# Molecular and Cellular Biology of *Leishmania* Development

Thesis submitted in accordance with the requirements of The University of Liverpool
for the degree of Doctor of Philosophy

By

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*If I have seen further it is by standing on the shoulders of giants.*

*Isaac Newton, 1675*
*(Letter to Robert Hooke)*

*Do not spoil what you have by desiring what you have not; but remember that what you now have was once among the things only hoped for.*

*Epicurus (341BC – 270BC)*

# Contents

## Chapter 2 Literature Review

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| aPPG | Amastigote proteophosphoglycan |
| α-Ara | Alpha arabinose |
| β-Gal | Beta galactose |
| β-Glc | Beta glucose |
| BME | Basal Medium Eagle |
| CL | Cutaneous leishmaniasis |
| cDNA | complementary deoxyribonucleic acid |
| CR | Complement receptor |
| CSPD | 3-(4-methoxyspiro{1,2-dioxetane-3-2'-(5'-chloro)tricycle [3.3.1.1$^{3.7}$]decan}-4-y1)phenyl phosphate |
| DCL | Diffuse cutaneous leishmaniasis |
| DEPC | Diethylpyrocarbonate |
| DIG | Digoxigenin |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| FBS | Foetal bovine serum |
| fPPG | Filamentous proteophosphoglycan |
| gDNA | Guide DNA |
| gp63 | Glycoprotein 63 (Leishmanolysin) |
| GPI | Glycosylphosphatidylinositol |

| | |
|---|---|
| HIV | Human Immunodeficiency virus |
| IFN-γ | Interferon gamma |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| kDNA | Kinetoplast DNA |
| *L.* | *Leishmania* |
| LPG | Lipophosphoglycan |
| *Lu.* | *Lutzomyia* |
| MAC | Membrane attack complex |
| MCL | Mucocutaneous leishmaniasis |
| MHC | Major histocompatibilty complex |
| mPPG | Membrane proteophosphoglycan |
| mRNA | Messenger ribonucleic acid |
| NO | Nitric oxide |
| NRAMP | Natural resistance associated membrane protein |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PG | Phosphoglycan |
| *Ph.* | *Phlebotomus* |
| PKC | Protein kinase C |
| PKDL | Post Kala-azar dermal leishmaniasis |
| PM | Peritrophic matrix |
| PPG | Proteophosphoglycan |

| | |
|---|---|
| pPPG | Promastigote proteophosphoglycan |
| PSG | Promastigote secretory gel |
| RBC | Red blood cell |
| RNA | Ribonucleic acid |
| SAP | Secretory acid phosphatase |
| SDS | Sodium dodecyl sulphate |
| Ser | Serine |
| SGC | Salivary gland content |
| SGL | Salivary gland lysate |
| SL RNA | Splice leader RNA |
| SGS | Salivary gland sonicate |
| SOC | Super optimal catabolite |
| SSC | Standard sodium citrate |
| Th | T helper cells |
| Thr | Threonine |
| UTR | Untranslated region |
| *V.* | *Vianna* |
| VL | Visceral leishmaniasis |
| WHO | World Health Organisation |

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I would like to extend my thanks to my supervisor, Dr. Paul Bates. Thank you for giving me the opportunity to study in your laboratory on an organism I already had a great interest in. Your guidance and support were fundamental throughout this study. Thank you for your patience and understanding during some of the difficult times.

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# Abstract

*Leishmania* species are protozoan parasites responsible for a spectrum of diseases, collectively termed the leishmaniases. Infection of humans results from the infective bite of a female phlebotomine sandfly. The life cycle of *Leishmania* involves the alternation between two major morphological forms. In the mammalian host the parasite exists as intracellular amastigotes, whereas in the sand fly vector they are found as extracellular promastigotes. There have been several forms of promastigote described in the sandfly, the most well known of these being metacyclic promastigotes, the mammalian infective stage.

For effective transmission to occur metacyclic promastigotes must be generated in sufficient numbers. This study provides evidence for two separate growth cycles during the development of *L. infantum* in *Lutzomyia longipalpis* sandflies, and *L. infantum* and *L. major* in cultures *in vitro*. Two independent cycles of multiplication were observed involving procyclic promastigotes and leptomonad promastigotes. The procyclic promastigotes are responsible for the expansion of the parasite population early in infection within the bloodmeal. A second round of division by leptomonad promastigotes, within the sugar meal phase, again expands the parasite population. This second round of division ensures the generation of sufficient numbers of leptomonad promastigotes, the precursors of infective metacyclic promastigotes. The two divisional cycles are linked together by a non-dividing form, nectomonad promastigotes. Taking these data into account a new revised *Leishmania* life cycle has been proposed.

A precursor-product relationship exists between the four major promastigote forms, whereby procyclic promastigotes differentiate into nectomonad promastigotes, which then transform in leptomonad promastigotes, which eventually differentiate into metacyclic promastigotes. Gene expression of several membrane and secreted proteins were analysed to determine a genetic difference between the various life cycle stages. Data is presented that shows each of the four major promastigote forms are not only morphologically distinct but also genetically distinct. These data indicate that each of the promastigote forms will have specific roles in the life cycle of *Leishmania* during development within the sandfly gut.

A common feature of *Leishmania*-infected sandflies is the formation of promastigote secretory gel (PSG) at the thoracic midgut and cardia regions. PSG is known to be of parasite origin and to be co-injected along with metacyclic promastigotes during blood feeding. *In vitro* macrophage infections using *L. mexicana* showed an exacerbation of infection in the presence of PSG. PSG did not increase uptake of parasites by macrophages but exacerbated infection by enhancing parasite survival in IFN-γ activated macrophages. Exacerbation of infection due to sandfly saliva was also observed but to a much lesser degree. The combination of both PSG and saliva gave an intermediate level of exacerbation.

# Chapter 1

# Introduction

Leishman (1903) first reported the discovery of "small round or oval bodies, 2-3μm in diameter", in 1900, from the post-mortem spleen of a young British soldier, who was stationed in Dum Dum, India. He suggested it was most likely that these bodies were degenerative forms of *Trypanosoma* following the death of the host. Donovan (1903) published similar findings from the enlarged spleen of a 12 year old boy in India. He also found these bodies in the splenic blood, indicating that they were not a consequence of host death. Following the above publications the genus *Leishmania* was created by Ross (1903) and the specific organism in question given the name *Leishmania donovani*.

The leishmaniases are a globally widespread spectrum of diseases caused by parasitic protozoa belonging to the order Kinetoplastida, family Trypanosomatidae and genus *Leishmania*. Most leishmaniases are zoonotic and humans become infected when accidentally exposed to the natural transmission cycle. However, there are anthroponotic forms of the disease where humans are the sole reservoir host. The following account of leishmaniasis disease, distribution, diagnosis, treatment and control is based on that of Ashford and Bates (1998), Bates (2001), Ashford (2000), and Markell *et al.* (1999).

## 1.1 Clinical Disease

Leishmaniasis infection can present itself in various forms with a range of clinical manifestations. This diversity of disease is a result of various factors including the species of infecting parasite, the host's response to infection and the restriction of parasites to specific organs of the body.

### 1.1.1 Cutaneous Leishmaniasis

Cutaneous leishmaniasis can result from infection with several species of *Leishmania*. Oriental sore, as it is known in the Old World, varies in its duration and severity depending upon the species involved. Usually it is painless and self-curing. A small papule at the site of the fly bite is the first sign of infection, the circumference of which gradually enlarges.

Infection with *Leishmania tropica* (Figure 1.1a) produces a chronic disease that can last for years without treatment. Infection is characterised by the production of a large, dry lesion that ulcerates only after several months. They are usually single lesions and occur primarily on the face. Infection with *L. aethiopica* presents very similarly to that of *L. tropica*. Lesions resulting from infection with *L. major* (Figure 1.1b) are often numerous and the centre of the wet lesion becomes quickly necrotic. These lesions normally self-cure in approximately 3 to 6 months and leave the individual with a fibrotic scar. Certain strains of *L. infantum*, a species normally associated with visceral leishmaniasis can give rise to cutaneous disease. This is also true for *L. donovani* in very rare cases.

In some infections involving *L. amazonensis* or *L. aethiopica* the cutaneous lesions do not heal but proliferate indefinitely, disseminating over a large area of the skin. Sometimes the entire body can become covered in severely disfiguring raised nodules and plaques. This condition is termed diffuse cutaneous leishmaniasis (Figure 1.1c) and is associated with a specific immunological anergy. The condition is difficult to treat successfully and often remains as a chronic infection for life.

Leishmaniasis recidivans is an unusual sequel to cutaneous leishmaniasis caused by *L. tropica* infection. The condition is characterised by small lesions that appear at the margins of a healed scar of oriental sore. The lesions are usually non-ulcerating and can last indefinitely. This condition resembles cutaneous tuberculosis and is associated with a poor cell-mediated immune response from the host.

In some cases cutaneous leishmaniasis can lead to permanent destruction of the host's tissue. When *L. mexicana* infection occurs in the ear (Chiclero's ulcer) the cartilage is invaded and the pinna gradually eroded away (Figure 1.1d). However the most serious destruction occurs as a result of *L. braziliensis* infection. Here a proportion of patients develop mucocutaneous disease following resolution of the primary lesion, sometimes several years later. Frequently the mucocutaneous lesions develop by metastasis to the mucosae of the palate and nasal area (Figure 1.2a). If left untreated the lesions gradually erode away the mucosa and underlying tissue, including cartilage, leading to gross disfigurement (Figure 1.2b). Advanced cases are difficult to treat and are often fatal.

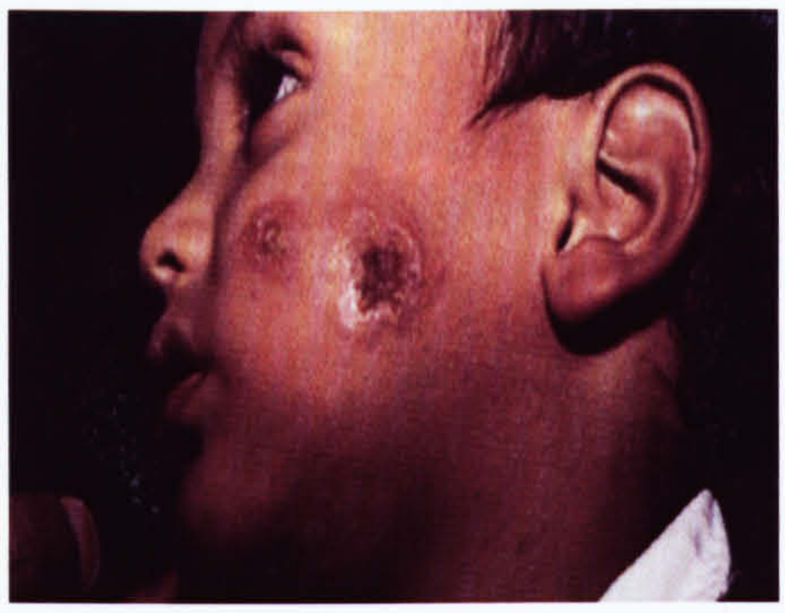


**Figure 1.1a** Dry lesion of *L. tropica*.

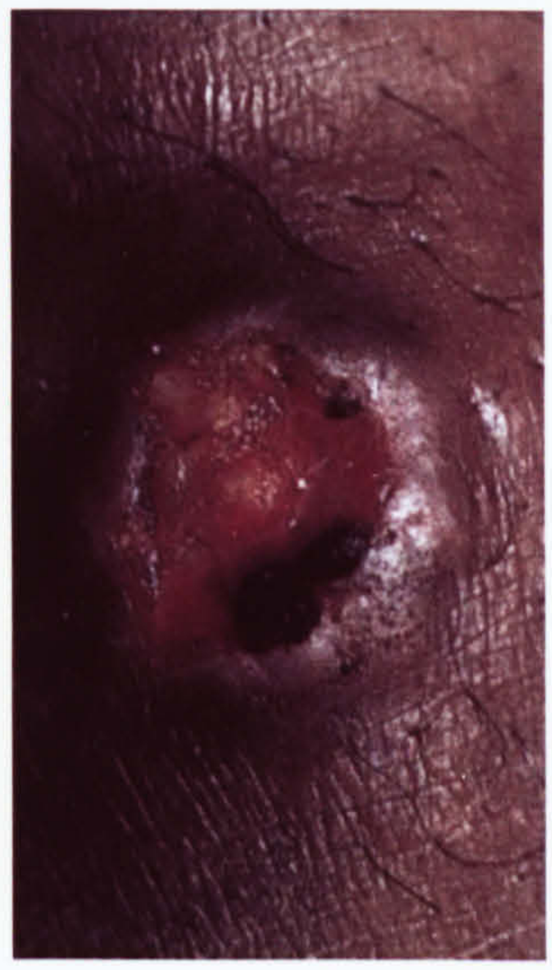


**Figure 1.1b** Nodulo-ulcerative lesion of *L. major*

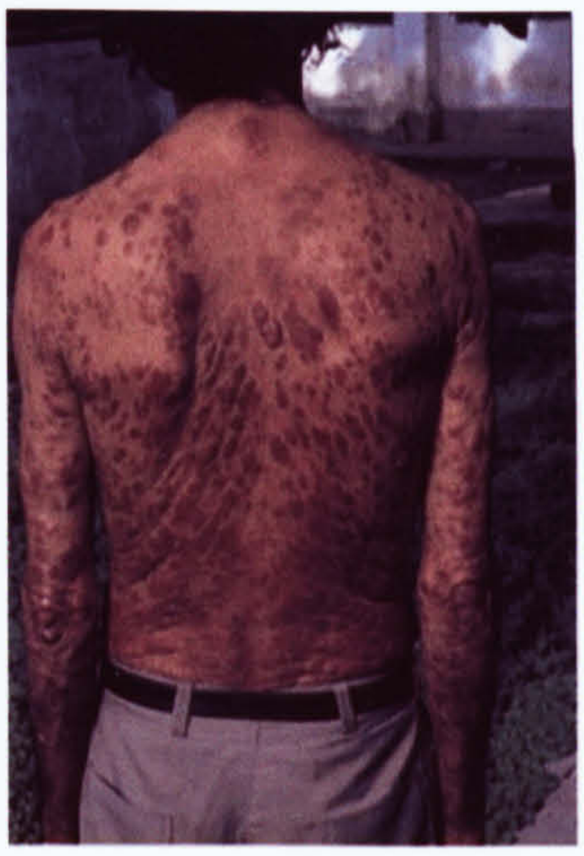


**Figure 1.1c** Diffuse cutaneous leishmaniasis in the New World following infection with *L. amazonensis*.

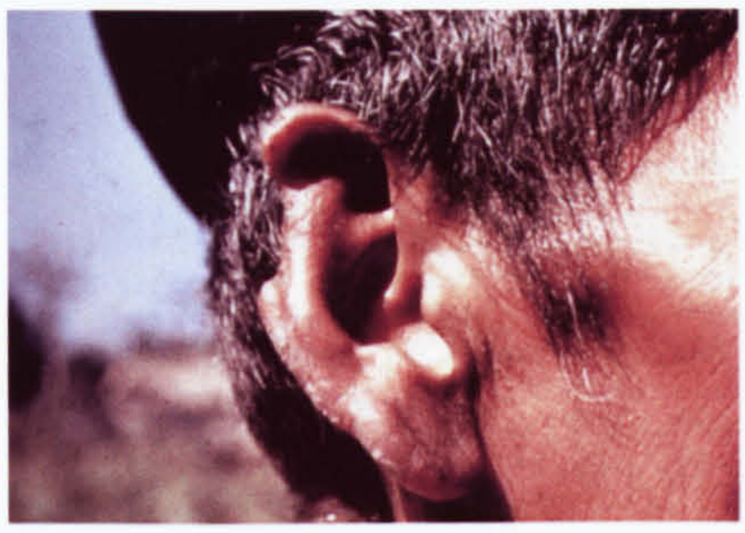


**Figure 1.1d** Chiclero's ulcer caused by *L. mexicana*

**Figure 1.1 (a-d)** reproduced from Peters & Gilles (1995)

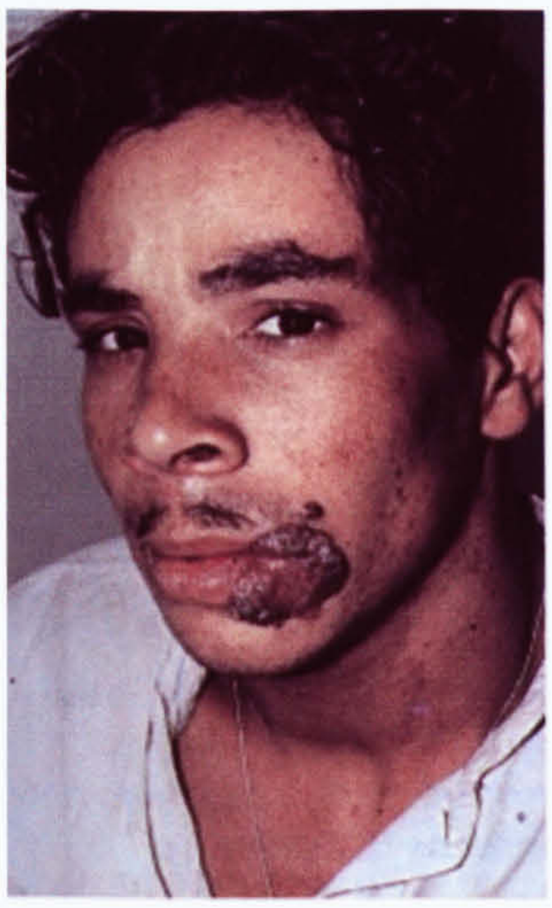


**Figure 1.2a** Early lesion of Espundia due to *L. braziliensis* infection

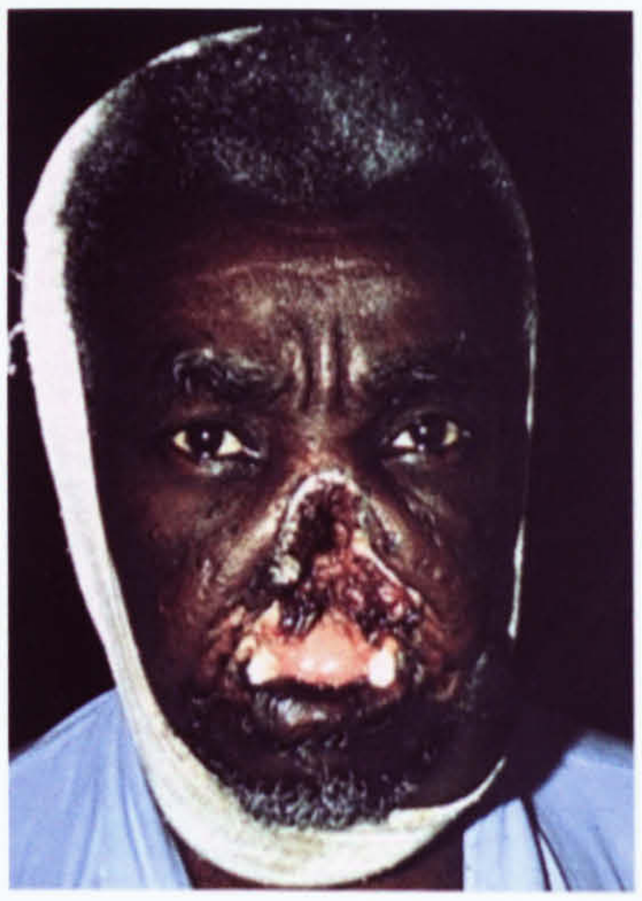


**Figure 1.2b** Destructive Espundia may follow inadequate treatment of *L. braziliensis* infection

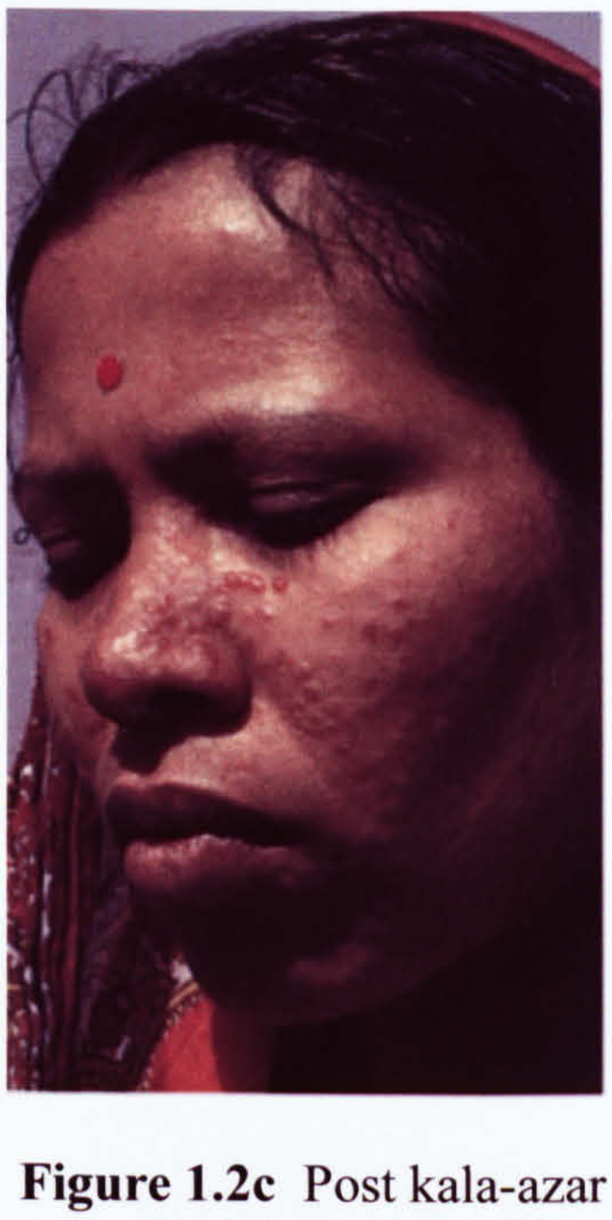


**Figure 1.2c** Post kala-azar dermal leishmaniasis, following treatment of *L. donovani* infection

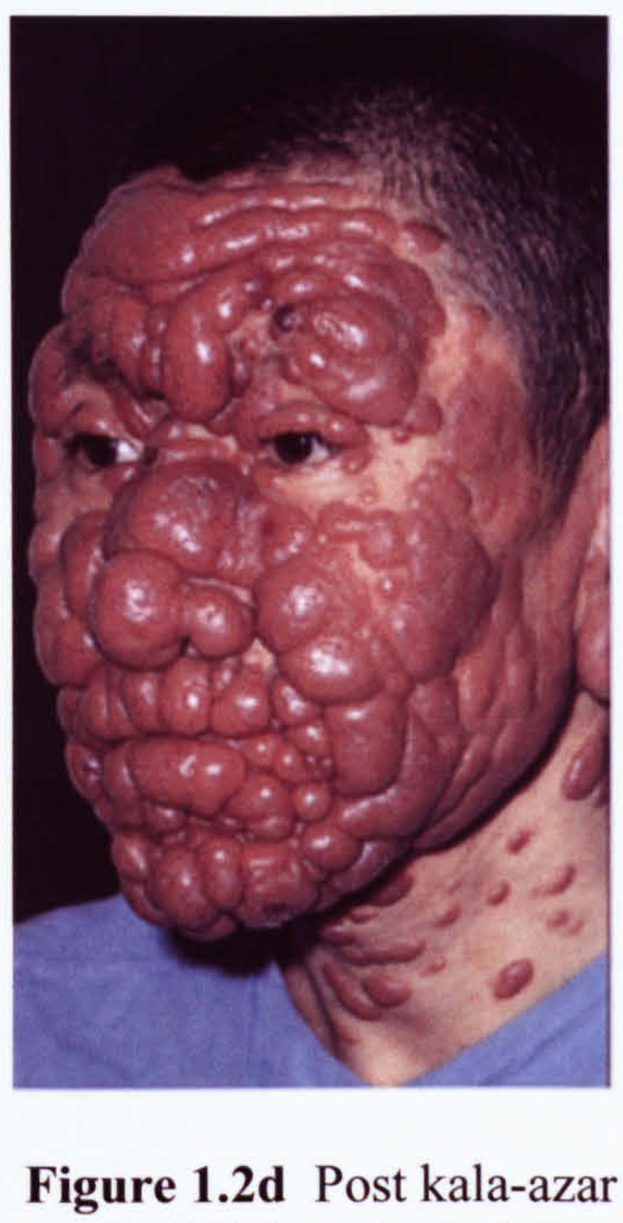


**Figure 1.2d** Post kala-azar dermal leishmaniasis following treatment of *L. donovani* infection

**Figure 1.2 (a-d)** reproduced from Peters and Gilles (1995).

## 1.1.2 Visceral Leishmaniasis

Visceral leishmaniasis generally results from infection with *L. donovani* and *L. infantum* (*L. chagasi*). In India the disease is known as kala-azar (black sickness). In most cases a primary skin lesion does not develop at the site of the fly bite. Visceral disease develops after an incubation period of 2 – 6 months and onset of clinical disease is accompanied by an irregular fever; sometimes mistaken for malaria. Present in most visceral infections is an enlargement of the spleen, which can also be accompanied by hepatomegaly. Parasites are also found in the bone marrow and can in fact be found in virtually any organ during advanced disease.

Malnutrition and immune defects have a major influence on the severity of the disease. It is, therefore, not surprising that epidemics of visceral leishmaniasis are often associated with poverty and immunosuppression. Once the disease has fully progressed it is almost invariably fatal if left untreated. If chemotherapy is successful the patient can suffer from post kala-azar dermal leishmaniasis (PKDL) (Figure 1.2c and 1.2d). PKDL is a relatively common consequence of therapeutic cure of visceral leishmaniasis caused by *L. donovani*. The patient's skin can become covered in papules or nodules that contain amastigotes. These papules can cover extensive areas of the body but the viscera are clear from parasites. The condition can persist for several years.

## 1.2 Epidemiology

Leishmaniasis is endemic in 88 countries on five continents: Africa, Asia, Europe, North America and South America (Figure 1.3; Table 1.1). Of these 88 countries, 76 belong to the developing world and 13 of them are amongst the least developed in the world. It is estimated that a total of 350 million people are at risk of the disease and worldwide approximately 12 million people are affected by leishmaniasis, with an estimated 1.5 – 2 million new cases occurring each year (WHO 2000). These consist of 1-1.5 million new cases of cutaneous leishmaniasis and 500 000 new cases of visceral leishmaniasis.

The most common and widespread of the leishmaniases is cutaneous leishmaniasis. This form of the disease is found on every tropical and subtropical continent with the exception of Australia. Of the current cases of cutaneous leishmaniases, 90% occur in the Middle East (Afghanistan, Iran, Saudi Arabia and Syria) and South America (Brazil and Peru). It is also in South America that 90% of mucocutaneous leishmaniasis occurs, in Brazil, Peru and Bolivia. Of the 500 000 new cases of visceral leishmaniasis that occur annually, 90% are in five countries; Bangladesh, India, Nepal, Sudan and Brazil.

In the past decade areas where leishmaniasis is endemic have significantly expanded, accompanied by an increase in recorded cases of the disease (Desjeux, 2001). Massive urban-rural migration together with agricultural and industrial projects bringing non-immune individuals into endemic rural areas are aiding this expansion.

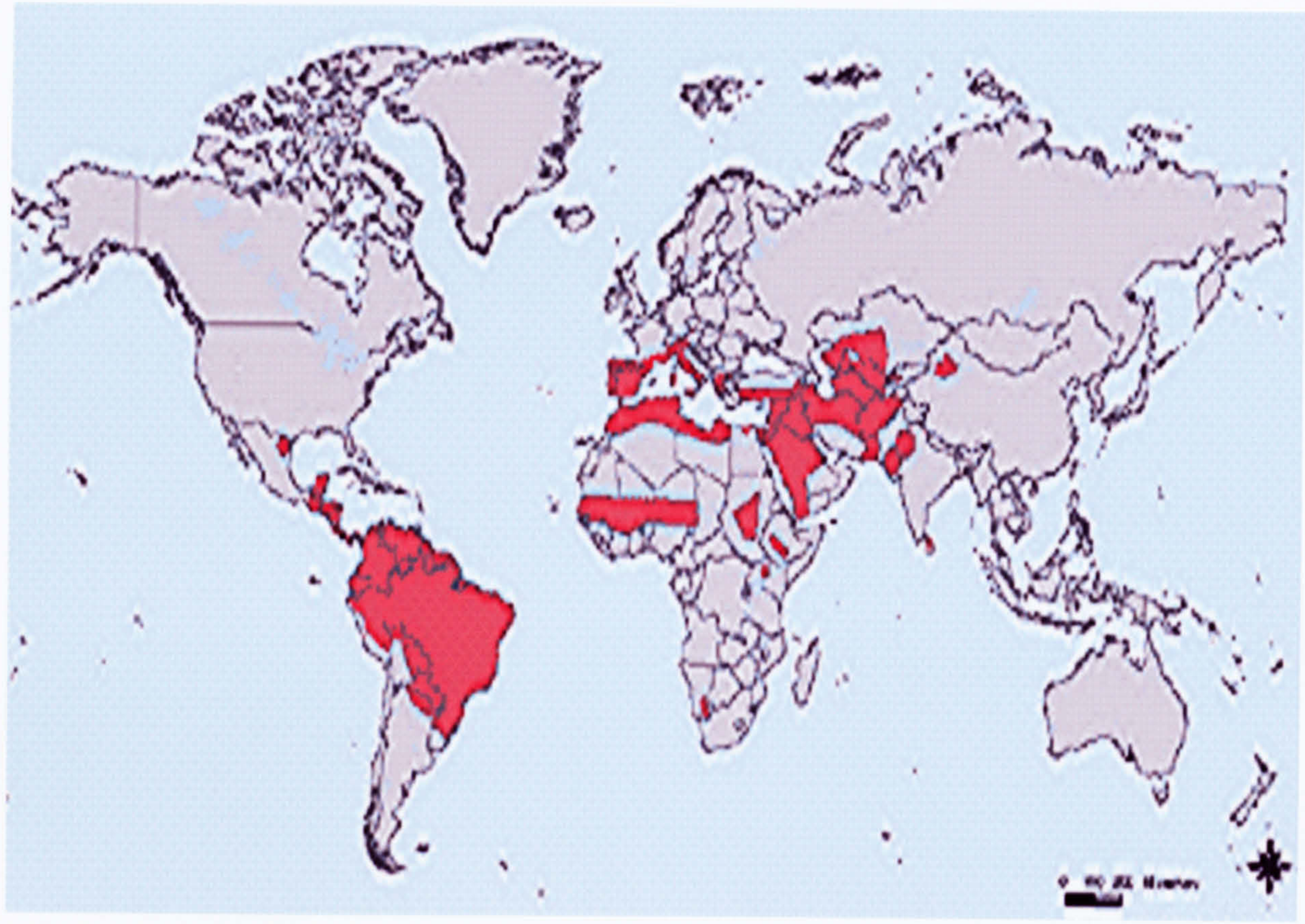


**Figure 1.3** Map showing the geographical distribution of 90% of cutaneous leishmaniasis.

(modified from WHO 2003)

**Figure 1.4** Map showing the geographical distribution of visceral leishmaniasis. (modified from WHO 2003)

Environmental impacts such as deforestation, dam building and irrigation systems are also contributing to the geographical spread of leishmaniasis. Leishmania/HIV co-infections are also facilitating the increase in reported cases of leishmaniasis. In Southern Europe 40% of visceral leishmaniasis cases have been presented by patients co-infected with HIV (Dereure *et al.* 1995). As both diseases attack cells of the immune system, this leads to a vicious circle where each reinforces the severity of the other.

## 1.3 Diagnosis

For effective treatment of leishmaniasis correct diagnosis is essential. Although none of the signs or symptoms of disease are diagnostic they are frequently regarded as sufficient in endemic areas. Diagnosis of visceral leishmaniasis in the presence of HIV infection is particularly difficult and clinical diagnosis cannot be relied on as presentation of the disease may be very atypical. However, where a presumptive clinical diagnosis is made disease should confirmed parasitologically.

Parasitological diagnosis can be confirmed by the demonstration of parasites in smears of infected tissue stained with Giemsa's stain at pH 7.2. Confirmation of cutaneous disease can be made by isolation of a small amount of material taken from the edge of the lesion. Sampling of deeper tissues by needle biopsy of lymph nodes, bone marrow or, mostly reliably, the spleen are required for the confirmation of visceral disease. Wherever possible it is desirable to culture the parasites and identify them to strain level using isoenzyme analysis.

| Species | Disease | Geographical Distribution | Important Mammalian Hosts |
|---|---|---|---|
| ***Leishmania Leishmania*** | | | |
| *L. major* | CL | North Africa, Sahel of Africa, Central and West Asia | Various rodents including great gerbil and fat sand rat |
| *L. tropica* | CL | Central and West Africa | Humans |
| *L. aethiopica* | CL, DCL | Ethiopia, Kenya | Rock hyraxes |
| *L. donovani* | VL, PKDL | Indian subcontinent, East Africa | Humans |
| *L. infantum* | infantile VL<br>CL | Mediterranean basin, Central and West Africa, Central and South America | Domestic dog, foxes |
| *L. mexicana* | CL | Central America | Forest rodents |
| *L. amazonensis* | CL | South America | Forest rodents |
| ***Leishmania Viannia*** | | | |
| *L. braziliensis* | CL, MCL | Central and South America | Forest rodents |
| *L. peruviana* | CL | Peru | Unknown reservoir host |
| *L. guyanensis* | CL | South America | Sloth, Anteater |
| *L. panamensis* | CL | Central America | Sloth |

**Table 1.1** Epidemiology of the main Old and New World *Leishmania* species. CL: cutaneous leishmaniasis, VL: visceral leishmaniasis, MCL: mucocutaneous leishmaniasis, DCL: diffuse cutaneous leishmaniasis, PKDL: post kala-azar dermal leishmaniasis. Adapted from Ashford and Bates (1998) and Bates (2001).

## 1.4 Treatment

Treatment of leishmaniasis is not always required. Simple cutaneous infection involving *L. major* will normally self cure within a few months and leave the patient with a solid immunity to reinfection. However, other species causing cutaneous infection require treatment as they can involve multiple lesions, which without treatment can be disfiguring for the patient. Symptomatic visceral leishmaniasis always requires treatment, as without it the mortality rate is almost 100%. Likewise mucocutaneous leishmaniasis should always be treated whenever possible.

Classic treatment of all types of leishmaniasis is pentavalent antimony in the form of meglumine antimoniate or sodium stibogluconate (Chance, 1995). Intralesional injection is used for simple single cutaneous lesions. Intramuscular injection is used in all other cases, and with visceral leishmaniasis requires prolonged courses of treatment. Pentamidine and amphotericin B are the drugs most commonly used in visceral cases that are unresponsive to treatment with antimonials. These can be extremely toxic and careful administration is essential. Amphotericin B while very effective against visceral disease, is far less effective in treatment of cutaneous and mucocutaneous leishmaniasis. A relatively new oral treatment, miltefosine, has proved extremely promising in the treatment of visceral leishmaniasis in India (Sundar *et al.* 2002) and more recently for the treatment of cutaneous leishmaniasis due to *L. panamensis* (Soto *et al.* 2004).

## 1.5 Immune Response

Immunity to *Leishmania* is essentially T cell-mediated. Most of the studies carried out to elucidate the mechanisms of immunity to *Leishmania* have been done in murine models using *L. major* and *L. donovani*. Although *Leishmania* parasites have several mechanisms to avoid the host's immune defences, it is possible for the host to mount a protective immune response and eliminate infection. This requires the host cell to become activated. Activation of macrophages is achieved by the action of cytokines from $CD4^+$ T helper cells (Th), most importantly interferon gamma (IFN-γ), which subsequently activates the production of nitric oxide (Liew & O'Donnell, 1993) and leads to parasite death.

In 1986, Mosmann and colleagues identified two subsets of T helper cells, Th1 and Th2. These subsets of T helper cells can be characterised by their cytokine profiles. Th1 cells release IFN-γ and interleukin (IL)-12, while Th2 cells release IL-4, IL-5, IL-6 and IL-10 (Roitt *et al.* 1996). Th1 cells that are necessary for the control of infection are present in healing tissues, whereas Th2 cells are associated with progression of disease (Heinzel *et al.* 1991).

One of the most important factors mediating Th cell development appears to be the cytokines that are present when the T cells are initially activated (Hondowicz & Scott, 1999). Scott (1991) showed that early release of IFN–γ drives Th1 development and subsequently control of infection. He found that C3H/HeN mice, (a strain normally resistant to *L. major* infection) given anti-IFN-γ monoclonal antibody in a single injection prior to infection produced significantly less IFN-γ and were unable to

control infection. These mice changed their cytokine profile early after infection and, when tested at day 3 post infection, were found to be producing IL-4 and IL-5, indicating a switch from a Th1 to a Th2 response.

In 1993 Heinzel and colleagues showed that IL-12 also played a role in protective T cell immunity. They found that following treatment with recombinant murine IL-12 during the first week of infection, 89% of BALB/c mice were able to cure. It was also observed that when anti-IFN-γ monoclonal antibody was administered the protective effect of IL-12 was abrogated. These data indicated that IL-12 promotes a Th1 response in an IFN-γ dependent manner.

Exacerbation of leishmaniasis is associated with a Th2 response that is driven by early release of IL-4 (Chatelain *et al.* 1992). Chatelain and colleagues observed that anti-IL-4, when injected into BALB/c mice prior to *L. major* infection, enabled control and healing of lesions. This was accompanied by increased production of IFN-γ. These mice were also completely resistant to reinfection when challenged 12 weeks later. This abrogated effect of anti-IL-4 was not observed if given later on in the infection (*i.e.* 2 weeks) and these mice produced relatively little IFN-γ. It was therefore concluded that IL-4 exerts its effect on Th2 differentiation very shortly after infection.

From the above studies it was obvious that host genetics have a major influence on the outcome of disease. Specific mouse strains show a predetermined variation in their response to infection. BALB/c mice are susceptible to infection, whereas C3H/HeN mice are resistant. Studies have been carried out to identify the genes responsible for this variation. Bradley and colleagues (1979) showed that the immune

response to *L. donovani* in mice is under the control of a single gene *Lsh*, recently renamed Nramp1 (Canonne-Hergaux *et al.* 1999). Exactly how these genes function in the control of leishmaniasis is still not fully understood.

## 1.6 Control

In the absence of an effective vaccine, control measures depend greatly on local knowledge. For example in the Amazonian forests, where transmission is sylvatic, control of the vector or reservoir hosts is impractical. In this situation avoidance of the transmission areas is the only effective strategy to reduce the risk of infection. Some national parks in Sudan are notorious for the transmission of *L. donovani* and are rarely visited in the transmission season. However, control of rodent reservoir hosts has had great success in central Asia (Sergiev 1977). Where peridomestic transmission occurs, as with *L. tropica* and *L. donovani* in Asia, insecticide spraying can greatly reduce the numbers of sandflies. Deltamethrin-impregnated dog collars have been shown to be effective in protecting dogs against sandfly bites in Brazil (David *et al.* 2001) Although it is possible to go a small way to controlling leishmaniasis many of the countries that are severely affected by the disease are poor and even war-torn. Add to this are the problems incurred from corrupt governments and unstable economies in endemic countries, and these control measures can prove difficult to get off the ground.

## 1.7 Molecular Biology

All members of the family Kinetoplastida have both nuclear and kinetoplast genomes and lack any detectable chromosomal condensation during metaphase (Vickerman & Preston, 1970). With the development of techniques, including pulsed-field gel electrophoresis (Schwartz & Cantor, 1984), it has been possible to generate molecular karyotypes for several *Leishmania* species. These data have revealed considerable size polymorphisms between homologous chromosomes within the same genome and between the genomes of different *Leishmania* species and strains. Differences in the number of repeat regions within the subtelomeric regions are responsible for these size polymorphisms (Bastien *et al.* 1992). The *Leishmania* genome appears to be diploid (Bastein *et al.* 1992) and this is confirmed by gene knock out experiments, where it has required two rounds of gene disruption to effectively generate a null mutant with the loss of both alleles (Souza *et al.* 1994).

It was initially reported that the *Leishmania* genome consisted of 36 chromosomes, ranging in size from 0.35 to ~3 Mb, using Old World species (Wincker *et al.* 1996). However, when the genome of New World species were examined, it was found that species of the subgenus *Viannia* had 35 chromosomes, while those of the *Leishmania* subgenus had 34 (Britto *et al.* 1998). These differences in chromosome number are the result of four chromosomal rearrangements, via either fusion or breakage. Britto *et al.* (1998) found that chromosomes 20 and 34 as identified by Wincker *et al.* (1996) had become a single chromosome in the subgenus *Viannia*, while chromosomes 20 and 36, and chromosomes 8 and 29 respectively had also become single chromosomes. However, they did observe a strong conservation in all of the un-

rearranged chromosomes and the genes were syntenic, thereby supporting the rearrangement mechanism of fusion and breakage.

### 1.7.1 *Trans*-splicing

The *Leishmania* genome has several properties that appear to be unique to the trypanosomatids. These include the absence of introns within transcription units and the organisation of genes into polycistronic transcription units (Stiles *et al.* 1999). Genes within these polycistronic units are co-transcribed under the control of a single 5' promoter giving rise to polycistronic precursor RNA. This arrangement is comparable to that seen in prokaryotes. As a consequence of this arrangement extensive post-transcriptional processing is required to produce mature mRNA. This occurs via the process of *trans*-splicing (Graham, 1995; Stiles *et al.* 1999), where a 39-nucleotide splice leader (SL) sequence containing an $m^7$Gppp residue at the 5' end is *trans*-spliced to the 5'end of each gene transcript. The SL is donated from the 5' end of a precursor SL RNA encoded elsewhere in the genome. The SL is ligated to the pre-mRNA via phosphodiester exchange reactions involving the formation of a branch Y intermediate (Figure 1.5). It is likely that *trans*-splicing occurs co-transcriptionally (Ullu *et al.* 1993), thereby preventing the formation of huge transcripts as each gene is processed immediately following transcription. Each mRNA is also polyadenylated at the 3' end to produce the mature mRNA. There is evidence that *trans*-splicing and polyadenylation are functionally linked (Ullu *et al.* 1993). As there seem to be no consensus polyadenylation sites in *Leishmania*, unlike most higher eukaryotes where the 3'UTR of an mRNA contains the sequence

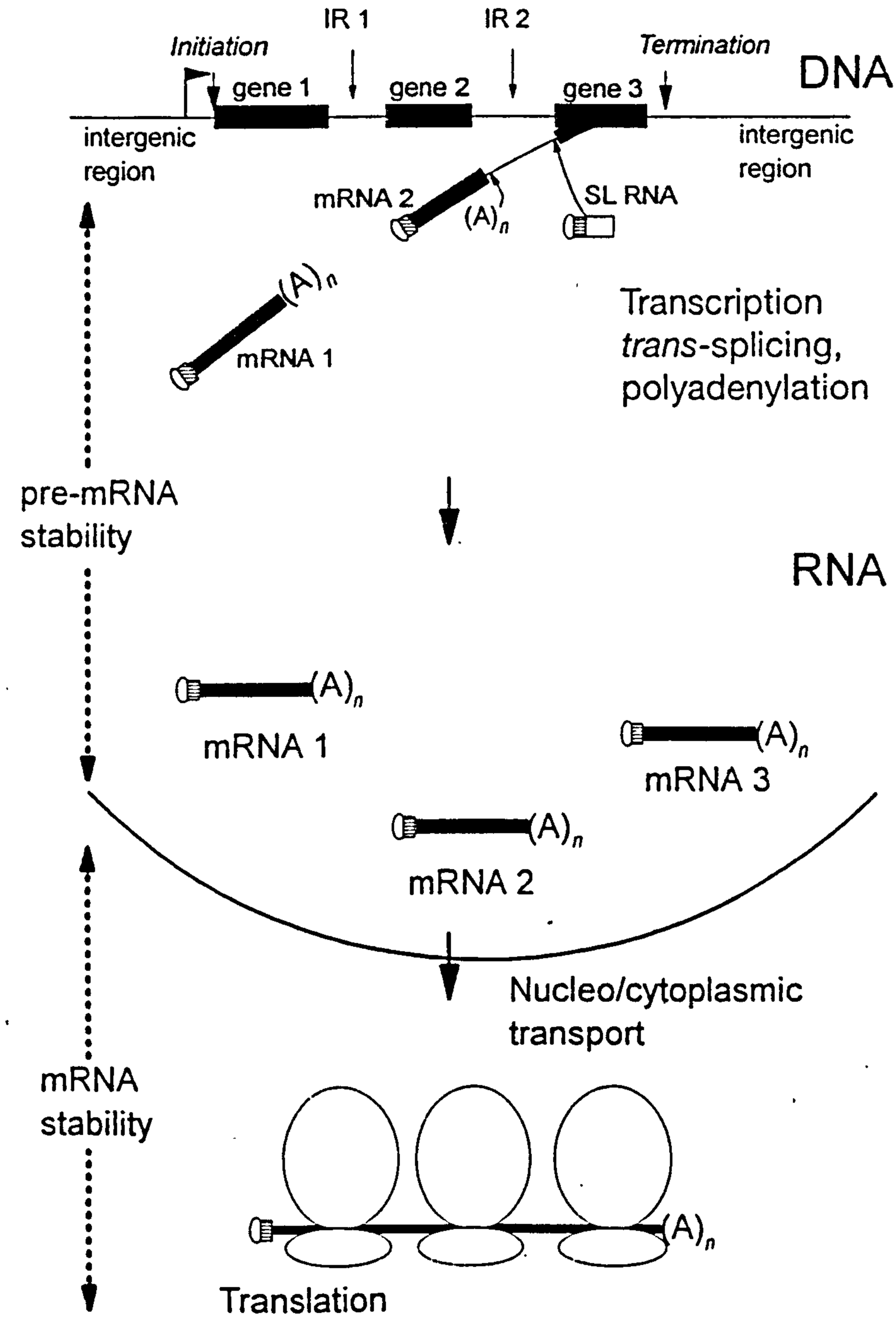



**Figure 1.5.** *Trans-splicing*, the mechanism used by trypanosomatids to generate monocistronic transcripts from polycistronic transcription units. The splice leader sequence (hatched box) carries a cap structure (open oval) which is donated from a discontinuously transcribed RNA sequence (SL RNA; open box). Adapted from Graham (1995).

AAUAAA to direct downstream polyadenylation, the linking of these processes may be a substitute for polyadenylation signals. A major consequence of polycistronic gene organisation is that gene expression cannot be regulated at the transcriptional level and all regulation must occur post-transcriptionally.

### 1.7.2 Kinetoplast DNA and RNA Editing

Kinetoplast DNA (kDNA) is a unique DNA structure of the Kinteoplastida. It is composed of an organised network of thousands of topologically locked DNA circles, of which there are two types. There are several thousand (5000-10 000) small minicircles and, 20-50 identical 20-35kb maxicircles (Shlomai, 1994). Maxicircles encode ribosomal RNAs and several proteins involved in mitochondrial energy transduction. However the expression of the proteins encoded by the maxicircles is extremely complex and involves the process of RNA-editing (Seiwert, 1995). The mRNA transcripts from kDNA maxcircles contain frameshifts and lack initiation codons, which, without further processing, cannot be translated. During RNA-editing uridine residues are added to or removed from the transcripts at precise positions, to create an open reading frame on the mature mRNA. The information required to drive RNA-editing is provided by small guide RNAs (gRNAs) (Blum *et al.* 1990). It is the small 1kb minicircles that contain the genetic information for the gRNAs, that are 40-70 nucleotide heterogeneous molecules (Sturm & Simpson, 1990).

# Chapter 2

# Literature review

## 2.1 *Leishmania* Life Cycle

*Leishmania* are parasites that undergo two developmental cycles in two different hosts. They exist in two major morphological forms, intracellular amastigotes found in mononuclear phagocytic cells including macrophages, monocytes and Langerhans cells, and extracellular promastigotes found in the alimentary tract of female phlebotomine sandflies. Amastigotes are small, non-motile, ovoid cells approximately 3-5μm in length (Figure 2.1a), whereas promastigotes are elongated motile cells of varying lengths ranging from 6-20μm (Figure 2.1b).

### 2.1.1 Development in the Vertebrate Host

Infection of vertebrate hosts, which are mostly mammals, occurs via the bite of infected female sandflies of the family Psychodidae, genus *Phlebotomus* or *Lutzomyia* found in the Old World and New World, respectively. The pre-adapted infective forms, termed metacyclic promastigotes (Sacks, 1989), are passed into the host during this process of blood-feeding.

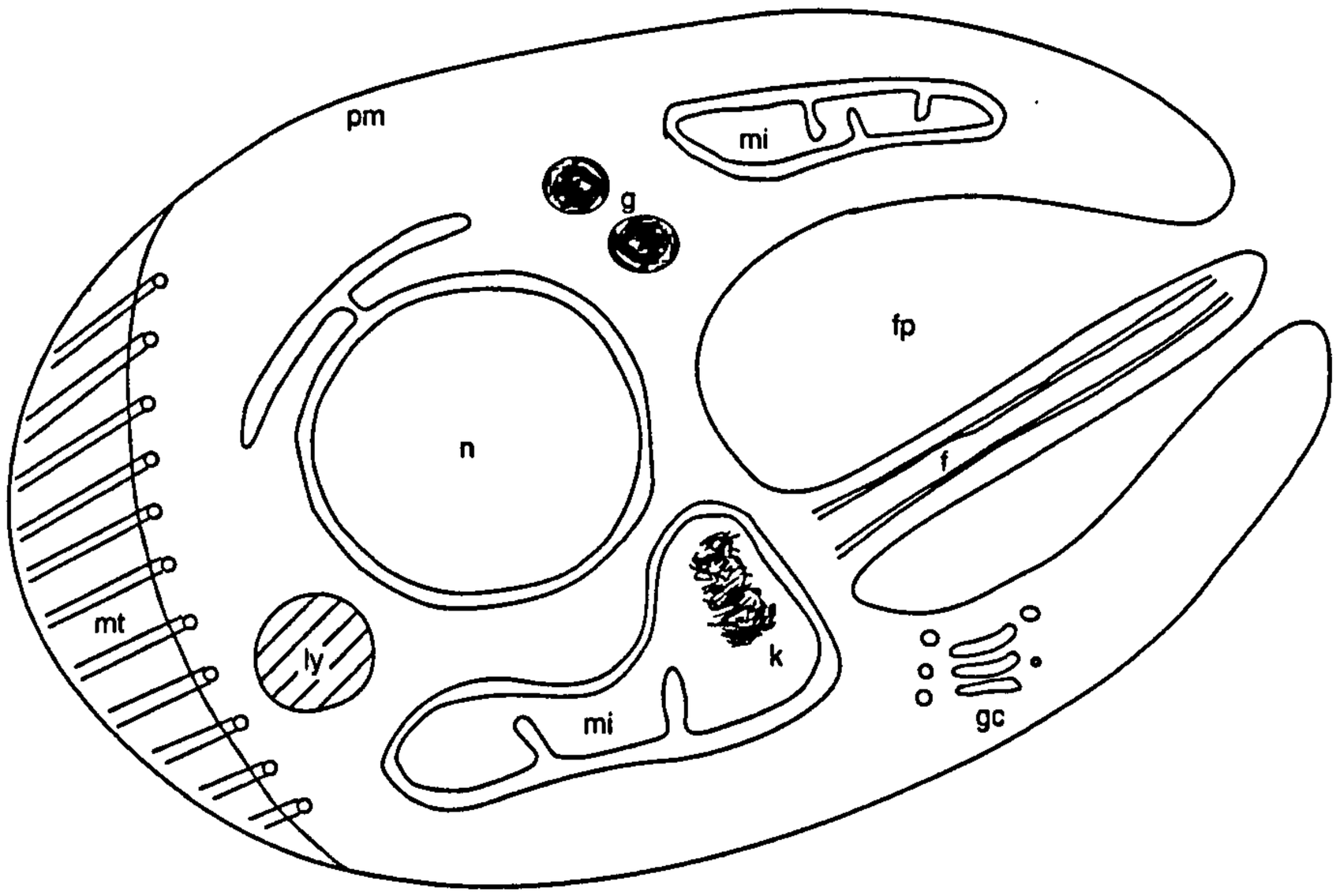



**Figure 2.1a** Ultrastructure of a *Leishmania* amastigote. Amastigotes possess a central nucleus (n) and adjacent kinetoplast (k) within a single branching mitochondrion (mi). The flagellum (f) arises from a flagellar pocket (fp) but does not extend beyond the cell body. Lysosomes (ly), glycosomes (g) and Golgi complex (gc) are found in the cytoplasm. Rows of microtubules (mt) run just below the plasma membrane (pm).

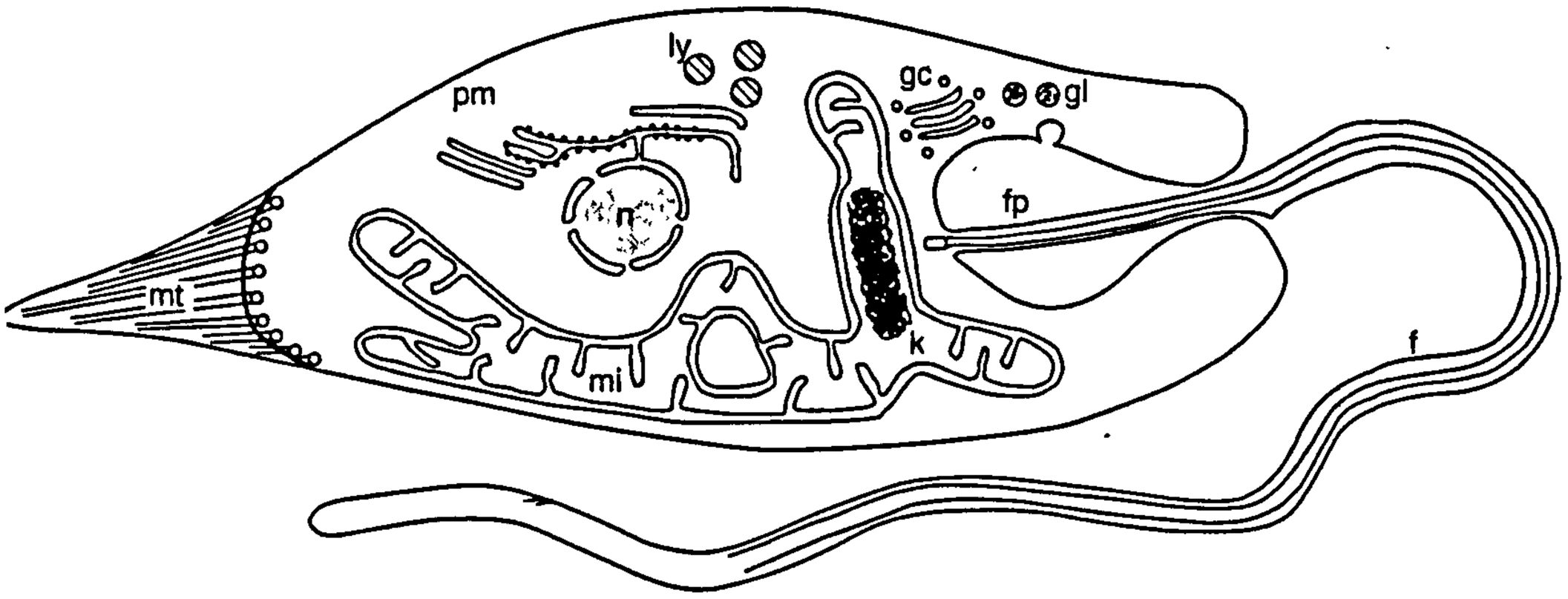



**Figure 2.1b** Ultrastructure of a *Leishmania* promastigote. Many of the features found in amastigotes are also found in promastigotes (see Figure 2.1a) for abbreviations). Some differences are that the flagellum extends beyond the cell body and the kinetoplast has a more anterior location relative to the nucleus.

**Figure 2.1a and 2.1b** reproduced from Ashford & Bates (1998).

It should be noted that there are also *Leishmania* parasites that infect reptiles, however, these belong to a different genus, *Sauroleishmania* (Lainson & Shaw, 1987), and are transmitted by sandflies belonging to the genus *Sergentomyia*. There is much debate regarding the classification of *Sauroleishmania* and new evidence indicates that they are actually closely related to the members of the subgenus *Leishmania* (Noyes *et al.* 1997; Croan *et al.* 1997; reviewed by Momen, 2001). For the purpose of this thesis and literature review only those parasites currently classified in the genus *Leishmania* will be discussed.

Once in the host, cells of the macrophage-monocyte series take up the metacyclic promastigote by receptor-mediated phagocytosis. Following uptake the promastigote is contained within a phagosome, to which lysosomes fuse to form a phagolysosome. Within this phagolysosome the metacyclic promastigotes undergo a morphological transformation to become amastigotes. This process of internalisation and transformation is complete within 12-24 hours (Alexander, 1975). The amastigotes then grow and divide by binary fission within the phagolysosome until a threshold of parasites is reached and the host cell ruptures releasing the amastigotes. New host cells then take up these free amastigotes. Infection of female sandflies occurs when they take a bloodmeal from an infected host and inadvertently take up parasite filled macrophages or free amastigotes from a cutaneous lesion or the peripheral blood system.

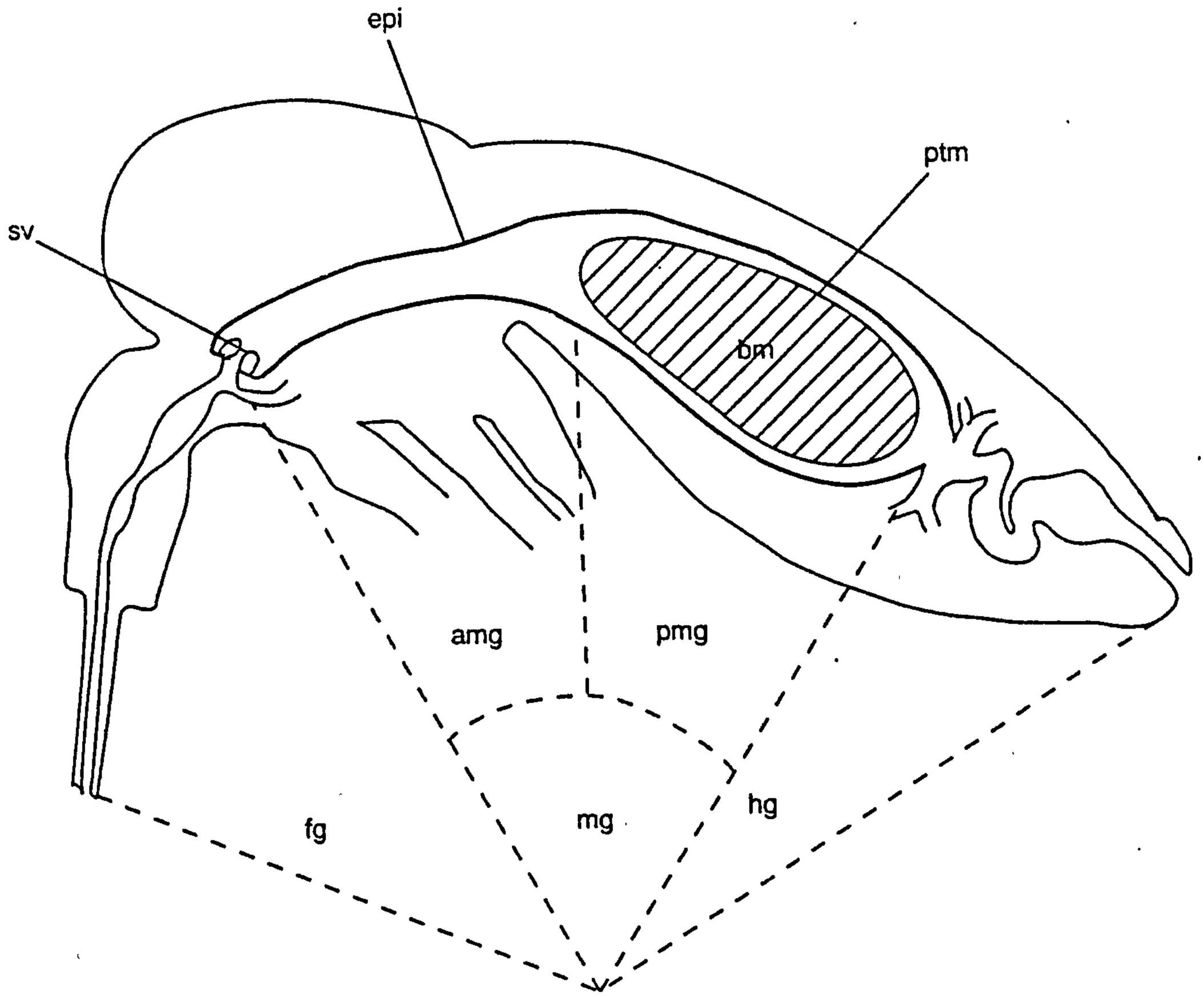



**Figure 2.2** Structure of the female sandfly gut. The gut can be divided into 3 sections: the foregut (fg), midgut (mg) and the hindgut (hg). The midgut is lined by epithelial cells (epi) with microvilli that project into the lumen of the gut, and is subdivided into the anterior or thoracic midgut (amg) and the posterior or abdominal midgut (pmg). The stomodeal valve (sv) forms the junction between the anterior midgut and the foregut. A chitinous cuticular layer lines the foregut, stomodeal valve and hindgut. Initial development of the parasite occurs within the bloodmeal (bm) encased by a peritrophic matrix (pm) in the posterior midgut. Reproduced from Ashford & Bates (1998).

## 2.1.2 Development in the Vector

There have been several studies carried out to investigate the development of various *Leishmania* species in both natural and experimental host-parasite combinations (including, Lawyer *et al.* 1987, 1990; Walters *et al.* 1987, 1989a, 1989b; Cihakova & Volf, 1997; Nieves & Pimenta; 2000, Rogers *et al.* 2002). These investigations have identified several morphological forms of promastigote within the sandfly including, procyclic promastigotes, nectomonad promastigotes, haptomonad promastigotes, leptomonad promastigotes, metacyclic promastigotes and paramastigotes. The parasites change from one form to another through a series of transformations, where one form gives rise to a new form that is a dividing population, and differentiations, where a dividing form gives rise to a non-dividing form.

During these studies two patterns of development have been observed, involving the hindgut (peripylarian development), and not involving the hindgut (suprapylarian development) (Figure 2.2). Those parasites that require a period of hindgut development, for example *L. braziliensis*, are placed into the subgenus *Leishmania* (*Viannia*), whereas those that require no hindgut development are placed into a separate subgenus *Leishmania* (*Leishmania*). Within the sandfly development of *Leishmania* parasites can be thought of as occurring during two separate phases, the bloodmeal phase and the sugarmeal phase.

## 2.2 *Leishmania* (*Leishmania*)

### 2.2.1 Development in the bloodmeal

Following ingestion the bloodmeal is encased by a type 1 peritrophic matrix (PM), composed of a meshwork of chitin fibrils, proteins and glycoproteins (Shao *et al.* 2001), within 1–4 hours. The PM is synthesised by the entire midgut digestive epithelium and completely envelopes the ingested food in the lumen of the gut. The trigger for the formation of the PM is thought to be distension of the gut. Schlein and Warburg (1985) observed the formation of a PM in sandflies that had been fed saline, supporting the distension theory. The PM forms a physical barrier between the gut contents and the digestive epithelial cells of the midgut. In doing so the PM serves to protect the midgut from pathogens and abrasive particles ingested while feeding. Pimenta and colleagues (1997) suggested that the PM may also play a role in protecting *Leishmania* parasites from the action of digestive enzymes, prior to their differentiation into protease resistant forms. They observed a decline in the survival of *Leishmania major* in *Phlebotomus papatasi* in the absence of a PM (Pimenta *et al.* 1997). The PM is also known to have digestive roles. Digestion within the insect gut involves a number of sequential steps and the PM plays a major role in this process by the partitioning of digestive enzymes. It is a semipermeable matrix that allows some newly synthesised digestive enzymes, such as endoproteinases and endoglycosidases, into the intraperitrophic space. The hydrolysed products of these digestive enzymes are allowed to pass in the opposite direction where digestion is continued by a new group of enzymes. Once digestion is complete the resulting molecules are absorbed by the epithelial cells.

It is within this intraperitrophic space that the first developmental event takes place; the transformation from amastigotes to procyclic promastigotes. Transformation into flagellated procyclic promastigotes is accompanied by intense multiplication (Walters *et al.* 1987, 1989a, 1993; Lawyer *et al.* 1987, 1990). Division of *Leishmania* parasites in sandflies is by binary fission. The specific sequence of division was outlined in a study by Walters and colleagues (1989a) using *L. chagasi* in *Lu. longipalpis*. They observed that division began with the development of a second flagellum, followed by nuclear division and subsequent division of the kinetoplast. Cell division began posteriorly resulting in a pair of promastigotes joined at their flagella tip.

Prior to this transformation an enlargement of amastigotes has been observed (Walters *et al.* 1989a). This transformation occurs relatively soon after blood-feeding and has been observed at 12–18 hours (Walters *et al.* 1987; Lawyer *et al.* 1987). Lawyer and colleagues (1987) noted that approximately 50% of the early forms of procyclic promastigotes were dividing. Several studies noted that procyclic promastigotes underwent two sequences of division (Walters *et al.* 1989a, 1993; Lawyer *et al.* 1990). Walters and colleagues termed the resulting parasites, "division promastigote I" which were small (6-10μm), oval and had a short flagellum, and "division promastigote II" which were elongated (9-12μm) and possessed a flagellum increased in length. Many of these dividing procyclic promastigotes were seen in rosettes of 4-12 parasites arranged radially with their flagella directed toward the centre (Lawyer *et al.* 1987, 1990; Walters e*t al.* 1989a, 1993).

Following this intense replication of procyclic promastigotes and, therefore, increase in parasite density, a new form of elongated (12-24μm), highly motile promastigote

emerges; the nectomonad promastigote. Walters *et al.* (1993) suggested that the elongated nectomonad promastigotes develop from procyclic or "stumpy forms", following the observation of mixed size rosettes indicating that growth occurred during division and that this form represented the terminal developmental form in the bloodmeal. The appearance of nectomonad promastigotes coincides with the breakdown of the PM. Walters *et al.* (1989a) observed the anterior breakdown of the PM as early as 36 hours post-feeding while studying the natural combination of *L. chagasi* in *Lu. Longipalpis*, whereas Lawyer *et al.* (1987, 1990) found breakdown of the PM to occur between 48-60 hours post-feeding, signified by the presence of small amounts of digested blood in the hindgut.

Prior to the breakdown of the PM the nectomonad promastigotes are seen to concentrate in the anterior region of the PM (Walters *et al.* 1989a). Within the vicinity of these concentrated parasites the dark colour of the blood haemoglobin disappears, this is most probably due to the digestion of haemoglobin by *Leishmania* proteases, possibly gp63 (Davies *et al.* 1990). It is at this stage in the development that the nectomonad promastigotes begin their escape from the PM, aided by the secretion of chitinase (Schlein *et al.* 1991). Schlein and Jacobson (1994) found that enzymatic activity of chitinase was reduced by 58-75% when parasites were grown in the presence of haemoglobin. It can therefore be assumed that during early infection within the bloodmeal, secretion of parasite chitinases is inhibited by the blood haemoglobin. However, as the bloodmeal is digested and the haemoglobin removed there is a gradual expression of chitinases and therefore degradation of the PM. Therefore, digestion of the blood haemoglobin in the vicinity of the nectomonads is a prequisite for the enzymatic function of chitinase.

## 2.2.2 Development in the Midgut (sugarmeal phase)

Nectomonad promastigotes that have penetrated and escaped the PM are seen in close association with the gut wall. This association is achieved by the insertion of the flagella between the microvilli of the gut epithelium (Warburg *et al.* 1986; Walters *et al.* 1987, 1989a, 1993). Walters *et al.* (1993) observed nectomonad promastigotes arranged in longitudinal rows or in groups perpendicular to the midgut epithelium, when studying the unnatural combination of *L. major* in *Lu. longipalpis.* This attachment behaviour is seen in the posterior and anterior midgut regions (Walters *et al.* 1989a, 1993) and prevents the loss of parasites via the peristaltic action of the gut during excretion of the digested bloodmeal. The attachment of nectomonad promastigotes to the midgut epithelium is therefore essential for establishment of infection past the bloodmeal phase of development. However, as described above, the parasites have to first navigate their escape from the PM to accomplish this. Studies by Lawyer *et al.* (1990) demonstrated that *L. major* was able to multiply in *Sergentomyia schwetzi* but could not establish infection past the bloodmeal phase, as a consequence of failure to escape the PM.

Flagellar attachment to the midgut epithelium is mediated by the major surface glycoconjugate, lipophosphoglycan (LPG). The specific structure of LPG is described in detail in section 2.6.1. Studies by Pimenta *et al.* (1992) using dissected midguts of *Ph. papatasi* showed that purified LPG from cultured procyclics of *L. major* inhibited the attachment of procyclic promastigotes to the midgut epithelium. The same study also showed that the adhesion of *Leishmania* parasites to the midgut epithelium is stage-specific. It was observed that purified LPG from cultured

metacyclic promastigotes showed no inhibition of procyclic binding. This stage-specific attachment is a result of changes in the terminating residues of the repeating side chains expressed on the LPG molecule. In procyclic promastigotes these terminated in β-Gal, whereas in metacyclic promastigotes the terminating residue changes to α-Ara or β-Glc. Another study by Pimenta *et al.* (1994) showed that *L. major* strains that express deficient amounts of LPG (L119) were unable to initiate a midgut infection past the bloodmeal phase and the parasites were excreted along with the digested bloodmeal. These data suggested that LPG is necessary for the successful colonisation of the midgut following PM breakdown. These findings were confirmed in a recent study (Sacks *et al.* 2000) using *LPG1* mutants of both *L. major* in *Ph. papatasi* and *L. donovani* in *Ph. argentipes*, both of which are natural parasite/vector combinations.

By day 2-3, following ingestion of an infected bloodmeal, the highly motile nectomonad promastigotes predominate within the midgut lumen. Killick-Kendrick *et al.* (1974) noted that these were a non-dividing form of promastigote while studying *L. amazonensis* in *Lu. longipalpis*. Later studies by Walters *et al.* (1989a) and Lawyer *et al.* (1990), observing *L. mexicana* in *Lu. abonnenci* and *L. major* in *Ph. duboscqi*, respectively, were both in agreement with this. However, several other studies using various parasite/sandfly combinations found that the nectomonad promastigotes did undergo division within the lumen of the midgut (Lawyer *et al.* 1987; Walters *et al.* 1989a). Walters *et al.* (1989a) observed that nectomonad promastigotes released into the anterior and posterior midgut initiated a third round of multiplication and noted a consequence of this division was the appearance of a population of smaller nectomonad promastigotes which also divided.

Massive forward migration of parasites is mediated by the nectomonad promastigote. A recent study by Rogers *et al.* (2002) showed that the nectomonad promastigotes are the first parasites to arrive at the thoracic midgut and appeared in small numbers as early as day 2 of infection, with increased migration at 3 days. Walters *et al.* (1987) also observed massive forward migration of infection at day 3.

The small dividing nectomonad promastigotes described by Walters *et al.* (1989a) have been observed by several other investigators (Walters *et al.* 1987, 1993; Lawyer *et al.* 1990; Warburg *et al.* 1986) and the forward migration of nectomonad promastigotes coincided with the increased appearance of these "short promastigotes" (Walters *et al.* 1987). The study of Warburg *et al.* (1986) indicated that the elongated nectomonad forms gradually transformed to shorter promastigotes. Using electron microscopy they observed the presence of short promastigotes with their flagella inserted between the microvilli of the thoracic midgut and termed these "intermediate forms". By day 4-5 of infection these are the predominant form found in the thoracic midgut (Rogers *et al.* 2002) and anterior cardia region (Lawyer *et al.* 1987; Walters *et al.* 1987, 1989a, 1993). It has been proposed by Rogers *et al.* (2002) that these short unattached promastigotes be termed leptomonad (*leptos* = small) promastigotes and should not be confused with haptomonad promastigotes that are short attached promastigotes (Lawyer *et al.* 1987, 1990; Nieves & Pimenta, 2000).

A common occurrence reported for several *Leishmania*/sandfly combinations including *L. donovani/Ph. argentipes* (Shortt & Swaminath, 1928), *L. mexicana/Lu. diabolica* (Lawyer *et al.* 1987), *L. mexicana/Lu. abonnenci* (Walters *et al.* 1987), *L. major/Ph. papatasi* (Kellick-Kendrick *et al.* 1988), *L. chagasi/Lu. longipalpis*

(Walters *et al.* 1989a), *L. braziliensis panamensis/Lu. gomezi* (Walters *et al.* 1989b), *L. major/Ph. duboscqi* (Lawyer *et al.* 1990), *L. major/Lu.longipalpis* (Stierhof *et al.* 1999) and *L. mexicana/Lu. longipalpis* (Rogers *et al.* 2002) is the formation of a mass of parasites embedded within a gel-like substance at the stomodeal valve. Walters *et al.* (1987) described this gel-like substance as a matrix of electron dense strands that exhibited a positive Periodic Acid-Schiff reaction for carbohydrates. The accumulation of this gelatinous matrix was seen as early as day 3 of infection (Lawyer *et al.* 1987), in *Ph. duboscqi* infected with *L. major*. However, more commonly it is seen on days 4-5 of infection (Lawyer *et al.* 1990; Walters *et al.* 1987, 1989a; Rogers, 2002). This coincides with the appearance of leptomonad promastigotes and Killick-Kendrick (1979) suggested that it could be parasitic in origin. Rogers *et al.* (2002) concluded that the leptomonad promastigotes are the parasite form responsible for the production of the gelatinous matrix. This conclusion was based on observations that dissected matrices from sandflies with mature infections were found to contain predominately leptomonad promastigotes. With the conclusion that the gel is parasite derived (Stierhof *et al.* 1999), Rogers *et al.* (2002) proposed the term promastigote secretory gel (PSG) to describe the gel-like matrix.

A recent study by Stierhof *et al.* (1999) using immunoflorescence and immunoelectron microscopy identified a major component of the gel-like matrix to be a parasite derived mucin-like filamentous proteophosphoglycan (fPPG). The specifics of fPPG are discussed in section 2.6.2.2. Motility of parasites within the PSG is greatly retarded, leading to a massive swelling of the cardia and stomodeal valve (Walters *et al.* 1989b; Lawyer *et al.* 1990), however when the PSG was diluted with

dissection medium the motility of the parasites increased (Lawyer *et al.* 1990; Rogers *et al.* 2002).

Haptomonads also play a role in the dysfunction of the stomodeal value. They are seen attached to the cuticular surface of the valve via the formation of hemidesmosome-like junctions (Killick-Kendrick *et al.* 1974). The origin of haptomonad promastigotes is unclear but Rogers *et al.* (2002) suggested that they may originate from leptomonad forms given their similar sizes. It could possibly be that they are leptomonad promastigotes that simply reach the stomodeal valve first and attach. It has been suggested that attached haptomonad promastigotes are altruistic in nature in order to help in the production of a "blocked fly" (discussed below).

The final step in the development of *Leishmania* parasites in the sandfly is the differentiation into metacyclic promastigotes. Adler and Theodor (1931) described very short flagellates with flagella longer than the body for *L. donovani* in *Ph. perniciosus*. They suggested that these highly active promastigotes were the end products of parasite development within the sandfly and considered them the most likely form to enter the host during feeding. In a study by Sacks and Perkins (1984) it was shown that *Leishmania* promastigotes sequentially develop into an infective form, both *in vivo* and *in vitro*. They found that promastigotes taken from the midguts of *Lu. anthophora* infected with *L. tropica* 3 days after infection were avirulent, whereas those that were taken from flies with day 7-10 old infections were able to generate footpad lesions in BALB/c mice. The same results were observed with logarithmic and stationary phase cultures, respectively.

Metacyclic promastigotes are generally seen in increasing numbers within the anterior midgut and foregut from days 4-5 of infection (Lawyer *et al.* 1987; Rogers *et al.* 2002). The origin of metacyclic promastigotes seems likely to be leptomonad promastigotes (Rogers *et al.* 2002; Walters *et al.* 1993), as these forms are seen trapped within the PSG which is probably the major site of metacyclogenesis (Rogers *et al.* 2002). Upon differentiation of leptomonad promastigotes into metacyclic forms within the PSG motility is unhindered (Lawyer *et al.* 1990) and metacyclic forms are seen to be extremely active.

The appearance of infective metacyclic promastigotes coincides with seeking of another bloodmeal by the sandfly. Taken together the timing and position of the metacyclic promastigotes along with the "blocked fly" phenomenon greatly facilitates their transmission. By the generation of a blocked fly it is thought that the sandfly experiences difficulty when feeding. The physical barrier that the PSG poses makes it almost impossible for the passage of blood into the midgut, leading to multiple probing (Beach *et al.* 1984). Schlein *et al.* (1992) showed that the stomodeal valves of *Leishmania* infected sandflies are damaged by the action of chitinase, and remain permanently open. This study suggested that regurgitation of blood during impaired feeding carries infective parasites from the midgut (PSG) into the host via the proboscis. Therefore, three factors are responsible for impaired feeding of infected sandflies; haptomonad attachment, PSG and damage to the stomodeal valve. As well as changes to the feeding behaviour of the sandfly aiding transmission of leishmaniasis, metacyclic promastigotes are pre-adapted for life in the vertebrate host. These adaptations are mainly the result of conformational changes to the metacyclic LPG (see below).

## 2.3 *Leishmania* (*Viannia*)

Members of the subgenus *Viannia* require a stage of development within the hindgut of the sandfly. Unfortunately very little research has been done to investigate the detailed development of *Leishmania* (*Viannia*) within their sandfly hosts. However, one significant study by Walters *et al.* (1989b) did examine the ultrastructural biology of *L.*(*V*). *panamensis* within *Lu. gomezi*, a natural parasite/vector combination. Initial development is similar to that involving species of the subgenus *Leishmania*, with "stumpy promastigotes" intensely multiplying for up to three days. After this time the peritrophic matrix begins to breakdown and the majority of parasites migrate to the hindgut, however, it should be noted that some parasites did migrate anteriorly to the midgut. By day 3 of infection nectomonad forms were found colonising the hindgut (Walters *et al.* 1989b, Rangel *et al.* 1992, Nieves & Pimenta, 2000) along with numerous haptomonad forms, attached to the cuticula intima via hemidesmosomal plaques. Day 6 of infection saw a massive anterior migration of parasites to the cardia region of the midgut. In light of the publication by Rogers *et al.* (2002), the leptomonad form of promastigote appears responsible for this forward migration, termed "short promastigotes" by Walters *et al.* (1989b). From here on the development is similar to that of the subgenus *Leishmania*, including the formation of the PSG.

## 2.4 *Phlebotomine* Sandflies as Vectors of *Leishmania*

All human leishmaniases are transmitted by phlebotomine sandflies, belonging to the order Diptera, family Psychodidae and subfamily Phlebotominae. There are

approximately 700 species on sandfly in five genera, although, only species within three genera feed of vertebrate blood, *Phlebotomus, Lutzomyia* and *Sergentomyia.* However, only species belonging to genera *Phlebotomus* in the Old World, and *Lutzomyia* in the New World, are of medical importance as disease vectors. Of the 700 species of sandfly approximately 70 are thought to be involved in the transmission of disease including various *Leishmania* parasites, bartonellosis and sandfly fever virus. Sandflies are found mainly in the tropics and subtropics, although some species are found in the temperate regions of both the northern and southern hemispheres. They occur in a wide range of habitats, from sea level to altitudes of 2800 m in the Andes and from hot dry deserts to tropical rainforests.

Adult flies are small in size, 1.3-3.5 mm in length, light brown to black in colour, have long stilt-like legs and hold their wings tent-like over the abdomen when at rest. Characteristically the head, thorax, abdomen and wings are densely covered with long hairs (Figure 2.3). Male and female flies can be distinguished by the prominent pair of claspers at the end of the male abdomen (Figure 2.3). Relatively little is known regarding egg and larval development in nature, although immature developmental stages have been found on occasions (Feliciangeli, 2004). Larvae of all known species have the characteristic matchstick hairs and caudal bristles, although their functions are not known.

Adult flies are crepuscular or nocturnal and most biting occurs during these periods. However, biting can occur during the day on darkened overcast days or if the flies are disturbed. They are relatively weak fliers and do not usually fly distances of more than a few hundred metres from their breeding places, although mark-release studies

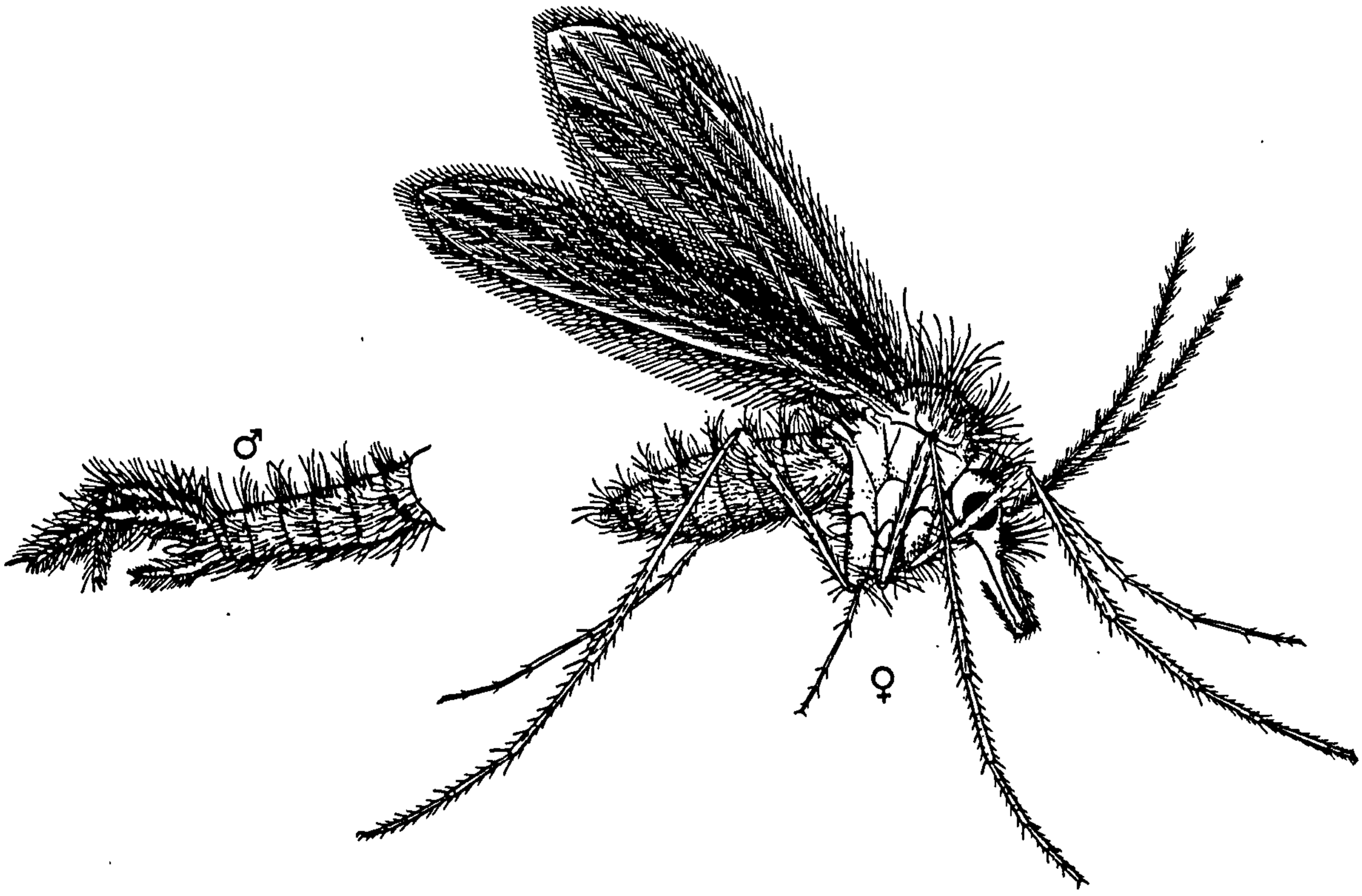



**Figure 2.3** Drawing showing the distinction of male (A) and female (B) sandflies by the presence of the external genitalia in males. Reproduced from Lane (1993).

have recaptured flies more than 2 km from the release point (Killick-Kendrick, *et al.* 1984).

Both males and females feed on sugary solutions, either sap or honeydew. However, only female sandflies feed on the blood of vertebrates as they require the proteins from a bloodmeal for egg development. Most species are gonotrophically concordant with oviposition occurring 3-8 days after feeding. However, there are species that are able to develop eggs without a bloodmeal (*Ph. papatasi*) (Lane, 1993). Sandflies are pool feeders, creating a small pool of blood on exposed areas of skin with their small serrated mandibles used in a scissor-like manner. Due to the small mouth parts of sandflies they cannot bite through clothing and can only feed on exposed areas of skin.

## 2.5 Species- specific Vector Competence

Killick-Kendrick (1985) proposed a close evolutionary relationship between *Leishmania* parasites and the sandflies that transmit them. It is known that only certain sandflies are capable of transmitting certain species of *Leishmania* species in nature (Table 2). For example, experimental infection of *Sergentomyia schwetzi* with *L. major* resulted in a total loss of infection by day 4, although up until this point infection was comparable with *L. major* in *Ph. duboscqi* (Lawyer *et al.* 1990). Much earlier experiments by Adler *et al.* (1928, 1938) showed that *Ph. papatasi* will support the full development of *L. major* but is refractory to infection with a number of other species. There are two major schools of thought regarding the development of *Leishmania* parasites in unnatural vectors, firstly susceptibility to midgut proteolytic

| *Leishmania* species | Distribution | Vector |
|---|---|---|
| *L. donovani* | Indian subcontinent<br>Sudan<br>Kenya | *Ph. argentipes*<br>*Ph. orientalis*<br>*Ph. martini*<br>*Ph. celiae* |
| *L. infantum* | Mediterranean basin<br>South America | *Ph. perniciosus*<br>*Ph. ariasi*<br>*Lu. longipalpis* |
| *L. tropica* | Central and West Asia<br>Kenya | *Ph.sergenti*<br>*Ph. guggisbergi* |
| *L. major* | Cental and West Asia<br>North Africa<br>Sahel | *Ph. papatasi*<br>*Ph. papatasi*<br>*Ph. duboscqi* |
| *L. aethiopica* | Ethiopia, Kenya | *Ph. longipes*<br>*Ph. pedifer* |
| *L. mexicana* | Central and South America | *Lu. olmeca olmeca* |
| *L. amazonensis* | South America | *Lu. flaviscutellata* |
| *L. braziliensis* | Central and South America | *Lu. whitmani*<br>*Lu. wellcomei* |
| *L. peruviana* | Peru | *Lu. peruensis*<br>*Lu. verrucarum* |
| *L. guyanensis* | South America | *Lu. umbratilis*<br>*Lu. anduzei* |
| *L. panamensis* | Central America | *Lu. trapidoi*<br>*Lu. ovallesi* |

**Table 2.1** *Leishmania* species, their distribution and proven natural vectors. Adapted from Ashford and Bates (1998) and Feliciangeli (2004).

enzymes within the bloodmeal (Schlein & Romano, 1986; Borovsky & Schlein, 1987), and secondly midgut attachment (Pimenta *et al.* 1994).

### 2.5.1 Modulation of proteolytic gut enzymes

The midguts of haematophagous insects are hostile environments, as bloodmeal digestion is accompanied by the release of proteolytic enzymes and can have important implications on the development of protozoa (Gooding, 1975). Studies by Dillon and Lane (1993a) reported that within 10 hours of a bloodmeal protease activity significantly increases in the midgut of *Ph. papatasi* and *Ph. langeroni*. They found that activity peaked at 24-34 hours post-feeding in *Ph. papatasi* but was delayed slightly in *Ph. langeroni*, peaking at 34-48 hours. Similarly, Schlein and Jacobson (1998) reported that the levels of alkaline protease, trypsin and aminopeptidase peaked at 28 hours in bloodfed *Ph. papatasi*.

It is known that many *Leishmania* infections fail in the initial stages with an unnatural vector (Killick-Kendrick, 1985). In 1938 Adler reported that plasma in bloodmeals artificially fed to *Ph. papatasi* prevented the growth of unnatural *Leishmania* parasites, and found that by reducing the levels of plasma within the bloodmeal the infection rate of unnatural *Leishmania* species were enhanced, presumably due to a reduction in the activity of proteolytic enzymes. This effect was confirmed using the unnatural combination of *L. donovani* and *Ph. papatasi* (Schlein & Jacobson, 1998). Three days following blood-feeding only 5% of sandflies were infected compared to 67% that had been feed an infected meal of erythrocytes in saline. The increase in infection rate corresponds to a decrease in the alkaline protease activity by 39.4%,

aminopeptidase activity by 35% and trypsin activity by 53.8%, possibly resulting from the 25% reduction in protein content. These results suggest that in nature the proteolytic enzymes are important in determining parasite/vector competence.

Schlein and Romano (1986) suggested that the reduced proteolytic activity observed in *L. major* infection was a result of the parasites ability to inhibit enzyme production. They found that flies infected with *L. major* had proteolytic activities approximately 28% below the control group, whereas, sandflies infected with *L. donovani* had proteolytic activity 32.4% above that of the control group. A subsequent investigation showed that the amount of trypsin and chymotrypsin-like enzymes in *L. major* infected *Ph. papatasi* was reduced to 34% of the control activity at 30 hours post-feeding, compared to 86% in *L. donovani* infected *Ph. papatasi* (Borovsky & Schlein, 1987). The addition of soybean trypsin inhibitor enabled *L. donovani* to survive within *Ph. papatasi*, thereby indicating that *L. major* is somehow able to naturally modulate trypsin-like activity in order to survive. Studies by Dillon and Lane (1993b) found that *L. major* infections were able to delay and lower the peak levels of protease activity in the midgut of *Ph. papatasi*. They suggested that a parasite induced delay in protease activity and therefore bloodmeal digestion, may allow an extension in the availability of nutrients to the growing parasites. These findings are in agreement to those of other investigators (Schlein & Romano, 1986; Borovsky & Schlein, 1987). However, it is difficult to make direct comparisons between these studies as different conditions were used to initiate sandfly infections, those of Dillon and Lane (1993b) being the closest to natural conditions.

The survival of an LPG-deficient strain of *L. major* in *Ph. papatasi* was shown to be enhanced by the addition of released glycoconjugate of the vector-specific strain of *L. major* (Schlein *et al.* 1990). Released glycoconjugates were isolated from *in vitro* cultures and are known to be composed of phosphoglycan-containing molecules including LPG, PPG and SAP (Ilg *et al.* 1994a). Infection rates of the LPG-mutant increased from 15.6% to 63%, on day 4, with addition of *L. major* released glycoconjugate, compared with 86% for the indigenous *L. major* strain. However, when the released glycoconjugate of *L. donovani* was added with the LPG-mutant, no enhancement of infection occurred, increasing to 17.7% from 15.6%. These data suggest that the glycoconjugates of *Leishmania* species are vector specific and may play a role in the modulation of proteolytic enzymes.

A more recent study by Sacks *et al.* (2000) has shown that LPG is not necessary for early infection and only plays a minor part in the modulation of proteolytic enzymes. This is in disagreement with earlier studies by Schlein *et al.* (1990). The reason for this is possibly due to the mutant strains of *Leishmania* used. Schlein *et al.* (1990) used *L. major* L119, a strain that is deficient in the synthesis of the disaccharide backbone of the repeat units of LPG (McConville & Homans, 1992), however, it is known that many PG containing molecules share a common backbone. It is therefore possible that L119 is also deficient in the synthesis of several other PG containing molecules as well as LPG. Two mutant strains were used by Sacks *et al.* (2000), *LPG1*$^{-}$ *L. major* and *L. donovani*; deficient in LPG but still able to assemble and secrete other PG molecules (Ryan *et al.* 1993; Huang & Turco, 1993), and *LPG2*$^{-}$ *L. donovani*; deficient in the synthesis of all PG molecules (Descoteaux *et al.* 1995; Ma *et al.* 1997). Data from this study while indicating that LPG is not required showed

that PG synthesis is necessary for early bloodmeal survival. This is in agreement with studies that observed a delay (day 3) in the expression of LPG on newly transformed promastigotes (Saraiva *et al.* 1993). This adds further to the suggestion that LPG plays little or no part in the protection of parasites against proteolytic enzymes in the sandfly midgut; as the peak of proteolytic activity is known to occur at approximately 30 hours post feeding (Borovsky & Schlein, 1987; Dillon & Lane, 1993b).

### 2.5.2 Midgut Attachment

Several studies have followed the development of *Leishmania* species in unnatural vectors and found no obvious inhibition in the early development and growth of the parasites. Infection rates for *L. major* in *Sergentomyia schwetzi* were comparable with those in its natural host *Ph. papatasi* (Lawyer *et al.* 1987), likewise infection rates with *L. tropica* were comparable between its natural vector *Ph. sergenti* and *Ph. papatasi* (Kamhawi *et al.* 2000a). However, infections within the unnatural vectors were completely lost immediately following defecation of the bloodmeal.

Developmental changes in the terminal sugar residues of LPG are known to occur between non-infective and metacyclic promastigotes (Sacks *et al.* 1985) and following experiments by Pimenta *et al.* (1992), LPG was shown to be involved in the interactions between parasites and sandfly midgut epithelial cells. Due to the extensive species-specific polymorphisms observed between different LPGs (see section on LPG for details) and its involvement in midgut attachment, it was suggested that this molecule could be responsible for vector specificity.

*Ph. argentipes* (a natural vector of *L. donovani*) supported the full development of several unnatural *Leishmania* species but infection with an LPG-deficient strain of *L. donovani* was completely absent by day 5 (Pimenta *et al.* 1994). In contrast *Ph. papatasi* would only support the complete development of *L. major*, infections with all other *Leishmania* species failed to maintain infections following bloodmeal digestion and passage. Studies using *L. major*, *L. donovani* and *L. tropica* have shown clear vector specificity of *Ph. sergenti* for *L. tropica* (Kamhawi *et al.* 2000a). Infections with *L. major* and *L. donovani* were lost closely following the passage of the digested bloodmeal, in agreement with the observations of Pimenta *et al.* (1994).

*In vitro* analysis of midgut binding of procyclic promastigotes revealed that only *L. major* was capable of binding to the midgut of *Ph. papatasi*, although this ability was absent in LPG-deficient strains (Pimenta *et al.* 1994). Similar findings were reported for *in vitro* binding of *L. tropica* by *Ph. sergenti* with no binding of *L. major* or *L. donovani* (Kamhawi *et al.* 2000a). In contrast, the midguts of *Ph. argentipes* were capable of binding several *Leishmania* species with the exception of an *L. donovani* LPG-deficient strain (Pimenta *et al.* 1994). Therefore, the *in vitro* binding pattern of promastigotes to different sandfly midguts is indicative of which combinations will result in survival of midgut infections. The role of LPG in midgut attachment of promastigotes has been conclusively proven in a recent study using an *L. major* LPG-deficient mutant (Sacks *et al.* 2000). The LPG-mutant strain was unable to retain its infection within *Ph. papatasi* following passage of the bloodmeal. However, when LPG expression was restored 40% of flies retained infections compared to 66% infection with the wild-type strain. The observation of Saraiva *et al.* (1993) that LPG

expression is delayed, coinciding with the appearance of nectomonad promastigotes and escape from the PM, is in agreement with LPG being responsible for midgut binding. The identification of a 65 kDa protein in *Ph. papatasi* as a receptor for *L. major* LPG (Dillon & Lane, 1999) adds further weight to this argument. It therefore seems likely that vector specificity is a combination of both modulation of midgut enzymes and LPG-dependent midgut binding.

## 2.6 Major Surface and Secreted Molecules

Several phosphoglycan molecules have been identified over the last two decades including lipophosphoglycan (LPG), secreted acid phosphatase (SAP), filamentous proteophosphoglycan (fPPG) and a non-filamentous proteophosphoglycan from amastigotes (aPPG). The parasites have a remarkable capacity to avoid destruction in the hostile environments encountered in both the sandfly vector and the mammalian host. The surface and secretory antigens form an interface between the parasite and its surroundings. It is therefore conceivable that these molecules play major roles in the transmission, virulence and survival of *Leishmania* parasites in these hostile environments.

### 2.6.1 Lipophosphoglycan

Lipophosphoglycan (LPG) was first described in *L. donovani* (Kaneshiro *et al.* 1982) and is the major surface glyconjugate found on all promastigotes studied to date. LPG is a major component of the glycocalyx that is distributed over the surface of the

parasite, including the flagellum and flagellar reservoir. It has been estimated that 1-5 x $10^6$ copies of LPG are found on each promastigote (Turco *et al.* 1987).

The LPG molecules characterised to date are constructed of four domains consisting of a lyso-alkylphosphatidylinositol membrane anchor, a phosphoglycan core, repeating disaccharide-phosphate units with saccharide side chains, and a terminating capped structure at the non-reducing end (Ilg *et al.* 1994a) (Figure 2.4). Structural analyses of LPG from several *Leishmania* species including *L. major* (McConville *et al.* 1990) and *L. mexicana* (Ilg *et al.* 1992) indicate that the lipid anchor, the phosphoglycan core and the backbone of the disaccharide-phosphate units are conserved, whereas extensive variability exists in the carbohydrate content of the side chains and the terminating cap.

The common backbone of LPG is composed of repeating disaccharide units of Galβ1-4Manα1 $PO_4$-6 (Sacks *et al.* 1995). In these repeating units the 3-position of the Gal can be substituted for a variety of saccharide side chains. These substitutions can either be complex as in *L. major* where Gal is substituted with side chains consisting of one to four saccharide residues of β-Glc, β-Gal or β-Ara (McConville *et al.* 1990), simple as in *L. mexicana* where the Gal is replaced with βGlc (Ilg *et al.* 1992) or non-existent as in *L donovani* where no substitutions are seen (Turco *et al.* 1987).

Developmentally regulated modifications are seen in LPG molecules during metacyclogenesis (Sacks *et al.* 1985). In a study by Sacks and colleagues (1990)

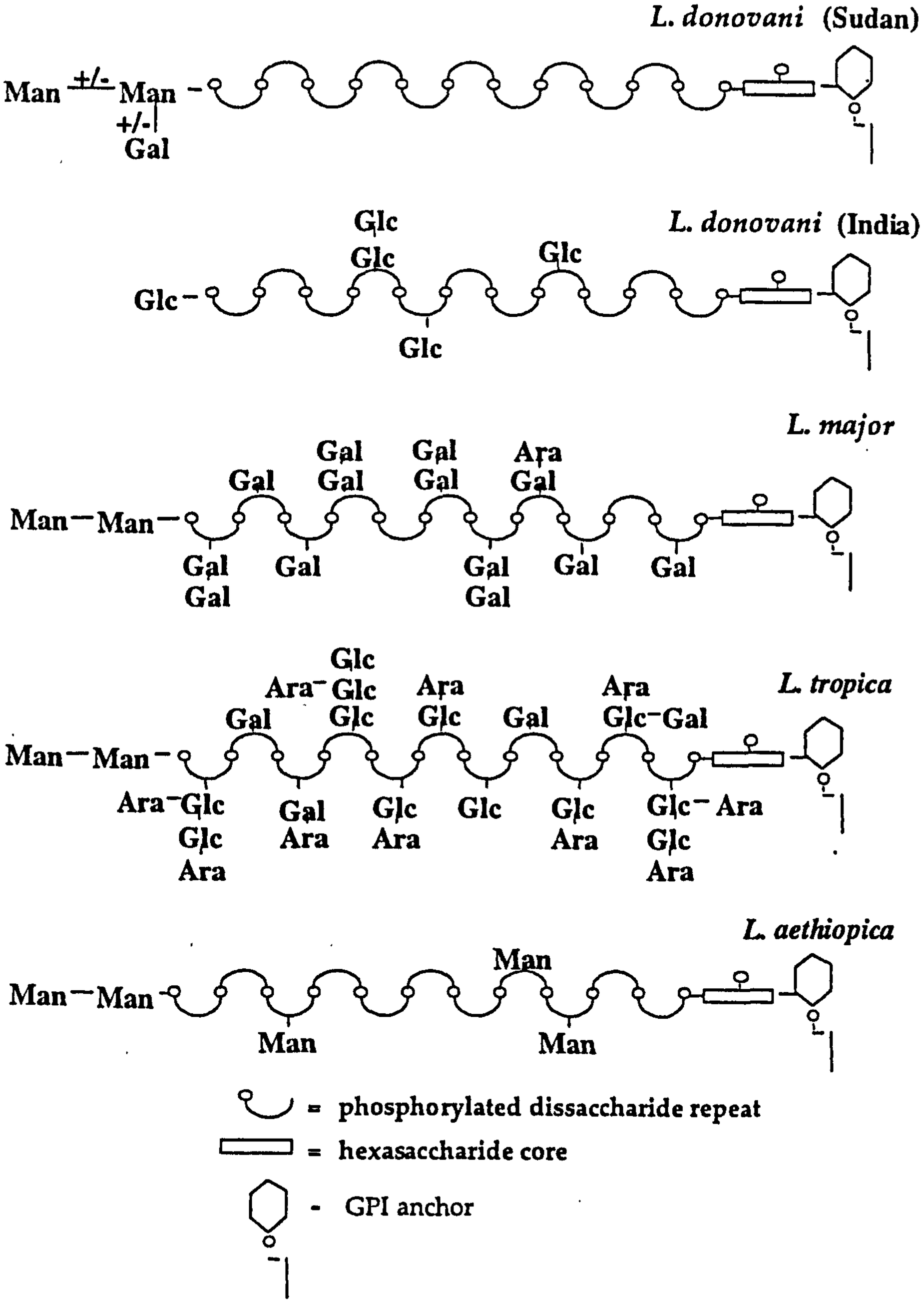



**Figure 2.4** Schematic representation of the species specific polymorphisms of LPGs from Old World *Leishmania* species. The oligosaccharide core and lipid anchor domains are conserved between the species. The phosphoglycan domains are highly diverse and species specific. Reproduced from Sacks (2001).

using *L. major*, it was demonstrated that the size of the LPG molecule observed on the metacyclic form of promastigote had approximately doubled in size from that of the procyclic form. This elongation was a direct result of a doubling in the number of repeating oligosaccharide-phosphate units per LPG molecule. In the same study it was also observed that the terminating residues of the repeating side chains differed between the two promastigote forms. In procyclic promastigotes these terminated in β-Gal, whereas in metacyclic promastigotes the terminating residue changes to α-Ara or β-Glc.

It is thought that this change in the terminating residues assists the migration of the metacyclic promastigotes to the anterior of the sandfly (Pimenta *et al.* 1992), a position that promotes transmission to the mammalian host. It has been shown that in *L. major*, adhesion of procyclic promastigotes to midgut epithelial cells of the sandfly is mediated by β-Gal terminating residues (Sacks *et al.* 1990). The up-regulation of terminal α-Ara or β-Glc allows anterior migration of the metacyclic promastigotes, since these residues do not act as ligands for the midgut epithelium. Similar studies on *L. donovani* (Sacks *et al.* 1995) have shown comparable developmental modifications in the LPG molecule. As well as having functional roles in the sandfly, a number of other roles have been proposed for LPG in the mammalian host.

### 2.6.2 Proteophosphoglycans

As well as lipophosphoglycan *Leishmania* species synthesise another family of molecules collectively termed proteophosphoglycans (PPG). Five different phosphoglycan-modified proteins have been identified: secreted acid phosphatase

(SAP); secreted filamentous PPG (fPPG); secreted non-filamentous amastigote PPG (aPPG); secreted promastigote PPG2 (pPPG2); and glycosylphosphatidylinositol (GPI) membrane anchored PPG (mPPG).

### 2.6.2.1 Secreted Acid Phosphatase

Gottlieb and Dwyer (1982) first identified SAP in the culture medium of *L. donovani*. The enzyme has subsequently been identified in all major *Leishmania* species infective to humans (Lovelace & Gottlieb, 1986, Shakarian & Dwyer, 2000). Using *L. donovani* Bates and Dwyer (1987) showed SAP to be the major secretory protein released into the culture medium by promastigotes during *in vitro* culture. Immunochemical localisation studies (Bates *et al.* 1989) provided direct evidence that SAP is exocytosed via the flagellar reservoir membrane and not through the general surface membrane of promastigotes.

The SAP of *L. donovani* is a highly glycosylated protein of 110-130 kDa (Bates & Dwyer, 1987). Modification of this enzyme was shown to occur by N-linked glycans in the endoplasmic reticulum (Bates & Dwyer, 1987) and by the post-transcriptional addition of an LPG related acid-labile phosphoglycan in the Golgi complex (Bates *et al.* 1990). Ilg and colleagues (1991) compared the composition and ultrastructure of the SAP from *L. donovani* and *L. mexicana*. In this study it was found that the two species secreted SAPs that differed in both of these properties. *L. donovani* SAP was shown to have the following composition: 57% protein, 28.5% carbohydrate and 14.5% phosphate, whereas the *L. mexicana* enzyme contained considerably more carbohydrate and less protein being composed of, 13.3% protein, 74.4% carbohydrate

and 12.3% phosphate. The same study revealed the structure of *L. donovani* SAP to be oligomeric whereas the SAP from *L. mexicana* was shown to have a polymeric structure.

The SAP from *L. mexicana* is probably the best studied of the SAPs. It was shown to be a complex composed of a 100 kDa proteophosphoglycan, SAP1, and a 200 kDa proteophosphoglycan, SAP2 (Ilg *et al.* 1991). Following monoclonal antibody analysis it was proposed that the components of the complex were secreted into the lumen of the flagellar reservoir, where they subsequently polymerised into filaments before being released into the culture medium (Ilg *et al.* 1991). Electron microscopy by Ilg and colleagues (1991) revealed that SAP from *L. mexicana* consists of long filaments composed of a central chain of protein subunits (SAP1) surrounded by a glycocalyx composed of proteophosphoglycan (SAP2).

Both components of the SAP complex of *L. mexicana* are glycosylated with short chains of O-linked phosphoglycans and phosphamannose/phosphomannans (Ilg *et al* 1994b). Using chemical analyses Ilg and colleagues (1994b) showed that these substituted oligosaccharides appear to be linked, via the sequence Manα-1-$PO_4$, to serine residues in the polypeptide backbone. This form of O-glycosylation is a novel and unique form not seen before and has been termed phosphoglycosylation. The phosphoglycosylation patterns of the SAP complex are similar to those found on LPG of the same parasite. There are, however, differences which make the phosphoglycosylation of SAP distinct. SAP has considerably shorter phosphoglycan chains than LPG and also possesses mannan oligosaccharides with longer chains ($Man_2$-$Man_6$) compared to those of LPG that are $Man_2$-$Man_3$ (Ilg *et al.* 1994b).

As well as this novel phosphoglycosylation both SAP1 and SAP2 also contain N-linked glycans.

The *L. mexicana* genes encoding the 100 kDa phosphoglycoprotein and a 200 kDa proteophosphoglycan, *lmsap1* and *lmsap2* respectively, have now been identified, cloned and sequenced (Wiese *et al.* 1995). *lmsap1* and *lmsap2* are tandemly arranged single copy genes that differ only in the length of the C-terminal Ser/Thr-rich domains, *lmsap1* having 32 and *lmsap2* having 383 amino acids. Post-transcriptional modification of SAP1 and SAP2 occurs via phosphoglycosylation in the Ser/Thr-rich domains (Wiese *et al.* 1995).

The function of this enzyme in the *Leishmania* life cycle still remains elusive. However, Wiese and colleagues (1995) showed that SAP is not essential for cultivation of *L. mexicana* promastigotes. In a later study, Wiese (1998), using deletion analyses, identified that SAP is not necessary for the infection of macrophages or mice. It is therefore conceivable that SAP has no relevant function in the mammalian host. This hypothesis is strengthened by the observation of Ilg and colleagues (1991) that *L. mexicana* amastigotes do not synthesise SAP. However, it has been observed that amastigotes of *L. donovani* produce SAP (Bates *et al.* 1989). This may possibly be a reason as to why one species causes a localised infection and the other is able to spread to the viscera. It could be that SAP is important for infection of the sandfly vector where it may have a nutritional function but knockout experiments are required to test this.

### 2.6.2.2 Filamentous PPG

During studies by Stierhof and colleagues (1994) characterising the release of SAP from *L. mexicana* another secreted phosphoglycan-modified filamentous product was identified. It was observed that this fibrous material consisted of complex phosphoglycans and was secreted via the flagellar reservoir (Stierhof *et al.* 1994). These fibrous filaments are found at the centre of promastigote aggregates where they form a network between and around the cells. In a subsequent study, column chromatography and ultracentrifugation were used to purify the filamentous phosphoglycan-modified compound from *L. major* (Ilg *et al.* 1996). Structural analysis of this novel secretory molecule characterised it as a proteophosphoglycan (Ilg *et al.* 1996). Biochemical analysis of this PPG showed that 95% of its mass consisted of phosphoglycans, whilst amino acids composed only 5% of the mass. Of the amino acids present, 52% were found to be serine, 21% alanine and 14% proline. It was also noted that large proportions of the serine residues (84-90%) were phosphoglycosylated by short phosphodiester-linked glycan chains. This phosphoglycosylation was the same as that previously seen in the SAP of *L. mexicana* (Ilg *et al.* 1994b).

A recent study by Stierhof and colleagues (1999), using *L. mexicana* in *Lu. longipalpis* and *L. major* in *Ph. papatasi*, has shown that filamentous proteophosphoglycan (fPPG) is a major component of the gel-like plug material found blocking the stomodeal valve of infected sandflies. Within the plug material the fPPG forms a gel-like three-dimensional network within which the parasites become embedded but still remain viable (Lawyer *et al.* 1990). Blockage of the stomodeal

valve in *Leishmania*-infected sandflies is a common event and has been observed in many parasite-fly combinations as described above. It is proposed that this blockage of the stomodeal valve aids transmission of the infective metacyclic promastigotes. Flies with this blockage find it difficult to fully engorge and this leads to multiple probing (Beach *et al.* 1984). As well as aiding transmission by multiple probing the occlusion of the stomodeal valve, caused by this blockage, may lead to a reflux of parasites from the midgut to the foregut and therefore deposition into the bite wound.

A recent study has shown that the PSG is responsible for an exacerbation of disease in mice (Rogers *et al.* 2004). Within this study the role that fPPG played in the observed exacerbation was examined. Mild acid hydrolysis was used to deglycosylate fPPG, this consequently resulted in the loss of exacerbative ability. However, they found that a chemically synthesised *Leishmania* PG (Nikolaev *et al.* 1995) was able to confer exacerbative properties in infections with an *L. mexicana* *lpg2*$^{-/-}$, while a chemically synthesised molecule that mimics the backbone of *L. mexicana* fPPG did not have any effect on the outcome of infection. These data therefore indicate that glycan moieties of fPPG are responsible for the exacerbation seen during infection.

### 2.6.2.3 Amastigote PPG/Promastigote PPG2

Bahr and colleagues (1993) were the first to identify a high molecular weight phosphoglycan from *L. mexicana* amastigotes and found it to be immunologically related to LPG and SAP. This phosphoglycan was eventually purified from mouse lesions resulting from *L. mexicana* infection, and following amino acid analysis identifying the presence of large amounts of serine residues on the polypeptide

backbone, was characterised as a proteophosphoglycan (Ilg *et al.* 1995). Ilg and colleagues (1995) demonstrated that aPPG is secreted from the flagellar reservoir as is SAP (Bates *et al.* 1989). Macrophage vesicles containing aPPG were detected either proximally or distally from the parasitophorous vacuole (Ilg *et al.* 1995) and could possibly have arisen via budding from the parasitophorous vacuole. It is, therefore, possible that these vesicles containing aPPG act as a transport mechanism, taking the aPPG to host cell membrane for secretion.

In another recent study Klein *et al.* (1999) identified a novel promastigote PPG, pPPG2. Using mAbs and electron microscopy this newly identified proteophosphoglycan was shown to be immunologically and morphologically distinct from SAP, fPPG and mPPG. Amino acid analysis of pPPG2 and aPPG suggested that the two might have the same protein backbone (Klein *et al.* 1999). However aPPG and pPPG2 differ in their molecular weight, ultrastructure and protease sensitivity and it is proposed that these are a direct result of stage-specific glycosylation of the shared protein backbone (Klein *et al.* 1999) (Figure 2.5). Phosphoglycosylation of serine occurs at approximately the same degree in both molecules but in pPPG2 the ser-linked glycans are of much shorter length than those observed in aPPG. A recent study (Gopfert *et al.* 1999) has identified and cloned the gene encoding aPPG and pPPG2 in *L. mexicana, ppg2.*

The function of pPPG2 has not yet been elucidated. It has however been shown that aPPG may contribute to the formation of the huge parasitophorous vacuole often seen in macrophages infected with *L. mexicana* (Peters *et al.* 1997a). It has also been

demonstrated that aPPG is a potent activator of the complement system via the mannan binding lectin pathway (Peters *et al.* 1997b). By activating complement with secreted aPPG, complement-mediated lysis of amastigotes may be prevented. As a result of complement activation the formation of the anaphylactic peptides C3a, C4a and C5a occurs, which consequently attract macrophages to the site of infection providing new host cells. As amastigotes are devoid of significant LPG production, aPPG may be an important virulence determinant for the mammalian stage of the parasite.

### 2.6.2.4 Membrane PPG

Another new addition to the proteophosphoglycan molecules of *Leishmania* parasites is the GPI anchored mPPG identified in *L. major* (Ilg *et al.* 1999). The same study has identified the gene responsible for encoding mPPG, *ppg1* and it was suggested that mPPG may extend 300nm above the plasma membrane. The function of this molecule is not yet known but due to its structure and numerous LPG-like phosphoglycans it may possibly function as a ligand for attachment to sandfly gut receptors and may also be a potential acceptor of complement.

Precise functions for PPG's in *Leishmania* parasites remain elusive, although it is conceivable that due to their LPG-like phosphoglycan repeats and cap structures they may be responsible for some of the functions previously attributed to LPG. Many of the LPG-specific mAbs used in these earlier studies are now known to cross-react with PPG. A recent study by Ilg (2000) showing that LPG is not required by *L. mexicana* for infection of mice or macrophages, strengthens this idea. Therefore

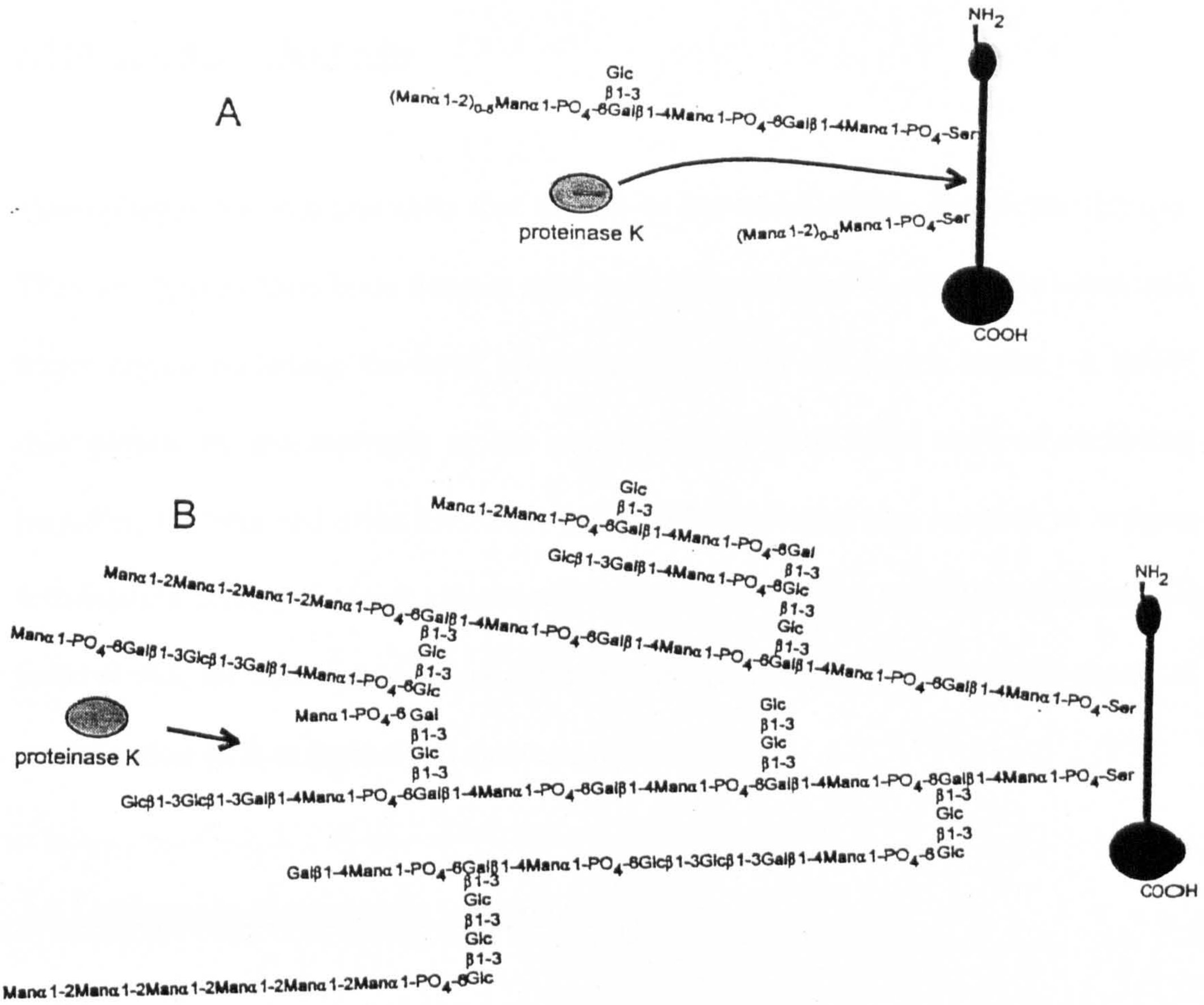



**Figure 2.5** Schematic representation of pPPG2 (A) and aPPG (B). (Reproduced from Klein *et al*. 1999).

many of these LPG functions need to be re-examined and with the identification, sequencing and cloning of the genes encoding the PPG's it should be possible using deletion analysis to retest these hypotheses.

## 2.7 Macrophages: host cells

Macrophages are immune cells that belong to the mononuclear phagocyte lineage. They are derived from bone marrow stem cells and are found in connective tissue and major organs including: the liver, bone marrow, spleen and lymph nodes. A major role played by macrophages is the engulfment of particulate material including parasites, bacteria and other infectious agents. Macrophages also function as antigen presentation cells; presenting antigen to T cells via MHC class I and II molecules. To achieve this, the macrophage must internalise the antigen and process it via chemical modification such as proteolytic cleavage (Roitt, 1996).

## 2.8 *Leishmania*-Macrophage Interactions

Upon entry into the host, metacyclic promastigotes encounter a major defence mechanism, complement. The complement system is composed of approximately 20 serum proteins that interact with each other via a complex array of proteolytic cleavages. The end result of this cascade of reactions is the formation of the membrane attack complex (MAC), which is inserted into the plasma membrane of the invading organism. As a consequence of MAC insertion lysis of the organism occurs, a process known as complement-mediated lysis.

The LPG on metacyclic promastigotes is efficient in activating the alternative complement pathway, resulting in the deposition of complement component C3b onto the metacyclic surface. This deposition generates the formation of the MAC but lysis of the metacyclic promastigote does not occur, prevented due to the developmentally regulated elongation of LPG (Sacks *et al* 1990), which is responsible for prevention of MAC insertion into the plasma membrane. Puentes and colleagues (1990) showed that the MAC is spontaneously shed from the surface of metacyclic promastigotes. The bound C3b that is not subsequently converted to C5 convertase acts as a ligand for interaction with the macrophage complement receptor CR1 (Da Silva *et al.* 1989) (Figure 2.6).

Another surface molecule, gp63, the major surface glycoprotein whose expression is upregulated in metacyclic promastigotes (Kweider *et al.* 1989), plays a crucial role in resistance to complement-mediated lysis (Brittingham *et al.* 1995). gp63 has proteolytic activity and can actively and can rapidly convert C3b to an antigenically similar inactive form iC3b. This iC3b is subsequently unable to form into C5 convertase and therefore prevents the formation of the MAC. Although iC3b is unable to participate in the complement cascade it remains opsonic and acts as a ligand for the macrophage complement receptor CR3 (Figure 2.6). By deposition of specific components of complement, namely C3b (Da Silva *et al.* 1989) and C3bi (Mosser & Edelson, 1985), on metacyclic promastigote surface molecules a high-affinity interaction with the macrophage surface receptors CR1 and CR3, respectively, is promoted. Therefore, not only are metacyclic promastigotes resistant to complement-mediated lysis, via disruption of MAC formation and insertion, but they exploit a mechanism designed to eliminate them to gain entry into the macrophage.

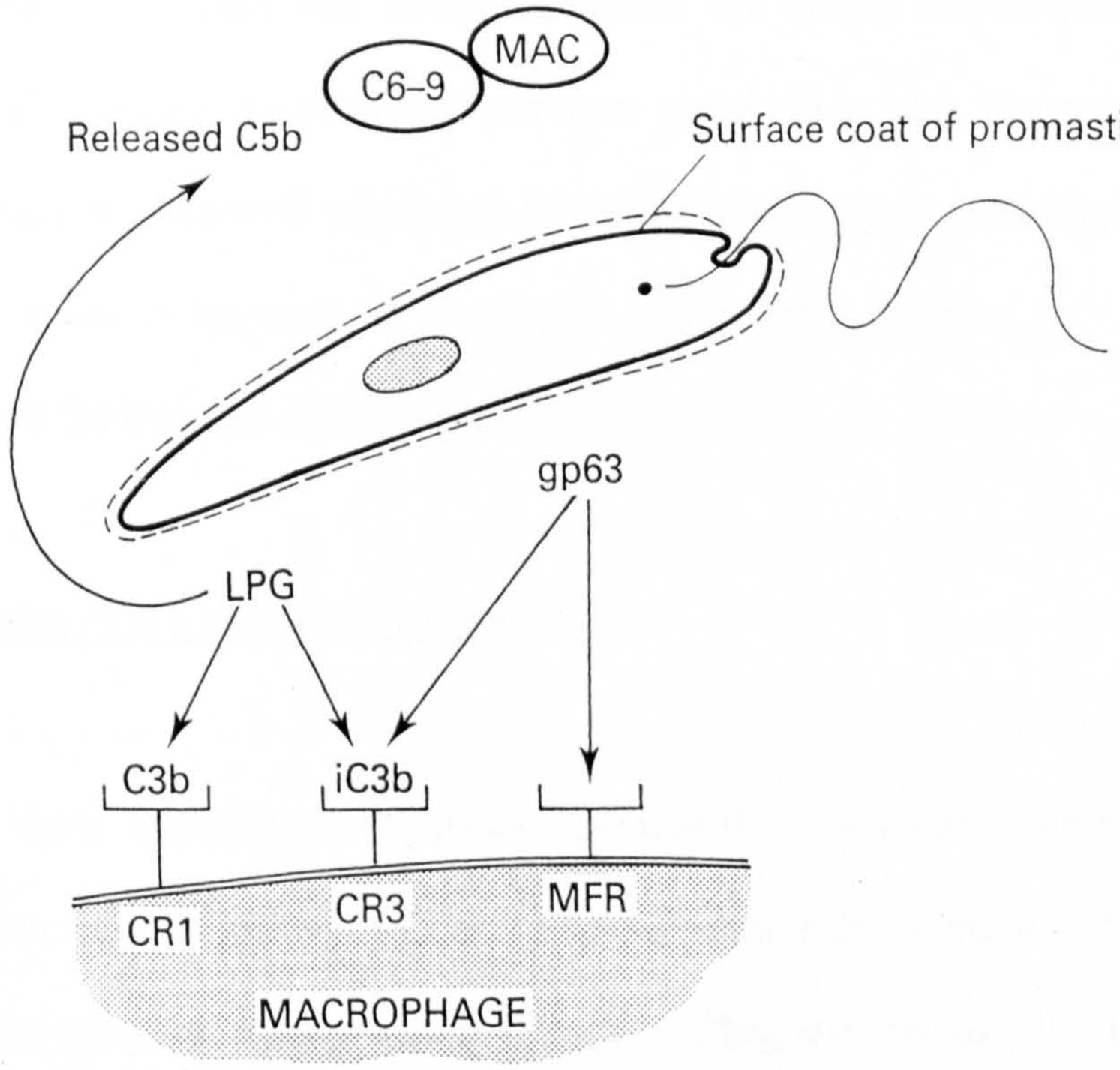



**Figure 2.6** *Leishmania*/macrophage surface interactions. (reproduced from Wakelin, 1996).

## 2.9 Intracellular Survival Strategies

Macrophages have many lysosomes, highly acidic organelles equipped with lysosomal enzymes. They use several methods for killing intracellular parasites including toxic oxygen metabolites, nitrogen metabolites and lysosomal killing. *Leishmania* parasites are well adapted to life within macrophages and employ several strategies to maintain intracellular survival. They have become very capable of survival within the immune cells designed to kill them.

### 2.9.1 Inhibition of the respiratory burst

One of the most important mechanisms employed by activated macrophages to accomplish intracellular killing of *Leishmania* parasites is the respiratory burst, which results in the production of oxygen radicals. Phagocytosis usually triggers the respiratory burst and the invading organism is subsequently killed. By using complement receptors (CR) *Leishmania* metacyclic promastigotes are able to enter into the macrophage without triggering the respiratory burst (Wright & Silverstein, 1983). This study observed that ligation to CR1 and CR3 failed to generate the release of hydrogen peroxide ($H_2O_2$) in phagocytes. Mosser & Edelson (1987) observed differences in the intracellular survival of parasites in the absence and presence of serum, where opsonized parasites were more successful in establishing intracellular infection. Therefore the acquisition of complement components C3b and C3bi by the metacyclic promastigotes not only enhances uptake by the macrophages but is also an important intracellular survival strategy.

## 2.9.2 Inhibition of Protein Kinase C

Protein Kinase C belongs to a family of $Ca^{2+}$ dependent and independent, closely related isozymes that play a major role in the transmission of extracellular signals from the cell surface to the nucleus (Nishizuka, 1988). Cytoplasmic protein kinase C is inactive and in the presence of elevated $Ca^{2+}$ is translocated to the inner leaflet of the macrophage plasma membrane (Turco, 1999). At the plasma membrane, protein kinase C interacts with another molecule, diacyglycerol, to become an active enzyme (Kishimoto *et al.* 1980). This active enzyme can subsequently phosphorylate target proteins in an ATP-dependent manner, resulting in the activation of the respiratory burst (Kiyotaki & Bloom, 1984). Descoteaux and colleagues (1992) showed that the presence of LPG inhibited the ability of protein kinase C to phosphorylate target proteins. The study observed that the translocation of protein kinase C to the plasma membrane occurred as normal, as did interaction with diacyglycerol but activation of the enzyme did not result.

LPG molecules are found on the surface of macrophages 5-10 minutes after infection (Tolson *et al.* 1989) and uniformly distributed over the entire surface within 30 minutes (Descoteaux & Turco, 1993). It has been shown that insertion of LPG into the plasma membrane increases the bilayer stability (Giorgione *et al.* 1996) and it has been suggested that this property of LPG is responsible for the inhibition of protein kinase C. It has also been proposed that the chelation of $Ca^{2+}$ by LPG may contribute to a decrease in the $Ca^{2+}$ dependent activity of protein kinase C (Turco, 1990). By

inhibiting activation of protein kinase C-mediated signal transduction pathways, *Leishmania* parasites can prevent macrophage activation.

### 2.9.3 Scavenging of Oxygen Radicals

In addition to inhibition of protein kinase C-mediated induction of the respiratory burst, LPG may also protect promastigotes from the toxic oxygen metabolites generated during the burst (Chan *et al.* 1989). Chan and colleagues showed LPG from *L. donovani* to be capable of completely preventing the death of the parasites due to hydroxyl radicals ($OH^{\cdot}$), in a cell free system. In the same study they also showed it was the repeating units of Galβ 1-4Manα1 $PO_4$-6 that gave LPG this protective ability. LPG can possibly therefore protect the invading parasites from the respiratory burst via three mechanisms; inhibition of the burst by the use of CR1 to gain entry into the macrophage, inhibition of PKC-mediated induction of the burst, and scavenging of oxygen radicals.

### 2.9.4 Inhibition of Phagolysosomal Biogenesis

Following phagocytosis into the macrophage *Leishmania* promastigotes are contained within a parasitophorous vacuole, which is bound by host cell membrane. Lysosomes subsequently fuse with the parasitophorous vacuole to create phagolysosomes. Lysosomes are membrane-bound vesicles produced by the Golgi body of the host cell and are found in the cytoplasm. The lysosomal membrane contains $H^+$ pumps that actively transport $H^+$ into the lysosome, which consequently creates an internal pH of

approximately 5. Upon fusion with the parasitophorous vacuole these enzymes are released onto the invading parasites and the environment is quickly turned acidic.

Desjardins and Descoteaux (1997) have shown that LPG can inhibit the formation of phagolysosomes. It has been proposed that LPG achieves this by its insertion into the lipid-bilayer of the parasitophorous vacuole membrane (Desjardins & Descoteaux, 1997). In doing so it stabilises the membrane (Giorgione *et al.* 1996) and this could reduce its fusogenic properties (Miao *et al.* 1995), resulting in prevention of fusion between the parasitophorous vacuole and lysosomes. It was observed that the transformation of metacyclic promastigotes into amastigotes, which produce little if any LPG, correlated with normal fusion events occurring between parasitophorous vacuoles and lysosomes (Desjardins & Descoteaus, 1997). This inhibition of phagolysosomal biogenesis may protect promastigotes from the lysosomal enzymes until transformation into amastigotes has occurred, as this form of the parasite is known to thrive in the acidic conditions of the phagolysosome.

## 2.10 Sandfly Saliva

Titus and Riberio (1988) first reported the disease enhancing effects of sandfly saliva. Early studies of *Ph. papatasi* demonstrated that saliva is injected during feeding and probing (Adler & Theodor, 1927). Titus and Riberio (1988) observed that *Lu. longipalpis* salivary gland lysates (SGL) had an exacerbative effect on infection with *L. major* when co-injected with the parasites. The exacerbative effect led to a 5 to 10 fold increase in lesion size, as well as an increase in the number of parasites within the lesion, in some cases by as much as 5000 fold. In the same study it was observed

that when parasites were injected into mice, in the absence of saliva, in numbers thought to reflect natural infection (10-100 parasites) (Warburg & Schlein, 1986), not only did they fail to generate a lesion but they did not survive. A recent study has estimated the bite of an infective sandfly to deliver approximately 1000 parasites per bite (Rogers *et al.* 2004), meaning previous studies were significantly underestimating the size of the infective inoculum. It should also be pointed out that previous studies used microcapillary force feeding to investigate the size of the infective inoculum whereas Rogers *et al.* (2004) used membrane feeding, a method that mimics natural feeding.

The exacerbative effect of sandfly saliva has been shown using several parasite/vector combinations (Theodos *et al.* 1991; Samuelson *et al.* 1991). Infections with *L. major* and *L. amazonensis* could both be enhanced, in a mouse strain known to be genetically resistant to infection with *L. major*, with saliva from *Lu. longipalpis* (Theodos *et al.* 1991). It was also shown that infections with *L. major* were also enhanced with saliva from its natural vector, *Ph. papatasi.* Theodos *et al.* (1991) justified the relevance of these studies by demonstrating that sandflies deliver the exacerbative components into the host during blood-feeding. They allowed uninfected sandflies to probe on exposed areas of mice prior to challenging the animals with *L. major.* The results were comparable with the data collected when parasites and SGL were co-injected. Warburg *et al.* (1994) proposed that the composition of *Lu. longipalpis* saliva influenced whether infection with *L. donovani chagasi* (*L. infantum*) resulted in cutaneous or visceral disease. They found that strains of *Lu. longipalpis* with lower maxadilan, responsible for vasodilatory activity (Lerner & Shoemaker, 1992), showed a reduced capacity to enhance cutaneous

infection and resulted in visceral disease. In contrast those strains with higher maxadilan, showed an increased ability to enhance cutaneous infection.

Several studies have focused on the mechanism by which sandfly saliva is able to enhance infection. It seemed that the effect was on the host immune system rather than a direct effect on the parasite itself. *In vitro* studies indicated that sandfly saliva had an inhibitory effect on the ability of macrophages to present parasite antigens to *L. major*-specific T cells (Theodos & Titus, 1993). Macrophages are incapable of killing invading *Leishmania* parasites alone, requiring activation via a series of Th1 cytokines, particularly IFN-γ (Liew *et al.* 1989). The production of parasite-specific T cells requires antigen presentation by macrophages, without which activation cannot occur. Other *in vitro* macrophage studies (Hall & Titus, 1995) showed that the saliva from *Ph. papatasi* did not enhance the uptake of parasites by macrophages but did inhibit IFN-γ-mediated killing of parasites. The inhibition of parasite killing directly correlated with a reduction in nitric oxide (NO) production. Waitumbi and Warburg (1998) confirmed the finding that *Ph. papatasi* saliva inhibited NO production. They showed that this was a result of a 50% reduction in the expression of the inducible nitric oxide synthase gene, which itself was a result of an inhibition of protein phosphatase activity and therefore signal transduction events.

Pre-exposure of mice to the saliva of *Ph. papatasi* resulted in the complete loss of any exacerbarative effect when the mice were subsequently challenged with a co-injection of saliva with *L. major* parasites (Belkaid *et al.* 1998). This effect was shown to be the result of antisaliva antibodies. Belkaid *et al.* (1998) observed that saliva incubated with serum IgG from sensitised animals completely removed the infection enhancing

properties of the saliva in mice. The investigators suggested that this is likely to occur in the natural world as individuals living in endemic areas will have prior exposure to the bites of uninfected sandflies before being bitten by an infected one. It was proposed that the exposure of individuals to vector saliva may influence the outcome of disease, as those mice pre-exposed to saliva failed to generate IL-4 and IL-5. The pre-exposure of mice to saliva in this study was via inoculation whereas a recent study used bites from uninfected sandflies (Kamhawi *et al.* 2000b). Kamhawi *et al.* (2000b) found that prior exposure to *Ph. papatasi* bites resulted in a significant reduction in the severity of dermal lesions resulting from subsequent bites from *L. major* infected *Ph. papatasi.* These observations were in agreement with the previous studies of Belkaid *et al.* (1998). However, the early IL-4 production seen previously with salivary gland sonicate (SGS) (Belkaid *et al.* 1998) was not observed in infections resulting from infective bites. This therefore indicated that IL-4 is not required for progression of mouse infections after transmission by bite and the molecule in SGS responsible for eliciting early IL-4 is absent from or only present in minute quantities in salivary secretions. These data indicate that infections initiated via inoculation should be interpreted with caution.

New evidence provided by Rogers *et al.* (2004) suggests that while saliva does produce a small to moderate exacerbation of infection, PSG produces a much greater exacerbation. Using an infective dose of 1000 *L. mexicana* metacyclic promastigotes they found that saliva did not exacerbate infection in BALB/c mice but did cause moderate exacerbation in CBA/Ca mice. In contrast to this PSG promoted substantial exacerbation of disease in both mouse strains. An interesting observation was that when both saliva and PSG were co-injected both BALB/c and CBA/Ca mice showed

an intermediate level of exacerbation, indicating that saliva and PSG act in an antagonistic manner. This study also identified fPPG as the active component of PSG (see section 2.6.2.2).

## Aims

The aims of this study were to

- observe the development of *L. infantum* within its natural vector *Lu. longipalpis*.
- determine the divisional status of each of the parasite life cycle stages identified.
- to identify stage-specific molecular markers for these life cycle stages
- to investigate the exacerbative effects of PSG and sandfly saliva using *in vitro* macrophage studies

# Chapter 3

# Materials and Methods

## 3.1 Lesion Amastigote Recovery

Parasite strains were maintained by infection in BALB/c mice. *Leishmania mexicana* (MNYC/BZ/62/M379) and *Leishmania major* (MHOM/IL/80/FV1) amastigotes were recovered from cutaneous lesions, and *Leishmania infantum* (MHOM/BR/76/M4192) amastigotes were recovered from spleen infections. Mice were asphyxiated using carbon dioxide in accordance with UK Home Office regulations. Ethanol (70%) was used to surface sterilize the mouse and all dissecting equipment.

For *L. mexicana* a small incision was made into the skin just above the lesion and the skin covering the lesion was removed. The exposed lesion was then removed intact and placed onto sterile 1mm gauge wire gauze, along with 5ml of M199 culture medium. The lesion was cut into small pieces and forced through the gauze using the plunger of a 10ml syringe. The homogenate was placed into a sterile 30ml universal tube and large debris allowed to settle. After 2-3 minutes the supernatant was transferred to a fresh universal. The suspension was drawn into a 10ml syringe coupled to a 19G needle and expelled to aid disruption of the cells. This was repeated several times, then repeated with a 25G needle, after which the suspension was expelled into a fresh universal tube.

To recover *L. infantum* amastigotes from the spleens of infected mice, a small incision was made into the skin, and the skin removed from the abdomen. The underlying peritoneal membrane was removed to expose the internal viscera and the spleen removed. Any fat was removed from the spleen and then the procedure outlined above was carried out.

For *L. mexicana* and *L. major*, amastigote numbers were calculated using an improved Neubauer haemocytometer under phase-contrast microscopy and the density of the suspension calculated. Due to the small size of the *L. infantum* amastigotes and the presence of spleen debris counting using a haemocytometer was difficult. Therefore a differential counting method was used. The numbers of red blood cells (RBC) were counted using a haemocytometer and a stained slide of the homogenate was also examined, where 500 RBC were counted and the accompanying amastigotes scored. These numbers were then used to calculate the density of the suspension.

## 3.2 Parasite *in vitro* Culture

### 3.2.1 Amastigote Culture

Freshly isolated lesion amastigotes were used to initiate amastigote cultures at a density of 5 x $10^5$/ml in Grace's insect tissue culture medium (GibcoBRL), supplemented with 20% (v/v) heat-inactivated foetal bovine serum (FBS; GibcoBRL) and 25μg gentamicin sulphate/ml (Sigma), pH 5.5 and incubated at 32°C.

### 3.2.2 Promastigote Culture

Freshly isolated lesion amastigotes were used to set up promastigote cultures at a density of $5x10^5$/ml in M199 culture medium (GibcoBRL) supplemented with 10% (v/v) FBS, BME vitamin solution (GibcoBRL) and 25µg gentamicin sulphate/ml, pH 7.2 and incubated at 26°C.

The cultures were monitored daily for the appearance of the various types of promastigote: procyclics, nectomonads and leptomonads. Once the cultures reached at least 75% homogeneity for a single promastigote form the cells were harvested by centrifugation.

### 3.2.3 Metacyclic Culture

Promastigote cultures were initially set up from lesion amastigotes as described above. Once exponential growth reached a density of $10^7$/ml, which usually occurred by day 2, the culture was subpassaged into Graces insect tissue culture medium (GibcoBRL), supplemented with 10% FBS (v/v) and 25µg gentamicin sulphate/ml, pH 5.5. The cultures were incubated at 26°C. Culture flasks remained sealed for the first 3 days to assist acidification of the medium, which encourages metacyclogenesis (Bates & Tetley, 1993). A near homogenous (85-90%) population of metacyclic promastigotes was usually reached following 5-6 days incubation.

### 3.2.4 Haptomonad Culture

Haptomonad promastigotes were cultured using scratched Melinex sheets as a substrate for attachment (Wakid & Bates, 2004). Melinex sheets were cut to size and the surface scratched using sandpaper. The sheets were then washed and left to soak in 70% ethanol for sterilisation. The Melinex sheets were then laid out and left to dry within a laminar flow hood.

Promastigote cultures were initially set up from lesion amastigotes as described above. After transformation of amastigotes to procyclic promastigotes had occurred, usually following a single overnight incubation, the cultures were transferred to a petri dish containing a scratched Melinex sheet. Once the cultures had been transferred the petri dish was sealed using parafilm and placed at 26°C for 5 days.

Using an inverted microscope the cultures were checked for the presence of attached promastigotes. The Melinex sheet was removed from the petri dish and washed several times with cold PBS to remove any unattached promastigote forms. The sheet was then placed in a clean petri dish with 5 ml of M199 and the cells were then scraped from the Melinex using a cell scraper. This process was repeated for each petri dish, and parasites pooled into a 50ml centrifuge tube. This was then centrifuged at 11 000*g* for 10 minutes and the cells resuspended in 10ml M199. The resuspension was then counted and used as required.

## 3.3 Sandfly Infections

Female *Lutzomyia longipalpis* sandflies were infected with amastigotes of *Leishmania infantum*. Infections were initiated via a membrane feeding system similar to that previously described by Ward *et* al. (1978) and Bates (1997). A chick skin, surfaced sterilised with 70% ethanol, was stretched over the membrane feeding apparatus and plucked. Lesion amastigotes recovered from the spleens of BALB/c mice were seeded into whole heparinised rabbit blood at a density of $2x10^6$/ml. The amastigote suspension was placed inside the reservoir of the feeding apparatus and warmed to 30-34°C via a water jacket attached to a water bath and pump. The membrane feeder was lowered into the cage of sandflies and breathing into the cage encouraged feeding.

Once most of the female sandflies had taken a bloodmeal the feeder was removed from the cage. These females were pooted into a fresh cage. A sugar meal and piece of filter paper propped against one side of the cage were placed into the cage and changed daily. The cage was then place inside a polythene bag along with a piece of dampened paper towel, to maintain humidity, and sealed. All infected flies were kept in a sealed room at an ambient temperature of 25 to 28°C, 70 to 80% humidity and on a 12 hour light/dark cycle.

## 3.4 Sandfly Dissection

Dissections were carried out in M199, using mounted entomological needles. Flies were knocked down by placing them on ice for 5-10 minutes. Immediately before

dissection, flies were washed in M199, to remove the external hairs and then placed on a clean slide in a drop of M199. The wings and legs were removed to immobilise the sandfly and the head was severed to free the gut from the foregut. The gut was then removed from the posterior end of the fly abdomen. This was achieved by placing the needles between segments seven and eight and slowly pulling these away from the abdomen. During the initial stages of bloodmeal digestion the gut was extremely distended and was gently massaged out of the abdomen, rather than pulled.

The dissected gut was picked up using a hooked needle and transferred into a microcentrifuge tube containing 40µl of M199 and 0.2mm glass beads. This was then homogenised using a Teflon pestle and 10µl of the resulting homogenate was transferred to a second microcentrifuge tube containing 10µl of formalin; 10µl of this was loaded into a haemocytometer and counted. Simultaneously 10µl of the homogenate was spotted onto a slide and fixed in methanol for two minutes.

## 3.5 Light Microscopy

Slides were stained for 12 minutes in 10% (v/v) Giemsa's stain in 10mM sodium phosphate buffer (pH 7.2) and examined under oil immersion at x1000. The morphology of the cells was observed and measurements of body length, body width and flagellum length in relation to body length were noted. The number of nuclei and kinetoplasts were also recorded.

## 3.6 DNA Isolation

*Leishmania mexicana* promastigotes were harvested in late log phase by centrifugation at 3000*g* for 10 minutes and washed twice with phosphate buffered saline (PBS). On completion of these washes the promastigote pellet was gently resuspended in lysis buffer (50mM NaCl, 50mM EDTA, 50mM Tris-HCl, 1% SDS: pH 8.0) and then incubated with proteinase K (100µg/ml) for 1 hour at 37°C.

The released DNA was extracted from the cell lysate by the addition of one volume of phenol. The upper aqueous layer was removed and one volume of chloroform added. The resulting aqueous layer was transferred to a new tube and the DNA precipitated by the addition of 0.3M sodium acetate, along with 2 volumes of ethanol. The DNA was collected by centrifugation and the resulting pellet was washed with 70% ethanol, air dried and resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA: pH 7.2).

## 3.7 RNA Isolation

Total RNA was isolated from pellets of $2x10^8$ cells. Cells were harvested by centrifugation, the pellets resuspended in 1ml of TRIzol® (GibcoBRL) and left at room temperature for 5 minutes. Then 200µl of chloroform (per 1ml lysate) was added, the tube shaken vigorously to mix and immediately transferred to ice for 5 minutes. This was then centrifuged at 12 000*g* for 5 minutes at 4°C and the upper aqueous phase transferred to a new tube. A volume of 250µl of isopropyl alcohol and 250µl of RNA precipitation solution (1.2M NaCl, 0.8M disodium citrate.$15H_2O$) were added to the transferred upper aqueous phase. The tube was inverted to mix and left

at room temperature for 10 minutes. The tube was then centrifuged at 12 000*g* for 10 minutes at 4°C. The supernatant was removed and 1ml of 75% ethanol added, the tube vortexed and centrifuged at 3350*g* for 5 minutes at 4°C. The ethanol was removed and the pellet was allowed to dry briefly for 10 minutes. The pellet was then resuspended in 30µl of DEPC-treated water.

## 3.8 PCR

PCR reactions were carried out using *Pfu* DNA polymerase (Promega), that possesses 3`- 5` exonuclease activity and produces high fidelity blunt ended products. Primers were designed using Primer 3 (**http://frodo.wi.mit.edu/cgi-in/primer3/primer3_www.cgi**) and manufactured by Invitrogen (listed below). Reaction mixes were set up in 50µl volumes as follows:

| | Final Conc. |
|---|---|
| 5µl 10X buffer | 1X |
| 1µl dNTP's (10mM each) | 200µM each |
| 0.5µl forward primer (25µM stock) | 0.25µM |
| 0.5µl reverse primer (25µM stock) | 0.25µM |
| 0.5µl *Pfu* DNA Polymerase | 1.5U |
| 1µl genomic DNA template | 200-500ng |
| 41.5µl sterile dd$H_20$ | |

PCR reactions were run using a Hybaid Thermocycler. An initial denaturation was performed at 95°C for 5 minutes, followed by a cycle of 94°C for 1 minute, 1 minute

annealing at temperatures dependent upon the primers in use (see below), and a 3 minute extension at 72°C, repeated for 35 cycles and a final extension step for 7 minutes at 72°C.

The following is a list of the primer pairs used and their annealing temperatures:

Alpha tubulin

Forward GCC GTA CCC GCG CAT CCA CTT

Reverse GAC TCG GCG CCA ACC TCC TCG TAG

Annealing temperature 64°C

PPG2a

Forward AAA TGG CGG CGG AAG TGG TAG CAG

Reverse AAA AAG AGG CCG CCG GAG TAG TCG

Annealing temperature 67.1°C

3`- nucleotidase/nuclease

Forward TGC ACA ACG TCA ACC TCT TC

Reverse CCT TGT TTT CCG TGT CCA CT

Annealing temperature 56.9°C

Secreted acid phosphatase 2

Forward CAA CAC GAC GGA GAT TTG TG

Reverse ATA CAC CGC CTT GAG ATT GG

Annealing temperature 48.5°C

Chitinase

Forward CTC GAC GGC ATA GAC TTC AAC TGG GAG TAC C

Reverse ATG GCG TAC GAC CAG GCT GTC GGG

Annealing temperature 51.5°C

DIG-labelled probes were generated using PCR. The reaction mix was as above with the addition of 5µl of PCR DIG-labelling mix (Roche). The water volume was adjusted to maintain a reaction mix of 50µl. PCR DIG-labelling mix contains the four conventional dNTP's as well as DIG-dUTP. Following the manufacturer's recommendations for the synthesis of highly sensitive probes, the addition of a PCR nucleotide mix was retained.

## 3.9 Cloning

Cloning of relevant PCR products was achieved using the Zero Blunt TOPO PCR cloning kit (supplied by Invitrogen). The kit employs the plasmid vector, pCR 4Blunt-TOPO, which is supplied linearized and permits the insertion of blunt ended PCR products. pCR 4Blunt-TOPO allows the direct selection of recombinants via a disruption of the lethal *E. coli* gene, *ccd*B. Insertion of a blunt end PCR product disrupts expression of this gene and therefore allows only positive recombinants to grow. Cells that contain non-recombinants are killed upon plating.

A ligation reaction consisting of 2µl of PCR product, 1µl of salt solution (1.2M NaCl, 0.06M $MgCl_2$), 1µl sterile water and 1µl of TOPO vector (10ng/µl plasmid DNA in:

50% glycerol, 50mM Tris-HCl (pH 7.4), 1mM EDTA, 2mM DTT, 0.1% Triton X-100, 100μg/ml BSA, 30μM bromophenol blue). This ligation reaction was mixed gently, incubated for 5 minutes at room temperature and then placed on ice. Transformation of chemically competent *E. coli* cells was achieved by the addition of 2μl of the ligation reaction. This was then incubated on ice for 30 minutes, followed by heat shocking for 30 seconds at 42°C. The cells were immediately transferred to ice for 2 minutes. After this 250μl of SOC medium (2% tryptone, 0.2% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added and this was incubated with shaking for 1 hour at 37°C, to allow the antibiotic resistance to be expressed before plating. Following this incubation the cells were then plated onto warm LB plates containing 100μg/ml ampicillin and incubated overnight at 37°C.

## 3.10 Northern Blot Analysis

All solutions used in the northern blot analysis were treated with diethylpyrocarbonate (DEPC). DEPC is an alkylating agent that destroys the enzymatic activity of RNase. Treatment of equipment was performed by soaking in DEPC-water, at 1ml/l, overnight. Following the overnight soaking the equipment was autoclaved. All tips and microcentrifuge tubes were guaranteed RNase free (Axygen).

### 3.10.1 Separation of RNA by gel electrophoresis

The gel tank and all related equipment were soaked in DEPC-water for 24 hours prior to use. The DEPC-water was drained off and the equipment allowed to dry in a

laminar flow hood.

RNA samples were prepared in microcentrifuge tubes and consisted of 3µl of 6M glyoxal, 12µl of dimethyl sulfoxide (DMSO), 3µl of 0.1M phosphate buffer (0.1M $NaH_2PO_4$, 0.1M $Na_2HPO_4$: pH=7.0), RNA sample and DEPC-water to a final volume of 30µl. The samples were then incubated at 50°C for 50 minutes and then chilled on ice for 10 minutes. While the samples were incubating, a 1% agarose gel was made using phosphate buffer and cast into the dried DEPC-treated gel tray. Immediately before loading the RNA samples into the gel, 2.5µl of RNA-loading buffer (50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol, 0.1M phosphate buffer, 6M glyoxal, DMSO) was added to each sample. The gel was then run at 60V in phosphate buffer. Magnetic stirrers were used to recirculate the buffer throughout electrophoresis to prevent the accumulation of $H^+$ and therefore maintain a neutral pH, this is necessary due to the fact that glyoxal will dissociate with RNA at pH 8.0 and above.

3.10.2 Transfer of RNA to a nylon membrane

Following electrophoresis the gel containing the RNA was equilibrated in 100ml of 20X SSC (3M NaCl, 0.3M Sodium citrate: pH 7.0) on a rocking platform at room temperature for 15 minutes. This was repeated twice. The RNA within the agarose gel was then transferred to a positively charged membrane (Boehringer Mannheim) by upward capillary transfer.

The capillary transfer was set up as described by Sambrook and Russell (2001). Whatman grade 3 filter papers, pre-soaked for 5 minutes in 20X SSC, served as wicks beneath the inverted gel. The vessel was filled to just below the level of the wick and gel support with 20X SSC. The nylon membrane, pre-soaked in 20X SSC was placed onto the inverted gel, ensuring no air bubbles were present. Two pieces of membrane-size Whatman grade 3 filter paper, pre-soaked in 2X SSC, were placed on top of the nylon membrane to generate an SSC gradient and encourage capillary transfer of the RNA. A stack of paper towels was placed on top of the 2X SSC filter papers to encourage the movement of SSC through the transfer system. The entire stacking system was weighed down with a 1kg lead ingot. The RNA was allowed to transfer overnight at room temperature. Once transfer was complete the RNA was cross-linked to the nylon membrane by exposure to ultraviolet radiation for 4 minutes.

### 3.10.3 Hybridisation and detection using the DIG system

The digoxigenin (DIG) system® is supplied by Roche and is a non-radioactive labelling system for the preparation and detection of probes used in nucleotide hybridisations. The DIG molecule is bound to dUTP, which can then be incorporated into a nucleic acid probe by random-primed Klenow extension (using the commercially available labelling mix, DIG High Prime, Roche) or polymerase chain reaction (PCR). These probes can then be used in standard hybridisations to bind to homologous nucleic acid sequences fixed on to nylon membranes. The hybridised probe is then detected by an anti-DIG antibody-alkaline phosphatase conjugate that binds to the DIG residues incorporated into the probe. A chemiluminescent substrate for alkaline phosphatase allows probe detection on X-ray film.

The cross-linked membrane was incubated in 200ml of 20mM Tris-HCl (pH 8.0) at 100°C, placed on a rocking platform and allowed to cool to room temperature. This removed any remaining glyoxal from the membrane. The membrane was then prehybridised with DIG Easy Hyb (Roche) (20ml/100cm$^2$ membrane), prewarmed to hybridisation temperature and incubated for 30 minutes at 50.5°C with agitation. Incubation was carried out in a sealed bag placed into a water bath and weighed down with a glass plate. DIG-labelled probe was denatured by boiling for 10 minutes and immediately transferred to ice water. The prehybridisation solution was removed from the membrane and replaced with 3-4ml of prewarmed DIG Easy Hyb containing 10µl of the denatured probe. The bag was resealed and incubated overnight with agitation at 50.5°C.

Following hybridisation the membrane was washed in four increasingly stringent solutions: twice in 100ml of 2X SSC/0.1% SDS at room temperature for 15 minutes and twice in 100ml of 0.1X SSC/0.1% SDS at 50°C. Each of the washes was carried out with agitation. Following the final stringency wash the membrane was washed in washing buffer (0.1M maleic acid, 0.15M NaCl, 0.3% v/v Tween 20: pH 7.5). The membrane was then incubated for 1.5 hours in 100ml of blocking solution (1% blocking buffer (Roche) in maleic acid: pH 7.5) at room temperature on a rocking platform. After blocking the membrane, it was incubated with anti-DIG alkaline phosphatase antibody (Roche) diluted 1:10,000 in 40ml of blocking solution, for 30 minutes on a rocking platform. Excess antibody was removed from the membrane by incubating in 50ml of washing buffer for 15 minutes, followed by a repeated wash for 1 hour. The membrane was equilibrated in 50ml of detection buffer (100mM Tris-

HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$) for 5 minutes prior to detection with CSPD (Roche). Following equilibration the membrane was incubated for 5 minutes in a sealed bag, containing the chemiluminescent alkaline phosphatase substrate diluted 1:100 in 4 ml of detection buffer. Excess CSPD was removed from the membrane and the bag resealed and incubated for 15 minutes at 37°C without agitation. The chemiluminescent signal was detected via X-ray film.

## 3.11 Preparation of PSG and sandfly saliva

Dr. Matthew Rogers generously provided both the PSG material and sandfly saliva used in this study. PSG material was dissected out of sandflies into phosphate buffered saline (PBS) and transferred into an eppendorf on ice containing PBS. The PSG material was then homogenised using a Teflon pestle and diluted with PBS to give an appropriate concentration of plug material per µl. The resulting homogenate was then centrifuged at 10 000*g* for 5 minutes and the supernatant removed. The supernatant was again centrifuged at 10 000*g* for 5 minutes and this was repeated a total of 4 times. Following the final centrifugation the supernatant was freeze-thawed 4 times in dry ice and methanol, to lyse any remaining parasites. The PSG was then stored at –70°C.

Salivary gland content (SGC) was prepared by dissecting out the sandfly salivary glands into PBS and transferring into a drop of PBS on a chilled IFAT slide. The two globes of each salivary gland were individually pierced and the SGC mixed into the PBS. The PBS was then transferred into a microfuge tube on ice and diluted with PBS to give an appropriate concentration of salivary glands per µl. The suspension

was then centrifuged at 10 000*g* for 5 minutes; the resulting supernatant was removed and stored at –70°C.

## 3.12 Isolation of Peritoneal Macrophages

Macrophages were obtained from healthy unstimulated CD1 female mice. Mice were killed by cervical dislocation and surface sterilised using 70% ethanol. The abdominal cavities of the mice were lavaged with 10ml of warm RPMI culture medium (GIBCO BRL Cat.N°. 52400-025), supplemented with 15% FBS (v/v) and 25μg gentamicin sulphate/ml, pH 7.2. The RPMI cell suspension was counted under phase-contrast as above and adjusted to the required density. The adjusted cell suspension was dispensed into 8 well chamber slides (Labtek) at $2x10^5$ cells per well, where 50% of cells are assumed to be macrophages. The slides were then incubated overnight at 32°C with 5% humidified $CO_2$ to allow the cells to adhere and spread.

## 3.13 *In vitro* infection of macrophages

Day 6 metacyclic promastigotes were counted as above, centrifuged at 11 000*g* for 5 minutes and resuspended in RPMI (15% FBS). The resuspended preparations were then recounted and the density adjusted as required. The parasite preparations were then distributed into the wells of the chamber slides, containing the adhered macrophages, at a volume of 0.1-0.4ml/well. Other material such as PSG, SGC and IFN-γ were added to the relevant wells as required. Final volumes within the wells was made up to 0.4ml with RPMI (15% FBS). Slides were then incubated for 4 hours at 32°C with 5% humidified $CO_2$, to allow uptake of the parasites by the

macrophages. Following incubation the slides were washed 3 times using RMPI (15% FBS), to remove any free or non-adherent parasites and 0.4ml of fresh RMPI added to each well. The slides were then left at 32°C with 5% humidified $CO_2$ and sampled at 4, 24, 48 and 72 hours.

# Chapter 4

# Results

## 4.1 Growth of *L. infantum* in *Lu. longipalpis*

Female *Lu. longipalpis* sandflies were infected with *L. infantum* amastigotes at a density of $2x10^6$/ml via a membrane feeding apparatus, as described in section 3.3. The engorged female flies were maintained in a separate cage. Ten flies were taken for 10 consecutive days and the guts removed by dissection. Dissected guts were homogenised and analysed. The numbers of parasites per sandfly were determined using a haemocytometer counting chamber. Combined results of three independent experiments are shown in Figure 4.1.

It was not possible to determine the exact amount of ingested parasites per fly due to the difficulties of distinguishing the tiny amastigotes within the dense bloodmeal. Therefore densities were only recorded from day 1 onwards. The data shows an initial decrease in parasite numbers from day 1 to day 2, with 13 100 and 7800 parasites per fly, respectively. This indicates that a proportion of the parasites are killed early in infection, possibly due to the action of trypsin. Following this initial decrease in density, parasites numbers steadily increased and reached a peak on day 7, with a density of 16 700 parasites per fly. Days 8 and 9 showed a decline in parasite numbers, however on

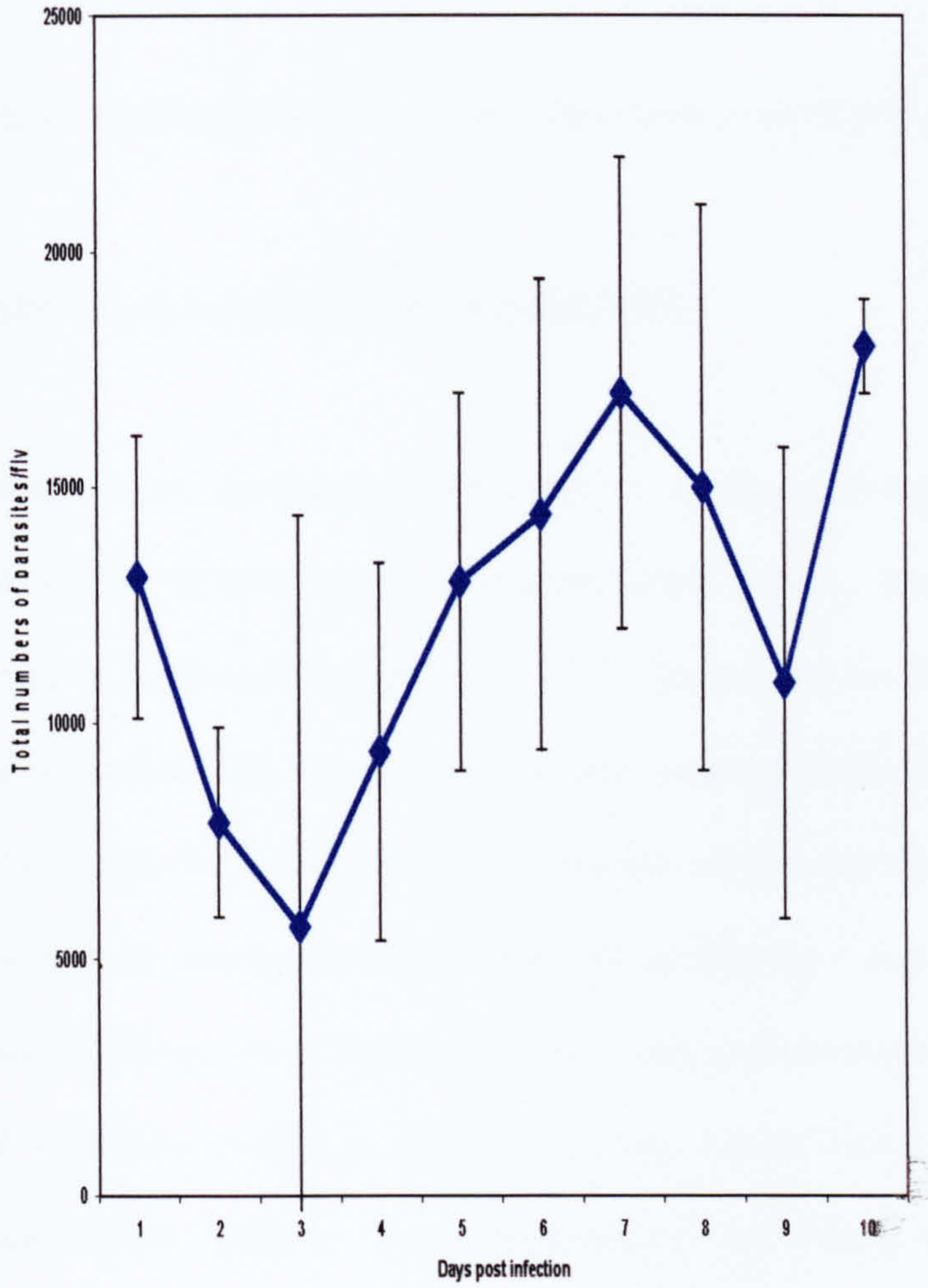



**Figure 4.1** Average *L. infantum* densities in *Lu. longipalpis*. Infections were initiated from spleen amastigotes. Data shown is a representation of three independent experiments.

day 10 parasite numbers had risen giving a final density of 18 600 parasites per fly.

## 4.2 Development of *L. infantum* in *Lu. longipalpis*

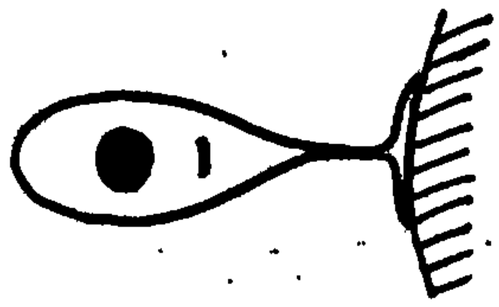
Several studies have provided detailed reports on the development of a variety of *Leishmania* species in both natural and experimental vectors. The data provided here is from the natural parasite/fly combination of *L. infantum* in *Lu. longipalpis*. Infections were initiated as described in section 3.3 and the progress of the infections monitored for 10 days. For each of the 10 days, 10 fed females were dissected and a slide was made from the whole gut homogenate and stained using Giemsa's stain. The morphology of the cells was observed, using light microscopy, and measurements of body length, body width and flagellum length in relation to body length were noted. Using these measurements each parasite was characterised as either, amastigote, procyclic promastigote, nectomonad promastigote, haptomonad promastigote, leptomonad promastigote or metacyclic promastigote (Figure 4.2). Combined data from three independent experiments is shown in Figure 4.3.

Transformation of amastigotes into procyclic promastigotes was seen as early as day one. At this time the parasite population was composed of 49% procyclic forms, the remainder being amastigotes. However, some amastigote forms had elongated body lengths, of 8μm, but were still devoid of protruding flagella. These are possibly the intermediate form between amastigote and procyclic. Those parasites that had transformed into

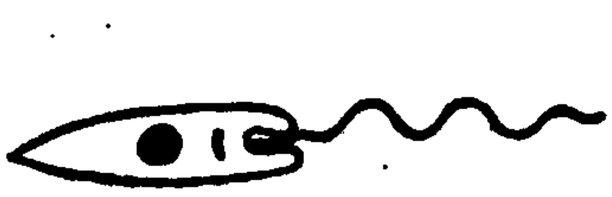
**Amastigote.** Ovoid body form, no flagellum protruding from the flagellar pocket.

**Procyclic.** Body length 6.5 – 11.5μm, flagellum<body length, body width variable.

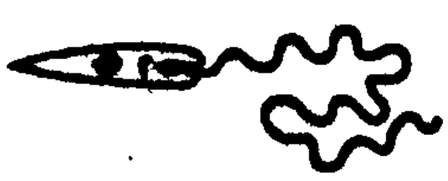
**Nectomonad.** Body length ≥ 12μm, body width and flagellar length variable.



**Haptomonad.** Disc-like expansion of flagellar tip, body form and flagella length variable.



**Leptomonad.** Body length 6.5 – 11.5μm, flagellum≥body length, body width variable.



**Metacyclic.** Body length ≤ 8μm, body width ≤ 1.0μm, flagellum > body length.

**Figure 4.2** Parasite morphologies outlined by Rogers *et al.* (2002).

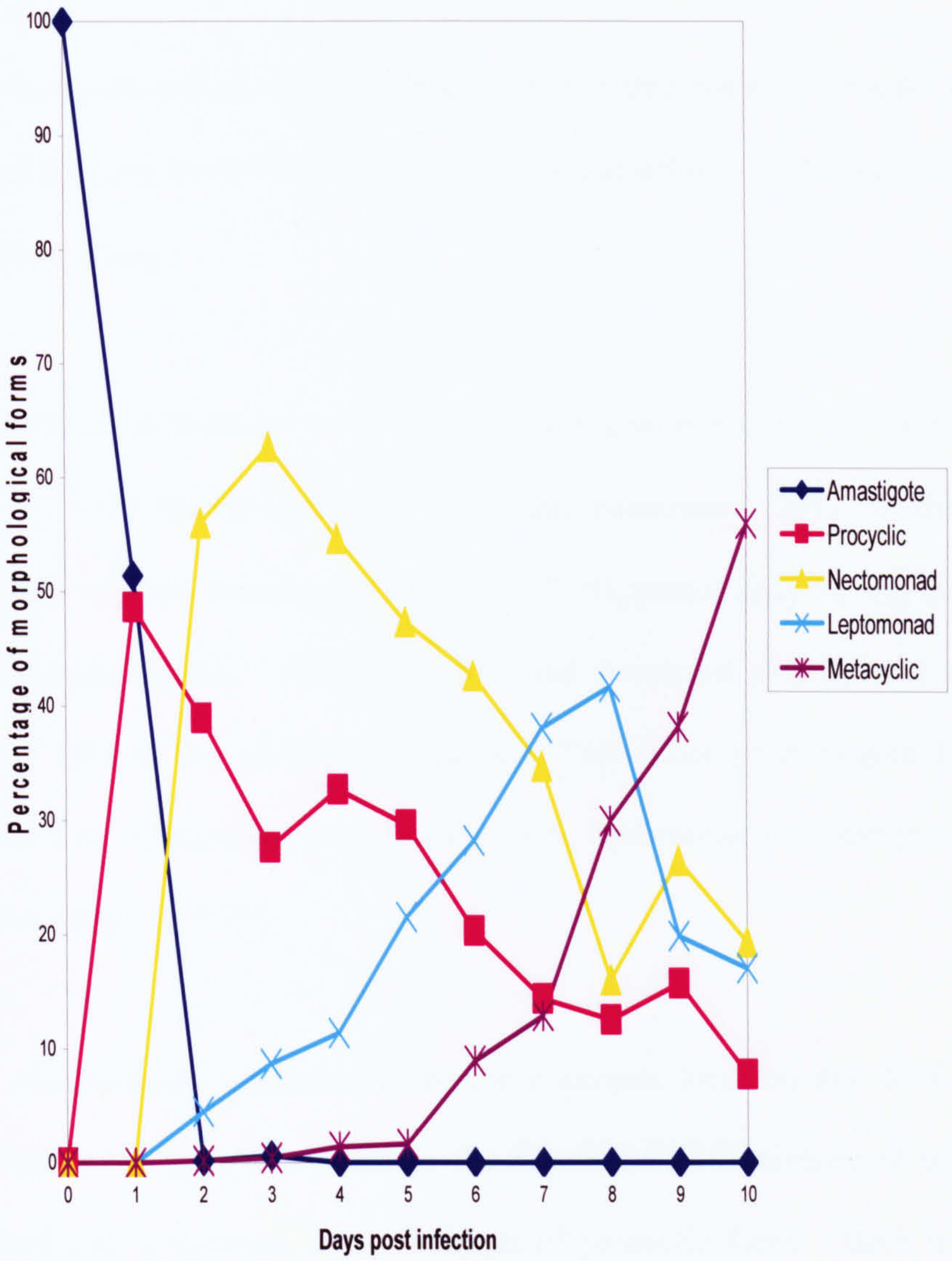



**Fig 4.3** Percentage of morphological forms present during *L. infantum* infection and development in *Lu. longipalpis*. Infections were initiated from spleen amastigotes. Data represents the combination of three independent experiments.

procyclic promastigotes possessed small flagella, shorter than the body length. They had body lengths of 6-11μm, however their body width remained like that of amastigotes and ranged between 1.5-3μm.

By day 2 of infection nectomonad promastigotes, having an elongated body ranging from 11μm upwards, were the predominant form and constituted 56% of the parasite population. Only very few amastigotes were seen, 0.2%, presumably having transformed into procyclic promastigotes. Procyclic forms had decreased slightly and were now responsible for 39% of the parasite population. Two other promastigote forms first appeared at day 2 of infection, albeit in low numbers, leptomonad promastigotes, 5% and metacyclic promastigotes, 0.2%.

Nectomonad promastigotes continued to be the dominant form on day 3 of infection, making up 63% of the parasite population. This increase in occurrence of nectomonad forms coincided with a decrease in the numbers of procyclic forms. Both leptomonad and metacyclic forms increased in prevalence making up 9% and 0.4% of the population, respectively. Amastigotes were again seen in very low numbers, however they were completely absent by day 4 of infection.

Nectomonad promastigotes, although still dominating the parasite population on day 4 of infection, had decreased in numbers, accounting for 55%. Leptomonad promastigotes were continuing to increase in numbers and were now responsible for 11% of the

population. Likewise the percentage of metacyclic promastigotes was also increasing and made up 1.4% of the total parasite population.

There was a sharp increase in the numbers of leptomonad promastigotes on day 5 of infection, rising to 22% of the parasite population. Nectomonad promastigotes continued to decrease in number, however, they remained the dominant form making up 47% of the parasite population. Metacyclic promastigote numbers increased only very slightly to 1.6% of the parasite population. Procyclic promastigotes declined in prevalence to 27% of the parasite population, following a slight increase in numbers on day 4 of infection.

By day 6 of infection leptomonad promastigote numbers had risen above that of procyclic promastigotes, making up 28% and 20% of the parasite population, respectively. Nectomonad promastigotes were still the dominant form although their numbers continued to decline and now made up 43% of the parasite population. The metacyclic promastigote population had a sharp increase in numbers to 8.9%. Leptomonad promastigotes became the dominant form on day 7 of infection, replacing nectomonad promastigotes, 38% and 35%, respectively. Day 7 was also the day when peak infection densities were reached and this can also be seen in Figure 4.4 showing the total number of morphological forms per fly. Metacyclic promastigote forms continued to increase in prevalence, while procyclic promastigotes numbers declined steadily.

Day 8 saw the continued dominance of leptomonad promastigotes and a sharp increase in metacyclic promastigotes to 30% of the total population. Metacyclic promastigote

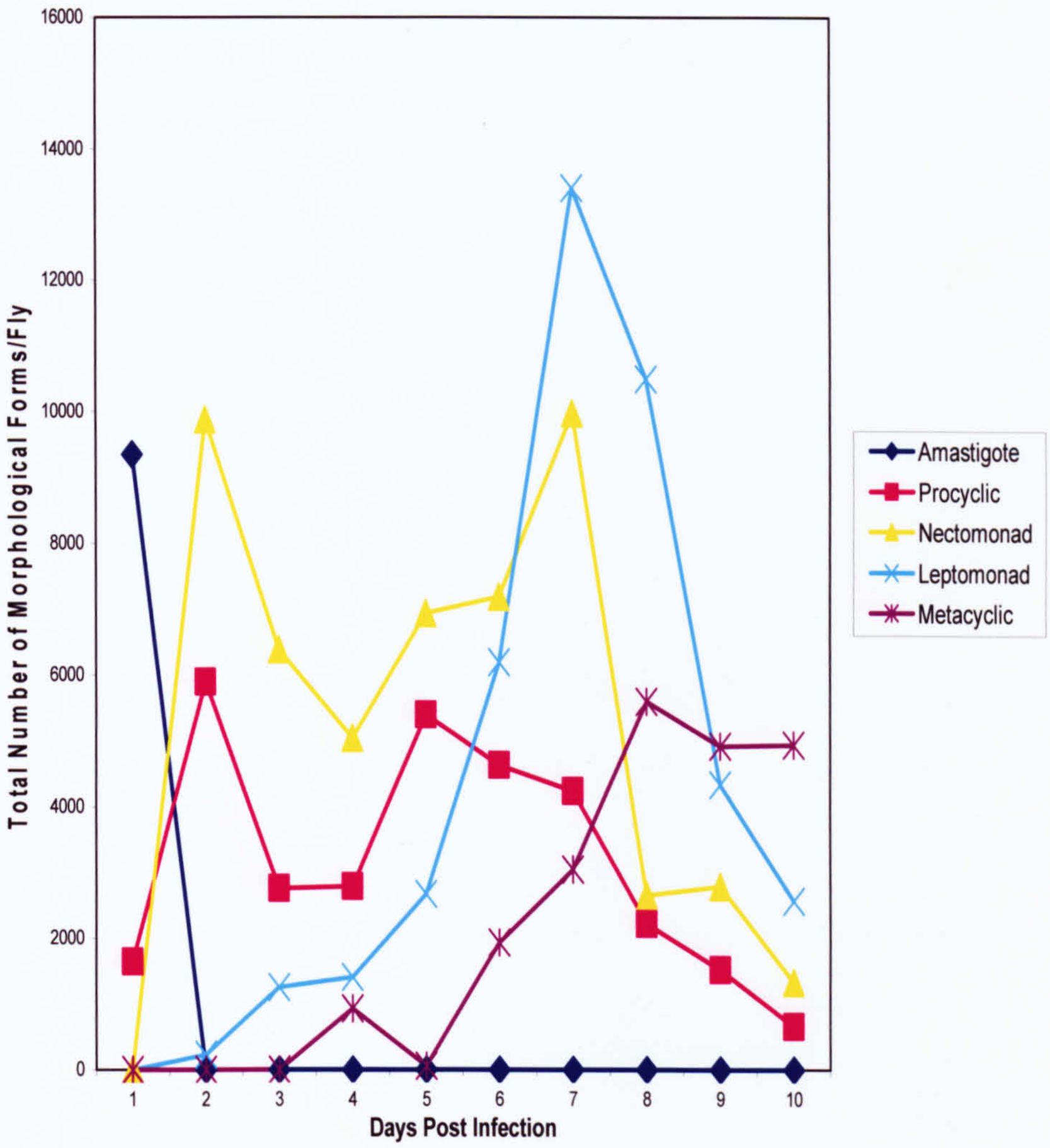



**Figure 4.4** Total number of morphological forms seen during *L. infantum* infection and development in *Lu. longipalpis*. Infections were initiated from spleen amastigotes. Data shown represents a combination of three independent experiments.

numbers continued to increase and by day 10 were responsible for 56% of parasites. This increase in metacyclic promastigotes was accompanied by a sharp fall in the prevalence of leptomonad promastigotes. Both procyclic and nectomonad promastigotes remained significant forms within the parasite population and by day 10 they were responsible for 8% and 19%, respectively. It should be noted that haptomonad promastigotes were observed in only one of the infected sandflies and were, therefore, not included within the analysis. Likewise, paramastigotes were very rarely seen.

This *in vivo* data outlines the sequential appearance of four major morphological forms of promastigote, namely; procyclic, nectomonad, leptomonad and metacyclic. It does appear that they comprise a series of precursors and products, whereby procyclic promastigotes are the precursors of nectomonad promastigotes and these in turn are the precursors of leptomonad promastigotes, which eventually give rise to metacyclic promastigotes. These data give no indication as to what extent these transformations are pre-programmed into the parasites and/or to what extent external stimuli *i.e.* the sandfly gut, contribute to this process.

## 4.3 Development of *L. infantum in vitro*

To investigate whether these transformations are pre-programmed into the parasites or influenced by external stimuli, a series of similar *in vitro* studies were conducted, thereby removing any influence the sandfly may be having on development of the parasites. Parasites from the same lesions used in each of the sandfly infections were used to set up

parallel *in vitro* cultures, as described in section 3.2.2. The data shown in Figure 4.5 is the combination of three independent experiments. Cultures were sampled for 10 days and 10μl was spotted onto a slide. Each slide was stained using Giemsa's stain and analysed under oil using light microscopy.

The overall appearance of the different morphological forms was the same as in the *in vivo* experiments. However, there are differences in the timing and appearance of the different morphologies. The first noticeable difference is the persistence of amastigote forms on day 1, remaining at 76% of the parasite population compared to 51% *in vivo*. Consequently, this lead to a delay in the peak of procyclic promastigotes, these not appearing until day 2 of infection. Amastigote forms were still found in significant numbers on day 2 and contributed to 16% of the parasite population. The peak of procyclic promastigotes seen *in vitro* was significantly higher than that observed *in vivo*. Although there was a peak of procyclic promastigotes seen *in vivo* they were never observed to be the dominating form, in contrast to that seen *in vitro* (day 2).

By day 3 procyclic promastigote numbers had fallen dramatically to 38% of the parasite population. It was now nectomonad promastigotes that dominated the parasite population at 60%. The first significant numbers of leptomonad promastigotes were seen on day 3 and made up 8% of the parasite population, compared to 5% on day 2 in *in vivo* infections. The same pattern of development of nectomonad promastigotes was seen *in vitro* and *in vivo*, although the nectomonad peak was not seen until day 5 *in vitro* compared with 3 *in vivo*. The increase in the percentage of nectomonad promastigotes

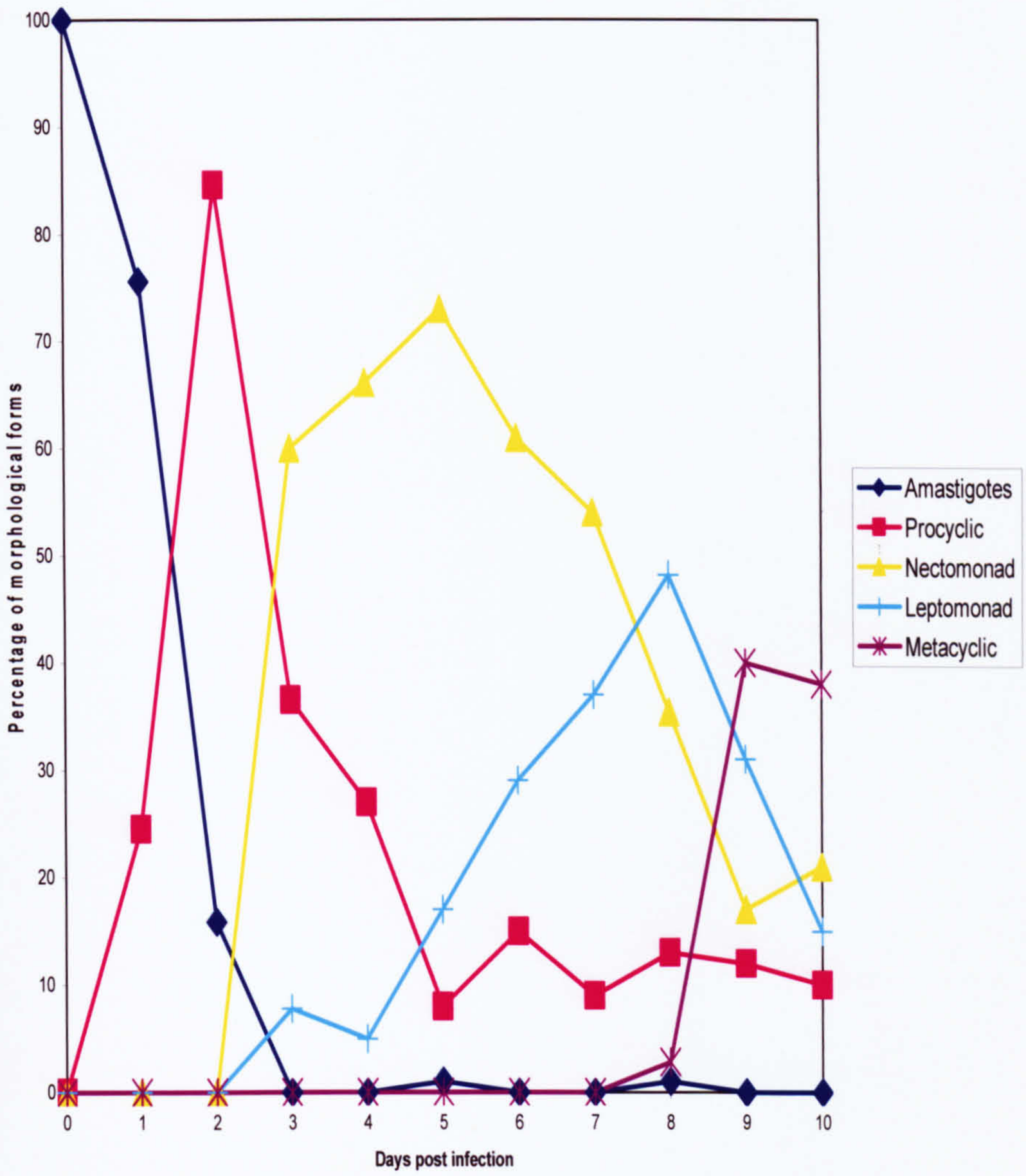



**Figure 4.5** Percentage of morphological forms of *L. infantum* during *in vitro* culture. All cultures were initiated with spleen amastigotes isolated from BALB/c mice. Data shown represents a combination of three independent experiments.

was again accompanied by a sharp decrease in the percentage of procyclic promastigotes, but these forms remained as a minor population for the duration of infection. Nectomonad promastigotes continued to be the dominant form for an extended period from day 3 through to day 7 and this is comparable with the *in vivo* data.

From day 4 through to day 8 leptomonad promastigotes steadily increased in number and were the dominant form within the cultures by day 8, as was the case in the sandfly infections. From day 6 to day 8 the progressive increase in leptomonad promastigotes was accompanied by a decrease in nectomonad promastigotes. A major difference at this point is the lack of a significant percentage of metacyclic promastigotes, with only 3% compared to the 30% present *in vivo*. However, by day 9 metacyclic promastigotes were the dominant form and had increased significantly making up 40% of the total population. This huge increase in metacyclic promastigotes was coincident with a decline in leptomonad promastigotes.

Overall the same general pattern of development was evident in both *in vivo* infections and *in vitro* cultures. The four major morphological forms of procyclic promastigote, nectomonad promastigote, leptomonad promastigote and metacyclic promastigote were present and displayed the same precursor-product relationships. These data indicate that the parasite itself is in some way pre-programmed for these developmental changes. However, there are differences in the timing and prevalence of the various morphological forms between *in vivo* infections and *in vitro* cultures, indicating that external stimuli

within the sandfly gut also contribute to the developmental biology of *Leishmania* parasites.

## 4.4 Development of *L. major in vitro*

The parasite used in the above experiments, *L. infantum*, is the causative agent of visceral leishmaniases in South America. Similar experiments by Rogers *et al.* (2002) also used a New World species, *L. mexicana*, a causative agent of cutaneous lesions. To test if the sequential appearance of the four major promastigote forms was also present in Old World species or if this was just a phenomenon seen with New World species, *in vitro* cultures of *L. major* were analysed as above. Since the *L. infantum in vitro* data showed the same sequential appearance of morphological forms as the *in vivo* infections, sandfly infections were not performed for *L. major*. Also the natural vector (*Ph. papatasi*) was not available.

Figure 4.6 shows the combined data from two independent experiments. Once again the overall sequential appearance of the different morphologies is the same as in *L. infantum*. There are, however, differences in timing and prevalence when compared to the *L. infantum in vitro* data. Procyclic promastigotes peak at day 1, compared to day 2, although they reach similar total percentages, 91% and 85% respectively. On day 2 the procyclic promastigote population sharply declined to 44% and nectomonad promastigotes became the dominant form at 49% of the parasite population. Leptomonad promastigotes were seen in significant numbers at day 1, responsible for 3% of the

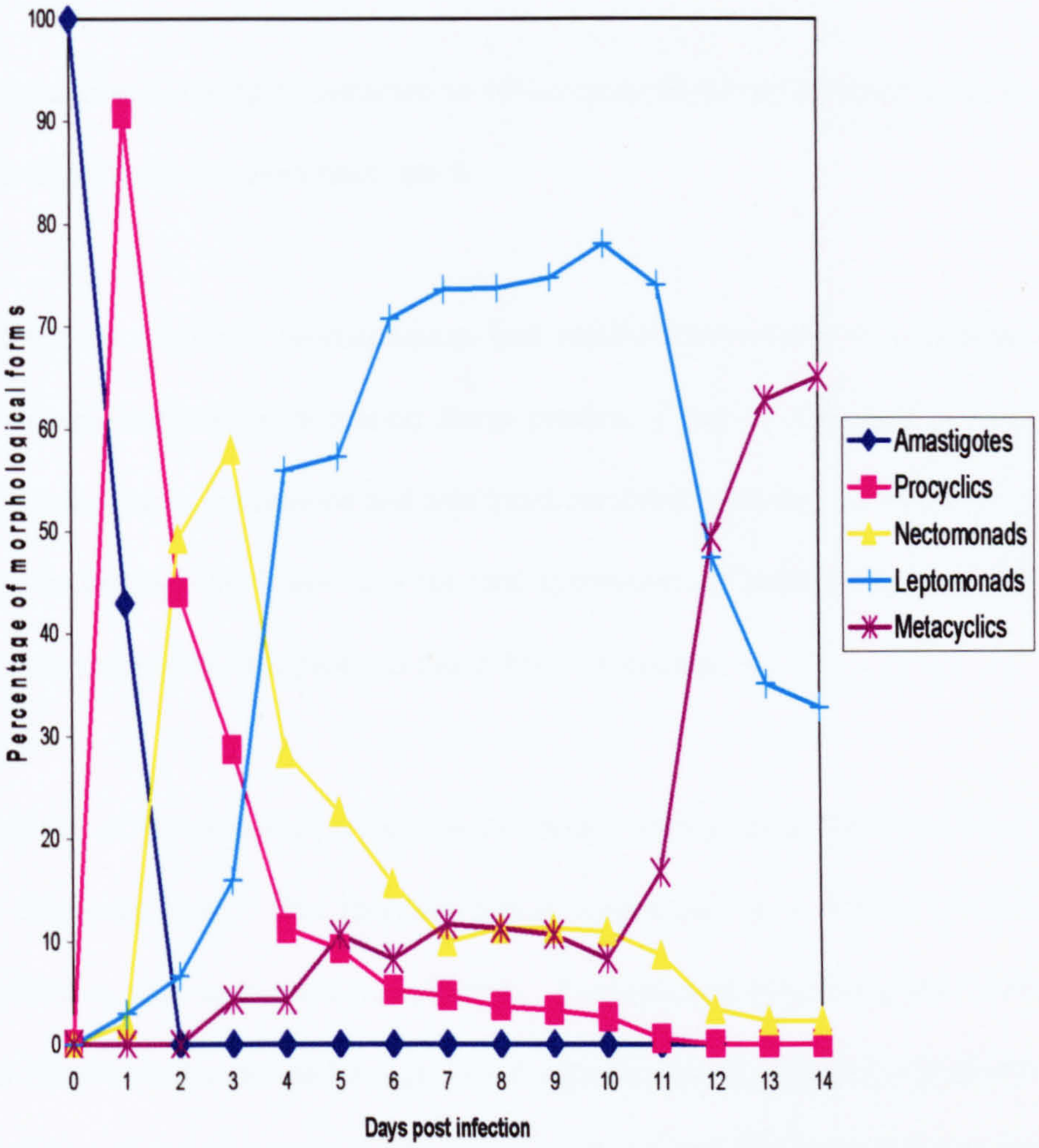



**Figure 4.6** Percentage of morphological forms of *L. major* during *in vitro* culture. All cultures were initiated from lesion amastigotes isolated from BALB/c mice. Data shown represents a combination of two independent experiments.

parasite population. During *L. infantum in vitro* culture leptomonad promastigotes were not seen in significant numbers until day 3.

By day 3 the nectomonad promastigotes had reached their peak, in contrast with *L. infantum in vitro* cultures nectomonad forms peaked at day 5. Procyclic promastigotes continued to decline in prevalence and this trend persisted until day 12 when no procyclic forms were recorded. Day 3 also saw the first appearance of metacyclic promastigotes in significant numbers, 4%, compared to day 8 for *L. infantum*.

The percentage of leptomonad promastigotes sharply increased by day 4 to 67%, making these the dominant form. This increase was accompanied by a decline in nectomonad promastigotes as was seen with *L. infantum*. Nectomonad promastigotes continued to decline in number but remained a significant member of the parasite population for the duration of the culture. Leptomonad forms continued to dominate the parasite population until day 11, reaching their peak on day 10. The early and prolonged dominance of leptomonad promastigotes is in contrast to *L. infantum* cultures where this form peaked and dominated the parasite population for a single day, day 8.

Although metacyclic promastigotes appeared as a significant proportion of the parasite population early on, day 3, they did not become the dominant form until day 12. In contrast, in the *L. infantum in vitro* cultures (Figure 4.5) metacyclic promastigotes appeared in significance numbers much later, day 8, but by day 9 they were the dominant

form. This delay of metacyclogenesis has also been observed in *L. major* infected *Ph. papatasi* (Dr. M.E. Rogers, a personal communication).

Collectively these data indicate the existence of a precursor-product relationship between the four major forms of promastigote. Data from *L. major in vitro* cultures confirm the idea that the parasites are in some way pre-programmed for these developmental transformations and differentiations. However, due to the differences in timing and prevalence of the various forms of promastigotes recorded between the two species studied, it is obvious that they do not share the same pre-programme. This could be a result of the close evolutionary development the parasites have with each of their natural vectors. Parasites may be pre-programmed to enter metacyclogenesis and produce infective promastigotes to coincide with the vectors requirement for a bloodmeal. When comparing the *L. infantum in vivo* and *in vitro* data, metacyclic promastigotes are the dominant forms on day 9 in both cases. This suggests that even without the influence of the vector the parasites still produce infective metacyclic promastigotes at a time when the sandfly would be taking another bloodmeal. The timing and prevalence of the pre-cursor promastigotes may be varied between *in vivo* and *in vitro* development, however, the overall outcome was the same, possibly meaning that the parasites have a pre-programmed deadline when infective metacyclic promastigotes must be generated.

## 4.5 Analysis of Dividing and Non-dividing Stages

During development within the sandfly the parasite density increases with time (Figure 4.1). This facilitates the generation of sufficient numbers of metacyclic promastigotes, which upon feeding, are transmitted to the mammalian host. Which of the promastigote forms contribute to this increased density was unclear. To answer this question, the potential of the four major promastigote forms to undergo cell division, and therefore increase the parasite population, was assessed.

Using Giemsa stained slides, observations were made and the divisional state of each parasite noted. The number of kinetoplast and nuclei per cell were recorded. A parasite was classed as being in the mitotic (M) phase of the cell cycle if it possessed two kinetoplasts and/or nuclei but had not yet completed cytokinesis. This data was collected for both the *in vivo L. infantum* infections and the *L. infantum* and *L. major in vitro* cultures. Results of this analysis are shown in Table 4.1.

The data indicates that for both *L. infantum* and *L. major* procyclic and leptomonad promastigotes are the major forms capable of cell division either *in vivo* or *in vitro*. Nectomonad promastigotes were very rarely seen in division in *L. infantum* both *in vivo* and *in vitro*, and this form was never seen in division in *L. major in vitro* cultures. It was therefore concluded that nectomonad promastigotes are essentially a non-dividing life cycle stage. No metacyclic promastigote was ever seen in a divisional state and are a strictly non-dividing form.

| Life Cycle Stage | Percentage of Dividing *in vivo* | Percentage of Dividing *in vitro* |
|---|---|---|
| *Leishmania infantum* | | |
| Procyclic | 6.0 (n=1253) | 9.9 (n=121) |
| Nectomonad | 0.1 (n=1701) | 0.2 (n=408) |
| Leptomonad | 10.0 (n=738) | 4.8 (n=180) |
| Metacyclic | 0.0 (n=1133) | 0.0 (n=78) |
| *Leishmania major* | | |
| Procyclic | N/A | 7.9 (n=464) |
| Nectomonad | N/A | 0.0 (n=505) |
| Leptomonad | N/A | 2.4 (n=1303) |
| Metacyclic | N/A | 0.0 (n=581) |

**Table 4.1** Percentage of dividing forms of *L. infantum* during infection and development in *Lu. longipalpis*, and both *L. infantum* and *L. major* development during *in vitro* culture. All infections and cultures were initiated from spleen/lesion amastigotes. n represents the number of parasites examined for each life cycle stage. Data shown is a combination of three independent experiments.

The data collected was also used to try to assess the sequence of division. It was assumed that if most dividing cells had 2 kinetoplasts and only 1 nucleus, the kinetoplast divided first and vice versa. The majority of cells that were classed as dividing for the purpose of this study, contained both 2 kinetoplasts and 2 nuclei. However, there were a small proportion of cells that contained only 2 kinetoplasts or 2 nuclei. The *in vivo* sandfly infections of *L. infantum* showed 94% of procyclic promastigotes to contain both 2 kinetoplast and 2 nuclei, although 5% contained only 2 nuclei and 1% 2 kinetoplasts. Similar findings were observed with the leptomonad promastigotes; 95% having both 2 kinetoplasts and 2 nuclei, and 5% containing 2 nuclei. The leptomonad promastigotes *in vivo* never contained only 2 kinetoplasts. Likewise, in the *L. major in vitro* cultures 91% of procyclic promastigotes contained 2 kinetoplasts and 2 nuclei, 7% had only 2 nuclei and 2% had only 2 kinteoplasts. However, all of the leptomonad forms contained both 2 kinetoplasts and 2 nuclei. In *L. infantum* cultures procyclic and nectomonad promastigotes contained both 2 kinetoplasts and 2 nuclei.

These data suggest that there is very little time difference in the division of the kinetoplast and the nucleus. It would appear that the nucleus begins division only slightly ahead of the kinetoplast. This would explain why the majority of cells contained 2 of each of these organelles. An increased sample size would allow a more detailed study of the divisional sequence, although these findings are in agreement with those of Walters *et al.* (1989a).

## 4.6 Analysis of Gene Expression

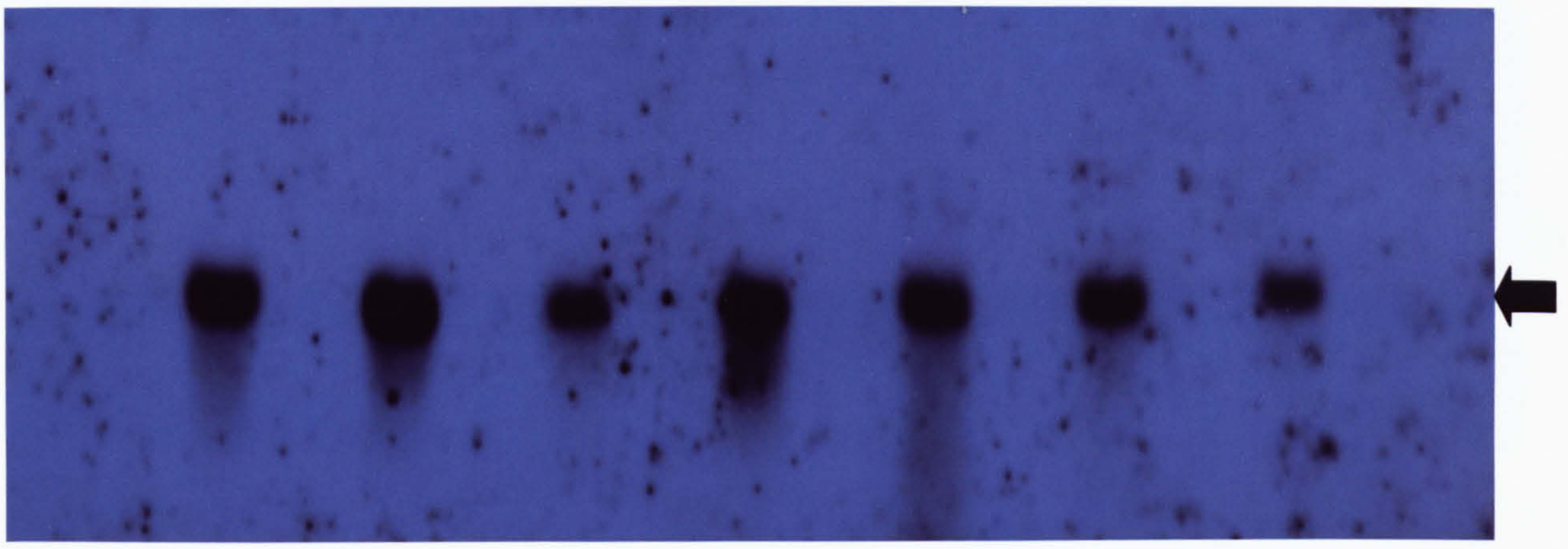
The above data clearly demonstrates the existence of four major promastigote forms that differ in their morphology. It also seems that they differ in the timing of their occurrence in infection. Procyclics were the first developmental form of promastigote seen during infection, followed by nectomonad promastigotes. Leptomonad promastigotes appear in significant numbers later in infection, with the infective metacyclic promastigotes appearing last as the terminal form. Therefore, it is likely that they contribute to the infection by having functions specifically related to their location within the sandfly gut. As a consequence of this the major promastigote forms should not only differ in morphology but also in their gene expression.

Differences in gene expression were investigated using northern blot analysis, described in section 3.10. Total RNA was isolated (see section 3.7 for details) from each of the following morphological forms; amastigotes, procyclic promastigotes, nectomonad promastigotes, haptomonad promastigotes, leptomonad promastigotes and metacyclic promastigotes. Aliquots of RNA were then electrophoresed and capillary transferred to a nylon membrane. DIG-labelled probes were generated using PCR with specific primers, hybridised with the membrane and the results visualised on X-ray film. All analyses were repeated at least twice and films were taken for several different exposure times, usually 20, 10, 5 and 2 minutes. All resulting films were analysed using a Syngene Gel Reader and densimetric measurements were taken for each of the bands. This was done

to ensure that the same pattern of expression appeared on all films and that any trend seen was not just a result of an extended exposure. Following densimetric analysis the bands were given a value above or below the average density of that particular film.

Expression of several genes was investigated, all of which coded for either surface or secreted proteins. The structural protein alpha tubulin was included in the analysis as a positive control. Figure 4.7 shows the data collected following hybridisation of the membranes with the alpha tubulin probe. The densities indicate that axenic amastigotes are expressing the most alpha tubulin mRNA, 44% above the average expression. Lesion amastigotes and nectomonad promastigotes also appear to express alpha tubulin at levels above the average, 28% and 16%, respectively. All other promastigote forms expressed levels below the average expression. The least expression was observed in the metacyclic promastigotes. It is possible that the amastigote forms of the parasite are producing high levels of alpha tubulin mRNA, as they are in a state that is ready for transformation into procyclic promastigotes. Although this may not be true for *in vivo* amastigotes it would certainly be true of those in culture. As amastigotes undergo the most significant of morphological changes, with elongation of the flagellum and an increase in body length and possibly width, they may be required to store more free tubulin mRNA than other life cycle stages. Nectomonad promastigotes are the largest of the life cycle stages and would therefore require more microtubules to provide support of the increase in body length. In contrast metacyclic promastigotes being the smallest of the promastigote stages would require less.

| LA | AA | P | N | H | L | M |
|---|---|---|---|---|---|---|



| +28% | +44% | -20% | +16% | -5% | -11% | -52% |
|---|---|---|---|---|---|---|

**Figure 4.7** Northern blot analysis of alpha tubulin expression. Each lane contains 3μg of total RNA. Lanes 1 through to 7 represent lesion amastigotes (LA), axenic amastigotes (AA), procyclic promastigotes (P), nectomonad promastigotes (N), haptomonad promastigotes (H), leptomonad promastigotes (L) and metacyclic promastigotes (M), respectively. The figure below each lane is the percentage of expression, above or below that of the average expression. The arrow indicates a transcript size of 2.7kb.

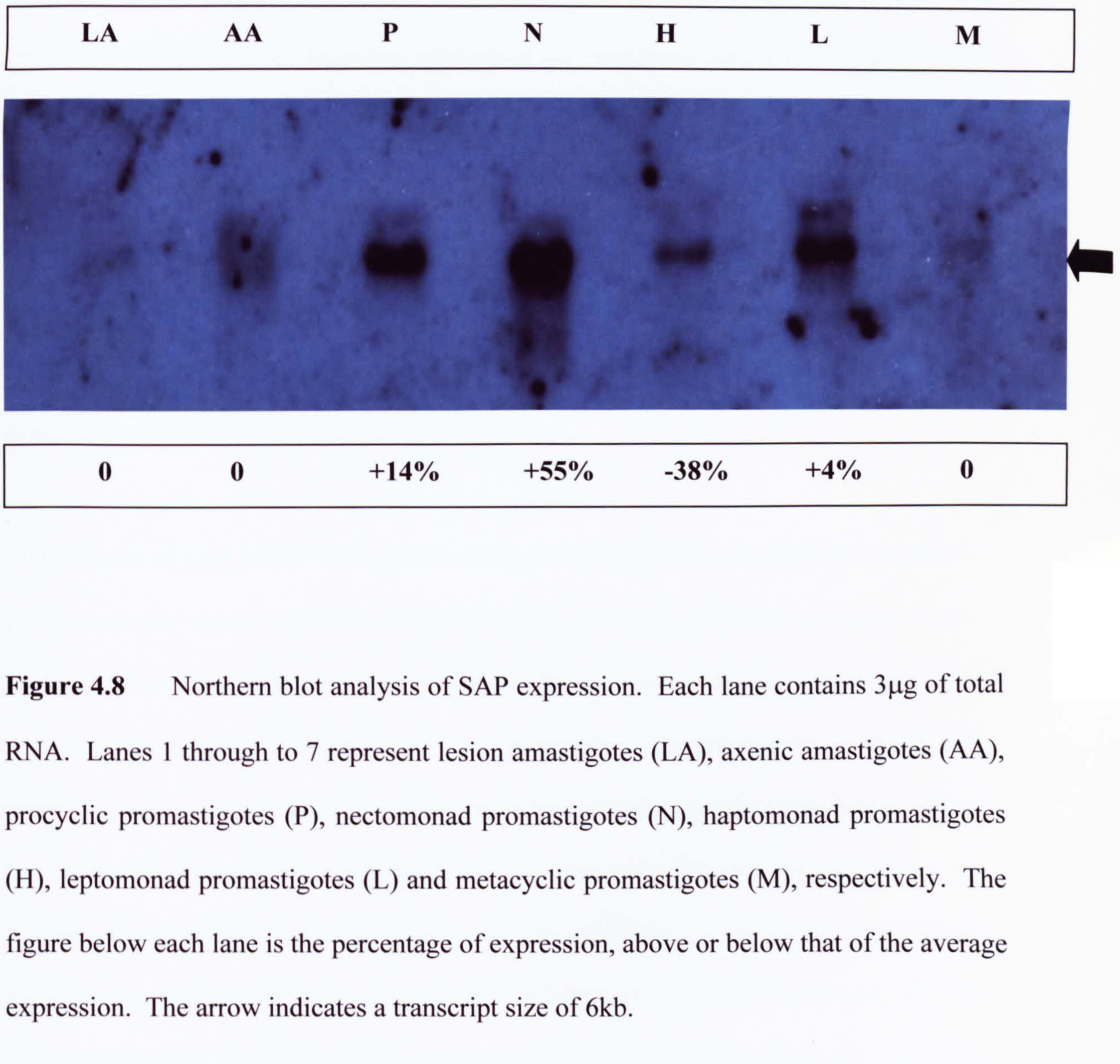



**Figure 4.8** Northern blot analysis of SAP expression. Each lane contains 3μg of total RNA. Lanes 1 through to 7 represent lesion amastigotes (LA), axenic amastigotes (AA), procyclic promastigotes (P), nectomonad promastigotes (N), haptomonad promastigotes (H), leptomonad promastigotes (L) and metacyclic promastigotes (M), respectively. The figure below each lane is the percentage of expression, above or below that of the average expression. The arrow indicates a transcript size of 6kb.

Expression of secretory acid phosphatase (SAP) was also examined using northern blot analysis. Section 2.6.2.1 gives a detailed description of SAP properties. Figure 4.8 shows the results obtained following probing of the membrane with a DIG-labelled SAP probe. No expression was detected in any of the two amastigote lanes, as was expected. Nectomonad promastigotes appear to be the dominant expresser of SAP mRNA, 55% above the average expression. Procyclic promastigotes and leptomonad promastigotes also showed levels of expression above that of the average, 14% and 4%, respectively. However, very little expression was detected for haptomonad promastigotes and expression was absent in metacyclic promastigotes.

The function of SAP is as yet still unknown but it could possibly be involved in nutrition. If this were the case it would be reasonable to expect high expression in highly motile nectomonad promastigotes, the form responsible for forward migration of infection. Due to their high motility it would be fair to assume that they expend more energy and therefore require more nutrients. Both procyclics and leptomonad promastigotes have been shown to be the forms responsible for the expansion of the parasite population via division and therefore would presumably also require nutrients. However, the nutrients available to each of these life cycle stages are different, namely blood and sugar, and it is therefore possible that SAP is only functional against one of these food sources. It is most likely that this would be blood, as most expression is seen in the two forms that are most likely to be in contact with the bloodmeal or its remnants. As expression is up-regulated in nectomonad promastigotes, the dominant form following defecation of the

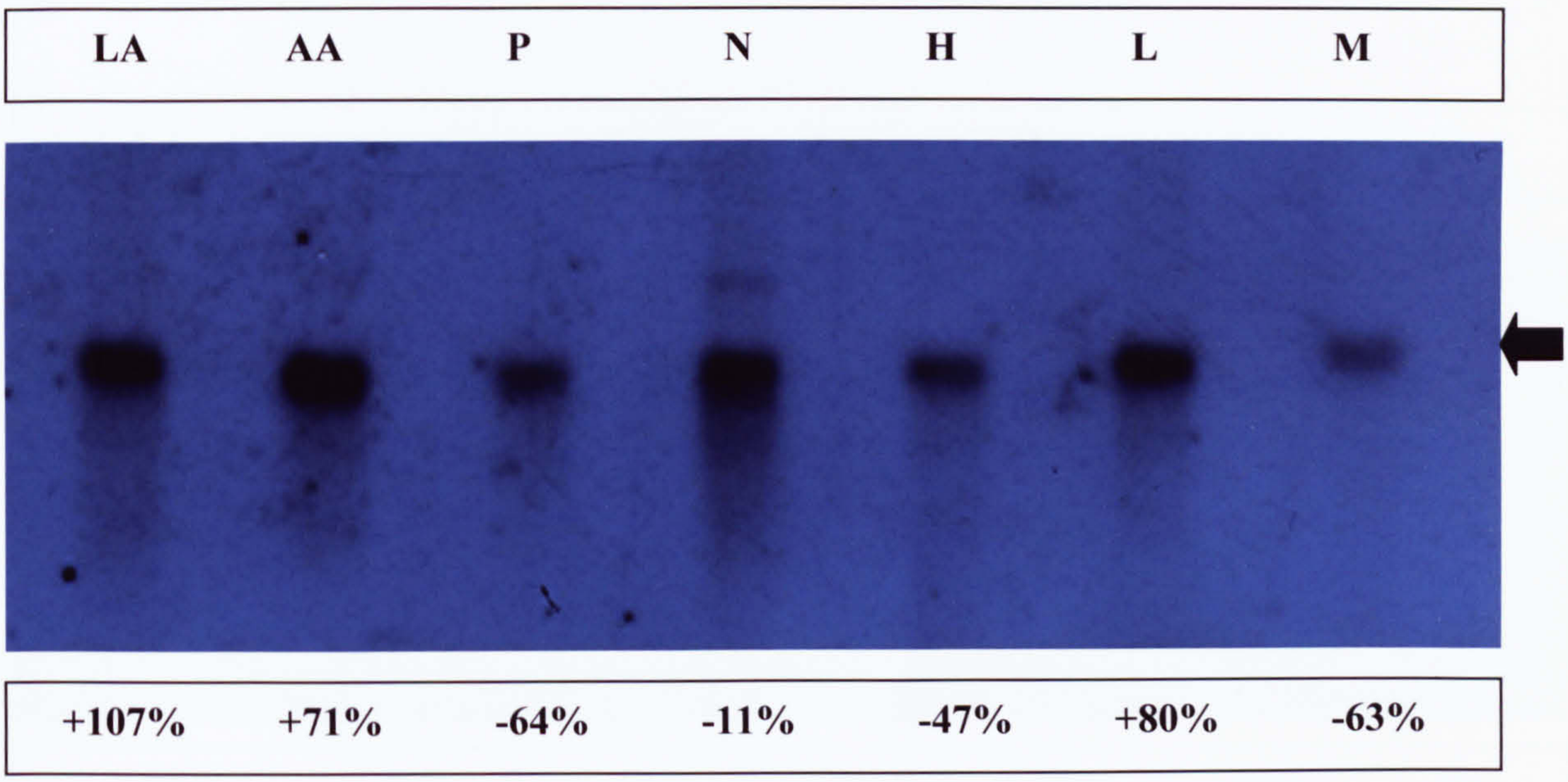
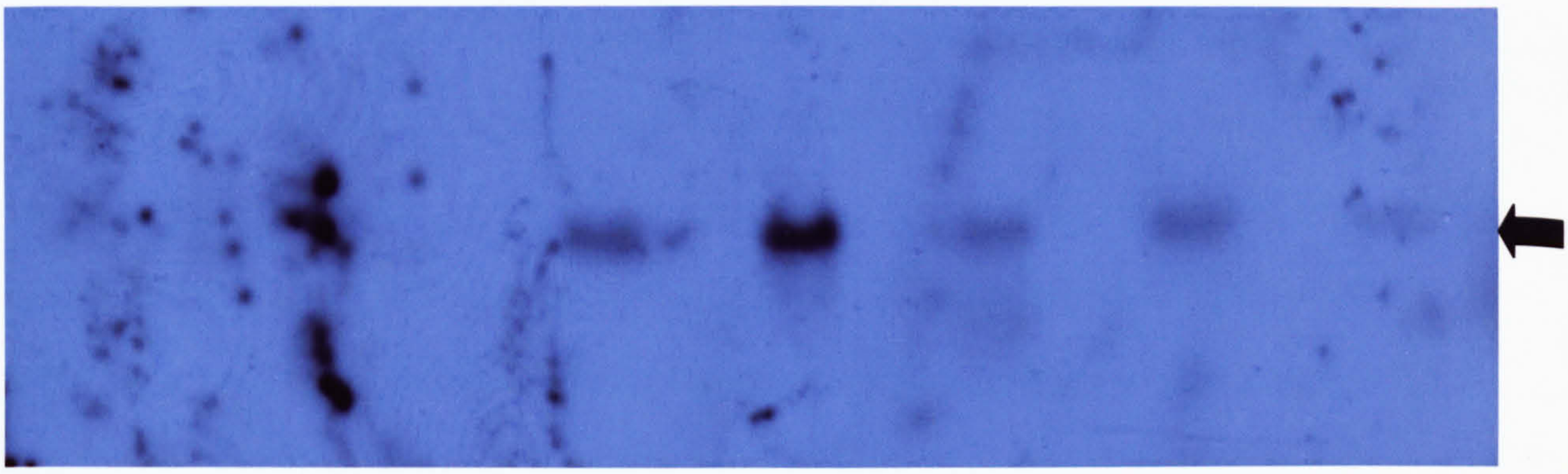



**Figure 4.9** Northern blot analysis of pPPG2a expression. Each lane contains 3µg of total RNA. Lanes 1 through to 7 represent lesion amastigotes (LA), axenic amastigotes (AA), procyclic promastigotes (P), nectomonad promastigotes (N), haptomonad promastigotes (H), leptomonad promastigotes (L) and metacyclic promastigotes (M), respectively. The figure below each lane is the percentage of expression, above or below that of the average expression. The arrow indicates a transcript size of 3.6kb.

| LA | AA | P | N | H | L | M |
|---|---|---|---|---|---|---|



| 0% | 0% | -17% | +72% | -35% | -30% | 0% |
|---|---|---|---|---|---|---|

**Figure 4.10** Northern blot analysis of 3'NT/Nu expression. Each lane contains 3μg of total RNA. Lanes 1 through to 7 represent lesion amastigotes (LA), axenic amastigotes (AA), procyclic promastigotes (P), nectomonad promastigotes (N), haptomonad promastigotes (H), leptomonad promastigotes (L) and metacyclic promastigotes (M), respectively. The figure below each lane is the percentage of expression, above or below that of the average expression. The arrow indicates a transcript size of 3.7kb.

bloodmeal, it could be possible that this is some kind of starvation factor, as nutrients would be in a state of limitation.

A second member of the proteophosphoglycan family was investigated for differential gene expression, pPPG2a. This molecule is a secreted PPG and shares a common protein backbone with aPPG (see section 2.6.2.3 for details). The resulting bands following hybridisation with the pPPG2a DIG-labelled probe are shown in Figure 4.9. Although pPPG2 is a promastigote form of PPG it was shown to be expressed at high levels in amastigotes, lesion amastigotes expressing 107% above the average expression and axenic amastigotes 71% above average. The only promastigote forms to express levels above the average expression were the leptomonad promastigotes, 80%. In all other promastigote forms pPPG2a mRNA was detected at levels below that of the average expression. An apparently unusual finding was that amastigotes were expressing high levels of mRNA for a promastigote secreted molecule. However, as mentioned previously both aPPG and pPPG share a common protein backbone, therefore the probe is detecting mRNA in the amastigotes that is coding for aPPG. The function of pPPG is not yet known, but as it was found to be up-regulated in leptomonad promastigotes in this study, and previous work has suggested that this form is responsible for the generation of PSG (Rogers *et al.* 2002), it could be possible that pPPG2 is a minor component of PSG.

Figure 4.10 shows the detected expression of the surface membrane 3'-nucleotidase/nuclease (3'NT/Nu). This protein functions in purine salvage as *Leishmania* parasites are incapable of synthesizing purine nucleotides *de novo* and has been shown to

be up-regulated in starvation conditions. The data shown in figure 4.10 indicates that there is an up-regulation of 3'NT/Nu in nectomonad promastigotes as these forms were expressing 72% more than the average expression. No mRNA for 3'NT/Nu was detected in amastigotes and there seemed to be a down regulation in procyclic promastigotes, haptomonad promastigotes and leptomonad promastigotes, with 7%, 35% and 30% expression below average expression, respectively. Expression in metacyclics was also not detected. As this gene has been shown to be up-regulated in conditions of starvation it seems plausible that the nectomonad promastigotes would be the most likely form to show increased expression of this gene, as these are the form subjected to low nutrient conditions.

The final gene to be investigated in this study was chitinase. Figure 4.11 shows the expression bands found during northern blot analysis. The data revealed that all life cycle stages produce mRNA for chitinase. However, the amount between each stage varied, the most obvious being that of the metacyclic promastigotes, which produced 63% below that of the average. Lesion amastigotes were found to contain the most chitinase mRNA and produced 41% above the average. Procyclic promastigotes and nectomonad promastigotes also showed an above average expression of chitinase, 15% and 11%, respectively. Leptomonad promastigotes expressed 2% below the average expression, whereas in haptomonad promastigotes expression was 25% below the average. A second band of 1.5kb was observed in haptomonad and metacyclic promastigotes and was seen in all repeats of this hybridisation. The protein product of this mRNA is unknown but it must have a similar sequence to the part of the chitinase

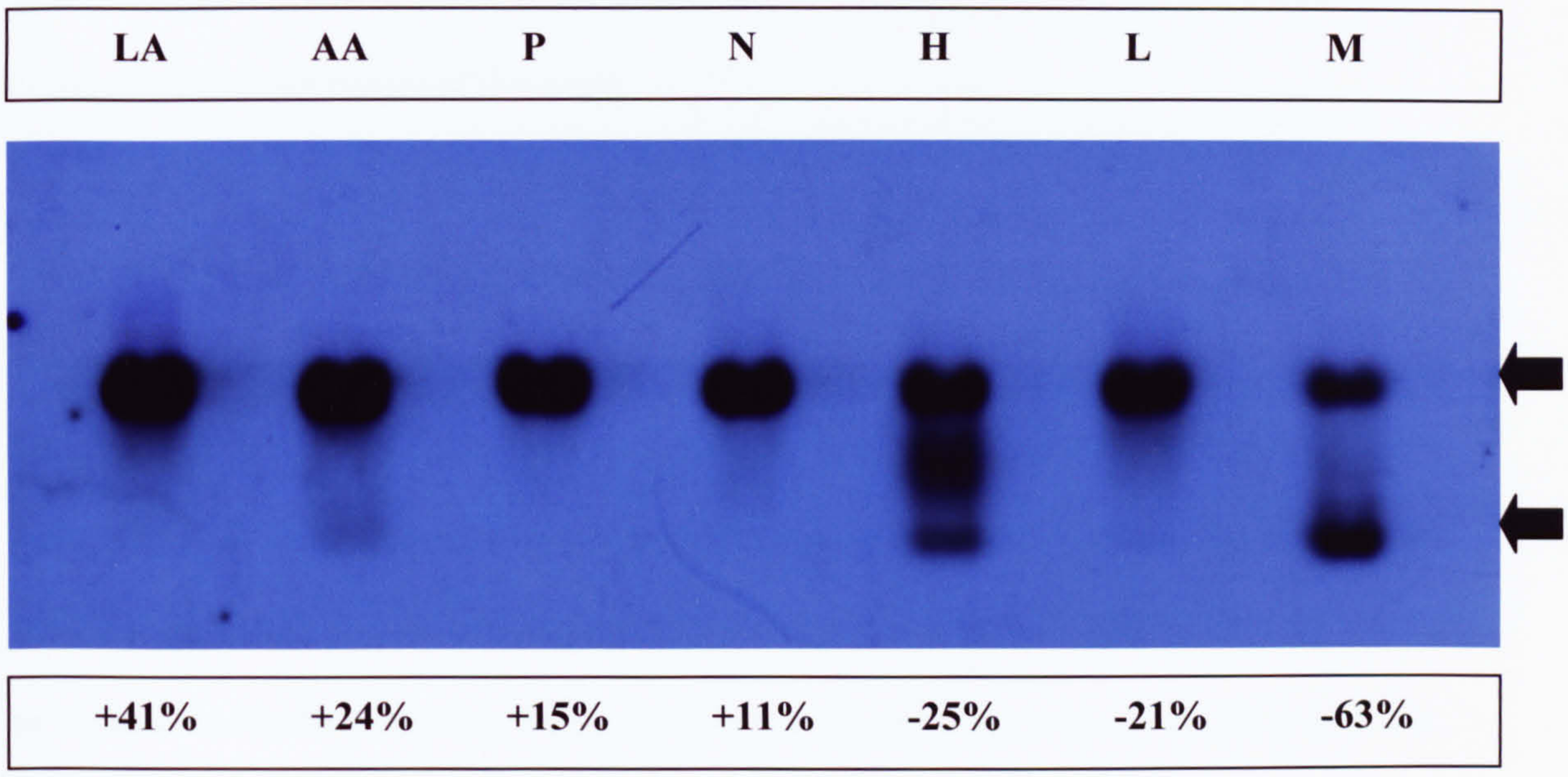



**Figure 4.11** Northern blot analysis of chitinase expression. Each lane contains 3μg of total RNA. Lanes 1 through to 7 represent lesion amastigotes (LA), axenic amastigotes (AA), procyclic promastigotes (P), nectomonad promastigotes (N), haptomonad promastigotes (H), leptomonad promastigotes (L) and metacyclic promastigotes (M), respectively. The figure below each lane is the percentage of expression, above or below that of the average expression. The top arrow indicates a transcript size of 3kb and the bottom arrow indicates a transcript size of 1.5kb

gene used as a probe in these experiments. This would require further investigation to determine the exact nature of this band.

As chitin is a polysaccharide found in the guts of insects the high expression of chitinase seen in amastigotes seems puzzling, as this is the stage found in the mammalian host. The function that amastigote chitinase has in the mammalian host is unknown; if at all there is one. It should be noted that the presence of mRNA does not necessarily indicate the presence of the protein, although this is true in most cases. Another possibility could be that as this is the first form found in the vector it somehow conditions the gut of the sandfly making it more accessible to infection. As chitinase is known to weaken the peritrophic matrix it could be possible that if chitinase is being excreted during PM formation this could lead to an already weakened structure. This would consequently make escape from the PM easier for the promastigote stages, which according to the data in Figure 4.10 express less chitinase than lesion amastigotes. Chitinase expression is significantly reduced in metacyclic promastigotes indicating that there is no role for this enzyme in mammalian infection, which would be in agreement of the above interpretation of the data.

## 4.7 *In vitro* Macrophage Infections

Rogers *et al.* (2002) proposed that a major function of leptomonad promastigotes is the production of PSG. Due to its anterior location and the feeding mechanism of infected flies by regurgitation a proportion of the PSG is co-inoculated into the mammalian host

along with the infective metacyclic promastigotes (Rogers *et al.* 2004). Sandfly saliva has long been of interest and several studies have concluded that saliva has an exacerbative effect on host infection, see section 2.10.

Experiments were designed to determine if PSG or sandfly saliva played any direct role in the uptake of parasites by host macrophages. Chamber slides were seeded with mouse peritoneal macrophages and allowed to adhere, as described in section 3.12. Any unattached macrophages were removed via washing. Metacyclic promastigotes were cultured as described in section 3.2.3 and the density was adjusted to give a ratio of 5:1 (parasites: macrophages), which was determined to be the most effective in a series of preliminary experiments. PSG and SGC preparations were prepared in RPMI (15% FBS), as described in section 3.11. Slides were incubated at 32°C with 5% humidified $CO_2$ and sampled at 4, 24, 48 and 72 hours. Slides were washed to remove any free parasites, stained with Giemsa's stain and analysed under oil using light microscopy.

Figure 4.12 represents the percentage of macrophages infected at the various time points. There is no major difference between the various conditions tested, with the percentage of infected macrophages relatively unchanged. The addition of neither PSG, saliva or a combination of both increased the uptake of parasites by macrophages above that of the control. This is particularly evident at 4 hours and 48 hours post-infection. At 4 hours post-infection the percentage of infected macrophages in the control group was 71% compared to 75%, 73% and 74% for the PSG, saliva and PSG/saliva groups respectively. At all time points both the control and PSG groups showed very similar percentages of

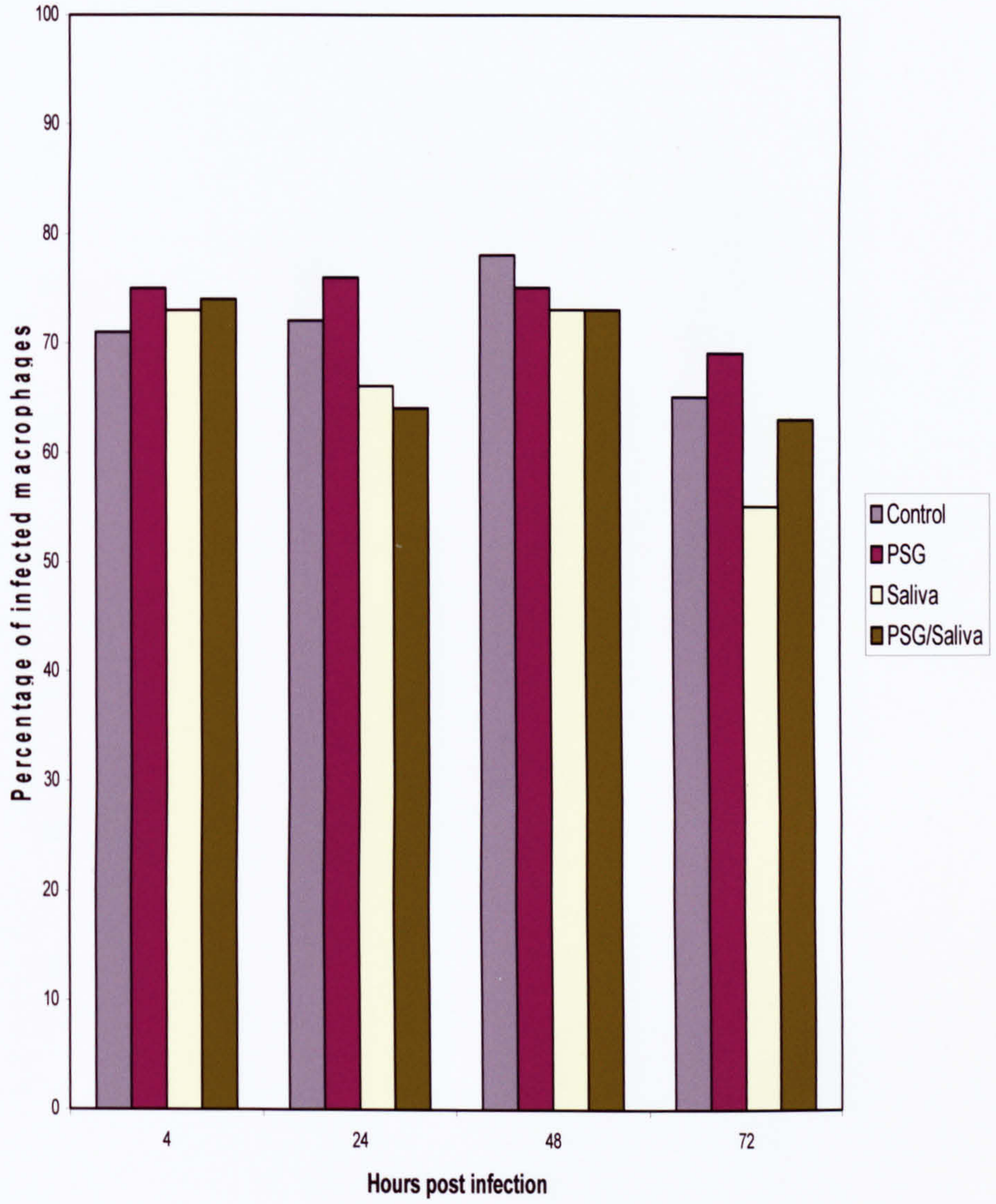



**Figure 4.12** Percentage of infected macrophages following incubation with PSG (1 plug/ml), SGC (2 glands/ml) or a combination of both PSG/SGC. Samples were taken at 4, 24, 48 and 72 hours post infection. For each sample 100 macrophages were analysed. The data shown is the mean of two independent experiments.

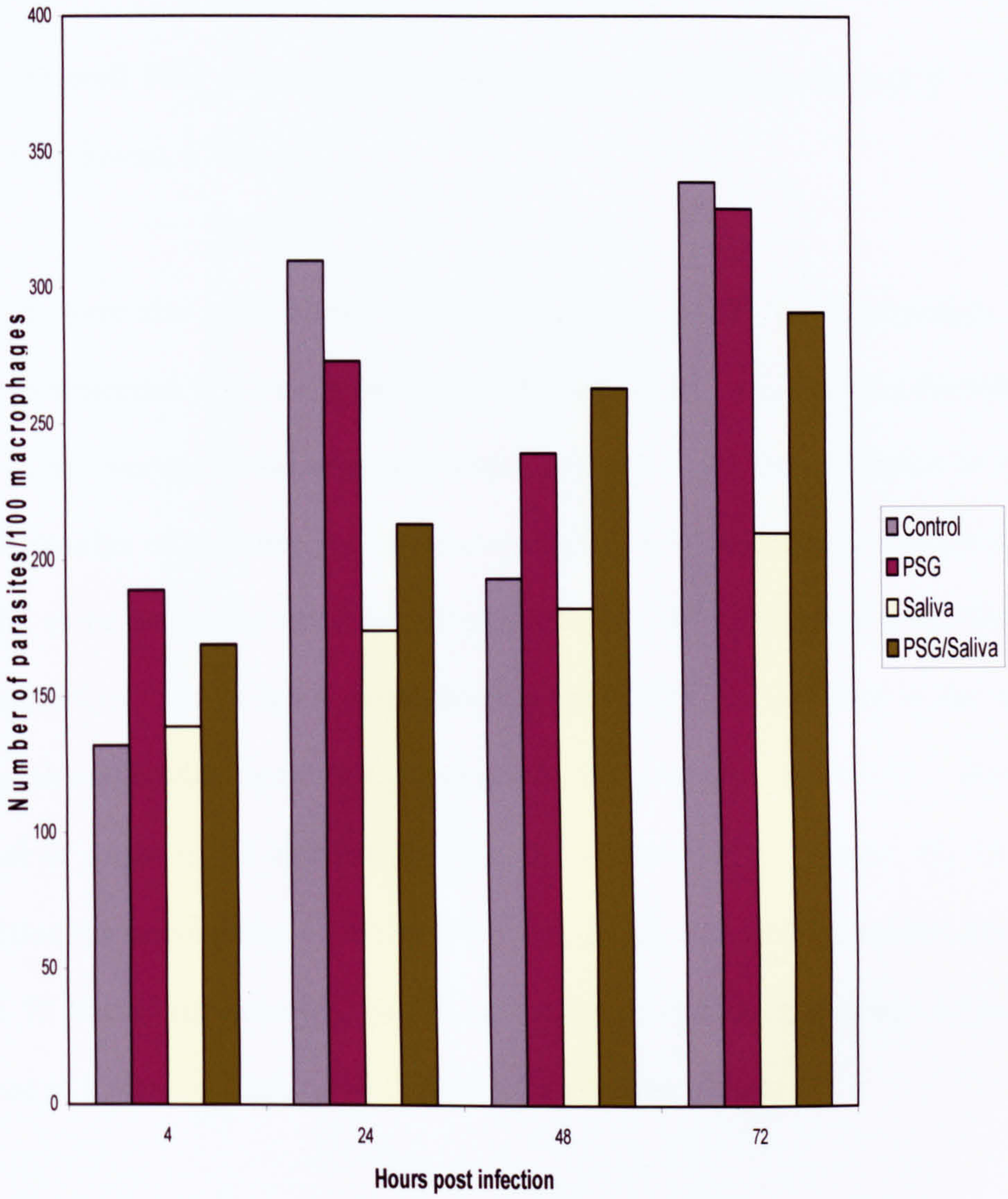



**Figure 4.13** Number of parasites per 100 macrophages following incubation with PSG (1 plug/ml), SGC (2 glands/ml) or a combination of both PSG/SGC. Samples were taken at 4, 24, 48 and 72 hours post infection. For each sample 100 macrophages were analysed. The data shown is the mean of two independent experiments.

infection. Overall PSG gave the highest percentage of infection, averaging 74% and saliva gave the lowest, 67%.

Observations were also made on the number of parasites within the macrophages. This was done to determine if it were possible for PSG and/or saliva to increase the infection in regard to the amount of amastigotes within each cell. This data is shown in Figure 4.13 as the number of parasites per 100 macrophages. At 4 hours post-infection there is only slight variation in the numbers of parasites per 100 macrophages between the various conditions. At 24 hours post-infection the number of parasites in the control group was almost double that in the saliva group, 310 and 174 respectively. However, the number of parasites did increase with time in all experimental groups. By 72 hours post-infection the saliva group contained 38% less amastigotes than the control group. At 4, 24 and 72 hours post-infection the PSG/saliva group contained numbers of parasites that seemed to be intermediate between the PSG and saliva groups.

Following the observation that neither PSG nor saliva appeared to play any direct role in the uptake of parasites by macrophages, their protective effects, if any, were investigated. To assess a protective effect macrophages were stimulated with 20U/ml IFN-γ at the time of infection. The data collected is shown in Figures 4.14 and 4.15. The percentage of infected macrophages (Figure 4.14) appears not to be affected by the addition of IFN-γ as all experimental conditions have similar rates of infection. At 24, 48 and 72 hours there was no difference between the two PSG groups (white and red bars) confirming that PSG gave no advantage to the parasites during uptake by the macrophages. Interestingly it

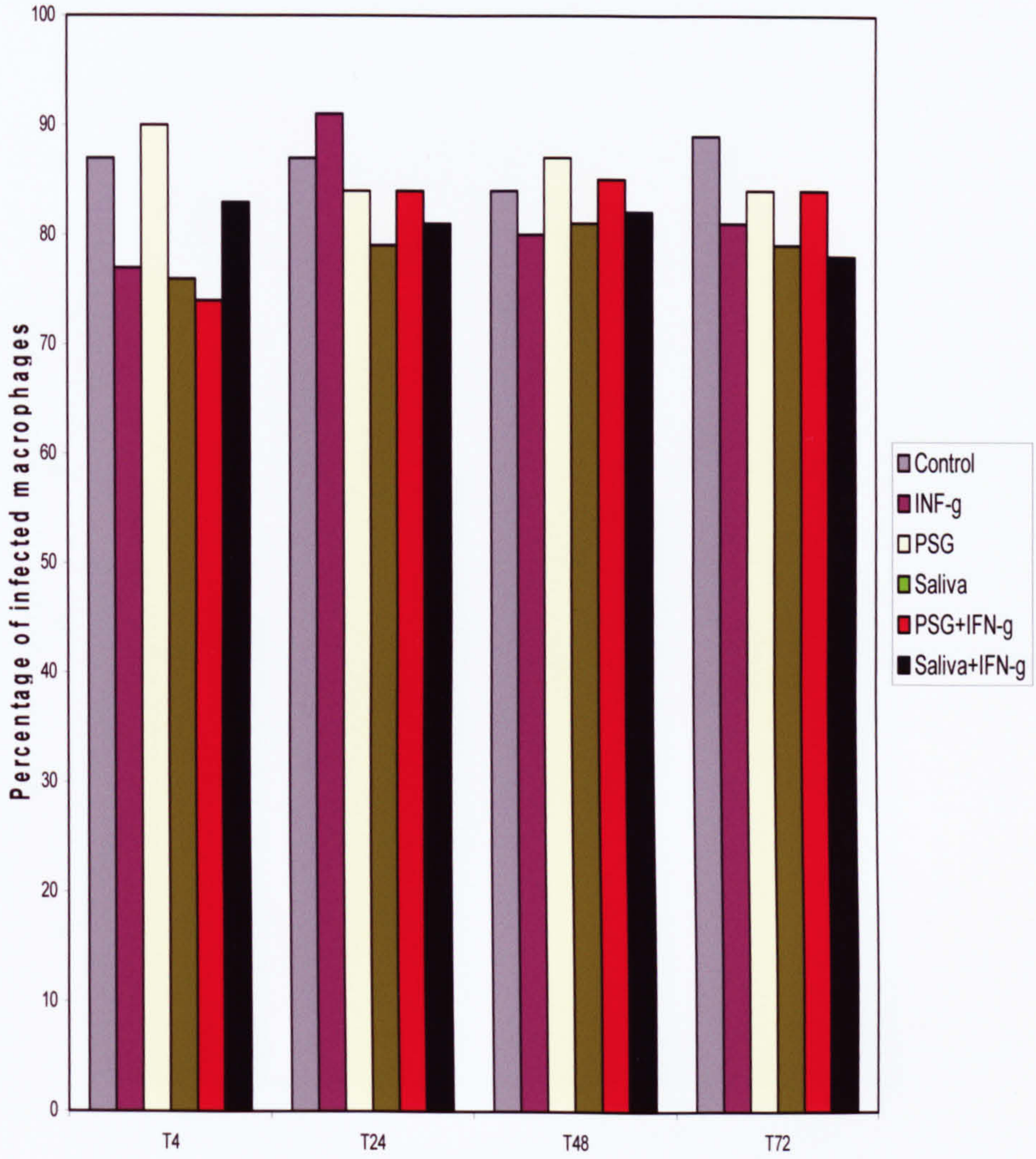



**Figure 4.14** Percentage of infected macrophages following incubation with PSG (1 plug/ml) or SGC (2 glands/ml) in IFN-γ activated macrophages. Samples were taken at 4, 24, 48 and 72 hours post infection. For each sample 100 macrophages were analysed. The data shown is the mean of two independent experiments.

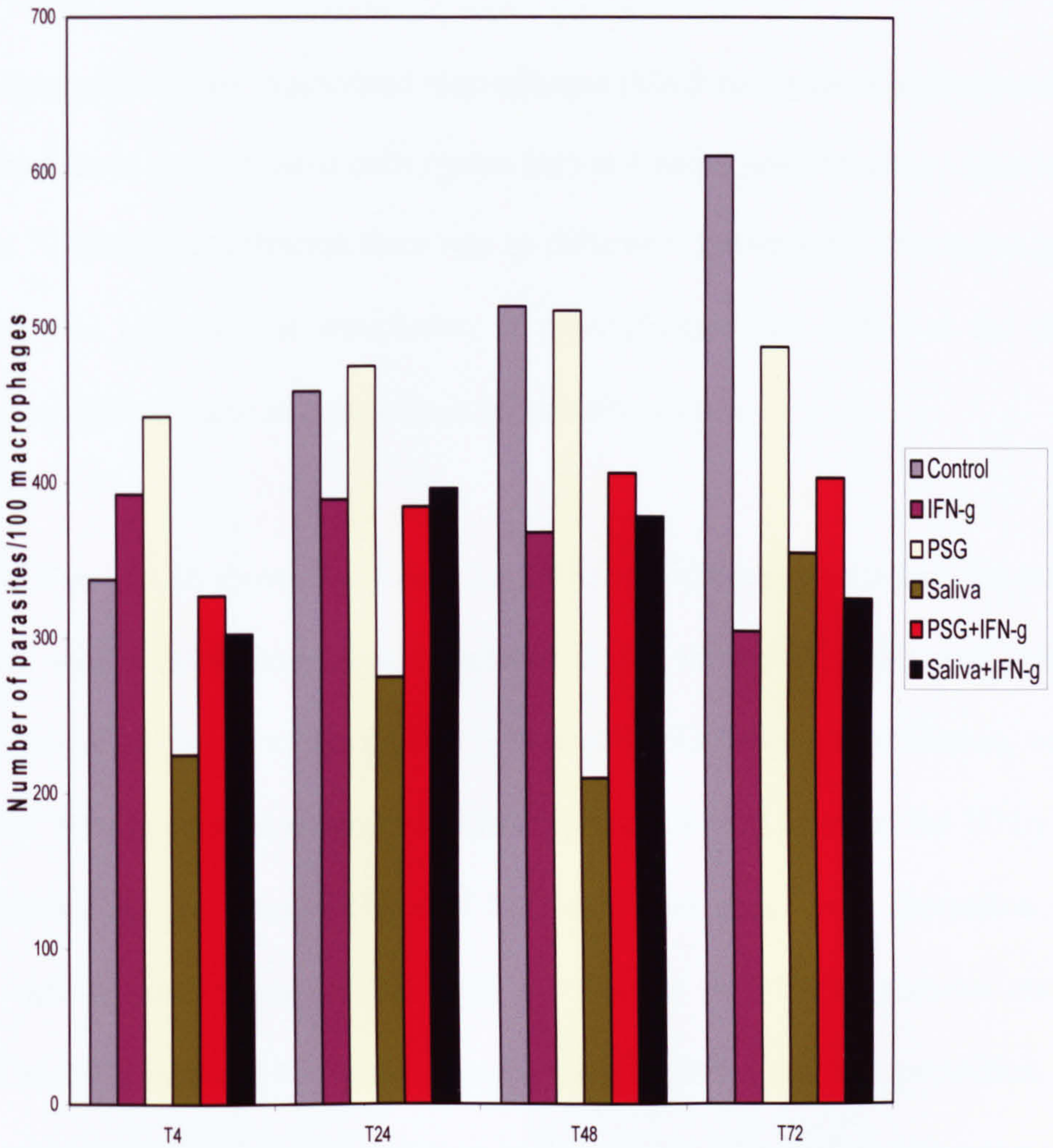



**Figure 4.15** Number of parasites per 100 macrophages following incubation with PSG (1 plug/ml) or SGC (2 glands/ml) in IFN-γ activated macrophages. Samples were taken at 4, 24, 48 and 72 hours post infection. For each sample 100 macrophages were analysed. The data shown is the mean of two independent experiments.

appeared that saliva added to activated macrophages (black bar) gave a higher percentage of infection than in non-activated cells (green bar) at 4 hours post infection. However, at 24, 48 and 72 hours post infection there was no difference between the two saliva groups. These data also indicate that stimulation of macrophages with IFN-γ at the time of infection does not have any adverse effects on parasite uptake.

The data in Figure 4.15 shows that following IFN-γ stimulation (purple bar) the numbers of parasites within the macrophages is reduced at 24, 48 and 72 hours post infection, compared to the control (grey bar). This is clearest at 72 hours post-infection, where a reduction of 50% in parasite numbers/100 macrophages is seen between the IFN-γ group and the control group. The addition of PSG (red bar) gave some protection to the parasites against macrophage killing, with a reduction of 34% in parasite numbers, compared to 50% in the IFN-γ group (purple bar). Saliva gave less protection to the parasites with a 47% reduction (black bar) in the number of parasites/100 macrophages compared with 50% in the IFN-γ group.

Parasite killing by stimulated macrophages should be more intense if they are stimulated prior to infection and therefore the protective effect of the PSG or saliva could be further tested. Therefore, further experiments were performed in which IFN-γ was used at 20U/ml to stimulate the macrophages at the time they were seeded into the chamber slides. Following an overnight incubation at 32°C with 5% humidified $CO_2$, the stimulated macrophages were infected with day 6 metacyclic promastigotes, under various experimental conditions. A group to include both PSG and saliva was added to

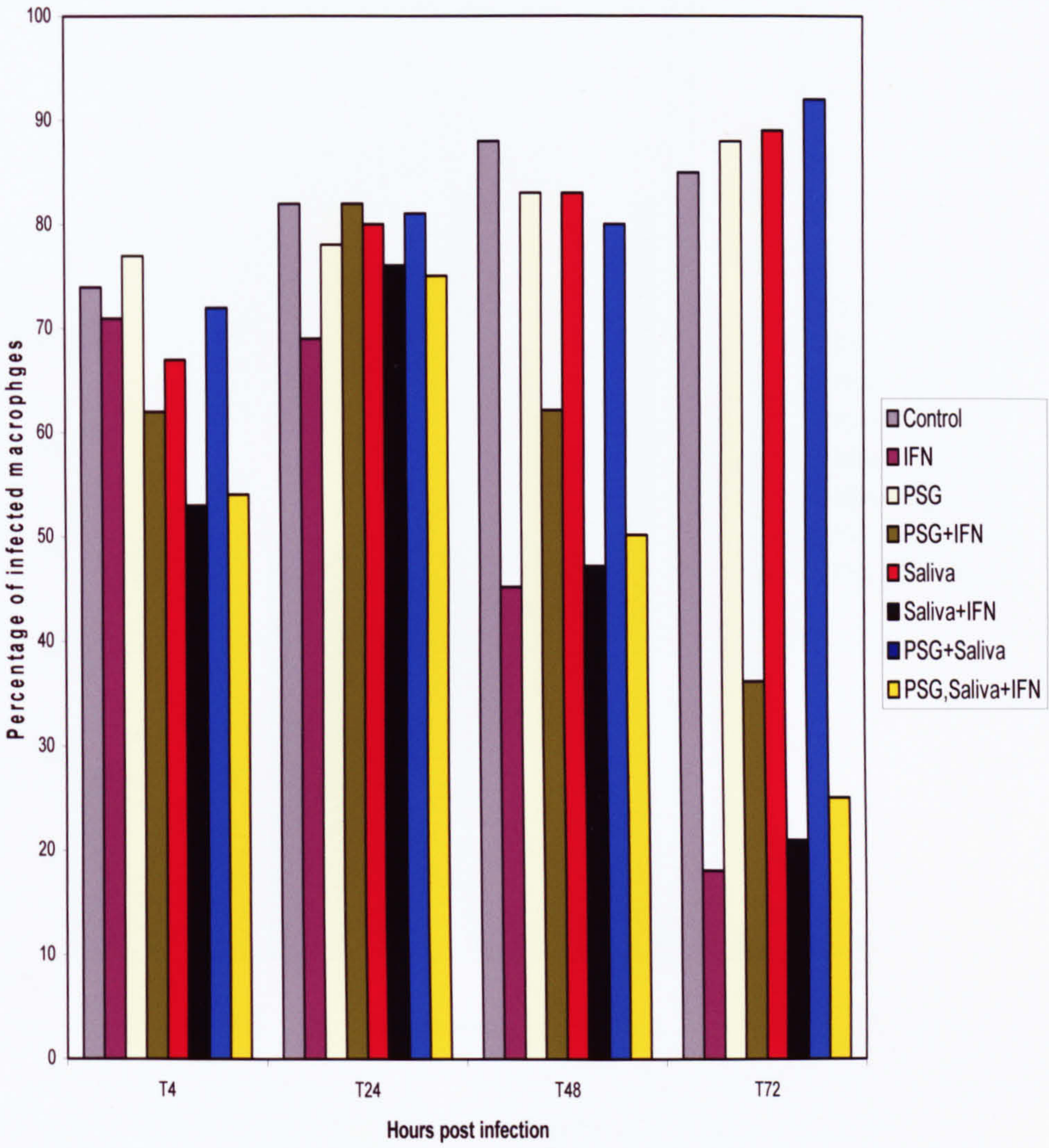



**Figure 4.16** Percentage of infected macrophages following incubation with PSG (1 plug/ml), SGC (2 glands/ml) or a combination of both PSG/SGC in IFN-γ activated macrophages. Samples were taken at 4, 24, 48 and 72 hours post infection. For each sample 100 macrophages were analysed. The data shown is the mean of two independent experiments.

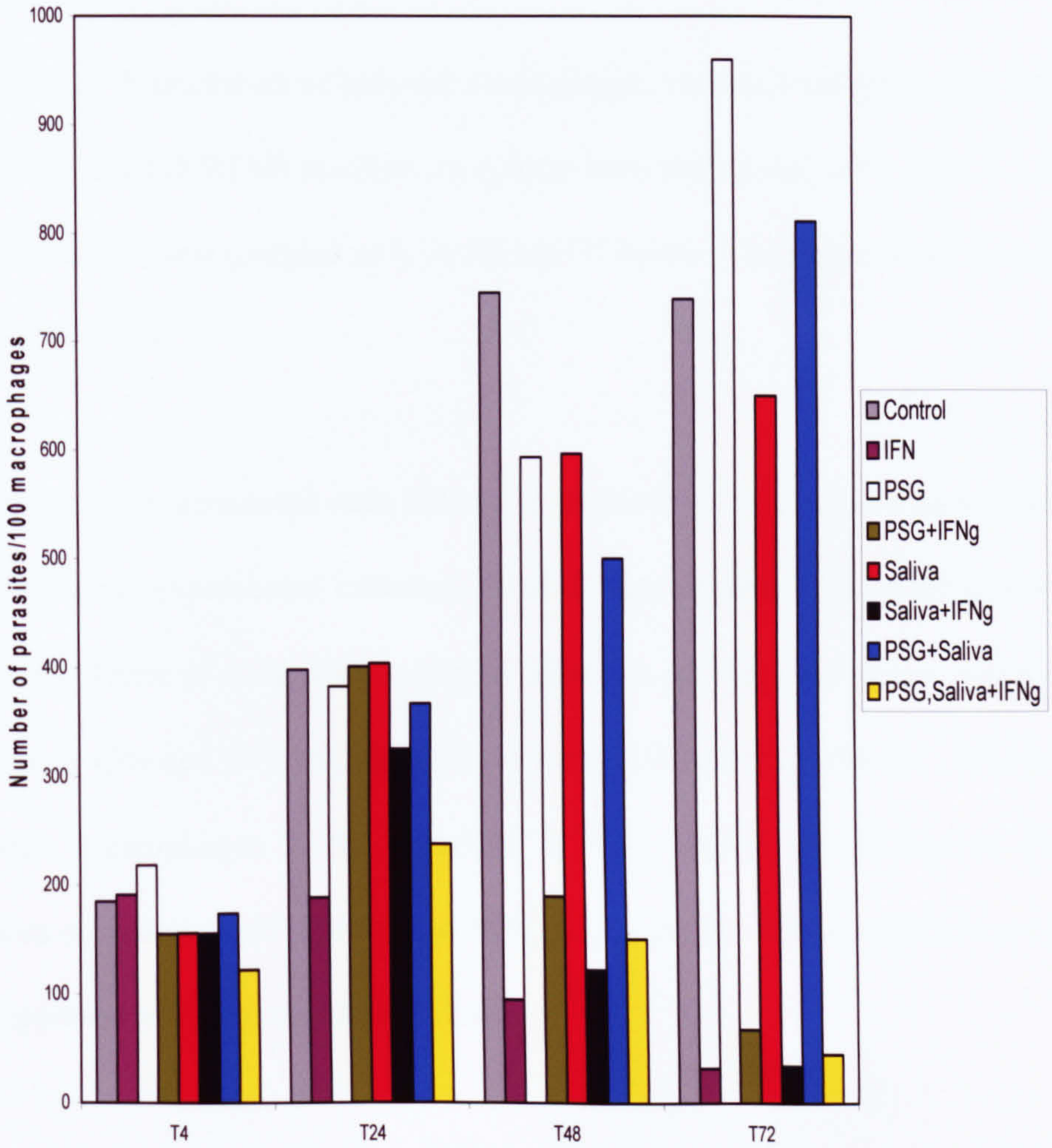



**Figure 4.17** Number of parasites per100 macrophages following incubation with PSG (1 plug/ml), SGC (2 glands/ml) or a combination of both PSG/SGC in IFN-γ activated macrophages. Samples were taken at 4, 24, 48 and 72 hours post infection. For each sample 100 macrophages were analysed. The data shown is the mean of two independent experiments.

this experiment. Stimulation of infected macrophages was maintained by the addition of 20U/ml IFN-γ in fresh RPMI medium on a daily basis and slides were kept at 32°C with 5% humidified $CO_2$ and sampled at 4, 24,48 and 72 hours. The data collected is shown in Figures 4.16 and 4.17.

In macrophages pre-stimulated with IFN-γ the presence of PSG, saliva or a combination of both PSG/saliva exacerbated infection. This is most evident at 48 hours post-infection (Figure 4.16). There is a significant loss of infection between the IFN-γ group and the control, where 45% and 88% of macrophages were infected, respectively. Whereas IFN-γ stimulated macrophages in the presence of PSG, saliva, or PSG/saliva showed a reduced loss of infection, 62%, 47% and 50%, respectively. This exacerbation of disease was also apparent at 72 hours post infection.

The data showing the numbers of parasites/100 macrophages, in Figure 4.17, also suggests that PSG and saliva have some exacerbative effect upon infection. At 48 hours post-infection an 87% reduction of parasite numbers occurred in the IFN-γ stimulated macrophages as compared to the control. However, in the presence of PSG this loss of parasites was reduced to 75% when compared to the control group (grey bar). Saliva only also reduced the level of parasite killing showing an 84% reduction in parasite numbers. Interestingly a combination of both PSG and saliva gave an intermediate level of protection between that of PSG and saliva. By 72 hours post infection the exacerbative effect of all three groups had significantly decreased and the numbers of parasites/100 macrophages was only 9% of the control group for the PSG group, 5% for

saliva and 6% for PSG/saliva. However these figures were still higher compared to the 4% observed in the IFN-γ group.

To determine how potent the protective effects of PSG were a further series of experiments using macrophages pre-stimulated with various concentrations of IFN-γ, 20U/ml, 100U/ml and 500U/ml were carried out. Stimulation was maintained as previously described above and samples were taken at 4, 24, 48 and 72 hours post-infection. The collected data is shown in Figures 4.18 and 4.19. At 72 hours post infection stimulation of macrophages with 20U/ml (white bar), 100U/ml (red bar) or 500U/ml (blue bar) gave 77%, 61% and 61% reduction in infection respectively compared to the control group. Interestingly it was the macrophages stimulated with 20U/ml IFN-γ that gave the most reduction in infection (Figure 4.18). The addition of PSG to infected macrophages stimulated with 20U/ml IFN-γ (green bar) provided some protection against loss of infection and reduced loss of infection from 77% to 58%. However, the addition of PSG to macrophages stimulated with 100U/ml (black bar) and 500U/ml (yellow bar) IFN-γ gave no protection to loss of infection at 72 hours post infection.

When parasite killing was assessed by way of number of parasites/100 macrophages (Figure 4.19) it was stimulation with 100U/ml IFN-γ (red bar) that produced the most parasite killing, 94%, compared with 91% for 20U/ml (white bar) and 57% for 500U/ml (blue bar), when compared to the control at 72 hours post infection. It would seem that there is a saturation level, after which further addition of IFN-γ has no effect on parasite

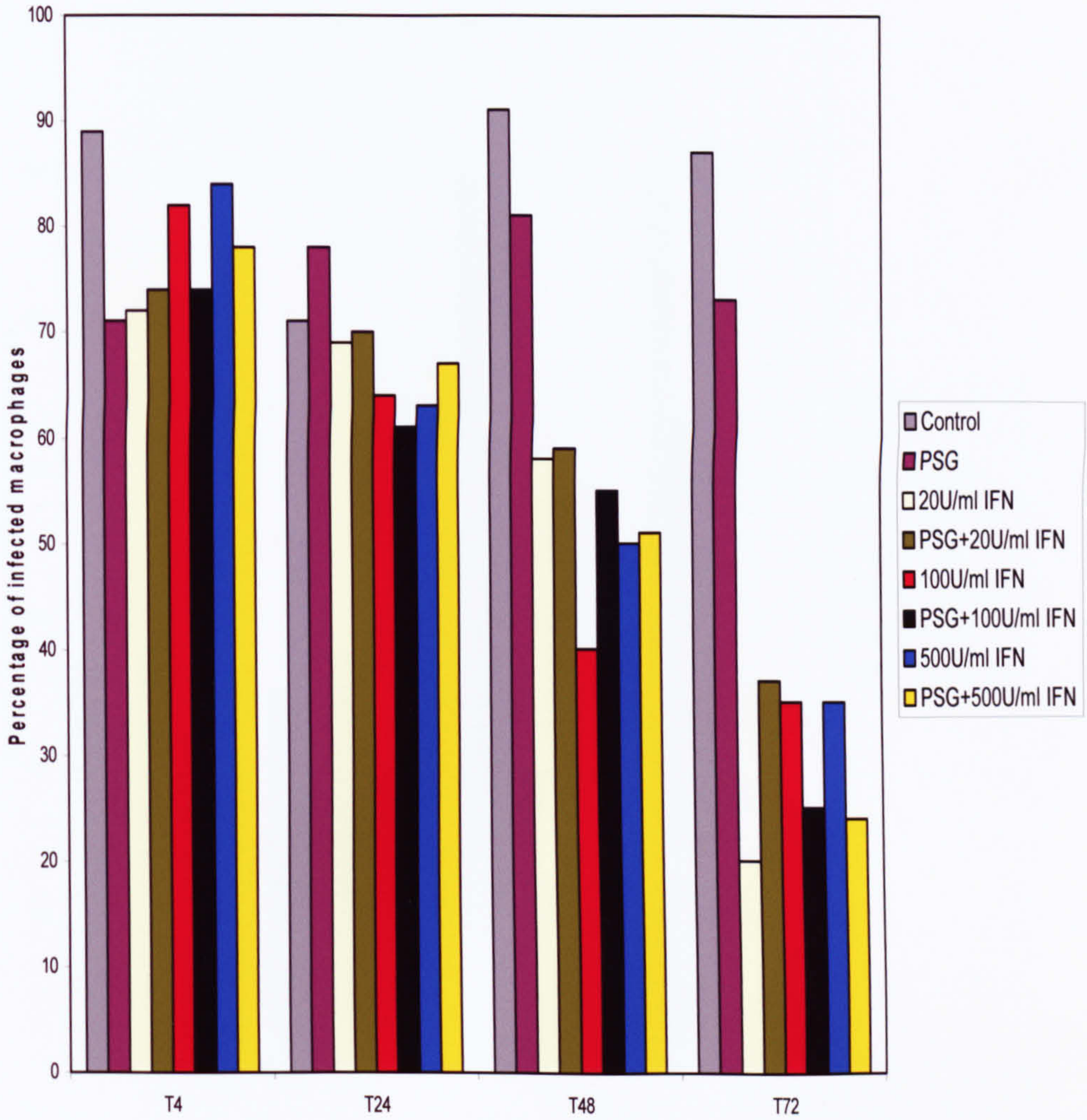



**Figure 4.18** Percentage of infected macrophages following incubation with PSG (1 plug/ml) and various concentration of IFN-γ 20U/ml, 100U/ml and 55U/ml. Samples were taken at 4, 24, 48 and 72 hours post infection. For each sample 100 macrophages were analysed. The data shown is the mean of two independent experiments.

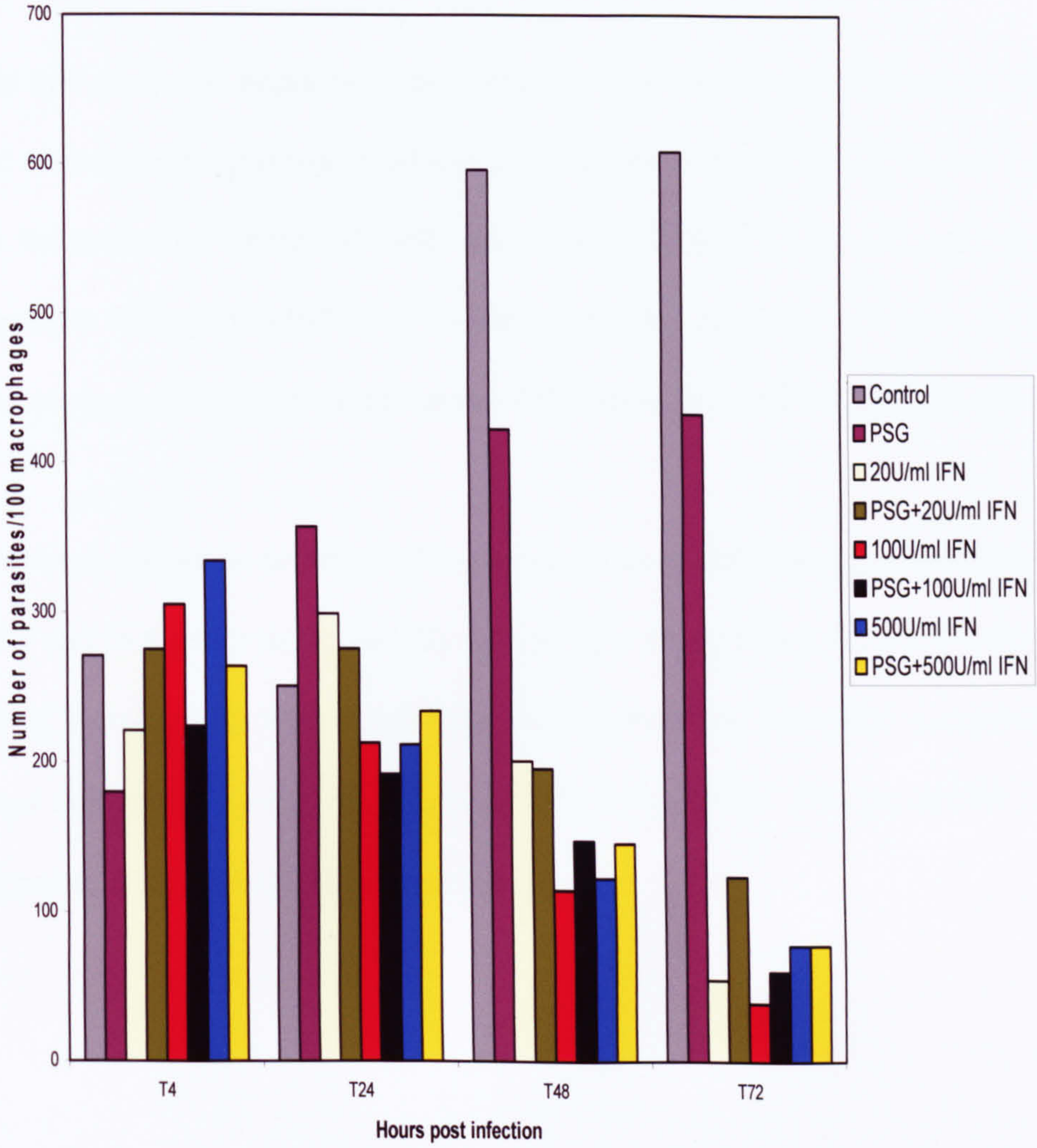



**Figure 4.19** Number of parasites per macrophages incubation with PSG (1 plug/ml) and various concentration of IFN-γ 20U/ml, 100U/ml and 55U/ml. Samples were taken at 4, 24, 48 and 72 hours post infection. For each sample 100 macrophages were analysed. The data shown is the mean of two independent experiments.

killing. In this study this appeared to be 100U/ml. The addition of PSG to the 20U/ml stimulated macrophages (green bar) reduced macrophage killing of parasites form 91% to 80%. In macrophages stimulated with 100U/ml, PSG (black bar) was only able to reduced parasite killing from 94% to 91% and completely unable to offer any protection against macrophage killing of parasites in the 500U/ml (yellow bar) stimulated cells.

The macrophage infections described above have shown a clear exacerbation of infection due to PSG and to a lesser extent sandfly saliva. This exacerbation does not seem to be the result of increased up take of parasites by macrophages but rather a reduction in macrophage killing of the parasites, following IFN-γ stimulation. The exacerbative effect is most apparent from 48 to 72 hours post infection.

# Chapter 5

# Discussion & Future Work

## 5.1 Discussion

This study aimed to determine the developmental sequence of the various morphological forms of parasite within *Leishmania* development. This was achieved by following the development of *L. infantum* in its natural sandfly host *Lu. longipalpis*. *In vitro* development of parasites was also examined for both *L. infantum* and *L. major*. The divisional status of each form of developing parasite, both *in vivo* and *in vitro*, was assessed to determine which life cycle stage was responsible for the expansion of the parasite population.

This study has provided evidence for the existence of four major morphological promastigote forms during *L. infantum* development in *Lu. longipalpis*. Four major promastigote forms; procyclic promastigote, nectomonad promastigote, leptomonad promastigote and metacyclic promastigote were observed in several repeated experiments. The data provided here confirms a precursor-product relationship between the various life cycle stages; amastigote-procyclic promastigote, procyclic-nectomonad promastigote, nectomonad-leptomonad promastigote and leptomonad-metacyclic promastigote (Figure 4.2). These relationships were proposed by Rogers *et al.* (2002) and the current data is in agreement with their observations

using *L. mexicana* in *Lu. longipalpis*. However, the data presented here has the extra advantage of being a natural parasite/vector combination.

Several earlier studies have examined the development of various species of *Leishmania* in a variety of vectors, some experimental and others natural combinations (see section 2.1.2). The findings of this study are in agreement with most of these previous investigations, although the parasite forms described in these earlier studies were not fully quantitated. The morphologies outlined by Rogers *et al* (2002) were followed within this study.

The results of this investigation indicate that *L. infantum* has two independent cycles of multiplication during development in *Lu. longipalpis*: the first as procyclic promastigotes (bloodmeal phase) and the second as leptomonad promastigotes (sugarmeal phase). These two cycles of division appear to be separated both in time and space. A non-dividing form, nectomonad promastigote, appears to link the two divisional cycles together.

Amastigote forms of *L. infantum* isolated from the midgut of *Lu. longipalpis* during this study were never observed in the process of division (data not included). This is in disagreement with observations by Lainson and Shaw (1988) using the same parasite/vector combination. They stated that amastigote forms can follow two lines of development, non-dividing and dividing. The dividing amastigotes were described as enlarged, and sometimes possessing a very short rudimentary

flagellum. Similar data was described for *L. panamensis* in *Lu. gomezi* where they were termed "amastigote-like". It is therefore possible that these are actually procyclic promastigotes, a stage shown here to be a dividing form. In contrast to Lainson and Shaw (1988) but in agreement with the data presented here, Walters *et al.* (1989a) observed no division of amastigotes during the development of *L. chagasi* (*infantum*) in *Lu. longipalpis*. Likewise division of amastigotes was not observed during the development of *L. mexicana* in *Lu. longipalpis* (Walters *et al.* 1987, Gossage *et al.* 2003) or *Lu. diabolica* (Lawyer *et al.* 1987) nor was it seen in *L. major* development in *Ph. duboscqi* (Lawyer *et al.* 1990). However, a later study using the experimental vector *Lu. longipalpis* reported the division of *L. major* amastigotes prior to transformation into stumpy promastigotes (Walters *et al.* 1993).

An interesting observation of this study was the dramatic fall in parasite numbers/fly early in infection (Figure 4.1). The reason for this is unclear and was not seen in similar studies by Pimenta *et al.* (1997) and Rogers *et al.* (2002). These previous studies used cutaneous species, *L. major* and *L. mexicana*, respectively. It is therefore possible that the spleen amastigotes used here are in a less productive state than amastigotes taken from cutaneous lesions. If this were the case then spleen amastigotes may require a prolonged period of adjustment before transformation to procyclic promastigotes occurs. This could possibly mean that trypsin levels are already high by the time that transformation begins, a time when the parasites are most vulnerable to the action of trypsin. However, in nature this

may be much less of a problem as sandflies become infected by *L. infantum* amastigotes found in the skin of dogs and perhaps these amastigotes are capable of much faster transformation.

Several studies have outlined the divisional status of procyclic promastigotes (Walters *et al.* 1987; Lawyer *et al* 1987, 1990). These studies are supported by the data presented here. Procyclic promastigotes are responsible for the initial expansion of the parasite population within the bloodmeal. Rogers *et al* (2002) showed that the appearance of procyclic promastigotes was co-incident with an increase in the number of parasites per fly. Within the current study this increase in parasite numbers per fly was not observed with the appearance of procyclic promastigotes and the data would actually suggest that nectomonad promastigotes are responsible for the increase in parasite numbers, Figures 4.1 and 4.3. However, this cannot be the case as nectomonad promastigotes were essentially shown to be a non-dividing form and therefore incapable of expanding the parasite population. Rogers *et al.* (2002) using *L. mexicana* in *Lu. longipalpis* showed that a peak of procyclic promastigotes occurred on day 2 of infection and this peak was responsible for approximately 80% of the parasite population and a similar peak representing nectomonad promastigotes was seen on day 3 of infection. In contrast the data presented here shows that the peak of procyclic promastigotes appears on day 1 of infection and accounts for approximately 50% of the parasite population (Figure 4.3), with nectomonad promastigotes peaking on day 3 making up approximately 60% of the parasite population. It is therefore possible that the

differentiation of procyclic promastigotes into nectomonad promastigotes is much faster with *L. infantum*. The initial peak of procyclic promastigotes (Figure 4.3) is a result of transformation from amastigote forms and from the data it seems possible that some of these differentiate into nectomonad forms fairly rapidly. It is only on day 4 that an increase in the proportion of procyclic promastigotes is seen, indicating division and this is co-incident with an increase in parasite numbers per fly (Figure 4.1).

This study has provided evidence that nectomonad promastigotes are essentially a non-dividing form. This supports similar findings by previous investigators. Nectomonad promastigotes of *L. mexicana* during development in experimental vectors *Lu. abonnenci* (Walters *et al.* 1987) and, *Lu. diabolica* and *Lu. shannoni* (Lawyer *et al.* 1987) were not observed in division. A similar finding was reported with the Old World parasite *L. major* in its natural vector *Ph.* duboscqi (Lawyer *et al.* 1990). In contrast to these studies and the present data, Walters *et al.* (1989a) reported that nectomonad promastigotes of *L. chagasi* in *Lu. longipalpis* initiated a third cycle of multiplication. Nectomonad promastigotes have been implicated in the maintenance of infection following bloodmeal passage (Sacks & Kamhawi, 2001) by attachment to midgut epithelia via their flagella. Nectomonad promastigotes are also the life cycle stage responsible for the forward migration of infection (Walters *et al.* 1993; Rogers *et al.* 2002).

In agreement with the outlined precursor-product relationship of Rogers *et al.* (2002), the data presented here indicates that leptomonad promastigotes are the product of nectomonad transformation. This finding and those of Rogers *et al.* (2002) support earlier observations by Walters *et al.* (1987), who observed that nectomonad promiastigotes transform into short promastigotes and that these forms were often found in division. This life cycle stage was also described by Lawyer *et al.* (1990), who termed them "haptomonad promastigotes". This terminology has continued to be used by other authors (Saraiva *et al.* 1993; Nieves & Pimenta, 2000) but these life cycle stages would be termed leptomonad promastigotes according to the current study and that of Rogers *et al.* (2002). For reasons of etymology it is recommended that the use of leptomonad promastigote be used to refer to such forms (lepto = small; hapto = attached). This will allow a distinction between these two life cycle stages, which quite possibly have different functions within the sandfly vector. This study has not been able to determine the exact origin of haptomonad promastigotes due to their relatively small numbers. However, this study and that of Rogers *et al.* (2002) indicate that they could possibly arise from nectomonad or leptomonad promastigotes.

Supporting the observations of previous investigations (Walters *et al.* 1987, 1993; Lawyer *et al.* 1987, 1990), leptomonad promastigotes within this study were found to be a dividing life cycle stage. These forms are responsible for a second round of division during the sugarmeal phase of development. This enables the parasite population to recover following defecation of the bloodmeal, a time when several

parasites may be lost. Leptomonad promastigotes may be required to increase the parasite density sufficiently to achieve a transmissible infection, after which they cease division and differentiate into metacyclic promastigotes, a strictly non-dividing form (Table 4.1). Based on this data a revised life cycle for *Leishmania* (*Leishmania*) species is proposed (Figure 5.1).

It should be pointed out that *Lu. longipalpis* is one of several species of sandfly that regularly take several bloodmeals during a single gonotrophic cycle (Elnaiem *et al.* 1992). A study by Elnaiem *et al.* (1994) reported that 24-72 hours after a second blood meal the percentage of metacyclic promastigotes was greatly increased. They suggested that this may mean that following a second bloodmeal transmission of the parasites by the vector may be more efficient. The data presented here does not take this discordant behaviour into account, as the female flies within this study were not offered a second bloodmeal.

The current investigation has not only provided data in support of earlier studies with regard to morphology but has also provided some molecular data, indicating genetic differences between the various life cycle stages. It has already been documented that certain genes are amastigote, procyclic or metacyclic specific. Within this study it has been possible to isolate all of the life cycle stages therefore making it possible to expand the procyclic category into the relevant promastigote stages: procyclic, nectomonad, haptomonad and leptomonad. This allowed a much more in depth analysis of gene expression between each form of parasite. The

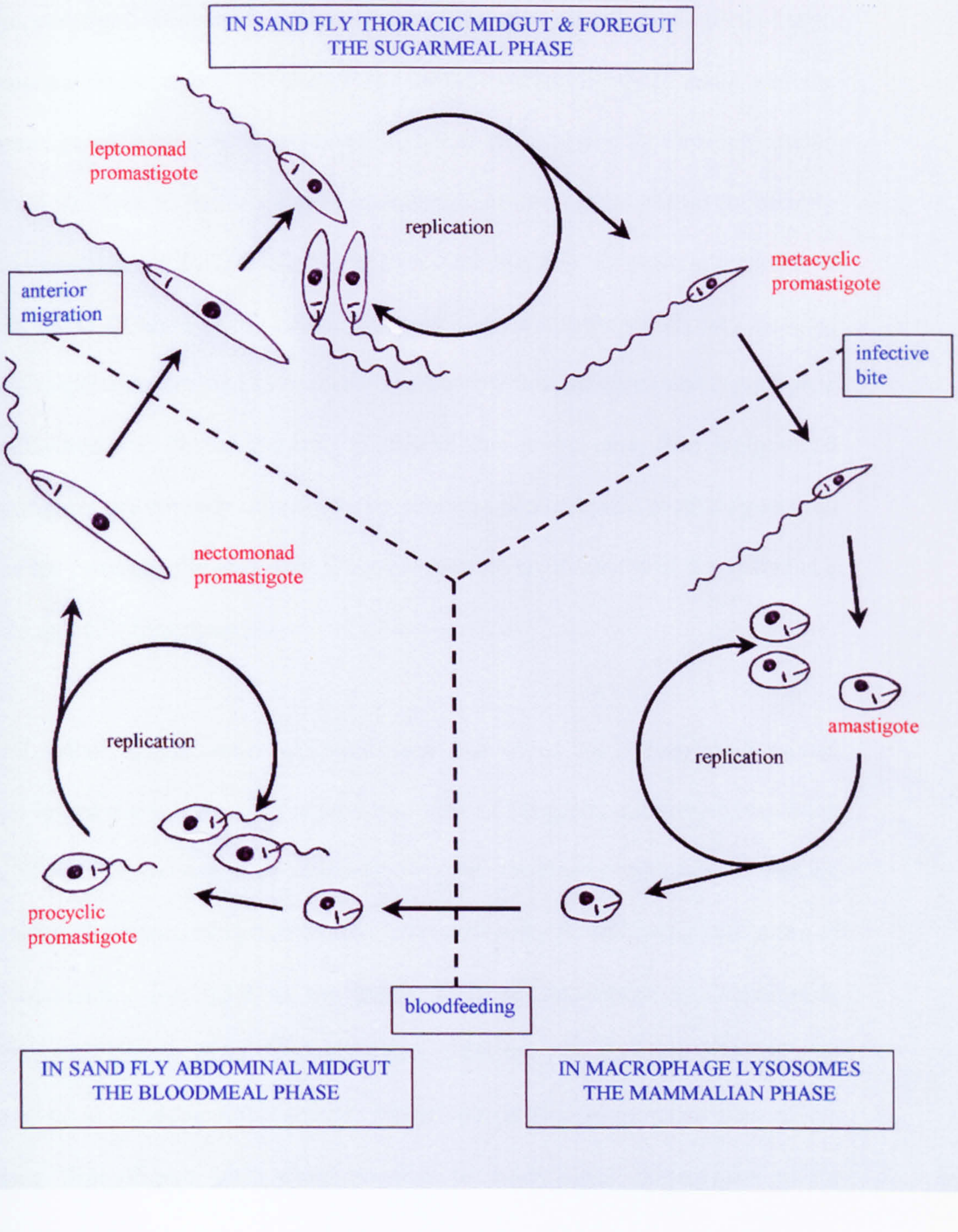



**Figure 5.1** Revised life cycle of *Leishmania* (*Leishmania*) species. Replication of parasites occurs at three points: amastigotes in macrophage phagolysosomes; procyclic promastigotes in the abdominal midgut; and leptomonad promastigotes in the thoracic midgut. These growth cycles are linked by various non-dividing or transmission stages as shown.

results presented here add further support for the introduction of the term leptomonad promastigote (Rogers *et al.* 2002). This life cycle stage was the dominant promastigote form expressing PPG2a, 80% above average expression (Figure 4.9). Due to the location of leptomonad promastigotes within the sandfly gut it is possible that PPG2a is a minor ingredient of PSG. A major constituent of PSG is fPPG (Rogers *et al.* 2004), however, it is unknown which promastigote form is responsible for its production. The current data indicates that leptomonad promastigotes may be the culprits. If indeed this is the case, then leptomonad promastigotes are not only important as precursors of metacyclic promastigotes but also as the promastigote form that alters the internal environment of the vector in a manner that aids transmission.

Several other genes were analysed and for three of these, nectomonad promastigotes showed the most expression. One of the genes is a known starvation factor, 3'NT/Nu, and would be advantageous to nectomonad promastigotes who are responsible for maintaining infection in low nutrient levels following defecation of the bloodmeal. For most of the genes analysed, expression in haptomonad promastigotes appears to be down regulated. This fits with the theory that this life cycle stage is altruistic. The smaller bands seen in the haptomonad lane on the chitinase film (Figure 4.11) could possibly be from partial degradation of the chitinase transcript.

As discussed above leptomonad promastigotes may have a vital function in the production of PSG. A recent study Rogers *et al.* (2004), provided evidence that PSG exacerbated *Leishmania* infection in mice. The exacerbative effect of sandfly saliva has long been of interest and has been the subject of several studies (see section 2.10). The data presented here supports that of Rogers *et al.* (2004) and confirms an exacerbative role for PSG in *Leishmania* infection. *In vitro* macrophage infections revealed that PSG did not increase the uptake of parasites by macrophages (Figure 4.16) but enhanced their survival in activated macrophages (Figure 4.17).

Zer *et al.* (2001) reported that *Lu. longipalpis* saliva increased the number of *L. infantum* amastigotes per macrophage. They suggested that sandfly saliva somehow accelerated the rate of amastigote division. Within the present study no difference in parasite numbers were observed between control groups and those containing saliva at 24 hours post infection (Figure 4.17) and on several occasions saliva actually reduced the number of parasites in the macrophages compared to the control (Figure 4.15). An earlier study observed that sandfly saliva did not alter the uptake of parasites by macrophages but appeared to inhibit parasite killing in IFN-γ activated macrophages (Hall & Titus, 1995). The present study is in agreement with this although the exacerbative effect appears to be much stronger for PSG than saliva. Rogers *et al.* (2004) reported an intermediate course of lesion development with a combination of both PSG/saliva and suggested this was a result of an antagonistic relationship between PSG and saliva.

The mechanism by which PSG confers this exacerbation is as yet unknown and requires further investigation. Sandfly saliva has been shown to reduce NO levels in activated macrophages (Hall & Titus, 1995) and it is therefore possible that PSG is able to do this more efficiently. An attempt was made in the present study to measure the nitric oxide (NO) levels present at 72 hours post infection, using the Griess reaction. Unfortunately this was unsuccessful. It is also possible that PSG provides infective metacyclic promastigotes with a protective coating, which is resistant to NO. The observed results could also be a combination of the two mechanisms. Whatever the mechanism, PSG provides metacyclic promastigotes with some protection from macrophage killing. Although it is unlikely that PSG persists indefinitely within the macrophage, it is possible that it persists long enough for metacyclic promastigotes to differentiate into amastigotes, a form more resistant to the killing effect of NO.

## 5.2 Future Work

The developmental profile of *L. major* has been examined *in vitro* within this study. However, to conclusively determine the sequential appearance of the various morphological life cycle stages and the division status of each of these, *in vivo* experiments would be required. The natural vector *Ph. papatasi* would be the obvious choice for these experiments, however, there are reported studies of *L. major* infection and development in *Lu. longipalpis* (Walters *et al.* 1993). It would

also be interesting to conduct similar studies using a *Leishmania* (*Viannia*) species to see if the same findings reported here also apply to this subgenus. Although it may not be possible to use the natural vectors, *Lu. whitmani* or *Lu. wellcomei*, it is possible to infect *Lu. longipalpis* with *L. brazilinesis* (Dr. P.A. Bates, personal communication). Some differences would be expected, since *Leishmania* (*Viannia*) species include a hindgut phase in their development (Walters *et al.* 1989b).

Haptomonad promastigotes are only briefly mentioned within this study due to their relatively small numbers. Their developmental origins are unknown, however, similar studies with increased sample sizes could possibly answer this question. It is possible that the small numbers of haptomonad promastigotes observed is due to the fact that they are still attached to the stomodeal valve and are therefore not being included within the analysis. If this is the case then an improved method for the homogenization of dissected sandfly guts is required. It may be that the attachment is so strong that the stomodeal valve itself needs to be dissected and examined at each time point. As these are possibly an essential life cycle stage involved in the occlusion of the stomodeal valve, their inclusion in the revised life cycle (Figure 5.1) is important.

A major research interest of this laboratory is the exacerbative effect of PSG on *Leishmania* infection. Gene expression analysis experiments will be able to determine which of the morphological forms are responsible for the production of PSG. As the current data suggests that leptomonad promastigotes are involved in

PSG production, the construction of a leptomonad specific cDNA library should identify the genes responsible for PSG production. This will also determine if a single life cycle stage is responsible for PSG production or if several are involved, maybe, haptomonad promastigotes, leptomonad promastigotes and metacyclic promastigotes.

Experiments to determine the mechanism by which PSG exacerbates infection would be of specific interest. Repeated *in vitro* macrophage infections would allow the NO levels to be monitored to determine if PSG is able to lower the production of NO. *In vivo* infections would allow the cytokine profiles of various experimental groups to be observed. Results from these experiments would possibly help to determine if PSG is able to influence the $CD4^+$ Th subset response. It would also be interesting to investigate any chemotatic properties of PSG, as maybe it functions by attracting macrophages to the site of initial infection thereby providing invading parasites with more host cells.

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