by the time of enrolment is shown in **Table 2.2**.

c) Sample collection

At each sampling round, dogs were collected from their homes and taken to the kennels at the laboratory in Salvaterra. The following day, they were anaesthetised with a cocktail of Medetomidine hydrochloride (Domitor®, SmithKline Beecham) and Ketamine (Vetalar®, Parke-Davis), at combined doses recommended for minor surgery. Blood (20mls) was collected by venepuncture (jugular) and defibrinated in a sterile polyproprylene tube with 30-40 glass-beads. Triplicate 2ml serum samples were taken after centrifugation and stored at -70ºC for subsequent testing for anti-*Leishmania* antibodies using an ELISA (see Serology). Bone marrow was aspirated from the iliac crest using a 16 x 25mm Klima needle (Veterinary Instruments, Newcastle) into a 20ml syringe containing 0.5% EDTA, and used for parasite detection, isolation and identification, as described below (see Parasitology). Each dog was then given a full clinical examination for signs of CVL and haematological samples collected as described in Chapter 4. Finally, all animals were permanently marked for individual identification with ink applied to both ear pinnae using a mechanical tattoo.

The entire sampling procedure including sample storage took approximately 25 minutes per dog. Dogs were awake within an hour without use of a reversal agent, with no casualties. Pregnant bitches were not anaesthetised due to the possible adverse effects of Medetomidine hydrochloride; therefore bone marrow aspiration was not attempted on these animals in these sample rounds.

The same or previous night, xenodiagnosis was performed on a proportion of the dogs using colony reared *Lu. longipalpis* (as described in Chapter 5).

d) Serology

***Immunofluorescent antibody-test* (IFAT)**

IFATs were performed on the Marajó sentinel dogs to confirm their seronegativity to anti-*Leishmania* antibodies. The tests were carried out following the procedures

described in Quinnell *et al.* (1997). Briefly, the test used an FITC-conjugated anti-dog IgG (Sigma) and *L. infantum* (MCER/BR/81/6445) amastigote antigen. Test sera were titrated at 2-fold dilutions from 1/20 to 1/320. Only dogs with reciprocal titres < 20 were permitted to enrol into the sentinel study. The usual negative cut-off titre for routine dog surveillance by IEC is < 40 (Courtenay *et al.*, 1994).

***Enzyme-linked immunosorbent assay* (ELISA)**

ELISAs were performed as described in Quinnell *et al.* (1997). To summarise, the study sera were tested against *L. infantum* (MHOM/BR/74/PP75) cultured promastigote antigen, using rabbit anti-dog IgG peroxidase conjugate. All the sera of individual dogs were tested on a single plate titrated from 1/50 to 1/800, in conjunction with a positive standard titrated from 1/50 to 1/3276800. Absorbance values were calculated as observed minus the mean background absorbance. Absorbancies of the test sera were then expressed in antibody units read off a standardised line fitted to the log-logit transformed positive control absorbency values.

The criteria to define seroconversion were based on the relative change in a dog's titre through time. Seroconversion was thus considered to occur on (1) the first occasion that an animal's (log unit) titre increased 4 fold to exceed 1208 units (which represents the lower boundary of the expected positive titre distribution). Otherwise, (2) for sera which had not undergone a 4 fold change but had reached >5500 units (representing the mean + 3 *S.D.* of the fitted negative distribution), seroconversion was taken at the point that it first exceeded 2253 units (representing the intersection or 'cut-off ' of negative and positive titre distributions).

e) Parasitology

Bone marrow samples were used to make 1-4 direct smears for microscopical examination (all rounds), and were inoculated into two sterile Difco blood-agar slopes (rounds 1-9), or into two golden hamsters (rounds 10-13). Blood-agar slopes were examined by microscopy on day 7, 14 and 28 following inoculation. Hamsters were sacrificed at 6-12 months and impression smears made of their livers and spleens; triturated samples were also cultured on blood-agar slopes. The 34 obtained isolates

(all from dogs) were identified by monoclonal antibodies as *L. infantum* (J. J. Shaw, pers. comm.).

2.2.2 Resident dog population

a) Demographic sampling

Demographical information was obtained on the resident dog population of 15 of the 25 study villages by questionnaire between 1993 and 1994. These data were combined with comparable information collected in the same villages in 1989–1992 (using the same procedures) (O. Courtenay and D. W. Macdonald, unpublished data). The methods are fully described in Chapter 3.

b) Serology

Simultaneous to the demographical surveys of 1989–1994, an ear blood-spot from each dog was collected onto a Whatman no. 4 filter paper, sun dried, and stored at -20ºC. Eluted blood samples were subsequently tested for anti-*Leishmania* antibodies using the IFAT described above. Resident dogs with titres ≥ 40 were considered positive (*cf.* Marajó recruited sentinel dogs), in order to be consistent with the routine surveillance procedures of IEC. In Chapter 3, the serological data of 1994 are combined with the 1989–1993 previously published data (Courtenay *et al.*, 1994) for comparative analysis.

2.2.3 Fox population

The study foxes comprised of animals living within a 12 km^2 area encompassing 14 of the 15 villages from which the resident dogs were sampled (illustrated in **Figure 2.1**). Baited box traps (Tomahawk Live Trap Co., USA) were set in 5 capture rounds between April 1994 and July 1995, detailed in Chapter 6. Captured foxes were immobilised *in situ* with anaesthetic at the dosages described for dogs, and then transported to the laboratory in Salvaterra. The serological, parasitological, clinical, and xenodiagnostic procedures were the same as those described for dogs above. Deviations from those methodological accounts are described in Chapter 6. Fox ages were estimated as described in Chapter 3. As for dogs, foxes were permanently marked by ear tattoo. Post sampling, animals were returned to the trap of capture while still

under sedation (to minimise stress and facilitate recapture), prior to their subsequent release. No casualties resulted from anaesthesia.

In Chapter 3, additional demographic data obtained for 23 foxes captured in the same area (Courtenay and Macdonald, unpublished data) were available for analysis. The ecology and spatial environment of this fox population has been previously described by Macdonald and Courtenay (1993; 1996).

2.3 Data analysis and statistical procedures

Univariate and multivariate data analyses were performed using generalised linear modelling techniques in either STATA (*v*.5.0) (StataCorporation, 1997), or GLIM (*v*. 4.07) (Crawley, 1993).

2.3.1 Correcting for autocorrelation

The longitudinal data in this thesis comprise repeated observations of the same individuals through time. In contrast to single observations such as cross-sectional data, which are assumed usually to be independent, repeated observations of the same individual are likely to be more closely related with each other than those from different individuals. The consequence of ignoring such autocorrelation or 'tracking' within subjects– canids in this case–, leads to an underestimation of the standard errors of the parameter estimates. To control for autocorrelation in multiple within-subject analysis, the Huber's 'robust' method (Huber, 1967) was used. This 'robust estimation' method effectively inflates the standard errors of parameter estimates without altering the coefficients, thus rendering statistical testing more conservative. This relatively simple approach has been shown to provide results comparable with other robust methods (including Jacknife, Bootstrapping, etc.), as well as more complex two-level random effects models (Stavola *et al.*, 1996). Robust estimation and statistics were performed using the XTGEE and ROBUST subroutine directives in STATA. Analytical procedures for multiple within-subject data were otherwise the same, as outlined below.

2.3.2 Multivariate analysis

a) Maximum model

Parameters were classified as either variables or factors, and entered into a maximum (ANOVA, ANCOVA or regression) model, specifying the appropriate error structure for the response parameter (e.g. Gaussian, binomial, binary, Poisson). For binomial and binary response data, either the logit link or complementary log-log link (transformation) was used according to which resulted in the lowest residual deviance in the minimum adequate model (MAM).

b) Model simplification

Model simplification to the MAM was achieved by backward stepwise elimination. Each explanatory term was tested by its removal from the maximum model. The absolute or relative change in residual deviance caused by its removal was examined for significance (see significance testing below); once all explanatory terms were tested, the insignificant parameters were then removed. Terms were assessed and removed in order of diminishing complexity, i.e. interaction terms before the main effects. When an interaction term was significant, its main effects parameters were not removed from the model. This procedure was repeated until the MAM, by definition, retained only explanatory variables which caused a significant increase in residual deviance when removed. In exceptional cases, when of biological interest, main effect terms were tested in the absence of its interaction.

c) Factor level simplification

The most parsimonious combination of factor levels was achieved by examining the change in residual deviance when replacing the more complex model with the simplified (combined factor level) model. An insignificant change in deviance indicated that the new grouping was appropriate. Factor levels were combined if insignificantly different from each other by inspection of their coefficients and attached errors.

d) Significance testing

For MAMs with binary or binomial error structures, changes in residual deviance were compared to χ^2 distribution; the F-ratio statistic was employed to test models against

the normal error distribution. In the event of overdispersion of the MAM residuals (i.e residual deviance >> residual degrees of freedom), the scale parameter was transformed using the Pearson χ^2, and the residual changes compared to the F, and not the χ^2, distribution (Crawley, 1993). Robust estimates obtained from STATA (including those corrected for overdispersion) were compared against the z-statistic, as appropriate. A significant deviation was defined as having a ≥ 95% probability of not occurring by chance (i.e. $P \leq 0.05$). P-values for multiple comparisons were adjusted following the Bonferroni method (Sokal and Rohlf, 1995).

2.3.3 Survival analysis

Survival analysis was performed in STATA using the Survival time (St) subroutine for single observation censored data, where St comprises time to death ("non-censored" = 1), or time (at sample) last observed alive ("censored" = 0). The relationship between survival and time (or age) was quantified by fitting a Weibull regression model which measures the "shape parameter", α, where $\alpha > 1$ and $\alpha < 1$ indicate increasing or decreasing survival with time, in contrast to $\alpha = 1$ which implies age-independent survival. The instantaneous mortality rate, μ, was thus calculated from the hazard rate function $h(t)$ as

$$h(t) = \alpha t^{(\alpha - 1)} e^{-\mu} \qquad \text{(equation 2.1)}$$

where t is the median survival time of the population. Where μ was not constant with age (i.e. $\alpha \neq 1$) the median age at death was used to calculate a median mortality rate, μ. Differences in values of α between populations were tested by a log-likelihood ratio test (LRT) (Cox and Oakes, 1984). Stratified survivorship functions were obtained by controlling for age ("entry time"), and compared by a non-parametric log-rank test and illustrated as Kaplan-Meier plots.

2.4 The epidemiological setting

In Marajó, 23 autochthonous cases of human ZVL were recorded between 1980 and 1992 (Shaw and Lainson, 1987; IEC unpublished data). Increased vigilance during the study (1993–1995) led to the positive diagnosis of a further 25 cases (22 successfully

treated) from a population of approximately 3,000. Of these cases, 19 were from the current study villages. VL control efforts in Marajó have relied on treatment of diagnosed human cases and sporadic spraying of houses and animal pens with DDT against vectors, the latter of which is part of the national anti-malaria campaign. The last spray-round was in 1990, but was not completed. Dog control (culling or treatment) has never been attempted in the study site, nor, as far as records show, in any other region of Marajó.

2.5 Collaborations

The data presented in this thesis were collected as part of a larger project funded by a Wellcome Trust grant to C. Dye and R. Quinnell. The IFATs were performed by technicians at Instituto Evandro Chagas (IEC) under the supervision of RQ, who also performed the ELISAs on the dog and fox sera. IEC technicians were responsible for the maintenance and/or examination of bone marrow preparations including the smears, culture and hamster inoculates. Analysis of the serological dog data from this study was performed by RQ and CD, presented in Quinnell *et al.* (1997). A copy of this publication is enclosed in the sleeve of this thesis.

3. RESERVOIR HOST POPULATION DYNAMICS, AND THEIR EPIDEMIOLOGICAL IMPLICATIONS

3.1 ABSTRACT

The population dynamics of dogs resident in 15 communities endemic for *L. infantum* in Marajó were studied between 1989 and 1994. Longitudinal and cross-sectional data were collected during 3 years (1989–1993) prior, and one year (1993–1994) after, the establishment of a cohort of sentinel dogs in the same villages. Estimates of the resident population age structure, life expectancy, growth, turnover, mortality, birth, and migration rates were calculated. A smaller number of demographic parameters were also measured on the sympatric population of free-ranging foxes, *C. thous*, and the sentinel dogs. The variation in demographic parameters of the resident dogs across villages was examined in relation to their infection rates based on a positive or negative anti-*Leishmania* antibody (IFAT) response, and also in relation to the density of peridomestic *Lu. longipalpis*. The resident population turnover rate was 0.42 per year (95% C.L. 0.35–0.50), driven by an immigration rate of 0.31 per year (95% C.L. 0.26–0.36) into the one-year age-class. This was matched by a mortality rate of 0.40 per year (95% C.L. 0.33–0.47). By comparison, birth and emigration rates were low (each < 0.12 per year), indicating that dog abundance was principally sustained by immigration whereby householders acquired new dogs to compensate for those that were lost from the population. Instalment of the sentinel dogs into these villages did not significantly alter the pre-trial dynamics of the resident population, though sentinel dog average life expectancy (1.45 years) was significantly lower than that of resident dogs (2.48 years). The variation in resident dog seroconversion rates between villages was positively associated with the mean ratio of humans to dogs per household (explaining 38.0% of the variation), sandfly abundance in houses (35.5%) and in animal sheds (32.8%), and negatively associated with the mean number of dogs per household (44.5%). Only the latter relationship held true when controlling for all these parameters in multivariate analysis. In turn, the variation in dog mortality between villages was associated with seroprevalence (24.2%), incidence (54.0%), and sandfly abundance in houses (77.7%)

and animal sheds (38.5%). Two of these variables, incidence and sandflies in houses, accounted for 85% of the variation in multivariate analysis, indicating *Leishmania* infection is an important cause of dog mortality in Marajó. Demographical information on the fox population yielded mortality rates of 0.314–0.319 per year, and population turnover times of 1.75 years, which were similar to the estimates for resident dogs. Unlike dogs, however, there was no evidence that foxes experienced additional mortality due to *Leishmania* infection. The implications of these results for ZVL epidemiology and control are discussed.

3.2 INTRODUCTION

Quantitative epidemiology has highlighted the importance of host population parameters which reflect conditions under which parasites become established and persist (e.g. Bartlett, 1960; Grenfell and Dobson, 1995; Scott and Smith, 1994; Anderson and May, 1991). Despite the role of dogs as the principal reservoir of many zoonotic pathogens, their population dynamics are poorly understood (reviewed by Coleman, 1998). Trends in existing data nevertheless reflect patterns in dog societies of different economic and geographical settings, such that compared to developed countries, developing countries show higher dog mortality rates (Nasser *et al.*, 1984; Cleaveland, 1996; Robinson *et al.*, 1996; Kitala *et al.*, 1993; Beran and Frith, 1988; Nasser and Mosier, 1980), higher human : dog ratios (Griffiths and Brenner, 1977), faster dog population turnover (Chomel *et al.*, 1988), higher dog densities per km^2 (Wandeler *et al.*, 1988; Kitala *et al.*, 1993; Kitala and McDermitt, 1995; Lengerich *et al.*, 1992), and a greater number of dogs per household (Butler, 1995; Kitala and McDermitt, 1995; Cleaveland, 1996). Within developing countries, urban *vs.* rural populations occur at higher densities per km^2 (Beran and Frith, 1988; Rangel *et al.*, 1981; Wandeler *et al.*, 1988; Kitala and McDermitt, 1995), though a lower proportion of households maintain dogs (Beran *et al.*, 1972; Rangel *et al.*, 1981; Fishbein *et al.*, 1992; Brooks, 1990). Some 90% of the epidemiological studies with dog demography components have been conducted in the context of rabies (see review by Coleman, 1998). In contrast, there are no detailed demography studies on dogs from an endemic focus of *L. infantum*; indeed there are only two studies which provide any demographic information for rural settings in Latin America (Fishbein *et al.*, 1992; Gurtler *et al.*, 1990), and the only available data for Brazil is an estimate of 10 for the national average urban human:dog ratio (Belotto, 1988).

In ZVL epidemiology, host demographic parameters of interest include mortality rates, dog densities, dog ownership patterns, and population turnover rates, depending on the specific area of interest. The replacement rate of susceptibles and mortality rate of infectious animals are key demographical processes which drive transmission. Thus one implication of the higher turnover rates in developing countries, for example, is that ZVL may be more difficult to control in Latin America than in Western Europe. This is

also suggested by the higher dog densities in developing *vs* developed countries since the density of (infectious) dogs is thought to constitute an important risk factor for human infection (Gavgani, 1998).

Of fundamental importance to ZVL control is knowledge of the basic reproductive number, R_0. For VL, a good approximation of R_0 is obtained from $R_0 = 1 + L / A$ (Quinnell *et al.*, 1997), where L is the average life expectancy of the infectious host, and A is the average age of acquiring infection. L may be calculated from the instantaneous mortality rate, μ, as $L = 1/\mu$, hence demographic studies are essential to measure the size of the control problem. In the event of a future anti-*Leishmania* vaccine, the proportion of the population which requires vaccination to provide effective herd immunity, *pc*, is reliant on estimating elements of R_0 since $pc = 1 - (1 / R_0)$. Knowledge of L is also required to predict the critical pulse vaccination interval, T_v, which is approximated by $T_v = L / R_0$ in a stationary host population (i.e. where the recruitment of new -susceptible- hosts equals the mortality rate) (Woolhouse *et al.*, 1997b). Under similar conditions, the rate at which susceptibles become infected (and infectious) in the population is dependant on the incidence rate $1/A$. Thus the critical pulse culling interval, T_c, to maintain *pc* at the appropriate level can be approximated by $T_c = A / R_0$.

Heterogeneities in host dynamics thus clearly influence the potential of different species, or populations, to maintain and transmit the parasite (e.g. wildlife *vs.* domestic reservoirs). The similarity in infection rates in foxes and dogs reported in Marajó, for example, could be genuinely due to equivalent forces of infection. Alternatively, their similarity may be the consequence of a greater disparity in mortality rates of seropositives and seronegatives in one species than in the other (Courtenay *et al.*, 1994). One major difference between domestic and sylvatic canids is their spatial distribution patterns and density; dogs are more likely to exist at higher densities than foxes, which implies that the domestic reservoir is more suited for the long-term persistence of the parasite. Although there are extensive data on the population dynamics of the suspected wildlife reservoir, *V. vulpes*, in Europe (Macdonald, 1980; Aubert, 1994; Anderson *et al.*, 1981), no such information is available for the Latin American host, *C. thous*.

This study is therefore the first to provide demographic data on canid reservoirs of ZVL in an endemic region. A major part of this thesis describes a sentinel domestic dog population recruited from a non-endemic region and introduced into the endemic villages of Marajó, and into households where local dogs already resided. Observations on such a large number of sentinel animals also represent a unique experiment in the study of leishmaniasis transmission. Such a study assumes that the instalment process does not alter the endemic environment, and that the experimental population is equivalent to, and will mimic, the infection outcome of the resident population. Questions generally arise concerning the extent to which experimental groups represent natural populations and, in the present context, the validity of selecting atypical breeds for a sentinel study (Vidor *et al.*, 1991). In this study the sentinel dogs certainly were typical of the resident breed (i.e. mongrels), but in number they were approximately half the size of the resident population. Monitoring the study population therefore is also important to identify changes in prevailing demographical and epidemiological processes which might arise through experimental manipulation.

3.2.1 Study aims

The aims of this chapter are **(1)** to quantify the dynamics of the resident dog population, for which values for migration, birth, mortality, life expectancy, and population turnover are estimated. **(2)** to confirm that the instalment of the sentinel population did not alter the endemic characteristics of the study-site, as reflected in the population dynamics pre-*vs.* post-trial instalment periods. **(3)** to confirm that the sentinel dogs responded typically to local conditions, their mortality rates were monitored and compared with those of the resident population. **(4)** to provide estimates of mortality, life expectancy and population turnover rates for the sympatric wildlife reservoir (fox) population. **(5)** to identify potential risk factors of dog infection and mortality across villages. Finally, the implications of the results to ZVL epidemiology and control are discussed.

3.3 METHODS

3.3.1 Resident dogs

a) Demographic measures

Sampling regime

Information on the resident dogs of the 15 study villages (described in Chapter 2) was obtained from their owners in September 1989, July 1990, January 1992 and 1993, and May 1994, by house to house visits and questionnaire (an example of the questionnaire is in **Appendix I**). Data were collected on age, sex, birth dates and mortality, and on dog migration between and within study villages, and to and from the study site. This provided both cross-sectional and longitudinal data from which demographical parameters could be estimated as described below.

Age estimation

Rigorous non-invasive methods of ageing dogs (especially those > 12 months old) are not available, and estimates provided by owners are likely to decline in accuracy with increasing dog age. Since 88% of all newly sampled dogs were identifiably young and reported ≤ 12m old, the ages obtained from owners at their first sample were taken as the best estimate, from which ages of dogs sampled on subsequent occasions were calculated.

Rates of population change

The annual rates, φ, of birth b, immigration i, mortality d, and emigration e, were each calculated from

$$\varphi = \left(\frac{n_{t+y}}{N_t}\right)\left(\frac{12}{y}\right) \qquad \text{(equation 3.1)}$$

where N_t is the total population size at time t, and n_{t+y} is the number of births, immigrants, deaths, or emigrants, as appropriate, recorded at time $t + y$ months. Using these estimates of φ, the annual rate of population growth, r, was calculated by

$$r = (b + i) - (e + d) \qquad \text{(equation 3.2)}$$

where values of $r > 0$ and $r < 0$ indicate positive and negative population growth, respectively.

Survival analysis and life expectancy

Instantaneous mortality rates, μ, were estimated by survival analysis described in Chapter 2. The ages of dogs on arrival to the study-site were used to control for the differential effects of age, entered into the model as the "entry time" parameter. Life expectancy, L, was calculated from the instantaneous mortality rate by $L = 1/\mu$, where μ represents the mean (age-independent) rate. Where μ was not constant with age, the median age at death was used to calculate L (as described in Chapter 2).

Population turnover

The annual instantaneous population turnover rate, ν, defined as the rate at which the population is replaced by new individuals, was calculated from the cross-sectional age distributions by

$$\nu = \ln(1 - f_0) \qquad \text{(equation 3.3)}$$

where f_0 is the proportion of the population in the zero age-class, when dogs are classified into discrete one-year categories labelled by their age, x, at their last birthday (i.e. 0, 1, 2...etc.). This estimation method assumes that the population age structure is stable through time, that the population growth rate, r, is constant, and that immigration is into the 0 age-class.

Host densities

Human and dog abundance data were obtained by serial questionnaire for 15 of the study villages. From these data, statistics on the human : dog ratio, number of dogs per household, and proportion of houses with dogs were derived. Dog densities per village were calculated from estimates of the village area measured using an adopted grid-cell method (White and Garrott, 1990): the X-Y co-ordinates of each house were plotted on a grid-map, and the area calculated as the cumulative size of grid-cells in which ≥ 1 houses were located. According to this method, the size of the grid cell is chosen (in

this case 300m x 300m) to demarcate realistically village boundaries by excluding excessively large unutilised areas outside the village.

b) Epidemiological measures

To test potential explanatory variables of dog infection and mortality, additional data were used from both published and unpublished sources.

Serological data

A filter paper blood-spot from the ear of each resident dog was obtained at the time of the house to house surveys. The eluted sera were then tested for anti-*Leishmania* antibodies by IFAT, and classified as either positive or negative, following the methods described in Chapter 2. Here, the published results of the 1989–1993 surveys (Courtenay *et al.*, 1994) are combined with additional data collected in 1994. Seroprevalences were thus known for dogs of each village from 5 independent cross-sectional surveys.

Sandfly abundance

Data on the abundance of *Lu. longipalpis* were available for 13 of the 15 study villages (Quinnell and Dye, 1994a). Sandfly numbers were measured by placing a single CDC light trap for one night in the house and animal (pig or chicken) pen of 180 households in 13 of the resident dog study villages between July and August, 1992. The mean log number of sandflies in houses (HF) and pens (SF) per village was used in the analyses described below.

c) Analysis

The parameters used to explain the variation in dog infection (seroprevalence and incidence) and mortality across study villages are shown in **Table 3.1**. Multiple estimates of seroprevalence and mortality were available for each cross-sectional sample, and were regressed against explanatory variables measured during the same period, labelled as "discrete" measures in **Table 3.1**. Since the numbers of dogs to seroconvert per village in any single sample interval were few, a second infection outcome parameter, mean incidence rate (i.e. seroconversion rate from negative to positive titre),

was calculated over the entire sample period, and regressed against mean explanatory values calculated for the same period. These are labelled as "period" measures in **Table 3.1**. Note that seroprevalence (PREV) and incidence (INCID) were also tested as potential explanatory variables of dog mortality. Since village sandfly abundance (HF and SF) is not expected to fluctuate greatly between years (Quinnell and Dye, 1994a), these data were assumed to represent relative differences between villages over the entire sample period; these were tested as "period" explanatory variables accordingly **(Table 3.1)**.

Table 3.1. Demographical and epidemiological parameters tested as potential explanatory variables of resident dog infection and/or dog mortality. "Discrete" measures represent estimates obtained in individual cross-sectional pre-trial sample years or inter-sampling intervals, whereas "period" measures represent values averaged over the pre-trial sampling period. Sentinel dogs were not included in these estimates.

explanatory variables tested	type of measure	abbreviation used in the text
***host density*:**		
human : dog ratio	discrete & period	RA
dogs per household	discrete & period	DH
humans per household	discrete & period	HH
dogs per village area	discrete & period	DDEN
humans per village area	discrete & period	HDEN
***mean sandlfy abundance*[1]:**		
per house	period	HF
per animal shed	period	SF
***dog infection*:**		
incidence	period	INCID
seroprevalence	discrete & period	PREV

[1] data for 13 villages only.

3.3.2 Sentinel dogs

Sampling regime

Sentinel dogs were observed at approximate bimonthly intervals as described in Chapter 2.

Age estimation

Ages of 75 of the 126 sentinel dogs were obtained from their owners at the time of acquisition. Otherwise, for the 16 dogs which appeared clearly ≤ 12m old, ages were estimated to the nearest month on the basis of tooth eruption, or for the 35 animals obviously >12m, to the nearest year based on inspection of toothwear. Guidelines for the estimation of age by tooth eruption and toothwear are shown in **Appendix II**.

Survival analysis and life expectancy

These parameters were estimated as described above.

Fecundity

Signs of whelping (gestation and lactation) for 76 potentially fecund sentinel dogs (defined as females ≥ 6 months old), were recorded during their bimonthly samples (see Chapter 2). The fecundity rate, f, at age x was calculated as

$$fx = \frac{p}{t} \qquad \text{(equation 3.4)}$$

where p is the total number of pups born over the total observation time, t, of potentially fecund dogs. The whelping rate is defined as the number of times the average breeding female whelps per year. Pups were counted, sexed and, if appropriate, their circumstances of death recorded. Data on the clinical signs associated with puppy mortality were obtained through a combination of direct observation and information provided by their owners.

3.3.3 Foxes

Sampling regime

Two samples of foxes were obtained from the study population using the mark-recapture trapping methods described in Chapter 2. Twenty-five animals were caught between 1988–1991 (Macdonald and Courtenay, 1996), and 37 animals captured during 1994–1995. Two of the latter group had been first caught between 1988–1991.

Age estimation

Fox ages were estimated on the basis of toothwear and tooth eruption (Harris, 1986), or, in the case of five 1988–1991 foxes, by knowing their precise birth month (Macdonald and Courtenay, 1996). Age estimation procedure were standardised for the two samples by cross-referencing the dentition of the 1994–1995 animals to 8 aged dental remains of the former group (Courtenay *et al.*, 1996).

Life expectancy and population turnover

Age at mortality was known for 44% of the 1988–1991 fox sample from intensive study using radio-telemetry and night vision equipment (Macdonald and Courtenay, 1996). For this sample, the mortality rate was obtained from survival analysis performed as described above; the only exception was that "last time observed alive" (censored data) here represents the ultimate radio-telemetry location, or last live-capture.

Since equivalent data were not available for the 1994–1995 fox sample, the instantaneous mortality rate, μ, was estimated from the cross-sectional age distribution, by

$$\ln (fx) = c - \mu x \qquad \text{(equation 3.5)}$$

where age, x, is discrete one-year categories, as described above for dogs. Since the sample was not strictly cross-sectional, the population age structure was obtained by adjusting ages at first capture to the mid-sampling date. This method assumes that μ is independent of age, that the population is stationary ($r = 0$), and that the population has a stable age distribution.

Values of parameters, l, and ν were calculated as described above for dogs.

3.4 RESULTS

3.4.1 Resident dogs

a) Demographic estimates

Distribution of hosts

Demographic information was obtained from 237–275 dogs from the 15 study villages in the 4 pre-trial years between 1989 and 1993, and in 1994 during the trial **(Table 3.2a)**. During the pre-trial years 71% (range: 50%–95%) of households owned at least one dog, with a mean of 1.5 dogs (range: 0.9–2.0). Villages maintained an average 16.8 dogs (range: 3.3–33.0) at a density of 9.5 dogs per km^2 (range: 3.4–15.9). The human : dog ratio was 3.61 (range: 2.2–5.2) **(Table 3.2b)**, with a M : F dog sex ratio of 0.91 (range: 0.81–1.01). Seventy-one Belém dogs were installed into these villages during the 1993–1994 sampling interval as part of the cohort study. Their inclusion in the village counts for 1994 (shown in **Table 3.2a**) did not cause dog abundance, nor any of the derived measures in **Table 3.2b,** to exceed the 95% confident limits of the pre-trial means.

Population structure, growth and turnover

The cross-sectional age distribution of the population was stable during the pre-trial years (test for deviation from zero slopes for age classes 0–9 & >9: $z < 0.95$, in each case), and no statistical differences were observed between combined pre-trial and trial years (comparison of slopes: $z = 0.35$, for each age-class).

The rates of birth, mortality, immigration and emigration are shown in **Table 3.3**. These values showed no consistent upward or downward trend over the pre-trial period ($t < 3.16$, for each parameter), giving estimates of population growth, r, which fluctuated around a mean of -0.010 (95% C.L. -0.125–0.104). This was clearly not significantly different to zero. The value of r for 1993–1994 trial period did not exceed the 95% C.L.s of the pre-trial mean **(Table 3.3)**, indicating that the instalment of the imported sentinel dogs did not affect the existing population abundance.

Table 3.2 Demographic statistics of the dog population in 15 Marajo study villages
Data shown include **(a)** dog abundance, and **(b)** host densities relative to village parameters
In (b) values represent the pre-trial period (1989-1993) only

(a)

village	number of dogs surveyed by questionnaire					total individual dogs recorded	pre-trial mean dogs per village	95% C.I.s
	pre-trial period				trial			
	1989	1990	1992	1993	1994[a]			
ALB	11	11	12	8	6	27	10.5	8.8 - 12.2
BD	4	3	2	4	3	8	3.3	2.3 - 4.2
BE	13	13	14	9	9	38	12.3	10.1 - 14.4
CA	42	33	52	39	35	114	41.5	33.7 - 49.3
CAP	11	7	17	4	5	28	9.8	4.2 - 15.3
CE	5	16	15	14	10	32	12.5	7.5 - 17.5
CH	22	17	19	17	18	41	18.8	16.4 - 21.1
CHA	18	22	25	16	15	47	20.3	16.3 - 24.2
ES	15	9	9	6	15	33	9.8	6.1 - 13.4
MD	12	16	18	19	16	38	16.3	13.2 - 19.3
MNG	31	25	22	24	25	66	25.5	21.7 - 29.3
PA	13	13	18	15	18	49	14.8	12.4 - 17.1
PD	34	32	30	36	29	87	33.0	30.4 - 35.5
SPC	2	2	5	8	9	15	4.3	1.4 - 7.1
TU	18	18	17	27	28	42	20.0	15.4 - 24.6
total	251	237	275	246	241	665	16.8	8.6 - 25.0

[a] includes 94 (71 Belem, 23 local) sentinel dogs recruited during the questionnaire dates

(b)

village	mean number of houses	mean proportion of houses with ≥1 dog	mean number of dogs /house	mean number of people per village	mean number of people /house	human dog ratio	village area (km^2)	dog density ($dogs/km^2$)
ALB	6.5	0.85	1.6	28.5	4.4	2.71	1.08	9.72
BD	2.0	0.63	1.6	9.0	4.5	2.77	0.64	5.08
BE	6.5	0.77	1.9	38.5	5.9	3.14	1.53	8.01
CA	36.0	0.53	1.2	177.0	4.9	4.27	4.41	9.41
CAP	5.5	0.95	1.8	22.0	4.0	2.26	1.26	7.74
CE	8.5	0.71	1.5	49.0	5.8	3.92	0.81	15.43
CH	10.0	0.95	1.9	54.5	5.5	2.91	1.98	9.47
CHA	12.0	0.65	1.7	69.5	5.8	3.43	1.98	10.23
ES	11.0	0.50	0.9	49.5	4.5	5.08	0.81	12.04
MD	11.0	0.64	1.5	68.5	6.2	4.22	2.25	7.22
MNG	16.0	0.83	1.6	82.5	5.2	3.24	1.98	12.88
PA	10.5	0.57	1.4	52.5	5.0	3.56	1.89	7.80
PD	32.5	0.69	1.0	171.0	5.3	5.18	2.07	15.94
SPC	4.0	0.63	1.1	22.0	5.5	5.18	1.26	3.37
TU	10.0	0.80	2.0	44.5	4.5	2.23	2.43	8.23
weighted mean	12.1	0.71	1.5	62.6	5.1	3.61	1.76	9.50
95% C.I.s	7.2 - 17.0	0.0 - 0.8	0.1 - 1.7	12.7 - 87.6	0.2 - 5.5	0.26 - 4.11	0.24 - 2.23	0.89 - 11.26

Table 3.3 Demographic changes in the resident dog population between 1989 - 1994 Annual rates of population input and output between sample years, categorised by pre- and post-instalment of sentinel dogs.

	pre-trial period			trial period		
sample period	1989-90	1990-92	1992-93	1993-94		
interval (months)	10	18	12	16		
total number dogs observed						
births	15	44	39	23[a]		
Immigrants	72	118	71	97[b]		
emmigrants	12	8	10	11		
deaths	85	117	124	120[c]		
not sampled	4	1	5	6		
rates per year					pre-trial mean	95% C.I.s
births	0.072	0.124	0.142	0.070	0.112	0.071 - 0.154
Immigrants	0.344	0.332	0.258	0.296	0.311	0.259 - 0.364
emigrants	0.057	0.023	0.036	0.034	0.039	0.019 - 0.059
mortality	0.406	0.329	0.451	0.366	0.395	0.326 - 0.465
growth rate, *r*	-0.048	0.104	-0.087	-0.034	-0.010	-0.125 - 0.104
sample year	1989	1990	1992	1993	1994	
population size	251	237	275	246	241	

[a] includes 18 locally recruited sentinel dogs born in the study site during the sampling interval.

[b] includes 71 Belem dogs installed during the sampling interval as part of the cohort study.

[c] includes 35 sentinel study dogs (14 local recruits) which died during the sampling interval.

Having confirmed the stable age distribution, the proportion of dogs in the zero age-class were used to calculate the instantaneous population turnover rate, giving a mean ν = 0.558 per year (95% C.L. 0.436–0.681).

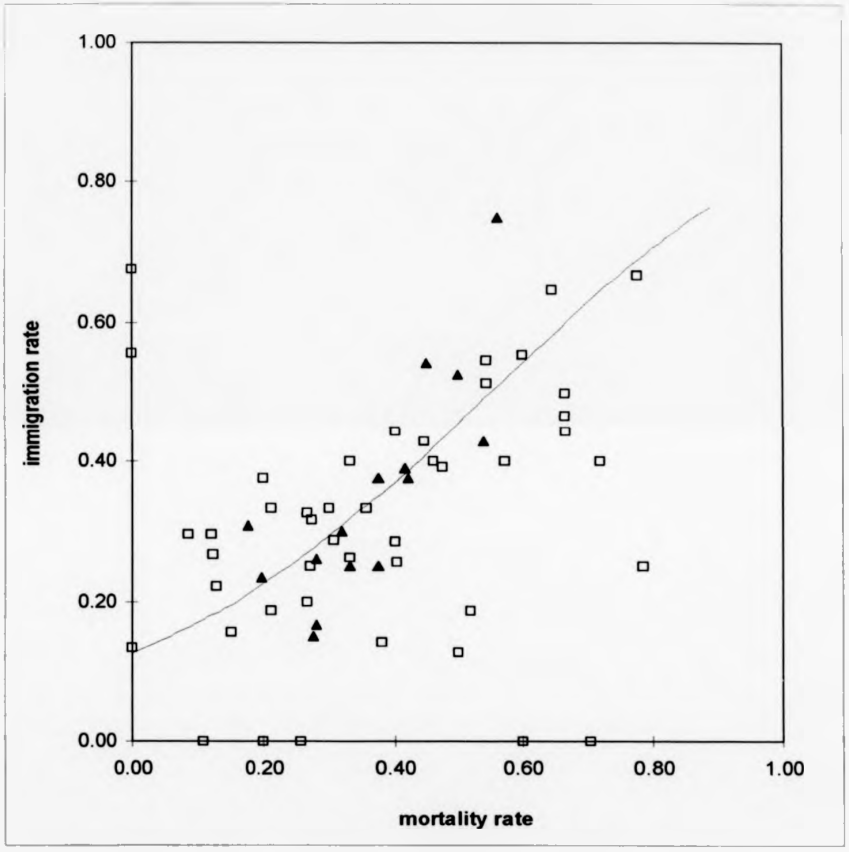
Population dynamics- birth, mortality and migration

Population turnover was principally driven by immigration. During the years prior to the cohort study, new dogs were recruited into villages at a mean rate of 0.311 per year (95% C.L. 0.259–0.364), the majority (84%) of which were ≤ 12 months of old upon arrival. Of the 56.4% dogs of known origin, 91% were acquired from communities and small towns on Marajó, and the remainder from the city of Belém (5.3%) and surrounding satellite towns (2.7%). The mean proportion of dogs which died was 0.395 per year (95% C.L. 0.326–0.465) which was similar to the immigration rate of 0.311 above.

At the village level, mortality rates ranged from 0.207 to 0.761 per year, which corresponded with the immigration rate during the same period (z = 2.77, P = 0.006) **(Figure 3.1)**. No statistical differences were detected between pre-trial years (comparison of slopes: z = 1.65; intercepts: z = 1.39). The apparent compensatory process of immigration was confirmed during 1993–1994 when the imported sentinel dogs were given to willing householders. In response, independent dog acquisition declined from the pre-trial mean of 0.311 to just 0.079, whereas inclusion of the sentinel dogs in this calculation gave an immigration rate of 0.296 per year, which was not significantly different to the pre-trial mean (comparison of pre-trial and trial slopes: z = 1.06).

The birth rate was only 0.112 per annum (95% C.L. 0.071–0.154), approximately a third (36%) of the immigration rate. Similarly, the emigration rate was low at 0.039 per year (95% C.L. 0.019–0.059). Changes of dog address within the study site during the pre-trial period were also infrequent: only 2.2% (S.E. 0.47) of animals moved between houses within the same village, and 0.94% (S.E. 0.35) moved between villages (all accompanied by their owners).

Figure 3.1 Relationship between dog mortality and immigration in 15 study villages between 1989–1994. The observed (symbols) and fitted (——) rates were not found to differ between pre-trial (□) and trial (▲) periods. Trial period data include imported sentinel dogs. Data points represent adjusted annual rates per village.



Life expectancy

The instantaneous mortality rate of the resident dogs estimated by survival analysis was $\mu = 0.404$ per year (95% C.L. 0.166–0.980), equivalent to a median life expectancy of $L = 2.48$ years (95% C.L. 1.02–6.01) (**Table 3.4**). No differences were apparent in mortality rates of the 3 pre-trial years periods (log-rank test for equality of survivorship functions: $\chi^2 = 2.93$), between pre-trial and trial periods (stratified log-rank test for equality of survivorship functions: $\chi^2 = 0.08$), nor local (76/117) and immigrant (278/427) resident dogs (log-rank test stratified by year, $\chi^2 = 1.12$), nor the sexes (log-rank test stratified by year, $\chi^2 = 0.48$). The shape parameter of the survival curves for each of these groups was $\alpha > 1$ (Weibull regression: H_0: $\alpha = 1$: $z > 5.70$, $P < 0.001$), indicating that the probability of death decreased with time in the study site.

b) The epidemiological significance of the variation in resident population parameters

Explaining dog infection

The seroprevalence of dogs in the 15 study villages was calculated from the 4 pre-trial cross-sectional surveys. The variation between villages ranged from 0.0 to 0.94 in any single year, and the incidence rate among seronegatives calculated by combining data for all 4 years ranged from 0.171 to 0.750 per village per year. To explain the variation in infection across villages, the potential explanatory variables tested included measures of both host (DDEN, HDEN, DH, HH, RA) and sandfly (HF, SF) abundance (see **Table 3.1**).

None of the test parameters (DDEN, HDEN, DD, HD, RA) was significantly associated with seroprevalence when controlling for sample year ($z < 2.55$, in each case), whereas village period incidence was positively associated with mean human : dog ratio (RA), and sandfly abundance in animal sheds (SF) and houses (HF), but negatively correlated with the number of dogs per household (DH) ($z > 2.27$, $P < 0.017$, in each case) (**Table 3.5**).

Table 3.4 Comparative demographical parameter estimates for the resident dogs, sentinel dogs, and fox populations

study population	μ instantaneous mortality rate / year[c]	μ 95% C.L.s	α shape parameter	L life expectancy (years)[c]	L 95% C.L.s	ν instantaneous turnover rate / year (95% C.L.s)	N sample size
resident dogs[a]	0.404	0.166 - 0.980	1.43	2.48	1.02 - 6.01	0.558	237 - 275
born locally	0.410	0.047 - 3.610	1.80	2.44	0.28 - 21.5	(0.436 - 0.681)	
immigrant	0.411	0.148 - 1.140	1.35	2.43	0.88 - 6.74		
sentinel dogs	0.691	0.545 - 0.868	1	1.45	1.15 - 1.84	--	126
local	0.633	0.003 - 159.1	0.67	1.58	0.01 - 397.0	--	99
Belem	0.679	0.036 - 12.90	1.46	1.47	0.08 - 27.90	--	27
foxes							
1988-91	0.325	0.180 - 0.587	1	3.08	1.70 - 5.56	0.844	25
1994-95	0.314[b]	0.194 - 0.507	--	3.19	1.97 - 5.16	0.844	35

[a] parameter values were calculated from the data collected during 1989 to 1994.

[b] calulated using equation 3.5 assuming $\alpha = 1$, and a stationary stable population

[c] estimates represent means except where $\alpha > 1 > \alpha$, in which case they represent medians calculated using a Weibull function (see Chapter 2)

Table 3.5 Relationship between dog incidence and individually tested host and sandfly parameters. Each "period" test variable (described in **Table 3.1**) was entered into a separate maximum model (with binomial errors and logit link function). The outcome and explanatory variables here are mean values for all sample years (see Methods).

explanatory variable (discrete)	parameter coefficient	*S.E.*	*P* <	r^2
RA	0.4888	0.1787	0.006	0.3804
DH	-1.5741	0.5023	0.002	0.4447
HF	1.4981	0.6303	0.017	0.3545
SF	1.0175	0.4482	0.023	0.3278
DDEN	-	-	*N.S*	-
HDEN	-	-	*N.S*	-
HH	-	-	*N.S*	-

As expected, the multivariate model to explain seroprevalence while controlling for all test parameters was not significant, whereas the equivalent MAM to explain period incidence (**Table 3.6**) retained only the single variable, DH, suggesting that the incidence amongst dogs in a village decreased with the mean number of dogs per household. This variable explained 44.5% (r^2 = 0.4447) of the total variation in incidence.

Table 3.6. Multivariate analyses to explain dog seroconversion rate (period incidence) across villages. The MAM resulting from the reduced full model (with binomial errors and logit link function) which included the test variables shown in **Table 3.5**.

explanatory variable	parameter coefficient	*S.E.*	*P* <	r^2
DH	-1.5741	0.5023	0.002	0.4447
MAM	total deviance	37.1		

Explaining dog mortality

The number of seropositive and seronegative dogs (estimated by IFAT) which died between sample rounds is shown in **Table 3.7**. The average survival time of seropositive dogs was significantly shorter than that of the seronegative dogs when controlling for age and stratifying by sample year (log-rank test, χ^2 = 5.81, *P* = 0.01). This result is partially due to the difference in shape parameters for the two groups (α =

1.85 *vs.* 1.56) (Log-likelihood ratio test: $\chi^2 = 8.40$, df. = 2, $P = 0.015$). However there was a difference between sample years (Weibull model: $z = 2.42$, $P < 0.001$): only in the year ending 1990 was there statistical evidence that the mortality rates differed between the serological groups when controlling for age (Log-likelihood ratio test: $\chi^2 = 4.51$, $P = 0.034$). Given the similarity in most years, the median mortality rate for serological groups combined was used to calculate I (as shown in **Table 3.4**).

Table 3.7. Mortality of resident dogs classified by serum anti-*Leishmania* antibody (IFAT) response between 1989–1994. Percentages are adjusted to annual rates.

	pre-trial period			trial period
	1989-90	1990-92	1992-93	1993-94
interval (months)	10	18	12	16
N dogs examined	240	228	251	288
serological response				
died (%)	42 (55.1)	61 (64.8)	65 (75.2)	97 (64.0)
positive *N*	114	98	123	169
died (%)	43 (50.4)	57 (38.4)	60 (63.2)	68 (63.6)
negative *N*	126	130	128	119

The proportion of resident dogs which died in the 15 villages between each of the 4 sampling rounds ranged from 0.0 to 0.786 per year. The test variables to explain the variation in "discrete" and/or "period" mortality estimates included measures of dog abundance, in addition to incidence (INCID) and seroprevalence (PREV), as described in **Table 3.1**.

Seroprevalence (PREV) was the only significant variable associated with the discrete measures of inter-village dog mortality, when controlling for sample year ($z = 2.78$, $P < 0.005$) **(Table 3.8a)**. Mean village dog mortality ("period" estimates) was positively associated with dog incidence (INCID), and sandfly abundance in houses (HF) ($z > 3.79$, $P < 0.001$, in both cases) **(Figure 3.2)**, and more marginally so with seroprevalence (PREV), and sandfly abundance in sheds (SF) ($z > 2.00$, $P < 0.05$, in both cases) **(Table 3.8b)**.

Table 3.8 Explanatory variables of dog mortality across villages. The outcome and test parameter values represent **(a)** "discrete" estimates measured at each sampling period , and **(b)** averaged over all years ("period" estimate) (described in **Table 3.1**). Each test variable was entered into separate maximum model (with binomial errors and logit link function), while in (a) controlling for sample year.

(a)

explanatory variable	parameter coefficient	*S.E.*	*P* <	r^2
PREV	1.5108	0.5434	0.005	0.1204
DDEN	-	-	*N.S*	-
RA	-	-	*N.S*	-
DH	-	-	*N.S*	-

(b)

explanatory variable	parameter coefficient	*S.E.*	*P* <	r^2
PREV	3.1383	1.5663	0.045	0.2415
INCID	2.5559	0.6736	0.001	0.5395
DDEN	-	-	*N.S*	
RA	-	-	*N.S*	
DH	-	-	*N.S*	
HF	1.7009	0.2863	0.001	0.7771
SF	0.8766	0.3397	0.010	0.3848

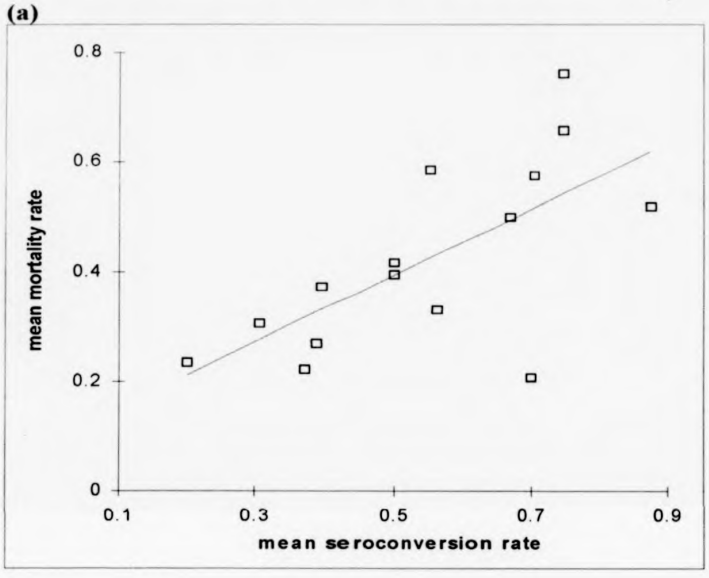
In multivariate analysis, PREV was the single parameter retained in the MAM to explain the annual ("discrete") estimates of village mortality ($z = 2.00$, $P < 0.005$, $r^2 = 0.2145$). The variation in "period" mortality was strongly associated with the mean abundance of sandflies in houses (HF) ($z = 4.52$, $P < 0.001$), and more marginally with the mean village incidence rate ($z = 2.20$, $P < 0.028$). The MAM explained 85% ($r^2 = 0.8500$) of the total variation in inter-village mortality; INCID and HF explained 7.3% ($r^2 = 0.0728$) and 32.0% ($r^2 = 0.3202$), respectively (**Table 3.9**).

Table 3.9. Multivariate analysis to explain dog mortality across villages. The MAM resulting from the reduced full model (with binomial errors and logit link function) which included the "period" test explanatory variables shown in **Table 3.8b**.

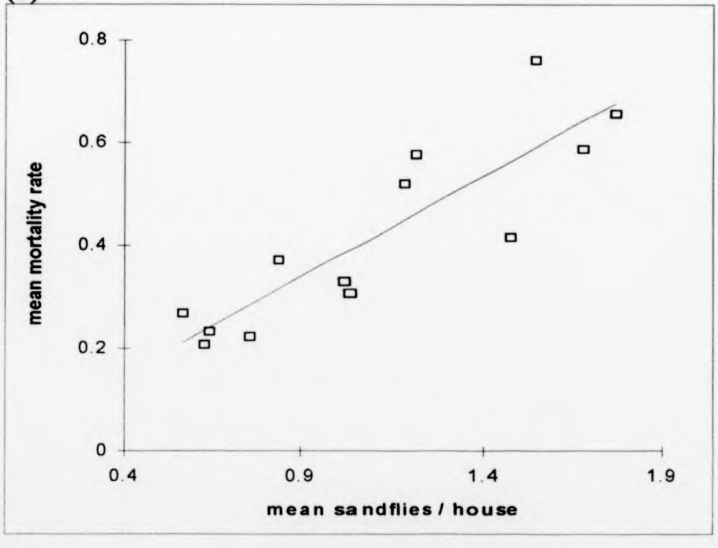
explanatory variable (period)	coefficient	*S.E.*	$P <$	r^2
INCID	1.3293	0.2944	0.028	0.0728
HF	1.1710	0.5317	0.001	0.3202
MAM	total deviance 93.1			0.8500

Figure 3.2. Explanatory variables of dog mortality recorded in the study villages between 1989–1993. **(a)** mean seroconversion rate calculated over the entire period for 15 villages, **(b)** geometric mean number of sandflies per house in 13 villages.

(a)



(b)



3.4.2 Sentinel dogs

Life expectancy

By the end of the study, 80.2% (81/101) of Belém recruits and 77.8% (21/27) of local recruits had died ($\chi^2 = 0.08$), with a similar proportion in each group, 25 (30.9%) and 6 (28.6%), due to accidental causes. Survival analysis detected no significant differences between Belém and local dog mortality, controlling for age (stratified log-rank test for equality of survivorship functions: $\chi^2 = 0.11$), giving an average life expectancy of $L = 1.45$ years (95% C.L. 1.15–1.84), calculated from an exponential mortality rate of $\mu = 0.691$ per year (95% C.L. 0.545–0.868) **(Table 3.4)**. No differences in α were detected prior to combining the data of the two recruit groups (Log-likelihood ratio test $\chi^2 = 3.1$, df. = 2).

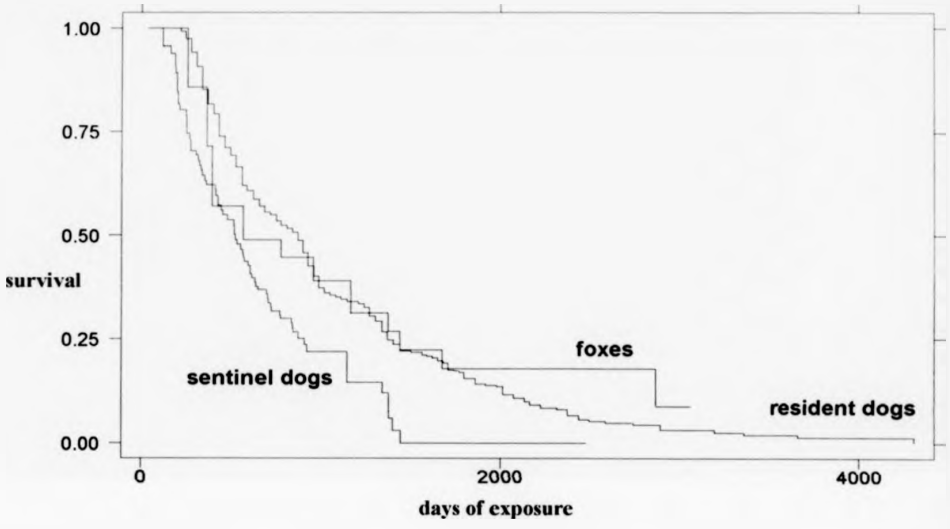
Compared to resident dogs, sentinel dogs survived for significantly shorter periods in the field (log-rank test: $\chi^2 = 27.1$, $P < 0.001$) **(Table 3.4; Figure 3.3)**, and at rates which differed with respect to age (comparison of shape parameters: Log-likelihood ratio test, $\chi^2 = 9.6$, df. = 2, $P < 0.01$).

The mean incidence of infection in the sentinel population was not dissimilar to that reported earlier for the resident population (1.33 vs. 1.56 per year), when calculated using the comparable method –an incidence-recovery model– (Courtenay *et al.*, 1994). These values, however, are expected to be gross underestimates of the absolute incidence since the prepatent period is ignored in their calculation (i.e. from infection to seroconversion) (see discussion in Chapter 6 and Quinnell *et al.*, 1997). A closer estimate of the true incidence in the sentinel dogs, accounting for the prepatent period, is described in Chapter 4. The infection and mortality rates of the sentinel dog were not tested relative to the resident host explanatory variables (dog and sandfly densities) due to the small number of sentinel dogs per village. However, the relationship between these two outcome parameters is fully addressed in Chapter 5.

Fecundity

The fecundity schedule of the female sentinel dogs (Belém and local recruits combined), and the survival rates of their offspring, are shown in **Table 3.10.** Of the 76 potentially

Figure 3.3 Survival of sentinel dogs, resident dogs, and foxes following initial exposure to *L. infantum*. Exposure was measured from the age of entry into the study-site (t_0); for foxes t_0 is set to zero



fertile bitches, 29 (38.2%) whelped during the average 9.64 month observation period, equivalent to 47.5% per year. The whelping rate (number of litters/per dam) was 1.34 (range: 1–2) per year each producing 3.9 (95% C.L. 0.37–7.43) pups per litter. The *per capita* fecundity rate increased with dog age **(Table 3.10)** with a mean of 1.80 per year. A total of 110 pups were born in 29 litters, of which only 7 (6.4%) survived to 2 months of age. This was the equivalent of a mortality rate of 0.468 per month, and average life expectancy of just 66.2 days.

Table 3.10. Age-specific fecundity schedule for sentinel dogs.

age-class (age at last birthday)	total potential fecund females (>6 m)	*t* total observation time in years (per breeding female)	proportion breeding pe r year	*p* total number of live offspring at birth	proportion of female offspring at birth	*f* fecundity rate per year
0	38	40	0.38	60	0.55	1.50
1	14	9	0.67	19	0.68	2.11
2–3	13	6.5	0.31	16	0.69	2.46
> 3	11	5.5	0.36	15	0.53	2.73
total or mean	76	61	0.48	110	0.59	1.80

Clinical signs preceding mortality were known for 97 (94.2%) of these pups. One or more signs including haemorrhagic diarrhoea, vomiting, and rapid onset of morbidity was seen or reported in 86/97 (88.7%) of cases, 54 (63%) of which were also emaciated, whereas the 11 other fatalities presented only the latter condition. Post mortum diagnosis was not performed.

3.4.3 Foxes

Life expectancy and population turnover

Data were available for 25 foxes caught between 1988–1991. By the end of that study, 12 (48%) foxes had died, confirmed by cadaver retrieval within a mean 10.3 days (95% C.L. 2.3–15.5) following their last live radio-location, or live capture. Survival analysis yielded a mortality rate μ = 0.325 per year (95% C.L. 0.180–0.587), which was

independent of age ($\alpha = 1$). This corresponded to a mean life expectancy $L = 3.08$ years (95% C.L. 1.70–5.56) (**Table 3.4**).

The cross-sectional age-structure of the 1988–1989 and 1994–1995 samples (25 and 37 animals respectively) were strikingly similar, with 57% of both samples ≤ 12 months of age. This implied that the population age-structure had been stationary between sample years. The instantaneous population turnover rate was 0.844 per year for both periods. The mortality rate of the 1994–1995 sample, calculated using **equation 3.5**, was $\mu = 0.314$ per year (95% C.L. 0.194–0.507), giving $L = 3.19$ year (95% C.L. 1.97–5.16) (**Table 3.4**), which was similar to the longitudinal estimate.

No differences was found between the survival rates of foxes (1989–1991 sample) and the resident dogs (log-rank test for equality of survivorship functions: $\chi^2 = 0.56$) (**Figure 3.3**), though the mortality rate of the former was constant with age ($\alpha = 1$) unlike that of the dogs (comparison of shape parameters: Log-likelihood ratio test, $\chi^2 = 5.62$, df. = 1, $P = 0.018$).

Data on the fox population infection rates are presented and compared to those in dogs, in Chapter 6.

3.5 DISCUSSION

3.5.1 The Marajó population relative to dog demographic world-wide

A resident population of *ca.* 250 dogs in rural Marajó was monitored between 1989 and 1994. The population had a stable age distribution and zero growth rate. A large proportion of the population were held in the < 1 year age-class indicating a rapid population turn-over rate. On average, new dogs acquired by householders entered the population at a rate of 31% per year, most (91%) from communities and small towns in Marajó. Dog acquisition appeared to be in response to dog losses, evidenced by a strong correlation between immigration and mortality rates across villages. Comparable rates of immigration (e.g. 26%–55%: Beran and Frith, 1988; Nasser and Mosier, 1980; Nasser *et al.*, 1984), and adult mortality (e.g. 19%–42%: Schnurrenberger *et al.*, 1961; Cleaveland, 1996) are known for dog communities elsewhere. The Marajó population is unusual however because it is sustained almost entirely by immigration: if the population had remained closed to migration, it would have decrease at a rate of 32% per annum. Only one dog community, in urban Kansas, USA, is identified from the literature as reliant on immigration (Nasser and Mosier, 1980; Nasser *et al.*, 1984), but this is due to dog owners having their pet dogs surgically spayed. Canine birth control is not practised in Marajó.

The more common picture in both developed and developing regions is that dog abundance is maintained by local births (e.g. Gurtler *et al.*, 1990; Cleaveland, 1996; Brooks, 1990). The surprisingly low input of such dogs in Marajó suggests poor fecundity and neonate survival. Fecundity data were not available for the resident dogs, but the proportion of sentinel bitches which whelped (0.48) was not low relative to dogs elsewhere (e.g. 0.18–0.60: Brooks, 1990; Robinson *et al.*, 1996), neither was the mean litter size of 3.9 (e.g. rural: 3.5–5.7, Cleaveland, 1996; urban: 2.1–4.1, Beran and Frith, 1988; Robinson *et al.*, 1996), or the whelping rate of 1.34 (e.g. 0.66: Coleman, 1998). Neonatal mortality, on the other hand, claimed 93% of the pups within their first 2 months. This is higher than the recorded maximum rate (84% to 3 months, Kitala and McDermit, 1995), and the more common range of 25%–60% (mean 39.2%: Cleaveland, 1996; Brooks, 1990; Beran, 1985; Barin and Frith, 1988).

No attempt was made to confirm the precise cause of pup mortality in this study, however up to 89% of the pups which died were reported or seen with signs consistent with canine parvovirus (CPV) infection (e.g. haemorrhagic diarrhoea and vomiting), known to be commonly fatal to 8–12 week old pups (Appel and Parrish, 1987). Although there was no evidence of a CPV epidemic, 7.7% of 39 resident adult dogs in Marajó tested seropositive for anti-CPV-2 antibodies (O. Courtenay, R. Quinnell & C. Dye, unpublished data). There are no published data on the effects of CPV on reproduction in endemic populations, though there are reports that the virus can reduce reproductive success in breeding kennels of susceptible dogs (Gooding and Robinson, 1982). The high prevalence of CVL among sentinel dams is a likely contributory factor to pup mortality since it certainly interfered with the provision of maternal milk, and presumably anti-CPV-2 antibodies. Assuming that the sentinel dog reproductive success mimics that of the resident dogs, then the low input of locally born dogs into the resident population is not surprising.

3.5.2 The effect of installing the sentinel population

Instalment of the imported sentinel dogs into the study villages did not significantly alter the pre-trial dynamics of the resident population, nor the population size, or village dog densities. This was principally due to the willingness of villagers to accept these dogs in the place of those that they would have ordinarily sought. The average life expectancy in the sentinel population, however, proved to be shorter than in the resident population (1.45 *vs.* 2.48 years). The reason is not obviously attributable to the study design since mortality was not dissimilar in imported *vs.* local sentinel dogs, nor immigrant *vs.* local born resident dogs (see **Table 3.4**). This fact would also seem to rule out a possible discrepancy in treatment of sentinel *vs.* resident dogs by householders; mortality was not density-dependent to suggest resource limitation at either the household or village level. Disproportionate susceptibility to *Leishmania* infection or CVL is also not inferred from the infection data since there was no difference in the incidence of infection or disease among Belém and local recruits over the extended study period (Quinnell *et al.*, 1997; and see Chapter 4), nor did the seroprevalences of resident dogs alter significantly between pre-trial and trial years (see **Table 3.7**). With no clear explanation for this

difference, the mortality rate of the resident dogs is clearly the more appropriate estimate to describe the endemic situation (see below).

3.5.3 Correlates of infection and mortality

Resident dog mortality across villages was strongly associated with seroprevalence and incidence, indicating *Leishmania* infection as a significant cause of death. Incidence in dogs was inversely correlated with the number of dogs per household (DH), and positively associated with the human : dog ratio (RA), and the numbers of sandflies in houses (HF), the latter being a crude index of transmission. The inference is that dogs are not the dominant factor attracting sandflies to the peridomestic environment, but that the risk of infection for a dog increases with a reduction in alternative bloodmeal sources in its immediate vicinity. Study dogs do not tend to sleep inside houses, but alongside houses and in open-sided dining huts attached to houses. The factors which govern host-sandfly contact and sandfly abundance are clearly complex. *Lu. longipalpis* abundance, for example, is influenced by the local habitat type, house construction, and the presence of animal sheds (Quinnell and Dye, 1994a; 1994b), whereas *Lu. longipalpis* attraction to hosts is thought to be predominantly a function of host size (Quinnell *et al.*, 1992). No relationship was found in this study between sandfly abundance and the number of hosts when correlating average values for houses per village, but Quinnell and Dye (1994a) reported a positive relationship with the number of dogs per household in analysis of these data at the household level.

These results further indicate that dog density patterns may influence the risk of human infection. Gavgani (1998) showed (in univariate analysis) that human infection rates were positively associated with dog abundance, and negatively correlated with the human : dog ratio, measured at the village level in North West Iran. The presence *vs.* absence of household dogs is also thought to be a risk factor for human transmission (Kotkat *et al.*, 1986; Gavgani, 1998), though this is not consistently observed in all foci (Evans *et al.*, 1992; Faris *et al.*, 1988; Navin *et al.*, 1985; Rab *et al.*, 1995).

3.5.5 Some implications for ZVL control

The high population turnover rate in Marajó implies that there is a large and steady supply of susceptible animals to drive transmission. Dogs which leave the population are replaced by others acquired by villagers. The consequence is that dog elimination is likely to increase the input rate of susceptibles, and thus the effort required to sustain this form of control. In these circumstances, ZVL control strategies which leave non immune dogs *in situ* (e.g. insecticides, future vaccine) should therefore have a distinct advantage. Accounting for the dog replacement process in a comparative simulation model of ZVL control options, Dye (1996) suggests that culling is likely to reduce human and dog incidence at a slower rate than achieved by residual insecticide, a dog / human vaccine, or by chemotherapy. Given the present estimate of $R_0 = 5.9$ (based on an estimated $L = 563$ days and $A = 115$ days, Quinnell *et al.*, 1997), an approximation of the critical pulse culling interval is $T_c = A / R_0 = 20$ days. This assumes that all infected dogs are infectious immediately upon seroconverting. This assumption is tested in Chapter 5. A programme of pulse vaccination would be required to maintain effective herd immunity at $100(1 - 1/R_0) = 89\%$, achieved by a pulse inter-vaccination interval of $T_v = L / R_0 = 95$ days.

The value of L in these calculations was obtained from μ for seropositive resident dogs (Quinnell *et al.*, 1997; shown in **Table 3.7**), where μ was assumed to be constant with age. In fact, the two parameter Weibull function fitted to the survival data in this chapter showed μ to decrease through time, giving a *median* life expectancy of $L = 905$ days (see **Table 3.4**). All else being equal, R_0 therefore may approach $R_0 = 1 + (905/115) = 8.9$, in which case the above value of T_c is an underestimate of the intervention effort, whereas the inter-vaccination interval is too conservative. One further assumption in calculating this parameter however is that *Leishmania*-induced mortality is the same for latent and infectious dogs (Quinnell *et al.*, 1997). While this appears to be true (see Chapter 5), it is less easy to precisely quantify *Leishmania*-induced *vs.* "natural" mortality (values for α and δ in equation A15 in Quinnell *et. al.*, 1997), not least due to the imprecision of existing diagnostic tools for *Leishmania*. Data presented in Chapter 4 indicates that *Leishmania*-related mortality does exceed natural mortality (i.e. $\alpha >> \delta$), in which case $L = 905$ days is probably an over-evaluation.

3.5.5 Comparison of dogs and foxes

Sixty foxes were caught in the two sampling periods. The age-distributions of the two samples were very similar, implying that the population had a stable age-distribution through time. The irregular trapping regime invalidated the use of mark-recapture techniques to estimate population size and growth rate (Caughley, 1977). However, given that the population is likely to be at carrying capacity, it is therefore likely to be stationary. On this assumption, a mortality rate (μ = 0.314 per year) was calculated using **equation 3.5** from the cross-sectional data of 1994–1995. This yielded a surprisingly similar value to that of 1989–1991 (μ = 0.325) obtained by survival analysis.

The life expectancy of foxes was similar to that for dogs (L = 3.14 *vs.* 2.48 years), despite evidence that foxes experience little additional mortality due to Leishmaniasis (see Chapter 6). None of the 37 foxes (73 sera) tested positive for antibodies to CPV-2 or canine distemper virus (CDV) (O. Courtenay, R. Quinnell & C. Dye, unpublished data). Alternatively, the high mortality rate appears to be due to hunting pressure, since this accounted for 83% of 12 deaths observed between 1989–1991 (Macdonald and Courtenay, 1996). This result implies that "recovery" in fox age-seroprevalence profiles (Courtenay *et al.*, 1994) are not due to a loss of anti-*Leishmania* antibody through disproportionate mortality of seropositives (this is further discussed in Chapter 6).

The observation that foxes had a population turnover rate, even higher than that of dogs (0.84 *vs.* 0.56 per year), suggests that foxes too had a steady input of susceptibles into the population. Unlike dogs, foxes live in typically small family groups (of 2–5 animals), arranged in spatially dispersed territories, and at a mean density of 0.55 animals per km^{-2} (*S.E.* 0.071, range: 0.273–0.769, n = 7 territorial groups). This makes direct access to foxes difficult for the purposes of practical control, in any case, their low densities questions their putative role as maintenance hosts for the parasite (Lainson and Shaw, 1987). This question is addressed directly in Chapter 6.

3.5.6 Conclusion

In conclusion, the data presented here describe some of the basic demographic processes contributing to the maintenance of the endemic in Marajó, and therein some clear implications for existing, potential, and future forms of ZVL reservoir control. Specific data on the clinical course and outcome of *Leishmania* infection are presented in Chapter 4 (for dogs) and Chapter 6 (for foxes), and in Chapter 5 the demographical estimates are used to mathematically explore the efficacy of present *vs.* potential strategies of culling. Finally, the data here have highlighted one potential short-coming of sentinel studies, in particular, the credibility of parameter estimates obtained in the absence of careful monitoring.

4. INFECTION AND DISEASE IN A COHORT OF SENTINEL DOGS

4.1 ABSTRACT

The course of *L. infantum* infection and clinical outcome in a population of 126 sentinel dogs established in 25 endemic villages in Marajó was monitored in 13 bimonthly sample rounds over a period of 24 months. Serological and parasitological samples were collected on all dogs through time, simultaneous to measuring seven clinical signs and two biochemical parameters on 116 of the dogs in a total of 562 independent clinical examinations. Correlates of disease outcome and mortality rate were quantified. By the end of the study, 80 dogs became infected with *L. infantum* confirmed by positive (ELISA) antibody titre (75 dogs), parasite detection in bone marrow (50 dogs) or both (45 dogs), representing 93% of the 86 dogs which remained in the study for > 3 sample rounds. Seventy-four infected dogs (92.5%) developed clinical conditions, whereas the point prevalence of symptomatics in the population was approximately 50%. This discrepancy was due to high rates of clinical recovery (87% of dogs) and sign re-acquisition (78% of dogs) between sample rounds. The variation in disease outcome was significantly associated with both antibody (ELISA) titre and parasite isolation success. Relative to dogs which were parasite negative and serologically negative (with titres $< 10^3$ log units, labelled "very low"), those with titres of $10^3 < 10^4$, $10^4 < 10^5$, and $> 10^5$ log units (labelled "low", "medium" and "high" responders, respectively) had significant additional risk (measured as odds ratios) of acquiring 1, 2 and 6 of the 7 clinical signs, respectively. The odds ratios of developing individual signs, if responding with "high" *vs.* "very low" titres, ranged between 2.8: 1 (for conjunctivitis) and 18.1: 1 (for lymphadenopathy). Dogs which also yielded parasites were 5.6 and 7.26 times more likely to acquire conjunctivitis and to be physically emaciated. Sixty-two of 92 dogs (67.4%) died of non-accidental causes, including 41 of 70 dogs (58.6%) with confirmed *Leishmania* infection. The risk of mortality was also

associated with both infection and disease. "High" titre responders and parasite positive dogs had an average 2.8 and 10.3 greater odds of dying than "very low" responders and parasite negative animals, respectively, whereas symptomatic dogs were 4.4 times more likely to die than asymptomatic dogs. Dogs which presented severe individual clinical signs were 7.5 to 22.3 times more at risk of death than those without the conditions. Sixty-four of the dogs were also classified by their longitudinal clinical profiles. Dogs classified as "sick" characteristically mounted "high" titres (70/230 sera samples) and experienced high mortality (22/34 dogs). By contrast those classified as "healthy" or "recovered" dogs neither mounted "high" titres (0/161 sera) nor suffered extensive mortality (1/19 dogs). Dogs which developed severe persistent clinical conditions ("sick" dogs) were statistically distinguishable from other clinical profile classes by their average antibody titre starting from 4 rounds (≈ 8 months) after initial exposure. The principal implications of these results are that **(1)** the dynamics of CVL in Marajó are similar to those in Europe, **(2)** dogs which develop persistent severe CVL can be identified by antibody titre in early infection for their selective control (e.g. drug therapy or culling), whereas **(3)** clinical condition is not a good predictor of immunological status. The applications of these findings for the reduction of transmission are discussed.

4.2 INTRODUCTION

Canine visceral leishmaniasis (CVL) is a systemic disease of the reticuloendothelial system which results in a spectrum of chronic to acute clinical and biochemical presentations, as described in Chapter 1. The outcome of infection is thought to be regulated by the animal's immunological status (Pinelli *et al.*, 1994; 1995; Killick-Kendrick, 1994; see Chapter 1), whereas population infection rates clearly reflect local epidemiological conditions. The dynamics of the disease in canid populations, however, are still poorly understood.

The available data indicate that the ratio of mild to severe clinical cases in the symptomatic population, revealed by cross-sectional survey, varies from 1: 0.67 to 1: 2.62 (Mancianti *et al.*, 1988; Gradoni *et al.*, 1988; Pozio *et al.*, 1981a; Saladrigas, 1992), with 32% to 90% of asymptomatic infections progressing to patent disease per year (Saladrigas, 1992; Pozio *et al.*, 1981a; Lanotte *et al.*, 1979). Up to 68% of patent cases may have a history of chronic infection (Saladrigas, 1992), with mortality rates due to CVL varying from < 10% to 62% and 88% per year (Dye *et al.*, 1993; Saladrigas, 1992; Pozio *et al.*, 1981a). Spontaneous recovery (clinical or serological) from severe disease, on the other hand, is typically rare at < 5% per annum (3/98: Lanotte *et al.*, 1979; Pozio *et al.*, 1981a; Saladrigas, 1992). While the few data from other endemic regions, including Brazil, indicate that the clinical signs commonly associated with *L. infantum* are similar throughout the parasite's range (**Table 1.2**; for Brazil see also Deane, 1956; Alencar and Cunha, 1963), the data are not sufficient for more detailed study of clinical development in relation to either immunological or epidemiological parameters.

Interest in the dynamics of CVL at the population level is two-fold. From the veterinary perspective, available antimony drugs usually provide only temporary remission, characterised by an inverse probability of cure with increasing disease severity (Mancianti *et al.*, 1988; Alvar *et al.*, 1994). The veterinary community is consequently advised to eliminate clinically severe cases and to treat asymptomatic or mild (oligosymptomatic) cases (Gradoni *et al.*, 1988; Oliva *et al.*, 1995). If severe cases could be differentiated during an earlier phase of infection, e.g. when asymptomatic or

oligosymptomatic, chemotherapy may be more effective. This is of particular interest in the European setting where dog elimination is less socially acceptable, and drug treatment is economically more viable.

From the epidemiological perspective, there is evidence that disease severity is associated with host infectiousness (Rioux *et al.*, 1972; Lanotte, 1975; Gradoni *et al.*, 1987), although this has recently been questioned (Molina *et al.*, 1994a). Similarly, anti-*Leishmania* antibody titre and parasite isolation success may or may not be reflected in CVL severity (Pozio *et al.*, 1981a; Dye *et al.*, 1993; Molina *et al.*, 1994a; Ferrer *et al.*, 1995). If severely infected dogs are in fact a better source of the parasite for human transmission, then control measures which reduce the mean disease severity in the population may also reduce ZVL incidence. Although chemotherapy has less immediate appeal in endemic Latin America (since treatment is not generally affordable, and control is primarily aimed at improving public health), differentiating infectious and uninfectious dogs using clinical or immunological marker is nevertheless desirable to target control.

Most of the available quantitative information on CVL comes from population studies in southern Europe. Canid disease dynamics, however, may not necessarily be the same in Europe as in Latin America, given, for example, the differences in dog ownership patterns. In Brazil, dogs are not generally well maintained, owners do not seek veterinary services, and pets are usually undernourished. One possible consequence of this is that dogs may be more susceptible to disease, as has already been shown for malnourished humans (Cerf *et al.*, 1987; Dye and Williams, 1993). Furthermore, diagnosis of CVL in Latin America may be complicated by non-specific signs due to additional pathogens. Fortunately the diseases which closely mimic CVL (e.g. systemic lupus erythematosus, ehrlichiosis) are few (Slappendel, 1988; Koutinas *et al.*, 1992; Ciaramella *et al.*, 1997); following the course of *Leishmania* infection using a range of diagnostic techniques, as in this study, should in any case facilitate differential diagnosis.

4.2.1 Study aims

In this chapter the aims are: **(1)** to describe the evolution of CVL in relation to infection parameters, **(2)** to assess the risk of mortality due to infection and disease among clinically defined groups, and **(3)** to test whether dogs with different clinical outcomes can be identified at an early stage in the course of infection. To achieve these aims, a population of sentinel dogs was installed into the endemic region in Marajó (as described in Chapter 2), and its infection and disease development followed through time. In Chapter 5, the questions concerning dog infectiousness are specifically addressed.

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4.3 METHODS

4.3.1 Study animals

Clinical records were obtained from 116 of the 126 sentinel dogs (described in Chapter 2), comprising 93 (93.9%) and 23 (85.2%) of the Belém and locally (Marajó) recruited populations respectively. The 10 sentinel dogs not included in this sample were lost from the study prior to clinical examination.

4.3.2 Sampling regime

The numbers of dogs clinically examined in each round following their instalment is shown in **Table 4.1**. The sampling dates are those shown in Chapter 2 (**Table 2.1**), coinciding with the acquisition of serological and parasitological data (following the methods described in that Chapter), and xenodiagnosis (Chapter 5). All dogs were visibly healthy when enrolled into the study. Clinical parameters were measured in detail only after August 1993, and, with the exception of 9 dogs, not in the first round (instalment). Consequently, for the 22 and 19 dogs enrolled in April and June 1993, clinical data were available only from rounds 3 and 2 respectively. Otherwise, dogs were examined in every round that they remained in the study.

Table 4.1. Sampling regime of sentinel dogs clinically examined in sample rounds 1–13.

sampling round	Belém dogs	local dogs	Total dogs sampled
instalment 1	4	5	9
2	58	15	73
3	59	12	71
4	62	16	78
5	59	12	71
6	49	11	60
7	40	11	51
8	29	9	38
9	23	10	33
10	22	6	28
11	17	6	23
12	12	5	17
13	6	4	10
Total samples	440	122	562

During the study, 13 dogs became seriously ill and were therefore painlessly euthanized using recommended dosages of Pentobarbitone Sodium (Euthanol®). It was considered that none of these dogs would have survived for more than *ca.* 10 days.

4.3.3 Samples

a) Clinical assessment

Seven clinical parameters usually associated with *L. infantum* infection were measured on each dog at each sample, defined as:

(1) dermatitis (DE): skin alteration excluding alopecia and chancres

(2) alopecia (AL): loss of hair

(3) chancres (CH): skin lesions/ulcers

(4) lymphadenopathy (NO): palpable popliteal lymph nodes

(5) onychogryphosis (ONY): excess nail growth

(6) conjunctivitis (CONJ): ocular discharge

The severity of each of these signs was scored on a relative scale of 0 (absent) to 3 (extensive). Note that chancre (CH) severity scores reflect the quantity, not quality, of skin lesions.

(7) body condition (COND):

An index of body condition was obtained by measuring the body fat coverage (depth) across the vertical posterior spinal-iliac process by applying gentle finger pressure, adapted from Wildman *et al.* (1982). Coverage was scored from 1 (emaciated) to 4 (above average), where a score of 3 represented a dog of average nutritional status. A score of 1, indicating anorexia, was defined here as a 7th clinical sign of CVL.

From the severity scores of signs 1–7 described above, 4 additional variables were generated for each sample, calculated as:

(8) total number of signs (NUM), the number of signs with severity scores > 1 (or 1 for COND),

(9) total skin disease (TD), the sum scores of dermatitis (DE) and alopecia (AL),

(10) total other disease (TE), the sum scores of chancres (CH), lymph nodes (NO), onychogryphosis (ONY), and conjunctivitis (CONJ),

(11) total disease score (TS), the sum of TD and TE.

b) Biochemical measures

***Packed cell volume* (PCV)**

PCV was measured as an indicator of anaemia. Whole blood was collected into duplicate heparinised microhematocrit capillary tubes, which were then centrifuged for 5 minutes. The packed cell volume (haematocrit) was measured as the percentage of the blood volume occupied by erythrocytes (Willard *et al.*, 1989). Samples were collected from 64 dogs on 212 occasions in rounds 8–13. On the few occasions that duplicate samples gave different values, the highest PCV value was used for analysis. The "normal" PCV value for adult dogs is 45.3% (95% C.L. 36.8 - 54.4) (Willard *et al.*, 1989). However, because anaesthesia using some drugs is known to cause physiological changes which can depress PCV values (Bennett *et al.*, 1991), the "normal" value range for this study was calculated from 17 samples taken from 7 healthy-looking anaethetised sentinel dogs collected 3 or more rounds prior to their known seroconversion dates (i.e. before the estimated pre-patent period). These data gave a mean of 35.1% (95% C.L. 31.5% - 38.7%), indicating that PCV < 31.5% were abnormally low, thus taken here to be indicative of "anaemia". In analyses where PCV was used as the binary outcome variable, values were reclassified as < 31.5% and ≥ 31.5% as condition present (1) or absent (0).

***Blood urea nitrogen* (BUN)**

Raised urea nitrogen in the blood is indicative of renal disorder (Palacio *et al.*, 1995). BUN was measured using clini-sticks (Azostix®, Bayer, Germany), which give alternative colour-coded values of 3.3, 7.5, 12.3, and 21.6 mg/dl following 1 minute exposure to whole blood. The respective values were interpreted as "average/below average", "average", "'slightly high" and "high" according to the manufacturer's recommendations. Samples were obtained from 56 dogs on 189 occasions in rounds 9–13. The "normal" values calculated from 10 samples taken from 6 sentinel dogs (prior to seroconversion, as for PCV above), was 3.3 mg/dl (range: 3.3–7.5). In analyses,

values > 7.5 mg/dl were taken to represent "above average" values for the population (complying with the quantitative class description above), and indicative of renal disorder. In analyses where BUN was used as the binary outcome variable, values were reclassified as (0) ≤ 7.5mg/dl, and (1) > 7.5mg/dl.

c) Ectoparasites

Dermatitis and alopecia may result from infestation with one or more mange mites including *Sarcoptes scabiei*, *Demodex canis* and *Cheyletiella yasguri*. Duplicate skin scrapes of dermal alterations were prepared in rounds 5 and 9 for 36 dogs on 45 occasions, and microscopically examined. Canine tungiasis caused by *Tunga penetrans*, results from penetration of the skin, usually the footpads, by impregnated female sand fleas. Heavy infestations generally render dogs less mobile, thus conceivably contributing to excessive nail growth (onychogryphosis). Footpads of dogs were visually inspected at all clinical examinations (see sampling regime above), and scored on a scale of 0 (absent) to 3 (heavily abundant). Heavy infestation with *T. penetrans* (scores of 3) was taken to be a potentially confounding agent of onychogryphosis (ONY).

d) Serology and parasitology

Serological and parasitological samples were collected as described in Chapter 2. Data for the entire sentinel population (126 dogs) are presented in the text to provide the background for the subsequent clinical analyses (116 dogs), which focus on the associations between serological, parasitological, clinical and biochemical parameters during the course of infection.

4.3.4 Analysis

The analytical variables, and their abbreviations used throughout the text, are described in **Table 4.2**.

Table 4.2 Continuous and categorical variables used in statistical analysis.

Variable	abbreviation used in text	Category values
dog identity	DOG	1-116
dog origin	ORI	1 Belém, 2 local
parasite isolation success	PARA	0 neg, 1 pos
parasite isolation technique	TECH	1 culture, 2 hamster, 3 slide
inter-round survival	SURV	0 alive, 1 dead
dermatitis	DE	0-3
alopecia	AL	0-3
chancres	CH	0-3
conjunctivitis	CONJ	0-3
onychogryphosis	ONY	0-3
lymph node enlargement	NO	0-3
body condition score	COND	0-4
total number of signs	NUM	0-7
total dermatitis score	TD	0-6
other clinical signs score	TE	0-12
total clinical score (TD+TE)	TS	0-18
blood urea nitrogen (mg/dl)	BUN	3.3, 7.5, 12.3, 21.6
haematocrit (%)	PCV	continuous
log ELISA titre	ELISA	continuous
time (days) since instalment	Tf	continuous
time (days) since seroconversion	Tc	continuous

a) Recovery and re-acquisition of clinical signs

Inter-round recovery from individual signs (1–7) was defined as a change in severity score from > 0 to 0 (1 to > 1 for COND) between rounds, whereas sign "re-acquisition" was defined as a score > 0 following prior recovery in any previous round.

b) Predisposition to infection

To test the possible effect of nutritional status on a dog's subsequent infection outcome, body condition score (COND) was measured -4, -2 and 0 months (rounds -2, -1 and 0) from seroconversion on 29, 60 and 71 dogs respectively. These data were used to explain the variation in subsequent level of infection, measured as **(1)** the maximum subsequent ELISA titre, and **(2)** the percentage of times that parasites were successfully detected in bone marrow aspirates. Only dogs which remained in the study for more than two rounds post-seroconversion (i.e. the minimum time observed to develop a maximum titre), were included in the analysis.

c) Classification of longitudinal clinical profiles

Round by round clinical assessment revealed whether dogs were diseased at any single examination. To describe the population in terms of its long-term clinical outcome, dogs were classified on the basis of their longitudinal development. This was achieved by scoring dogs at each round as symptomatic (1) or asymptomatic (0) for any of signs 1–7. To discount mild conditions (i.e. to increase the probability of characterising *L. infantum* infection), original scores of 1 for individual signs 1–6 (see Clinical assessment) were treated as zero. Using these data it was then possible to categorise dogs into 4 clinical outcome classes based on their longitudinal score sequences, as follows:

(a) "sick": uninterrupted score sequences of 1 from the time of initial clinical development. An asymptomatic round in between a minimum of 2 symptomatic rounds in sequence (e.g. 1,1,0,1,1) was permitted so long as the overall asymptomatic / symptomatic ratio did not exceed 1/5. This class represented dogs that became sick and remained so until the end of the study or until their death.

(b) "healthy": the reverse sequence of (a) (e.g. 0,0,1,0,0), representing dogs that never or rarely presented signs.

(c) "recovered": a minimum of 2 sequential symptomatic scores followed by at least two asymptomatic scores (e.g. 1,1,0,0). Representing dogs that became sick and then clinically recovered.

(d) "fluctuating": score sequences which did not fit the patterns of **(a)–(c)** above (e.g. 0,1,0,1), indicative of dogs which fluctuated between sick and not sick.

64 dogs were classified using this method; the clinical profiles of dogs sampled < 4 times were not considered sufficient for reliable classification and were therefore excluded from the sample.

(c) "recovered": a minimum of 2 sequential symptomatic scores followed by at least two asymptomatic scores (e.g. 1,1,0,0). Representing dogs that became sick and then clinically recovered.

(d) "fluctuating": score sequences which did not fit the patterns of **(a)**–**(c)** above (e.g. 0,1,0,1), indicative of dogs which fluctuated between sick and not sick.

64 dogs were classified using this method; the clinical profiles of dogs sampled < 4 times were not considered sufficient for reliable classification and were therefore excluded from the sample.

4.4 RESULTS

4.4.1 Description of serological and parasitological courses of infection

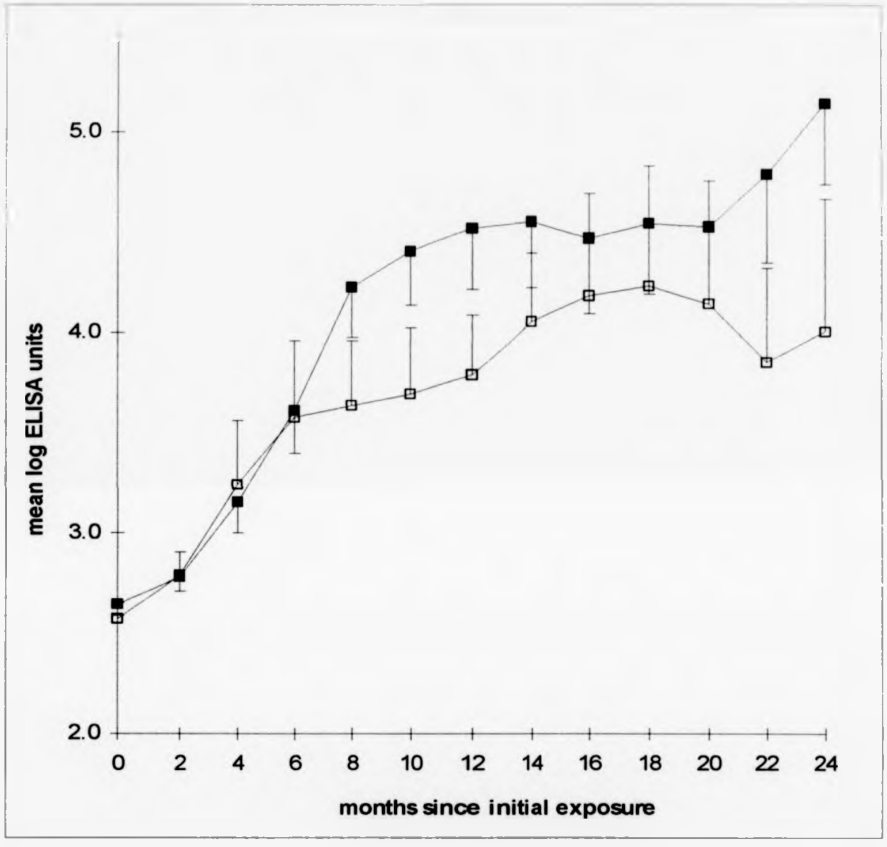
A total of 756 sera samples from the complete sentinel population (126 dogs) were tested for anti-*Leishmania* antibody by ELISA, and 514 bone marrow aspirates from 117 dogs were examined by microscopy of direct smear preparations (100 dogs, 237 samples), or indirect smears following inoculation into hamsters (39 dogs, 71 samples) or culture (104 dogs, 360 samples).

Following the methods described in Chapter 2 from Quinnell *et al.* (1997), 75 dogs seroconverted with a median period from infection to seroconversion (the pre-patent period) of 94 days (95% C.L.s. 82–111 days). The instantaneous incidence was 3.16 per year, which was based on an estimated mean time from instalment to infection of 115 days (95% C.L. 107–126 days). As expected, the population mean antibody titre increased with time from initial exposure **(Figure 4.1)**. Only 3 dogs [B12, C19 & C20] were considered to have recovered serologically by the end of the study, having shown positive titres which dropped below the threshold of 3.35 units, in 1/1, 3/3 and 6/8 ultimate rounds, respectively. The serorecovery rate was 3/221 per sampling interval, or 0.007 per year.

Amastigotes were detected in the bone marrow of 45 of the 75 seroconverted dogs, and an additional 5 dogs which did not seroconvert, giving a total of 89 positive bone marrow samples. Thus, 80 dogs were infected all of which belonged to the 116 clinically sampled dogs, or 80/86 (93.0%) of those which remained in the study for > 3 rounds.

The sensitivity of parasite isolation from seropositive dogs was greater by hamster (23/65 samples = 35.4%) than culture inoculation (49/224 = 21.9%) ($z = 4.57$, $P < 0.001$), or by direct smear examination (9/145 = 6.2%) ($z = 4.4$, $P < 0.001$). Parasite isolation success reached a maximum 2 months after seroconversion and declined thereafter (see **Figure 6** in Quinnell *et al.*, 1997). The mean antibody titre of parasite positive dogs was consistently greater than parasite negative dogs after seroconversion

Figure 4.1 Mean anti-*Leishmania* antibody response of the Belém (■) and locally recruited (□) sentinel dogs in samples rounds 1–13 following time of instalment. Bars represent 95% C.L.s.



(z = 3.63, P < 0.001), but not in the pre-patent phase (z = 1.54). The sensitivity of serology to detect parasite positive dogs was nevertheless low at 84.3% (75/89).

The only detectable difference in infection between Belém and locally (Marajó) recruited sentinel populations was that the former mounted a higher mean antibody response in 8/13 sample rounds, reflected in a significantly greater titre slope through time (comparison of slopes: z = 3.17, P < 0.002) (**Figure 4.1**), and yielded amastigotes more

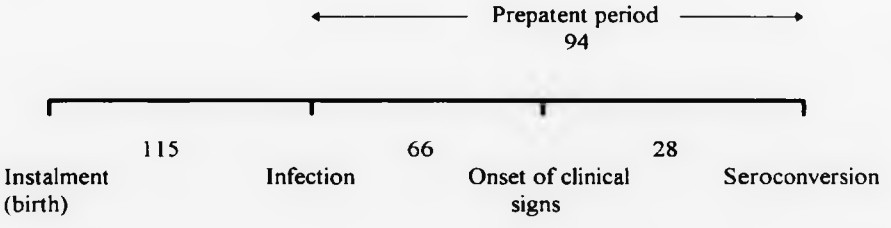
frequently in 4/13 sample rounds (test for difference in isolation success through time $z = 2.85$, $P < 0.004$). In all, 42/92 (45.7%) Belém and 6/25 (24.0%) local dogs were parasite positive on 74/405 (18.3%) and 8/101 (7.9%) sample occasions respectively.

4.4.2 Description of clinical course of infection

A subset of 116 sentinel dogs were clinically examined on a total of 562 occasions in 13 sample rounds over a period of approximately 24 months. Ninety-seven (83.6%) of them developed one or more clinical signs by the end of the study period, which included 74 (92.5%) dogs with confirmed *Leishmania* infection, and 23 (63.9%) of the 36 apparently uninfected dogs. Of the 19 dogs (7 *Leishmania* positive) which did not develop signs, only 2 (both *Leishmania* positive) remained in the study for > 2 rounds (for 4 rounds each).

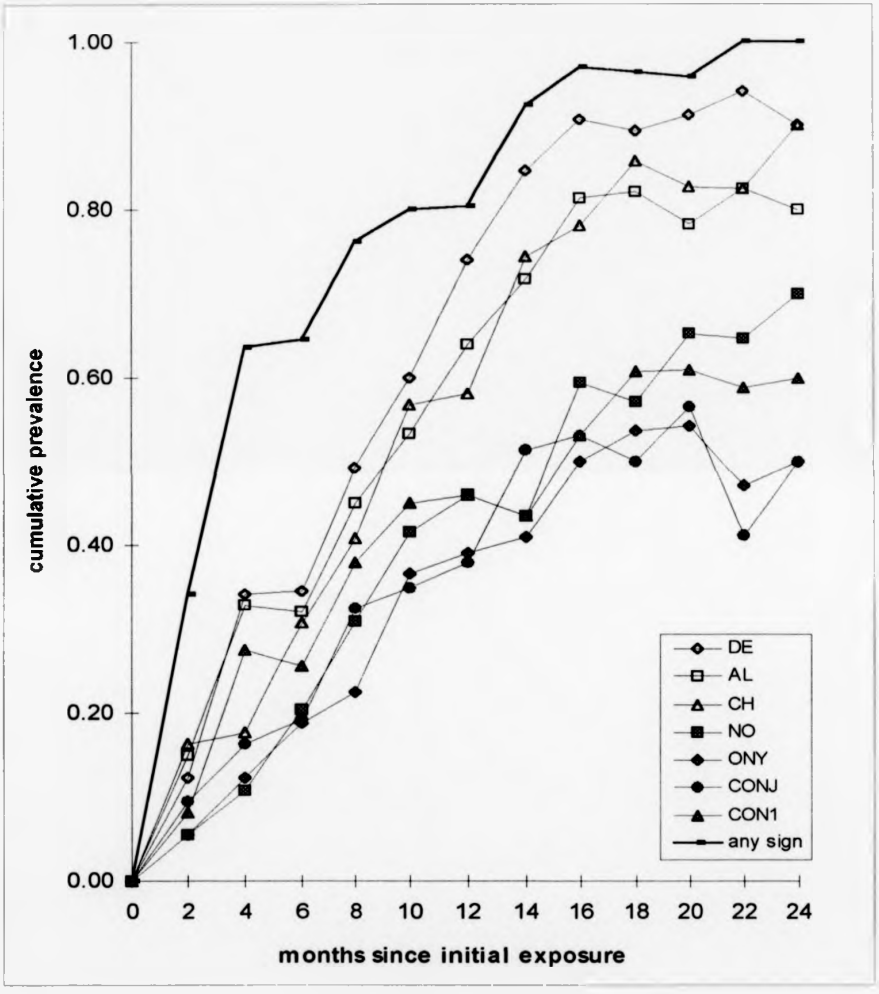
Thirty-four dogs had full sequential clinical records from the first round after instalment to the time of seroconversion. Disease signs were initially presented an average 181 days post instalment and 28 days (95% C.L.s. 9–65 days) prior to seroconversion. Subtracting the median 94 day pre-patent period gave a median incubation period (from infection to disease onset) of 66 days. The course of infection and disease is summarised in **Figure 4.2.**

Figure 4.2. The course of infection and disease in days from instalment.



The cumulative prevalence of signs 1–7 (DE, AL, CH, CONJ, NO, ONY & COND) in the population from time of instalment (**Figure 4.3**), indicated that all dogs became symptomatic by 24 months.

Figure 4.3 Cumulative prevalences of clinical signs 1–7 in the population from time of instalment



The cumulative prevalences of skin conditions (82%–94% for DE, AL and CH) were generally higher than those (54%–70%) of the other conditions. In contrast, the point prevalence of symptomatic dogs at any single cross-sectional sample round was approximately 50% following an initial *ca.* 4 month period of increase (**Figure 4.4**). Signs were strongly correlated with each other, both in terms of sign presence (*vs.* absence), and severity (**Table 4.3**). The frequency of total signs (NUM) acquired by dogs in each sample round did not suggest a dichotomy in the population's clinical response through time (**Figure 4.5**).

The point (cross-sectional) prevalence estimates were lower than the period estimates due, in part, to substantial spontaneous clinical recovery between sample rounds. Sixty-six (86.8%) of 76 afflicted dogs recovered from one or more signs on 260/671 (38.8%) of occasions, and 78% (46/59) of dogs re-acquired the signs, usually within 2 rounds (*ca.* 4 months) (**Table 4.4**). As expected, the odds of recovering from each condition was inversely related to the severity score (Mantel-Haenszel summary χ^2 for weighted odds ratios: $\chi^2 > 5.96$, $P < 0.015$, for each sign), though not dissimilar between individual signs (indicated by the 95% C.L.s, not shown). Inspection of the 95% C.L.s in **Table 4.4** indicate that the rates of sign recovery and reacquisition were generally similar irrespective of severity score. Neither sets of rates were dependent on the time from initial clinical onset (comparison of sign slopes through time: recovery: $z < 1.88$; re-acquisition $z < 2.14$, in each case).

Figure 4.4 Point prevalences of clinical signs 1–7 in the population from time of instalment

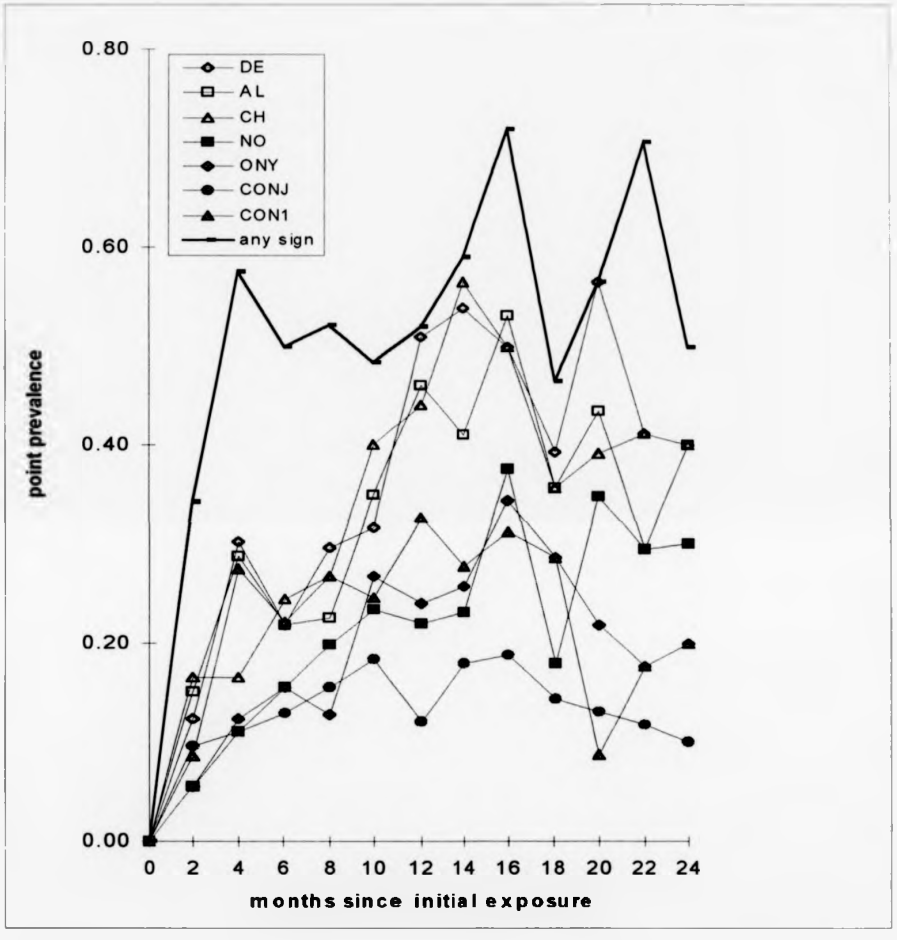


Table 4.3. Pairwise covariance coefficients (R) of clinical and biochemical measures. Values are shown for **(a)** sign presence/absence, and **(b)** clinical severity scores and biochemical values. R-values are shown in the upper diagonals and their Bonferroni-adjusted significance levels in the lower diagonals. Values were estimated controlling for time since instalment (Tf), and within-dog auto-correlation.

(a)

	DE	AL	CONJ	CH	NO	ONY	COND
DE	1	0.6649	0.2396	0.4385	0.3148	0.2925	0.3247
AL	0.001	1	0.2083	0.3433	0.2719	0.3050	0.3219
CONJ	0.015	0.001	1	0.1495	0.0856	0.0971	0.1577
CH	0.001	0.001	0.021	1	0.3548	0.3034	0.2807
NO	0.001	0.001	*NS*	0.001	1	0.3800	0.2552
ONY	0.001	0.001	*NS*	0.001	0.001	1	0.3831
COND	0.001	0.001	0.024	0.001	0.012	0.001	1

(b)

	DE	AL	CONJ	CH	NO	ONY	COND	PVC	BUN
DE	1	0.7533	0.3357	0.5019	0.3774	0.3605	-0.4652	-0.3721	0.0788
AL	0.001	1	0.2985	0.4653	0.3210	0.3700	-0.4727	-0.3898	0.1149
CONJ	0.001	0.001	1	0.3656	0.1952	0.2217	-0.2415	-0.3178	0.1799
CH	0.001	0.001	0.001	1	0.3942	0.3701	-0.4088	-0.3631	0.2050
NO	0.001	0.001	0.011	0.001	1	0.4430	-0.3843	-0.2648	0.1432
ONY	0.001	0.001	0.001	0.001	0.001	1	-0.5078	-0.3429	0.1502
COND	0.001	0.001	0.001	0.001	0.001	0.001	1	0.4271	-0.1763
PCV	0.001	0.001	0.024	0.001	0.018	0.001	0.001	1	-0.3691
BUN	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	0.001	1

Figure 4.5. Frequency distributions of the number of clinical signs (NUM) presented by dogs in sample rounds 1–13 (running top left to bottom right) post instalment

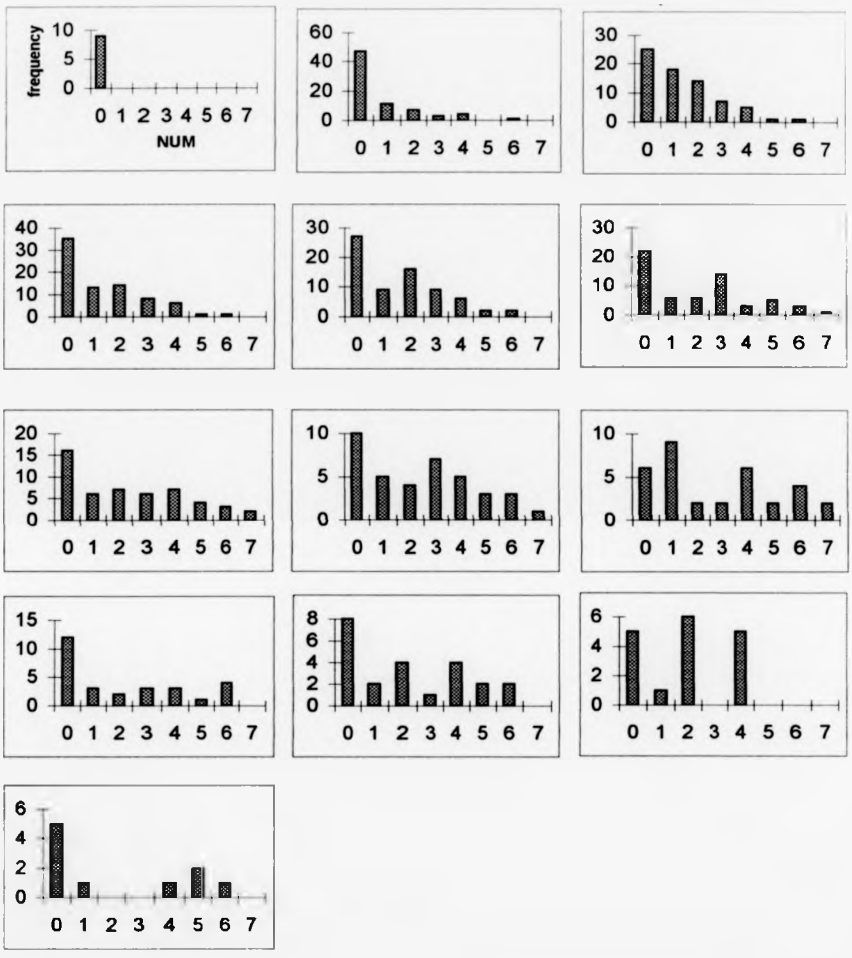


Table 4.4 The *per capita* rates of recovery and re-acquisition of signs 1–7 calculated from their presence or absence between consecutive sample rounds. Frequencies represent the number of dogs which recovered from at least one sign on one occasion, and dogs that re-acquired a sign following recovery in any previous sample. Rates are for the mean sampling interval of 67 days.

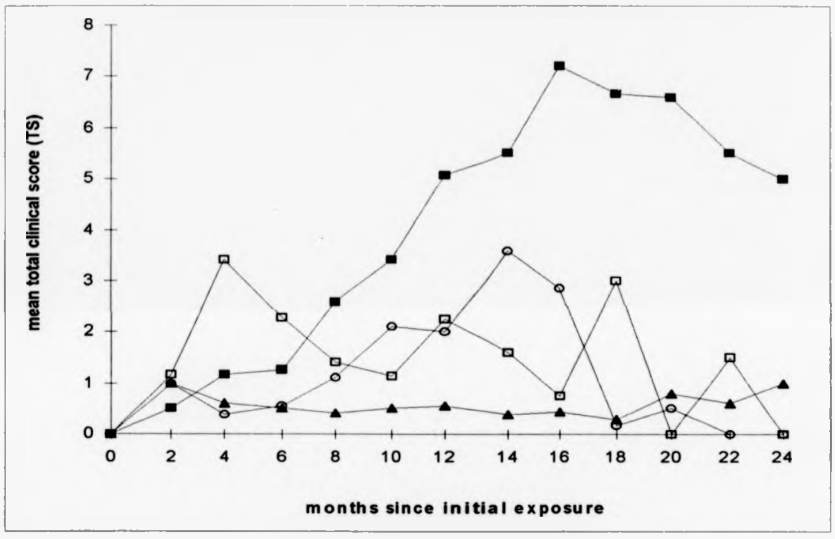
	recovery			re-acquisition		
clinical sign	number of dogs (% of total with sign)	rate per sampling interval (95% C.L.s)	N follow-up rounds	number of dogs (% of total recovered)	rate per sampling interval (95% C.L.s)	N follow-up rounds
DE	58 (63.8)	0.31 (0.23-0.39)	135	31 (61.3)	0.22 (0.12-0.36)	88
AL	58 (62.1)	0.34 (0.25-0.44)	125	27 (55.6)	0.20 (0.12-0.31)	76
CONJ	34 (85.3)	0.59 (0.45-0.72)	53	25 (36.0)	0.14 (0.06-0.29)	64
CH	50 (72.0)	0.34 (0.27-0.42)	134	28 (67.9)	0.34 (0.23-0.47)	59
NO	37 (86.5)	0.53 (0.42-0.65)	74	26 (86.5)	0.38 (0.32-0.40)	60
ONY	34 (70.6)	0.38 (0.27-0.51)	77	21 (52.4)	0.29 (0.20-0.39)	42
COND	46 (56.5)	0.36 (0.26-0.46)	81	20 (50.0)	0.23 (0.12- 0.39)	56
totals	66 (86.8)		671	46 (78.0)		445

The apparent differences in infection between Belém and Marajó sentinel recruits were not detectable in any of the clinical outcome measures. For example, no differences were observed in the cumulative prevalences of signs that the two groups acquired (comparison of slopes: $z < 2.06$, for each sign 1-7), their mean severity scores through time (comparison of slopes: $z < 1.46$, for each sign 1-11), nor in their abilities to recover from disease (comparison of slopes: $z < 1.51$, for signs 1-7 when controlling for ELISA titre or not).

Sixty-four dogs were also classified by their longitudinal clinical profiles. Of these 34 (53.1%) became and remained diseased ("sick"), 10 (15.6%) never or rarely presented signs ("healthy"), 9 (14.1%) became sick and clinically recovery ("recovered"), and 11 (17.2%) remained in a state of flux, for the most part symptomatic ("fluctuating"). The mean total severity scores (TS) of these clinical classes are illustrated in **Figure 4.6**. The "very low", "low", and "medium" antibody responders did not differ significantly from each other in their mean total clinical scores (TS) through time (comparison of TS

slopes during model reduction: $z < 0.87$; means: $z = 1.57$), whereas the "high" responders had significantly greater TS scores than the three other serological groups combined (comparison of grouped TS slopes: $z = 2.26$, $P < 0.02$).

Figure 4.6. Mean severity of disease of sentinel dogs categorised by clinical profile in rounds 1–13 post instalment. Clinical profiles: 'sick' (■), 'fluctuating' (□), 'recovered' (○), and 'healthy' (▲), as defined in the text.



4.4.3 Correlates of disease outcome

a) Serology and parasitology

Dogs were classified at each sample round into one of four serological categories reflecting 10-fold increments in log unit titre, labelled as "very low" (1–999 units), "low" ($10^3 < 10^4$), "medium" ($10^4 < 10^5$), and "high" ($> 10^5$). The odds of acquiring individual signs was associated with the magnitude of anti-*Leishmania* antibody response: relative to "very low" responders, "medium" responders were more likely to contract two of the seven signs (chancres, odds = 2.7; enlarged lymph nodes, odds = 4.9), whereas "high" responders had an odds of 2.8 to 18.1 of acquiring each of the seven individual signs (**Table 4.5**). Successful parasite isolation increased the odds of

developing conjunctivitis (CONJ) and/or body emaciation (COND) by a factor of approximately 2 (see Table legend for derivation of odds values).

Table 4.5. The risk of disease associated with infection status. Values represent the odds of dogs presenting individual clinical signs or indicated biochemical values in response to the specified level of infection. The odds are calculated relative to the ("baseline") group which here are dogs with "very low" titres (as defined in the text) and which are parasite negative. The titre category columns show the risk of disease among parasite negative dogs with the indicated titre, and the parasite column shows the additional risk if also parasite positive. The latter values represent a mean across titre categories since the interaction term (titre x parasite isolation success -ELISA x PARA) was not significant ($z < 0.89$, for each sign). The odds ratios in corresponding titre and parasite columns are multiplicative, thus, for example, the odds of a "high" titre parasite positive dog being emaciated (COND) was 3.3 x 2.2 = 7.26. Only values which deviated from the null model at $P < 0.05$ were considered significant. Analyses were performed by transforming sign scores into binary data (disease presence/absence) as the response variables in the series of logistic ANCOVAs, while controlling for the effects of time since instalment (Tf), parasite isolation technique (TECH), and within-dog auto-correlation. Note that the *P*-values shown were not Bonferroni-adjusted since the Table seeks to demonstrate general trends rather than absolute risk.

	ELISA titre category					
binary outcome variable	low	medium	high	high titre 95% C.L.s	parasite positive	parasite 95% C.L.s
ANY SIGN	1	1	10.2	3.4–30.6	1	1
DE	1	1	3.5	1.7–7.1	1	1
AL	1	1	3.9	1.8–8.3	1	1
CH	1	2.7	6.2	2.5–15.8	1	1
CONJ	1	1	2.8	1.1–7.5	2.0	1.1–3.7
NO	3.8	4.9	18.1	5.1–63.9	1	1
ONY[3]	0.3	1	1	1	1	1
COND	1	1	3.3	1.5–7.5	2.2	1.2–3.9
TD	1	1	3.3	1.6–7.0	1	1
TE	1	1	7.7	3.3–17.9	1	1
PCV[1]	1	1	4.2	1.4–12.2	1	1
BUN[2]	1	1	7.1	1.3–37.5	1	1

[1] odds calculated relative to values ≤ 32%.
[2] odds calculated relative to values ≥ 7.5 mg/dl.
[3] controlling for *Tunga penetrans* (see Co-infection with ectoparasites), the odds for "high" titre dogs was 3.0 (95% C.L.s. 1.2–7.4) ($z = 3.1$, $P = 0.002$), whereas significant changes to the other titre categories were not indicated.

In order to investigate the association between disease severity (as opposed to presence/absence) and infection, the antibody titre and parasite isolation success

parameters were treated as the outcome variables and the clinical severity scores as explanatory variables.

Controlling for the extensive covariance between signs (indicated in **Table 4.3**), the significant clinical markers retained in the MAM included body condition score (COND), chancres (CH), and lymph node enlargement (NO) (**Table 4.6a**), which explained 35.9% (r^2 = 0.3585) of the total variation in antibody titre. The signs indicative of parasite positivity when controlling for antibody titre were body emaciation (COND), conjunctivitis (CONJ) and onychogryphosis (ONY) (**Table 4.6b**). The MAM in this case explained 19.1% (r^2 = 0.1913) of the total variation in parasite isolation success. Neither biochemical variable PCV or BUN were significant when repeating these analyses on the appropriate reduced datasets (PCV: $z < 1.28$; BUN: $z < 1.18$, for each of the infection outcomes). None of the individual signs accounted for > 8% (r^2 = 0.0710) of the variation to suggest that they are reliable markers of infection (**Table 4.6a** and **b**).

Of the 64 dogs classified by longitudinal clinical profile, 63 seroconverted, and all but the three dogs (the three serorecoveries already mentioned) were serologically positive at their last sample. Two of these dogs were classified as "healthy", the other as "sick". "Sick" dogs accounted for 87.5% (70/80 samples) of the recorded "high" titres (as defined above), in contrast to "recovered" and "healthy" dogs which were never observed to mount 'high' titres (**Table 4.7a**). Parasite isolation success was not significantly different between the clinical groups (z = 0.75) when controlling for the effects of antibody titre and Tf (**Table 4.7b**).

Table 4.6 The association between infection and clinical severity. The infection outcome parameters were **(a)** log unit antibody titre (ELISA) as a continuous variable, and **(b)** parasite isolation success (PARA) as a binary variable. The full ANCOVA models (with (a) Gaussian errors and identity link, or (b) logistic with binomial errors and logit link), comprised of explanatory clinical signs 1–7, time since instalment (Tf), in addition in (b) to parasite isolation technique (TECH) and log unit titre (ELISA). For the categorical explanatory variables, the positive or negative association (+ or −) with the response variable represents a trend across categories rather than a slope.

(a)

explanatory variables	association	$P <$	r^2
Tf	+	0.000	0.1780
CH	+	0.005	0.0280
NO	+	0.012	0.0139
COND	-	0.004	0.0710
MAM			0.3585
total deviance	541.8		

(b)

explanatory variables	association	$P <$	r^2
ELISA	+	0.001	0.1272
CONJ	+	0.026	0.0086
ONY	-	0.015	0.0134
COND	-	0.011	0.0218
MAM			0.1913
total deviance	404.69		

The usefulness of antibody titre to distinguish dogs of different clinical outcome was examined by comparison of the 64 dogs with clinical profiles. Seropositive *vs.* seronegative antibody titres did not distinguish the four clinical groups in any round post instalment ($z < 1.47$, for each round). Neither were there any detectable differences in the average titres of "healthy", "recovered" and "fluctuating" dogs following instalment (comparison of group titres during model reduction: $z < 1.22$, in each round). "Sick" dogs, however, mounted significantly higher average titres than the other clinical classes combined, in rounds 5–11 (or 8–20 months after initial exposure) (comparison of mean titres: $z > 2.55$, $P < 0.002$, in each round) **(Figure 4.7)**, corresponding to 2–12 months

Table 4.7. Infection and disease outcome relative to longitudinal clinical profile. Associations between **(a)** anti-*Leishmania* antibody titre category (as described in text), **(b)** parasite isolation success, and **(c)** mortality.

(a)

clinical profile class	N dogs in class	titre category: very low (proportion of samples)	low	medium	high	N sera samples tested
healthy	10	9 (0.11)	46 (0.56)	27 (0.33)	0 (-)	82
recover	9	10 (0.13)	32 (0.41)	37 (0.47)	0 (-)	79
fluctuate	11	22 (0.31)	30 (0.42)	9 (0.13)	10 (0.14)	71
sick	34	42 (0.18)	51 (0.22)	67 (0.29)	70 (0.30)	230
totals	64	83 (0.18)	159 (0.34)	140 (0.30)	80 (0.17)	462

(b)

clinical profile class	N parasite +ve dogs (prop. per class)	titre category: very low (proportion of samples)	low	medium	high	N parasite samples (prop. +ve)[1]
healthy	2 (0.20)	0 (-)	2 (0.07)	2 (0.07)	0 (-)	29 (13.8)
recover	4 (0.44)	0 (-)	3 (0.10)	4 (0.13)	0 (-)	31 (22.6)
fluctuate	9 (0.82)	0 (-)	3 (0.09)	7 (0.20)	4 (0.11))	35 (40.0)
sick	22 (0.65)	1 (0.01)	3 (0.03)	16 (0.16)	25 (0.25)	102 (44.1)
totals	37	1 (0.01)	11 (0.06)	29 (0.15)	29 (0.15)	197 (35.5)

(c)

clinical profile class	N dogs died (prop. per class)	titre category: very low	low	medium	high	mean number of rounds observed
healthy	0 (0)	0	0	0	0	7.2
recover	1 (0.11)	0	1	0	0	7.6
fluctuate	3 (0.27)	0	1	0	2	5.5
sick	22 (0.65)	0	3	5	14	5.8
totals	26	0	5	5	16	

[1] sum of bone marrow aspirates over all rounds (1 per round).

after seroconversion (1–6 rounds after instalment)(comparison of mean titres aligned by seroconversion: $z > 3.60$, $P < 0.002$, in each round). A significant divergence in the mean total clinical scores (TS) of "sick" *vs.* other profile categories was detected 8–20 months after instalment (comparison of mean titres: $z > 2.83$, $P < 0.005$, in each round) **(Figure 4.8)**, though this is clearly not independent of the choice of profile classification method.

Figure 4.7. Mean anti-*Leishmania* antibody titres of sentinel dogs categorised by clinical profile in rounds 1–13 post instalment. Clinical profiles: 'sick' (■), 'fluctuating' (□), 'recovered' (○), and 'healthy' (▲), as defined in the text.

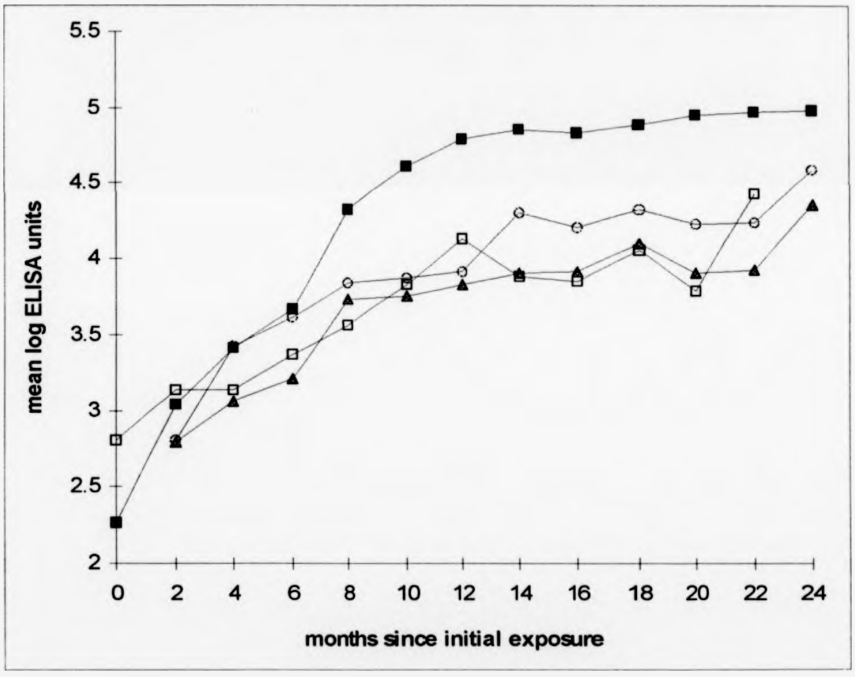
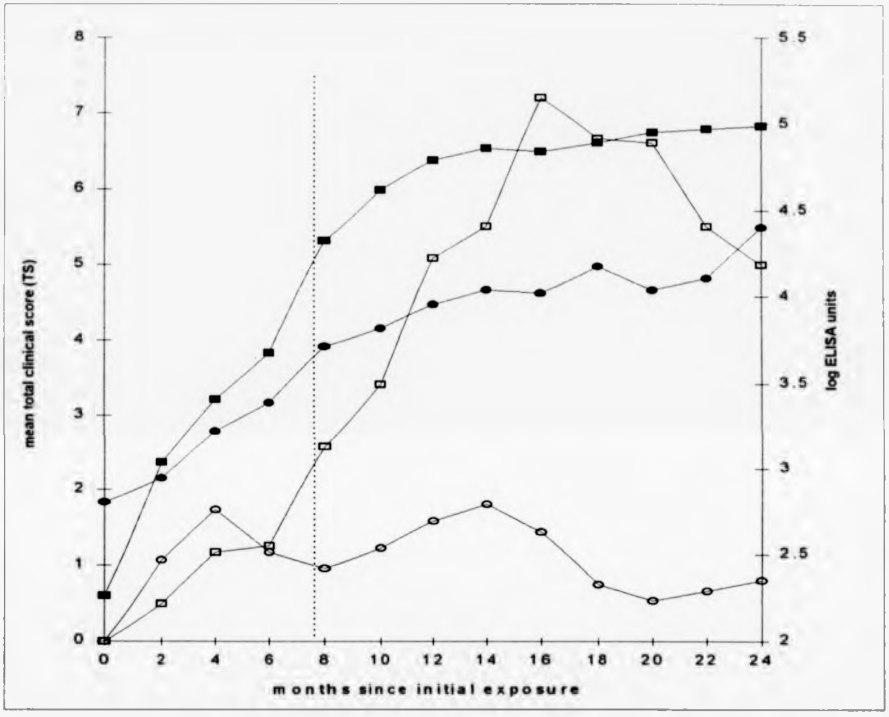


Figure 4.8. Divergence in mean anti-*Leishmania* antibody response (solid symbols) and disease severity scores (open symbols) of sentinel dogs clinically classified as 'sick' (■, □) or other (●, ○), following instalment. The dashed line (-----) represents the point of statistical distinction in each of the outcome parameters (clinical severity and titre) assessed independently.



The effect of malnutrition on susceptibility

To test whether malnourishment had a significant influence on the subsequent course of infection, the body condition score data of dogs at 0, -1 and -2 rounds prior to seroconversion were regressed against the infection outcome parameters, i.e. treating the immunological and parasitological measurements as surrogate markers of susceptibility. There was no evidence of any association between prior body condition and either maximum antibody response ($z < 0.84$, for each explanatory term) or parasite isolation success ($z < 1.26$, for each explanatory term) to suggest a causal relationship.

b) Co-infection with ectoparasites

The occurrence of four ectoparasites, *S. scabiei*, *D. canis* and *C. yasguri*, and *T. penetrans*, which were perceived to potentially confound interpretation of the clinical response to *Leishmania* were monitored in a subset of dogs. The prevalences of each of the parasites are shown in **Table 4.8.** *S. scabiei* and *T. penetrans* were the most common parasites observed, whereas *C. yasguri* was not detected in any sample. Dogs with confirmed *Leishmania* infections were no more likely than negative dogs to be infested with either *T. penetrans* ($z = 0.51$), *S. scabiei* (Fisher's Exact, *NS*), or *D. canis* (Fisher's Exact, *NS*) (comparison of samples in each case), suggesting that ectoparasite infestation did not predisposed them to *Leishmania* infection. The odds of dogs with dermatitis (DE) and alopecia (AL) being infested with *S. scabiei* were 9.5 and 8.1 respectively, over dogs without these skin complaints ($z > 2.62$, $P < 0.009$, in both cases). Dogs with onychogryphosis (ONY) were 1.8 times more likely to have *T. penetrans* ($z = 4.07$, $P < 0.001$). As potential confounders of these clinical conditions, the analyses described in **Table 4.5** were repeated while controlling for these ectoparasite variables (where appropriate). *T. penetrans* infestation significantly altered the odds of dogs presenting onychogryphosis (ONY) (see note in **Table 4.5**); whereas *S. scabiei* did not have a significant effect on the odds of presenting either DE or AL. (Data were too few to test for the effect of *D. canis*).

Table 4.8. Prevalence (%) of ectoparasites causing mange and tungiasis in confirmed and unconfirmed *Leishmania* infected dogs and samples. Total sample sizes are shown in parentheses.

	Sarcoptes scabiei	*Demodex canis*	*Cheyletiella yasguri*	*Tunga penetrans*
samples				
Leishmania negative	50.0 (4)	0.0 (4)	0.0 (4)	7.6 (66)
Leishmania positive	46.3 (41)	2.4 (41)	0.0 (41)	5.1 (490)
total population	46.7 (45)	2.2 (45)	0.0 (45)	5.3 (562)
dogs				
Leishmania negative	66.6 (3)	0.0 (3)	0.0 (3)	11.4 (35)
Leishmania positive	51.5 (33)	3.0 (33)	0.0 (33)	16.1 (81)
total population	52.8 (36)	2.8 (36)	0.0 (36)	14.7 (116)

Twelve of the 23 unconfirmed *Leishmania* cases which developed signs presented medium to severe conditions associated with CVL. Only one of these was found to harbour mites and *T. penetrans* which could entirely account for its clinical presentation (i.e. DE and ONY); all other 11 dogs presented signs additional to DE, AL or ONY.

4.4.4 Correlates of the mortality rate

Eighty-six deaths, 62 from non-accidental causes, were observed among the 116 clinically examined dogs. The risk of mortality was strongly associated with both infection and disease.

a) Infection

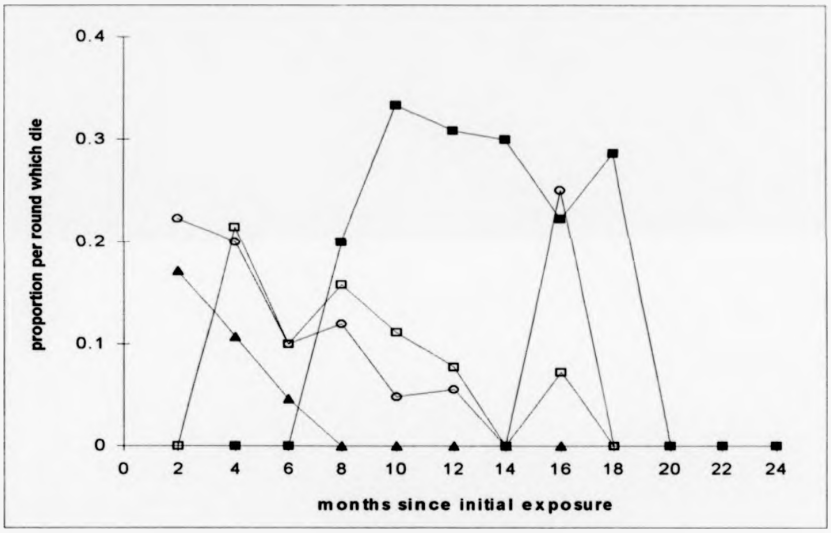
A total of 41/70 (58.6%) dogs with confirmed *Leishmania* infection and 21/22 (95.5%) of negative dogs died of non-accidental causes. Eighteen of the 21 *Leishmania* negative deaths occurred in the initial phase (round 3) of the study. The crude mortality rates of "very low" to "medium" responders declined through time from round 2 post instalment (**Figure 4.9**). Mortality among "high" responders rapidly peaked at approximately 30% in the fifth round (10 months post instalment), and remained relatively constant thereafter. No statistical differences in mortality rates through time were observed among "very low" "low" and "medium" responders (comparison of mortality rates through time during model simplification: $z < 0.91$), whereas the odds of "high" titre dogs dying relative to the 3 other classes combined was an average 2.8 (95% C.I.s 1.2–6.5) ($z = 2.47$, $P = 0.014$). Of 26 deaths of seronegative dogs, 7 and 19 were asymptomatic and symptomatic, respectively.

Death followed 22 (29.3%) of the 75 occasions when parasites were detected, in contrast to 19/320 (5.9%) when they were not ($\chi^2 = 35.8$, $P < 0.001$). Controlling for antibody titre, the odds of a parasite positive dog dying was 10.3 (95% C.I. 3.7–28.8) over a parasite negative dog ($z = 4.46$, $P < 0.001$).

b) Disease

The risk of mortality was also associated with disease. Dogs were 4.4 times more likely to die when symptomatic than when asymptomatic ($z = 2.32$, $P = 0.02$). The difference was most pronounced among dogs presenting severe disease (**Table 4.9**). Relative to parasite negatives with "very low" titres, the odds associated with severe conditions ranged from 5.3 (for NO) to 45.8 (for CONJ), irrespective of antibody titre. Mortality

Figure 4.9. Proportion of sentinel dogs categorised by anti-*Leishmania* antibody titre which died in rounds 1–13 post instalment. Antibody titre categories: 'high' (■), 'medium' (□), 'low' (○), and 'very low' (▲), as defined in the text.



was also more likely (odds of 12.9) among dogs with below average PCV values (< 32%) ($z = 4.41$, $P < 0.001$) (**Table 4.9**).

Controlling for sign covariances in multivariate analysis (**Table 4.10**), the only significant clinical signs were body emaciation (COND) and conjunctivitis (CONJ). This held true irrespective of controlling for parasitological status (compare **Tables 4.10a** and **b**), whereas ELISA was not retained in either MAM. Body emaciation (represented as category 3 in both **Tables 4.9** and **4.10**) explained the greatest

proportion of the variance (r^2 values of 0.1047 and 0.0675). Both PCV and BUN were significant additional explanatory terms when these analyses were repeated on the smaller datasets (PCV: $r^2 = 0.0259$; $z = 2.29$, $P = 0.02$; BUN: $r^2 = 0.0437$; $z = 2.48$, $P = 0.013$).

Table 4.9 The risk of mortality associated with disease severity. Values represent odds of dying between sample rounds relative to asymptomatic parasite negative dogs, unless otherwise stated (see "baseline" values in Table notes). The full logistic ANCOVA models were performed controlling for infection magnitude (ELISA titre, PARA), Tf, and TECH. Since the interaction terms ELISA x explanatory variable were not significant ($z < 0.87$, in each case), the additional risk associated with parasite positivity is multiplicative, as described by example in **Table 4.5**. ELISA was not retained in any of the MAMs ($z < 1.3$, in each case).

	clinical score or category[2]					
explanatory variable	1	2	3	score 3 95% CL.	parasite positive	parasite 95% CL.
DE	1	4.2	5.9	2.2–16.0	11.1	4.7–26.3
AL	1	9.3	7.2	2.6–20.3	12.6	5.3–30.0
CH	1	1	9.6	3.0–30.6	12.2	5.2–28.3
CONJ	1	9.0	45.8	11.5–182.7	11.8	4.9–28.4
NO	3.2	8.4	5.3	1.8–35.5	14.3	5.9–34.7
ONY	1	3.9	6.0	1.7–21.0	14.5	6.5–32.5
COND	1	1	9.1	2.4–34.5	5.4	2.7–10.6
NUM[1]	1	3.8	19.7	5.5–71.1	10.9	4.3–27.4
TS	1	1	12.3	3.9–39.1	10.9	4.5–26.6
TD	1	5.4	7.1	2.6–19	11.9	4.9–28.8
TE	1	1	23.1	7.3–73.5	11.8	5.0–27.6
BUN	–	1	1	–	1	–
PCV	1	1	12.9	2.3–72.5	1	–

[1] odds refer to categorised numbers of signs (see below), not severity scores.
[2] category and "baseline" values for which odds ratios were calculated were:

		categories		
explanatory variable	baseline values	1	2	3
COND	4	3	2	1
NUM	0	1-2	3-4	5-7
TS	0	1-3	4-6	7-16
TD	0	1-2	3-4	5-6
TE	0	1-2	3-4	5-10
BUN (mg/dl)	3.3	-	7.5	12.3 & 21.6
PCV (%)	41-56	37-40	33-36	9.5-32

Table 4.10 Association between mortality and disease severity, controlling for sign covariances. The odds of inter-round mortality was tested by logistic ANCOVA (binomial errors and logit link) comprising signs 1–11 in addition to **(a)** log unit titre (ELISA), and **(b)** ELISA, parasite isolation success (PARA), and isolation technique (TECH). Note that the clinical scores and baselines used for calculating odds ratios are as shown in **Table 4.9**, unless otherwise stated (see Table notes). The interaction terms PARA x sign were not significant, hence the results shown in (**b**) indicate that parasite positivity increased the odds of mortality by a factor of 4.8, irrespective of clinical severity.

(a)

	clinical score				
explanatory variables	1	2	3	95% CL.	r^2
CONJ	1	1	16.0	4.8–53.5	0.0378
COND	1	1	8.9	2.6–30.6	0.1047
MAM					0.1903
total deviance	404.6				

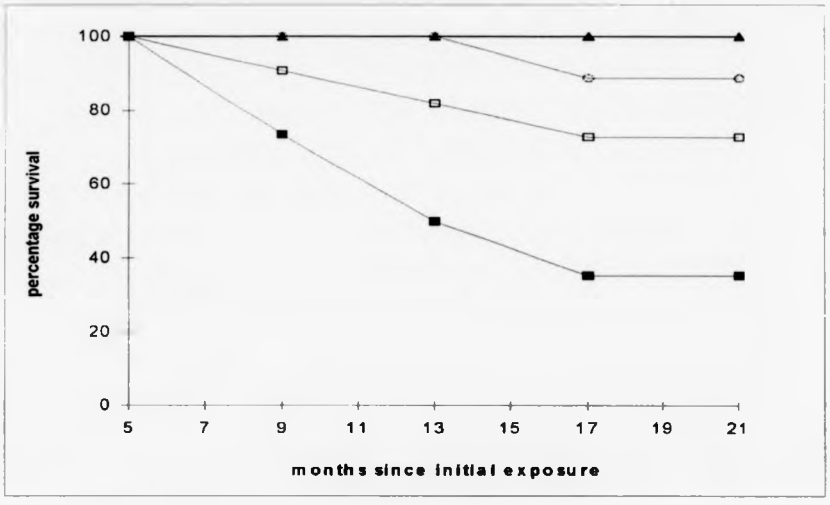
(b)

	clinical score				
explanatory variables	1	2	3	95% CL.	r^2
CONJ	1	1	22.3	4.6–109.1	0.0333
COND	1	1	7.5	2.0–28.5	0.0675
PARA	–	–	4.8[a]	2.4–9.8	0.0558
MAM					0.2467
total deviance	270.8				

[a] odds represent parasite positives *vs.* negatives

The three measured variables expected to most directly reflect *Leishmania*-related mortality were anorexia (COND score of 1), anaemia (low PCV) and renal malfunction (high BUN). These conditions were associated with 61.3% (38/61), 56.5% (13/23) and 31.6% (6/19) of the non-accidental deaths respectively. High BUN concentrations were indicated in 23.2% (13/56) of the clinical examinations (all *Leishmania* positive). Of the dogs which died with anorexia, 41.7% (5/12) had BUN values > 7.5 mg/dl, and 70.6% (12/17) also had PCV values < 32%. In all, 32/42 confirmed *Leishmania* cases compared to 7/15 with no evidence of infection had one or more of these conditions immediately prior to death (Fisher's Exact, $P = 0.05$).

Figure 4.10. Survival of sentinel dogs categorised by clinical profile from round 3 post instalment. Clinical profiles: 'sick' (■), 'fluctuating' (□), 'recovered' (○), and 'healthy' (▲), as defined in the text.



Twenty-six (40.6%) of the 64 dogs classified by clinical profile died by the end of the study, including 22 (65.0%) "sick" and, by contrast, 1/19 (5.3%) "healthy" and "recovered" combined classes (**Table 4.7c**). A decrease in survival was observed through "healthy", "recovered", "fluctuating" to "sick" categories (χ^2 = 19.1, P < 0.001) (**Figure 4.10**). Sixty-two percent (16/26) of all deaths were among dogs with "high" titres; controlling for antibody response and parasite isolation success, these dogs had an odds of 2.7 (95% C.L. 1.3–5.4) of mortality relative to dogs in all other clinical classes combined (test of grouped categories following model reduction: z = 2.74, P = 0.006). These results confirm those obtained for the larger sample above.

The precise mortality rate due to *Leishmania* infection is not known. An approximation is obtained by making the assumption that mortality in dogs with "high" titres is predominantly due to infection. In this case, the rate was 68/87 per sampling interval, giving 0.362/month or average life expectancy having developed a titre of $>10^5$ log units of L = 2.77 months.

4.5 DISCUSSION

4.5.1 Course of infection

A total of 116 sentinel dogs were clinically examined on 562 occasions following instalment into an endemic area. By the end of the study, 80 dogs were identified as *Leishmania* positive by demonstration of specific antibody and/or by parasite detection. All dogs developed clinical signs within 24 months of initial exposure. The prevalences of individual signs in the symptomatic population conformed to the general picture of CVL in other regions as summarised in **Table 4.11**. Twenty-three of the 36 uninfected sentinel dogs also presented signs, 20 of which remained in the study for less than the expected average time to seroconversion (6.7 months). A proportion of these could have been pre-patent infections; relatively few alternative parasitological agents were monitored during the study, thus alternative aetiological agents cannot be entirely ruled out.

The mean incubation period of clinical development in the known *Leishmania* cases was 66 days, which preceded the end of the median 94 day prepatent period by approximately 1 month. Similar, though varying, incubation periods of 1, 2.7 and 7.5 months have been reported for naturally infected dogs in Europe (Gradoni *et al.*, 1988; Lanotte, 1975; Vidor *et al.*, 1991). The risk of developing disease was clearly associated with the magnitude of antibody response, being most pronounced among dogs with "high" titres and among those which were parasite positive. Both the severity and prevalence of CVL have been associated with serological titre and parasite positivity in European populations (e.g. Pozio *et al.*, 1981a; Fisa *et al.*, 1991; 1992; Saladrigas, 1992; Dye *et al.*, 1993).

Following the onset of the disease, there were surprisingly high recovery (0.31–0.59) and re-acquisition rates (0.14–0.38) of individual signs between sample rounds, observed in 87% and 78% of the dogs, respectively. This accounted for the relatively low proportion of symptomatics (≈ 50%) in any single round (*cf.* cumulative prevalence), which is consistent with European studies (e.g. Abranches *et al.*, 1991; Neogy *et al.*, 1992; Pozio *et al.*, 1981a; Marzochi *et al.*, 1985; Dye *et al.*,

1992), implying that cross-sectional data may underestimate the true abundance of symptomatics.

Table 4.11. Prevalence of clinical and clinicopathologic signs among serologically and/or parasitologically confirmed infected dogs. Data from the present study in Marajó are compared to other endemic regions. Values represent the percentage of dogs with condition; nd = not reported; R "rare", C "common" as quoted in the original source.

location	Marajó	S. Brazil	Greece	Italy	Europe	France	Portugal	Iran
source[a]	this study	1	2	3	4	5	6	7
clinical sign								
lymphadenopathy	51	86	96	89	90	100	66	60
alopecia	73	86	nd	14–18	89[b]	84	38[c]	71
dermatitis	74	43	75	56	89[b]	nd	38[c]	nd
chancres	68	43	nd	40	9	49	34	57
onychogryphosis	48	43	40	24	20	73	61	54
conjunctuivitis	48	14	50	11	33	20	R	50
weight loss	nd	nd	31	32	64	nd	R	61
anorexia	68	100	37	17	33	nd	R	nd
hepatosplenomegaly	nd	nd	R	53	33	nd	nd	76
renal failure	27[e]	nd	19	16	33–45	nd	nd	nd
hyperthermia	nd	nd	nd	6–35	35	nd	nd	nd
biochemical sign								
anaemia	69[f]	nd	94	60	C	nd	nd	nd
proteinuria	nd	nd	68	4	85	nd	nd	nd
azotemia	nd	nd	19	4	45	nd	nd	nd
leukopenia	nd	nd	6	0	22	nd	nd	nd
hypoalbuminaemia	nd	nd	nd	68	94	nd	nd	nd
hyperglobulinaemia	nd	nd	nd	71	100	nd	nd	nd
albumin/globulin	nd	nd	nd	76	nd	nd	nd	nd
N cases	81	7	52	150	80	45	61	24[d]

[a] 1 Marzochi *et al.*, (1985); 2 Kontos and Koutinas (1993); 3 Ciaramella *et al.*, (1997); 4 Slappendel, (1988); 5 Lanotte *et al.*, (1979); 6 Abranches *et al.*, (1991); and 7 Gavgani (1998).
[b] dermatitis and alopecia not differentiated in source, but referred to as "skin involvement".
[c] stated to include dermatitis and/or depilation.
[d] confirmed parasitological cases only.
[e] samples size, $N = 49$
[f] samples size, $N = 52$

Temporal variation in clinical presentation of this nature has not been reported elsewhere, since the more common unit of study is the asymptomatic, oligosymptomatic and symptomatic dog, defined by the association and/or number of signs presented (e.g. Saladrigas, 1992; Mancianti *et al.*, 1988; Pozio *et al.*, 1981a; Molina *et al.*, 1994a; Fisa *et al.*, 1991).

In contrast to the short-term transience of individual signs, full longitudinal clinical recovery was not commonly observed. Nine of the 64 clinical profiles were categorised as "recovered", though some of these, due to the classification method, included dogs which retained mild conditions. In fact, only five dogs totally resolved all of their signs, each dog having been only mildly afflicted post infection. Saladrigas (1992) reported only 1 clinical recovery among 35 oligosymptomatic dogs observed over a year, and none of eight dogs with advance disease. Clinical or serological recovery are not common in severe cases undergoing antimony drug treatment either (Mancianti *et al.*, 1998; Alvar *et al.*, 1994), whereas 10% or more of asymptomatic / oligosymptomatic infections may recover serologically (Lanotte *et al.*, 1979; Pozio *et al.*, 1981a; Fisa *et al.*, 1991; Saladrigas, 1992). Only 3/75 (4.0%) dogs were observed to serologically recover in this study, two of them asymptomatic, the other oligosymptomatic.

4.5.2 Mortality

Relative to dogs with no evidence of either infection or disease, the risk of mortality was greatest for those with severe CVL (odds ratios of 7.5–22.3: 1), "high" antibody titres (2.8: 1), and parasite positives with "high" titres (10.3: 1). These results strongly suggest that fatalities among "sick" and/or "high" antibody responders are predominantly due to *Leishmania*. A similar conclusion can be drawn from analysis of the data presented by Fisa *et al.* (1991), working in Spain, which shows dog mortality to significantly increase with anti-*Leishmania* antibody titre (proportion dead regressed against reciprocal DOT-ELISA titre classes < 100 (seronegatives), 100-200, 400, and 800, $z = 6.3$, $P < 0.001$). This yields an odds ratio of 13.0: 1 for "high" titre *vs.* seronegative dogs. An odds ratio of 7.0: 1 (DAT titres > 1/800: 21/34 *vs.* > 1/800: 3/34) is calculated using the data from another focus in Spain

(Saladrigas, 1992). Comparative data for the sentinel dogs indicates a not dissimilar odds ratio (of 9.5: 1) for "high" relative to "very low" serological responders.

Further similarities in mortality patterns between Europe and Marajó were found relative to clinical outcome. The odds of Spanish dogs dying from severe disease is 3.3: 1 relative to asymptomatic and oligosymptomatic dogs combined (15/23 *vs.* 9/45) (Saladrigas, 1992). This is somewhat lower than the equivalent estimate (12.3: 1) for Marajó dogs (22/34 "sick" *vs.* 1/19 "recovered" and "healthy"). Considering both clinical and immunological measures together, deaths among "sick" dogs with "high" titres amounted to 53.9% (14/26) of those longitudinally classified in Marajó, which is comparable to the crude estimates of 44.4% (8/18) and 46% (19/41) for similarly afflicted dogs in Europe (Fisa *et al.*, 1991; Pozio *et al.*, 1981a).

CVL is considered a wasting disease with renal failure the major cause of mortality (Slappendel, 1988; Biewenga and Gruys, 1986; Mancianti *et al.*, 1989). Three conditions were measured during this study which were expected to reflect more directly *Leishmania*-related mortality: anorexia (COND score of 1), renal malfunction (BUN), and anaemia (PCV). The three conditions were associated with 61%, 32% and 57% of the deaths respectively, though none of them proved to be reliable predictors. In accordance with others studies (e.g. Slappendel, 1988), serious loss of condition was not always associated with renal incompetence, though at least 5 (41.7%) of the 12 anorexia-related deaths in this study were. The prevalence of renal disease in the sentinel population was similar to that in other populations (16%–45%) (**Table 4.11**), but was probably underestimated here since BUN is not considered a very sensitive diagnostic technique (Palacio, 1993, cited in Palacio *et al.*, 1995).

Anorexia has clear nutritional implications, though cause and effect are not always clear (Pearson *et al.*, 1992; Weigel *et al.*, 1995). Malnutrition has been shown to increase the risk of *Leishmania* infection and disease progression in humans (Harrison *et al.*, 1986; Badaro *et al.*, 1986b; Cerf *et al.*, 1987; Dye and Williams, 1993), and to impair the ability of animals to recover clinically (Perez *et al.*, 1979;

1984). Furthermore, some micro-nutrients (e.g. Vitamin A and Zinc) are known to be important modulators of the immune function (Chandra, 1997). In this study, there was no evidence that body condition, measured up to a mean 42 days prior to the pre-patent period, predisposed dogs to infection severity used here as a surrogate marker of susceptibility. More sensitive measures of nutritional status, however, are needed to confirm that this is the case.

4.5.3 Differentiating dogs

In common with studies of natural (Dye *et al.*, 1993; Vidor *et al.*, 1991) and experimental (Abranches *et al.*, 1991; Carrera *et al.*, 1996) *Leishmania* infections, sentinel dogs across the clinical spectrum responded with positive antibody titre. There was no apparent dichotomy in clinical and/or serological response during the first *ca.* 20 months of infection, by which to extend the findings of Pinelli *et al* (1994) and Killick-Kendrick *et al.* (1994). Their data suggested that from 3 years post infection, the presence *vs.* absence of anti-*Leishmania* antibody is sufficient to distinguish symptomatic from asymptomatic infections, which they equate with "susceptibility" and "immunity" (see Chapter 1). However, the notion that dogs of different clinical outcome can be identified in early infection was indicated in the Marajó study, by finding that "sick" dogs mounted higher mean antibody titres than dogs of other clinical classes, starting 8 months after instalment (i.e. 1 round after seroconversion), and preceded development of advanced CVL. Heterogeneity in antibody response has been shown for clinically defined dogs in a study of kennelled sentinel beagles in France (Dye *et al.*, 1996). Coincidentally, their mean (IFAT) antibody titres diverged also about 8 months after the beginning of the transmission season. Dogs of the other clinical categories in the present study ("healthy", "recovered" and "fluctuating"), were not distinguishable at any time.

4.5.4 Conclusion

The results in this chapter show that the clinical outcome and mortality due to *Leishmania* infection is similar in Marajó and southern Europe, at least in the parameters measured in this study. Dogs which are destined to develop severe disease may be identifiable for selected treatment or euthanasia during an early stage

of clinical progression. If this were the case, treating such dogs might improve the prognosis (Mancianti *et al.*, 1988). This approach may also help reduce the incidence of severe CVL as suggested by past and current studies in Italy (Gradoni *et al.*, 1988, Gradoni, 1998), though the precise mechanisms of this have yet to be described. To reduce transmission, dogs would clearly need to be selected prior to the onset of infectiousness. The epidemiological significance of treating or eliminating clinically defined groups first requires identification of which type of clinical and/or serological responder contributes most to transmission. This and related questions are fully addressed in the next chapter.

5. HETEROGENEITY OF INFECTIOUSNESS IN THE SENTINEL DOG POPULATION, AND SOME IMPLICATIONS FOR ZVL CONTROL

5.1 ABSTRACT

Infectiousness of the host to the vector is perhaps the most important epidemiological parameter governing the dynamics of the infection in a susceptible host population. Few data on the infectiousness of reservoir hosts of ZVL are available, and certainly no estimates of population infectiousness parameters are available. To improve the choice of ZVL control options, quantitative data on the course of infectiousness and vigorous methods to identify infectious dogs are needed. Fifty sentinel dogs were experimentally exposed to laboratory-bred *Lu. longipalpis* in 173 trials conducted in 12 sample rounds over a period of 24 months. None of the 6,002 flies dissected was infected by seronegative animals, whereas 18/40 (45%) seropositive dogs infected a mean 28% (95% C.L. 20–36%) of flies through time. Dogs became infectious a median 128 days after seroconversion, corresponding to a latent period (time from infection to infectious onset) of 222 days. The point prevalence of infectious dogs in the population was constant at 30% (95% C.L. 23–38%) from *ca.* 2 months after seroconversion. The proportion of flies infected varied extensively between dogs and between trials. Although infectious dogs did not form statistically definable groups, 20% of the infected (i.e. seropositive) population was shown to account for 80% of the transmission potential, in accordance with host-vector systems elsewhere. Infectiousness was positively associated with antibody titre (ELISA), intensity of skin disease (dermatitis, alopecia and chancres), and inversely correlated with conjunctivitis and packed cell volume. Antibody titre proved the best predictor of infectiousness (r^2 = 0.25–0.43), with 72% of all infectious trials associated with titres > 5.0 log units, corresponding to 28% of all trials above this threshold. The clinical and biochemical parameters, in

contrast, each explained < 9% of the total variation in infectiousness, when controlling for sign covariances with time.

Using the empirically derived infectiousness estimates, a compartmental model was designed to compare the simulated outcomes of dog culling strategies, both current (mass elimination) and potential (targeted), on population infectiousness. The models predicted that **(1)** targeting control at infectious dogs has a greater impact in reducing transmission than a strategy of mass elimination of all seropositive animals, and **(2)** that antibody (ELISA) titre, as the most promising surrogate marker of infectiousness identified in this study, can not reliably distinguish infectious and uninfectious animals. It is concluded that more sensitive diagnostic field tools are needed before selective control can be considered a feasible option. The reasons for the current failure of culling strategies to reduce ZVL transmission in Brazil are discussed.

5.2 INTRODUCTION

The ability of one host to infect another host is the central tenant of epidemiology. Infectiousness measures are important to identify high transmission risk individuals and to characterise the relative capacity of different reservoir populations. Surprisingly, the course of infectiousness has not been documented for any host species of *Leishmania*. The few available data on canine infectiousness with *L. infantum* indicate that not all dogs are infectious (prevalences reach 81%), and that there is extensive variation in the proportion of sandflies that an individual infects (Deane and Deane, 1955; Molina *et al.*, 1994a). The notion that infectiousness (defined hereafter as the proportion of sandflies infected from a single bloodmeal) is associated with the disease (as opposed to infection) dates back to the 1930s when Parrot *et al.* (1930) first identified promastigotes in 4/53 *P. perniciosus* fed on a skin lesion of a dog in Algeria. More recent studies suggest that infectiousness is associated with the severity of CVL (Rioux *et al.*, 1972; Gradoni *et al.*, 1987), dermal macrophage intensity (Adler and Theodor, 1935; Slappendel, 1988; Ciaramella *et al.*, 1997), and immunological parameters, the latter inferred from positive correlation's between parasite isolation success and anti-*Leishmania* antibody titre (Pozio *et al.*, 1981a; Abranches *et al.*, 1991; as cited in Dye *et al.*, 1993).

The nature of these associations, however, require verification, not least because the studies cited represent only a small number of cross-sectionally sampled dogs. For example, contradictory results indicate that asymptomatic dogs might in fact be as infectious as symptomatic dogs (Deane, 1961; Molina *et al.*, 1994a; Vexenat *et al.*, 1994), and Molina *et al.* (1994a), having examined 16 infected dogs in Spain, suggested that infectiousness was not correlated with either antibody titre or clinical severity. Certainly, none of the published accounts describe the course of infectiousness at the population level, nor provides estimates of the latent period (time between infection and infectiousness), or the duration of infectiousness. Nor is it yet possible to define the sensitivity and specificity of measurable immune responses with respect to infectiousness. Consequently, basic data are needed to determine **(1)** what proportion of infected dogs become infectious, **(2)** whether such dogs can be identified in the mixed population, **(3)** how strategies aimed at reservoir control precisely affect population

infectiousness, and, therefore, **(4)** what intervention method is likely to be the most efficacious.

Mass elimination of seropositive dogs has not been effective in long term control of ZVL (Ashford *et al.*, 1998, Dietze *et al.*, 1997; Evans *et al.*, 1992- see Chapter 1), undoubtedly because the effort required to drive $R_0 < 1$ for eradication has been under-estimated. Control theory suggests that parasite eradication using a blanket approach, such as mass culling, will require less effort in a heterogeneously than equivalent homogeneously infectious population (Anderson and May, 1991). Moreover, empirical data show that heterogeneities in vector-host contact generally conform to a pattern of aggregation whereby 20% of hosts are responsible for 80% of the transmission potential (Woolhouse *et al.*, 1997a; and references therein). This further suggests that a strategy of culling which targets only infectious dogs, or such "high transmission" groups, might be more successful.

Targeting infectious dogs first requires that they can be identified. Ideally this would be based on xenodiagnosis, but despite the relative ease with which some sandfly species adapt to laboratory rearing conditions (e.g. *Lu. longipalpis*, Killick-Kendrick *et al.*, 1977), the practicalities of processing large quantities of flies condemns its application to small scale studies. Consequently, detection of infectious hosts must rely on surrogate marker(s). In practical terms, these might include specific antibody responses, clinical signs, or DNA-based parasitological techniques, or some combination of these. But, as described above, there are outstanding questions concerning the first two of these parameters, while the third remains untested. Even in the event that infectious dogs could be identified, it is not clear what intervention regime would be required to reduce transmission. In Chapter 3 it was shown that dogs which leave the population are replaced by new animals. Therefore, even selective culling strategies may be unsustainable approaches to ZVL control.

5.2.1 Study aims

In **Part I** of this chapter, baseline estimates of population parameters, including infectious prevalence, the latent period, duration of infectiousness, mortality rates, and

heterogeneities in infectiousness, are calculated from data collected by longitudinal xenodiagnosis of sentinel dogs in Marajó. In search of potential surrogate markers of infectiousness, these data are related to serological, clinical, biochemical and parasitological measures over the time course of infection. In **Part II**, culling strategies are simulated mathematically using a deterministic compartmental model of infectiousness based on parameter estimates calculated in **Part I** and Chapters 3 and 4 of this thesis. The theoretical outcomes of targeted *vs.* mass culling regimes on transmission are compared, and related to the performance of (ELISA) antibody titre as a potential surrogate marker of infectiousness.

5.3 METHODS

5.3.1 Study animals

Fifty of the 126 sentinel dogs were examined by xenodiagnosis, representing 48% (13/27) and 37% (37/99) of the local and Belém recruits, respectively.

5.3.2 Sandfly colonies

Dogs were exposed to *Lu. longipalpis* from three laboratory bred colonies, two of which were established with flies caught in the study site, the third with flies from Santarém (also in Amazonia). The Marajó colonies were replenished at bimonthly intervals to provide first and second generation adults. These were supplemented by the smaller Santarém colony which was in its 80th generation at the beginning of the study. The two source populations belong to the same sibling group, being morphologically indistinguishable (Ward *et al.*, 1983; Phillips *et al.*, 1986), and producing the same sex pheromones (J. G. C. Hamilton, pers. com.); the age of the colony was not expected to affect the flies' capacity of infection (Goncalves *et al.*, 1985). The methods of colony maintenance were those described by Killick-Kendrick *et al.* (1977), with the exceptions that **(1)** wild caught females were offered hamster, not human, blood, **(2)** larvae were reared in petri dishes lined with damp filter paper, **(3)** adult flies were not fed sugar solution prior to xenodiagnosis, and **(4)** colonies were maintained at ambient temperature (25–30°C).

5.3.3 Xenodiagnosis

Xenodiagnosis was performed in the laboratory in Salvaterra. Dogs were placed into individual cages measuring 0.75cm x 0.75cm x 2m sheathed in sandfly proof netting. In each trial, an average of 75.6 (*S.E.* 4.05, range: 8–160) 2–3 day old adult female flies, and approximately equal number of males, were introduced into the cage, and the females allowed to feed over night (1900–0700 hrs). Post exposure, visibly engorged flies were removed and placed into a smaller cage (20 cm^3), or tubed individually, and offered fresh sugar solution until day 4–5 post-feeding when dissected and microscopically examined for promastigotes (Killick-Kendrick, 1987). An average of 34 (*S.E.* 1.92, range: 1–96) female flies survived per trial to dissection. Infectiousness was estimated as the number of infected flies divided by the total dissected.

5.3.4 Sampling regime

a) Bimonthly interval trials

Dogs were experimentally exposed according to the availability of adult colony flies when housed overnight at the laboratory for serological, parasitological and clinical sampling (described in Chapter 2). In practice, 50% of the exposed dogs were from the groups installed into the study area in rounds 1 and 2. Xenodiagnosis was repeated on as many dogs and as frequently as the colony yields permitted. Dogs were thus exposed to flies on 1–12 occasions (mean 3.5, *S.E.* 0.22), at a mean inter-trial interval of 91 days (*S.E.* 5.4, range 33–474) days (**Table 5.1**). The 173 data points obtained from these trials were used to estimate population parameters, and to search for correlates of infectiousness, as described below.

Table 5.1. Sampling regime: **(a)** the frequency that individual dogs were exposed experimentally to sandflies, and **(b)** the number of dogs that were exposed in each sample round from the time of their enrolment into the study.

(a) frequency of exposures to sandflies	number of dogs
1	20
2	6
3	4
4	6
5	2
6	2
7	3
8	4
9	2
10	0
11	0
12	1

(b) sample round	number of dogs exposed
instalment 1	0
2	15
3	15
4	21
5	20
6	19
7	22
8	15
9	12
10	12
11	11
12	6
13	5

b) Short-interval trials

For 8 dogs (10 occasions), 2–5 consecutive trials were performed at inter-trial intervals of 6.1 days (*S.E.* 1.58, range: 1–20 days, n = 29), which permitted examination of the variation in infectiousness in the short-term. The mean values from each 10 sets of trials (calculated as the total number of infected/ the total number of dissected flies, and dated as the first in the series), formed part of the 173 points described above.

5.3.5 Parameter estimation

a) Onset of infectiousness

From the longitudinal data the average instantaneous rate that infected dogs became infectious, σ, was calculated from

$$\sigma = -\ln(1 - p/n)/t \qquad \text{(equation 5.1)}$$

where p the is the number of seropositive dogs which become infectious over a period of t months. The latent period (time from infection to infectiousness) is $1/\sigma$.

b) Mortality and life expectancy

Mortality rates, μ, were calculated by survival analysis as described in Chapter 2.

5.3.6 Statistical analysis

Statistical procedures are outline in Chapter 2. The variables used in the statistical analyses, and their abbreviations, are as described in Chapter 4 (see **Table 4.1**).

5.3.7 Mathematical model

A flow diagram of a compartmental model of infectiousness (**Figure 5.1**) was designed, based on the information presented in this thesis and already published (Quinnell *et al.*, 1997). The movements of dogs between the different compartments are described in the legend, and the model parameters described in **Table 5.2**.

Figure 5.1 Flow diagram of the compartmental model of infection and infectiousness of dogs exposed to *L. infantum*. Dogs enter the population of size D as susceptibles (S), and become infected (indicated by a positive serological response), of which a proportion (ϕ) become latently infectious (L_i) and then infectious (I) at a rate σ ($1/\sigma$ = the average latent period). The other fraction of the infected population ($1-\phi$) do not become infectious (L_u). Dogs enter the population through birth (β) and replacement (κ_{input}) and are lost through death (δ) and culling (κ). Dog replacement is instantaneous and directly proportional to culling, where $\kappa_{input} = (\kappa I + \kappa L_i + \kappa L_u)$. The symbols are described in **Table 5.2**.

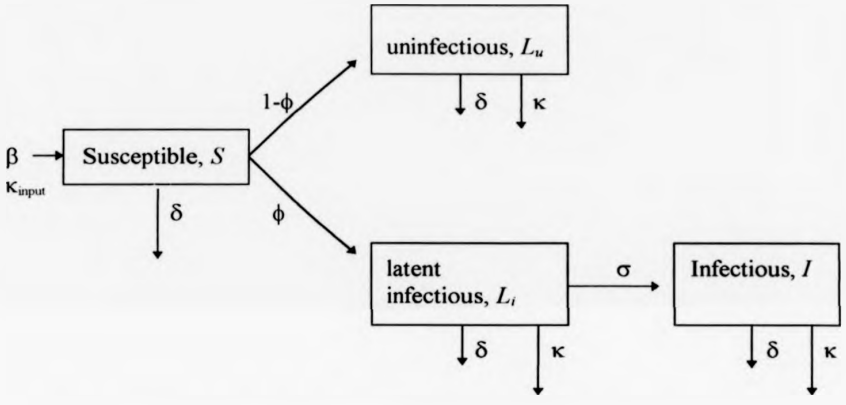


Table 5.2 Variables and parameters used in the model

S	Number of susceptible dogs
L_u	Number of infected dogs which never become infectious
L_i	Number of infected dogs which become infectious = latent infections
I	Total number of infectious dogs
D	Total number of dogs ($S + L_u + L_i + I$)
α	Proportion of susceptible dogs which acquire infection
β	Birth rate (the equivalent of δ in a stationary stable population)
C	Vectorial capacity of the sandfly population transmitting infection between dogs
δ	Death rate of dogs ($1/\delta$ = life expectancy)
κ	Proportion of I, L_i and L_u class which are culled and replaced as susceptibles by a program of culling
ϕ	Proportion of susceptible dogs which become latently infected
σ	Rate at which latent dogs, L_i, become infectious

Movement through the model compartments are quantified by the following set of first order differential equations, based on Dye (1996), and Cleaveland and Dye (1995):

$$\frac{dS}{dt} = \alpha\beta I D - CI\frac{S}{D} - \delta S + (\kappa L_u + \kappa L_i + \kappa I)$$

$$\frac{dL_u}{dt} = (1-\phi)CI\frac{S}{D} - \delta L_u - \kappa L_u$$

(equation 5.2 a-d)

$$\frac{dL_i}{dt} = \phi CI\frac{S}{D} - (\delta+\sigma)L_i - \kappa L_i$$

$$\frac{dI}{dt} = \sigma L_i - \delta I - \kappa I$$

The basic reproduction number, R_0, and the proportion of the population that was infectious, I/D, were calculated from

$$R_0 = \frac{(1-\phi)C\alpha\sigma}{\delta(\delta+\sigma)} \qquad \text{(equation 5.3)}$$

and

$$\frac{I}{D} = \left(1-\frac{1}{R_0}\right)\left(\frac{\alpha\sigma(1-\phi)}{\sigma+\delta}\right) \qquad \text{(equation 5.4)}$$

The purpose of the model is to compare the relative effects of pulse culling dogs of different status (L_i, L_u, and I classes- see **Table 5.2**), at varying magnitudes of κ, on population infectiousness index (I/D), measured as the proportion of infectious dogs in the population. Since dogs that leave the population are known to be replaced by their owners (see Chapter 3), culled dogs in the model are replaced instantaneously as susceptibles at equivalent values of κ. The term susceptible here refers to newly exposed dogs which mount a positive antibody response, irrespective of their longer-term infection outcome. The proportion of dogs which acquire infection, α, was set to 1 since seropositivity was observed in 96.2% (75/78) of the sentinel dogs that remained in the study for long enough to seroconvert (i.e. approximately 6 months) (Quinnell *et*

al., 1997); no assumption was made about the fraction of "susceptible" or "resistant" dogs (as defined by Pinelli *et al.*, 1994).

Prior to simulation, the model was run to a steady state, at which the index of transmission was $I/D = 0.31144$. The assumptions of the model are that the population is stationary (i.e. zero growth rate: input = output), that there is no serological recovery from infection, and that infectious dogs are homogeneously infectious for life following infectious onset.

5.3.8 Monte Carlo simulation

Seropositive dogs were ranked in diminishing order of the mean proportion of sandflies that they infected over all seropositive trials. The infectiousness of each dog was defined as a binomial distribution in logits (output from STATA- see Chapter 2). A Monte Carlo simulation was then performed for 2000 iterations, with the value of infectiousness for each dog chosen at random from the specified binomial distribution. The four dogs for which a binomial distribution could not be calculated (i.e. those exposed on only one occasion when seropositive) were excluded from the simulation. Based on the same ranking, the cumulative proportion of all sandfly infections due to dog 1 to n (where n is an integer between 1 and 36) was also calculated. Monte Carlo simulation was performed using @risk (Palisade, 1997).

5.4 RESULTS

PART I POPULATION INFECTIOUSNESS

A total of 6,002 blood-fed flies were dissected after feeding on 50 dogs in 173 trials performed over 22 months in 12 sample rounds. Twenty-eight dogs when seronegative were exposed to sandflies in 53 trials without infecting any of the 1,823 flies dissected. Forty dogs were exposed to flies when seropositive, of which 22 were uninfectious to 1,570 dissected flies from 53 trials. The other 18 seropositive dogs, however, infected 501 (19.2%) of 2,609 flies in 36/67 (53.7%) trials (**Table 5.3**).

Table 5.3 Infectiousness of dogs experimentally exposed to *Lu. longipalpis* relative to their serological status.

sero-conversion status	number of dogs	number of trials	number of dissected *Lu. longipalpis*	number of infected dogs (%)	number of positive trials (%)	number of infected flies (%)
non-converters	9	9	291	0	0	0
pre	19	44	1,532	0	0	0
post	40	120	4,179	18 (45)	36 (53.7)[a]	501 (19.2)[b]
totals	50	173	6,002	18	36	501

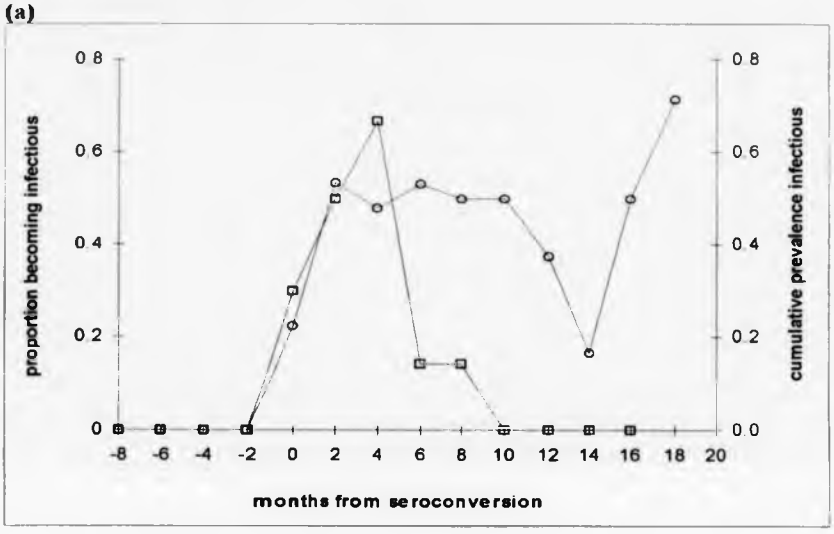
[a] n = 67 trials performed on infectious dogs.
[b] n = 2,609 *Lu. longipalpis* fed on infectious dogs.

5.4.1 Prevalence

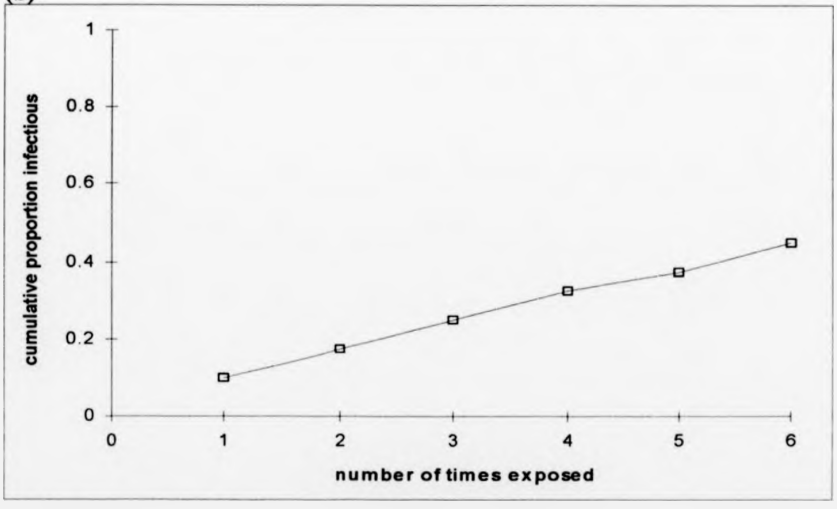
The rate at which dogs first became infectious peaked approximately 4 months after seroconversion and declined thereafter to zero over a period of approximately 6 months (**Figure 5.2a**). The cumulative prevalence of infectious dogs in the population during this period asymptoted at 49.1% (95% C.L.s 41.9–56.3%) (z = 0.13) (**Figure 5.2a**), which was similar to the asymptote reached at 10 months from the time of instalment (not shown). In accordance with the observed cumulative prevalence of 45% (18/40) among seropositives (see above), these data indicated that the majority of infectious animals had been detected. The mean number of times that infectious and uninfectious animals were experimentally exposed to flies when seropositive was not dissimilar (unpaired t-test, t = 2.06). However, the probability of any individual dog being infectious clearly increased with the number of times that it was experimentally exposed

Figure 5.2 **(a)** Cumulative prevalence of infectious dogs in the population (○), and the rate of infectious onset (□), relative to the time of seroconversion. **(b)** cumulative prevalence of infectious dogs in the seropositive population relative to the number of xenodiagnosis trials performed per dog.

(a)



(b)



(z = 2.25, P < 0.025), but without reaching an obvious asymptote (**Figure 5.2b**), suggesting, in fact, that more than 49% of the population was infectious. The point (cross-sectional) prevalence of infectious dogs was constant through time at 30.0% (95% C.L.s 23.2–37.9) (z = 0.06) starting from the round following seroconversion.

5.4.2 Variation in infectiousness

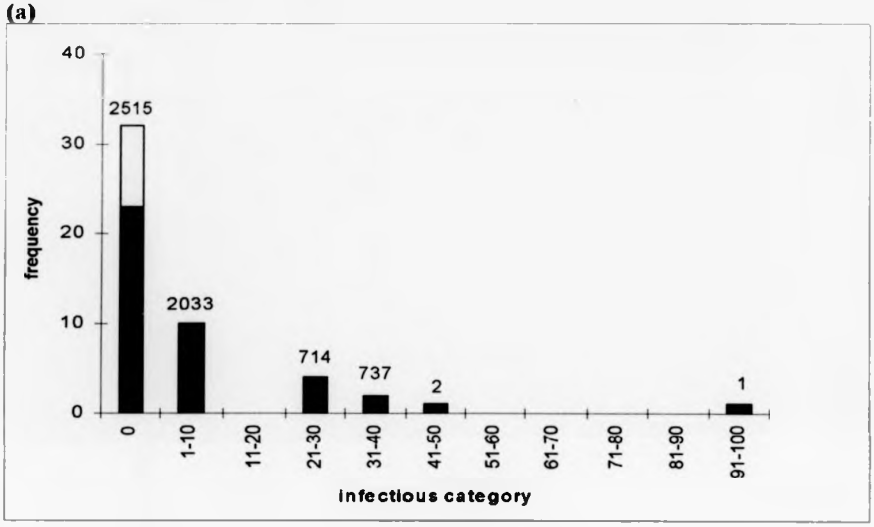
Infectiousness (the percentage of flies infected) in the population was over-dispersed, characterised by a variance/mean ratio of 37.1 (333.6/9.0) among individual dogs (**Figure 5.3a**), and 43.6 (254.7/7.8) for individual feeding trials (**Figure 5.3b**). The median percentage of flies infected by dogs when infectious was 23.9%.

Comparison of the mean proportion of flies infected per seropositive trial by individual dogs revealed that they were not equally infectious (z = 2.51, P = 0.01). Neither were the 18 dogs which had become infectious by the end of the study a homogeneous group either (comparison of means per dogs: z = 2.48, P < 0.01; **Table 5.4**). Exclusive sub-groups were not statistically identified; instead infectiousness represented a continuum, as illustrated in **Figure 5.4a**: this shows the mean percentage of all sandfly infections resulting from each dog calculated by Monte Carlo simulation. The shown values are calculated bounded by the empirically derived binomial error distributions recorded for 14 of the 18 dogs ranked in diminishing order of mean infectiousness. In 97.5% of all iterations, the minimum number of dogs responsible for at least 80% of all the infections was 7, representing 19.4% (7/36) of the infected (seropositive) population (**Figure 5.4b**).

Through time, the mean probability that a dog was infectious in any trial post onset was no greater than 50% (z = 1.77). The percentage of flies infected by the population was also constant, whether aligned by time from seroconversion (z = 1.35), or from infectious onset (z = 0.58), giving a fitted mean population infectiousness (when infectious) of 27.5% (95% C.L. 20.2–36.2).

Figure 5.3 Frequency distributions of infectiousness of **(a)** dogs, and **(b)** trials, when exposed to *Lu. longipalpis*. Infectiousness is defined as the percentage of infected flies of the total exposed per dog or trial. Dogs which never seroconverted and seronegative trials are indicated (unshaded). Numbers above the columns represent total flies dissected.

(a)



(b)

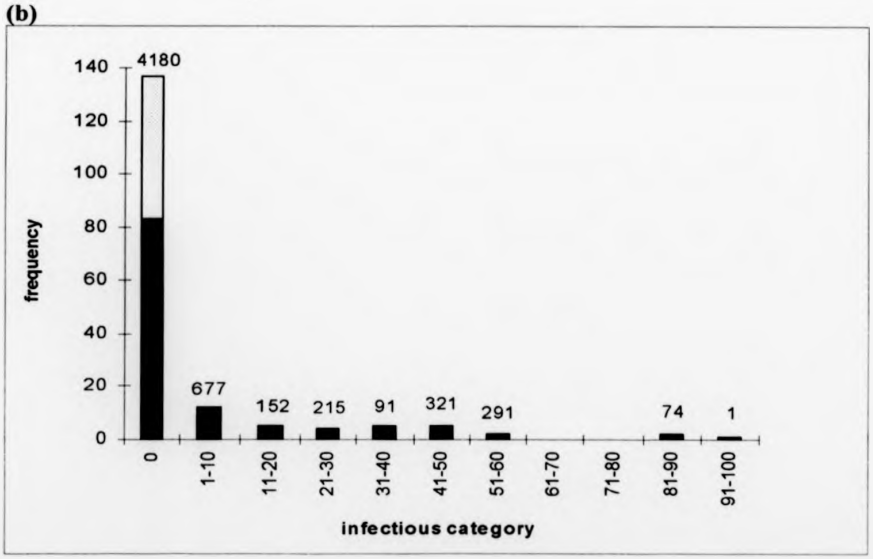
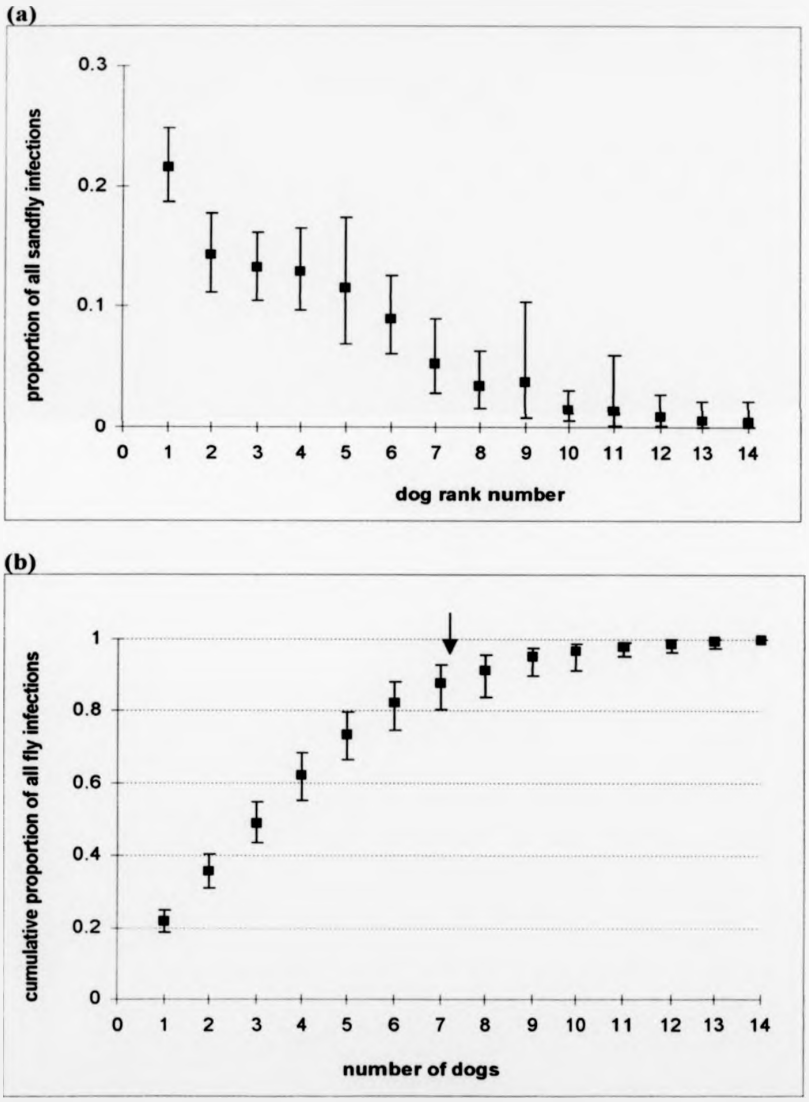


Table 5.4 Percentage of flies infected by the 18 identified infectious dogs in each trial from initial onset. Trials are presented in sequential order aligned by infectious onset; sampling intervals are not necessarily equal. Total flies dissected are shown in parenthesis.

dog ID	trial 1 (onset)	2	3	4	5	6	7	8	9	10	mean % of flies infected	binomial *S.E.*	total flies dissected
A37	24 (108)[a]	59 (273)[a]	16 (31)	0 (24)	43 (105)[a]	83 (30)	40 (5)	24 (21)	0 (22)	28 (78)	39.5	1.85	697
A40	2 (52)	0 (65)	0 (22)	0 (1)	0 (13)	0 (7)	0 (10)				0.6	0.59	170
B24	4 (27)	0 (38)	0 (32)	0 (18)	0 (7)	0 (3)					0.8	0.80	125
A78	6 (65)	4 (71)	37 (49)	45 (88)[a]							23.8	2.58	273
C10	1 (77)	0 (76)	0 (28)	15 (27)							2.4	1.06	208
A43	33 (21)	0 (25)	15 (13)								15.3	4.72	59
A29	2 (44)	19 (32)[a]	0 (43)								5.9	2.17	119
A31	10 (20)	14 (49)[a]	47 (32)								23.8	4.26	101
C14	5 (40)	56 (18)									20.7	5.37	58
A79	84 (44)	10 (103)[a]									32.0	3.86	147
A83	43 (94)[a]	3 (63)									26.8	3.54	157
C21	3 (39)	0 (27)									1.5	1.52	66
E40	25 (8)	0 (28)									5.6	3.87	36
C02	3 (76)										2.6	1.85	76
C08	40 (5)										40.0	24.49	5
C09	100 (1)										100.0	-	1
E04	50 (2)										50.0	50.00	2
E06	36 (11)										36.4	15.21	11

[a] weighted mean values based on 1-5 non-independent trials over 1-20 days (as shown in Table 5.5a)

Figure 5.4 Monte Carlo simulations of infectiousness, showing **(a)** the mean proportion of all sandfly infections due to each dog **(b)** the cumulative proportion of all sandfly infections relative to the cumulative number of seropositive dogs, both ranked in diminishing order of mean infectiousness. The minimum number of dogs responsible for at least 80% of all infections (in 97.5% of the 2000 simulations) is indicated (arrow). Error bars represent 95% C.L.s. Data represent seropositive trials only.



Differences between dogs accounted for only 17.8% (r^2 = 0.1776) of the total variation in infectiousness. The percentage of flies that infectious dogs infected when exposed on 2–5 consecutive occasions at 1–20 day intervals varied extensively, with differences in infectiousness ranging from 1–45% in 2 days to 8–80% in 4 days (**Table 5.5a**). This was compared to the variation among infectious dogs exposed to flies at intervals of 33–474 days (**Table 5.5b**). Analysis of standardised variances of all 55 paired consecutive trials (excluding paired uninfectious trials) was not found to depend on the length of the inter-trial interval (z = 1.51).

Table 5.5b The variance (s^2) in infectiousness of dogs exposed to *Lu.longipalpis* in paired consecutive trials of increasing inter-trial time intervals.

inter-trial interval (days)	paired trials *N*	s^2 percentage of flies infected	s^2 95% C.L.
1-20	17	14.0	7.8–32.4
>20-62	13	10.8	5.7–28.0
>62	15	9.2	4.5–22.0

Table 5.5a Percentage of flies infected by dogs exposed in 1-5 consecutive trials conducted over periods of 1-20 days. Data are presented as described in Table 5.4. Mean values for the trial series of infectious dogs were entered as discrete values in the larger dataset (indicated in **Table 5.4**)

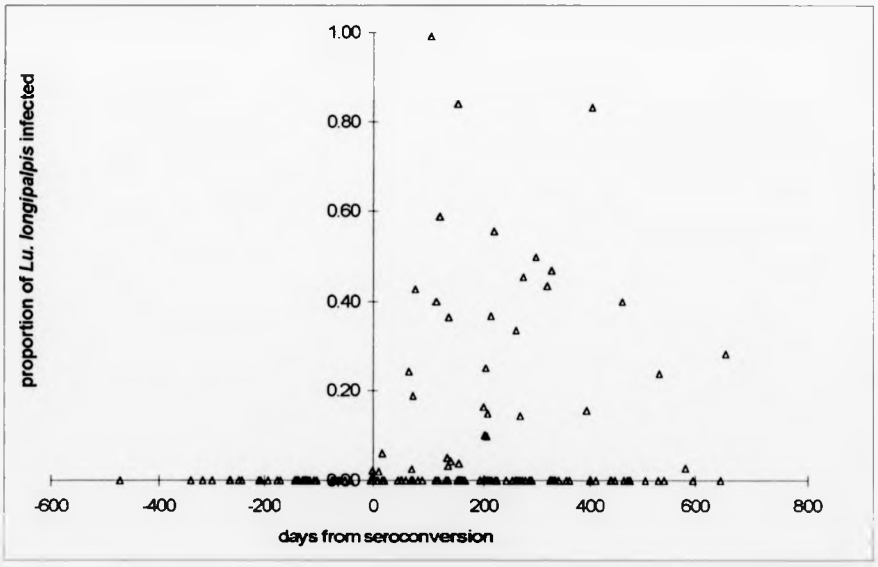
dog	consecutive trial 1	2	3	4	5	mean trial interval and range (days)	mean % of flies infected	binomial *S.E.*	total flies dissected
A37	4(75)	70 (33)				20 (-)	24.1	4.13	108
A38	0 (10)	0 (23)				20 (-)	0.0	0.00	33
A37	80 (30)	38 (55)	67 (63)	66 (76)	49 (49)	1 (-)	59.0	2.98	273
A38	0 (13)	0 (50)				3 (-)	0.0	0.00	63
A83	9 (35)	63 (59)				17 (-)	42.6	5.13	94
A79	27 (30)	0 (38)	6 (35)			6.5 (1-12)	9.7	2.93	103
A29	0 (10)	27 (22)				1 (-)	18.8	7.01	32
A37	0 (38)	8 (26)	20 (10)	100 (8)	74 (23)	5.8 (2-15)	27.6	4.38	105
A78	74 (19)	29 (7)	47 (17)	36 (45)		1.7 (1-3)	45.5	5.34	88
A31	50 (4)	11 (45)				1 (-)	14.3	5.05	49

5.4.3 Parameter estimates

a) Latent period

Forty of the dogs which seroconverted were exposed to a total of 5,711 flies in 164 trials conducted from 471 days before to 657 days after seroconversion **(Figure 5.5)** Infectious onset for the 18 dogs occurred a median 128 days (range 1–582 days) after seroconversion. When added to the estimated 94 day pre-patent period (Quinnell *et al.*, 1997), gave a latent period (time from infection to infectiousness) of 222 days (= 7.2 months), and median rate of infectious onset of 1/222 days = 0.00451/ day. Estimated by equation 5.1, 18/40 latently infected dogs at seroconversion became infectious in a median time of 134 days (i.e. 2 sample rounds x 67 day intervals), giving an instantaneous rate of $\sigma = 0.00446$/day, and latent period, $1/\sigma = 224$ days. These 2 sets of values were similar.

Figure 5.5. Proportion of *Lu. longipalpis* infected per trial relative to the time of seroconversion.



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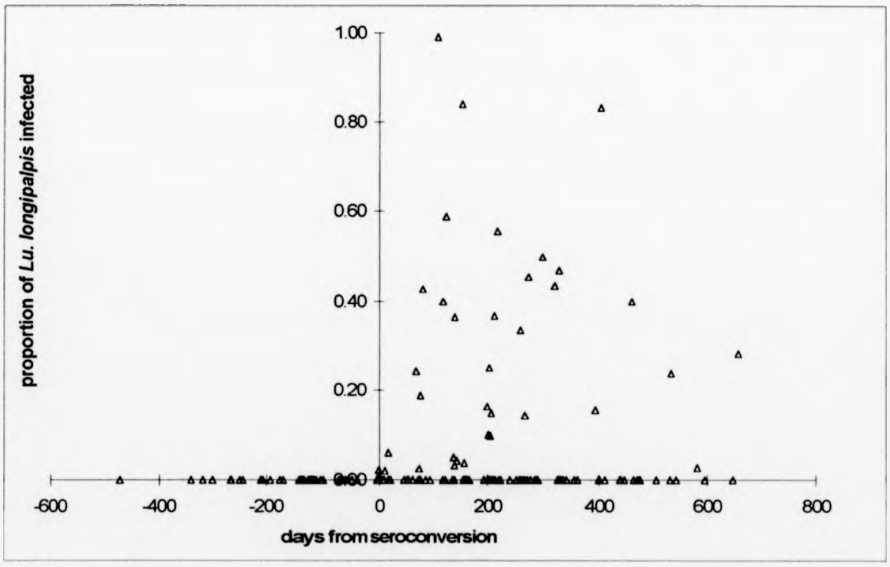


b) Duration of infectiousness

Recovery from infectiousness was defined as the failure to infect flies in > 2 consecutive trials following infectious onset. Only 2 of the 8 infectious dogs exposed to flies on 3 or more occasions fitted these criteria, having been uninfectious in 5 and 6 trials, respectively (**Table 5.4**). These data were too few to calculate an average duration of infectiousness, but nevertheless suggest that reversion to an uninfectious state is a rare event. The timing of infectiousness relative the course of infection and disease is summarised in **Figure 5.6**. Note that the duration of infectiousness shown in the Figure is based on the estimated mortality rate of dogs which achieve "high" titres (see Chapter 4) since most infectious trials corresponded with "high" titres (i.e. $>10^5$ log units; see Correlates of infectiousness below).

c) Life expectancies

Twelve (66.7%) of the 18 infectious dogs and 7/20 (35%) uninfectious dogs died (excluding accidents) by the end of the study. No statistical distinction was detected in their survival rates (log-rank test for equality of survivorship function: $\chi^2 = 2.91$). Nor was there any evidence of a difference between the mortality rates of infectious and uninfectious dogs between rounds when classified as such by their previous trial result ($z = 0.67$). Thus the average mortality rate of uninfectious and infectious dogs combined was $\delta = 0.00083$/ day, which was not significantly different from that calculated for the sentinel population as a whole (see **Table 3.4** in Chapter 3), equivalent to $L = 2.48$ years.

5.4.4 Correlates of infectiousness: infection and disease

a) Serology

A total of 173 sera from the 50 dogs exposed to flies were tested for anti-*Leishmania* antibodies. None of the dogs was infectious when seronegative, as already reported. Post seroconversion, infectiousness positively increased with antibody titre ($z = 5.15$, $P < 0.001$), with 72% of all infectious trials associated with titres > 5.0 log units (**Figure 5.7**).

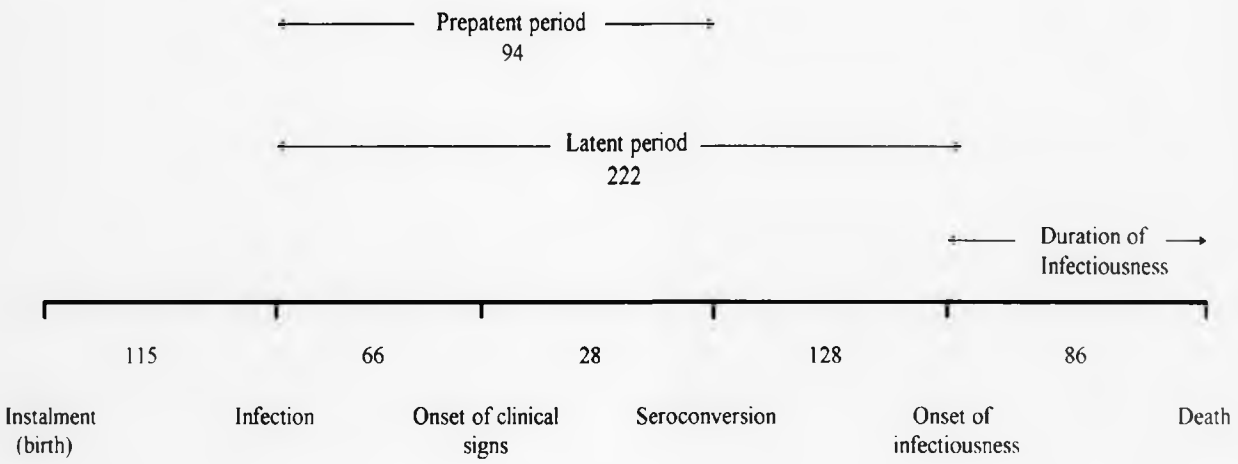
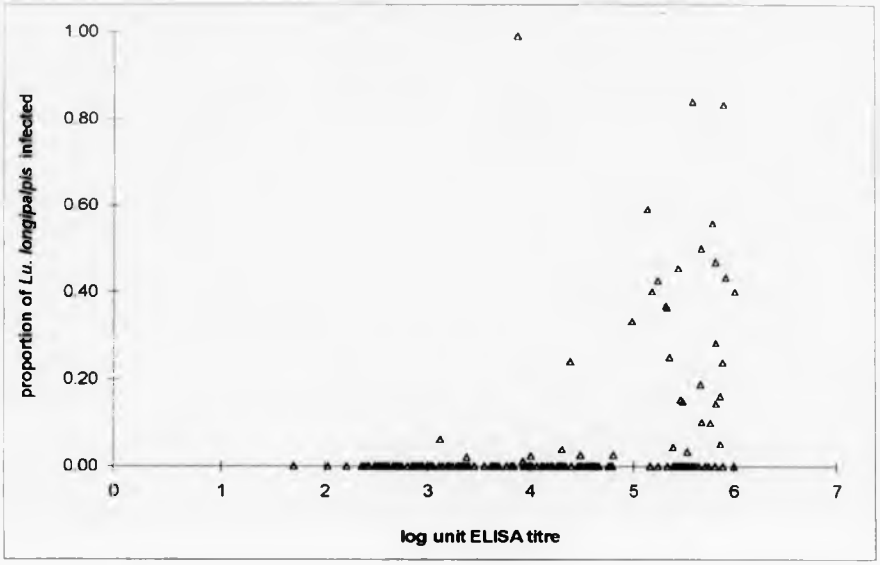


Figure 5.6 Infectiousness relative to the course of infection and disease in days from instalment

Figure 5.7. Proportion of *Lu. longipalpis* infected per trial relative to the anti-*Leishmania* antibody titre (log units) at the time of exposure.



b) Parasitology

Xenodiagnosis was performed on 48 dogs (155 occasions) when *Leishmania* isolation was attempted, 21 (43.8%) of which were parasite positive in 35 (22.6%) trials. Dogs were infectious on a greater proportion of occasions when parasites were isolated (17/35 = 48.6%) than when they were not (15/120 = 12.5%) (χ^2_1 = 21.5, $P < 0.001$). They were also infectious to a greater mean proportion of sandflies (0.2445, 95% C.L. 0.118–0.439 *vs.* 0.022, 95% C.L. 7.9^{-03}–0.058), irrespective of time since initial exposure ($z = 0.58$). The timing of infectious onset in the 13 dogs for which data were available, corresponded to the round of initial parasite isolation, with a median difference of only 3 days (95% C.L. 1.2–41.2).

The sensitivity of parasite isolation to detect infectious dogs and trials was low at 72.2% (13/18) and 53.1% (17/32), respectively, and independent of the time since seroconversion ($z < 0.56$, in each case). On 51.4% (18/35) of occasions when parasites were isolated, dogs were not observed to be infectious. Controlling for antibody titre

and time since instalment (Tf), the probability of successful parasite isolation from uninfectious and infectious dogs was not significantly different ($z = 1.32$).

c) Clinical and biochemical measures

A total of 146 clinical records (of the 14 variables described in Chapter 4) were available for 44 of the dogs fed to sandflies. Twenty-two dogs were xenodiagnosed when asymptomatic of which 9 (40.9%) were infectious in 9/53 (17.0%) trials. The 18 identified infectious dogs infected a similar proportion of flies during their asymptomatic and symptomatic phases when controlling for ELISA titre ($z = 0.15$), and on a similar proportion of occasions when exposed (9/16 *vs.* 27/40, $\chi^2 = 0.62$). However, relative to the long-term clinical outcome (as classified in Chapter 4), only 16.7% (1/6) of "healthy" dogs, compared to 72.7% (8/11) of "sick" dogs, were infectious (Fisher's Exact, $P < 0.05$).

The relationship between infectiousness and disease intensity was not uniform for the measured signs of CVL. Infectiousness among seropositive dogs significantly increased with DE, AL, CH, TS, TD and PCV intensity when the signs were tested individually (**Table 5.6a**). Controlling for Tf and the covariances between signs, the MAM retained only CH, TD, and CONJ, the latter which was inversely related to the proportion of flies infected (**Table 5.6b**). PCV was also significant, inversely related to infectiousness, when the analysis was repeated to include the 2 biochemical measures (BUN and PCV) (coefficient: -0.094, *S.E.* 0.0333, $z = 2.83$, $P = 0.005$).

Table 5.6 Associations between infectiousness and clinical severity in seropositive dogs. The MAMs represent **(a)** each of the clinical and biochemical variables tested individually while controlling for time since instalment (Tf), and **(b)** all clinical variables tested simultaneously, controlling for Tf. For ease of interpretation, categorical variables were treated here as continuous variables in full regression models (with binomial errors and logit link function); the coefficient of each explanatory variable represents a positive or negative trend with infectiousness. Estimates are shown as logits.

(a)

explanatory variable	parameter coefficient	*S.E.*	*P* <	Tf coefficient	*S.E.*	*P* <
DE	0.449	0.1916	0.019	-0.004	0.0009	0.001
AL	0.502	0.2098	0.017	-0.004	0.0009	0.001
CH	0.410	0.1869	0.028	-0.002	0.0008	0.011
CONJ	-	-	ns	-	-	ns
NO	-	-	ns	-0.002	0.0008	0.024
ONY	-	-	ns	-0.002	0.0008	0.006
COND	-	-	ns	-0.002	0.0008	0.042
NUM	-	-	ns	-0.002	0.0008	0.001
TS	0.114	0.0577	0.049	-0.003	0.0009	0.001
TD	0.248	0.1021	0.015	-0.004	0.0009	0.001
TE	-	-	ns	-0.002	0.0008	0.005
BUN	-	-	ns	-	-	ns
PCV	-0.089	0.0340	0.009	-	-	ns

(b)

explanatory variable	parameter coefficient	*S.E.*	*P* <	r^2
DE	-	-	ns	-
AL	-	-	ns	-
CH	0.521	0.2240	0.02	0.0621
CONJ	-0.852	0.3684	0.021	0.0156
NO	-	-	ns	-
ONY	-	-	ns	-
COND	-	-	ns	-
NUM	-	-	ns	-
TS	-	-	ns	-
TD	0.293	0.1152	0.011	0.0492
TE	-	-	ns	-
Tf	-0.005	0.0009	0.001	0.1301
MAM deviance: 316.2				
total deviance: 1409.4				0.2244

5.4.5 Predicting infectiousness

In search of potential markers of infectiousness, the serological, clinical, biochemical, and parasitological data, and Tf, were simultaneously entered into a series of ANCOVA models as the explanatory variables (**Table 5.7**). The first analysis included the data of both seronegative and seropositive dogs, thereby representing the "mixed" population. The MAM explained 50% ($r^2 = 0.5009$) of the total variation, with ELISA titre revealed as the strongest predictor ($r^2 = 0.4320$). The other significant variables (AL, CONJ, and Tf) accounted for < 15% each (**Table 5.7**). Parasite isolation success (PARA) was not significant in the equivalent analysis on the reduced dataset, whereas PCV was retained in association with ELISA, AL and CONJ ($z = 2.28$, $P < 0.023$, $r^2 = 0.0525$). Again, in this model, ELISA explained the greatest proportion of the variation ($r^2 = 0.3678$; $z = 2.85$, $P < 0.001$).

Table 5.7. Potential clinical surrogate markers of infectiousness. The explanatory variables were tested by multivariate analysis controlling for Tf. The full models are as described in **Table 5.6b** but with the inclusion of ELISA.

explanatory variable	parameter coefficient	*S.E.*	$P <$	r^2
DE	-	-	ns	-
AL	0.643	0.1927	0.001	0.0660
CH	-	-	ns	-
CONJ	-0.693	0.2712	0.011	0.0345
NO	-	-	ns	-
ONY	-	-	ns	-
COND	-	-	ns	-
NUM	-	-	ns	-
TS	-	-	ns	-
TD	-	-	ns	-
TE	-	-	ns	-
Tf	-0.007	0.0017	0.001	0.1438
ELISA	1.800	0.2459	0.001	0.4320
MAM deviance: 860.8				
total deviance: 1718.7				0.5009

The diagnostic potential of ELISA was further examined by including only seropositive dogs in the analysis (not shown). In this case, the MAM retained the same parameters as shown in **Table 5.7**, but the predictive power of ELISA dropped to 25% (r^2 = 0.2526). Neither PARA, PCV or BUN were significant ($z < 1.26$, in each case).

Finally the ability of ELISA titre to differentiate infectious and uninfectious dogs was tested by comparing the average titres of the two groups at each sample round, aligned firstly, by time from instalment and, secondly, by time from seroconversion. No statistical differences were detected in any round irrespective of alignment (from instalment: $z < 1.97$; from seroconversion: $z < 1.84$, for each round). Similar attempts to differentiate the 7 "high risk" dogs responsible for 80% of sandfly infections (see above) were equally unsuccessful (from instalment: $z < 1.13$; from seroconversion: $z < 2.09$, for each round).

PART II MATHEMATICAL MODEL

5.4.6 Predicting the outcome of targeting infectious and uninfectious dogs

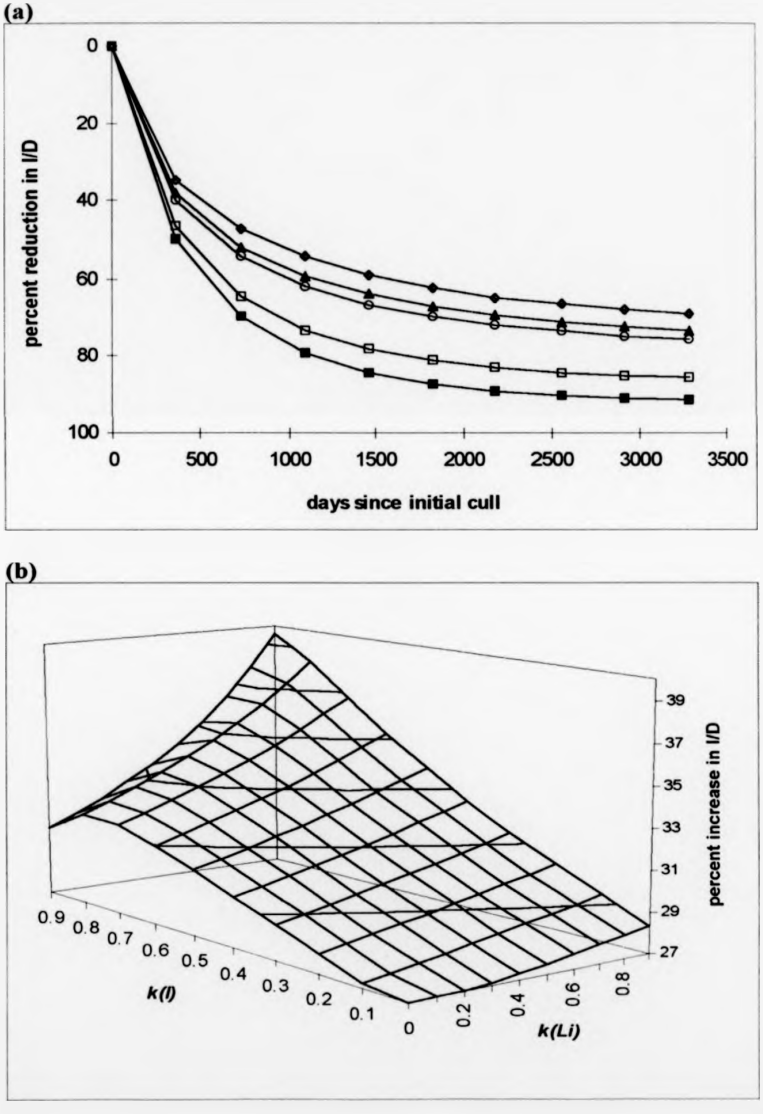
The parameter values used to model population infectiousness are listed in **Table 5.8**. The compartmental model (**Figure 5.1**; **Table 5.2**) was designed to simulate pulse culling of uninfectious (L_u), latent (L_l), and infectious (I), dogs at specified values of κ, starting at day 1, and repeated at yearly intervals for an arbitrary 8 years. Vectorial capacity, C, was set to yield transmission equivalent to R_0 = 5.9, and the birth and mortality rates were equalised to reflect a demographically stationary population (as shown in Chapter 3).

Table 5.8. Parameter estimates used in the mathematical simulation.

Parameter	parameter description	value or rate
$1/\sigma$	latent period	224 days
σ	rate that L_l dogs become infectious	0.005/day
δ	mortality rate	0.001/day
ϕ	Proportion L_l dogs	0.45
α	Proportion of dogs which acquire infection	1
β	Birth rate	0.001/day
C	Vectorial capacity	0.01573/day
R_0	Basic reproduction number	5.9

The first set of simulations examined the expected outcomes of culling dogs at different combinations of κ, where dogs in each class were precisely identified by a hypothetical diagnostic tool. By varying $\kappa(L_u)$, and holding $\kappa(I)$ and $\kappa(L_l)$ constant (at a moderate κ = 0.6), the first set of simulations showed that there was an inverse relationship between the percentage reduction in the transmission index, I/D, and the proportion of L_u dogs that were included in the cull (**Figure 5.8a**). By comparison, a non-selective culling strategy (in this example where $\kappa(I) = \kappa(L_l) = \kappa(L_u) = 0.6$), was clearly less effective than targeting only I and L_l dogs (comparison of $\kappa(L_u) = 0.6$ and $\kappa(L_u) = 0.0$ in **Figure 5.8a**). Irrespective of the values of $\kappa(I)$ and $\kappa(L_l)$, eliminating L_u dogs limited the potential reduction in I/D achieved when not culling L_u dogs. **Figure 5.8b** shows the percent change in I/D relative to culling 90% *vs.* 0% of the L_u dogs in the population.

Figure 5.8 Mathematical simulation of population infectiousness showing percent changes in transmission index (*I*/*D*) following pulse culling I, I_{-u}, and I_{-l} dogs at rates, (**a**) $\kappa(I) = 0.6$, $\kappa(I_{-l}) = 0.6$, $\kappa(I_{-u}) = 0.0$ (■), 0.2 (□), 0.6 (○), 0.7 (▲), 0.9 (◆), (**b**) the difference between $\kappa(I_{-u}) = 0.9$ and $\kappa(I_{-u}) = 0.0$ at varying values of $\kappa(I_{-l})$ and $\kappa(I)$ as shown. In (**b**) the results represent the initial cull only. The data shown are the maximum values of (*I*/*D*) during the recovery phase following each cull



As expected, I/D was greatly more sensitive to the value of $\kappa(I)$ than to either values of $\kappa(L_l)$ or $\kappa(L_u)$ **(Figures 5.9a-d)**. Thus relative to the potential reductions in I/D achieved by culling I dogs (especially at higher values of $\kappa(I)$), the inclusion of L_l or L_u dogs in the cull had relatively insignificant additional effect on I/D. This is shown in **Figures 5.9a & b** where κ is varied for the 2 classes of dog. Increases, as opposed to decreases, in the transmission index occurred only at values of $\kappa(I) \leq 0.2$, generally irrespective of $\kappa(L_u)$ and $\kappa(L_l)$ **(Figures 5.9c & d)**.

Against these predictions based on an exact theoretical surrogate marker, the ability of antibody cut-off titres to detect infectiousness in the population was quantified by calculating the sensitivity of each 0.5 log unit step in cut-off titre with respect to the 3 classes of dog **(Table 5.9)**. These estimates were entered into the model as values of κ, as appropriate. The results indicated that the greatest reduction in I/D was achieved by applying the lower cut-off titre (3.5 log units) **(Figure 5.10a)**, which is the equivalent of a non-selective culling strategy (i.e. $\kappa(I)$, $\kappa(L_l)$ and $\kappa(L_u) > 95\%$, in **Table 5.9)**.

Table 5.9. Sensitivities of ELISA cut-off titres to identify sentinel dogs of known infectious status.

log unit cut-off titre >	Infectious, I	latent, L_l	uninfectious, L_u
3.5	0.99	0.99	0.96
4.0	0.94	0.63	0.61
4.5	0.89	0.5	0.44
5.0	0.72	0.25	0.30
5.5	0.50	0.13	0.17

The absolute number of dogs in the selective cull is clearly determined by the population size and the magnitude of R_0. The culling effort is also related to the selection of cut-off titre, illustrated by the inverse relationship with the proportion of the total population selected **(Figure 5.10b)**, and the ratio of uninfectious to infectious dogs within that

Figure 5.9. Mathematical simulation of population infectiousness (**a**) $\kappa(I_{Li}) = 0.6$, (**b**) $\kappa(I_{Lu}) = 0.6$, (**c**) $\kappa(I_{Lu}) = 0.6$, (**d**) $\kappa(I_{Li}) = 0.6$, otherwise values of κ as shown. The conditions under which culling *I* dogs causes an increase in *I/D* in (**a**) and (**b**) are shown in (**c**) and (**d**) respectively. Data are plotted as described for **Figure 5.8**.

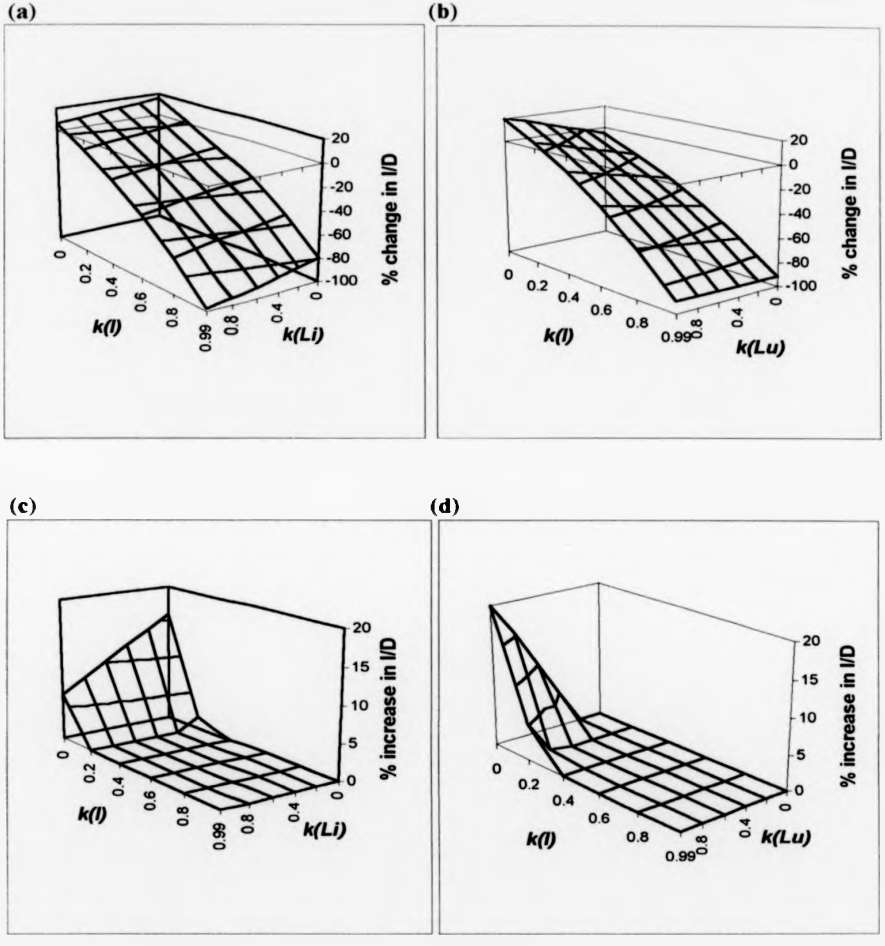
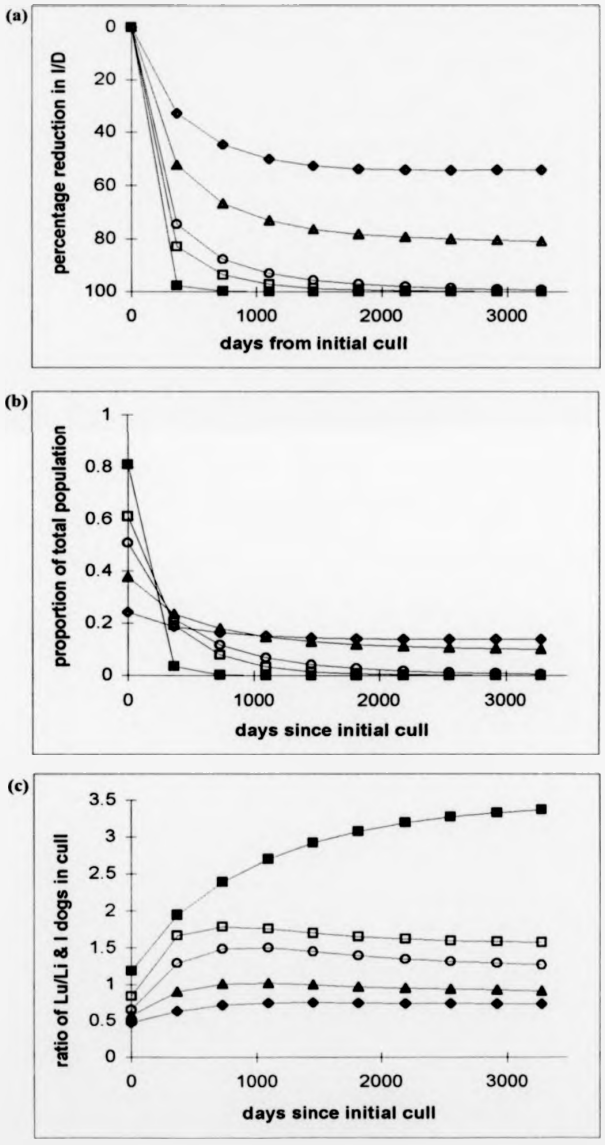


Figure 5.10. Mathematical simulation of **(a)** population infectiousness where values of κ are defined by the sensitivity of each 0.5 increase in log unit cut-off titre to identify I, I_{Lu}, and I_{Li} dogs (see **Table 5.9** for values). The relative culling effort required to achieve these reductions are measured as **(b)** the proportion of the total population culled, and **(c)** the ratio of Lu to Li & I dogs included in the cull. Cut-off titres 3.5 (■), 4.0 (□), 4.5 (○), 5.0 (▲), 5.5 (◆). Data are plotted as described for **Figure 5.8**.



selection (I_{-u} to I & I_{+} dog ratio) (**Figure 5.10c**). Thus, following the initial cull, the proportion of the total population required to be eliminated was smaller at the lower cut-off titres, but the ratio of I_{-u} to I & I_{+} dogs (i.e. "wrong" *vs.* "right" dogs) was < 1. To minimise simultaneously both the total number of dogs in the cull, and the proportion of uninfectious dogs in the selection, a higher cut-off titre than 3.5 log units is indicated.

5.5 DISCUSSION

5.5.1 Course of infectiousness

Fifty dogs were experimentally exposed to *Lu.longipalpis* in 173 trials from which 6,002 female flies were dissected. None of the dogs was infectious when seronegative, whereas 18 (45%) dogs infected flies on 54% of occasions when seropositive. The median latent period (infection to infectiousness) was 224 days. The prevalence of infectious dogs in any round after seroconversion was 30% (95% C.L. 23–38%), infecting a mean 28% (95% C.L. 20–36%) of flies. Only 2 dogs ceased to be infectious (in 5 and 6 bimonthly trials), suggesting a small degree of recovery. Despite the high risk of mortality associated with parasite positivity, higher antibody titres, and severe CVL (see Chapter 4), infectiousness did not significantly increase the mortality rate above that of uninfectious dogs.

Infectious onset corresponded with the time of initial parasite isolation, inferring that the time of *Leishmania* dissemination around the skin and to the bone marrow was similar. This does not conform to the expectation that transmission to sandflies commences at the time of initial infection, as shown by restricting blood-feeding *Lu. longipalpis* on the site of inoculation (Vexenat *et al.*, 1994). The sensitivity of parasite isolation to detect infectious dogs was constant from the time of seroconversion (at 72% and 53% for trials and dogs, respectively), suggesting that parasite load in the skin is relatively stable. Whether parasite detection in skin biopsies is a more sensitive indicator of infectiousness than bone marrow isolations has yet to be evaluated. Deane and Deane (1955) observed parasites in the skin of 38/49 (77.6%) dogs, of which 12/16 (75%) among the 49 were also infectious to *Lu. longipalpis*. Unfortunately the authors failed to report the precise correlation. Adler and Theodor (1935) demonstrated, however, that the proportion of *P. perniciosus* infected by 2 experimentally inoculated dogs (exposed on 3 occasions at 1–2 monthly intervals), increased with the intensity of amastigote infiltration in the dogs' skin.

The average proportion of flies infected by the sentinel population (median 24%, mean 28%), was similar to the equivalent crude estimates for 13 dogs experimentally exposed to *P. perniciosus* in Spain (median 36%; mean 37%, Molina *et al.*, 1994a), and 12 dogs

exposed to *Lu. longipalpis* in north-east Brazil (mean 25%, Deane and Deane, 1955). The estimated prevalences of infectious dogs in the population, on the other hand, were surprising low compared to the 81% and 75% reported in those cross-sectional studies (13/16, Molina *et al.*, 1994a; 12/16, Deane and Deane, 1955), particularly given the greater sampling effort in Marajó. The comparability of infection level between the populations cannot be confirmed to explain this, since Deane and Deane (1955) do not provide serological data, and Molina *et al.*, (1994a) used an IFAT. Clinical comparability is equally difficult to assess due to the lack of documented information, though certainly the proportion of seropositive dogs exposed solely when asymptomatic in this study (4/40) was less than in the mentioned cross-sectional studies (5/18 and 3/16). The cumulative prevalence of infectious sentinel dogs increased linearly with the sampling effort which suggests that the number of infectious dogs in the population in fact was underestimated.

5.5.2 Correlates of infectiousness

The strongest correlate of infectiousness in this study was antibody titre. While seropositivity was a necessary condition of infectiousness, clearly not all seropositive dogs were infectious. Positive xenodiagnosis corresponded to a 100-fold range in antibody titre, with 72% corresponding to > 5.0 log units (see **Figure 5.7**). Molina *et al.* (1994a) found no such association with IFAT titre in their cross-sectional sample, nor, similarly, Gradoni *et al.* (1987) or Alvar *et al.* (1994), for the few dogs that they followed pre and post chemotherapy. Titres (IFAT) in those studies, however, were limited to a 4-fold range of dilutions which is likely to have obscured such a relationship.

Controlling for covariances between clinical signs (with or without ELISA), the clinical correlates consistently associated with infectiousness included skin alterations- alopecia (AL), dermatitis (by contribution to TD), and chancres (CH), haematocrit (PCV), and conjunctivitis (CONJ). Skin abnormalities and hair loss are common in CVL (Koutinas *et al.*, 1992; Ferrer *et al.*, 1988; see Chapter 4), known to be due to the direct action of amastigote infiltration, and immunocomplexes deposition around hair follicles (Adler and Theodor, 1932, Hommel, 1978, Ridley and Ridley, 1983). Different types of

dermatitis may represent disproportionate sources of parasites to sandflies since parasite densities are reportedly higher in the nodular than the ulcerative form of the disease (Ferrer *et al.*, 1988; Ridley and Ridley, 1983; Fondevila *et al.*, 1997). Although chancres (skin lesions), in contrast to nodular dermatitis, were common in this study (82% of the 50 exposed dogs; see also Chapter 4), sandflies were not restricted to feed on specific skin alterations to test their potential infectiousness. Sentinel dogs were also more infectious when anaemic (PCV values $\leq$ 31.5%), but this relationship did not remain significant after controlling for the covariances in antibody titre and clinical signs. Dogs were not more infectious at the time of death than at other times post onset.

Infectiousness was not exclusively associated with the disease, however. From the time of the pioneering studies of CVL, parasite infiltration of externally healthy skin has been reported (Parrot *et al.*, 1930; Adler and Theodor, 1935; Deane 1956). Deane (in Deane, 1961) observed 2/3 such dogs to infect 23% and 25% of *Lu. longipalpis* fed on them, while a similar proportion of flies was infected by 3/5 asymptomatic dogs in the study of Molina *et al.* (1994a). Vexenat *et al.* (1994) also reported "lesion free" skin to be infectious, though to less sandflies (4/65) than symptomatic skin (57/163). The present results show that while dogs may be equally infectious prior and post clinical development, only a relatively small proportion of dogs with chronic infection (i.e. the "healthy" and "recovered" dogs as classified in Chapter 4) become infectious. This suggests that, in accordance with the clinical associations shown above, the contribution of chronic cases to transmission is relatively small.

The extensive variation in the proportion of *Lu. longipalpis* infected by individual dogs exposed at short (1–20 day) inter-trial intervals was not expected. Indeed, the variation was as great between 2 consecutive days as between bimonthly trials. The implication is that xenodiagnosis performed cross-sectionally may not provide an accurate measure of an individual's state of infectiousness. Short-term variation is obviously not associated with large alterations in clinical and/or serological parameters, but are probably explained by heterogeneities in amastigote accessibility to probing sandflies (e.g. patchy distributions associated with skin disease), and sandfly feeding behaviour. It is not

known whether females show preferences for probing sites, or indeed what governs the choice of lekking sites by males, although both are presumably mediated by a range of host-vector interactions including olfactory and visual cues (Ready, 1978; Hamilton and Ramsoondar, 1994). However, even when sandflies are limited to feed on a single lesion over a period of a few days, infectiousness has been shown to vary between 59% and 89% (Vexenat *et al.*, 1994).

5.5.3 Targeting infectious dogs

Sustained intervention targeted at infectious dogs is expected to reduce the population infectiousness. A deterministic compartmental model was therefore designed to examine the relative change in population infectiousness resulting from varying the culling effort (values of κ) directed at the 3 classes of dog in this study: infectious, latent (i.e. pre-infectious), and uninfectious. Secondly, the model was used to test the performance of ELISA cut-off titres to distinguish infectious dogs in the mixed population.

The first set of simulations revealed that culling uninfectious dogs invariably limited the potential reduction in transmission achieved when such animals were precisely identified and thereby excluded from the cull. The principal reason for this is that the input of susceptibles into the population is proportional to the rate at which dogs are lost (as shown in Chapter 3). Indiscriminate culling effectively increases the mortality rate, so that the 55% uninfectious portion of the culled population is replaced by susceptibles, 45% of which become infectious, thus increasing the net population infectiousness. Moreover, the turnover rate of susceptibles is inversely related to the specificity of the diagnostic marker. A program of mass elimination, which inevitably includes a large proportion of uninfectious dogs, is therefore likely to be less efficacious than targeting only infectious dogs.

In practice, infectious dogs would have to be identifiable in the field. Although antibody titre was more closely correlated with infectiousness than any of the clinical or biochemical parameters, its predictive power was low (r^2 = 0.25–0.43). This was reflected in its inability to distinguish infectious from uninfectious dogs in any round

post instalment. Its potential as a surrogate marker was tested by calculating the sensitivities of a range of ELISA cut-off titres to detect the 3 classes of dogs, as values of κ in simulation. Because high sensitivities were associated with low specificities (see **Table 5.10**), its performance was poor: to eliminate sufficient I dogs to achieve relatively large reductions in transmission, for example, required applying the lower test cut-off titre (3.5 log units), which led to the inclusion of > 95% of the uninfectious and latent population in the cull. This clearly approximates the mass elimination scenario. Given that ELISA, when carefully defined, is one of the more sensitive serological tests for *Leishmania* infection in dogs (Evans *et al.*, 1990; Garcez *et al.*, 1996), the results here are clear: the mean antibody titres are not sufficiently different to distinguish infectious, uninfectious, and latent dogs.

One important potential caveat is that in this model, like previous CVL models (Hasibeder *et al.*, 1992; Quinnell *et al.*, 1997), infectious dogs were assumed to be homogeneously infectious. This was clearly not the case. Although such dogs did not form statistically exclusive groups, there was good evidence that only 20% of the infected population was responsible for 80% of the transmission potential. This conforms nicely to the observed statistical patterns of host-vector aggregation for a number of vector-bourne and STD pathogens (Anderson and May, 1991; Woolhouse *et al.*, 1997a), suggesting that in fact only a fraction of the infectious dogs require identification. Unfortunately the ELISA also failed to distinguish the 20% ("high risk") transmission group from the other responders.

Given the sensitivity of the infectiousness parameter to the proportion of infectious dogs culled, mass dog elimination should by all accounts interrupt *L. infantum* transmission. This is not the experience in countries such as Brazil where dog culling is practised (see Chapter 1). One likely explanation is that the pulse intervention interval is too short to prevent transmission from reverting to endemic equilibrium (Koella, 1991). Furthermore, a proportion of infectious dogs are likely to be missed using the current seropositive selection criteria due to the combination of a long pre-patent period, the high rate of infectious onset close to the time of seroconversion, and consequently, the possibly that infectiousness precedes a detectable antibody response, depending on the

choice of serological test/cut-off titre. Ashford *et al.* (1998), working in Brazil, showed that following elimination of seropositive dogs, canine incidence declined from 36 to 6 percent in two years, but started to increased again to 11% and 14% in the two years post intervention. The simulations here represent a culling interval set at one year, whereas the apparent following-up time of the Brazilian Ministry of Health (FNS) is reported to be 6 months (Lacerda, 1994). Even a 6 month intervention interval, however, has not been successful in reducing human or canine seroconversion rates (Dietz *et al.*, 1997). From data presented earlier in this thesis (see Chapter 3), it was shown that based on the current estimate of R_0 = 5.9 (for Marajó), the critical pulse culling interval would have to be, in fact, no less than $T_c = A / R_0$ = 20 days. This assumed that all infected dogs become infectious at the time of seroconversion, which, as revealed here, is not the case. Substituting A in the equation for the average latent period of 222 days gives the more accurate estimate of T_c = 38 days. Even this interval, however, is clearly not sustainable in a large scale control program.

5.5.4 Conclusion

In conclusion, these results show that only a proportion of the infected domestic reservoir population contributes to transmission, and that the heterogeneity in infectiousness follows a pattern of aggregation (akin to other host–vector systems). Both of these observations could be exploited for ZVL control by targeting a sub-group in the population. At present, no proportion of infectious dogs is identifiable on the basis of antibody titre. Since serological tests are the only cheap, practical surrogate tool for large scale surveillance of infectiousness, targeted control will not be feasible until a more sensitive marker/tool becomes available. The present strategy of dog culling in Brazil and elsewhere is unlikely to be successful, even as part of an integrated approach to ZVL eradication, since the effort required exceeds practicality.

6. INFECTION, DISEASE AND INFECTIOUSNESS IN THE FOX, *C.thous*, AND ITS COMPARATIVE ROLE IN TRANSMISSION.

6.1 ABSTRACT

The effort required to control *L. infantum* in dogs may be seriously underestimated if an additional reservoir contributes to the peridomestic transmission cycle. In Brazil, one potentially significant wildlife host is the crab-eating fox, *C. thous*. To assess the role of this species in transmission relative to dogs, 37 free-ranging foxes from the study area were (re)captured for serological, parasitological, and clinical sampling on 74 independent occasions (1–4 times each). Xenodiagnosis using colony-bred *Lu. longipalpis* was also performed on 26 of the foxes (1–3 times each). Seventy-eight percent of the foxes had anti-*Leishmania* antibody (ELISA) titres >3.35 log units, and 38% were parasite positive. None of the foxes was infectious to any of the 1,469 female flies that were dissected from 44 trials. Mild clinical signs of CVL (dermatitis, onychogryphosis, and lymphadenopathy) were observed in only one fox, though these signs started to self-resolve over a period of four months. In comparison with dogs, foxes developed significantly less disease following infection, and were significantly less infectious to the vector. The incidence of infection in foxes was from 0.068–0.240 per month, according to which of three criteria were chosen to define infection. There was no evidence of serorecovery, nor *Leishmania*-related mortality, to indicate a loss of seropositivity in the population through time. Comparative incidence estimates calculated from age-seroprevalence data were similar for foxes and dogs (0.097 *vs.* 0.113 per month). These data suggest that the domestic and wildlife reservoirs experience similar infection rates, but that the contribution of *C. thous* to peridomestic transmission (in Marajó) is relatively insignificant.

6.2 INTRODUCTION

In search of effective strategies to interrupt the dog-sandfly-human transmission cycle, it is prudent to confirm that there are no additional reservoir species which could amplify transmission rates, or re-establish a transmission cycle in the event that the parasite is successfully eradicated in dogs. In either of these events, targeting only the dog population could lead to a gross underestimate of the size of the control problem.

Of the known mammalian hosts of *L. infantum* (see Chapter 1), foxes have been implicated as potentially important wildlife reservoirs: namely the crab-eating fox, *C. thous*, in Latin America, and the red fox, *V. vulpes* in western Europe (where it replaces *C. thous*) (**Table 6.1**). The initial evidence incriminating *C. thous* came from parasite isolations from the skin, viscera or blood of 4/33 animals caught in an endemic foci in north-east Brazil (Deane and Deane, 1954a; Deane, 1956), followed soon after by isolations from a further 7/173 animals caught in the same region (Alencar, 1959; 1961). In Amazonia, 14 of 49 (28.6%) *C. thous* were similarly found to be infected, including 11/26 animals from the study area in Marajó (Lainson *et al.*, 1969; 1987; Lainson and Shaw, 1971; Silveira *et al.*, 1982; Lainson *et al.*, 1990). The ability of this host to infect the sandfly vector was shown for a single naturally infected animal in advanced stages of the disease, which infected 10/10 *Lu. longipalpis* fed on it (Deane and Deane, 1954b). The infectious nature of the fox was later confirmed by Lainson *et al.* (1990) who observed an asymptomatic animal to infect 4/54 *Lu. longipalpis* 15 weeks after its experimental inoculation (with promastigotes of a local *L. infantum* fox strain via the bite of *Lu. longipalpis*).

These studies thus indicated the potential importance of this wildlife host for human infection. That foxes have contact with the peridomestic environment initially came from anecdotal reports that they predate domestic fowl from animal huts known to harbour large numbers of domicillary *Lu. longipalpis* (Lainson, 1988). This prompted studies of the fox's spatial and behavioural ecology in Marajó, with the primary aim of quantifying its degree of contact with both peridomestic and sylvatic habitats (Macdonald and Courtenay, 1993; 1996). The results from those studies revealed that the average fox entered 1–4 dispersed villages on 43% of nights, with an average

Table 6.1 Natural infections of *L. infantum* in *C. thous* in Latin America, and *V. vulpes* in Western Europe.

number of +ves /total (%)	number with clinical signs/ confirmed +ves	region, state, country	source
Cerdocyon thous			
parasitology			
11/26[a] (42)	0/11	Marajó, Pará, Brazil	Silveira *et al.*, 1982; Lainson *et al.*, 1990
3/23[a] (13)	0/3	Belem, Para, Brazil	Lainson *et al.*, 1969, 1987, Lainson and Shaw, 1971
4/33[b] (12)	3/4	Sobral, Ceará, Brazil	Deane and Deane, 1954a, 1955, Deane 1956
1/11 (9)	0/1	Corumba, Mato Grosso, Brazil	Mello *et al.*, 1988
7/173 (4)	0/7	Ceará, Brazil	Alençar, 1959; 1961
0/3 (0)	–	Ceará/Pernambuco, Brazil	Ponde *et al.*, 1942
0/27[b] (0)	–	Jacobina, Bahia, Brazil	Sherlock, 1996
0/50 (0)	–	North Central Venezuela	Torrealba and Torrealba, 1964
serology			
13/25 (52)	0/13	Marajó, Pará, Brazil	Courtenay *et al.*, 1994
Vulpes vulpes			
parasitology			
4/5 (80)	0/4	Setubal region, Portugal	Santos *et al.*, 1996
9/50 (18)	0/9	Imperia, Liguria region, Italy	Mancianti *et al.*, 1994
4/71[a] (6)	0/4	Setubal region, Portugal	Abranches *et al.*, 1982, 1983; 1984
3/64 (5)	0/3	Alhama, Spain	Marin Iniesta *et al.*, 1982
2/99 (2)	1/2	Cevennes, France	Rioux *et al.*, 1968
2/150 (1)	1/2	Cevennes, France	Lanotte, 1975
≥1/ 68[a,c] (–)	0/1	Grosetto, Tuscany, Italy	Bettini *et al.*, 1980; Pozio *et al.*, 1981b
0/169 (0)	–	Alcacer do Sal, Portugal	Abranches *et al.*, 1982, 1983; 1984
0/24 (0)	–	Lisbon region, Portugal	Abranches *et al.*, 1982; 1983
serology			
4/5 (80)	0/4	Setubal region, Portugal	Santos *et al.*, 1996
14/61 (23)	0/14	Setubal region, Portugal	Abranches *et al.*, 1982; 1983; 1984
11/16 (7)	0/11	Priorat, Tarragona, Spain	Saladrigas, 1992
0/22 (0)	–	Alcacer do Sal, Portugal	Abranches *et al.*, 1984
0/7 (0)	–	Lisbon region, Portugal	Abranches *et al.*, 1984

a isolates from 1 *C. thous* (Silveira *et al.*, 1982), and 4 *V. vulpes* (Abranches *et al.*, 1984) typed by isoenzymes as *L. infantum* zymodeme MON–1; 1 isolate from *V. vulpes* (Gramiccia *et al.*, 1982) typed by isoenzymes as *L. infantum* zymodeme variant MON–18; isolates from 4 *C. thous* and 2 *V. vulpes* considered indistinguishable from dog isolates and MON-1 by one or more of the following methods: RFLP, RAPD and partial DNA sequencing of gp63 gene (Mauricio *et al.*, 1998a; 1998b).

b originally cited as *Dusicyon vetulus* (= *Lycalopex vetulus*), but now considered to have been *C. thous* (= *Dusicyon thous*) (Courtenay *et al.*, 1996). To avoid confusion, the nomenclature adopted here follows that described and discussed by Ginsberg and Macdonald (1990).

c the number of positive animals could not be determined, as the author writes: "...a single hamster was inoculated, at different times, with spleen homogenates of 39 foxes...".

peridomestic exposure time of 30 minutes (range: 2–143) per fox per night, the equivalent of 4 hours of fox-prompted link per village per night. Moreover, these studies suggested that the foxes experienced similar infection rates to the dogs from these villages (Courtenay *et al.*, 1994).

Despite the relatively high infection prevalences reported for *C. thous* (up to 42%, **Table 6.1**), none of the studies so far has answered one of the more fundamental epidemiological questions: whether this wildlife host is generally infectious to the vector, and, if so, what is its relative contribution to peridomestic transmission. Deane and Deane (1955) concluded that the two species are equally important in maintaining transmission based on a comparison of the infectiousness of dogs (25% of 238 sandflies), and that of the diseased fox (as described above). While this view is clearly not based on a rigorous sample size, it has nonetheless acquired general acceptance in the literature. The possibility that dogs and foxes do not in fact represent equally effective reservoirs is implied by their apparently different courses of clinical infection: compared with dogs (see Chapter 5), CVL as a disease of foxes is apparently rare, reported in only 3/26 (11.5%) *C. thous*, and 2/25 (8.0%) *V. vulpes*, with confirmed infections **(Table 6.1)**.

6.2.1 Study aims

The two principal aims of this chapter are **(1)** to quantify infectiousness in the fox population in relation to its course of infection and disease, and **(2)** to compare these three epidemiological parameters between foxes and dogs (using data in Chapters 4 & 5) in order to assess their relative contributions to peridomestic transmission. To achieve these aims, longitudinal samples were obtained from a population of free-ranging foxes in Marajó (already described in Chapter 2 & 3), using a proven system of live capture-recapture.

6.3 METHODS

6.3.1 Study population

The study animals comprised 37 free-ranging *C. thous* from a population living within the study area (see Chapter 2). Their spatial distribution relative to the study communities is shown in **Figure 2.1**.

6.3.2 Trapping and sampling regime

Foxes were captured and recaptured on a total of 128 occasions in 5 sample rounds (**Table 6.2a**). To ensure independent sampling, foxes were examined only once per round (at first capture), thus each fox was sampled on 1–4 occasions, giving a total of 74 serological, parasitological and clinical samples collected at a mean interval of 4.3 months (*S.E.* 0.29, range: 1.3–8.9 months). One blood sample obtained in round 5 was lost prior to testing. Xenodiagnosis was performed on 26 (70.3%) of the foxes on 44 occasions, or a median of 2 (range: 1–3) times each (**Table 6.2b**). The mean time interval between consecutive trials was 5.0 months (*S.E.* 0.50, range 3.5–9.0).

6.3.3 Samples

The sampling procedures were as described for dogs in previous chapters: serology and parasitology (Chapter 2), haematology (Chapter 4), clinical examination for signs of CVL (Chapter 4), and xenodiagnosis (Chapter 5). Fox ages were estimated as described in Chapter 3. Exceptions to these accounts are described below.

a) Serology

Note that the fox sera was tested using a rabbit *anti-dog* IgG peroxidase conjugate (Sigma).

b) Parasitology

Bone marrow aspirates were inoculated into culture slopes (capture rounds 1–3), or 2 golden hamsters (rounds 4–5) and used to prepare direct microscopical smears (rounds 1–5).

Table 6.2. Sampling regime of foxes, showing **(a)** the number of animals caught and sampled per capture round, and **(b)** the frequency of samples obtained per animal

(a)

capture round	mid-sample date	trapping period (days)	mean inter-round interval (days)	total captures	number of foxes sampled	number of foxes experimentally exposed to *Lu. longipalpis*
1	29 April 1994	33		31	9	2
2	17 June	23	49	23	12	7
3	15 November	25	151	26	22	16
4	16 March 1995	15	121	32	21	11
5	26 July	11	132	16	10	8
totals				128	37	26

(b)

frequency of samples	number of foxes examined	
	serology[1], parasitology, clinical	xenodiagnosis
1	13	12
2	14	10
3	7	4
4	3	0
total	74	44

[1] one sample lost prior to testing

c) Haematology

Hematocrit (PCV) was measured in round 3 (8 samples). The normal range of PCV for non-endemic captive *C. thous* is 39%–42% (Bennett *et al.*, 1991).

d) Xenodiagnosis

Foxes were anaesthetised for xenodiagnosis. In 28 of the 44 trials animals were placed into individual cages measuring 0.3m x 0.3m x 1m, sheathed in sandfly proof netting. In the other 16 trials only the animal's head was exposed by placing it in a smaller gauze cage (0.2m x 0.2 x 0.2m). In each trial, an average of 92 (*S.E.* 6.5) 2–3 day old adult female *Lu. longipalpis*, and an approximately equal number of males, were introduced

into the cage and allowed to feed for 1–2 hours in darkness (under black hessian). One hour proved sufficient time for all female flies to obtain a bloodmeal. Post exposure, blood-fed flies were treated in the same way as those fed on dogs (Chapter 5). An average of 34 (*S.E.* 2.9) flies from each trial were dissected. Selection of individual animals for (re-)exposure depended on the availability of colony sandflies at the time when foxes were brought to the laboratory for sampling.

6.3.4 Data analysis

a) Serological cut-off titre

None of the methods to select an intrinsic cut-off titre to define positive anti-*Leishmania* antibody sera could be applied to the fox samples (due to reasons described in the Discussion). Alternatively, the two cut-off titres, 2253 and 5500 antibody units (equivalent of 3.35 and 3.74 log units), which best discriminated negative from positive sentinel dog sera were used, representing the intersection of the fitted negative log normal and positive titre distributions, and the mean + 3 *S.D.* of the fitted log negative distribution (described in Quinnell *et al.*, 1997). Estimates of infection parameters were then calculated from the serological data as described below.

b) Incidence and recovery

The instantaneous incidence (λ) (force of infection) was calculated from changes in consecutive antibody titres of individual foxes by

$$\lambda = 1 - \ln\left(1 - \frac{I}{S}\right) / t \qquad \text{(equation 6.1)}$$

where I is the number of foxes among the seronegatives, S, which seroconvert to positive over a period of t months. Instantaneous serorecovery (ρ) was calculated where I is the number of positives which revert to negative, and S is the total number of positives.

A second estimation method for λ and ρ was based on the cross-sectional age-seroprevalence data, calculated by finding the proportion of seropositive foxes $p(a)$, at age a, from

$$p(a) = \frac{\lambda}{\lambda + \rho}\left(1 - e^{-(\lambda+\rho)a}\right) \qquad \text{(equation 6.2)}$$

where $\lambda/(\lambda + \rho)$ is the asymptotic proportion of positive animals. The model was fitted by maximum likelihood and by varying λ and ρ following Williams and Dye (1994). The model assumes that incidence and recovery are constant with age, that the population is homogeneously exposed, and that seroconversion immediately follows exposure. Recovery in this model may indicate a loss of seropositivity with age (time).
A third estimate of incidence was obtained by identifying the proportion of negatives (titres < 2253) which seroconverted, defined as the time at which the titre had increased 4-fold (+ 0.6 log units), following Quinnell *et al.* (1997).

The 95% C.L.s of the parameter estimates obtained by the two longitudinal methods were calculated following Zar (1984), and that for the cross-sectional method as described in Williams and Dye (1994).

6.4 RESULTS

6.4.1 Infection, disease and infectiousness

a) Serology

Seventy-three sera from 37 foxes were tested by ELISA. The frequency distribution of the log unit titres was unimodal (**Figure 6.1a**). Seventy-four percent of the sera had titres ≥ 3.35 log units, while 43.8% exceeded 3.74 log units. These represented 78% (29/37) and 46% (17/37) of the foxes which achieved these titres on one or more occasion. As expected, titres increased with fox age (**Figure 6.2**).

b) Parasite isolation

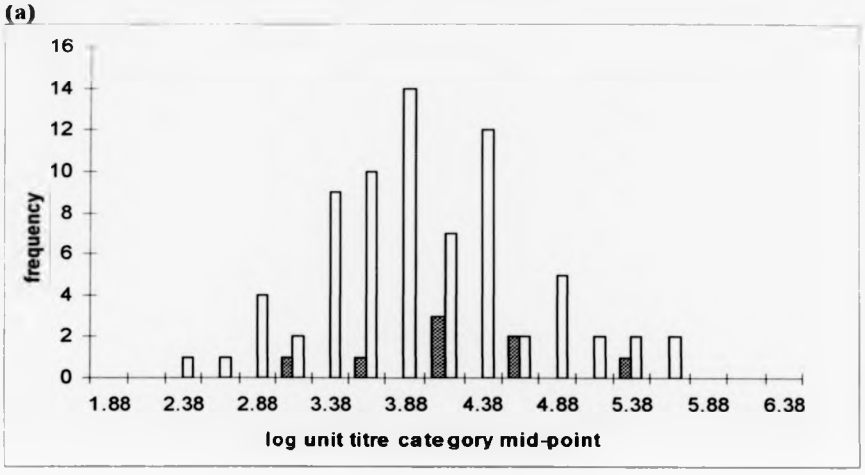
Bone marrow aspirates were obtained on 74 occasions (1–4 times per fox). Of the 31 samples from 21 foxes inoculated into culture slopes or hamsters, 8 (25.8%) were positive, representing 38.1% of the foxes. None of the 74 directly prepared smears was positive.

The 8 positive bone marrows included 6 (30%) of 20 attempts using hamsters (for 15 foxes), and 2/11 (18.2%) using culture (11 foxes). Parasite isolation success did not significantly differ according to the inoculation technique employed (TECH: $z = 0.57$), and asymptoted at approximately 30% between 10–20 months of age (**Figure 6.3**).

Parasite isolation success increased with antibody titre ($z = 3.14$, $P = 0.002$) when controlling for age (=Tf), with all positive samples associated with titres ≥ 3.10 log units (mean 4.24, *S.E.* 0.667, range: 3.19 - 5.32) (**Figure 6.1a**). The sensitivity and specificity of the ELISA to detect parasite positives and negative samples was 88% (7/8) and 30% (7/23) using the lower threshold of 3.35 log units, and 75% (6/8) and 61% (14/23) according to the higher cut-off of 3.74 log units.

Figure 6.1 Frequency distribution of anti-*Leishmania* (ELISA) antibody titres in the population of **(a)** foxes, and **(b)** sentinel dogs. Titres of parasite positive samples are indicated as shaded columns. The line in (b) represents the best logistic fit to the proportion of *Lu. longipalpis* infected in xenodiagnosis trials corresponding to the shown titre at the time of exposure (see **Figure 6.6**).

(a)



(b)

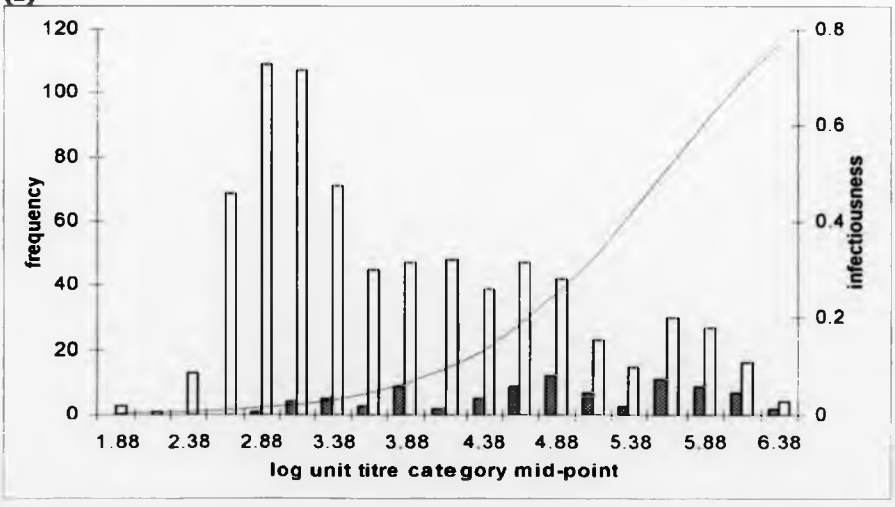


Figure 6.2 Fox anti-*Leishmania* antibody (ELISA) titre relative to age at sampling. Titres of the parasite positive samples are indicated (■)

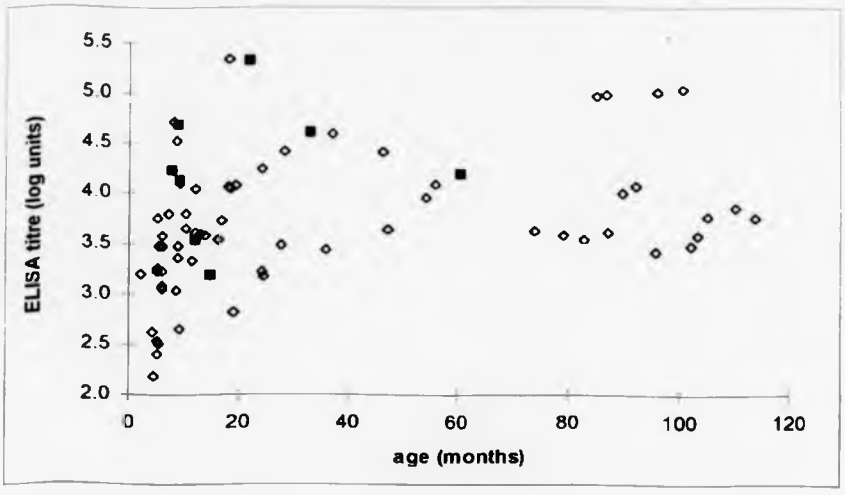
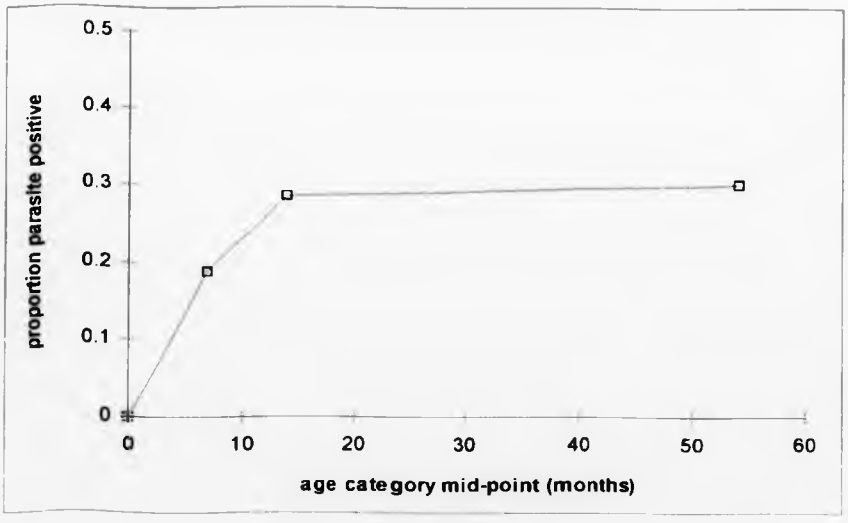


Figure 6.3. Parasite isolation success relative to age at sampling.



c) Xenodiagnosis

Xenodiagnosis was performed on 26 foxes; 12, 10 and 4 individuals were exposed on 1, 2 and 3 occasions each. Thirty-five (81.4%) of the 43 trials for which there were serological data, and 20 (80%) of the foxes in those trials, were performed on animals with log unit titres ≥ 3.35, and 18 (41.9%) had titres ≥ 3.74 (10 foxes). Five of the 8 parasite positive foxes were xenodiagnosed at the time of isolation.

Of the 1,469 sandflies dissected from 44 trials, none was infected (**Table 6.3**).

d) Haematology

Packed cell volume (PCV) values for 8 foxes (measured once per fox) ranged from 38.5 to 51.0 (mean 42.6, *S.E.* 1.44), indicating that none of the sampled foxes was suffering from anaemia. There was no association between PCV and either antibody titre (z = 0.55), or age (z = 0.21). Parasites were not detected in any of these animals, either by culture inoculation (2 samples) or by direct smear (8 samples).

e) Clinical signs

Only one fox (male R26) showed any clinical signs of CVL. On first examination (when 18 m old) the animal showed slight popliteal lymph node enlargement, excessive nail growth on one rear foot, and mild dermatitis on one pinna. *Demodex* or *Sarcoptes* mite infestation (the aetiological agents of mange) was not detected by skin scrape. The animal's log unit titre was 5.34. Four months later, the skin and nail conditions had spontaneously recovered, though slight prescapular and popliteal (but not premaxillar) lymphadenopathy was noted. The animal's titre was similar (5.32 log units), and parasites were isolated by hamster inoculation. The 7.4% reduction in the animal's weight between samples (from 5.4 kg to 5.0 kg) was no greater than the average variation in weight recorded for healthy age-matched foxes (95% C.L.s -9.3–5.5%, n = 12). Its body condition score (of 3) was stable between rounds; PCV was not measured. Xenodiagnosis (dissection of 26 and 49 exposed *Lu. longipalpis*) was negative on both occasions.

Table 6.3. Anti-*leishmania* antibody response, infectiousness, and parasite isolation success, of foxes

serology			xenodiagnosis				parasite isolation success			
							hamster & culture		direct smears	
ELISA titre category (log units)	number of foxes	number of samples	number of foxes	number of trials	number of dissected flies	% positive	number of foxes (% positive)	number of samples (% positive)	number of foxes (% positive)	number of samples (% positive)
1 - 3.35	14	19	7	8	260	0	7 (14.3)	8 (12.5)	14 (0)	19 (0)
3.35 - 3.74	14	22	11	17	453	0	6 (16.7)	8 (12.5)	14 (0)	22 (0)
3.75 - 4.48	12	21	8	12	495	0	8 (37.5)	8 (37.5)	12 (0)	21 (0)
>4.48	6	11	3	6	209	0	4 (75)	6 (50)	6 (0)	11 (0)
untested	1	1	1	1	52	0	1 (0)	1 (0)	1 (0)	1 (0)
total	37	73	26	43	1469	0	21 (38.1)	31 (25.8)	37 (0)	74 (0)

f) Infection incidence and recovery

Consecutive sera samples were obtained for 23 of the foxes on 35 subsequent occasions (**Table 6.4**). Estimates of incidence, λ, and recovery, ρ, were first calculated according to changes in longitudinal titre defined by the two cut-off titres (using equation 6.1), or by a 4-fold increase in titre from < 3.35 log units. Of the 8 foxes with initial titres < 3.35 log units captured and recaptured at a mean interval of 179 days, 6 subsequently exceeded this threshold, giving a force of infection λ = 0.2401/month (**Table 6.5**). Four of these 8 seronegative animals also showed a 4-fold increase in titre during the same period, giving λ = 0.120/month. Applying the higher cut-off titre to these same data, 5 of 15 foxes with initial titres < 3.74 log units seroconverted during an average 186 day interval, giving a third estimate λ = 0.0676/month (**Table 6.5**). None of the seropositive animals, including 16 with titres > 3.35 log units, and 8 with titres > 3.74 log units, observed for 218 and 463 days respectively, dropped below the cut-off values to indicate serorecovery (i.e. ρ = 0). Additional values of λ and ρ were obtained by fitting the cross-sectional age-seroprevalence data to equation 6.2 (**Table 6.5**).

Table 6.4. Anti-*Leishmania* antibodies (ELISA) titres of 23 foxes captured in two or more capture rounds. Data presented as titre log units.

	capture round				
	1	2	3	4	5
sampling interval (days)		49	151	121	132
fox ID					
R18			2.18	4.53	
R22			2.51	4.69	
R27			2.64	4.24	
R12		2.83	3.17		
R10		3.05	3.34	3.19	
R2	3.08	3.04	3.60	3.73	
R24			3.21	3.48	
R19			3.27	3.36	
R9	3.42		3.48		
R21			3.48	3.49	
R31				3.49	3.80
R34				3.54	3.54
R29				3.59	3.66
R8	3.61		4.04		
R14		3.63	3.58	3.54	3.61
R1	3.76	3.80	4.05		
R11		3.77	3.86	3.76	
R5	3.96	4.09	4.20		
R3	4.01	4.08			
R7	4.07	4.08		4.42	
R15		4.60	4.60		4.42
R4	4.97	4.99		5.01	5.05
R26			5.34	5.32	

Table 6.5. Estimates of incidence, λ, and recovery, ρ, in the fox population calculated by 3 criteria based on changes in anti-*Leishmania* antibody titre. Values represent monthly rates (95% C.L.s are shown in parentheses).

	cut-off titre (log units)			
	3.35		3.74	
estimation method	λ	ρ	λ	ρ
longitudinal (equ. 6.1)	0.240 (0.074–0.597)	0 (–)	0.068 (0.021–0.160)	0 (–)
4- fold change in titre	0.120 (0.030–0.321)	0 (–)	–	–
cross-sectional (equ. 6.2)	0.097 (0.060–0.134)	0 (–)	0.055 (0.018–0.092)	0.034 (0.007–0.075)

The age-seroprevalence projections based on the longitudinal titre changes are firstly compared to the cross-sectional data, for threshold seronegative titres < 3.35 log units (**Figure 6.4a**). All the profiles asymptoted at unity though the estimated values of λ varied between 0.097–0.240. The value calculated using equation 6.1 was 2–2.5 times greater than values calculated using either the cross-sectional data or the criterion of a 4-fold titre increase. This is illustrated by the difference in the rising curves in **Figure 6.4a**. None of the 3 methods indicated any serorecovery ($\rho = 0$).

Comparison of the profiles predicted by the higher cut-off titre (**Figure 6.4b**) gave closer estimates of λ at younger ages but diverged substantially at older ages. This is reflected in a recovery rate of ρ = 0.034/month in the cross-sectional profile not observed in the longitudinal data.

There was no loss of seropositivity due to a higher mortality rate, or lower catchability, of seropositive than seronegative animals: although fox mortality was not monitored directly in this study, the number of times that foxes were captured was neither related to their maximum log unit titre (z = 1.04), nor to their age (compared at first capture) (z = 0.66). Clearly, approximately half of the population was insufficiently susceptible to reach the higher (*cf.* lower) cut-off titre. The seroconversion rate was not observed to statistically differ with age (z = 0.30), though there was some evidence that it was higher among younger (≤ 6 months: 4/9) than older animals (> 6 months 1/6).

Additional comparisons were made with previous estimates of λ and ρ for this population (different foxes) examined in 1989–1991, calculated using an IFAT cut-off titre of 1/80 (Courtenay *et al.*, 1994). **Figure 6.5** illustrates the close similarity in both longitudinal (**Figure 6.5a**) and cross-sectional (**Figure 6.5b**) parameter values of that

Figure 6.4 Seroprevalence profiles of the fox population, calculated using cut-off titres of **(a)** 3.35 and **(b)** 3.74 log units, calculated from observed changes in longitudinal titres (—), a 4-fold increase in titre from titres < 3.35 units (– –), and the best fit (----) to the cross-sectional age-seroprevalence data (□). The values of λ and ρ describing these profiles are shown in **Table 6.5**.

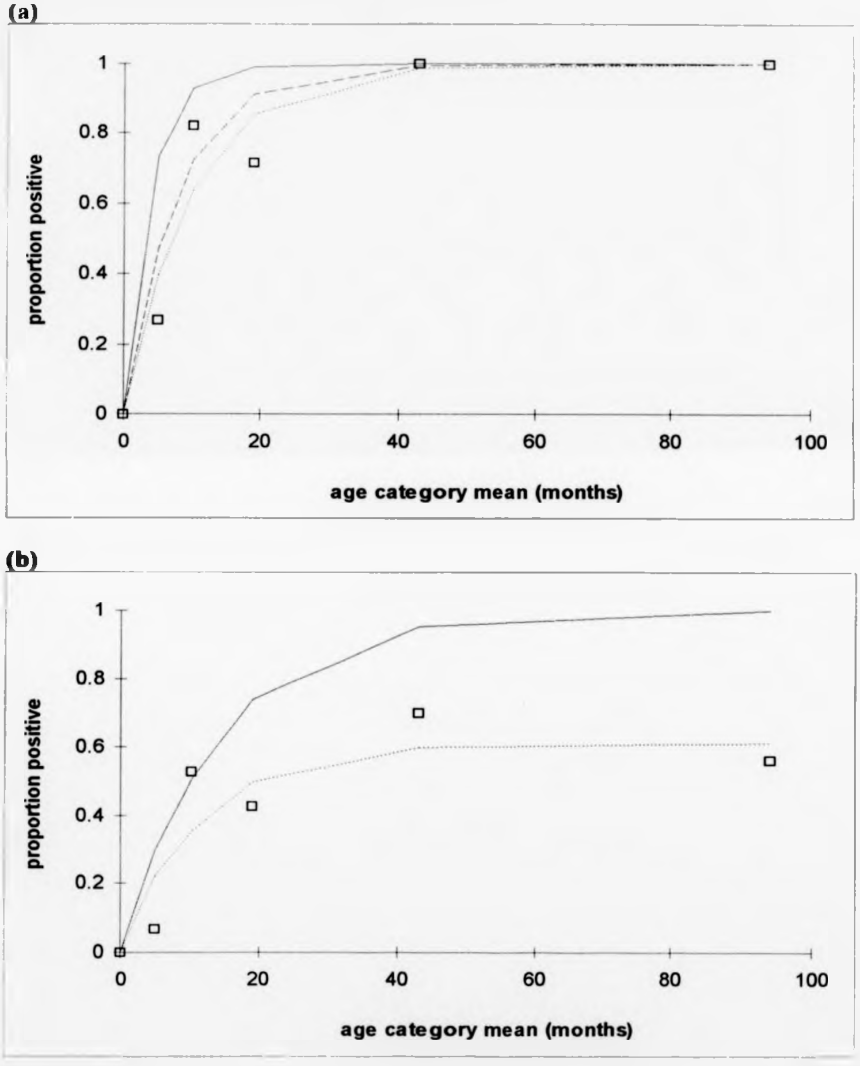
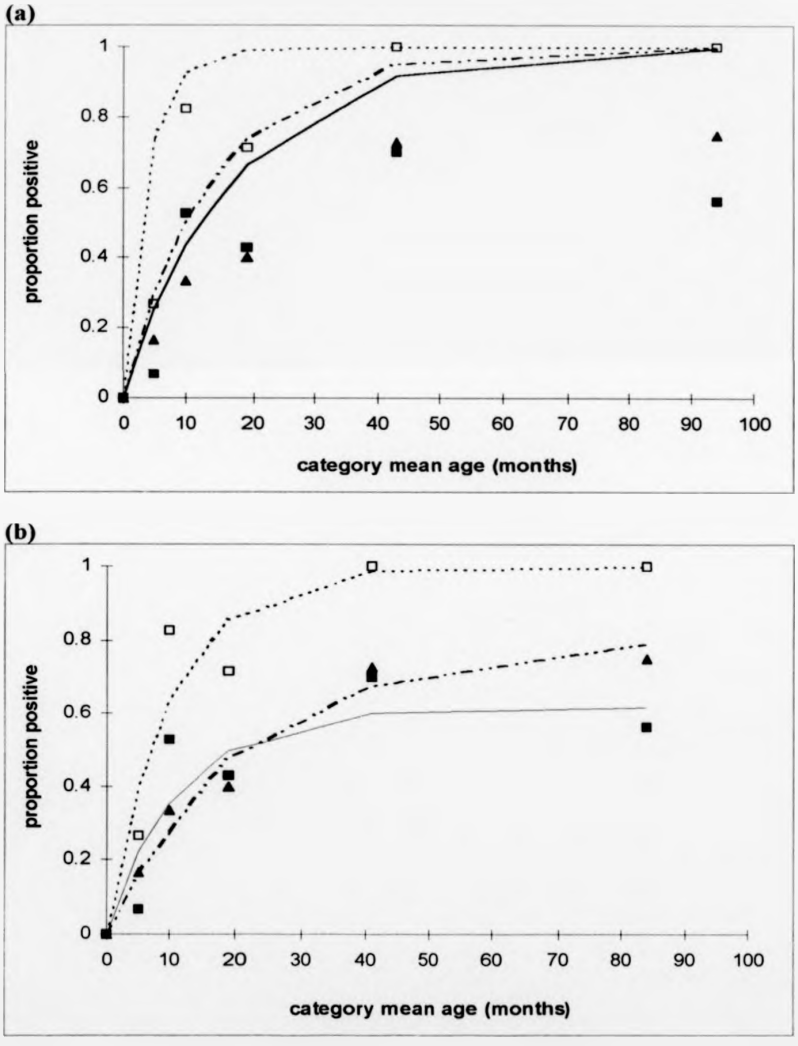


Figure 6.5 Seroprevalence profiles of the fox population, past and present. The infection profiles (lines) calculated from **(a)** the observed changes in longitudinal antibody titre (equation 6.1), and **(b)** the maximum likelihood fit (equation 6.2) to the cross-sectional age-seroprevalence data (symbols). These are compared to the estimates of λ and ρ calculated using the same methods for the 1989–91 sample of foxes (see text). The illustrated profiles are based on ELISA cut-off titres of 3.35 (---, □), and 3.74 (– – –, ■), and IFAT cut-off of 1/80 (—, ▲). The values of λ and ρ for 1994–95 are as in **Figure 6.4**; the 1989–91 estimates are $\lambda = 0.0364$; $\rho = 0.00481$.



study, and those of the present study when applying the higher, not lower, cut-off titre. (The values λ and ρ for the 1988–91 sample are given in the legend of **Figure 6.5**)

Two of the foxes in this study were first captured during 1989–1991. One (R03) was seropositive during an initial 19 months (with IFAT titres 1/80–1/320), and again (ELISA log units titres > 4.0) when captured 37 and 39 months later during the current study. The other fox (R06) was seronegative for 14 months during the original study (IFAT < 1/20), but seropositive (ELISA titre 3.59) when tested 56 months later, at approximately 8 years of age.

6.4.2 Comparisons between fox and dog infection and infectiousness

a) Infection

Allowing for the high frequency of seronegative titres in the sentinel dog population due to the enrolment process (see Chapter 2), the frequency distribution of fox titres was not dissimilar to the dog titres, except for a small extension to right hand tail of the distribution (compare **Figure 6.1a** and **b**).

The probability of parasite isolation success from dogs and foxes with similar antibody titre was not significantly different (test for difference in species intercepts: culture z = 0.71, hamster: z = 1.09; species slopes: culture: z = 1.35; hamster: z = 1.00).

b) Infectiousness

The parasitological, clinical and infectiousness records for dogs and foxes categorised by antibody titre are shown in **Table 6.6**, and the results of the xenodiagnosis trials relative to antibody titre illustrated in **Figure 6.6**. Multivariate comparisons of species infectiousness based on the variances in the response data were not reliable due to the inflated errors attached to the zero outcome for foxes (i.e. proportion of sandflies infected = 0). Alternatively, a more crude set of analyses was performed on the categorised data, stratified by titre response or parasite isolation success. The absolute numbers of dogs and foxes found infectious were not statistically different when including seronegatives (titres < 3.35 log units) (stratified Mantel-Haenszel: χ^2 = 3.01). However, significantly more dogs than foxes were infectious with titres >3.35 log units

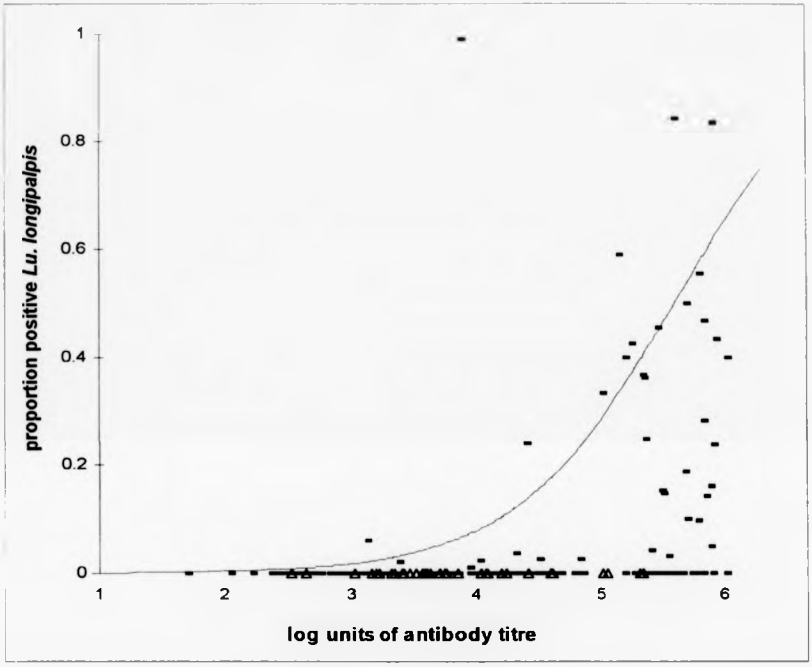
(individuals 18/40 *vs.* 0/20: χ^2 = 12.6, $P < 0.001$; trials 36/67 *vs.* 0/35: χ^2 = 29.1, $P < 0.001$), and with titres > 3.74 log units (individuals 0/10 *vs.* 17/37: Fisher's Exact, $P = 0.008$; trials 0/18 *vs.* 34/102: χ^2 = 8.4, $P < 0.004$). Sandflies were exposed to only the fox's head in a proportion of the xenodiagnosis trials. Comparisons of these data with dogs showed similar differences using either cut-off titre (individuals: χ^2 > 10.6, $P < 0.001$; trials: Fisher's Exact, $P < 0.031$, for both cut-offs). Dogs were also infectious in a greater proportion of parasite positive trials than foxes (17/35 *vs.* 0/5: Fisher's Exact, $P = 0.05$).

Table 6.6 Infection, infectiousness and disease in the fox and sentinel dog populations compared

	serology		parasite isolation[1]		xenodiagnosis			clincial signs	
antibody titre category (log units)	number of individuals (% of total)	number of samples (% of total)	positive individuals (% of total)	positive samples (% of total)	positive animals/*n* (%)	positive trials/*n* (%)	mean % infectiousness (*S.E.*, *n* flies dissected)	% symptomatic individuals (*n* sampled)	% symptomatic examinations (*n* sampled)
foxes									
untested	1 (2.7)	1 (1.4)	- (-)	- (-)	0/1 (0)	0/1 (0)	0 (-, 52)	0 (1)	0 (1)
1 - 3.35	14 (37.8)	19 (25.7)	1 (14.3)	1 (12.5)	0/7 (0)	0/8 (0)	0 (-, 260)	0 (14)	0 (19)
3.36 - 3.74	14 (37.8)	22 (29.7)	1 (16.7)	1 (12.5)	0/11 (0)	0/17 (0)	0 (-, 453)	0 (14)	0 (22)
3.75 - 4.48	12 (32.4)	21 (28.4)	3 (37.5)	3 (37.5)	0/8 (0)	0/12 (0)	0 (-, 495)	0 (12)	0 (21)
> 4.48	6 (16.2)	11 (14.9)	3 (75)	3 (50)	0/3 (0)	0/6 (0)	0 (-, 209)	16.7 (6)	18.2 (11)
total	37 (100)	74 (100)	8 (38.1)	8 (25.8)	0/26 (0)	0/44 (0)	0 (-, 1496)	2.7 (37)	2.7 (74)
dogs									
untested	7 (5.6)	7 (0.9)	- (-)	- (-)	- (-)	- (-)	- (-)	100 (1)	100 (1)
1 - 3.35	126 (100)	392 (51.9)	10 (9.3)	10 (4.2)	1/29 (3.5)	1/57 (1.7)	6.2 (-, 1996)	56.7 (104)	49.3 (205)
3.36 - 3.74	44 (34.9)	73 (9.7)	9 (25.7)	9 (16.1)	1/10 (10)	1/14 (7.7)	2.0 (-, 502)	58.5 (41)	54.2 (68)
3.75 - 4.48	51 (40.5)	129 (17.1)	14 (34.2)	15 (14.6)	5/18 (27.8)	5/35 (14.3)	26.0 (18.7, 920)	72.0 (50)	50.0 (128)
> 4.48	49 (38.9)	162 (21.4)	30 (71.4)	48 (40)	15/28 (53.6)	29/67 (43.3)	26.3 (3.5, 2584)	93.9 (49)	85.0 (160)
total	126 (100)	756 (100)	67 (27.5)	82 (15.9)	18/50 (36)	36/173 (20.8)	25.6 (5.7, 6002)	83.6 (116)	59.9 (561)

[1] from culture or hamster inoculation only

Figure 6.6 Comparative infectiousness of dogs and foxes to *Lu. longipalpis* relative to their anti-*Leishmania* antibody titres at time of exposure. Data points represent individual xenodiagnostic trials for dogs (▬) and foxes (Δ); the best logistic fit to the dog data is shown (—).



6.5 DISCUSSION

Thirty-seven free-ranging foxes were sampled on 74 occasions during 5 independent trapping rounds. The cumulative prevalence of parasite positive animals in the population was 38% (8/37), with point prevalences of 30% through time. These were similar to the equivalent estimates for the sentinel dog population (39% and *ca.* 30–40%, see **Figure 6** in Quinnell *et al.*, 1997). Due to the known insensitivities of these parasitological techniques, detection of anti-*Leishmania* antibody should be a better measure of infection. A common difficulty is the identification of the appropriate threshold titre to define true infection. Two methods are commonly used: **(1)** if the distribution of antibody titres of the endemic canid population is bimodal then the trough between the two peaks is interpreted to be the highest titre of negative responders. The distribution of anti-*Leishmania* antibody titres in endemic populations is rarely bimodal (e.g. Dye *et al.*, 1993; Badaro *et al.*, 1986a; Evans *et al.*, 1990; for exception see Lanotte *et al.*, 1979); that of the fox population was no exception. **(2)** Select a cut-off titre which exceeds those of non-endemic or endemic negative control sera by 2 or 3 *S.D.*s (e.g. Harith *et al.*, 1989). Neither type of control sera was available for foxes in this study. A third and novel method was applied to the sentinel dog sera: **(3)** define the cut-off titre by the truncation point of the lognormal curve fitted to the left hand tail of the distribution of endemic titres (Quinnell *et al.*, 1997). This requires a population of known negatives.

In the absence of an independent method of defining a threshold titre for foxes, the two ELISA cut-offs (3.35 and 3.74 log units) calculated from the sentinel dog data (as described in Chapter 2) were adopted. These yielded similar sensitivities with respect to parasite positive foxes (75% and 88%), sentinel dogs (85%) (Chapter 4; Quinnell *et al.*, 1997). Two immediate objections arise, however, when applying a 'dog-derived' threshold titre to fox sera. First, infected foxes are unlikely to produce the same quantities of detectable antibody to antigenic challenge as dogs. Second, anti-dog conjugates, generally used to test fox sera (Abranches *et al.*, 1983; Saladrigas, 1992; Courtenay *et al.*, 1994), may not adhere as well to fox antibody as they do to dog antibody. In the latter event, the cut-off titre would have been set too high, thereby rendering the test conservative for foxes. That they may in fact respond with similar

amounts of measurable antibody is suggested by the similarities in their detectable IgG at comparable parasite loads (parasite isolation success)

The two test cut-off titres gave infection seroprevalences of 78% (29/37) and 46% (17/37) respectively. The maximum likelihood fit of the incidence-recovery model (equation 6.2) to the cross-sectional data, and the simulated curves from the longitudinal estimates of λ and ρ, indicated that over time all the foxes attain titres of 3.35 log units, whereas only *ca.* 50% of the population exceeded titres of 3.74 log units. Two hypotheses can be offered to explain this. Either titres >3.35 log units are actual infections, and 50% of the population do not mount responses > 3.74 log units, or, only 50% of the population is infected and animals with maximum titres between 3.35 and 3.74 log units are false positives. Whichever of these hypotheses may be true, the cause of the heterogeneity in the population might be due to the fact that **(1)** a proportion of foxes are relatively resistant to infection and therefore do not seroconvert to high titres, or **(2)** all foxes are susceptible but the probability of seroconverting to high titres declines with age. An additional explanation in the case of the second hypothesis could be that **(3)** not all foxes are exposed. The possibility that older animals are more resistant was suggested by a trend of higher seroconversion rates (to >3.74 log units) in younger than older animals, though this could not be rigorously tested due to the small sample sizes.

Certainly the recovery term does not reflect loss of antibody through time, since neither these foxes (8–15 seropositives observed during 2.2–15 months), nor the five animals monitored over a mean of 16 months during the previous study (Courtenay *et al.*, 1994), showed any significant reduction in titre. Indeed, one of the seropositive foxes (R03) from the initial study was also seropositive when recaptured, suggesting that it had retained a positive antibody response for 4.8 years.

Some difference in the longitudinal and cross-sectional incidence estimates is expected due to variation in seasonal transmission (Courtenay *et al.*, 1994; Quinnell *et al.*, 1997). The longitudinal sampling times were biased towards the intermediate to high transmission season (May–December), whereas the cross-sectional data reflect average

(i.e. lower) rates across all seasons. Again, the data were insufficient to calculate separate estimates for different seasons.

The longitudinal data gave estimates of λ between 0.068–0.240 per month according to the criteria selected. The equivalent estimates for dogs and foxes using the incidence-recovery model, while not dissimilar (0.097 *vs.* 0.113 per month), are not expected to be reliable absolute estimates. This is because the model does not account for the pre-patent period (from infection to seroconversion), the penalty of which, as shown for the sentinel population, is to underestimate λ by as much as 50% (Quinnell *et al.*, 1997). The study design did not permit calculation of a pre-patent period for foxes. However, assuming it to be the same in both species, dog and fox infection rates do not appear to widely differ.

Irrespective of the cut-off titre, foxes were clearly less infectious than dogs, as was also true, though more marginally, for parasite positive animals. Xenodiagnosis was performed on 26 foxes on 44 occasions without them infecting any of the sandflies dissected. This is in stark contrast to the infectiousness prevalence of 30–45% observed among dogs (Chapter 5). Only 4 foxes (all xenodiagnosed) had titres > 5.0 log units above which dogs were generally more infectious (see **Figure 6. 6**). Due a lack of sick foxes in this study, it was not possible to confirm whether diseased animals would mount higher titres and/or be more infectious, as shown for dogs (Chapters 4 & 5). CVL as a disease of either New or Old World foxes appears to be rare (see **Table 6.1**). The single clinical case in this study was considered extraordinary since none of the foxes in this region, including 24 seropositive and 14 parasite positive animals from previous studies (see references in **Table 6.1**), showed any signs of clinical abnormality. Intense study of the ecology of this population using radio-telemetry and night vision equipment did not indicate that putative sick foxes avoided capture thus biasing the study sample, nor that foxes with high and negative (IFAT) titres differed significantly in spatial behaviour (Courtenay *et al.*, 1994; Macdonald and Courtenay, 1993; 1996). Difference in species infectiousness cannot be entirely attributed to the respective clinical courses of infection in dogs and foxes, however, since a significant proportion of asymptomatic dogs can be infectious (Chapter 5), and both symptomatic and

asymptomatic foxes have been shown capable of infecting the vector (Deane and Deane, 1954b; Lainson *et al.*, 1990).

The only technical differences between the xenodiagnostic procedures for the two species was the use of anaesthetic in foxes, and the exposure of only their heads in a proportion (36%) of the trials. Comparison of only fox and dog body-exposures did not alter the outcome of these analyses. It seems unlikely that the anaesthetic interfered with amastigote accessibility to probing sandflies in the skin since a number of studies have achieved consistent infections in flies exposed to anaesthetised dogs (Molina *et al.*, 1994a; Killick-Kendrick *et al.*, 1994; Alvar *et al.*, 1994). The mean proportion of fox-fed flies that survived to dissection (0.43, 95% C.L. 0.36–0.51) was no lower than dog-fed flies (0.47, 95% C.L. 0.43–0.51) to suggest that the anaesthetic might have killed all fox-infected flies either.

6.5.1 Conclusion

The results in this chapter suggest that the contribution of *C. thous* to peridomestic transmission is relatively insignificant when compared with that of dogs. Their capacity to infect probing sandflies is low despite the ease with which the parasite can be detected in their bone marrow, viscera, and both healthy and diseased skin (Lainson and Shaw, 1971; Silveira *et al.*, 1982; Deane, 1956; Deane and Deane, 1954a; 1955). In modifying an earlier hypothesis (Lainson *et al.*, 1990), the results also imply that foxes are likely to acquire infection in the peridomestic, rather than the sylvatic, environment. This would be facilitated by the regularity of their nocturnal visits to endemic villages (Macdonald and Courtenay, 1993). Although foxes frequent sylvatic habitats where *Lu. longipalpis* are known to occur (Lainson *et al.*, 1990), there was no indication that contact with such environments increased their probability of infection (Courtenay *et al.*, 1994). Moreover, there is no evidence that dog and fox parasites differ which would indicate two independent transmission cycles: isolates from dogs, *C. thous*, and the proposed European wildlife host, *V. vulpes*, have been considered indistinguishable on the basis of isoenzyme patterns, restriction fragment length polymorphisms (RFLP) of 4 different inter-genic regions, random amplification of polymorphic DNA (RAPD), and by partial DNA sequencing of the gp63 gene (Mauricio *et al.*, 1998a; 1998b). With

the exception of one parasite isolated from a *V. vulpes* in Italy which was identified as MON-18 (Gramiccia *et al*., 1982), all the other 11 fox isolates are considered the same as *L. infantum* MON-1, which is responsible for the human and canine disease throughout the geographical distribution of ZVL. If foxes are infected principally in the vicinity of villages, then successful reduction in peridomestic transmission could simultaneously reduce fox infection rates. Meanwhile, the potential roles of other mammalian hosts, including humans, in transmission have not been fully investigated. This will be considered in the final discussion.

7. GENERAL DISCUSSION

7.1 Overview

Quantitative data on the course of infection, disease and infectiousness in canid reservoir populations naturally infected with *L. infantum* are expected to help identify the merits and flaws of existing (treatment, culling) and future (e.g. anti-*Leishmania* vaccine) options of reservoir control. Data of this quality are not generally available for regions where ZVL is a major public health problem. Clinical, immunological, and infectiousness parameters were therefore measured through time in a population of sentinel dogs and a population of foxes. Additionally, demographic and epidemiological data were collected on the sympatric population of resident dogs.

7.1.1 Dogs

The demographic studies (**Chapter 3**) showed that the resident dog population was stationary and stable, with a high turnover rate driven by the immigration of new dogs acquired by owners in compensation for those which died. The short average life expectancy of both resident and sentinel dogs (≈ 2.5 and 1.5 years, respectively) reflected a high risk of mortality, which was correlated with infection rates (**Chapters 2** and **4**), sandfly abundance (**Chapter 2**), and CVL severity (**Chapter 4**). This led to the conclusion that *Leishmania* is the principal cause of dog mortality in this endemic region. In line with conventional wisdom, few dogs with advanced signs of CVL clinically recovered (**Chapter 4**). This is also generally true for dogs undergoing chemotherapy treatment, whereby the chance of either spontaneous or drug-induced cure is inversely related to the severity of the disease (Mancianti *et al.*, 1988). In this study, dogs which developed enduring progressive conditions were distinguishable from those with more transient clinical outcomes by the magnitude of their antibody (ELISA) titre, in the early phase of the infection (at *ca.* 8 months of age) (**Chapter 4**). This suggests that dogs which would ordinarily progress to advanced, often fatal, CVL could be treated early on in infection, i.e. when asymptomatic or oligosymptomatic. Although this may improve prognosis for individual animals, its usefulness in reducing transmission is unclear. For example, a proportion of these dogs may never mount a protective immune response to avoid relapse or reinfection (Oliva *et al.*, 1995;

Slappendel *et al.*, 1988), and clinically improved cases may still be infectious (Alvar *et al.*, 1994). Prolonged treatment against CVL, on the other hand, can lead to chronic, presumably infectious, conditions (Deplazes *et al.*, 1995), and encourages selection of antimonial-resistant *Leishmania* strains (Gramiccia *et al.*, 1992). As a strategy of ZVL control, therefore, it may be more effective to eliminate severe clinical cases and to restrict treatment to asymptomatic and oligosymptomatic infections. Such an approach using Glucantime® resulted in reductions in canine incidence during a trial conducted in the Isle of Elba (Gradoni *et al.*, 1988), and is reported to reduce successfully human incidence in an ongoing trial in southern Italy (Gradoni, 1998), based on a combined drug regime of antimony and aminosidine (Oliva *et al.*, 1998).

Mass chemotherapy of dogs is possible in Europe where dogs are valued and treatment is sought. In Latin America where treatment is not generally affordable, control of canine infection has relied on mass elimination of seropositive dogs irrespective of clinical condition, (Lacerda, 1994). There no evidence, however, that mass culling in the absence of insecticide spraying reduces canine or human incidence in the long-term (Dietz *et al.*, 1997; Ashford *et al.*, 1998).

Serial xenodiagnosis of the sentinel population (**Chapter 5**) revealed that the onset of infectiousness occurred after (by an average of 4 months), rather than prior to, seroconversion, confirming that seropositivity is an adequate criterion for the selection of infectious dogs. However, only half (45%) of the seropositive population was sufficiently infectious to be detected, and of which only 20% were responsible for 80% of all sandfly infections (**Chapter 5**). These results indicated that ZVL control strategies could be directed at only a proportion of the infected population. Mathematical simulation demonstrated that a regime of targeted culling as opposed to mass culling is theoretically more efficient, due to the mortality-driven dog replacement process already discussed (**Chapter 3**). Furthermore, it was predicted that if a regime of mass culling was implemented under conditions $R_0 = 5.9$ (e.g. in Marajó), the effort required to interrupt transmission (measured as the intervention interval, T_c) would need to be many fold greater than currently practised in Brazil (i.e. $T_c = 1$ *vs.* $T_c = 6$ months) (Lacerda, 1994).

Thus, in an attempt to exploit the heterogeneities in population infectiousness to target control, clinical and immunological parameters were assessed for their potential as surrogate marker(s) of infectiousness (**Chapter 5**). Infectious dogs were shown more likely to respond with high ELISA antibody titres (72–75% of all infectious trials were associated with titres ≥ 5.0 log units), and to present advanced clinical signs (73% of "sick" dogs). However, none of the quantified measures was sufficiently heterogeneous between infectious and uninfectious dogs to differentiate the two groups through time (**Chapter 5**), implying that targeted treatment or elimination is not presently possible using these markers. This does not exclude the possibility that other diagnostic tools, e.g. DNA-based techniques, may be more sensitive and specific than serum antibody. For example, a study of dogs in Marseilles showed that immunoblotting detected *Leishmania* in skin-snips of 6/9 PCR confirmed asymptomatic cases, whereas none of them was positive by IFAT (Berrahal *et al.*, 1996).

7.1.2 Foxes

In comparison with domestic dogs, the transmission potential of the fox population was surprisingly low. None of the sampled foxes was infectious in xenodiagnosis (**Chapter 6**), despite indications that both symptomatic and asymptomatic animals are able to infect the vector (Lainson *et al.*, 1990; Deane and Deane, 1954b). Foxes neither presented clinical signs of CVL nor, in consequence, did they appear to suffer mortality due to *Leishmania* infection, despite a high population turnover rate due to local hunting pressure (**Chapter 3**).

The general absence of the disease in foxes complies with the idea of a non-pathogenic parasite-host relationship in an indigenous wildlife reservoir (Lainson *et al.*, 1987). The *L. infantum* strains identified in the New World are not dissimilar to those in the Old World (see references in Chapter 6), which supports the view that *L. infantum* was introduced into the Americas from the Old World (Momen *et al.*, 1993). Importation might have occurred via an infected domestic dog in post-Colombian times (Killick-Kendrick, 1985), or via a wild canid host (Lainson *et al.*, 1987b), presumably during the radiations of the Canidae in the late Miocene to early Pleistocene period (Martin, 1989; Berta, 1987). This remains entirely speculative.

Given the relatively poor ability of this wildlife host to transmit the parasite, and the low densities at which it occurs in Marajó (range: 0.27–0.77 animals per km^2) (**Chapter 3**), the fox is unlikely to maintain a transmission cycle independent of domestic dogs. Indeed, *Leishmania* strains specific to foxes, or any other wildlife host for that matter (see **Tables 1.1** and **6.1**), have not been identified to suggest the existence of an independent wildlife cycle. Although sylvatic populations of *Lu. longipalpis* have yet to be screened for infection (Lainson *et al.*, 1990), the current results imply that foxes acquire infection in the peridomestic, rather than sylvatic, environment. If this is the case, then interruption to the peridomestic transmission cycle should also reduce fox infection rates. This would also implicate dogs rather than foxes as the more likely route of importation into Latin America.

7.1.3 Summary of implications for reservoir control

The principal implications for public health and reservoir control can be summarised as follows:

(1) identifying infectious dogs for selective treatment or elimination is not feasible using clinical signs or anti-*Leishmania* antibody titre.

(2) indiscriminate culling of seropositive dogs is unsustainable at the levels required to attain substantial reductions in incidence.

(3) early treatment of would-be irreversible / fatal cases based on a calibrated antibody titre is likely to be possible for improving prognosis for valued dogs (thus of interest to the veterinary community), though it is unlikely to reduce population infectiousness.

(4) foxes are not important in the human transmission cycle given the presence of infected (infectious) dogs.

7.2 Further considerations

7.2.1 Additional reservoir hosts

The potential roles of other mammalian hosts for peridomestic transmission need quantifying. The best data for a wildlife species (**Table 1.1**) focuses on opossums. *L. infantum* has been isolated from 32% and 23% (n = 37 and 22) of *D. marsupialis* in two ecologically distinct endemic foci in Colombia (Corredor *et al.*, 1989a; 1989b; Travi *et al.*, 1994), in contrast to only 2% (2/119) of *D. albiventris*, captured in Jacobina, Brazil (Sherlock *et al.*, 1984; Sherlock, 1996). Three of five experimentally inoculated *D. marsupialis*, and one naturally infected *D. albiventris*, have also been shown capable of infecting *Lu. longipalpis* (Travi *et al.*, 1998; Sherlock, 1996). The behavioural characteristics which implicate opossums as better reservoirs than foxes for peridomestic transmission include **(1)** their high density around human dwellings (Corredor *et al.*, 1989a; Sherlock, 1996; Adler *et al.*, 1997), **(2)** their sedentary spatial activity patterns relative to those of foxes (Adler *et al.*, 1997; Macdonald and Courtenay, 1996), and **(3)** their habit of consuming domestic fowl in the aggregation site (chicken huts) of blood-feeding *Lu. longipalpis* (pers. obs.). The average infectiousness of opossums compared to foxes is not, however, dissimilar (**Table 7.1**).

The few available data on human infectiousness are no less intriguing. Deane and Deane (1955) found 16% (7/43) of symptomatic patients with parasitized skin, and showed that 4 (29%) of 14 cases infected 15% of 81 *Lu. longipalpis* fed on them. A similar proportion of *Lu. longipalpis* (32/201) were infected by 2/6 clinical patients according to Sherlock (1996). Subclinically infected patients may also be infectious, though their general status is not known (Sherlock, 1996). The possible involvement of man in transmission is further suggested by the epidemiologic clustering of leishmanin-positive cases in China (25% in infected households *vs.* 3.8% in non-infected households) (Ho *et al.*, 1982), and associations between active and prior infection among households in north-east Brazil (Evans *et al.*, 1992; Cabello *et al.*, 1995), both of which follow patterns of anthroponotic Indian kala-azar due to *L. donovani s.s.* (Nandy *et al.*, 1987). These patterns, however, may be the result of shared infection risk rather than reflect a causal relationship.

Table 7.1 Xenodiagnosis of mammalian hosts of anthroponotic and zoonotic visceral leishmaniasis. The sandfly species used in these experiments were the proven vectors *Lu. longipalpis* (*L. infantum*) and *P. argentipes* (*L. donovani, s.s.*), unless otherwise indicated. Total sandflies dissected represent those exposed on infectious hosts, identified as infectious in at least one trial[a].

number infectious individuals[a] (no. infectious trials /total)	total infected flies	total flies dissected	% infected	clinical description	source
Anthroponotic (*L. donovani s.s.*)					
H. sapiens[b]					
4 (nd/8)	32	60	53.3	PKDL	Addy & Nandy, 1992
1 (nd/2)	4	8	50.0[c]	PKDL	Napier *et al.*, 1933
1 (nd/2)	4	28	14.3[d]	PKDL	Napier *et al.*, 1933
1 (nd/2)	8	57	14.0[c]	PKDL	Napier *et al.*, 1933
1 (nd/2)	1	20	5.0	PKDL	Napier *et al.*, 1933
>1 (nd)	205	236	86.9	kala-azar	Shortt *et al.*, 1931
>1 (nd)	43	102	42.2	kala-azar	Napier & Smith, 1927
3 (nd/6)	5	13	38.5	kala-azar	Christophers *et al.*, 1925
>1 (nd)	152	587	25.9	kala-azar	Shortt *et al.*, 1927
>1 (nd)	76	403	18.9	kala-azar	Smith *et al.*, 1940
5 (nd/10)	5	258	1.9	kala-azar	Mukhopadhyay & Mishra, 1991
total	535	1772	30.2		
unweighted mean			31.9		
median			25.9		
Zoonotic (*L. infantum*)					
H. sapiens					
2 (2/6)	32	201	15.9	sym	Sherlock, 1996
4/14[a] (4/14)	12	81	14.8	sym	Deane & Deane, 1955
total	44	282	15.6		
unweighted mean			15.4		
median			15.4		
C. familiaris					
2 (2/2)	94	134	70.1	sym	Gradoni *et al.*, 1987
1 (-)	33	49	67.3[e]	sym	Vexenat *et al.*, 1994
1 (nd/>1)	17	26	65.4	sym	Adler & Theodor, 1932
1 (nd/>1)	265	424	62.5	sym	Adler & Theodor, 1932
13/16[a] (13/16)	149	404	36.9	sym/asym	Molina *et al.*, 1994a
5 (nd/2)	61	167	36.5[f]	sym	Vexenat *et al.*, 1994
1 (nd/>1)	11	34	32.4	sym	Adler & Theodor, 1932
8 (13/20)	107	368	29.1	nd	Sherlock, 1996
18/40[a] (36/173)	501	2609	19.2	sym/asym	this study
12/16[a] (12/16)	59	238	24.8	sym/asym	Deane & Deane, 1955
20 (-)	2	16	12.5[e]	sym/asym	Vexenat *et al.*, 1994
1 (1/1)	4	53	7.5	sym	Parrot *et al.*, 1930
1 (1/1)	61	73	83.6	sym (treat)	Rioux *et al.*, 1972
1 (1/1)	72	89	80.9	sym (treat)	Rioux *et al.*, 1972

Table 7.1 *continued...*

number infectious individuals[a] (no. infectious trials /total)	total infected flies	total flies dissected	% infected	clinical description	source
1 (1/1)	44	61	72.1	sym (treat)	Rioux *et al.*, 1972
1 (3/3)	60	198	30.3	sym (treat)	Gradoni *et al.*, 1987
1 (3/3)	28	157	17.8	sym (treat)	Gradoni *et al.*, 1987
1 (1/1)	7	63	11.1	sym (treat)	Rioux *et al.*, 1972
1 (3/4)	nd	nd	8.0[g]	sym (treat)	Alvar *et al.*, 1994
1 (1/4)	nd	nd	4.0[g]	asym (treat)	Alvar *et al.*, 1994
1 (3/3)	48	73	65.8[f]	sym (exp)	Vexenat *et al.*, 1994
1 (3/3)	47	120	39.2	sym (exp)	Adler & Theodor, 1935
1 (2/3)	35	130	26.9	sym (exp)	Adler & Theodor, 1935
total	1712	5549	30.9		
unweighted mean			38.1		
median			31.3		
C. thous					
1 (1/1)	10	10	100.0	sym	Deane & Deane, 1955
0/26[e] (0/44)[h]	0	1469	0.0	asym	this study
1 (2/3)	6	186	3.2	asym (exp)	Lainson *et al.*, 1990
total	16	1665	1.0		
unweighted mean			34.4		
median			3.2		
Didelphis spp.					
1 (2/8)	27	193	14.0	asym	Sherlock, 1996
1 (1/1)	5	52	9.6	sym (exp)	Travi *et al.*, 1998
1 (1/3)	2	135	1.5	asym (exp)	Travi *et al.*, 1998
1 (1/3)	1	125	0.8	asym (exp)	Travi *et al.*, 1998
total	35	505	6.9		
unweighted mean			6.5		
median			5.5		
R. rattus					
2[j] (1/1)	17	73	23.3	asym (exp)	Gradoni *et al.*, 1983
2[j] (1/1)	3	92	3.3	asym (exp)	Gradoni *et al.*, 1983
total	20	165	12.1		
unweighted mean			13.3		
median			13.3		

a where indicated, data represent number of infectious hosts / total number of hosts exposed.
b patients selected on basis of high parasitaemia, in most cases; flies usually exposed twice to subject, once before, once after oviposition.
c flies restricted to feed on depigmented skin only.
d flies restricted to feed on nodules only.
e flies caught by CDC light-trap set in kennel housing the dogs indicated.
f flies restricted to feed on lesion only.
g mean proportions of flies infected estimated from Figure presented in source.
h data represent all trials on all foxes.
i exposed to *P. perniciosus*.
j same rats as for (i) exposed to *P. perfiliewi*.
Abbreviations: PKDL, post kala-azar dermal leishmaniasis; sym, symptomatic; asym asymptomatic; treat, receiving chemotherapy treatment during or soon prior to xenodiagnosis; exp, experimental infection; nd, data not provided by source.

When comparing the crude average infectiousness of hosts (**Table 7.1**), human *L. infantum* cases appear to be only half as infectious as either kala-azar or post-kala-zar dermal leishmaniasis (PKDL) patients (15–16% *vs.* 26–32%), or dogs infected with *L.* infantum (31–38%), whereas they are more infectious than either of the wildlife hosts of *L. infantum* (foxes and opossums) for which data are available (**Table 7.1**). These data indicate than man is more than simply an incidental host for *L. infantum*. According to the original data sources, the majority of *L. donovani* xenodiagnosis experiments were conducted on patients selected for high parasitaemia and therefore the values probably represent upper estimates. Nevertheless, they are similar to the average values for dogs, the principal reservoir of zoonotic VL. Furthermore, almost all of the *L. donovani* cases had received or were receiving antimony treatment. In the context of the discussions above, it is interesting that the average infectiousness of treated and untreated dogs was not different (24–42% *vs.* 29–37%), although it was slightly higher in experimentally than naturally infected dogs (39–44% *vs.* 30–37%). The role of *R. rattus* in transmission has not received much attention. Despite an apparently high level of infectiousness in experimentally infected animals (**Table 7.1**), the prevalence of infection in natural populations is relatively low (**Table 1.1**).

A more substantial role for anthroponotic transmission of *L. infantum* has developed with the changing patterns of *Leishmania* epidemiology, particularly in southern Europe, where distinct strains of *L. infantum* have been associated with HIV co-infected patients (Alvar *et al.*, 1997, and references therein). This strongly suggests the existence of a transmission cycle where the zoonotic reservoir is excluded and, in certain circumstances, where the vector is replaced by shared syringes (Molina *et al.*, 1994b; Alvar *et al.*, 1997). Molina *et al.* (1994b) infected sandflies indirectly on the blood of 10/10 VL/HIV co-infected patients, and a further 6/6 by direct xenodiagnosis (Molina *et al.*, unpublished, in Alvar *et al.*, 1997). The unweighted average and median proportions of sandflies infected during the former experiments were 36% and 30% (range 8–93%), which were not dissimilar to the estimates for *L. donovani* patients (**Table 7.1**). The densities of infectious co-infected patients are not likely to reach that of infectious dogs, but their role in transmission in specific foci may still be of epidemiological significance.

7.2.2 Strategies of control

The results of this thesis go part way towards explaining the ineffectiveness of mass elimination of dogs. This adds to the rational (Tesh, 1995) and quantitative (Dye, 1996) arguments for shifting the emphasis of ZVL control away from blanket culling towards an integrated approach based on sustainable methodology. In modelling the potential impact of alternative control methods (drug treatment, a future anti-*Leishmania* canine vaccine, and insecticide), Dye (1996) demonstrated that chemotherapy should perform better than culling if it induces long-term cure and immunity. A prophylactic vaccine would be advantageous since it would be administered prior to infection and thus the onset of infectiousness (unlike case-indicated treatment, and possibly culling – see discussion above). It would also substantially reduce dog mortality due to *Leishmania* and thus the immigration rate of susceptibles. A vaccine is likely to have delivery problems however; whether incentives would be effective in encouraging dog owners to visit vaccination posts would need to be investigated.

On theoretical grounds residual insecticides should be the best of these options due to the non-linear ways in which killing sandflies affects components of vectorial capacity (Garret-Jones and Grab, 1964; Dye, 1994; 1996). Intervention using residual insecticides has been successful in reducing sandfly abundance in the past (see Chapter 1) though it clearly needs sustained application to have any impact in the long-term. Recent studies on the behaviour of *Lu. longipalpis* stress the importance of thorough insecticide coverage of both houses and animal pens (the latter being the main aggregation site for *Lu. longipalpis* in the peridomestic environment in Brazil): it has been suggested that disruption (i.e. killing or repelling) of pheromone-producing males, which are the primary attractant of blood-seeking females to susceptible hosts, in theory could divert flies to houses and dining huts, thus increasing rather than decreasing the human biting rate (Dye *et al.*, 1991; Kelly and Dye, 1997; Kelly *et al.*, 1997).

A novel method of applying insecticide in the form of plastic deltamethrin-impregnated collars was recently shown to provide protection against 94% of the bites of *P. perniciosus*, resulting in 21–60% fly mortality (compared to 0–12% in controls), with biological activity persisting for up to 34 weeks (Killick-Kendrick *et al.*, 1997). The

Chinese version of this idea has been to dip the entire dog in insecticide (an unspecified quantity of water containing 25mg of deltamethrin) resulting in a 61.5% reduction in dog attractiveness to the vector, *P. chinensis*, relative to 2.8% in controls (Xiong *et al.*, 1995). Reducing transmission in this way has, like a vaccine, the advantage over culling of reducing canine infection and related mortality.

Protecting people at risk using insecticide is also in its infancy, with tested options including impregnated bednets (Alexander *et al.*, 1995a; Oliveira-Filho and Melo, 1994), curtains (Maroli and Majori, 1991; Alexander *et al.*, 1995a), and (mud) house bricks (Oliveira-Filho *et al.*, 1995). Improving house construction to physically reduce sandfly access is also indicated (Kumar, 1995; Quinnell and Dye, 1994a). The continued use of insecticide, however, is not without potential problems. Insect resistance to DDT and pyrethroids has increased (WHO, 1992; Curtis *et al.*, 1998), and is now known to exist in a growing number of peridomestic populations of *P. papatasi* and *P. argentipes* in the Old World (Joshi *et al.*, 1979; Kaul *et al.*, 1978; Das-Gupta *et al.*, 1995; Mukhopadhyay *et al.*, 1990; 1996).

In reality, leishmaniasis control in Brazil is of low priority relative to other health issues. It is therefore likely that a sustainable cost effective control scenario would form part of a broader national health package.

7.3 Future research

Suggestions for future research leading from these discussions include:

7.3.1 Diagnostics

(1) quantify the sensitivity and specificity of alternative surrogate markers of infectiousness such as parasite detection by DNA-based techniques (e.g. PCR) in skin-snips, and detection of anti-*Leishmania* antibodies using the cloned antigen recombinant rK39 antigen (Houghton *et al.*, 1998; Badaro *et al.*, 1996; Ozensoy *et al.*, 1998). The latter would be less expensive for large scale use. The ultimate aim would be to target control.

7.3.2 Epidemiology

(2) perform xenodiagnosis studies to define the contribution of humans and *Didelphis* to peridomestic transmission relative to that of dogs. For humans this can be facilitated by exposure of flies to pre-collected blood samples of ZVL cases (Molina and Alvar, 1996). *D. marsupialis* is ubiquitous in Marajó and could be easily captured for serial sampling.

(3) calculate the critical threshold of reservoir population density for parasite persistence to further explore the relative importance of domestic and wildlife hosts. Models already exist for two reservoir systems (e.g. for *Trypanosomiasis*, Rodgers, 1988).

(4) calculate the appropriate intervention-intervals for different integrated approaches of control, and match these data with cost-effectiveness studies. Integrated measures might include a combination of:

(a) insecticide application to houses and animal pens,

(b) protection of dogs using impregnated dog collars or pour-ons,

(c) education to persuade people to use impregnated-bednets or curtains,

(e) education to improve house construction techniques,

(d) improve child nutritional health to lower the risk of disease progression (Dye and Williams, 1993).

7.3.3 Implementation

(5) conduct intervention trials based on the quantitative predictions of **(1)**–**(4)** above

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APPENDIX I

Questionário- Marajó

Data

Codigo da casa
Nome da família

número pessoas
número cachorros

1. humanos

Quantas pessoas moram na casa?

<15 anos

Morava na casa no último

a) Sim
b) Não

b) Não
Onde morava antes?
Data que chegou na casa

2. Cachorro

cachorro e novo para esse estudo?

a) Sim
b) Não

a) Sim
Data que chegou na casa
Data de Nascimento
Local

História passada

sexo

M
F

idade (m) ☐

b) Não

Codigo do Cachorro ☐

Se ainda permanece nesta mesma casa? a) Sim ☐
b) Não ☐

b) Se morreu? Sim ☐
Não ☐

Como morreu? ☐

Data morreu ☐

b) Se mudou de endereco? Sim ☐
Não ☐

Novo local ☐

data mudou ☐

b) Se sumiu? Sim ☐
Não ☐

data sumiu ☐

3. Serologia

Numeros de amostras ☐

Se foi coletada a amostra no ultimo Sim ☐
Não ☐

Codigo de amostra ☐

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outras informações

APPENDIX II

GUIDELINES FOR AGE ESTIMATION BY TOOTH WEAR AND ERUPTION

Dental formula

Dog	
Deciduous teeth	$DI_{3/3}$ $DC_{1/1}$ $DP_{3/3}$ = 28
Permenant teeth	$I_{3/3}$ $C_{1/1}$ $P_{4/4}$ $M_{2/3}$ = 42
Fox	
Deciduous teeth	$DI_{3/3}$ $DC_{1/1}$ $DP_{3/3}$ = 28
Permanent teeth	$I_{3/3}$ $C_{1/1}$ $P_{4/3}$ $M_{3/4}$ = 44

Tooth eruption schedule

3-4 weeks	temporary canines $DC_{1/1}$
4-5 weeks	temporary incisors $DI_{3/3}$ first 3 temporary cheek teeth $DP_{3/3}$
4 months	permanent premolar $P^{1}/_{1}$ (appears with the deciduous dentition) then fourth cheek tooth $P^{4*}/_{4}$
5-6 months	permanent canines $C^{1}/_{1}$ corner incisors $I^{3}/_{3}$ first 3 cheek teeth $P^{3}/_{3}$ then 5th cheek tooth $M^{1}/_{1*}$
6-8 months	6th cheek tooth (often absent in superior mandible)

*carnassials/sectorial teeth (P^{4} and M_{1})

***Tooth-wear** (depending on diet)*

1 yr	incisors in wear but retain the triple crown ("fleur-de-lys")
2 yr	triple crown wearing off or disappeared