REACTIVE OXYGEN SPECIES AND ANTIOXIDANT ENZYMES IN THE *LUTZOMYIA-LEISHMANIA* SYSTEM

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

By

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"I want to stay as close to the edge as I can without going over. Out on the edge you see all kinds of things you can't see from the centre."

Kurt Vonnegut, Jr.

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DECLARATION

In chapter three figures 3.3.1 to 3.6.2, amino acid sequence alignment and phylogeny trees were done by Dr Fernando Genta, Instituto Oswaldo Cruz, Fiocruz. Also in chapter three, microarrays were designed and performed by Rod Dillon. Programming of Image SMX ParaMorph was done by Prof Steve Barrett, Department of Physics, University of Liverpool. Development of ParaMorph V. 3.0 was done by Paul Mulligan and V 3.1 was done by Hannah Delemare, Department of Physics, University of Liverpool.

This declaration confirms that the work described within this thesis is my own with the exception of the methods mentioned above.

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ABSTRACT

Female phlebotomines are the vectors of *Leishmania* protozoa. *Leishmania* reside in the gut of the sand fly and they share this niche with different microbes that interact with either sand fly or *Leishmania*. Reactive Oxygen Species (ROS) are a major component of the insect innate immune system regulating gut-microbe homeostasis in other insects but the importance of this component in sand flies and its impact on *Leishmania* is unknown. The sand fly ROS system was initially investigated by examining the expression of antioxidant genes in the midgut of *Lu. longipalpis* throughout blood digestion using semi-quantitative RT-PCR. Antioxidant genes were differentially expressed throughout digestion and exhibited a peak at 48 h after blood feeding. Catalase was the most upregulated gene. Sand fly fecundity was affected by age and redox balance, as suggested by a significant reduction in egg numbers from older flies as well as after RNAi-mediated silencing of catalase. ROS detoxification appeared to be important during egg development as suggested by the accumulation of catalase in developing oocytes as well as an increase in egg numbers after antioxidant *per os* supplementation. Sand fly longevity was affected by redox balance, as shown by a significant reduction in survival after RNAi-mediated abrogation of catalase. Dietary addition of antioxidant failed to rescue early mortality, but this group also showed higher levels of phenoloxidase, a potential indicator of bacterial infection. Antioxidant genes were differentially expressed in *Leishmania* and *Serratia* colonised guts. Overall, midguts exhibited downregulation of ROS-detoxifying enzymes while *Serratia*-infected ones displayed the opposite trend. RNAi-silencing of catalase reduced *Leishmania* populations in the midgut suggesting that oxidative stress is deleterious to this protozoan. Dietary addition of the antioxidant uric acid in *Serratia*-infected flies increased sand fly mortality as in previous experiments with vitamin C. Although *Serratia* CFUs were significantly lower in the group with the highest mortality, the population of the resident microbiota was significantly higher in the same group. Interestingly, the numbers of resident microbiota were even higher in flies not infected with *Serratia*. The implications of the results are discussed in relation to gut immune homeostasis in other insect-microbe systems as well as the possibility of applying some of this information towards understanding the systems governing adult longevity in relation to vectorial capacity and the improvement of sand fly control.

CHAPTER 1

Introduction

1.1. Phlebotomine sand flies

Phlebotomine members of the family Psychodidae, commonly known as sand flies, are of great medical and veterinary relevance since they are responsible for the transmission of the leishmaniases. This group is widely distributed in tropical and subtropical regions of both the Old and New World (Lane and Crosskey 1993). Approximately 700 species have been described so far, around ten percent of which have been incriminated as leishmaniasis vectors; sufficient evidence of vectorial capacity has been shown for around thirty species (Bates 2007; Antinori *et al.* 2011). Male and female adults feed on natural sources of sugar from plants through their lifetime, (Schlein and Warburg 1986; Cameron *et al.* 1995). However, females also feed on blood to provide nutrition for the developing eggs (Killick-Kendrick 1999) and this behaviour provides the only confirmed natural infection route of the *Leishmania* parasite (Bates and Rogers 2004). It is at this stage, where parasites and sand flies meet, that an intricate set of interactions occur. Both species may be considered as a combined element; in effect The *Leishmania*-Sand fly System. However, sand flies, in common with other animal and plant feeder, have to survive and develop in the midst of a myriad of microbes that may be beneficial, commensalistic or entomopathogenic and subsequently may benefit or suffer from this relationship. *Leishmania* enter this potentially hostile ecological niche and yet they have evolved to survive and thrive in the environment of the sand fly gut. A series of relevant aspects emerging from this interaction in time and space will be reviewed in the following sections.

1.2. *Leishmania* life cycle

Leishmania enters its phlebotomine host after the female fly has fed on blood from an infected vertebrate. The parasite is initially present as a non-flagellated

amastigote (Figure 1.1a) found intracellularly in phagocytes such as macrophages (Handman and Bullen 2002), which are recruited into the bite site upon bite injury caused by skin abrasion. Sand flies are pool-feeders that cut through the skin and capillaries with their mouthparts (Lane and Crosskey 1993). Changes in temperature and pH are responsible for this transformation of the non-flagellated to the flagellated forms (Bates and Rogers 2004; Kamhawi 2006).

1.2.1. Overview

Development inside the invertebrate host starts with the transformation of the sessile amastigotes into the longer, flagellated procyclic promastigote (Figure 1.1b) that replicates within the bloodmeal, all of which are separated from the midgut epithelium by a type I peritrophic matrix (PM) (Adler and Theodor 1926; Adler and Theodor 1957; Kamhawi 2006). Towards the end of blood meal digestion (72-94 hours), procyclics transform into long, slender and very motile nectomonads (Figure 1.1b) and migrate towards the anterior part of the PM, (for a detailed time-line of parasite development inside the sand fly refer to Figure 1.2). Nectomonads are able to disrupt PM integrity by secreting a chitinase (Schlein *et al.* 1991; Shakarian and Dwyer 2000). Some nectomonads attach to microvilli in the midgut while others establish at the stomodeal valve and transform into the replicative leptomonads (Figure 1.1b) (Gossage *et al.* 2003). Leptomonads produce promastigote secretory gel, a gel-like matrix that plays a key role in transmission (Rogers *et al.* 2002; Rogers *et al.* 2004). A peculiar parasite subpopulation emerges from either nectomonads or leptomonads (it is still uncertain (Bates 2007)) named haptomonads that attach to the cuticle-lined surface of the stomodeal valve due to expansion of the flagellar tip into hemi-desmosome-like structures (Killick Kendrick *et al.* 1974; Wakid and Bates 2004). Some leptomonads transform into metacyclic promastigotes (Figure 1.1b) (Rogers *et al.* 2002), free swimming, fast , highly motile and complement-resistant forms that are highly adapted to infect the vertebrate host (Kamhawi 2006).

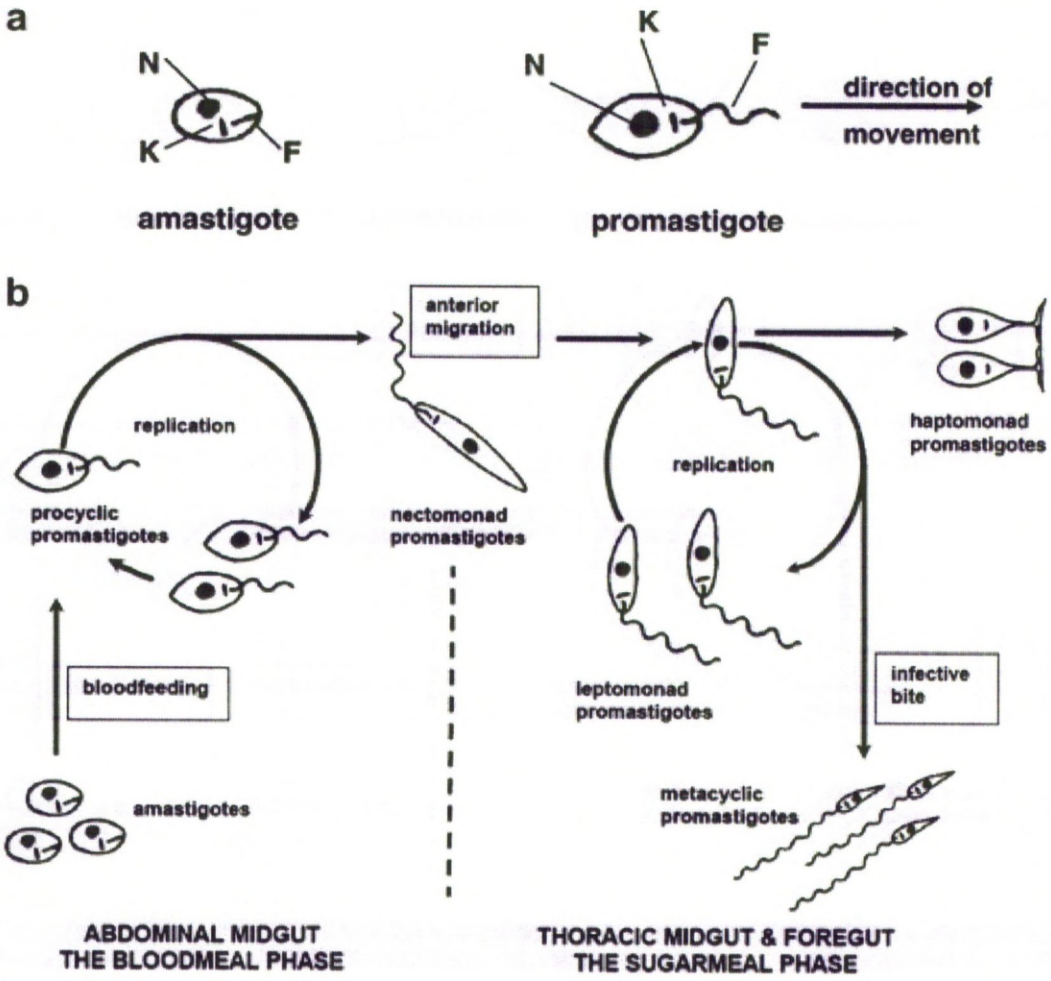


Figure 1.1: Development of *Leishmania* (*Leishmania*) species in the sand fly vector. (a) The morphology of amastigotes and promastigotes. Each form has a nucleus (N), kinetoplast (K) and flagellum (F). The kinetoplast is the mitochondrial genome. The flagellum in amastigotes is internal and non-functional; in promastigotes the flagellum extends from the cell body, beats and pulls the organism in the direction shown, emerging from the anterior end of the cell. (b) The developmental sequence of the five major promastigote forms: procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, haptomonad promastigotes and metacyclic promastigotes. Adapted from Bates, (2007).

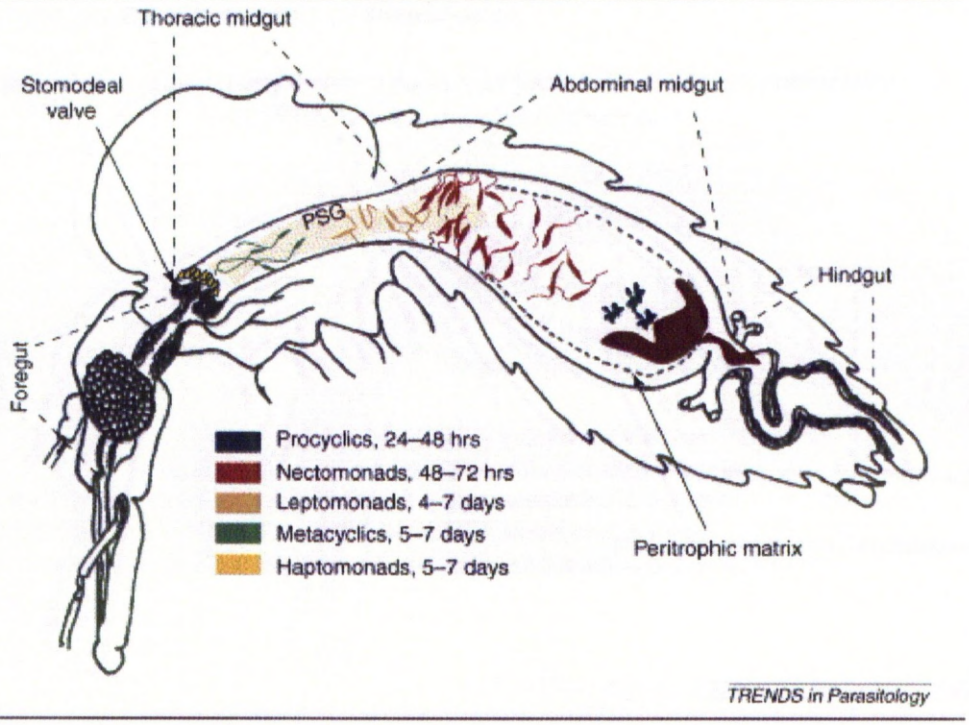


Figure 1.2: The life cycle of *Leishmania* in a competent vector, illustrating the time-dependent appearance of distinct morphological forms of promastigotes within the sand fly midgut. PSG=Promastigote secretory gel. Adapted from Kamhawi, (2006).

1.2.2. Physiology of parasite establishment

Suprapylarian *Leishmania* parasites develop within a gut which possesses chemical and physical barriers to prevent pathology due to microbial infections. Each developing stage bears a distinctive morphology and carries out particular functions to successfully establish and develop inside the sand fly. Once the digestion process begins, amastigotes are liberated from the macrophages in the blood meal and rapidly transform into promastigotes (Bates 2007). The resulting procyclics have to deal with deleterious hydrolytic enzymes released by the midgut epithelial cells (Pimenta *et al.* 1997). Parasite development in the midgut and digestion appear to be strongly correlated: experimental inhibition of digestive enzymes in *Phlebotomus papatasi* increased the number of *L. donovani* (Borovsky and Schlein 1987). Similar results were found in *L.major*-infected *P. duboscqi* after oral supplementation of galactosamine, a lectin-binding carbohydrate that negatively affected the activity of alkaline proteases and

trypsin in the midgut (Volf *et al.* 2001). Promastigotes have been able to survive *in vitro* in a trypsin-supplemented medium, but these enzymes may harm *in vivo* if *Leishmania* development is not coordinated with the digestive process, (Dillon and Lane 1993b). During the early stages of digestion, survival has proven to be species-specific in experimental infections of sand flies with non-compatible *Leishmania* strains, (Lawyer *et al.* 1990; Schlein and Jacobson 1998). A significant number of parasites are killed during these first stages (Pimenta *et al.* 1997; Rogers *et al.* 2002); in different combinations of sand fly and parasite species (Nieves 2002; Rogers *et al.* 2002). It has been demonstrated that the peritrophic matrix (PM) plays a determinant role in parasite survival by limiting the exposure of amastigotes to proteolytic enzymes and providing time for them to develop into the more resistant promastigotes (Pimenta *et al.* 1997). Promastigotes overcome proteolytic damage by the expression of glycoconjugates, a group of phosphoglycans either attached to the cell surface via glycosylphospatidylinositol (GPI) lipid anchors (lipophosphoglycans (LPG) and phosphoglycans (PPG)) or secreted as protein-containing phosphoglycans (secreted phosphoglycan (sPPG) and secreted acid phosphatase (sAP)) which appear to confer protection to the parasite from the activity of proteolytic molecules (Sacks *et al.* 2000; Secundino *et al.* 2010). Altogether, these investigations suggest that timing is extremely important in parasite development and that *Leishmania* coordinates its development to take advantage of the sand fly behaviour and physiology to successfully complete its life cycle.

Two major events happen once the parasites had successfully survived the early stages of digestion: degradation of the PM and development of procyclics into nectomonads. The peritrophic matrix might play a protective role, presumably by slowing the diffusion of certain enzymes and allowing the diffusion of others. But the parasites have to cross this physical barrier since failure to do so results in their inability to establish an infection because trapped parasites are expelled with the rest of the digested meal (Walters *et al.* 1992; Coutinho-Abreu *et al.* 2010). Lysis of the PM is facilitated by parasite-secreted chitinases that are able to break down the chitin-rich network of the PM, (Schlein *et al.* 1991; Shakarian

and Dwyer 2000). When adding allosamidin, a chitinase inhibitor, to a blood meal, parasites were not able to escape the PM, (Pimenta *et al.* 1997). Sand flies are also able to express chitinases (Ramalho-Ortigao *et al.* 2005) which seem to facilitate *Leishmania* colonisation and to enhance transmission to the mammalian host (Rogers *et al.* 2008). Procyclic transformation into nectomonads confers physical advantages to escape the PM, since these forms are longer, slender and strongly motile (Bates 2007). However, higher motility is not enough to survive to the next stages of development; further attachment of parasites to the midgut wall is vital and it is mediated by the expression of phosphoglycans.

1.2.2.1. Parasite LPG-mediated colonization

Phosphoglycans not only confer protection against enzymatic activity, as discussed above, but also play a key role in the successful establishment of infection once the nectomonad promastigotes migrate outside the PM. Lipophosphoglycan (LPG) is the major glycoconjugate on the surface of promastigotes. Several studies in Old and New World species of *Leishmania* have examined the role of LPG in the binding process to the midgut epithelium,(Sacks *et al.* 2000; Ilg 2001; Sacks and Kamhawi 2001; Soares *et al.* 2002). In *Phlebotomus papatasi*, the LPG of *L. major* attaches to a midgut epithelial galectin (Pelletier *et al.* 2003; Kamhawi *et al.* 2004; Soares *et al.* 2004)and this specific binding appears to account for species-specific vector competence in sand fly species which are termed 'restricted, *i.e* if the *Leishmania* species is able to develop transmissible infections in that particular sand fly species. After binding to the wall, nectomonads develop into leptomonads, which are of particular importance, since they recommence replication into the infective metacyclic promastigotes (Gossage *et al.* 2003) and secrete promastigote secretory gel (PSG). Although successful binding to the midgut avoids expulsion during defaecation, it can be a "double-edged sword", since the parasite must liberate themselves when the sand fly feeds on the next mammal (Beverley and Dobson 2004). Metacyclics rely on the expression of a non-attaching LPG to allow anterior migration and subsequent transmission (Kamhawi *et al.* 2004). The mechanism of detachment is unknown although the binding process may be a more active

process rather than a sessile one since microscopic observation of the flagella between the microvilli suggests that the nectomonads are actively swimming against the epithelium with the flagellar tip burrowing between the microvilli (Dillon unpublished observations).

1.2.2.2. Parasite non LPG-mediated colonization

The LPG-mediated *Leishmania* microvillar binding process was previously thought to be the main mechanism of binding and explanation for species specificity. Recently this central tenant was challenged and a study confirmed that it was not the only molecular mechanism for *Leishmania* attachment inside the midgut (Volf and Peckova 2007). The possibility of an alternative mechanism emerged after experiments with permissive vectors such as *Lu. longipalpis* infected with LPG-deficient *L. mexicana* or *L. arabicus* resulted in survival in the midgut similar to those from wild type parasites (Rogers *et al.* 2004; Myskova *et al.* 2007). It was also suggested that midgut O-glycosylated proteins with N-acetylgalactosamine (GalNAc) epitopes might also play a role in parasite attachment in permissive vectors such as *Lu longipalpis* (Svobodova *et al.* 2006; Myskova *et al.* 2007; Volf and Peckova 2007). This shows parasite establishment inside the gut is more complex than initially proposed. Another possibility is that parasites remain inside the gut without attaching at all. *Leishmania* display chemotaxis to different molecules as well as to pH and sugar gradients (Bray 1983; Van Zandbergen *et al.* 2002), and are also able to reduce peristalsis in the host (Vaidyanathan 2004). An alternative hypothesis for a mechanism of establishment without attachment would involve *Leishmania* movement in an anterior direction in the gut with reduced peristalsis. Whatever strategies *Leishmania* utilises for successful completion of the 'gut phase' of its' life cycle, it seems that the mechanisms are more diverse than the 'lock and key' (LPG-galectin) attachment system.

1.2.3. Promastigote secretory gel (PSG)

Probably the latest and most relevant finding so far on Leishmaniasis transmission (and the last key step in parasite gut development) is the secretion of the PSG. This high molecular weight filamentous glycoprotein has been proven

to enhance transmission of the metacyclics in the mammalian host,(Rogers *et al.* 2002; Rogers *et al.* 2004). It seems to promote successful infection when some of the plug, is regurgitated by the "blocked fly" before it can feed on the next host (Bates 2007). The PSG plug also embeds the majority of the metacyclic subpopulation and its acidic, low-oxygen conditions might induce metacyclogenesis of leptomonads (Rogers *et al.* 2002). It is possible that the PSG might be playing other additional roles, like protection of the parasites against gut immune effectors or potentially harmful bacteria from incoming sugar meals (Dillon, unpublished data). It has also been shown that the major constituent of PSG, the filamentous proteophosphoglycan, leads to long-term disease exacerbation (Rogers *et al.* 2004). The PSG causes partial blockage of the gut and the fly has difficulty in feeding thus promoting transmission of the metacyclic *Leishmania* (Rogers and Bates 2007).

How *Leishmania* manages to survive and thrive inside the midgut is still far from being fully understood. Recent studies, (Cohen-Freue *et al.* 2007; Leifso *et al.* 2007) showed an interestingly low differential expression of mRNA between amastigotes and promastigotes. A study of this kind has not been performed in other promastigote subpopulations such as nectomonads, leptomonads, and haptomonads where production *in vitro* and separation in enough numbers is difficult (Rogers *et al.* 2002; Gossage *et al.* 2003). Such data could provide a better understanding of the survival of the parasite inside its insect vector.

1.3. Insect immunity

Insects are able to mount a robust innate immune response against potentially pathogenic microbes. They have evolved different strategies to sense the pathogenic non- self from self and they are able to recognize different microorganisms and to mount different immune defence mechanisms accordingly. Recent work has started to examine how insects are able to react differently to beneficial microbes compared to pathogenic ones in their guts and to regulate their microbiota in a molecular cross-talk (Ha *et al.* 2005a; Ha *et al.*

2009a; Ryu *et al.* 2010) .The following section will review pathogen recognition, signalling pathways and antimicrobial response of systemic and epithelial immunity.

1.3.1. History

The early interest in insect immunity was associated with human activities of economical relevance, specifically the silk industry of the 19th century. It was in 1835 that Agostino Bassi proved that the fungus *Beauveria bassiana* was the etiological agent of the white muscardine disease in the silkworm *Bombyx mori*. Bassi not only provided the first experimental proof of the germ theory of disease, his works are also the first evidence of microbe pest control (Steinhaus 1957; Lord 2005). Pasteur himself spent several years studying two different silk worm diseases and noted differential susceptibility of insects to the disease, probably the first scientific record of pathogen resistance in insects (Bordenave 2003). The first practical use of insect susceptibility to pathogens came from Elie Metchnikoff. After analysing a beetle's population fluctuation and its relationship with disease outbreaks, he suggested to apply conidia of the green muscardine *Metarhizium anisopliae* as pest control (Lord 2005).

Research on insect immunity during the following decades also led to a better understanding of vertebrate immunity. In 1895, Cuénot studied phagocytosis of pathogens in hematocytes of the domestic cricket *Gryllus domesticus* (Thompson 1930). Stephens demonstrated that the hemolymph of *Galleria mellonella* displayed microbicidal properties after injection of *Pseudomonas aureuginosa* (Stephens 1962). Observations as early as 1898 of haemolymph peculiar behaviour of darkening upon exposure to air (Biedermann and Moritz 1898) eventually lead to the discovery of the role of phenoloxidase in immunity and nonself recognition and (Nappi 1973; Pye 1974). Ratcliffe and Rowley also provided the first detailed evidence of phagocytosis in haematocytes (Ratcliffe and Rowley 1974). Another notable discovery was that of Boman *et al.*, who demonstrated that injection of the fruit fly *Drosophila melanogaster* with bacteria could confer protection to a subsequent infection (Boman *et al.* 1972). Although

the biochemistry of this phenomenon was not clear at the time, further research culminated with the isolation of attacin and cecropin, the first antimicrobial peptides ever described (Steiner *et al.* 1981; Hultmark *et al.* 1983). A few years later, it was found that insects express immunosuppressive factors that were quite similar to mammalian NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Sun and Faye 1992). In 1996, Lemaitre *et al.* reported that such similarities extended even further, they found that Toll receptors, only known for their role in embryo development, played an important role in immune response against fungi in *Drosophila*. (Lemaitre *et al.* 1996). Very shortly afterwards, a Toll-like receptor was cloned in humans. This receptor was found to activate NF-κB (Medzhitov *et al.* 1997). Perhaps one of the latest relevant discoveries is the role of reactive oxygen species (ROS) in epithelial immunity. Ha *et al.* showed that *Drosophila* effectively uses ROS as a first line of defence against pathogens and that ROS play a major role in midgut homeostasis and regulation of microbial population (Ha *et al.* 2005a; Ha *et al.* 2005b; Ha *et al.* 2009a).

1.3.2. Systemic immune response

Insects are able to mount a robust immune response against pathogenic microbes. They have evolved different strategies to sense the pathogenic non-self from the self and they are able to recognize different microorganisms and to mount different immune defence mechanisms accordingly (Lemaitre and Hoffmann 2007). Insects are even able to identify entomo-'beneficial' microbes from entomopathogenic ones in their guts and to regulate their microbiota via molecular cross-talk.

1.3.2.1. Anti-microbial peptides (AMPs)

Insects are able to mount a systemic antimicrobial humoral response upon septic injury. This response consists mainly of a major release of antimicrobial peptides (AMPs) into the haemolymph after synthesis in different tissues such as the fat body and haemocytes. AMPs are small (>10 kDa, except for the 25kDa Attacin), usually cationic peptides with high specificity against fungi and bacteria (Imler and Bulet 2005). Fungi are sensitive to Drosomycins and Metchnikowins

(Fehlbaum *et al.* 1994; Levashina *et al.* 1995), Gram-positive bacteria are labile to Defensins (Dimarcq *et al.* 1994), while Cecropins, Diptericins, Drosocins and Attacins attack Gram-negative bacteria (Wicker *et al.* 1990; Bulet *et al.* 1993; Åsling *et al.* 1995). AMPs have also been found to exhibit activity against protozoan parasites like *Crithidia*, *Trypanosoma*, *Plasmodium* and *Leishmania* (Dimopoulos *et al.* 1997; Boulanger *et al.* 2002; Boulanger *et al.* 2004; Boulanger *et al.* 2006). The microbicidal mechanisms of AMPs are not fully understood, however it is believed that these peptides are detrimental to microbes by altering the cell membrane structure as well as inhibiting nucleic acid and protein synthesis (Yang *et al.* 2000; Brogden 2005).

1.3.2.2.Regulation of humoral response

AMPs gene expression is regulated by DIF (dorsal-related immunity factor) and Relish in *Drosophila* and they both belong to the nuclear factor-κB (NF-κB) family of inducible transactivators: DIF (dorsal-related immunity factor) and Relish. Gram-positive bacteria and fungi are mainly responsible for DIF activation, while Gram-negative microbes activate Relish in *Drosophila*. DIF and Relish are activated through two different signalling cascades: the Toll and Immune deficiency (IMD) pathways (Lemaitre *et al.* 1996; Ferrandon *et al.* 2007), which will both ultimately activate a nuclear factor-κB (NF-κB)/reticuloendotheliosis (Rel) family transcription factor (Hoffmann and Reichhart 2002; Leclerc and Reichhart 2004).

1.3.2.3. Toll pathway

The Toll pathway is an evolutionarily conserved signalling cascade which was originally found to participate in the establishment of dorso-ventral patterning in *Drosophila* (Belvin and Anderson 1996), but a few years later it was found to play a major role in the regulation of the immune response (Lemaitre *et al.* 1996). The Toll pathway is reminiscent of the TLR/interleukin – 1 pathway which regulates mammalian inflammatory response (Silverman and Maniatis 2001). This pathway is triggered upon fungal or Gram-positive bacterial infection (Figure 1.3, left) (Leclerc and Reichhart 2004). Toll is a transmembrane receptor which is activated

after binding of Späetzle, a secreted protein which circulates the haemolymph as a pro-protein and is turned functional by a serine protease (Weber *et al.* 2003). Späetzle induces dimerization of Toll and further recruitment of adaptors MyD88 and Tube as well as the kinase Pelle. Pelle activates proteosomal degradation of Cactus, which allows translocation of Dif and Dorsal transactivators. This translocation results in induction of expression of immune-related genes such as *Drosomycin* (Belvin and Anderson 1996; Tauszig-Delamasure *et al.* 2002; Broderick *et al.* 2009).

1.3.2.4. Imd pathway

The Imd was pathway was initially deduced after research on a mutation in *Drosophila* defined as *immune deficiency* (*imd*). This mutation abrogated expression of different AMPs genes except for *Drosomycin* (Lemaitre *et al.* 1995; Corbo and Levine 1996; Levashina *et al.* 1998). Gram-negative bacteria kill *imd* mutant flies and show higher resistance to fungi and Gram-positive germs (Lemaitre and Hoffmann 2007). The Imd pathway is activated mainly by Gram-negative bacteria (Fig 1.3, right). This pathway is mediated by the peptidoglycan recognition protein LC (PGRP-LC), a transmembrane PRR (Choe *et al.* 2002; Gottar *et al.* 2002) with a death domain similar to a mammalian receptor interacting protein that plays a role in both NF-κB activation and apoptosis (Georgel *et al.* 2001). Upon infection, PGRP-LC recruits Imd which in turn recruits the dFADD adaptor and caspase Dredd, which is thought to associate with Relish. Translocation of Relish induces the expression of immune-related genes such as *Diptericin* (Broderick *et al.* 2009).

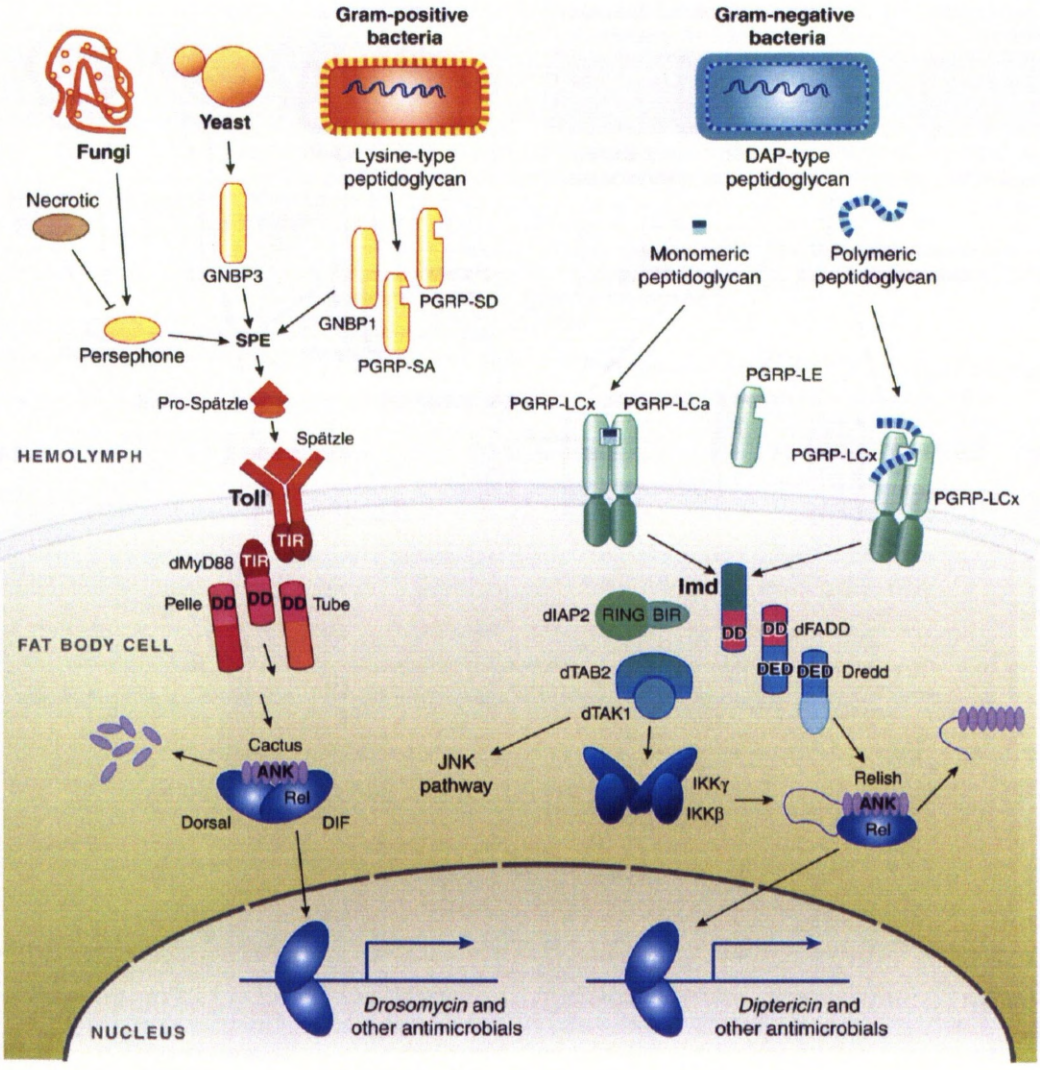


Figure 1.3: Model of Toll and Imd pathway activation. Antimicrobial peptide genes are regulated by a balance between two signalling pathways: the Toll pathway that is largely activated by fungi and Gram-positive bacteria, and the Imd pathway that is mainly activated by Gram-negative bacteria. According to the κB sites present in their promoters, antimicrobial peptide genes are more sensitive to either the Toll cascade (*e.g.*, Drosomycin) or the Imd cascade (*e.g.*, Diptericin) or are coregulated. Modified from Lemaitre and Hoffman, (2007)

1.3.2.5. Melanization

In *Drosophila*, as well as other insects and arthropods, physical injury of the cuticle leads to a physiological process known as melanization, a *de novo* expression and deposition of melanin. This immediate defence response occurs in the wound site and is also observed on the surface of foreign objects in the haemocoel. Melanization is involved in a plethora of immune reactions such as injury repair, microbial sequestration, encapsulation of parasites and synthesis of toxic compounds with putative antimicrobial properties (Muta and Iwanaga 1996; Soderhall and Cerenius 1998).

Melanization occurs through the oxidation of mono- and diphenols to orthoquinones by the enzyme prophenoloxidase (proPO) and the non-enzymatic polymerization of orthoquinones to melanin (Fig 1.4). proPO exists as a proform and is activated to phenoloxidase (PO) by a serine protease known as prophenoloxidase activating enzyme (PPAE). Activation of PPAE is also mediated by serine proteases since this enzyme exists as an inactive zymogen. Research preformed in different invertebrate models has shown that melanization is started by either injury or recognition of Microbe-Associated Molecular Patterns (MAMPs) such as PGN, β(1,3)-glucan, and LPS (Ochiai and Ashida 1999; Ma and Kanost 2000; Ochiai and Ashida 2000; Lee et al. 2004) .

1.5.3.6. Nitric oxide and immunity

Over the past two decades, nitric oxide (NO) has been acknowledged as one of the most versatile components of vertebrate immunity, performing a dual role as immune effector molecule as well as major signalling molecule (Bogdan et al. 2000; Pryor et al. 2006). Studies performed in different arthropod models such as the horseshoe crab *Limulus polyphemus* as well as the dipterans *Drosophila* and *Anopheles* have shown that NO plays similar roles in the invertebrate immune response (Radomski et al. 1991; Luckhart et al. 1998; Nappi et al. 2004). In different species of invertebrates, NO *per se* and after interaction with ROS and

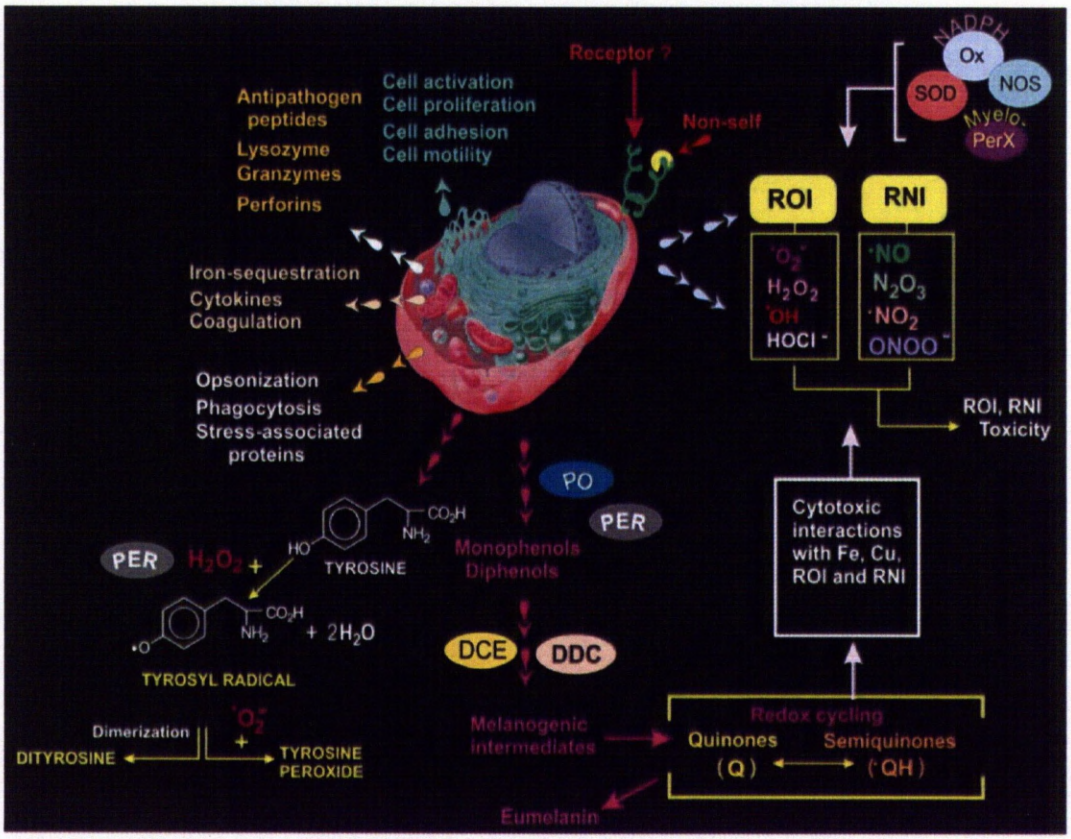


Figure 1.4: Overview of some toxic molecules manifested in the innate immune responses of various invertebrates. Non-self recognition may involve plasma membrane receptors independently functioning, or cooperatively engaging non-self binding molecules in the host's hemolymph. Melanotic encapsulation, which is a common manifestation of the defense reaction made by arthropods infected with eukaryotic pathogens, involves activation of one or more of the following enzymes; dopa decarboxylase (DDC), dopachrome conversion enzyme (DCE), phenylalanine hydroxylase (PAH), and phenoloxidase (PO). Enzymes capable of generating reactive intermediates of oxygen (ROI) and nitrogen (RNI) include myeloperoxidase (Myelo-Px), NADPH oxidase (NADPH Ox), nitric oxide synthase (NOS), and superoxide dismutase (SOD). Melanogenic intermediates such as quinones and semiquinones can react with ROI, RNI and the active centers of certain metaloenzymes to contribute additional toxic molecules. Modified from Nappi (2010)

reactive nitrogen intermediates (RNIs) constitutes a common defence response against pathogens (Fig 1.4), showing cytotoxic activity against bacteria, virus and parasites (Torreilles and Guérin 1999; Weiske and Wiesner 1999; Nappi et al. 2000; Beck et al. 2001; Novoa et al. 2002; Hao et al. 2003; Jiang et al. 2006). NO is catalyzed by nitric oxide synthase (NOS), an enzyme which activation upon parasitic challenge in invertebrate disease vectors seems to play a role in regulation of parasitemia in the host (Dimopoulos et al. 1998; Luckhart et al.

1998; Bayne et al. 2001; Hahn et al. 2001). NO also acts as a signalling molecule in insect immunity (Stefano and Ottaviani 2002; Kumar et al. 2004). Upon bacterial challenge, NO is synthesised in haemocytes of *Drosophila*, which leads to activation of the Imd pathway and further expression of diptericin (Nappi et al. 2000; Foley and O'Farrell 2003). Expression of diptericin and antibacterial activity in response to bacterial LPS also increases after experimental overexpression of NOS in *Drosophila* Malpighian tubule cells (McGettigan et al. 2005).

1.3.3. Microbe recognition

1.3.3.1. Microbe-Associated Molecular Patterns (MAMPs)

The immune system effectively recognizes and combats a plethora of pathogenic microbes in the environment associated with insects. It has been suggested that recognition of potentially hazardous bacteria relies on molecular structure patterns that are: a) shared by several pathogens, b) conserved metabolic products, and c) completely distinguishable from the insect-self; these structures are called pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway 1997a), a more inclusive term that includes commensal and beneficial microbes is microbe associated molecular patterns (MAMPs). Some of the best studied MAMPs are lipopolysaccharides and peptidoglycans expressed by Gram-negative and gram-positive bacteria, respectively. Other MAMPs include double stranded RNA from viruses and mannans from yeasts (Medzhitov and Janeway 1997a). These are all signature molecules produced exclusively by pathogens which are effectively recognized by receptors of the innate immune system called pattern recognition receptors (PRRs) (Janeway 1989; Medzhitov and Janeway 1997b; Medzhitov and Janeway 2002). PRRs differ in both structure and function for recognition of a wide variety of MAMPs, as well as induction of different immune response mechanisms. *Drosophila* recognizes bacteria throughout particular forms of a glucopeptidic polymer denominated peptidoglycan (PGN). This is a cell wall component common to both Gram-positive and Gram-negative bacteria (Mengin-Lecreulx and Lemaitre 2005). Peptidoglycan shows two major differences between the two groups of bacteria. In Gram-negative bacteria it is known as DAP-type PGN, it has *meso*-diaminopimelic acid (DAP) instead of a

lysine in the peptide chain. DAP-type PGN is also single-layered and located underneath the lipopolysaccharide layer and the outer membrane. In Gram-positive bacteria, Lys-type PGN consists of several layers and it is found on the bacterial surface. (Ferrandon *et al.* 2007; Lemaitre and Hoffmann 2007). *Drosophila* is able to distinguish between both types of PGN and launch an immune response accordingly. Gram-negative DAP-type PGN induces the Imd signalling pathway, whereas the Toll pathway is induced by Lys-type PGN (Leulier *et al.* 2003). This differential sensing is possible due to specific peptidoglycan recognition receptors in *Drosophila*.

1.3.3.2. Peptidoglycan-recognition proteins (PGRPs)

Microbe sensing is achieved by recognition of particular petidoglycan patterns by PPRs called Peptidoglycan-recognition proteins (PGRPs). Gene sequences coding for these proteins have been identified in a wide variety of species, from insects to mammals (Kang *et al.* 1998; Werner *et al.* 2000; Liu *et al.* 2001) and show a common 160 amino acid domain similar to bacteriophage T7 lysozyme (Yoshida *et al.* 1996; Kim *et al.* 2003; Royet and Dziarski 2007). Recognition of Lys-type PGN Gram-positive bacteria is achieved by PGRP-SA, PGRP-SD and GNBP1 (Gram-negative binding protein1, a historical application of a wrong name) (Lemaitre and Hoffmann 2007). GNBP1 is a PPR with a glucan-binding and a mutated glucanase domain (Lee *et al.* 1996), it has been suggested that this receptor hydrolyzes Lys-type PGN for recognition by PGRP-SA (Filipe *et al.* 2005). Activation of these PPRs triggers proteolytic cascades that result in the cleavage of Späetzle and further activation of the Toll pathway. Detection of DAP-type Gram-negative bacteria is mediated by PGRP-LC and PGRP-LE (Ferrandon *et al.* 2007). DAP-type PGN can be detected by PGRP-LC and PGRP-LE either intact or as shorter fragments, such as tracheal cytotoxin (TCT)(Leulier *et al.* 2003; Kaneko *et al.* 2004; Stenbak *et al.* 2004; Ferrandon *et al.* 2007) and results in activation of the Imd signalling pathway. Fungal recognition and further Toll pathway activation is mediated by Gram-negative binding protein 3 (GNBP3)(Gottar *et al.* 2006) as well as Persephone, a serine protease (Ligoxygakis *et al.* 2002). This PPR

domain binds to fungi upon identifying long chains of β-1,3-glucans in the fungal cell wall as a major ligand (Mishima *et al.* 2009)

1.3.4. Epithelial Immunity

The first studies on microbe-insect host interactions utilised cuticular wound-like model infections to analyse the immune response. However, although insects might face the challenge of a wound in nature (*i.e.* surviving a predatory attack or fungal pathogens), it is their natural orifices and connecting tracts that are the regions most likely to be challenged by microbes on a regular basis, specifically, the digestive and respiratory tissue. Gut and trachea are equipped with physical and physiological features against potential pathogens. The respiratory tract is coated with a protective chitinous lining (Merzendorfer and Zimoch 2003). The foregut and hindgut are protected by a cuticle layer, whereas the midgut epithelium secretes a peritrophic matrix that protect against abrasion and bacteria(Lehane and Billingsley 1996; Vallet-Gely *et al.* 2008). The midgut epithelium defence repertoire includes lysozymes that destroy microbes during digestion (Hultmark 1996), as well as anti-microbial peptides and reactive oxygen species (Lemaitre and Hoffmann 2007).

1.3.4.1. Anti-Microbial Peptides (AMPs)

Oral infection of the model insect *Drosophila* with Gram-negative bacteria triggers the Imd pathway in gut epithelium and induces the expression of different AMPs in a tissue-specific manner (Tzou *et al.* 2000). Infection with *Erwinia carotovora* activated the Imd pathway and induces the expression of Drosomycin and Diptericin in respiratory and digestive tissues (Basset *et al.* 2000). The Imd pathway plays a major role in oral infection, as confirmed by an increase in mortality of imd pathway mutants upon infection with *Serratia marcescens* (Nehme *et al.* 2007). It has also been demonstrated that bacteria have evolved strategies to overcome AMPs inside the digestive tract. *Pseudomonas entomophila* secretes ApraA, a zinc metalloprotease that protects it against Diptericin (Liehl *et al.* 2006). Recognition of PGN and subsequent activation of the Imd pathway upon oral infection is achieved by PGRP-LC, the same

transmembrane receptor responsible for triggering the Imd signalling cascade in the systemic immune response (Zaidman-Remy *et al.* 2006). So far, there is no evidence of Toll pathway activation in epithelial immune response, neither do fungi or Lys-type PGN Gram-positive bacteria stimulate AMP production in such tissues (Lemaitre and Hoffmann 2007; Ryu *et al.* 2010).

1.3.4.2. Reactive Oxygen Species (ROS)

AMPs production per se is not sufficient to mount a complete immune response in the midgut. Mutant *Drosophila* flies unable to express AMP were able to exhibit resistance to oral infection except when infected with ROS-resistant bacteria (Ryu *et al.* 2006). ROS are metabolic bi-products that exhibit microbicidal properties in the gut epithelium when released after bacterial infection (Ha *et al.* 2005a; Ha *et al.* 2005b). Flies unable to express a secreted, immune regulated catalase (IRC) exhibited higher mortality after oral infection, as well as after challenge with dead bacteria. The latter mortality was proposed to be caused by oxidative stress through accumulation of ROS (Ha *et al.* 2005a). It has been shown that Dual oxidase (DUOX) is the main source of ROS in *Drosophila* (Ha *et al.* 2005a; Ha *et al.* 2005b). DUOX is part of a conserved family of NADPH-oxidases with a N-terminal peroxidase domain capable of regulated ROS production(Ritsick *et al.* 2004). Silencing of DUOX by RNA-interference reduces the levels of ROS in the gut and also increases the mortality after oral infection with *E. carotovora* (Ha *et al.* 2005a). ROS release by DUOX is not triggered by PGN, but by a non-PGN ligand (Ha *et al.* 2009a). These non-PG microbial ligand(s) appear to be recognized by unknown G protein coupled receptor (GPCR) and to transmit the signals to Gαq and phospholipase Cβ (PLCβ) that lead to the mobilization of intracellular calcium via generation of inositol 1,4,5-trisphosphate (IP3). This PLCβ/IP3-dependent calcium mobilization is sufficient for spontaneous DUOX activation and subsequent ROS generation to kill the bacteria (Ha *et al.* 2009b)

1.3.5. Microbiota regulation in the midgut

Bacteria have been present on Earth for at least 2700 million years (Brocks *et al.* 1999) and organisms that appeared afterwards had to evolve within an environment dominated by microbes. Millions of different species of microbes co-habit with and within metazoans (Hooper and Gordon 2001). The intersection between microbes and the highly diverse Class Insecta over the millennia provided a particularly rich opportunity for the development of a diversity of microbe-host interactions leading to maintenance of immune homeostasis (Dillon and Dillon 2004). Insects like *Drosophila* host a simpler commensal microbiota (five to twenty species), compared to their vertebrate counterparts (Cox and Gilmore 2007; Ren *et al.* 2007). However, it is this simpler condition that makes some insects good models to study bacterial interactions. The traditional paradigm regards microbes as elicitors of the immune system (Ryu *et al.* 2010). However, gut microbes do not appear to trigger an antagonistic immune response in their metazoan hosts under regular conditions. Actually, vertebrates and invertebrates reared in experimental, germ-free conditions exhibit a reduced life-span (Dillon *et al.* 2005; Cheesman and Guillemin 2007). It has been shown that gut microbiota plays a beneficial role in human nutrient absorption (Gordon *et al.* 2005; Turnbaugh *et al.* 2006) as well as in protection against fungal pathogens in insects (Dillon and Charnley 1988; Currie *et al.* 2003).

However, commensal microbiota exhibits some of the same immunostimulator molecules (MAMPs) that are produced by pathogenic bacteria (Ryu *et al.* 2008; Salzman *et al.* 2010). How can the insect midgut differentiate between both? Some commensal bacteria are able to induce the expression of MAMP-degrading molecules in the midgut, hence avoiding stimulation of the immune response (Zaidman-Remy *et al.* 2006; Ryu *et al.* 2008). But one of the major differences between residents and pathogens is that the latter will ultimately damage the host. Pathogen associated damage will stimulate the release of danger signals within the host tissue, which together with the presence of MAMPs results in full activation of the immune response (Ha *et al.* 2005b; Liehl *et al.* 2006; Buchon *et al.* 2009; Lazzaro and Rolff 2011). This differential response may also explain the

dilemma posed by some microbes that may be beneficial or pathogenic depending on the physiological status of the insect host.

1.3.5.1. Sensing of pathogen-like behaviour of microbes in the gut

In *Drosophila*, it has been proposed that PGRPs with amidase activity participate in bacterial tolerance by degrading microbe-derived PGN to a non-immune stimulatory form (Bischoff *et al.* 2006; Zaidman-Remy *et al.* 2006; Ryu *et al.* 2008) and that commensals differ from pathogens in the amount of released PGN due to higher growth rates of pathogens (Zaidman-Remy *et al.* 2006). It has been suggested that this PGRP amidase negative feedback protects the host from damage derived from a prolonged immune activity (Bischoff *et al.* 2006). Other immune regulators include PGRP-LC-interacting inhibitor of Imd signalling (PIMS). PIMS present at basal levels during commensal microbiota-host interactions suppresses the activation of the Imd pathway (Lhocine *et al.* 2008). One of the latest models of immune homeostasis suggests that the interplay of Relish (NF-kB insect homologue) and Caudal (patterning gene in *Drosophila*) is responsible for regulation of AMPs expression in the gut (Ryu *et al.* 2008).

1.4. Immunity in phlebotomine sand flies

Knowledge of phlebotomine sand fly immunity is still in its infancy. Much of the information available is inferred from better-studied species such as *Drosophila* and mosquitoes where genome sequence data is available. Mosquitoes are a particularly rich source of information for the elucidation of the sand fly immunity since they fall within the Nematocera and the adults exhibit the dual feeding mode for plant carbohydrate and blood.

Insects, unlike vertebrates, do not bear the outstanding and complex antigen-antibody system of adaptive immunity; they rely on innate immunity to survive the challenge of pathogens and parasites (Boulanger *et al.* 2006; Lemaitre and Hoffmann 2007). As mentioned in a previous section insects such as sand flies discriminate particular microbe-associated molecular patterns (MAMPs),that are

typical of microbes, (Nurnberger *et al.* 2004) via pattern recognition receptors (PRRs),(Medzhitov and Janeway 2002). The major and best-studied PPRs in insects are peptidoglycan recognition proteins (PGRPs) and Gram-negative bacteria binding proteins (GNBPs) (Osta *et al.* 2004). Other PPRs include, but are not limited to, galectins (GALE), thioester-containing proteins (TEPs) and scavenger receptors (SCRs), (Dimopoulos *et al.* 1998; Christophides *et al.* 2002). Analysis of a cDNA library of *Lutzomyia longipalpis* (Dillon *et al.* 2006) identified the expression sequences with a predicted function for all of the aforementioned pattern recognition receptors. GALEs had been reported in similar studies of *Phlebotomus papatasi* (Ramalho-Ortigão *et al.* 2007). Further research on the interaction of these PPRs and *Leishmania* infection needs to be performed.

Activation of PPRs by PAMPs starts a cascade of signals that orchestrates a series of molecular events against the pathogen. This innate humoral response model had been best studied in *Drosophila*, (Hoffmann 2003). One of the outcomes of this cascade is the expression of antimicrobial peptides (AMPs). *Drosophila* synthesizes specific AMPs against different pathogens, (Tzou *et al.* 2002; Cherry and Silverman 2006). The role of AMPs in parasite infection has been discussed in mosquitoes (Osta *et al.* 2004; Meister *et al.* 2005; Luna *et al.* 2006) and tsetse flies (Boulanger *et al.* 2002; Lehane *et al.* 2004a; Hu and Aksoy 2006). These blood-sucking insects are able to express AMPs, such as defensins, against *Plasmodium* and *Trypanosoma*, respectively. In the sand fly *Phlebotomus duboscqi*, a defensin has been identified (Boulanger *et al.* 2006). This AMP was found to be specifically active against promastigotes *in vitro*; its activity *in vivo*, with an infection using *Leishmania* mutants lacking the proper formation of LPG, suggested that parasite surface antigens could account for a weakening in recognition of *Leishmania* by the sand fly.

Another mechanism that helps in controlling pathogens is the production of reactive oxygen species (ROS). *Drosophila* has been used as a model to study this host protection response,(Ha *et al.* 2005a). The homeostasis of redox balance, mediated by an immune-related catalase (IRC), was found to play a key role

during permanent host-microbe interactions in the gut environment. The impact of ROS and antioxidant expression in pathogen-insect interactions will be discussed in the final section.

Antioxidant systems in blood-sucking insects are extremely important in other biological issues than pathogen control. The following section will review some of the most important adaptations that blood-sucking insects had to undergo during their evolution to cope with the challenges derived from such a specialised lifestyle. The biochemical features of dealing with toxic blood by-products, particularly on antioxidant systems, will be discussed later in this chapter.

1.4.1 Sand fly Immunity and *Leishmania*

There is evidence of ancestral *Leishmania* forms (*Paleoleishmania*) present in the midgut of reptilian-bloodfed phlebotomines from as early 100 million years ago. The *Leishmania* has probably eluded insect immunity ever since, evolving inside the insect gut from the time they stopped being free-living organisms and started colonising insects (Poinar Jr 2007; Tuon *et al.* 2008). *Leishmania* does not seem to elicit a specific immune response inside its phlebotomine host. The only phlebotomine AMP isolated is not *Leishmania*-specific and it only kills the protozoan *in vitro* at a high concentration (Boulanger *et al.* 2004). Also, sand flies seem to be able to mount a ROS response against pathogenic bacteria but *Leishmania* does not seem to elicit this biochemical defence mechanism (chapter 5). Although *Leishmania* has developed different strategies to survive inside the gut, these seem to be more focused on enduring digestion and defecation rather than on the insect immune response (Kamhawi 2006). What is the net impact of *Leishmania* in the sand fly? Experimental colonisation of phlebotomines by *Leishmania* led to a reduction of survival of flies after oviposition but had no effect on fecundity (Rogers and Bates 2007). Moreover, these artificially infections used inocula that might be considerably higher compared to those expected in the wilderness and the effect of low vs. high inocula in transmission is significant (Lira *et al.* 2000). Perhaps under natural conditions the impact of *Leishmania* on sand fly survival might not be significant, at least not significant

enough to mount an immune response against the protozoan. It could also be possible that *Leishmania* relationship with its host shifts from parasitic to commensal or even beneficial depending on the insect immune status in the same way gut microbes do in *Drosophila* (Lemaitre and Hoffmann 2007). Recent experiments are re-defining this relationship suggesting that *Leishmania* may 'benefit' the sand fly host by creating an alliance against the entomopathogenic and *Leishmania* lytic *Serratia marcescens: L. mexicana*-colonised flies exhibit a significantly lower mortality after oral infection with *Serratia* compared to non-colonised sand fly controls (Sant'Anna, Diaz-Albiter, Genta and Dillon, unpublished).

1.5. Physiology and biochemistry of blood meal digestion

Of all the feeding habits displayed by insects, blood feeding or haematophagy, is relatively rare. Approximately 14, 000 species distributed in five orders are hematophagous, (Adams 1999). If haematophagy is considered as a feeding habit of insects that bear piercing/sucking mouthparts and prey on significantly bigger animals, then it is mainly restricted to four orders: Phthyraptera (lice), Hemiptera (true bugs), Siphonaptera (fleas) and Diptera (true flies), (Lukashevich and Mostovski 2003; Lehane *et al.* 2004b). The earliest known example amongst insects of a structure specialized for blood sucking comes from the Late Carnian (Late Triassic, ca. 220 Ma) of Virginia, USA (Blagoderov *et al.* 2007). Since that time, these insects have evolved a multiplicity of adaptations in morphology, biochemistry, behaviour to deal with the challenge of blood-feeding. Of particular interest for the present research are the biochemical adaptations featured in the digestive tract, specifically during the digestion process.

Many hematophagous species are able to feed on large amounts of blood in a single meal (Graca-Souza *et al.* 2006). Mosquitoes and kissing bugs can take as much as three to ten times their own mass (Friend *et al.* 1965; Lehane *et al.* 2004b; Graca-Souza *et al.* 2006). However, dealing with a blood meal is not only a matter of size. Blood is a very rich source of proteins. The most abundant in

mammalian blood, reaching concentrations as high as 150 mg/ml, is haemoglobin (Hb). Degradation of this protein during the digestion process initiates a massive release of haem. This prosthetic group of Hb has the potential to have an adverse impact on midgut physiology (Graca-Souza *et al.* 2006).

1.5.1. Haem degradation and toxicity

The iron protoporphyrin IX, or haem, is a molecule involved in generation of the highly toxic reactive oxygen species (ROS). Iron is the central molecule of haem; when not coupled to oxygen, it constitutes a stable-state iron, the addition of oxygen might lead an electron to delocalize between both molecules and create the free radical superoxide (O_2^-), (Halliwell and Gutteridge 1985). Although this superoxide can be further modified by superoxide dismutase (SOD), resulting in oxygen and hydrogen peroxide (H_2O_2), the latter is still dangerous since it can lead to the production of more ROS via a Fenton-type reaction,(Graca-Souza *et al.* 2006):

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^{\bullet}$$

The reaction is facilitated by the presence of iron which can be found as a bi-product of haem metabolism by haem oxygenase in blood feeding insects like *Aedes* and *Rhodnius* (Graca-Souza *et al.* 2006). To avoid the production of an even more reactive hydroxyl radical, insects (like all eukaryotes) are able to express catalase (CAT) and glutathione peroxidase (GPx), an organic hydroperoxide that uses glutathione (GSH). Both CAT and GPx break down hydrogen peroxide into water and other non-reactive oxygen species (Davies 1995).

Reactive oxygen species release is the price to pay for aerobic life. Superoxide and hydroxyl radicals and hydrogen peroxide are common products of life in an aerobic environment. These molecules have important deleterious effects on life systems which can lead to degradation of proteins, lipids, carbohydrates, DNA, as well as modifications of membrane permeability and selectivity (Gutteridge and Smith 1988; Schmitt *et al.* 1993). Blood sucking insects not only live in an aerobic environment, but also feed on a meal that is a significant source of ROS. To overcome its toxicity insects have developed different strategies: In *Rhodnius*

prolixus, a haem aggregation process has been found to account for the insolubilization of a significant amount of free haem (Oliveira *et al.* 2000). Haem dimers are linked together by reciprocal iron carboxylate bonds, these dimers are held together by hydrogen bonds(Slater *et al.* 1991) The resulting aggregate is called haemozoin (Hz) and it is found in *Plasmodium* as well as in other non-insect species (Oliveira *et al.* 2000). Formation of Hz has not been reported for other insects so far. Haem aggregation also occurs in mosquitoes; however, this process is mediated by the peritrophic matrix in an independent mechanism of Hz formation(Pascoa *et al.* 2002).

Aggregation by itself cannot account for all the free-haem clearance. Blood-sucking insects posses a set of antioxidant enzymes that constitute a strong line of defence against haem toxicity. Most of the traditional studies have focused on catalases, superoxide dismutases (Cu, Zn and Mn) and glutathione peroxidases(Graca-Souza *et al.* 2006). However, other antioxidant proteins found in insects have been described and studied. Thioredoxins (Trxs), for instance; are small, ubiquitous monomeric proteins with both reductase and peroxidase functions (Wagner *et al.* 1978). Thioredoxin reductase uses nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor, and is thought to make up for the absence of glutathione reductase in insects,(Kanzok *et al.* 2001). Another group that has raised interest in the past years is an expanding family of thiol-specific antioxidant proteins called peroxiredoxins (Prxs). These molecules are present in a wide range of species ranging from archaea to animals and, to some extent, may overlap the peroxide functions of glutathione peroxidases and catalases (Wood *et al.* 2003).

It is quite interesting to note that sequences with predicted functions for many of these enzymes have been found in blood-sucking insects like *Glossina morsitans morsitans* (Munks *et al.* 2005), *Aedes aegypti* (Sanders *et al.* 2003), *P. papatasi* (Ramalho-Ortigão *et al.* 2007) and *Lutzomyia longipalpis* (Dillon *et al.* 2006; Jochim *et al.* 2008). The increasing amount of gene-mining resources available

has shed some light on how similar antioxidant enzymes are expressed in different blood-sucking insects.

1.6. Redox homeostasis and parasites/pathogens

Although the Duox system has not been described in hematophagous insects so far, it is interesting to note that antioxidant enzymes expression and ROS have been found to play a role in pathogen-insect interactions. In *Anopheles stephensi*, the development of *Plasmodium berghei* was enhanced after dietary supplementation of mosquitoes with a strong oxidant scavenger, showing that oxidative stress has a negative impact on parasite development (Peterson *et al.* 2007). In another study with a susceptible and a refractory strain of *An. gambiae*, levels of ROS were dramatically different between both. This was proved by the differential induction of midgut-expressed Cu/Zn SOD and catalase mRNA after blood feeding. The refractory strain was able to block *Plasmodium* development by keeping higher levels of midgut ROS and promoting parasite melanization. Catalase seemed to be responsible for this enhancement in encapsulation, (Kumar *et al.* 2003). Knocking down of this enzyme in *An. gambiae* resulted in a significant diminution in the numbers of *P. berghei* present in the midgut(Molina-Cruz *et al.* 2008). Brennan *et al.*, (Brennan *et al.* 2008) showed and upregulation of other antioxidant enzymes in an *Aedes albopictus* cell line. It was suggested that the induction on expression of Cu/Zn SOD, Prx and glutathione peroxidase was caused by the infection with the endosymbiont *Wolbachia pipientis*. In the tse tse fly *Glossina morsitans mositans*, a range of antioxidants added in the blood meal dramatically increased survival of *Trypanosoma brucei*, suggesting that antioxidant-mediated reduction of the midgut environment can protect trypanosomes from death induced by ROS,(MacLeod *et al.* 2007b).

Although there is no evidence so far of the role of antioxidant enzymes in sand fly-*Leishmania* interactions, three different cDNA libraries show sequences with putative oxidative-stress functions. In whole-body *Lu. longipalpis*, Dillon *et al.*,(Dillon *et al.* 2006) found Cat, peroxidase, SOD, Prxs and thioredoxin reductase

expressed sequence tags. In a different study Cat, Prxs and glutathione s-transferase were found in the analysis of the midgut transcriptome of the same species(Jochim *et al.* 2008). Another midgut transcriptome analysis, but in *P. papatasi*, showed the expression of putative glutathione s-transferase and Prxs (Ramalho-Ortigão *et al.* 2007). Current validation of a cDNA microarray of *Lu. longipalpis* (Dillon, unpublished data), shows an upregulation of antioxidant enzymes in *Leishmania*-infected sand flies. Taken altogether, these investigations suggest that the presence of *Leishmania* may somehow lead to the modification of the expression profile of certain sand fly transcripts and that such modification is important for the parasite successful development inside the vector.

1.7 Aims of this Study

This study was aimed at providing knowledge regarding the role of reactive oxygen species (ROS) in female phlebotomine sand flies during Leishmania and bacteria interactions. In particular, it was attempted to explore the expression of antioxidant enzymes during digestion of infected and non-infected blood to find out potential candidates for RNAi-mediated gene knockdown in the sand fly model as well as exploring regulation of ROS and its potential role in and midgut immunity. Another major aim of this research included the development of a computer program able to determine *L. mexicana* subpopulations based on morphometric data extracted from image analysis. The specific aims of this study per experimental chapter are the following:

- Chapter 3-Differential expression of putative ROS-detoxifying genes in female *Lutzomyia longipalpis*: The aim of this chapter was to obtain a gene expression profile of different midgut antioxidant genes during digestion of non-infected and *Leishmania*-infected blood.

- Chapter 4-The effect of ROS-scavenging by catalase on fecundity and mortality of female *Lutzomyia longipalpis*: The aim of this chapter was to analyze the effect dietary supplementation of ROS-scavengers

(antioxidants) in fecundity and mortality. Another aim included the study of ROS regulation and its role in the aforementioned fitness components using RNAi-mediated silencing of catalase.

- Chapter 5-Reactive Oxygen Species-mediated immunity against *Leishmania mexicana* and *Serratia marcescens* in *Lutzomyia longipalpis*: The aim of this chapter was to explore the ROS-midgut immunity against microbes, specifically a pathogenic bacterium and a protozoan. Specifically, midgut gene expression of infected sand flies and ROS biochemical assays were performed to understand differential ROS activation by microbes. Another aim was to perform gene knockdown of catalase to find out its relevance during *Leishmania* colonisation. The final aim of this chapter was to analyse ROS regulation of gut microbiota by modifying midgut ROS levels using exogenous dietary antioxidants.

- Chapter 6-Development of software for analysing *Leishmania* morphometrics: The aim of this chapter was to develop an automated method for classification of *L. mexicana* subpopulations together with colleagues from the Department of Physics. This would be achieved by generating digital photographs of parasite smears and obtaining manual morphometric data. This would be compared with morphometric data calculated by the computer-based algorithm.

Chapter 2

Methods

2.1 General Methods

2.1.1 Sand fly rearing

All experiments were performed using insectary-reared *Lu. longipalpis* from a colony first started with individuals caught in Jacobina, Brazil. Insects were kept under standard laboratory conditions (Modi 1997). Sand flies were fed with 70% w/v sucrose solution in cotton wool (unless stated differently in experiments), kept under a photoperiod of 8 hours light/16 hours darkness, temperature of 27°C (±2) and a relative humidity of >80 % inside the rearing cages. The females in the colony were fed on rabbit blood via a Hemotek membrane feeder (Discovery Workshops, UK) at 37°C. All procedures involving animals were performed in accordance with UK Government (Home Office) and EC regulations.

2.1.2 Parasites

L. mexicana MNYC/BZ/62/M379 promastigotes were kindly donated by Prof. P. Bates and kept at 26° C in M199 medium supplemented with 25 µg/ml gentamicin sulphate (Sigma), 1x BME vitamins (Gibco) and 20% foetal calf serum (PAA). Promastigotes were sub-passaged into fresh medium when cultures reached late-log phase.

2.1.3 Parasite infections

Axenic amastigotes were obtained from promastigotes as previously described with some modifications (Bates 1994). Briefly, promastigotes were centrifuged at 671 x *g* for 10 min, resuspended in Graces medium supplemented with 25 µg/ml gentamicin sulphate (Sigma), 1x BME vitamins (Gibco) and 20% foetal calf serum (PAA) at pH 5.5 and incubated at 32°C until fully transformed amastigotes were present in the flask. Axenic amastigotes were maintained and sub-passaged in supplemented Graces medium at 32°C. For sand fly infections, amastigotes were

resuspended in 1mL of rabbit blood ($2x10^6$ parasites/mL) and fed to the insects through a chick skin membrane via a Hemotek feeder at 37 °C. Insects were kept under standard laboratory conditions until required for experimental work.

Table 2.1. Oligonucleotides for dsRNA synthesis and Reverse Transcriptase PCR I

Oligonucleotide	5'-3'sequence	Size (bp)
dsCAT1472 Forward	**TAATACGACTCACTATAGGG**GCTCGCGGTCCAGCTGAAGA	1472
dsCAT1472 Reverse	**TAATACGACTCACTATAGGG**TGGCCCAAGCTTGCATCGAC	
dsGFP Forward	**TAATACGACTCACTATAGGG**ACGTAAACGGCCACAAGTTC	693
dsGFP Reverse	**TAATACGACTCACTATAGGG**CTTGTACAGCTCGTCCATGCC	
RT CAT484 Forward	TGTTGCAGGGACGTCTCTTTGCC	484
RT CAT484 Reverse	AGGTTGGAGCACTTCTTGCGTTCG	
RT Ribo60S Forward	TCTCATCGGAAGTTTTCTGC	850
RT Ribo60 Reverse	GGCTTGTGACACCCTTGAAT	
RT SOD295 Forward	ATCCTGCACAGAACCCACAT	295
RT SOD295 Reverse	CACAGCACGTCCGATGATAC	
RT Prxr200 Forward	AGTGATTGCCTGCAGTGTTG	200
RT Prxr200 Reverse	AAATGCCTCGGTGGTCAATA	
RT OXR424 Forward	TGAGCCATTAGCGCCGCAGG	424
RT OXR424 Reverse	ACCCCCAATCGATACTCACGCACA	

2.1.4. RNA extraction and gene relative expression profile by RT-PCR

Sand flies were infected with either *Leishmania* or *Serratia* after 3 DPE (days post-emergence). Control group was bloodfed, non-infected flies. Insects were dissected at 1, 24, 48, 72 or 96 h post-infection (PI). At each time point, 8 midguts were homogenised in 50 µl of TRI Reagent® (Ambion, Austin, TX) and kept at -80°C until needed. RNA was extracted following the manufacturer's protocol. Total RNA was quantified using a Nanodrop® (NanoDrop Technologies, Wilmington, USA) and normalised to 10 ng/µl. RT-PCR was carried out with SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase Kit (Invitrogen, San Diego, CA) performing 25 cycles and following the manufacture's protocol (primers listed in table 2.1). Relative expression was normalised using a housekeeping gene (GenBank Accession number: AM088777, 60S ribosomal protein L3). RT-PCR products were analysed by 1.5 % w/v agarose/ethidium bromide gel electrophoresis and changes in gene expression were determined by densitometric measurement of bands using GeneSnap/GeneTools software

(Syngene, UK). Putative gene sequences of *Lu. longipalpis* catalase (CAT), Cu/Zn superoxide dismutase (SOD) and peroxiredoxin (PrxR) were obtained from a midgut-specific EST library (ABV60342, ABV60343, ABV60347, respectively) (Jochim *et al.* 2008) . A putative gene sequence for oxidative resistance protein-1 (OXR-1) was obtained from a cDNA library constructed from sand fly whole bodies (AM097733) (Dillon *et al.* 2006). BLAST was used to compare these sequences with the National Center for Biotechnology Information data base (Altschul *et al.* 1990). Conserved residues in those protein families were retrieved from the CDD database (Marchler-Bauer *et al.* 2011). Multiple alignment, phylogenetic analysis and Neighbor Joining cladograms were performed with MEGA package (Tamura *et al.* 2007). Relative expression data were shown as mean ± SEM (standard error mean) from three biological replicates.

2.1.5. Statistical analysis

Comparisons between means of two independent groups were carried put using a pair-wise t-test. Multiple comparisons were done by one-way ANOVA. Survival curves were analyzed with the Kaplan-Meier Log Rank χ^2 test. Relative frequencies were compared with the chi-square test. For nonparametric data, multiple comparisons were done with Kruskal-Wallis and pair-wise comparisons done with Mann-Whitney test. Significance was considered when $p<0.05$ unless stated otherwise. All data were analysed with the use of the SPSS Data Editor software (version 17.0, SPSS Inc).

2.2. Specific Methods

2.2.3 Chapter 3

2.2.3.1 Fecundity assays

Female *Lu. longipalpis* were allowed to mate under regular rearing conditions and fed with rabbit blood at three, six and nine days post-emergence (DPE). A batch of >500 flies was released into a large (20 m^3) rearing cage and groups of ~100 individuals were transferred to medium sized cages (5 m^3) at 3, 6 and 9 DPE and

blood-fed as above. Fifteen fully-engorged females were then transferred to a new medium rearing cage. Insects were dissected five days later to count developing oocytes.

2.2.3.2. Ascorbic Acid feeding

Fecundity assays were carried out as described above with female *Lu. longipalpis* fed on a 70% sucrose solution supplemented with 20 mM ascorbic acid and blood-fed at 9 DPE. Supplemented sucrose-meal was freshly changed daily and continued after blood-feeding. A 9 DPE control group was reared under the same conditions but fed with a 70% sucrose solution. Only fully engorged insects from both groups were selected for the experiments.

2.2.3.3. Ovarian Catalase Activity

Ovaries were collected from 5 female sand flies at 24 and 48 hrs post blood feeding (PBF). Samples were homogenised in 50 ul of 0.15 M NaCl solution, kept on ice and transferred to a -80 °C freezer until needed. Before assays, samples were centrifuged at 2700 x *g* for 2 minutes and 1 µl of the supernatant was diluted in 24.9 µl of 0.15 M NaCl solution. Catalase activity was determined using Amplex Red Catalase Assay Kit (Invitrogen Ltd) following the manufacture's protocol. Enzyme-specific activities were expressed as units/mg of protein. One unit of catalase activity was defined as 1 µM of H_2O_2 consumed per minute. All assays were carried out in triplicate. Fluorescence was measured using a Varioskan fluorescence spectrometer (Thermo Electron) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Ovarian catalase activity was normalised using the total amount of protein in the whole body (minus dissected ovaries) using the BIORAD® Protein assay reagent following the manufacturer's protocol and using bovine serum protein as standard. Endpoint absorbance was measured at 595 nm in a 96 well plate with a microplate reader (VersaMax Microplate Reader, Molecular Devices Inc.).

2.2.3.4. Ovarian Catalase Expression

Six DPE sand flies were blood fed and ovaries from 10 sand flies (two pools of 5 flies) were dissected at 12, 24 and 48 hours PBF, homogenised in 50 µl of TRI Reagent® (Ambion, Austin, TX) and kept at -80°C until needed. RNA was extracted following the manufacturer's protocol. Total RNA was quantified using a Nanodrop®(NanoDrop Technologies, Wilmington, USA) and normalised to 10 ng/µl. RT-PCR was carried out with SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase Kit (Invitrogen, San Diego, CA) performing 19 cycles and following the manufacture's protocol (primers listed on Table 2.2). Relative expression of catalase was normalised using a housekeeping gene (AM088777, 60S ribosomal protein L3). RT-PCR products were analysed by 1.5 % agarose/ethidium bromide gel electrophoresis and reduction in catalase expression was determined by densitometric measurement of bands using the software GeneSnap/GeneTools (Syngene, UK).

2.2.3.5. Age-related expression of ovarian catalase

To measure catalase LlongKat1 mRNA expression levels in different age groups, 3, 6 and 9 DPE sand flies were blood fed and ovaries from 10 sand flies (two pools of 5 flies) were dissected at 48 hours PBF. Additionally, to evaluate the effect of feeding a ROS-scavenger in age-related expression of ovarian catalase, a group of 9 days old sand flies was fed with ascorbic acid-supplemented sucrose solution as described above, blood fed and dissected at 48 hours. RNA was extracted and checked for catalase relative expression as above.

Table 2.2
Oligonucleotides for dsRNA synthesis and Reverse Transcriptase PCR II

Oligonucleotide	5'-3'sequence	Size (bp)
dsCAT484 Forward	**TAATACGACTCACTATAGGG**TGTTGCAGGGACGTCTCTTTGCC	524
dsCAT484 reverse	**TAATACGACTCACTATAGGG**AGGTTGGAGCACTTCTTGCGTTCG	
dsGFP Forward	**TAATACGACTCACTATAGGG**ACGTAAACGGCCACAAGTTC	693
dsGFP Reverse	**TAATACGACTCACTATAGGG**CTTGTACAGCTCGTCCATGCC	
RT CAT484 Forward	TGTTGCAGGGACGTCTCTTTGCC	484
RT CAT484 Reverse	AGGTTGGAGCACTTCTTGCGTTCG	
RT Ribo60S Forward	TCTCATCGGAAGTTTTCTGC	850
RT Ribo60 Reverse	GGCTTGTGACACCCTTGAAT	

2.2.3.6. RNAi-mediated catalase knockdown

Sense and anti-sense catalase-specific primers flanked by the T7 promoter site (Table 2.2) PCR amplified a 484 bp product from a plasmid obtained from a whole body *Lu. longipalpis* normalised cDNA library (Dillon *et al.* 2006) that was used as template for double-stranded RNA synthesis dsRNA. Transcription reactions and column purification were carried out using the Megascript RNAi Kit (Ambion®) following the manufacturer's protocol. dsRNA purity was assessed by 1.5 % agarose/ethidium bromide gel electrophoresis and dsRNA was quantitated using a Nanodrop ND-1000 Spectrophotometer (LabTech, UK). dsRNA was eluted with nuclease-free water at 65°C, concentrated to 4.5 µg/µL with a Christ® RVC 2-25 rotational vacuum concentrator and stored at -80°C until needed. Enhanced Green Fluorescent protein (eGFP) dsRNA was produced from a 653 bp amplicon of the pEGFP-N1 expression plasmid (Clontech) and used as a 'mock' injected control. RNAi was achieved by dsRNA injections as previously described (Sant'Anna *et al.* 2008). After injections, sand flies were transferred to cages and kept with access to 70 % sucrose solution *ad libitum*. Developing oocytes were dissected and counted 48 hours after blood feeding. Non-injected flies of the same age and kept under the same conditions were used a second control. Three

pools of three whole sand flies were collected from each group to evaluate knockdown by RT-PCR.

2.2.3.7. Survival assays

To assess sand fly survival mediated by ROS-scavenging related to catalase activity, RNAi-mediated catalase knock down was carried out in a group of 50 sand flies. Flies were injected with dsRNA for catalase (dsCAT) as described above. To exclude wound-related mortality, all dead flies at 24 hrs post-injections were removed and were not included in the experiment. Dead sand flies were counted and removed from the cage daily from day 2 to 7 after injection. Flies injected with dsRNA for GFP (dsGFP) and a needle-pricked group were used as controls. To assess exogenous ROS-scavenging related survival, 50 female *Lu. longipalpis* were collected upon emergence and sugar fed on a 70 % w/v sucrose solution supplemented with 20 mM ascorbic acid. Dead sand flies were counted and removed from the cage every day until day seven. A group of sand flies fed with 70 % sucrose was used as a control.

2.2.3.8. Phenoloxidase assays

Phenoloxidase activity was determined by measuring the production of dopachrome from 3,4 dihydroxy-DL-phenylalanine (DOPA) (Pomerantz 1963; Genta *et al.* 2010). Briefly, single flies were homogenized in 60 µL of PBS and centrifuged at 25,000g for 5 min at 4^{0}C to recover the soluble fraction. 20 µL of supernatant was mixed with 10 µL of PBS (spontaneous PO) or trypsin solution (for total PO activity; 1 mg/mL in PBS, FLUKA cat. no. 93614), incubated for 20 min at 37˚C followed by the addition of 20 µL of a saturated solution of DOPA (4 mg/mL in PBS) and absorbance (490 nm) measured by kinetic assay for 1h at 5 minutes intervals in a microplate reader at 30^{0}C. PO activity was measured to ensure that activity was proportional to protein concentration and incubation time. Independent experiments showed that the PO activity was stable in the conditions above. Controls with no enzyme or no substrate were included. One unit of enzyme (U) is defined as the amount that produces 0.001 unit of absorbance/min.

2.2.3.9. Sequence analysis

The coding sequence of LlonKat1 was analyzed using the algorithms pI/Mw tool (Walker 2005) , signal IP (Emanuelsson *et al.* 2007), PTS1 Predictor (Neuberger *et al.* 2003), PeroxiP (Emanuelsson *et al.* 2003), TargetP (Emanuelsson *et al.* 2007) based at the EXPASY Proteomics Server (http://expasy.org/). Selected amino acid sequences of catalases were aligned with catalase LlonKat1 using the ClustalW Multiple Alignment tool in BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Alignment was generated using Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

2.2.3.10. Microarrays

DNA from the sequencing of the EST's was used as a template to generate PCR products to make the spotted array. A C6 amino modified T7 forward primer and a T3 reverse primer were used (T7 5' C6-TAATACGACTCACTATAGGG, T3 5' ATTAACCCTCACTAAAGGGA) (Invitrogen Ltd, Paisley, UK) PCR products were checked by agarose gel and any multiple band products were discarded. PCR products were filtered with Sodium Phosphate buffer final concentration 250mM pH8.5 (1M Sodium Phosphate pH8.5, 0.001% sarkosyl) prior to spotting in duplicate on CodelinkTM Activated slides(GE Healthcare UK Ltd., Little Chalfont, UK) with a BioRobotics MicroGrid II robot (Genomic Solutions® Inc, Huntingdon, UK). 0.1ug RNA was amplified using Amino Allyl MerssageAmpTM II aRNA Kit according to manufacures instructions (Applied Biosystems/Ambion, Warrington, UK) aRNA was labeled with CYTM dye post-labelling reactive dye (GE Healthcare UK Ltd., Little Chalfont, UK) Labeled samples were purified using RNeasy mini kit (Qiagen,Crawley, UK) 2.5ug Cy3 and 2.5ug Cy5 labeled aRNA samples were co precipitated with poly A DNA and Herring sperm DNA, then re suspended in hybridization buffer (50% formamide, 5 X SSC, 0.1% SDS, 0.1 mg/ml BSA) prior to placing on the array and hybridizing overnight at 49OC. The arrays were washed at room temperature (W1 2 X SSC, 0.2% SDS, W2 0.2 X SSC. W3 0.1 X SSC, W4 0.01 X SSC) and scanned using a GenePix® 4000B Laser scanner (Molecular Devices, Sunnyvale, CA, USA). Resulting files were process using GenePix® Pro software (Molecular Devices, Sunnyvale, CA, USA) prior to analysis.

2.2.4. Chapter 4

2.2.4.1. Bacterial infections

S. marcescens (NCIMB 1377) was inoculated on LB agar plates and incubated at 26°C for 20 hours. Bacterial suspensions were prepared by transferring a colony of *S. marcescens* into 5 mL of LB broth and incubating overnight at 37°C under shaking. The suspension was centrifuged at 19000 x *g* for 5 minutes, resuspended in PBS and diluted to a concentration of $5.7x10^6$ CFU/ml. Bacteria were then diluted in heat-inactivated blood to a final concentration of $1.14x10^4$ CFU/ml and offered to sand flies via a Hemotek feeder as explained above.

2.2.4.2. H_2O_2 profile

Sand flies were infected with either *Leishmania* or *Serratia* after 3 DPE (days post-emergence). Insects were dissected at 24, 48, 72 and 96 h PI. Control group was bloodfed, non-infected flies. H_2O_2 was also measured in sugar-fed flies one day before infection/bloodfeeding. At each time point two pools of four midguts were homogenised in 60 µl of PBS per pool containing 2mg/ml of the catalase inhibitor 3-amino-triazole (AT). Samples were flash-frozen in liquid N_2 and kept at -80°C. Prior to assay, samples were thawed and centrifuged at 25 000 x *g*, 5 min at 4°C. Five µl of the supernatant were assayed for H_2O_2 using the Amplex Red® hydrogen peroxide/peroxidase Assay Kit (Invitrogen Ltd) following the standard protocol as recommended by the manufacturer. All assays were carried out in triplicate. The experiment was performed twice.

2.2.4.3. *In vivo* detection of ROS

Sand flies were infected with either *Leishmania* or *Serratia* after 3 DPE and dissected at 1, 24, 48, 72 h and 7 days PI. Control group was bloodfed, non-infected flies. ROS production was also measured in sugar fed flies, one day before infection/bloodfeeding. At each time point five midguts were dissected for *in vivo* detection of ROS as previously described (Owusu-Ansah *et al.* 2008). Briefly, midguts were dissected in L-15 (Leibovitz) medium (Sigma) and incubated with 30µM dihydroethidium (DHE) in L15-medium for 5 min in a dark chamber on

a mini orbital shaker (70 RPM) at room temperature. After 3x5 minute washes under the same conditions to remove DHE, individual midguts were transferred to 10-well slides. ROS production was monitored via an inverted fluorescence microscope using a U-MWG fluorescence cube (excitation: 530–560 nm; emission: 590 nm). Images were captured using a x10 objective and analyzed by Image J. A semi-quantitative approach was used to compare fluorescence between samples. Based on tiff image files saved using the NIS-Elements BR 3.00 imaging software (Nikon), the sand fly midguts were delimited using the paintbrush tool of the Image J program and mean intensity was measured inside the midgut, minimising interference from background fluorescence. Mean intensity values were then used to compare fluorescence between samples after incubation.

2.2.4.4. Midgut Catalase Activity

Sand flies were infected with *Leishmania* after 3 DPE and dissected at 1, 24, 48, 72 and 96 h post blood feeding (PBF). 10 Individual midguts were homogenised in 50 µl of 0.15 M NaCl solution, kept on ice and transferred to a -80 °C freezer until needed. Before assays, samples were centrifuged at 2700 x *g* for 2 minutes and 5 µl of the supernatant was diluted in 25 µl of 0.15 M NaCl solution. Catalase activity was determined using Amplex Red Catalase Assay Kit (Invitrogen Ltd) following the manufacturer's protocol. Enzyme-specific activities were expressed as units per midgut. One unit of catalase activity was defined as 1 µM of H_2O_2 consumed per minute. All assays were carried out in triplicate. Fluorescence was measured using a Varioskan fluorescence spectrometer (Thermo Electron) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Experiment was performed once.

2.2.4.5. dsRNA-mediated gene knockdown, insect survival and parasite count

Sense and anti-sense catalase and OXR1-specific primers (Table 2.3) flanked by the T7 promoter site amplified by PCR a 1472 and a 800bp product (respectively) obtained from a normalised whole body *Lu. longipalpis* cDNA library (Dillon *et al.* 2006) that were used as template for double-stranded RNA synthesis. Transcription reactions and column purification with the Megascript RNAi Kit

(Ambion®) followed the manufacturer's protocol. dsRNA purity was assessed by 1.5 % w/v agarose/ethidium bromide gel electrophoresis and dsRNA was quantitated using a Nanodrop ND-1000 Spectrophotometer (LabTech, UK). dsRNA was eluted with nuclease-free water at 65°C, concentrated to 4.5 µg/µL with a Christ® RVC 2-25 rotational vacuum concentrator and stored at -80°C. Enhanced Green Fluorescent protein (eGFP) dsRNA was produced from a 653 bp amplicon of the pEGFP-N1 expression plasmid (Clontech Ltd) and used as a 'mock' injected control. RNAi was achieved by dsRNA injections as previously described (Sant'Anna *et al.* 2008). After injections, sand flies were transferred to cages, kept under standard rearing conditions and infected with *Leishmania* 72 h after injections. Insects were dissected 94 h post-infection and 15 midguts were homogenised individually in 50 ul of PBS and parasites were counted using a haemocytometer. Three whole sand flies were reserved for individual RNA extraction and knockdown evaluation by RT-PCR. Survival was recorded in dsOXR1-injected flies up to 5 days after injections (n=50). Catalase-silencing experiment was performed three times, OXR1 experiment was performed twice.

Table 2.3
Oligonucleotides for dsRNA synthesis and Reverse Transcriptase PCR III

Oligonucleotide	5'-3'sequence	Size (bp)
dsCAT1472 Forward	**TAATACGACTCACTATAGGG**GCTCGCGGTCCAGCTGAAGA	**1472**
dsCAT1472 Reverse	**TAATACGACTCACTATAGGG**TGGCCCAAGCTTGCATCGAC	
dsOXR1 800 Forward	**TAATACGACTCACTATAGGG**GCCCCTACCCACCTCGGTCAT	**800**
dsOXR1 800 Reverse	**TAATACGACTCACTATAGGG**ATGCTGCGATCGCCCCTGATT	
dsGFP Forward	**TAATACGACTCACTATAGGG**ACGTAAACGGCCACAAGTTC	**693**
dsGFP Reverse	**TAATACGACTCACTATAGGG**CTTGTACAGCTCGTCCATGCC	
RT CAT484 Forward	TGTTGCAGGGACGTCTCTTTGCC	**484**
RT CAT484 Reverse	AGGTTGGAGCACTTCTTGCGTTCG	
RT Ribo60S Forward	TCTCATCGGAAGTTTTCTGC	**850**
RT Ribo60 Reverse	GGCTTGTGACACCCTTGAAT	
RT SOD295 Forward	ATCCTGCACAGAACCCACAT	**295**
RT SOD295 Reverse	CACAGCACGTCCGATGATAC	
RT Prxr200 Forward	AGTGATTGCCTGCAGTGTTG	**200**
RT Prxr200 Reverse	AAATGCCTCGGTGGTCAATA	
RT OXR424 Forward	TGAGCCATTAGCGCCGCAGG	**424**
RT OXR424 Reverse	ACCCCCAATCGATACTCACGCACA	

2.2.4.6. H_2O_2 feeding

Sand flies were kept under standard rearing conditions and allowed to feed *ad libitum* on a cotton wool soaked in 5 mM H_2O_2 in 70% w/v sucrose solution since emergence and until the end of the experiment. Hydrogen peroxide solutions were freshly prepared from a H_2O_2 30% w/w stock solution (Sigma). Insects were infected with *Leishmania* 3 DPE. Control group was fed on plain 70% w/v sucrose solution. Flies were dissected 94 h post-infection and 15 midguts were homogenised individually in 50 µl of PBS and parasites were counted in a haemocytometer. Experiments were repeated twice.

2.2.4.7. Uric acid feed (UA), insect survival and bacteria counts

Sand flies were kept under standard rearing conditions and allowed to feed *ad libitum* on a cotton wool soaked in 10 mM uric acid in 7% w/v sucrose solution (pH=8.9) since emergence and until the end of the experiment. Uric acid solution was freshly prepared every day. Control group was fed on sucrose 7% w/v, no UA (pH=8.9). Insects were infected with *Serratia* 3 DPE as explained above. Control flies fed on non-infected blood. Survival was recorded every day. Twelve flies were collected at 48 h post-infection, dissected and four pools of three midguts were homogenised in 50 µl of PBS per pool. Serial dilutions were inoculated onto LB agar plates and colony forming units (CFUs) were counted after incubation at 26°C for 24 h. Experiments were performed three times.

2.2.5 Chapter 6

2.2.5.1. Analysis of subpopulations of parasites

Fifty parasites per smear were randomly selected and analysed using bright-field microscopy. Parasites were observed and measured under oil immersion at 1000x magnification with the aid of an eyepiece graticule. For the sake of consistency with the software, parasites were placed into 1 of the 5 more relevant categories out of the seven (Table 6.1), based on previous descriptions (Killick-Kendrick *et al.* 1974; Molyneux and Killick-Kendrick 1987; Rogers *et al.* 2002), namely

amastigotes, procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes and metacyclic promastigotes.

2.2.5.2. Analysis of subpopulations of parasites *in silico*

Twenty photographs from a single smear per time point were taken at 400x magnifications and saved as Tagged *Image* File Format (TIF) with a resolution of 2590x1920 pixels. To calibrate the software (i.e., convert pixels into µm) a haemocytometer grid was photographed at the same magnification and conditions and included in every set of parasite pictures. Images were run through two different versions of the software. ParaMorph V 3.0 and ParaMorph V 3.1, a newer version of the software that was modified to identify metacyclics that were not correctly located and classified by ParaMorph V 3.0 in preliminary runs.

2.2.5.3. Validation of software output vs. manual classification

Relative frequencies of parasite subpopulations obtained from manual counts were compared against relative frequencies reported by the software from both Paramorph V 3.0 and ParaMorph V 3.1. Further validation of parasite classification was performed by randomly selecting one photograph from the software set per time point and by manually selecting all parasite forms in the photograph that would have been selected during manual counts (*i.e.* non-overlapping, non-dividing).

2.2.5.4. Induction of metacyclogenesis and sampling

Metacyclogenesis was induced as previously described with some modifications (Bates 1994). Briefly, cultured promastigotes were centrifuged at 671 x *g* for 10 min, re-suspended in Graces medium supplemented with 25 µg/ml gentamicin sulphate (Sigma), 1x BME vitamins (Gibco) and 20% foetal calf serum (PAA) at pH 5.5 and incubated at 32°C until metacyclic promastigotes were present in the flask. To collect a heterogeneous mixture of subpopulations, cultures were sampled at early (24 h, 48 h) and late (7d, 8 d) metacyclogenesis. Cultures were sampled by pipetting 10 µl volumes x 3 onto microscope slides, fixed with

methanol and stained with 10% (v/v) Giemsa's stain. Smears were scanned systematically and 50 parasites were randomly sampled.

Chapter 3

Differential expression of putative ROS-detoxifying genes in female *Lutzomyia longipalpis*

3.1. Introduction

The gut of blood feeding insects constitutes a niche for different species of microbes, ranging from potentially beneficial and pathogenic bacteria to parasitic protozoa using the insect as a vehicle for transmission. A few species of the latter are transmitted to humans by hematophagous insects and cause morbidity and mortality affecting millions around the world. These 'blood dwelling' protozoa have evolved different strategies to overcome several barriers and insults from both vertebrate and invertebrate hosts. One of the major challenges happens during blood meal digestion, when a plethora of digestive enzymes and other compounds turn the midgut into a potentially hostile environment for incoming microbes. Reactive oxygen species (ROS) are a set of highly reactive molecules which seem to play an important role during blood-meal digestion as well as during insect-microbe interactions. The aim of this chapter was to analyse the expression of putative ROS detoxifying genes in *Leishmania mexicana* infected female *Lu. longipalpis* and to discuss gene differential expression in the context of blood meal digestion inside the phlebotomine sand fly.

Haematophagous arthropods are able to ingest an amount of blood many times their own body size in a single meal(Graca-Souza *et al.* 2006). This represents a particular physiological challenge considering the biochemical nature of vertebrate blood. Up to ninety percent of the protein in red blood cells is made up of haemoglobin (Barnhart and Steinmetz 1986), which is an oxygen-binding protein made up of four polypeptide chains, each with a haem prosthetic group (Hames and Hooper 2005). As a consequence of haemoglobin digestion, larger quantities of haem are released in the gut. Haem promotes the formation of toxic reactive oxygen species and leads to oxidative damage of lipids, proteins and DNA (Gutteridge and Smith 1988). Haem is a low-molecular-mass form of iron

(Gutteridge and Smith 1988), capable of inducing oxidative stress through two different pathways. In the presence of hydrogen peroxide (H_2O_2) it generates the strong oxidant hydroxyl radical ($OH^{\bullet}$) via a Fenton-like reaction. Additionally, $OH^{\bullet}$ can also react with other molecules and ultimately lead to the production of highly reactive alkoxyl and peroxyl radicals (Fig 3.1).

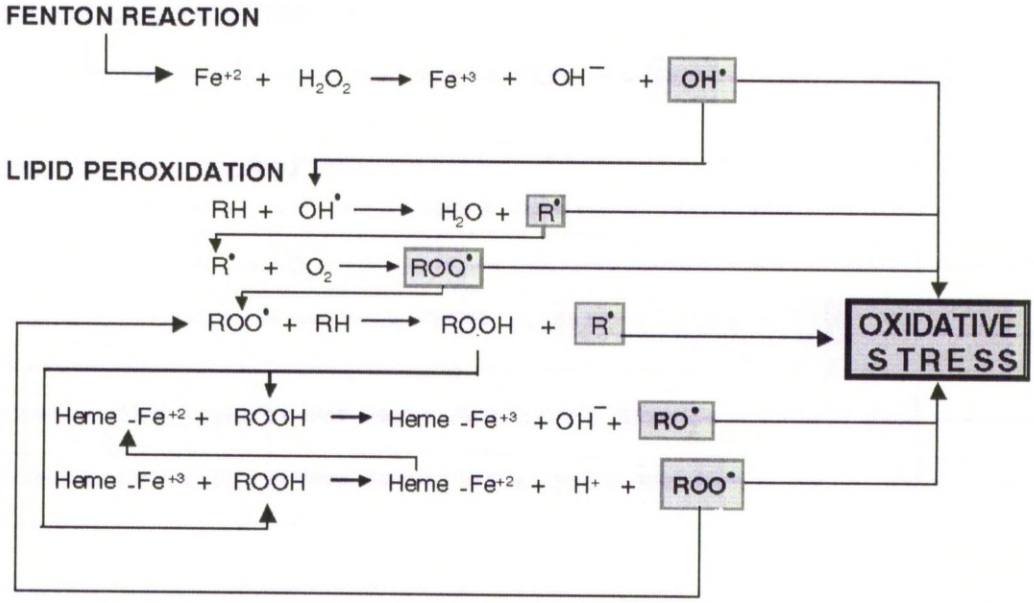


Figure 3.1: Haem and iron promote lipid peroxidation by different mechanisms. Iron-induced oxidative stress is thought to be mediated by the Fenton reaction which generates hydroxyl radicals ($OH^{\bullet}$) that can initiate lipid peroxidation chains by abstracting electrons from other molecules such as an unsaturated fatty acid (RH), generating an alkyl radical ($Rd^{\bullet}$). In contrast, haem-induced formation of radical species relies on the conversion of low-reactive organic hydroperoxides (ROOH) into highly reactive alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals. Adapted from Graca-Souza (Graca-Souza *et al.* 2006)

Arthropods avoid haem-derived oxidative damage during blood digestion through different mechanisms including haem aggregation and degradation, as in the case of the kissing bug *Rhodnius prolixus* or the cattle tick *Boophilus microplus* (Graca-Souza *et al.* 2006), or peritrophic matrix-mediated concentration, as in the case of the mosquito *Aedes aegypti* (Pascoa *et al.* 2002). The other strategy developed to regulate oxidative balance in the host tissue is the production of antioxidant enzymes. This is an array of molecules produced by aerobic cells that are able to

detoxify ROS. The main antioxidant enzyme systems are catalase (CAT), superoxide dismutases (SOD), and glutathione peroxidases (GPx) (Fridovich and Freeman 1986), which activate within the cell for the catalysis of toxic ROS such as superoxide anion ($O_2^{\bullet -}$)and H_2O_2 into innocuous water, oxygen and reduced glutathione (Fig 3.2) (Matés *et al.* 1999). However, other enzymes are also able to detoxify ROS. For example, peroxiredoxins (PrxR) are an expanding and ubiquitous family of thiol-specific antioxidant proteins (Wood *et al.* 2003). PrxRs reduce and detoxify H_2O_2, peroxynitrite and organic hydroperoxides (ROOH) throughout their peroxidase activity ($ROOH+2e^- \rightarrow ROH+H_2O$) (Wood *et al.* 2003). Although they are less efficient compared to other traditional antioxidant enzymes, they seem to be the major ROS scavengers in taxa that lack catalase, such as kinetoplastids including *Leishmania* (Hofmann *et al.* 2002). Additionally, there is evidence for upstream gene regulation of antioxidant enzymes in insects. In *Anopheles gambiae*, oxidation protein 1 (OXR1) regulates gene expression of catalase and glutathione peroxidase (Jaramillo-Gutierrez *et al.* 2011) .

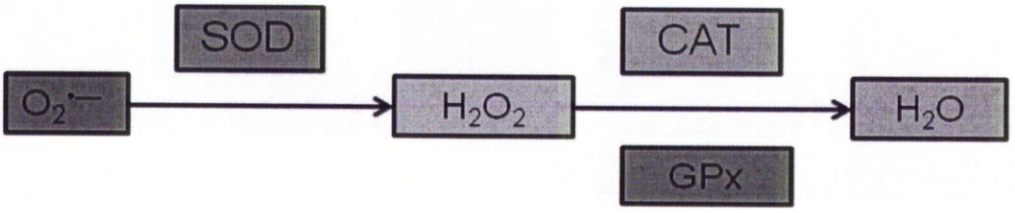


Figure 3.2: ROS regulation by antioxidant enzymes. Superoxide dismutase (SOD) catalyzes de dismutation of superoxide ($O2^{\bullet -}$) into hydrogen peroxide (H_2O_2), which is in turn reduced to water and oxygen by catalase (CAT), or decomposed by glutathione peroxidase (GPx) into water and oxidized glutathione.

Haematophagous insects exhibit an increment in expression or activity of antioxidant enzymes upon blood-feeding. Catalase and SOD levels were significantly higher in the midgut of *R. prolixus* compared with other tissues and organs (Paes *et al.* 2001); catalase expression was found to be upregulated upon blood-feeding in *Anopheles gambiae* (Kumar *et al.* 2003) and *Aedes aegypti* (Sanders *et al.* 2003). The use of gene silencing tools has also highlighted the importance of antioxidant enzymes in oxidative damage. Double-stranded RNA-

mediated knockdown of catalase and superoxide dismutase induced a higher mortality in sugar fed *Culex pipiens* (Sim and Denlinger 2011). Similar results were observed in blood fed *An. gambiae* after silencing of catalase only (Magalhaes *et al.* 2008).

Over the past few years, an increasing number of studies have discussed the relevance of midgut redox homeostasis and successful transmission of blood parasites. The malaria parasite *Plasmodium* cannot survive inside the continuous oxidative stress of a refractory strain of *An. gambiae* with high levels of hemolymph H_2O_2 and $O_2^{\bullet -}$ (Kumar *et al.* 2003). A set of different antioxidants fed to the tsetse fly *Glossina morsitans morsitans* inhibited cell death of the sleeping sickness parasite *Trypanosoma brucei brucei,* suggesting a lethal effect of bloodmeal-induced ROS against trypanosomes (Macleod *et al.* 2007a; MacLeod *et al.* 2007b). In *Trypanosoma cruzi,* the etiological agent of Chagas disease, virulence is associated to in vitro resistance of epimastigotes and metacyclic trypomastigotes to H_2O_2. Both forms are found in the triatomine bug stage of the parasitic cycle (Piacenza *et al.* 2009).

Female sand flies must feed on blood to complete egg development. They are exposed to the same challenges presented by blood digestion like any other haematophagous arthropod. The generation of cDNA libraries from *Lutzomyia longipalpis* has allowed the identification of different putative ROS detoxifying genes. In a whole body cDNA library a putative copper-zinc SOD and a catalase were identified (Dillon *et al.* 2006). Additionally, results from a midgut-specific cDNA library allowed to identify catalase (CAT), copper-zinc superoxide dismutase (SOD) and a peroxiredoxin (PrxR) (Jochim *et al.* 2008). Species-specific expression profiling microarray hybridization of *Leishmania*-infected vs. bloodfed female *Lu. longipalpis* suggested differential expression of putative ROS detoxifying genes (Dillon unpublished data). To investigate the effect of *L. mexicana* infection on female *Lu. longipalpis* ROS detoxifying gene expression, four putative antioxidant/ROS-regulatory genes were selected to explore changes in expression throughout the course of blood digestion using reverse transcriptase PCR (RT-

PCR)-based semi-quantitative profiling. Over the following sections, sequence phylogeny, intraspecific comparative alignment, microarray and RT-PCR-based gene expression profiling results are shown and discussed.

3.2. Results

3.2.1. *Lu. longipalpis* catalase

Contig 142e04.q1k from a whole body cDNA library (GenBank AM105518) exhibited the most significant similarity (99%) to a putative catalase from a midgut-specific EST library (Table 3.2). *Lu. longipalpis* CAT mRNA sequence contains 1930 bp and encodes a predicted peptide of 510 aminoacids (GenBank ABV60342). An alignment of four available catalases from four dipteran species revealed that this protein is highly conserved within the regions predicted to bind haem and NADPH (Marchler-Bauer and Bryant 2004; Marchler-Bauer *et al.* 2009; Marchler-Bauer *et al.* 2011) and that *Ae. aegypti* has the highest identity score(73.3%) (Fig 3.3.1). To examine these relationships more closely a phylogenetic analysis with other insect species was performed and it grouped *Lu. longipalpis* catalase within a clade formed with catalase amino acid sequences from Suborder Nematocera (*An. gambiae* and *Ae. aegypti*) (Fig 3.3.2).

3.2.2. *Lu. longipalpis* SOD

Contig NSFM-39d09.p1k from a whole body cDNA library (GenBank AM095907) exhibited the most significant similarity (99%) to a putative Cu/Zn SOD from a midgut-specific EST library (Table 3.2). *Lu. longipalpis* SOD mRNA sequence contains 833 bp and encodes a predicted peptide of 205 amino acids (GenBank ABV60343). An alignment of four available SODs from four dipteran species revealed that this protein is highly conserved within the regions predicted to bind Zn^{+2} and Cu^{+2} (Marchler-Bauer and Bryant 2004; Marchler-Bauer *et al.* 2009; Marchler-Bauer *et al.* 2011) and that *An .gambiae* has the highest

Table 3.2. Putative ROS-regulatory genes

Contig	Accession number	Best match to non-redundant protein database (NRPD) excluding *Lu. longipalpis*	NRPD E value	Best match to *Lu. longipalpis* midgut EST library	Accession number	E value	Identity (%)	Similarity (%)
NSFM-142e04.q1k	AM105518	putative catalase [*Phlebotomus perniciosus*]	3e-11	putative catalase	ABV60342	1e-13	99	99
NSFM-39d09. p1k	AM095907	putative Cu/Zn superoxide dismutase [*Phlebotomus perniciosus*]	2e-73	putative Cu/Zn superoxide dismutase	ABV60343	1e-95	99	99
NSFM-34h03.q1k	AM102380	GI16636 [*Drosophila mojavensis*]	7e-98	putative peroxiredoxin	ABV60347	3e-126	99	99
NSFM-22f08.p1k	AM097733	oxidation resistance protein [*Glossina morsitans morsitans*]*	5e-101	14.5 kDa midgut protein	ABV60314	1.1	30	44

Best matched results and corresponding lowest BLASTX E value together with putative function based on this homology of a GenBank derived non-redundant protein database and a *Lutzomyia longipalpis* midgut cDNA library. The contig is given for each gene. A "q" following the clone identifier indicates that sequencing was from the 3' end of the clone. The lowest BLASTX E value (most significant similarity) together with putative function based on this homology is given. Asterisk represents best match with a putative oxidation resistance protein function.

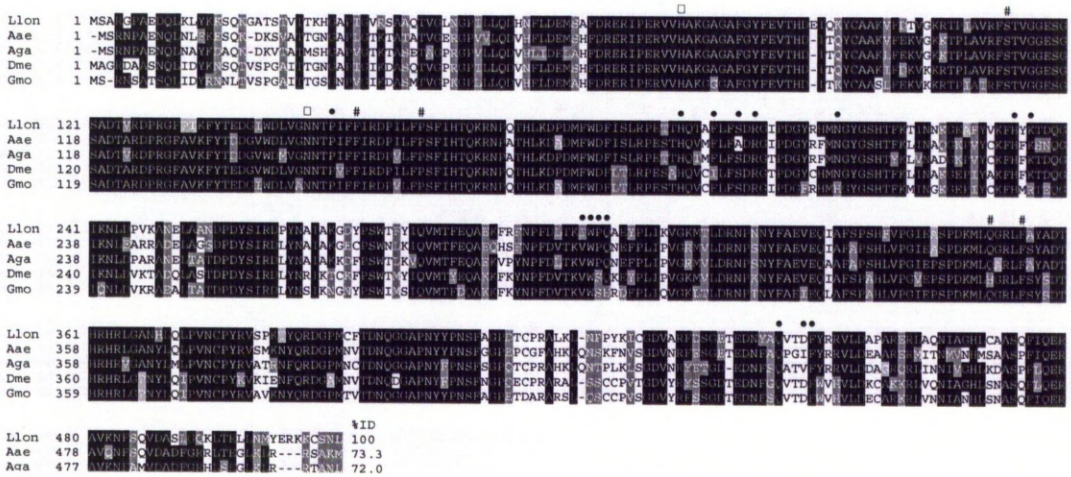


Figure 3.3.1. Amino acid sequence alignment of selected catalases. Sequences were retrieved from GenBank (GB). The listed proteins are respectively from *Lu. longipalpis* (ABV60342.1), *Aedes aegypti* (XP_001663600.1), *Anopheles gambiae* (ABL09376.1), *Drosophila melanogaster* (NP_536731.1), *Glossina morsitans morsitans* (ADD20421.1). Conserved residues are with black background, consensus alternatives are shaded. The symbols □, # and • mark catalytic, haem binding and NADPH binding residues, respectively.

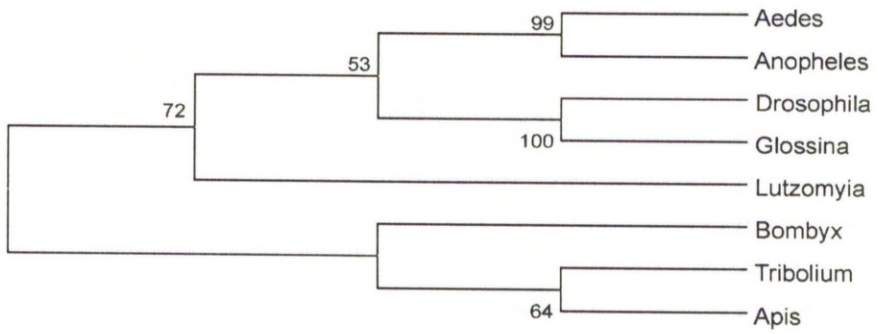


Figure 3.3.2. Phylogeny of selected catalases. Non-rooted neighbour-joining consensus trees of selected sequences from catalase protein families. Sequences are from *Aedes aegypti* (Diptera: XP_001663600), *Anopheles gambiae* (Diptera: ABL09376), *Drosophila melanogaster* (Diptera: NP_536731), *Glossina morsitans morsitans* (Diptera: ADD20421), *Lutzomyia longipalpis* (Diptera: ABV60342), *Bombyx mori* (Lepidoptera: BAD38853), *Tribolium castaneum* (Coleoptera: NP_001153712), *Apis mellifera* (Hymenoptera: NP_001171540). Bootstrap values (above 50, 10000 replicates) for each branch point are given.

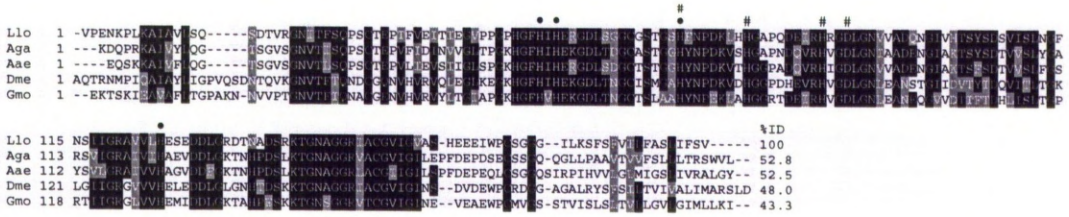


Figure 3.4.1: Amino acid sequence alignment of selected superoxide dismutases. Sequences were retrieved from GenBank (GB). Partial sequences corresponding to the mature proteins (signal peptides removed) were used in the alignment. The listed proteins are respectively from *Lu. longipalpis* (ABV60343.1), *Aedes aegypti* (XP_001651857.1), *Anopheles gambiae* (XP_314137.4), *Drosophila melanogaster* (NP_001036536.1), *Glossina morsitans morsitans* (ADD19264.1). Conserved residues are with black background, consensus alternatives are shaded. The symbols # and • mark catalytic Zn^{+2} and Cu^{+2} binding residues at the active site, respectively.

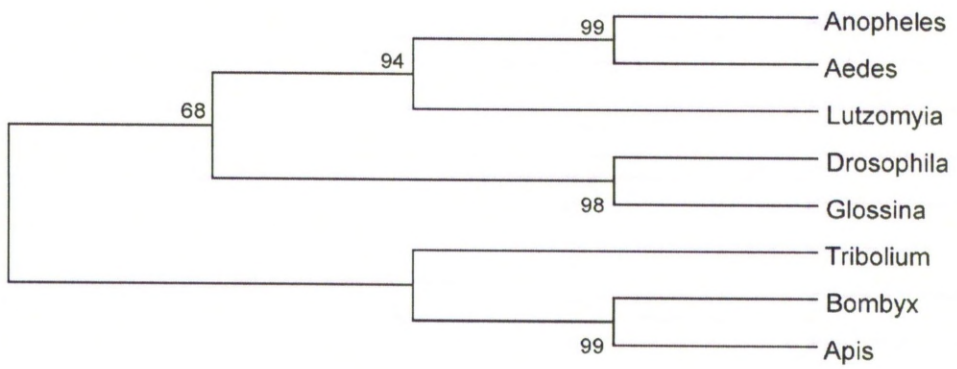


Figure 3.4.2: Phylogeny of selected superoxide dismutases. Non-rooted neighbour-joining consensus trees of selected sequences from superoxide dismutase (SOD) protein families. Sequences are from *Aedes aegypti* (Diptera: XP_001651857), *Anopheles gambiae* (Diptera: XP_314137,), *Drosophila melanogaster* (Diptera: NP_001036536), *Glossina morsitans morsitans* (Diptera: ADD19264), *Lutzomyia longipalpis* (Diptera: ABV60343), *Bombyx mori* (Lepidoptera:, NP_001037084), *Tribolium castaneum* (Coleoptera: NP_001164126), *Apis mellifera* (Hymenoptera: NP_001171498). Bootstrap values (above 50, 10000 replicates) for each branch point are given.

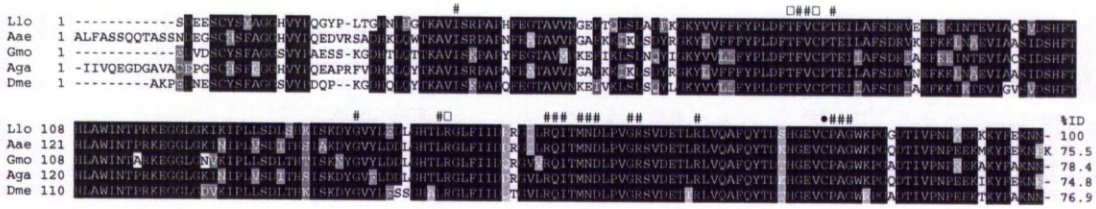


Figure 3.5.1: Amino acid sequence alignment of selected peroxiredoxins. Sequences were retrieved from GenBank (GB). Partial sequences corresponding to the mature proteins (signal peptides removed) were used in the alignment. The listed proteins are respectively from *Lu. longipalpis* (ABV60347.1), *Aedes aegypti* (XP_001648972.1), *Anopheles gambiae* (XP_308336.4), *Drosophila melanogaster* (NP_525002.1), *Glossina morsitans morsitans* (ADD19060.1). Conserved residues are with black background, consensus alternatives are shaded. The symbols □, # and • mark the catalytic triad (including the peroxidatic cysteine), binding residues at the dimer interface and the resolving cysteine, respectively.

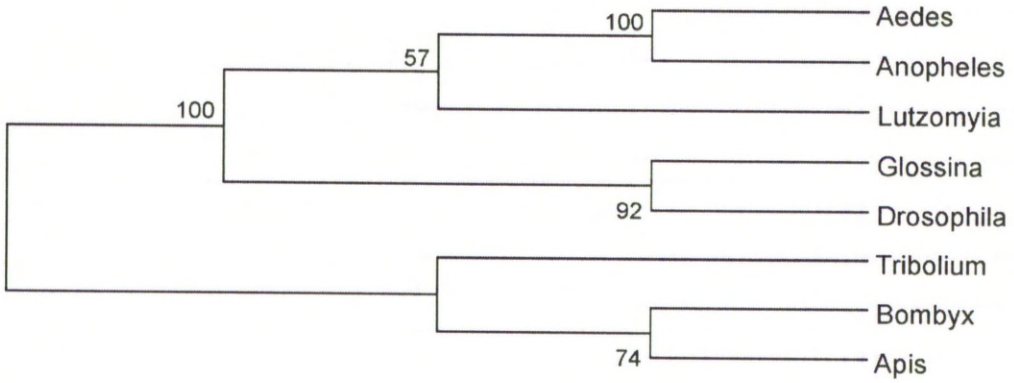


Figure 3.5.2: Phylogeny of selected peroxiredoxins. Non-rooted neighbor-joining consensus trees of selected sequences from peroxiredoxin (PrxR) protein families. Sequences are from *Aedes aegypti* (Diptera: XP_001648972), *Anopheles gambiae* (Diptera: XP_308336), *Drosophila melanogaster* (Diptera: NP_525002,), *Glossina morsitans morsitans* (Diptera: ADD19060), *Lutzomyia longipalpis* (Diptera: ABV60347), *Bombyx mori* (Lepidoptera: AAR15420), *Tribolium castaneum* (Coleoptera: XP_970797), *Apis mellifera* (Hymenoptera: XP_393445). Bootstrap values (above 50, 10000 replicates) for each branch point are given.

identity score (52.8 %)(Fig 3.4.1). To examine these relationships more closely a phylogenetic analysis with other insect species was performed and it grouped *Lu. longipalpis* SOD within a clade formed with SOD amino acid sequences from Suborder Nematocera (*An. gambiae* and *Ae. aegypti,*) (Fig 3.4.2).

3.2.3. *Lu. longipalpis* PrxR

Contig NSFM-34h03.q1k from a whole body cDNA library (GenBank AM102380) exhibited the most significant similarity (99%) to a putative PrxR from a midgut-specific EST library (Table 3.2). *Lu. longipalpis* PrxR mRNA sequence contains 874 bp and encodes a predicted peptide of 248 amino acids (GenBank ABV60347). An alignment of four available PrxRs from four dipteran species revealed that this protein is highly conserved within the regions predicted for the catalytic triad, dimer interface binding and resolving cysteine (Marchler-Bauer and Bryant 2004; Marchler-Bauer *et al.* 2009; Marchler-Bauer *et al.* 2011) and that *Ae. aegypti* has the highest identity score (75.5 %) (Fig 3.5.1). To examine these relationships more closely a phylogenetic analysis with other insect species was performed and it grouped *Lu. longipalpis* PrxR within a clade formed with PrxR amino acid sequences from Suborder Nematocera (*An. gambiae* and *Ae. aegypti*) (Fig 3.5.2).

3.2.4. *Lu. longipalpis* OXR1

Contig NSFM-22f08.p1k from a whole body cDNA library (GenBank AM097733) exhibited the most significant similarity (E value=5e-101) to a putative oxidation resistance protein from *Gl. morsitans morsitans* (Table 3.2). *Lu. longipalpis* PrxR mRNA sequence contains 1881bp and encodes a predicted peptide of 394 aminoacids (GenBank AM097733). An alignment of four available OXR1 from four dipteran species revealed that this protein is moderately conserved within the regions predicted for the LYS1, OXR1 and TLDc domain (Schultz *et al.* 1998; Letunic *et al.* 2009) (Fig 3.6.1 and 3.6.2) and that *D. melanogaster* has the highest identity score (28.3%)(Fig 3.6.2). To examine these relationships more closely a phylogenetic analysis with other animal species was performed and it grouped *Lu. longipalpis* OXR1 within a clade formed with OXR1 amino acid

acid sequences from Suborder Brachycera (*D. melanogaster and Gl. morsitans morsitans*) (Fig 3.6.3).

3.2.5. ROS-regulatory genes are differentially expressed in *Leishmania*-infected flies

Comparison of fold changes of whole-body putative ROS-detoxifying genes from microarray data shows differential gene expression in *L. mexicana*-infected flies from 8 to 188 h PBF. Putative CAT shows expression levels below the mean fold change (MFC) from 8 to 24 h PBF and above MFC from 48 to 144 h PBF (Fig 3.7A). SOD exhibits expression levels above MFC at 8 hrs PBF and a reduction from 24 to 48 h PBF followed by an increase above MFC from 27 to 144 h PBF (Fig 3.7B). PrxR shows an increment of ~ 60 % above MFC at 8 h PBF followed by a reduction below MFC from 24 to 144 h PBF (Fig 3.7C). Finally, putative OXR1 exhibited expression levels below MFC at 8 h PBF, a further increment above MFC from 24 to 72 h and an expression level lower than MFC at 144 h PBF (Fig 3.7D). To analyse midgut- specific changes in expression of ROS-regulatory genes, sand flies were infected with *L. mexicana*. Expression of four ROS-regulatory genes was assessed by semi-quantitative RT-PCR. Catalase exhibited the highest variation in expression among all ROS-detoxifying gene sequences analysed. Catalase from non-infected midguts showed significant changes in expression from 1 to 96 h PBF (Fig 3.8A; ANOVA, $p<0.001$). Catalase was downregulated in *Leishmania*-infected flies at 48 h PBF compared to control group ($p<0.05$, Fig 3.8A). A putative SOD was down regulated at 48 h PBF in *Leishmania*-infected flies compared to control group ($p\leq0.016$) (Fig 3.8B). A *Lu. longipalpis* peroxiredoxin (PrxR) displayed a trend of upregulation at 1 and 24 h PBF in non-infected flies relative to the infected group but such differences were not statistically significant.(Fig 3.8C). OXR1, a gene described in *A. gambiae* that regulates the expression of catalase and glutathione peroxidase (Jaramillo-Gutierrez *et al.* 2011) did not exhibit significant changes in expression after *Leishmania mexicana* infections (Fig 3.8D).

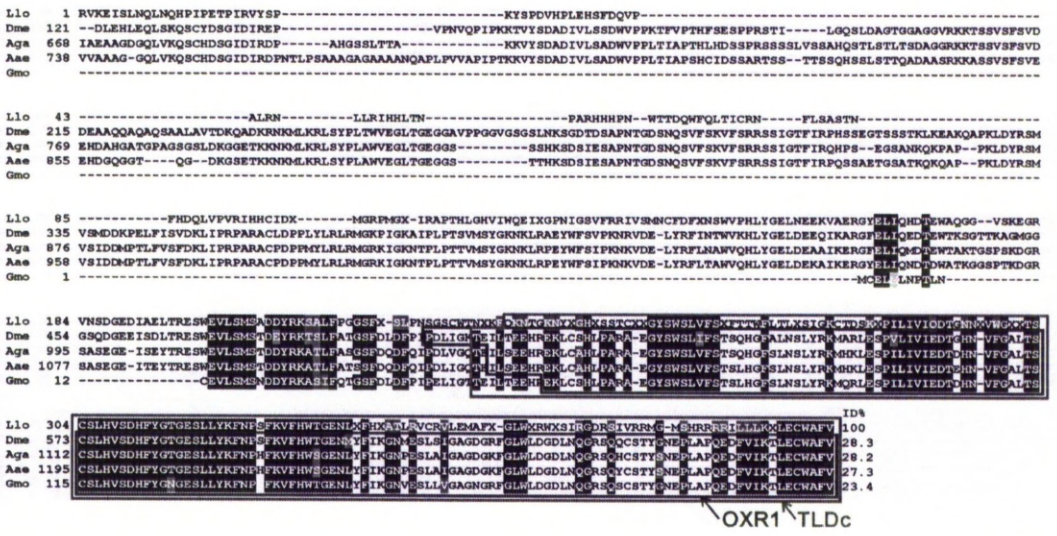


Figure 3.6.1: Amino acid sequence alignment of selected oxidation resistance proteins. Sequences were retrieved from GenBank (GB). The listed proteins are respectively from *Lu. longipalpis* (AM097736.1), *Aedes aegypti* (XP_001662202.1), *Anopheles gambiae* (XP_321331.4), *Drosophila melanogaster* (ACV53876.1), *Glossina morsitans morsitans* (ADD20017.1). Black background represents conserved residues whereas consensus alternatives are shaded.

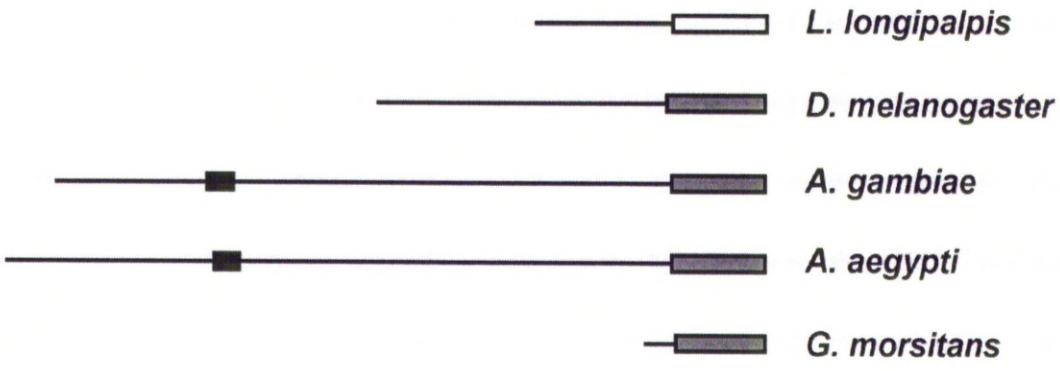


Figure 3.6.2: Protein domains present in selected oxidation resistance proteins from various animal species. Sequences are same from Fig 3.4.1. Black, gray and white boxes correspond to LYS1 (smart00584), OXR (COG5142) and TLDc (smart00584) domains from SMART and COG databases, respectively.

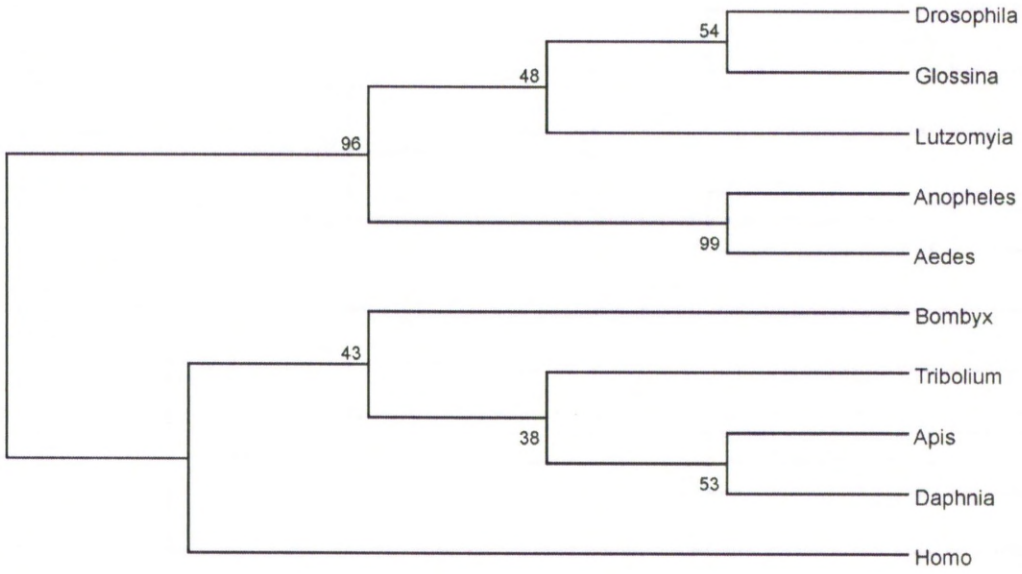


Figure 3.6.3. Phylogeny of selected oxidation resistance proteins. Non-rooted neighbour-joining consensus trees of selected sequences OXR1 protein families. Sequences are from *Aedes aegypti* (Diptera: XP_001662202), *Anopheles gambiae* (Diptera: XP_321331), *Drosophila melanogaster* (Diptera: ACV53876), *Glossina morsitans morsitans* (Diptera: ADD20017), *Lutzomyia longipalpis* (Diptera: NSFM-22f08), *Bombyx mori* (Lepidoptera: NP_001139127), *Tribolium castaneum* (Coleoptera: XP_967175), *Apis mellifera* (Hymenoptera: XP_393372), *Daphnia pulex* (Crustacea: OXR, EFX81439), *Homo sapiens* (ORX, AAQ76813). Bootstrap values (above 50, 10000 replicates) for each branch point are given.

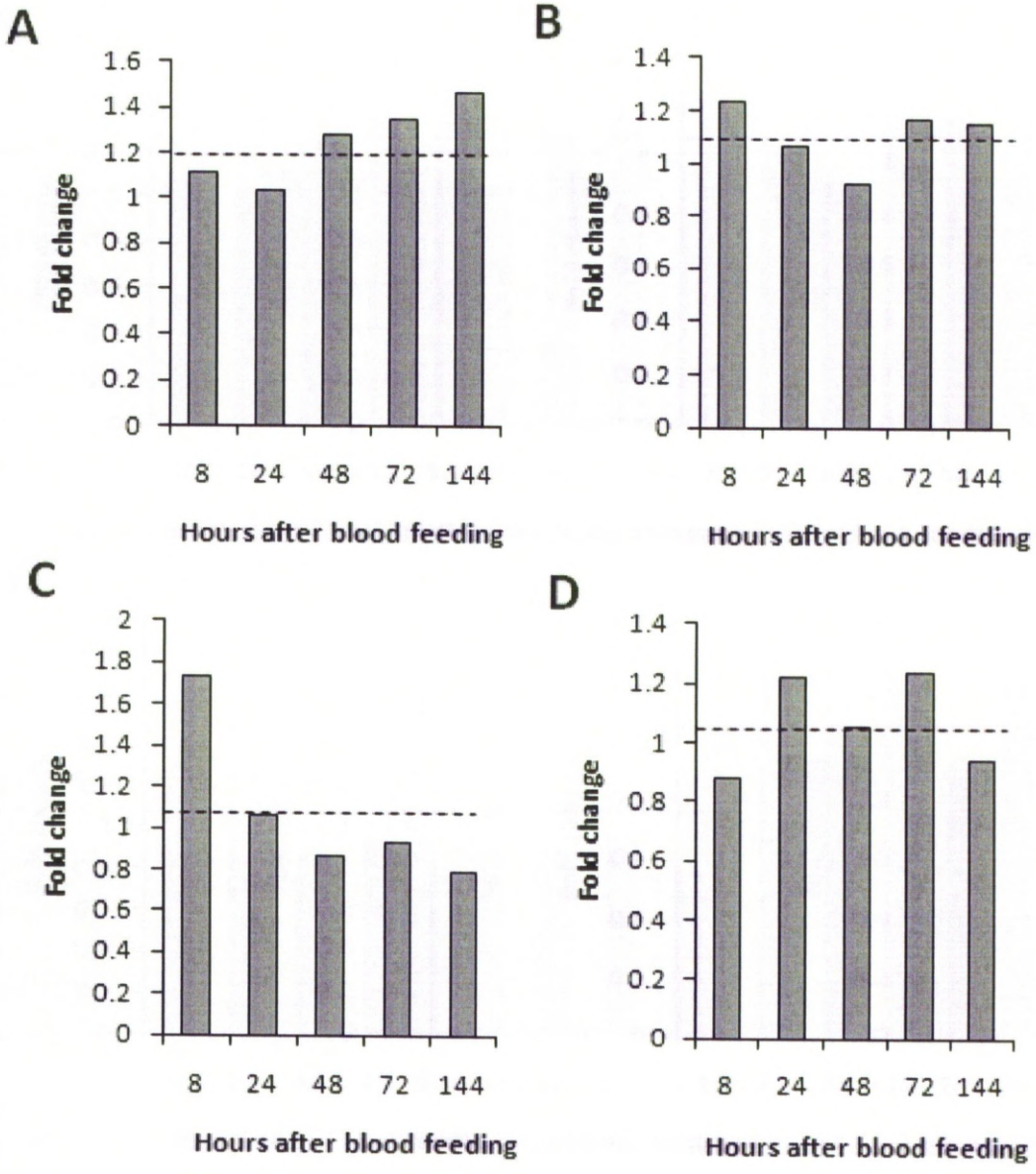


Figure 3.7: Putative ROS-detoxifying gene expression profile change in flies infected with *L. mexicana*. Bars represent fold change values from data by Dillon *et al.* (unpublished) derived from a microarray of *L. mexicana*-infected female *Lu. longipalpis* versus blood fed, non-infected flies. A. Putative catalase. B. Putative superoxide dismutase. C. Putative peroxiredoxin. D. Putative oxidation resistance protein. Dotted line represents mean of fold change values.

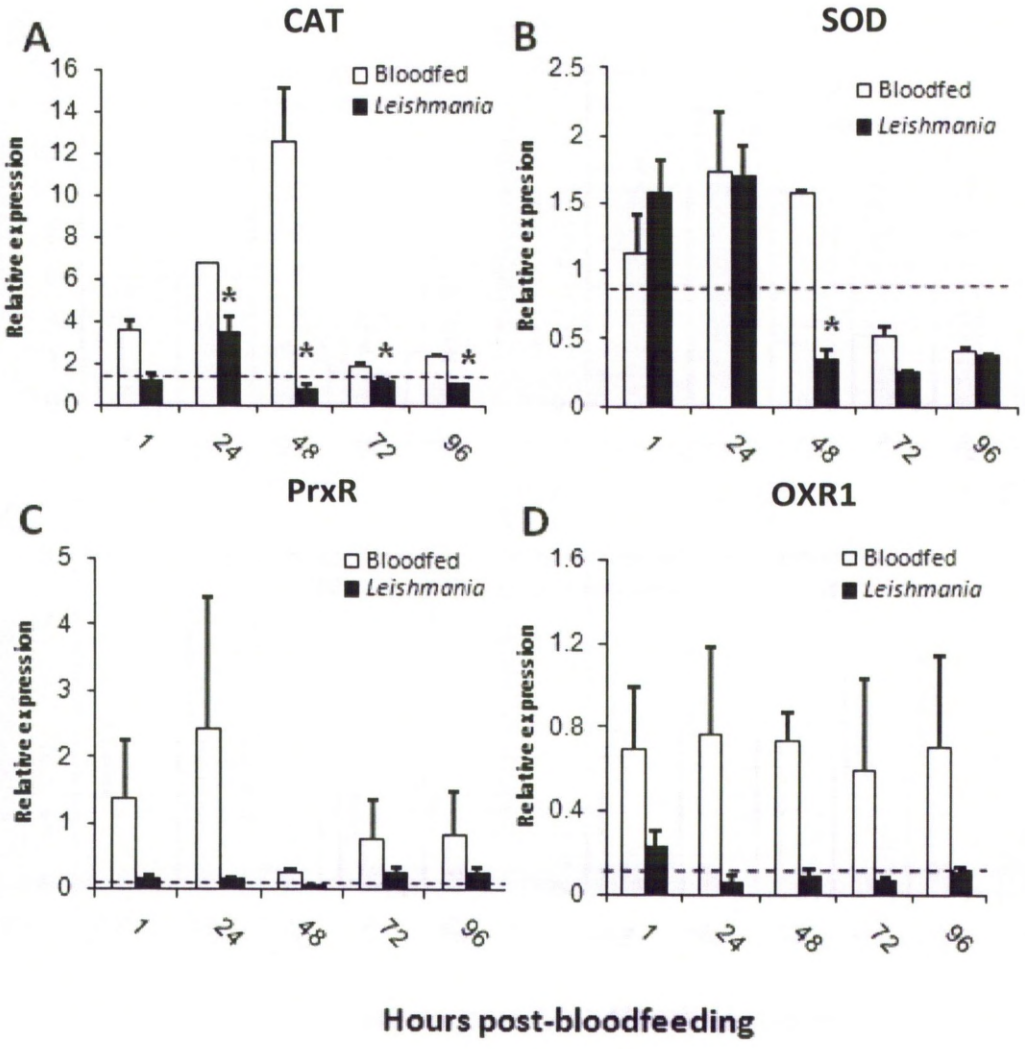


Figure 3.8: Midgut-specific relative expression profile by semiquantitative RT-PCR of ROS-regulatory genes in infected *Lu. longipalpis*. Female flies were fed with either a non-infected blood meal (Control), or a *Leishmania mexicana*-infected bloodmeal. Midguts were pooled (N=8) and relative expression of the following genes was assessed: *A*, catalase (CAT), *B*, Cu/Zn superoxide dismutase (SOD), *C*, peroxiredoxin (PrxR) and *D*, oxidation resistance 1(OXR1). Bar charts represent mean ± SEM of combined samples from at least 2 independent experiments. Asterisk indicates statistical difference at $p<0.05$. Dotted line represents mean of relative expression values of *L. mexicana*-infected samples.

3.3. Discussion

The work presented in this chapter shows that *Lutzomyia* ROS-regulatory gene expression changes throughout digestion and that gene expression of SOD and catalase is modulated in the sand fly midgut after infection with the flagellate protozoan *L. mexicana*. Oxidation resistance1 (OXR1) and peroxiredoxin (PrxR) did not show statistically significant differences in relative expression between bloodfed and *Leishmania*-infected groups.

3.3.1. Microarray vs. RT-PCR

Gene expression analysis from both microarray data and RT-PCR show differential expression in infected vs. non-infected flies. However, expression profiles from the same putative genes exhibit a different pattern when comparing microarray fold-change data with RT-PCR relative expression (Fig 3.7A-D and 3.8A-D). This is not surprising if we consider that microarray data emerged from a whole-body cDNA library with infection using *L. infantum* (Dillon *et al.* 2006), while midgut cDNA was chosen for semi-quantitative RT-PCR gene expression profiling. Although present in the midgut, these putative genes are probably expressed in other tissues. In *Drosophila*, catalase has found to be highly expressed in the midgut, but also in oenocytes, fat body, testes and ovaries (Klichko *et al.* 2004). CAT and SOD are highly expressed in the ovaries, flight muscle and spermatheca of the honey bee *Apis mellifera* (Collins *et al.* 2004). In the silk worm *Bombyx mori*, CAT is expressed in the fat body, silk gland and haemocytes (Yamamoto *et al.* 2005). Actually, ROS-detox enzymes have been found in a wide variety of tissues and organs in insect species large enough to allow multiple tissue-specific measurements. In *Rhodnius prolixus* CAT and/or SOD have been found in many of the aforementioned tissues as well in the cuticle, heart, crop, midgut content and salivary glands (Paes *et al.* 2001). The size of the sand fly precluded comparative measurements using other tissue.

The microarray data was derived from a whole-body cDNA library and although it is not completely accurate for specific-tissue level expression comparisons it still

provides an informative picture of changes in gene expression. Semi-quantitative RT-PCR, real-time RT-PCR and Northern blot analysis are still the tools of choice for microarray data validation, however, it has been stated that validation consistency is only possible when genes possess more than a four-fold difference (Chuaqui *et al.* 2002). It should be noted that none of the transcripts from the microarray data set had differences higher than four-fold (Fig 3.7). We could, nonetheless, measure significant differences in expression in at least two of the putative ROS-detox genes analysed.

3.3.2. Gene expression and blood digestion

Sequences corresponding to catalase (CAT), Cu/Zn Superoxide Dismutase (SOD) and Peroxiredoxin (PrxR) retrieved from a sand fly whole body cDNA library exhibited a high level of identity (>99%) and similarity (>99%) against sequences obtained from a midgut specific library (Table 3.2)(GenBank ABV60342, ABV60343 and ABV60347) (Jochim *et al.* 2008), suggesting that these genes are also expressed in the midgut of *Lu. longipalpis.* OXR1 could not be found in the midgut EST library, but specific oligonucleotides targeting the TLD_C conserved domain from OXR1 amplified the expected product when included in the midgut expression profile. Also, a BLAST search performed in a preliminary *Lu. longipalpis* genome data base recently available on-line, after the completion of this work, has identified several sequences with significant alignments for *An. gambiae* OXR1, (http://www.hgsc.bcm.tmc.edu/project-species-i-Lutzomyia_ longipalpis. hgsc).

Catalase was differentially expressed from 1 to 96 h post-blood feeding in non-infected *Lu. longipalpis* (ANOVA. $P<0.001$) (Fig 3.8A) in an expression profile that correlates with the process of bloodmeal digestion (Fig 3.9). It has been previously shown that expression of the main 'late' trypsin gene is detectable from 2 to 72h PBF, reaching its peak at 12 h (Telleria *et al.* 2007). Similar trypsin enzymatic profiles have been found in other species of sand flies such as *Phlebotomus papatasi* and *Ph. langeroni* (Dillon and Lane 1993a; Dillon and Lane 1993b).

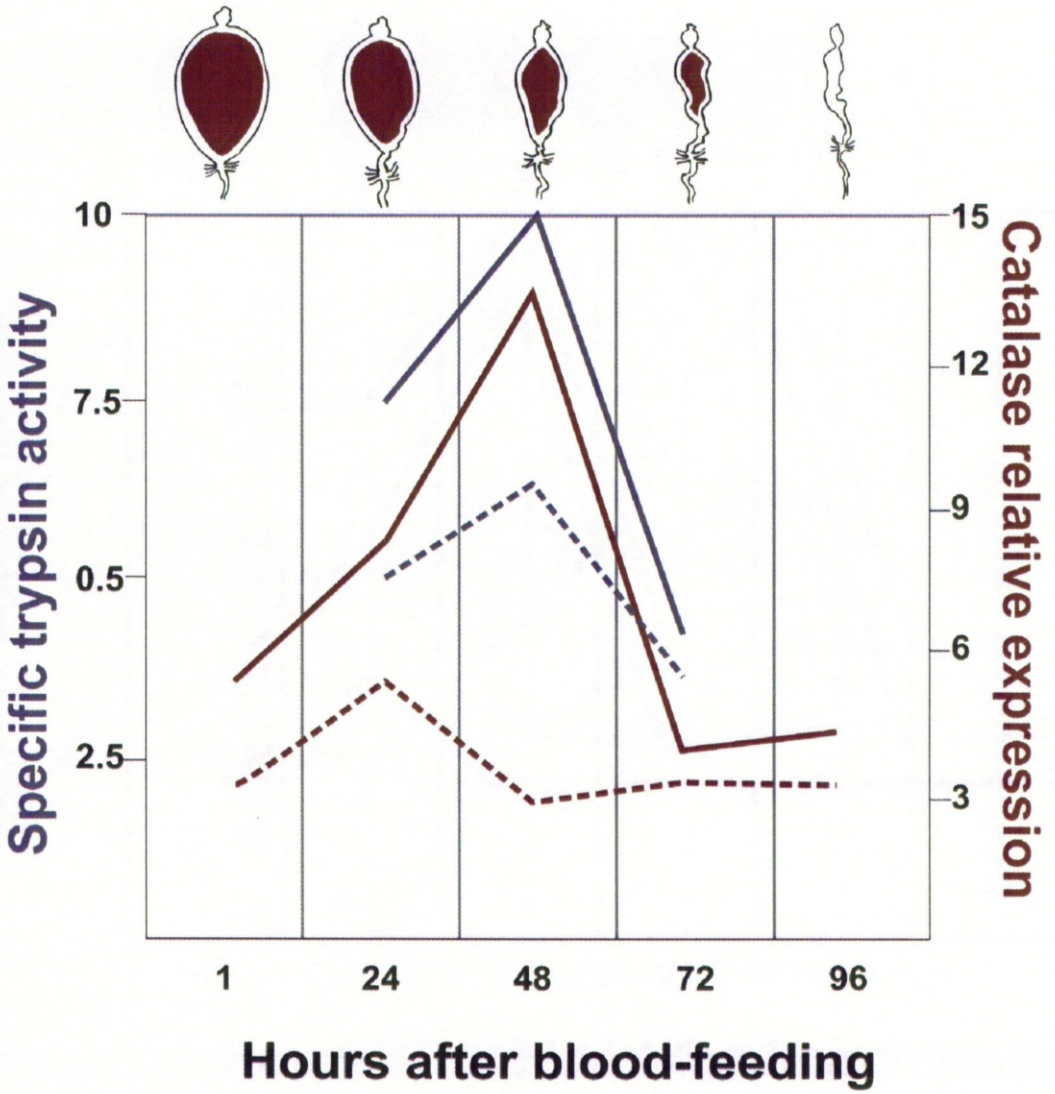


Figure 3.9: Relative profiles of catalase expression and bloodmeal digestion in female *Lu. longipalpis*. Continuous purple line represents trypsin activity (μM/min/mg protein) in in midgut extracts after a bloodmeal, dashed purple line represents midguts infected with *L. mexicana,* (Sant'Anna *et al.* 2009). Red continuous line represents midgut catalase expression in *Lu. longipalpis*, dashed red line represents *L. mexicana*-infected midgut catalase expression (data from Fig 3.8A). Cartoons on top represent sandlfy midguts during digestion timepoints from 0 to 96 h after blood-feeding.

A similar expression pattern of ROS-detoxifying enzymes occurs in other blood feeder taking discrete meals, since ROS are largely produced during blood digestion (Graca-Souza *et al.* 2006). In different blood-sucking insects, catalase is expressed upon blood ingestion (Paes *et al.* 2001; Sanders *et al.* 2003; Munks *et al.* 2005; Jochim *et al.* 2008; Magalhaes *et al.* 2008; Molina-Cruz *et al.* 2008). It has been postulated that this enzyme plays an important role detoxifying ROS derived from haemoglobin breakdown (Graca-Souza *et al.* 2005; Graca-Souza *et al.* 2006). An increasing expression of catalase could reflect the use of this enzyme during digestion. In *Anopheles gambiae*, mRNA levels of digestive enzymes peak around 20 to 24 h after blood feeding but immunoblot analysis of protein lysates displayed peak levels from 30 to 48 h after blood ingestion (Muller *et al.* 1995; Dana *et al.* 2005). In *Drosophila*, differences in catalase mRNA expression and protein accumulation profiles suggested that catalase expression might be affected by transcriptional and post-transcriptional regulation (Radyuk *et al.* 2000). Catalase could be present as an inactive storage proform for fast release to avoid ROS-induced damage during blood digestion.

There were no significant changes in OXR1 expression in the midgut of *Lu. longipalpis* during blood digestion and *Leishmania* infections. In *An. gambiae*, OXR1 regulates the expression of ROS-detoxifying enzymes, specifically catalase and glutathione peroxidase (GPx) (Jaramillo-Gutierrez *et al.* 2011). In our study, primers were also designed to cover the carboxyl terminal TLD_C domain, the most highly conserved region of the gene (Fig 3.6.1 and 3.6.2). However, gene expression between *Lu longipalpis* OXR1 and catalase was not related and there was no indication that OXR1 expression controls catalase expression in a similar way previously described in *A. gambiae*.

3.3.3. Modulation of ROS-detox enzymes by *Leishmania*

Leishmania-infected sand flies displayed a constant and significant reduction in catalase expression in comparison to bloodfed controls up to 96h post infection with the highest fold-difference at 48 h (Fig 3.8A). Interestingly, SOD was also significantly reduced in infected midguts at this time point (Fig 3.8B). Previous

studies in *Anopheles gambiae* have shown an increase in systemic expression of ROS detoxification enzymes at 24 and 48 h PBF (Molina-Cruz *et al.* 2008). During *Plasmodium* infection, catalase was downregulated 24h post-infection and it has been suggested that such suppression of catalase expression might be a response against the parasite that would ultimately lead to higher intracellular levels of H_2O_2, protein nitration and finally, apoptosis of infected cells (Molina-Cruz *et al.* 2008). However, as *Leishmania* development is restricted to the sand fly midgut (Bates 2007), any reduction in catalase expression would result in increased oxidative stress, ultimately leading to an impact on *Leishmania* survival within the sand fly midgut. Although it is possible that a reduction in catalase expression in *Leishmania*-infected midguts represents a response against the parasite (as suggested in *Plasmodium*-infected Anopheline mosquitoes), this would not explain how *Lu. longipalpis* is able to harbour heavy *Leishmania* infections in both experimental and field conditions (Deane and Deane 1954; Freitas *et al.* 2002; Killick-Kendrick and Rioux 2002).

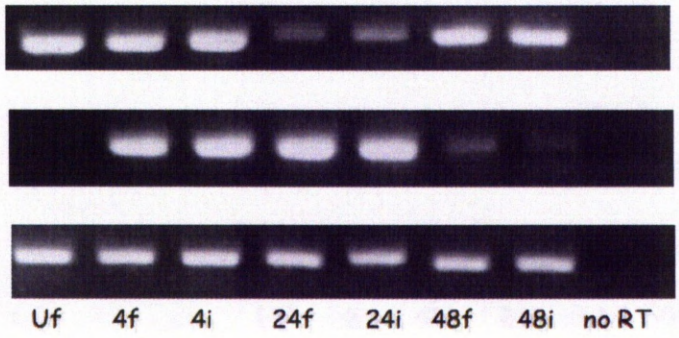
What could be driving such a reduction in catalase? It has been demonstrated that *Leishmania* are able to modify the enzymatic environment inside the sand fly midgut during digestion. Experiments as early as the mid 1980s showed that *L. major* and *L. donovani* reduced the proteolytic effect of midgut homogenates in bloodfed *Ph. papatasi* (Schlein and Romano 1986; Borovsky and Schlein 1987). Specifically, alkaline protease, trypsin and aminopeptidase activity was found to be suppressed by *L. major* at the peak of digestion (30 h PBF), not only in *Ph. papatasi* but also in *Ph. langeroni* (Dillon and Lane 1993b). Digestive enzyme suppression confers protection to *Leishmania* during blood-digestion and has been found to play a major role in vector competence (Schlein and Jacobson 1998; Volf *et al.* 2001). Although the exact mechanism is still elusive, evidence so far suggests that protease suppression happens even before translation occurs. The presence of *Leishmania* modified the abundance of a transcript coding for a chymotrypsin molecule in the midgut of *Ph. papatasi* and *Lu. longipalpis* ((Ramalho-Ortigão *et al.* 2007; Jochim *et al.* 2008) and supplementary Fig 3.1, Appendix 1). Suppression of PperTryp3, the major bloodmeal-induced trypsin

occurred in the presence of *L. infantum*-infected midguts of *Ph. perniciosus* (Dostalova *et al.* 2011).

Trypsin activity in *Lu. longipalpis* peaks at 48 h PBF (Sant'Anna *et al.* 2009), which means that major proteolytic events are expected to occur at that time point inside the midgut. Massive release of blood cell contents is expected. It is possible that *Leishmania*-mediated suppression of digestive enzymes in *Lu. longipalpis* might have an impact in blood-derived ROS release and therefore, a decrease in expression of ROS-detox enzymes would be expected as postulated in Figure 3.9. It has already been shown that *Leishmania* is susceptible to hydrolytic enzymes inside the midgut, this susceptibility is stage-specific and highlights the importance of digestion dynamics and successful *Leishmania* colonisation (Pimenta *et al.* 1997) . Regulation of digestion in sand flies by *Leishmania* might not just protect the protozoans from enzymatic breakdown, but also from oxidative stress caused by release of ROS from the bloodmeal. The importance of catalase and midgut ROS balance will be approached in the following chapters.

Appendix 1

A



B

Array fold change	8h	24h	48h	72h	144h
Early	-4	-8	-2	-1.7	
Late	6	8	8	2.1	1.4

Effect of feeding

	8h	24h	48h	72h	144h
Early	2.1		3	1.2	
Late	-4		-2.6		

Effect of infection

Supplementary Figure 3.1: Effect of blood-feeding and *L. mexicana* infection in expression of early and late trypsins in female *Lu. longipalpis*. A. Gel picture from RT-PCR in bloodfed (f) and uninfected (u) whole female *Lu. longipalpis* at 4 24 and 48 h after blood-feeding/infection. Uf represents sugarfed negative control. B. Analysis of microarray data for early and late trypsin expression fold-change in *L. mexicana* infected and bloodfed sand flies. Early trypsin is downregulated after bloodfeeding and upregulated after infection. Late trypsin is upregulated after bloodfeeding and downregulated after infection. Data from Lewis and Dillon (unpublished).

Chapter 4

The effect of ROS-scavenging by catalase on fecundity and mortality of female *Lutzomyia longipalpis*

4.1. Introduction

Phlebotomine sand flies in the wilderness have a lifespan barely longer than a week (Dye *et al.* 1987; Ferro *et al.* 1995; Killick-Kendrick and Killick-Kendrick 1999; Schlein and Jacobson 1999). In those 8 to 10 days, sand flies have to find a sugar meal, a suitable mammalian source of blood to feed on and ultimately lay eggs; these tasks will employ most of the adult life and many flies will die soon after egg laying (Volf and Volfova 2011). A few, unknown, number of individuals, however, will live long enough to feed again on blood. *Leishmania* requires this second bloodfeed to be successfully transmitted to a mammalian host; therefore even a small reduction in sand fly life expectancy will have a significant impact in vectorial capacity. Better understanding of the biological factors involved in sand fly mortality and fecundity will provide new information towards reduction of *Leishmania* transmission.

Ageing or senescence can be defined as a time-dependant reduction in fitness and performance and is a common feature of all multicellular species (Ricklefs 1998; Hughes and Reynolds 2005). Theories of ageing have focused on different mechanisms such as cellular signalling, dietary control of life span extension and oxidative stress resistance (Hughes and Reynolds 2005). It has been over 50 years since Harman first discussed his theory of ageing where he postulated that accumulation of free radicals derived from cell metabolism would lead to an increment in oxidative stress in macromolecules with a concomitant reduction in longevity (Harman 1956; Harman 2009). Since oxidative stress can also be caused

by non-free radicals such as peroxides and aldehydes, this model is currently known as the oxidative stress theory of ageing.

The fruit fly *Drosophila melanogaster* is the multicellular model *par excellence* for studying the role of ROS in longevity using a variety of approaches. For example, its genetic plasticity has been exploited to study the oxidative stress theory of ageing by modifying the expression of genes encoding for antioxidant enzymes (Aigaki *et al.* 2002; Muller *et al.* 2007). The majority of the work has focused on superoxide dismutase (SOD), which protects the cell from oxidative stress by transforming highly reactive superoxide anion (O_2 •) into hydrogen peroxide (H_2O_2). The first studies demonstrated that homozygote fruit flies for a mutant strain carrying a SOD-null mutation survived until adult stages but caused infertility and a reduced life-span (Phillips *et al.* 1989). Subsequent research focused on the overexpression of *Sod1* using a constitutive promoter. Interestingly, overexpression of this gene led to an increase in average lifespan in one study (Reveillaud *et al.* 1991) but had the opposite effect in another (Seto *et al.* 1990).

Drosophila has also been used to study the important antioxidant enzyme catalase, which is the most efficient H_2O_2 detoxifying enzyme in nature (Zamocky *et al.* 2008). Studies performed in catalase-null mutants showed that an 86% reduction in catalase activity did not have an effect on either lifespan or metabolic potential (Orr *et al.* 1992), whereas *Drosophila* overexpressing *Catalase* exhibited high levels of activity and tolerance against H_2O_2 but had no significant effect on lifespan (Orr and Sohal 1992). Overexpression of both *Sod1* and *Catalase* prolonged the metabolic life of flies (Sohal *et al.* 1995). Evidence so far shows that overexpression of antioxidant enzymes might not increase lifespan, but a depletion or reduction certainly diminishes survival.

Interestingly, oral delivery of antioxidant compounds to *Drosophila* has shown contrasting effects on survival, even when the same antioxidant was administered to different experimental flies (Table 4.1) (Le Bourg 2001). Although

Le Bourg *et al.* discussed that that such variation could be explained by differences in lots and strains of fruit flies, it is also true that these studies were performed before the discovery of the role of antioxidant balance in regulating microbes in the gut of *Drosophila* (Ha *et al.* 2005a). It is possible that oral administration of the same antioxidants had an effect in gut microbiota which in turn could have a positive or negative impact in *Drosophila* mortality.

Table 4.1
Contrasting effect on longevity of male *D. melanogaster* with antioxidants added in food

Antioxidant	Percent change
Vitamin E	13.7*
Dinitrophenol	12.3*
Thiocentrophenoxine	14.7*
Ascorbic acid	1.7*
Propyl gallate	34.2*
N-Acetylcysteine	-1.9
N-Acetylcysteine	26.6*
Xanthine	1.8*
Vitamin A palmitate	17.0*
Ascorbyl palmitate	-3.2

The compound was given during both the developmental (from egg to pupae) and the adult stages or during the adult stage only. Asterisk denotes significant positive effects in longevity, negative values denote the opposite. When different doses have been used, the best result is given. Note that the same antioxidant may appear twice in the table, with contrasting results. Adapted from Le Bourg (2001).

Data derived from *Drosophila* has been successfully used to design studies on physiology of longevity in other insect species. Enzymes like catalase and SOD exhibit amino acid sequences that are well conserved throughout different insect taxa, including blood-feeding insects (Figs 3.3.1 and 3.4.1, Chapter 3). In contrast to fruit feeding flies, haematophagous arthropods not only have to detoxify ROS produced by cellular respiration, but also ROS released during digestion of the blood meal (Graca-Souza *et al.* 2006). Blood-derived ROS are also deleterious for the insect and antioxidant enzymes seem play a role in detoxifying blood-derived

ROS. RNAi-mediated silencing of catalase increased mortality of *Anopheles gambiae* after a bloodmeal (Magalhaes *et al.* 2008). Also, the number of oviposited eggs decreased after a bloodmeal (DeJong *et al.* 2007). In the same study, it was shown that catalase accumulated in the developing eggs and it was speculated that such accumulation could be involved in oocyte protection against potential H_2O_2 damage. Moreover, RNAi-mediated silencing of catalase significantly reduced fecundity. Interestingly, although oral administration of an antioxidant restored fecundity of old flies, it produced a high mortality in an experimental mosquito strain that possessed high systemic levels of H_2O_2 (DeJong *et al.* 2007).

To analyse the biological role of ROS-scavenging in fecundity and survival of the sand fly *Lu. longipalpis*, gene expression of the antioxidant enzyme catalase was analysed in different age groups of female sand flies. Also, changes in catalase activity and expression in the developing oocyte were assessed. ROS-scavenging via gene silencing of the endogenous antioxidant enzyme catalase and its effect on fecundity were analysed, as well as dietary supplementation of the exogenous antioxidant ascorbic acid. Mortality of antioxidant supplemented flies was explored.

4.2. Results

4.2.1. Age-related decrease of fecundity

To evaluate the effect of ageing on fecundity, females of different age groups were blood fed and dissected to examine potential differences in developing oocyte numbers. Female *Lu. longipalpis* from the older age group showed a decrease in the number of developing oocytes dissected five days after blood feeding in comparison to younger sand flies (Fig 4.1). Female *Lu. longipalpis* that were bloodfed 3 and 6 days post-emergence (PE) showed no significant difference in number of oocytes. However, sand flies bloodfed at 9 days PE showed a significant decrease in number of oocytes after dissecting 5 days after blood feeding (Fig 4.1; $p < 0.005$, ANOVA).

4.2.2. ROS-scavenging reverses age related loss of fecundity

To evaluate the role of ROS scavenging in age-related decrease of fecundity, 9 day old female *Lu. longipalpis* were fed a sucrose meal supplemented with a ROS-scavenger upon emergence until end of the experiment. In similar experiments previously performed by Molina Cruz *et al.* (2008) in mosquitoes, ascorbic acid was used as ROS-scavenger and it was decided to follow the same direction in the sand fly model. In a preliminary experiment, the concentration of ascorbic acid fed to *Anopheles* (0.14 M) proved to be inadequate for *Lutzomyia* (*i.e.*, flies did not feed). Sand flies were offered lower concentrations of 100, 50, 20, 10 and 5 mM ascorbic acid in 70% sucrose to evaluate feeding and mortality and 20 mM ascorbic acid was the highest concentration with the lowest effect on mortality (data not shown). Sand flies were offered a 70% sucrose solution supplemented with 20 mM ascorbic acid and subsequently blood-fed on day 9 PE. The number of developing oocytes dissected 5 days after blood feeding was significantly higher (Fig 4.2; $p < 0.0001$, t-test) in sand flies that received a sugar meal supplemented with 20 mM ascorbic acid in comparison to control sand flies fed on 70% sucrose solution. This suggests that exogenous ROS-scavenging can reverse age-related loss of fecundity in sand flies blood fed 9 days PE.

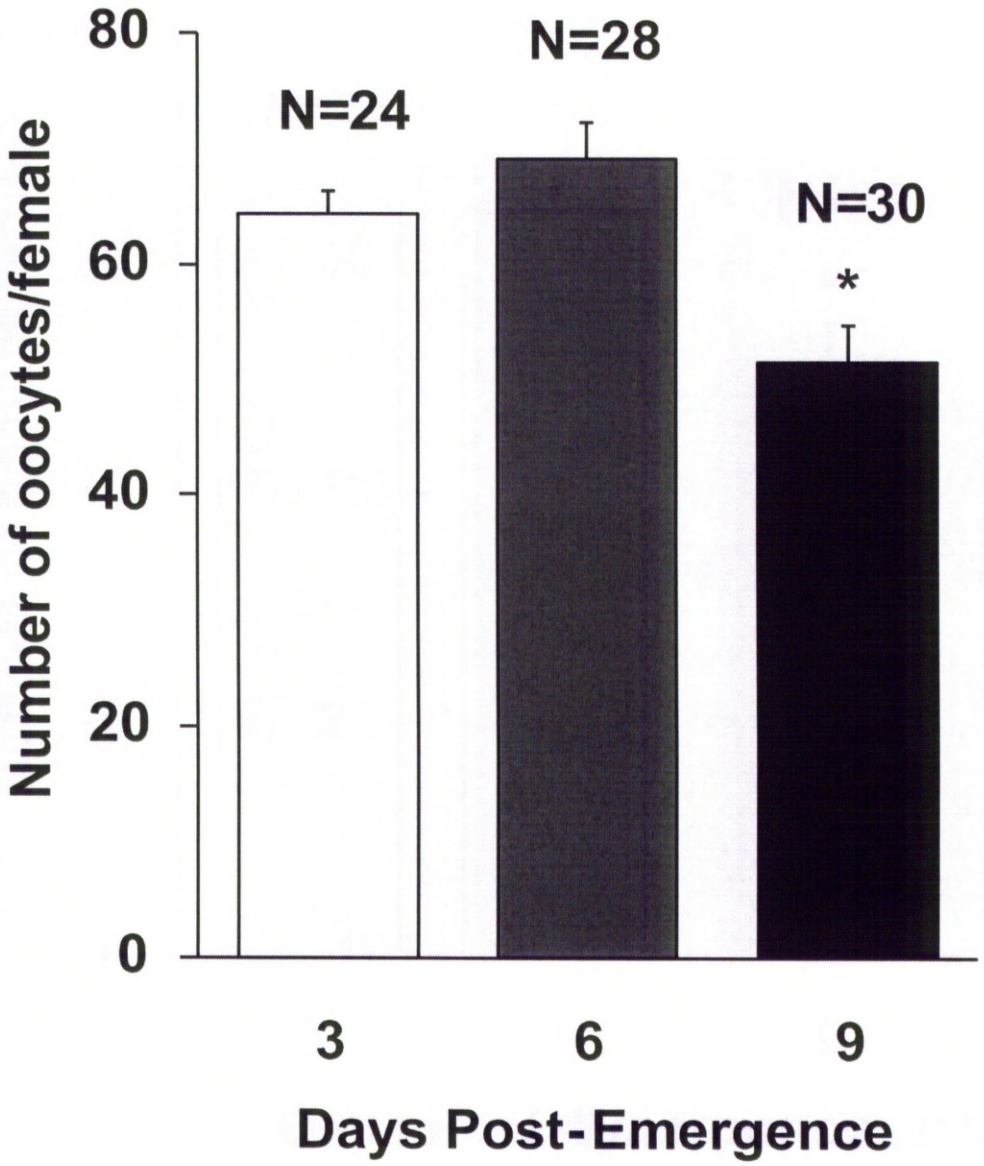


Figure 4.1: Effect of age at blood feed on subsequent fecundity of female *Lu. longipalpis*. Bars represents average number of oocytes dissected 5 days after blood meal ± SEM Sand flies were blood-fed at 3, 6 and 9 days Post-Emergence. Asterisk indicates statistical difference at $p<0.005$ (ANOVA). Results represent two independent biological replicates.

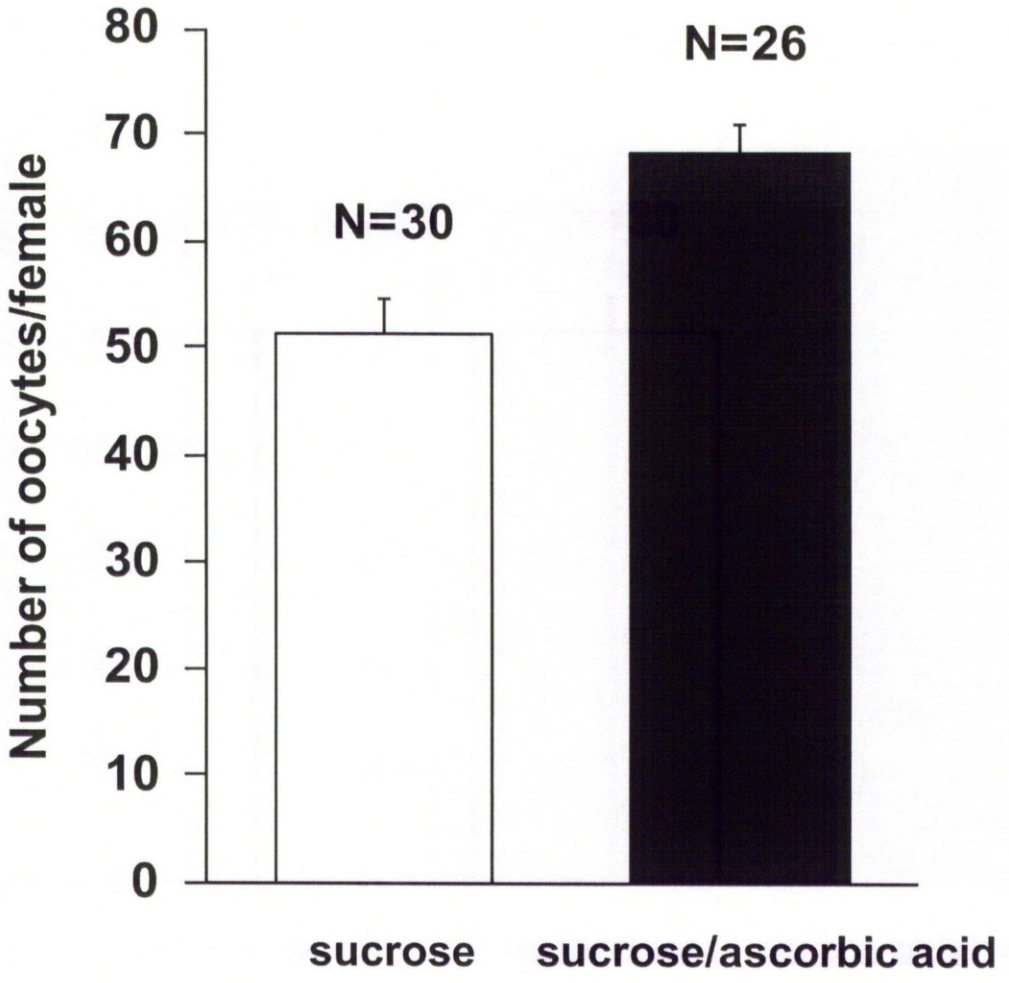


Figure 4.2: Effect of ascorbic acid supplementation on fecundity in *Lu. longipalpis*. Flies were blood-fed 9 days Post-Emergence and bar chart represents average number of oocytes dissected 5 days after blood meal ± SEM (combined samples derived from 2 independent experiments). Sand flies fed on 20 mM ascorbic acid-supplemented 70% sucrose solution show significantly higher oocyte numbers in comparison to control sand flies (p <0.0001 , t-test).

4.2.3. Catalase activity is reduced in developing oocytes of older flies and ROS scavengers reverse catalase depletion

Flies were assayed at 24h and 48h to find out if catalase accumulated in developing oocytes. Ovaries of *Lu. longipalpis* dissected 6 days PE contained higher catalase enzymatic activity at 48h compared to 24h after blood feeding (Fig 4.3A; $p<0.0001$, t-test). Moreover, mRNA expression of catalase increased with oocyte development from 12 to 48 hours after blood feeding (Fig 4.3B).

To further understand the role of endogenous ROS-scavenging and ageing, flies from different age groups were assayed for catalase LlonKat1 expression. Flies from different age groups (3, 6 and 9 days PE) showed a decrease in expression in ovaries dissected at 48 hours after blood feeding (Fig 4.3C; $p<0.001$, ANOVA). Interestingly, when 9 day old sand flies were fed with a 20 mM ascorbic acid supplemented sugar meal, catalase LlonKat1 mRNA expression was significantly higher compared to flies of the same age fed on sucrose only (Fig 4.3C; $p<0.002$, t-test). The results show that a) catalase accumulates in the developing oocyte as shown by increase in enzymatic activity and relative expression, b) catalase expression is age-dependant and is lower in older flies and c) the dietary supplementation with an exogenous ROS-scavenger increases catalase expression in older flies.

Lutzomyia longipalpis catalase sequence was already described (Dillon *et al.* 2006; Jochim *et al.* 2008) and was retrieved from the GenBank (ABV60342.1). It codes for a protein (named LlonKat1 in this chapter) with molecular mass of 57682 Da and isoelectric point of 8.28, without a signal peptide and mitochondrial or peroxisomal targeting sequences of types 1 and 2. LlonKat1 has high identity (ranging from 46 –73%) to catalase sequences from other insects, crustaceans, yeast and mammals and lower identity to the bacterial catalase from *Pseudomonas syringae* (Fig 4.4.1). LlonKat1 sequence contains the conserved residues His73 and Asn147 (catalytic), Ser113, Val115, Phe152, Phe160, Leu298, Met349, Arg353, Tyr357 (haem binding/coordination) and His193, Arg202, Ile301, Gln304 (putative NADPH binding pocket) (Fig 4.4.1).

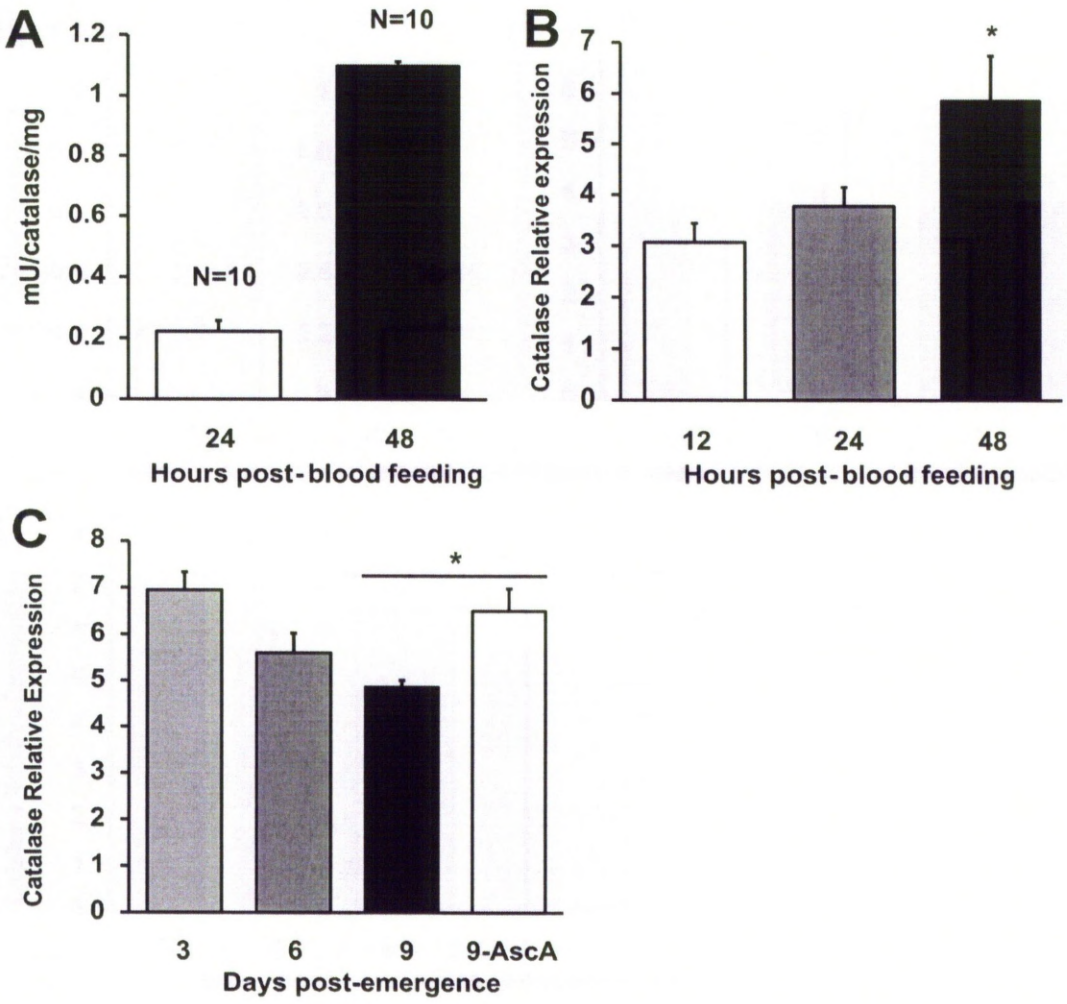


Figure 4.3: Changes in catalase in the developing oocytes of *Lu. longipalpis*. (A) Catalase activity of developing oocytes after blood feeding. Six day old female *Lu. longipalpis* were blood-fed and dissected at 24 and 48 hours. Enzymatic activity in the developing oocytes was significantly higher at 48 hours compared to 24 hours after blood feeding (p <0.0001, t-test). Bar charts represent mean ± SEM of combined samples from 2 independent experiments. (B) Relative expression of catalase LlongKat1 mRNA in developing oocytes dissected at 12, 24 and 48 hours from 6 days-old blood-fed female *Lu longipalpis*, (n=three groups of 20 females each). Asterisk indicates statistical difference at p<0.05 (ANOVA) Bar charts represent mean ± SEM of combined samples from 2 independent experiments. (C) Age-related decrease of catalase mRNA relative expression in developing oocyte. Flies were blood-fed at 3, 6 and 9 days Post-Emergence (n=three groups of 15 females each) and whole ovaries were dissected 48 hours after blood feeding. Relative expression was statistically different in all 3, 6 and 9 days old flies (p<0.001, ANOVA). A 4th group (n=15 females) fed on an ascorbic acid-supplemented sugar solution upon emergence (9-AscA) showed catalase relative expression levels similar to groups of younger flies fed on 70% sucrose solution, and statistically higher than the non-treated, 9 DPE group (p <0.002, t-test). Bar charts represent mean ± SEM of combined samples from 2 independent experiments.

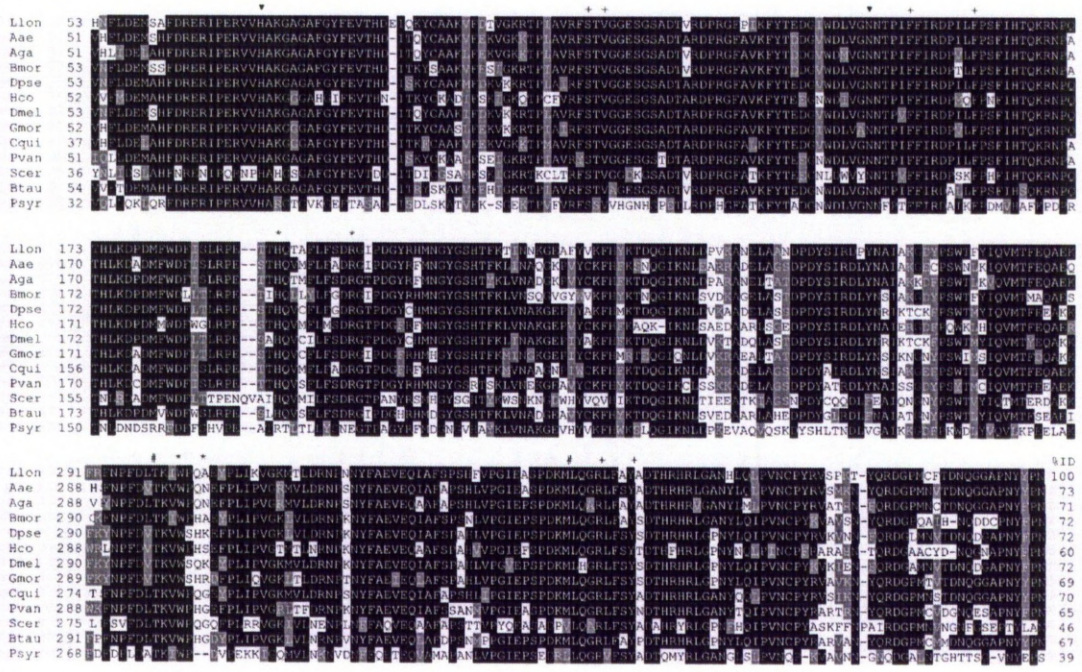


Figure 4.4.1: Amino acid sequence alignment of selected catalases. Sequences were retrieved from GenBank (GB), Protein Data Bank (PDB) or from Peroxibase (PB). The listed proteins are respectively from *Lutzomyia longipalpis* (GB:ABV60342.1), *Aedes aegypti* (PB:5267), *Anopheles gambiae* (PB:5269), *Bombyx mori* (PB:5266), *Drosophila pseudoobscura* (PB:5273), *Haemonchus contortus* (PB:5270), *D. melanogaster* (GB:NP_536731.1), *Glossina morsitans morsitans* (GB:ADD20421.1), *Culex quinquefasciatus* (GB:XP_001848573.1), *Penaeus vannamei* (PB:5278), *Saccharomyces cerevisiae* (PDB:1A4E), *Bos taurus* (PDB:8CAT), *Pseudomonas syringae* (PDB:1M7S). Conserved residues in catalases are with black background, consensus alternatives are shaded. The symbols ▼, +, and * mark catalytic, haem binding and NADPH binding residues, respectively. The symbol # mark residues that define haem orientation. All sequences are from clade 3 of monofunctional catalases, with the exception of Psyr, which is a clade 2 enzyme. In catalases from clade 2 (Psyr numbering), haem orientation (His-IV) is defined by residues 301 (never Leu) and 350 (frequently Leu). In catalases from clade 3, these positions are commonly occupied by Leu and non-Leucine residues, respectively. NADPH binding catalases have the signature (Btau numbering) His 193, Arg 202, Val 301 and His 304, which is not present in catalases from clades 1 (not shown) and 2 (Psyr). Insect catalases share some of the NADPH binding residues, but not all. However, catalytic residues and haem binding residues are fully conserved in all sequences.

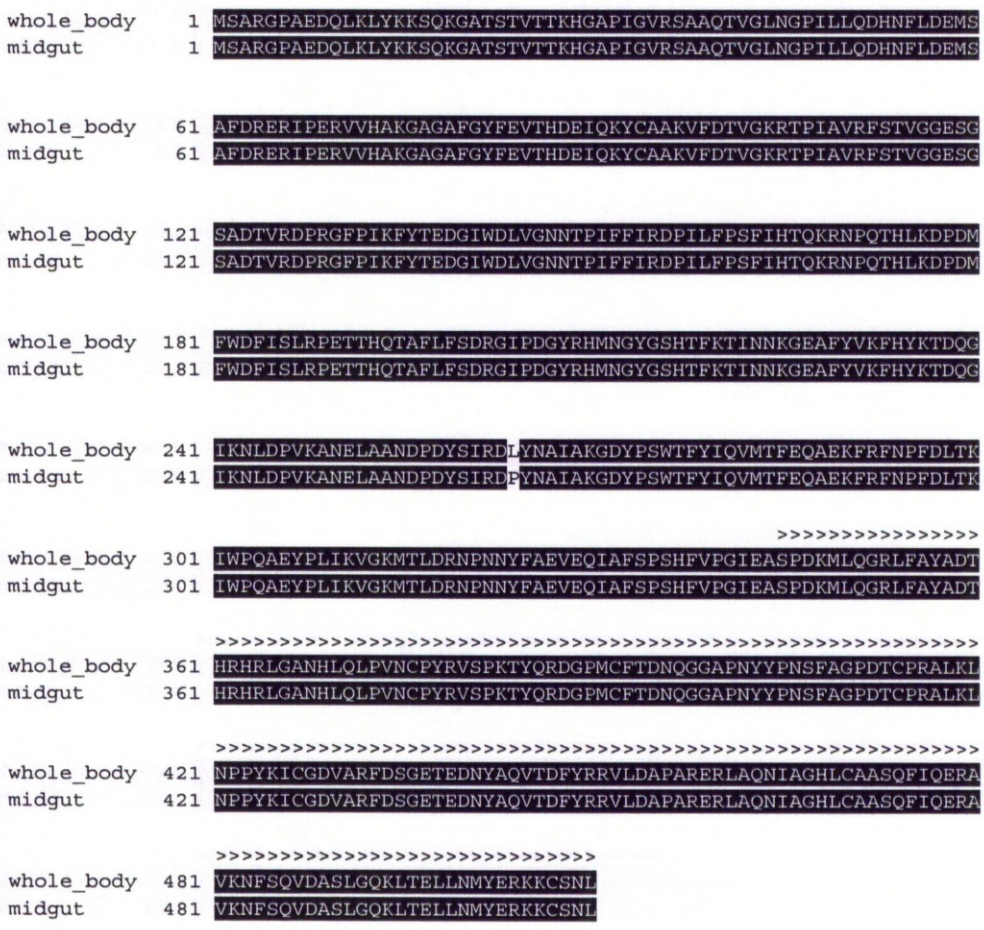


Figure 4.4.2: Alignment of the aminoacid sequence of *Lutzomyia longipalpis* catalase, translated from a whole body (GenBank AM105518)(Dillon *et al.* 2006)) and a midgut-specific (GenBank EU124624.1) cDNA library. Sequences show a 99% identity and a 99% similarity, '>>>>' represents the targeted region for dsRNA-mediated gene silencing.

4.2.4. Catalase gene RNAi mediated depletion leads to a decrease in sand fly fecundity

The gene sequence of *Lu. longipalpis* catalase was obtained from a cDNA library constructed from sand fly whole bodies (GenBank AM105518(Dillon *et al.* 2006)) and aligned with a previous described catalase obtained from *Lu. longipalpis* midguts (GenBank Accession number: EU124624) , showing a high level of identity (99%) and similarity (99%) (Fig 4.4.2). To confirm the role of endogenous ROS-scavenging in fecundity catalase was depleted using RNAi. Flies injected with 144 ng of dsRNA for catalase (dsCAT) showed a dramatic decrease in oocyte number dissected 48 hours after blood feeding (Fig 4.5A; $p<0.005$, ANOVA) compared to sand flies injected with a non-related dsRNA (dsGFP) and uninjected sand flies. A change in appearance of ovaries was observed during dissections with matured ovaries. In sand flies injected with dsRNA for catalase they appeared underdeveloped in comparison with both mock-injected and uninjected controls (Fig 4.5B). A dsRNA-mediated significant reduction in catalase expression in whole flies was observed by RT-PCR (Fig 4.5C). These results confirm that endogenous ROS-scavenging in developing oocytes plays a major role in female *Lu. longipalpis* fecundity.

4.2.5. Effect of ROS-scavenging in the survival of sand flies

To evaluate the role of exogenous ROS scavenging in survival, female *Lu. longipalpis* were fed with an antioxidant-supplemented sugar meal upon emergence. Mortality was recorded from day 1 PE up to day 7 PE. Survival curves depict an increase in mortality due to exogenous ROS-scavenging by an exogenous antioxidant (Fig 4.6A). In order to assess whether the higher mortality rate was related to an effect on sand fly immune homeostasis, phenoloxidase (PO) activity was measured in control and antioxidant-supplemented females. Spontaneous PO is defined as the activity measured upon reaction with 3,4 dihydroxy-DL-phenylalanine (DOPA), and corresponds to the enzyme that is already activated in physiological conditions and total activity was the activity observed after *in vitro* activation of the enzyme, by pre-incubating the sample with bovine trypsin. Sand flies fed on ascorbic acid-supplemented sucrose

showed a significant increase in spontaneous PO (Fig 4.6B; $p<0.05$, t-test) but no difference in total PO activity. To further investigate if ROS-scavenging was implicated in increased mortality, catalase LlonKat1 was depleted via RNAi injection in female *Lu. longipalpis* and mortality was recorded from day 1 PE up to day 7 PE. Mortality rates were higher in knocked down (dsCAT) sand flies (Fig 4.7), compared to flies injected with a non-related dsRNA (dsGFP) and non-injected flies. These results show that ROS-scavenging by either endogenous or exogenous antioxidants play an important role in female *Lu. longipalpis* survival.

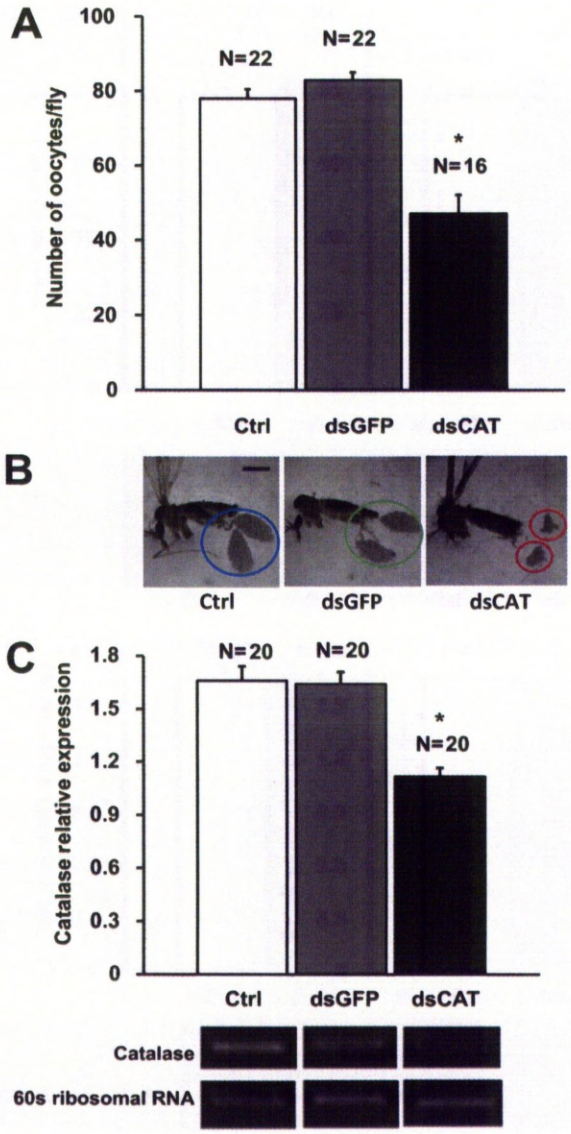


Figure 4.5: RNAi-mediated depletion of catalase LlonKat1 in female *Lu. Longipalpis* and its effect on fecundity. (A) Average number of developing oocytes dissected 48 hours days after blood meal ± SEM of combined samples from at least 2 independent experiments. (B) Relative development of female *Lu. longipalpis* ovaries observed upon catalase gene knockdown by RNAi, in comparison to mock-injected and uninjected control sand flies. Bar=1mm. (C) Relative expression of catalase LlongKat1 mRNA in whole fly homogenates from dsRNA-injected catalase knock-down sand flies. Bar charts represent mean ± SEM of combined samples from at least 2 independent experiments. Asterisk indicates statistical difference at $p<0.05$ (ANOVA).

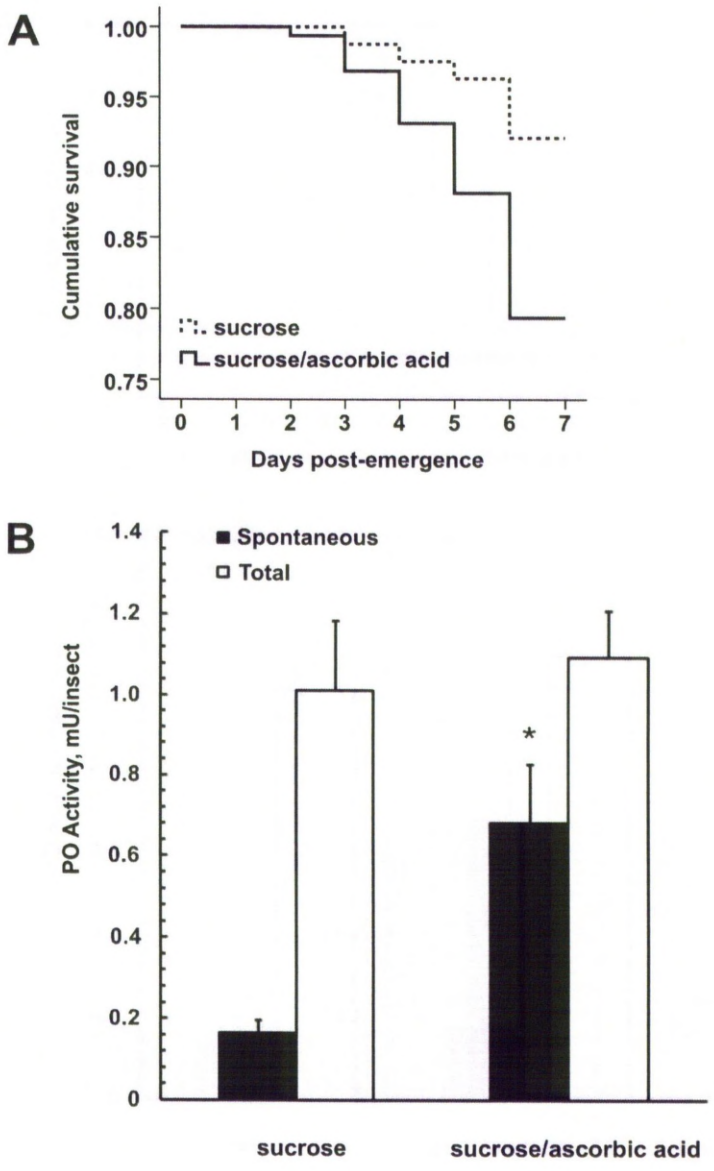


Figure 4.6: Effect of dietary supplementation of ascorbic acid on mortality of sugar fed *Lu. longipalpis*. (A) Female sand flies were offered a 70 % sucrose solution supplemented with 20 mM ascorbic acid or a non-supplemented sucrose solution. Experimental flies (sucrose + 20 mM ascorbic acid) exhibited a significantly lower survival rate compared to control flies, ($p< 0.001$, Kaplan-Meier, Log Rank χ^2 test). **(B)** Spontaneous and total phenoloxidase (PO) activity in *Lu. longipalpis* females after 7 days of feeding with 70% sucrose solution or 70% sucrose solution supplemented with 20mM ascorbic acid. Spontaneous PO activity in ascorbic acid supplemented flies was significantly higher than control flies ($p <0.05$, t-test). Results are mean ± SEM from 3 independent experiments with 10 sand flies per experiment.

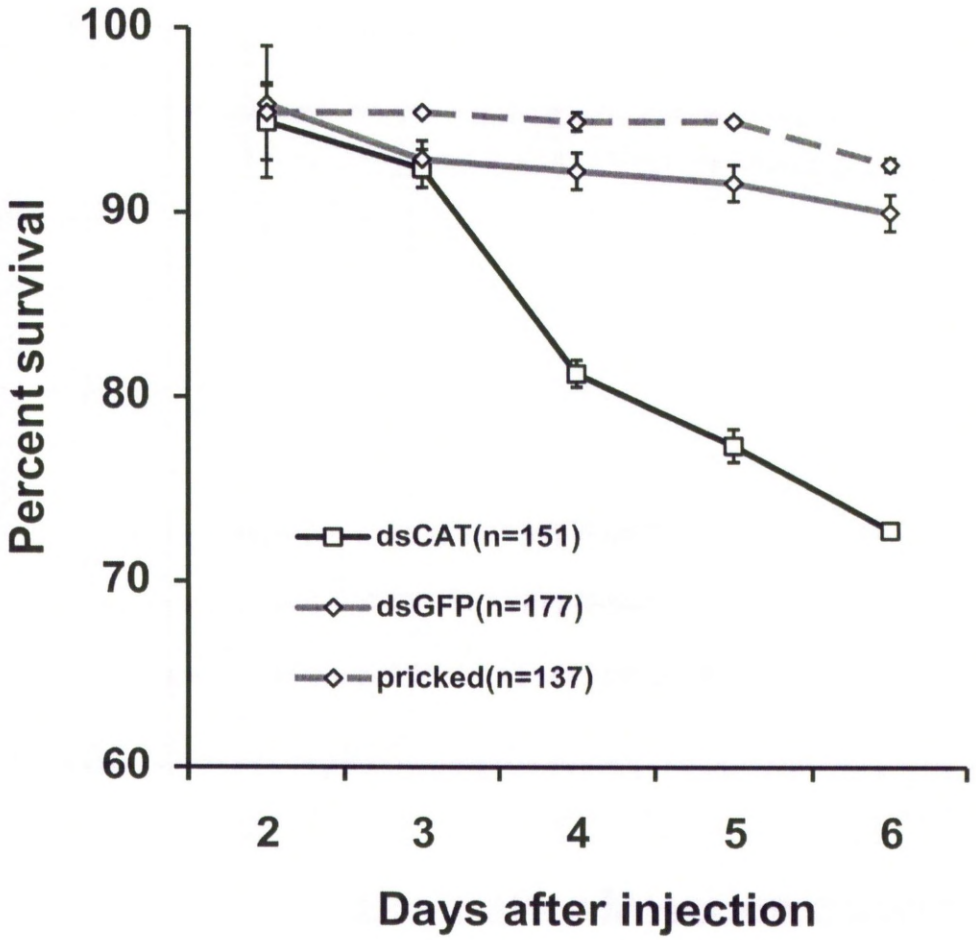


Figure 4.7: Survival in female *Lu. Longipalpis* after RNAi-mediated depletion of catalase LlonKat1 . Experimental group (dsCAT) exhibits a significantly lower survival rate compared to both dsGFP and pricked control groups, (p<0.0001, Kaplan-Meier, Log Rank χ^2 test). Results represent mean ± SEM of 3 independent biological replicates.

4.3. Discussion

The present results suggest that catalase-mediated ROS scavenging has a significant impact on female *Lu. longipalpis* fecundity and survival. Female *Lu. longipalpis* from different age groups showed differences in developing oocytes numbers, with the oldest (9 days PE) presenting the lowest number of oocytes (Fig 4.1). The age-related loss of fecundity could be reversed with dietary supplementation of a potent exogenous ROS-scavenger (Fig 4.2). This underlines the importance of catalase in the reproductive success of blood sucking phlebotomines. Evidence from other dipterans show that aging results in an increase of oxidative stress and loss of enzymatic antioxidant efficiency (Sohal *et al.* 1990; Yan and Sohal 2000; Das *et al.* 2001). Moreover, inactivation or silencing of catalase in *Drosophila melanogaster* (Mackay and Bewley 1989), *Musca domestic*a (Allen *et al.* 1983), *Rhodnius prolixus* (Paes *et al.* 2001)and *Anopheles gambiae* (Magalhaes *et al.* 2008) led to increased mortality due to increase in ROS levels. It is likely that accumulation of ROS in older flies could account for the decrease of female sand fly fecundity due to an increase in oxidative stress, loss of antioxidant enzymatic efficiency or both. In *An gambiae*, fecundity of female mosquitoes declined with age, with reduction of number of eggs oviposited and number of larvae hatched per female (DeJong *et al.* 2007). We did not measure differences in fecundity in terms of larval development but it is likely that the age-related differences in fertility would have resulted in less viable larvae being produced from older flies, as they would be presumably exposed for a longer periods to oxidative damage.

Catalase enzymatic activity as well as catalase LlonKat1 mRNA relative expression increased in the ovaries of older female sand flies (6 days PE) after the blood feeding (Fig 4.3A and B). Protein expression and accumulation increased upon blood feeding in maturing ovaries of mosquitoes due to nutrient allocation for egg production (Wheeler 1996; Ahmed *et al.* 2002). It has been shown in different insect species that antioxidant activity increases in the ovaries to

protect the embryo from oxidative damage (Logullo *et al.* 2002; Freitas *et al.* 2007). It is conceivable that such accumulation of catalase in sand fly ovaries also provides the means to protect developing eggs from oxidative damage. Additional support for this hypothesis was given by the dramatic decrease in developing oocyte numbers upon successful catalase gene depletion by RNAi in female sand flies (Fig 4.5A and B).

Interestingly, oral delivery of ascorbic acid seemed to stimulate catalase LlonKat1 mRNA expression in older flies to levels similar to that of younger flies (Fig 4.3C). It has been shown that age-related accumulation of ROS/oxidative stress leads to loss of efficiency in cellular processes (Sohal *et al.* 1990; Sohal *et al.* 1993; Yan and Sohal 2000; Das *et al.* 2001; Ferguson *et al.* 2005), therefore it is possible that ROS-scavenging by an exogenous antioxidant slowed or lowered such deleterious effects in either catalase LlonKcat1 mRNA or in other molecules involved in its upregulation. On the other hand, it has been shown that (Orr 1967a) ascorbate is a potent inhibitor of catalase, the inhibition being independent of substrate concentration and pH and strongly influenced by temperature. Furthermore, catalase incubation with ascorbate leads to degradative changes to the catalase molecule (Orr 1967b). In our experiments, the increase in catalase gene expression might reflect a compensation response to replenish normal catalase levels in the sand fly body after catalase was degraded, by an unknown mechanism, during ascorbic acid supplementation with the sugar meal.

Catalase LlonKcat1 does not have a signal peptide or targeting sequences to mitochondria or peroxisomes. These features suggest a cytosolic location but this needs confirmation. Based on the identities with other catalases retrieved from Peroxibase (Koua *et al.* 2008), LlonKat1 seems to belong to the monofunctional clade 3 of catalases, which includes sequences from bacteria, archaebacteria, protists, fungi, plants and animals. These enzymes have small subunits with molecular mass ranging from 43-75 kDa (Zamocky *et al.* 2008), which is consistent with LlonKat1 monomer predicted molecular mass (57.7kDa). All conserved catalytic and haem binding residues are present in LlonKat1 sequence, suggesting

a full catalytic activity, and the presence of residues Leu298, Met349 indicate that His70 is above the ring III of the haem molecule (His-III orientation), as seen in other clade 3 catalases (Chelikani *et al.* 2004).

ROS-scavenging by dietary supplementation of ascorbic acid (Fig 4.6A) led to a reduction in sand fly survival. When antioxidants were provided to a susceptible strain of *Anopheles gambiae* to *Plasmodium* infection, a similar but more drastic effect was observed with female mosquitoes (DeJong *et al.* 2007). Magwere *et al.* (Magwere *et al.* 2006) observed that antioxidant supplementation did not extend the lifespan of wild type *Drosophila*. Similarly, Bayne *et al.* (Bayne *et al.* 2005) showed that overexpression of MnSOD and catalase, despite protecting *Drosophila* from oxidative stress, were detrimental for lifespan and physical fitness of the insects. Kang *et al.* (Kang *et al.* 2008) observed a reduction in the lifespan of *Anopheles stephensi* when the mosquitoes were bloodfed with the antioxidant MnTBAP in comparison with the buffer control. It has been hypothesized that a minimal level of ROS might be required to maintain the balance of the gut microbiota and that a baseline level of ROS activity might be crucial for basic midgut physiology. Previous studies done with other dipteran species had showed that ROS release constitutes a first line of defence against pathogens in the midgut (Hoffmann 2003) . Experiments in *D. melanogaster* have demonstrated the existence of a midgut-specific active ROS releasing system against orally delivered bacteria (Ha *et al.* 2005a). In the present study, higher activities of spontaneous PO were recorded and this might be due to an increase in microbial infection associated with sand flies that fed on an ascorbic acid-supplemented sugar meal. In insects, PO activation is often related to bacterial or fungal infections (Pye 1974; Ratcliffe *et al.* 1984; Leonard *et al.* 1985; Cerenius *et al.* 2008; Kanost and Gorman 2008; Eleftherianos and Revenis 2010). Since only the soluble form of PO was measured, it is more likely that the activity was related to the immune response rather than to the melanisation of the adult cuticle or egg shell. PO has already been described in gut tissues or adhered haemocytes in other dipterans (Gillespie *et al.* 2004). It is possible that mortality in our experimental group fed with sucrose supplemented with ascorbic acid

may be due to a decrease in ROS production inside the midgut and that ROS activity, similar to the events in certain strains of mosquitoes, may play a role in sand fly immunity towards opportunistic microbes or be involved in important cellular signalling pathways (Kamata and Hirata 1999; Morey *et al.* 2003). The putative role of ROS in immunity against bacteria will be further investigated in chapter 4.

There is evidence of other antioxidant enzymes with catalase-like functions found in the sand fly midgut, such as peroxiredoxins (Dillon *et al.* 2006; Jochim *et al.* 2008). These are a family of thioredoxin-dependent peroxidases, found in several insect species (Radyuk *et al.* 2001; Kim *et al.* 2005; Wang *et al.* 2008; Hu *et al.* 2009), that function as ROS-scavengers as well as other cellular processes. However their efficiency in converting H_2O_2 was found to be significantly lower compared to catalase (Wood *et al.* 2003). The role of dietary antioxidants in regulating microbial populations and its effect in sand fly mortality will be approached in chapter 5 of this thesis.

Recent studies on transgenic *Anopheles stephensi* (the leading malaria vector in India and parts of Asia and the Middle East) overexpressing the protein kinase AKT gene increased the insulin signalling in the mosquito midgut, significantly reducing mosquito lifespan and inhibiting *P. falciparum* development (Corby-Harris *et al.* 2010). The role of genes involved in stress responses in *Plasmodium* survival within the mosquito midgut was investigated by Jaramillo-Gutierrez *et al.*(Jaramillo-Gutierrez *et al.* 2011) . RNAi gene knockdown of the OXR1 gene (oxidation resistance gene) in *Anopheles gambiae* showed that this gene regulates the basal levels of catalase and glutathione peroxidase expression and that OXR1 gene knockdown decreased *Plasmodium berghei* oocyst formation. An OXR1 gene homologue for *Lu. longipalpis* was identified after completion of this study (unpublished); investigating this gene would shed further light on the regulation of ROS production within the sand fly gut and will also help to understanding how ROS production impacts on *Leishmania* development in the sand fly midgut.

Current sand fly vector control strategies rely on spraying of residual insecticides to control vector population. Insect transgenesis and paratransgenesis are novel strategies that aim at reducing insect vectorial capacity by using genetic manipulation of disease vectors, rendering them incapable or less efficient to transmit a given pathogen (Coutinho-Abreu and Ramalho-Ortigao 2010) or even reducing the longevity and fecundity of a given insect vector. This chapter confirms that catalase is a key gene in determining survival and fecundity of phlebotomine sand flies and future developments may warrant this gene being included as a potential target to reduce female sand fly fitness and reproductive capacity in the field.

CHAPTER 5

Reactive Oxygen Species-mediated immunity against *Leishmania mexicana* and *Serratia marcescens* in *Lutzomyia longipalpis*

5.1. Introduction

In recent years, evidence from other dipteran species has highlighted the immunity role of reactive oxygen species (ROS) in regulating potential insect pathogens and influencing the profile of the commensal gut microbiota (Ha *et al.* 2005a; Munks *et al.* 2005; Molina-Cruz *et al.* 2008).

ROS are oxygen-derived radical species formed during cell respiration, mainly derived from mitochondrial electron transport. This group includes superoxide anion (O_2 •), the hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2). Although H_2O_2 does not have unpaired electrons, it is usually considered as a ROS since it can be easily transformed into the highly reactive •OH via a Fenton-like reaction (Thannickal and Fanburg 2000; Bonekamp *et al.* 2009). ROS production in excess has deleterious effects in the cell, damaging lipids, proteins and DNA (Freeman and Crapo 1982). Eukaryotic cells are able to regulate ROS levels through the production of antioxidant enzymes. O_2 • is produced by a NADPH oxidase and transformed to H_2O_2 by superoxide dismutase, whereas H_2O_2 is reduced to H_2O by catalase (Thannickal and Fanburg 2000).

ROS are actively produced in the midgut of *Drosophila melanogaster* at a basal level in the presence of a commensal microbiota (Ha *et al.* 2005a; Ha *et al.* 2005b) and highly generated upon allochthonous bacterial oral challenge (Ha *et al.* 2009b). In *Anopheles gambiae*, ROS modulate immunity against bacteria and *Plasmodium* (Kumar *et al.* 2003; Molina-Cruz *et al.* 2008). Studies done with *A. gambiae* showed that *Plasmodium* refractory strains were in a constant oxidative

stress state exacerbated by bloodfeeding and contributed to a higher *Plasmodium* melanisation rate in comparison to strains susceptible to the malaria parasite(Kumar *et al.* 2003). Superoxide anions are secreted into the midgut lumen of the adult *Aedes aegypti* mosquito and bloodmeal ingestion decreased ROS levels via blood haem activated protein kinase C (Oliveira *et al.* 2011). The complexity of the role of ROS in gut microbe homeostasis was further underlined by the suggestion that gut bacterial derived ROS may kill *Plasmodium* in the Anopheline mosquito (Cirimotich *et al.* 2011).

In chapter 4 it was shown that ROS scavenging by means of antioxidant supplementation decreased survival of adult *Lu. longipalpis* and led to activation of the phenoloxidase cascade, which was proposed to be due to bacterial proliferation (Diaz-Albiter *et al.* 2011). The purpose of the work presented in this chapter was to investigate the ROS activities in the gut of *Lu. longipalpis* after feeding *Leishmania mexicana* and *Serratia marcescens* - an insect pathogen also found in wild sand fly populations (Gouveia *et al.* 2008). We analysed the expression of ROS-regulatory enzymes during infection and also manipulated ROS balance in infected flies to analyse its effect on gut- microbe homeostasis. The results suggest that *Leishmania* infections do not elicit ROS production within the *Lu. longipalpis* midgut, whereas *Serratia* infections increase ROS generation inside the sand fly gut.

5.2. Results

5.2.1. ROS-regulatory genes are differentially expressed in the midgut of *Leishmania* and *Serratia*-infected flies

To analyse changes in expression of ROS-regulatory genes in the midgut of *Lu. Longipalpis* the sand flies were infected with either *S. marcescens* or *L. mexicana*. Expression of four ROS-regulatory genes was assessed by RT-PCR. Non-infected blood-fed flies were used as negative control. Catalase (CAT) exhibited the highest variation in expression among all ROS-detoxifying gene sequences analysed. CAT was downregulated in *Leishmania*-infected flies at 1, 24 and 48 h

PBF compared to control group ($p<0.05$, Fig 5.1A). *Serratia*-infected flies exhibited CAT upregulation at 24 and 96 h PBF compared to *Leishmania*-infected flies ($p<0.05$, Fig 5.1A). A putative superoxide dismutase (SOD) was down regulated at 48 h PBF in *Leishmania*-infected flies compared to control group ($p\leq0.016$) (Fig 5.1B). A *Lu. longipalpis* peroxiredoxin (PrxR) displayed a trend of upregulation at 1 and 24 h PBF in non-infected flies relative to the infected groups but such differences were not statistically significant (Fig 5.1C). OXR1, a gene described in *A. gambiae* that regulates the expression of catalase and glutathione peroxidase (Jaramillo-Gutierrez *et al.* 2011) did not exhibit significant changes in expression after *L. mexicana* and *S. marcescens* infections (Fig 5.1D).

5.2.2. *Serratia* and not *Leishmania* induces changes in H_2O_2 concentration in the midgut

Catalase degrades toxic H_2O_2 into water and oxygen. To understand whether these changes in catalase expression have an effect on hydrogen peroxide levels, midgut-specific H_2O_2 concentration was measured in *L. mexicana* and *S. marcescens*-infected *Lu. longipalpis*. Sand flies were infected and midguts were assayed for H_2O_2 at 24, 48, 72 and 94 h PBF. Time zero was considered as H_2O_2 concentration of non-blood-fed flies before infection. Only *Serratia*-infected flies exhibited a significant increase of H_2O_2 concentration at 48 h PBF compared to the *Leishmania*-infected group ($p<0.05$, Fig 5.2). These results show that *L. mexicana* infection does not induce changes in hydrogen peroxide concentration in the midgut of *Lu. longipalpis* from 24 to 96 h PBF, in contrast to *S. marcescens* which induced a significant increase in H_2O_2 concentration at 48 hours after inoculation.

5.2.3. *Leishmania* induces changes in catalase activity in the midgut

To investigate whether a reduction in midgut-specific catalase expression would be reflected in a concomitant decrease in catalase activity, midguts were dissected at different time points and assayed for enzymatic activity. *Leishmania*-colonised flies exhibited a significantly lower midgut-specific catalase activity at 48 h PBF (Fig 5.3, $p<0.05$).

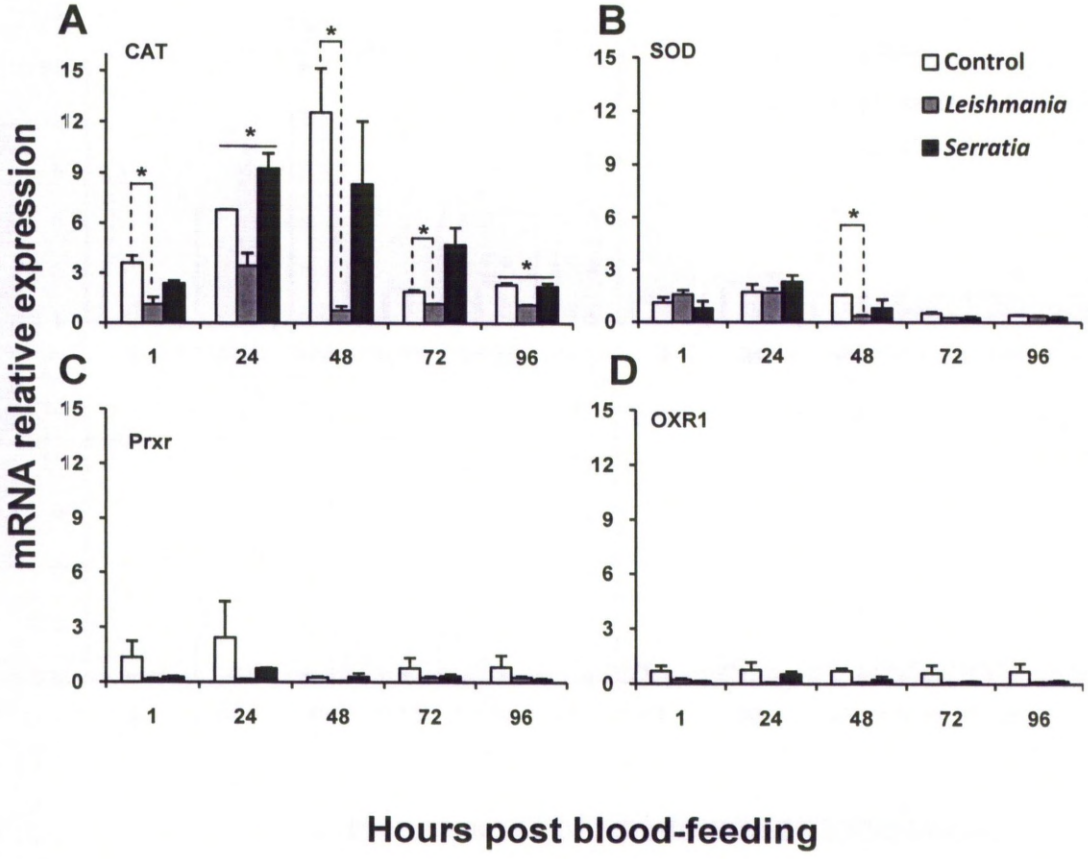


Figure 5.1: Midgut-specific relative expression profile by semiquantitative RT-PCR of ROS-regulatory genes in infected *Lu. longipalpis*. Female flies were fed with either a non-infected bloodmeal (Control) or a *Serratia marcescens*-infected bloodmeal. Midguts were pooled (N=8) and expression of the following genes was assessed: *A* catalase (CAT), *B* Cu/Zn superoxide dismutase (SOD), *C* peroxiredoxin (PrxR), and *D* oxidation resistance protein 1(OXR1). Bar charts represent mean ± SEM of combined samples from at least 2 independent experiments. Asterisk indicates statistical difference at $p<0.05$ between groups under solid line. Charts combine expression data from *Serratia*-infected flies with previous ones from *Leishmania*-colonised midguts (Fig 3.8, chapter 3).

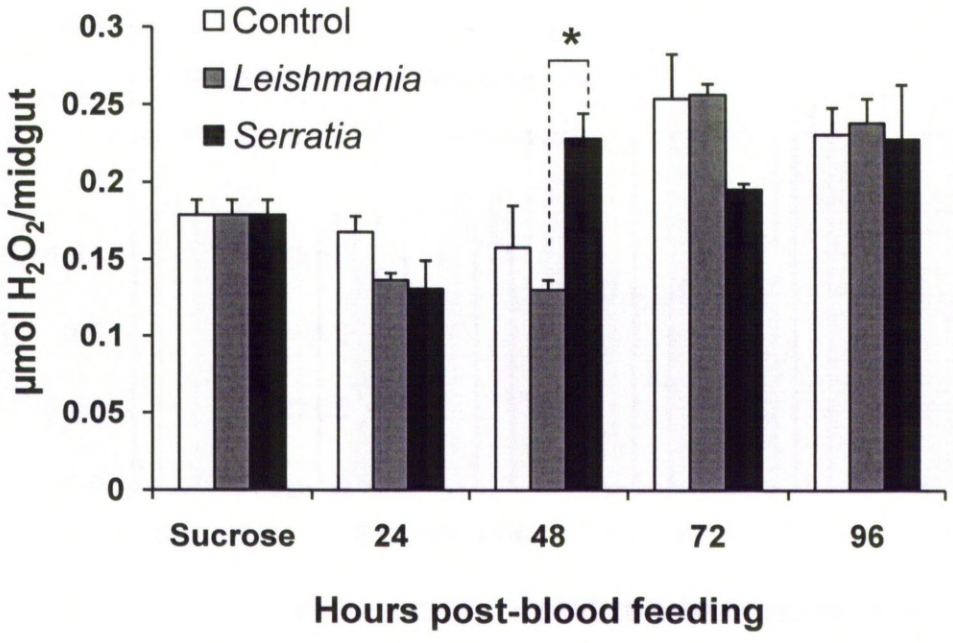


Figure 5.2: Hydrogen peroxide concentration in the midgut of *Leishmania* and *Serratia*-colonised sand flies. Female flies were fed with either a non-infected blood meal (Control), a *Leishmania mexicana* or *Serratia marcescens*-infected bloodmeal. Midguts of sucrose fed sand flies were dissected 24 h before blood feeding and included in the analysis. Midguts were pooled (N=8) and assayed for H_2O_2 concentration. Bar chart represents mean ± SEM of combined samples from 2 independent experiments. Asterisk indicates statistical difference at $p<0.05$.

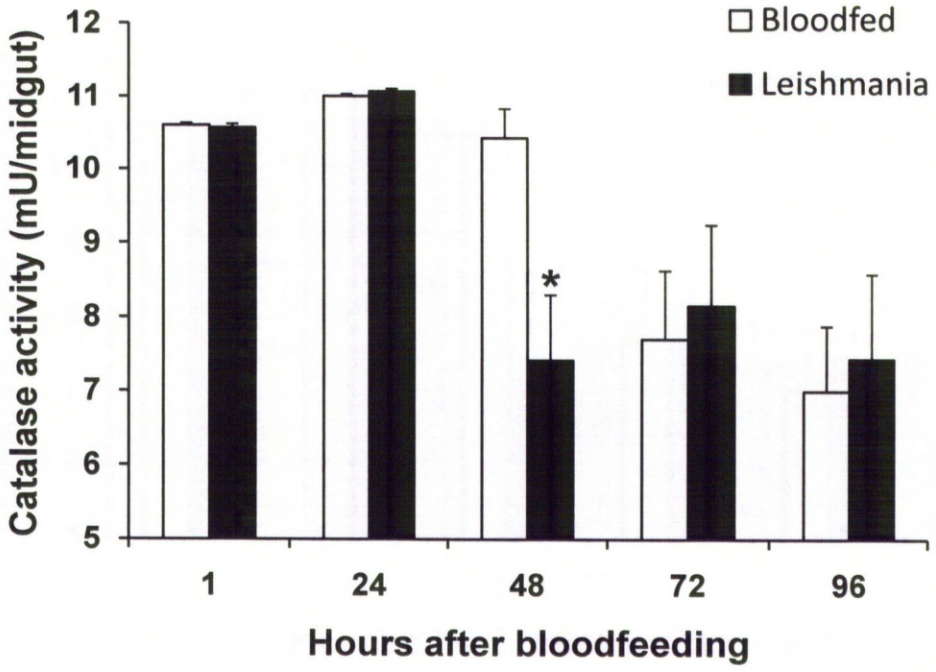


Figure 5.3: Catalase activity in the midgut of *Leishmania*-colonised sand flies. Female flies were fed with either a blood meal (Bloodfed) or a *Leishmania mexicana* - inoculated bloodmeal. Individual midguts and assayed for catalase activity (N=10). Bar chart represents mean ± SEM of combined samples from one experiment. Asterisk indicates statistical difference at $p<0.05$.

5.2.4. *Serratia* and not *Leishmania* induces changes in midgut ROS production *in vivo*

To further investigate in more detail ROS generation within *Lu. longipalpis* midguts, superoxide anion production was monitored *in vivo* in *Leishmania* and *Serratia*-infected sand flies. *Serratia*-infected midguts showed a significant increase in superoxide production at 24, 48, and 72 h PBF compared to both *Leishmania*-infected and negative control midguts ($p<0.02$, Fig 5.4A). *Leishmania*-infected midguts did not show significant differences in ROS production compared to bloodfed negative controls (Fig 5.4A). All *Serratia*-infected flies were dead at 7 days post-infection. ROS production significantly decreased at 24 and 48 h after blood feeding compared to sugar fed flies (Fig 5.4A, $p<0.05$) returning to similar values from sugar fed flies after 72 h and 7 days after blood feeding (Fig 5.4A). These results show that *Serratia* inoculation dramatically increases superoxide production within *Lu longipalpis* midgut, whereas activity of superoxide in *Leishmania* infections did not increase in comparison to blood-fed control insects up to seven days post-infection.

5.2.5. Continuous H_2O_2 feeding to sand flies negatively affects *Leishmania* survival *in vivo*

To analyse whether H_2O_2 has a negative effect in *Leishmania* survival inside the midgut, sand flies were allowed to feed *ad libitum* throughout the experiment on a 5 mM H_2O_2-supplemented 70% w/v sucrose solution followed by *Leishmania* infections to determine the effect of chronic H_2O_2 feeding on *Leishmania* survival. Midgut homogenates of 96 h PBF sand flies fed on H_2O_2 had significantly fewer parasites compared to negative controls fed on plain sucrose ($p<0.05$, Fig 5.5). These results show that H_2O_2 exposure *in vivo* decreases *L. mexicana* survival within the *Lu. longipalpis* midgut.

5.2.6. dsRNA-mediated knockdown of catalase negatively affects *Leishmania* survival in the midgut

To further understand the deleterious effects of ROS on the development of *L. mexicana* within the sand fly midgut, a *Lu. longipalpis* catalase was knocked down

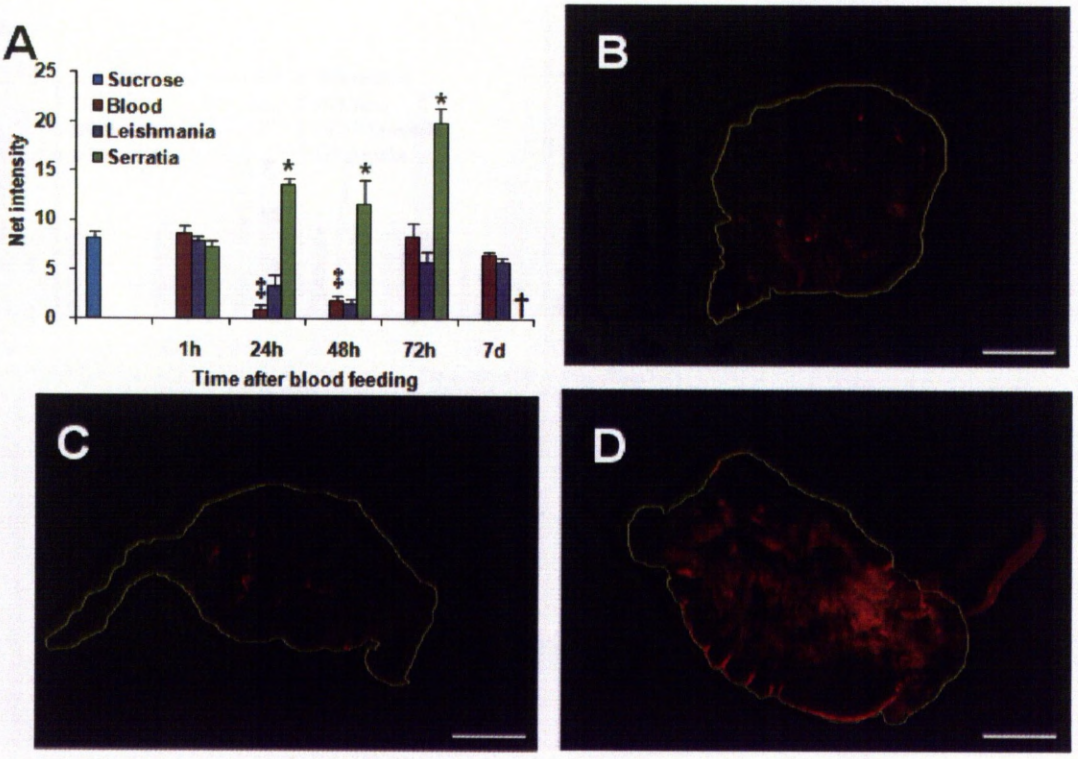


Figure 5.4: ***In vivo*** **detection of ROS in *Leishmania* and *Serratia*-infected sand flies.** Female flies were fed with either a non-infected, a *Leishmania mexicana* or a *Serratia marcescens*-infected bloodmeal. Individual midguts were DHE-stained and photographed to analyse superoxide production. Midguts of sucrose fed sand flies were dissected 24 h before blood feeding and included in the analysis. *A*, Bar charts represent mean values of net colour intensity ± SEM of at least five individual midguts. Asterisk indicates statistical difference at $p<0.05$.Symbol ‡ indicates statistical differences at $p<0.05$ compared to Sucrose group. Dagger represents no survivors (*Serratia* 7days). *B-D*, selected representative images of non-infected, *Leishmania* and *Serratia* infected midguts, respectively. Scale bar represents 200 µm.

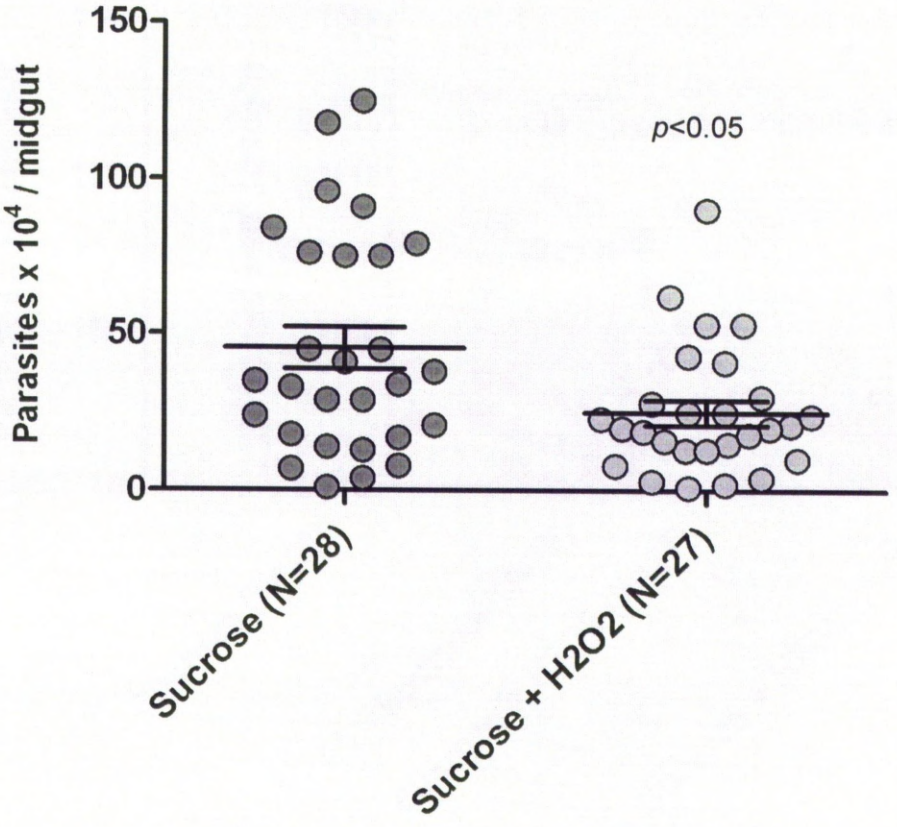


Figure 5.5: *Leishmania* infection after continuous feeding of female *Lu. longipalpis* with a hydrogen peroxide-supplemented sucrose meal. Flies were fed *ad libitum* from emergence on either 70 % sucrose (Sucrose) or a 5mM H_2O_2-supplemented 70% sucrose solution (Sucrose+ H_2O_2) and infected 3 days post-emergence. Individual midguts were dissected and sampled for *L. mexicana* at 96 h after infection. Circles represent number of parasites per individual midgut. Horizontal line represents mean ± SEM of combined samples from 2 independent experiments. Groups were compared using the Mann-Whitney U test.

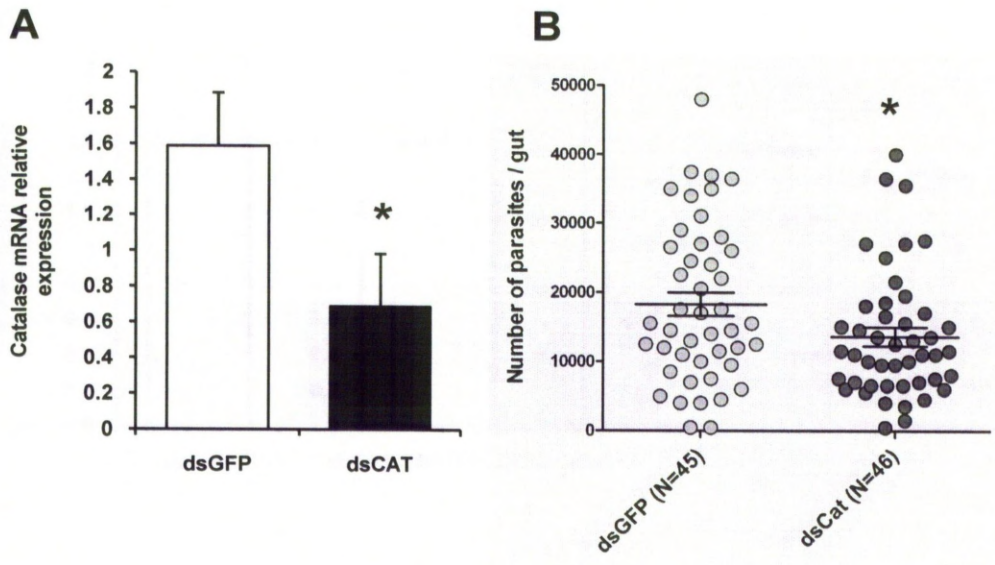


Figure 5.6: dsRNA-mediated knock down of catalase reduces *Leishmania* population in the midgut. *A*, relative expression of catalase mRNA in whole fly homogenates from catalase dsRNA-injected and "mock-injected" sand flies. *B*, effect of dsRNA-mediated catalase silencing on *Leishmania* infection. Individual midguts were dissected and sampled for *L. mexicana* at 96 h after infection. Circles represent number of parasites per individual midguts. Horizontal line represents mean ± SEM of combined samples from 3 independent experiments. Groups were compared using the Mann-Whitney U test. Asterisk indicates statistical difference at $p<0.05$

by RNA interference (RNAi). A dsRNA-mediated catalase knockdown of >50% was achieved at 96h post injection ($p<0.001$, Fig 5.6A). Catalase gene depletion was detrimental to *L. mexicana* survival within *Lu. longipalpis* midgut at 4 days after infections ($p<0.05$, Fig 5.6B), suggesting that changes in catalase activity within the sand fly gut had a negative outcome on *Leishmania* survival and development.

5.2.7. Chronic feeding of a potent ROS scavenger reduces sand fly survival in *Serratia*-infected flies and increases naturally-occurring microbiota

To test if ROS depletion by chronic feeding of an antioxidant would have an effect on *Lu. longipalpis* survival after *Serratia* inoculation, insects were fed from emergence and throughout the experiment on an uric acid-supplemented sucrose solution in cotton wool and then infected with *S. marcescens* in rabbit blood. *Serratia*-infected sand flies fed with uric acid-supplemented sucrose (Serr+UA) exhibited a significant decrease in survival compared to *Serratia*-infected flies fed on plain sucrose solution (Serr) ($p<0.001$, Fig 5.7A). Uric acid-supplementation had no effect on survival as no significant reduction could be observed in bloodfed control flies chronically fed with uric acid (Ctrl+UA) in comparison to blood-fed control flies (Ctrl) (Fig 5.7A). To analyse whether reduction in insect survival was due to an increase in *Serratia* bacterial cells within the sand fly gut, midguts of sand flies inoculated with *Serratia* with or without UA were dissected at 48 h PBF and homogenates were diluted in PBS and inoculated onto LB agar plates. *Serratia* population in flies fed on UA-supplemented sucrose were significantly lower compared to controls ($p\leq0.012$, Fig 5.7B). However, control flies (non-infected) fed on UA-supplemented sucrose exhibited larger population of resident microbiota, ($p\leq0.009$, Fig 5.7C). To investigate whether naturally-occurring microbial growth displayed a similar behaviour in *Serratia*-infected samples, colony counts of resident microbes were performed. UA supplementation significantly increased resident microbiota numbers in *Serratia*-infected flies ($p\leq0.037$, Fig 5.7B).

5.2.8. dsRNA-mediated depletion of OXR1 affects sand fly survival but does not affect *Leishmania* development in the midgut

To investigate the putative role of OXR1 in redox homeostasis and *Leishmania* colonisation, OXR1 was knocked down by RNA interference (RNAi). Gene silencing was extremely efficient as a significant reduction in relative expression was achieved up to 7 days after dsRNA injections ($p<0.05$, Fig 5.8A). However, silencing of OXR1 did not decrease expression of SOD and CAT transcripts (Fig 5.8B) nor did it affect development of *Leishmania* in the midgut (Fig 5.8C). However, OXR1-knockdown sand flies exhibited lower survival compared to 'mock' injected controls ($p<0.05$, Fig 5.8D).

5.2.9. H_2O_2 has a deleterious effect on *Leishmania mexicana in vitro*. To investigate the effect of hydrogen peroxide in *L. mexicana* cultures, promastigotes were incubated for 24 h in different concentrations of H_2O_2-supplemented M199 medium. Parasites numbers in 500 µM and 5 mM-supplemented groups were significantly lower than both negative control and 5 µM-supplemented groups ($p<0.01$, Fig 5.9).

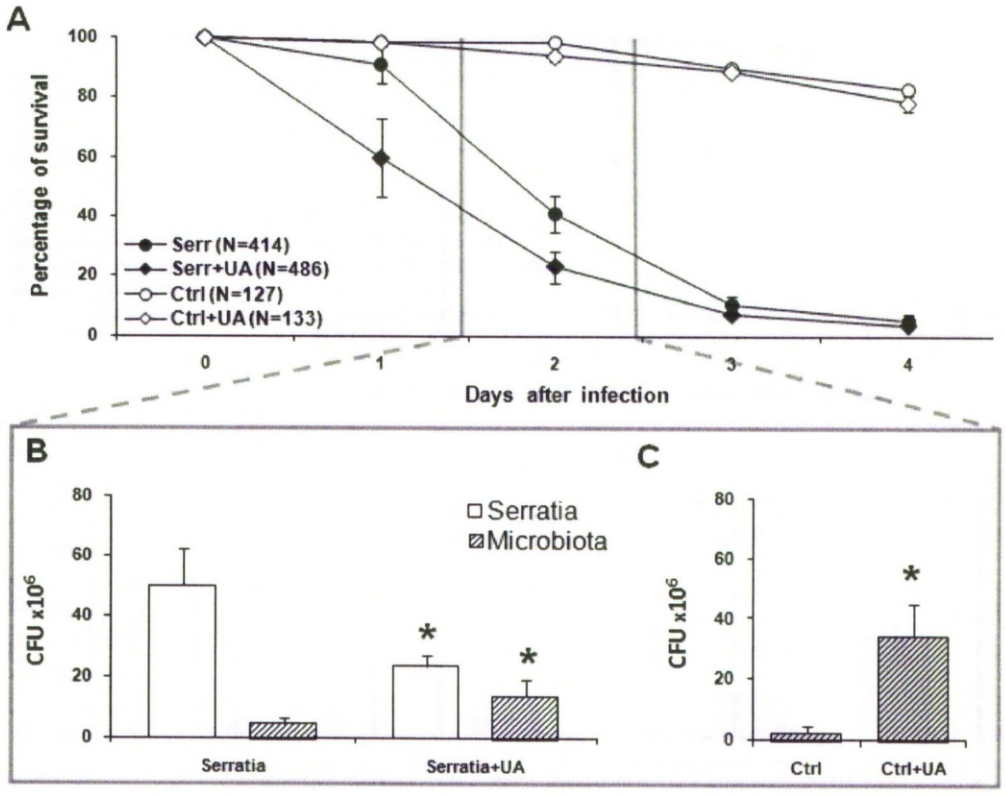


Figure 5.7: Chronic feeding of a uric acid-supplemented sugar meal reduces survival in *Serratia*-infected flies and increases naturally-occurring microbiota. *A*, percentage survival of sand flies fed with: *Serratia* diluted in blood and 70% sucrose solution *ad libitum* (Serr); *Serratia* diluted in blood and 10 mM uric acid-supplemented 70% sucrose solution *ad libitum* (Serr+UA); non-infected blood and 70% sucrose solution *ad libitum* (Ctrl); and non-infected blood and 10mM uric acid-supplemented 70% sucrose solution *ad libitum* (Ctrl+UA). *B*, effect of uric acid-supplemented sucrose feeding on *Serratia* and resident microbiota within the sand fly midgut; *C*, effect of uric acid-supplemented sucrose feeding on resident microbiota in non-inoculated sand flies. Bar charts represent mean ± SEM of combined samples from at least 3 independent experiments (N=60). Groups were compared using the Mann-Whitney U test. Asterisk indicates statistical difference at $p<0.05$. Survival curves were compared using the Kaplan-Meier Log Rank χ^2 test

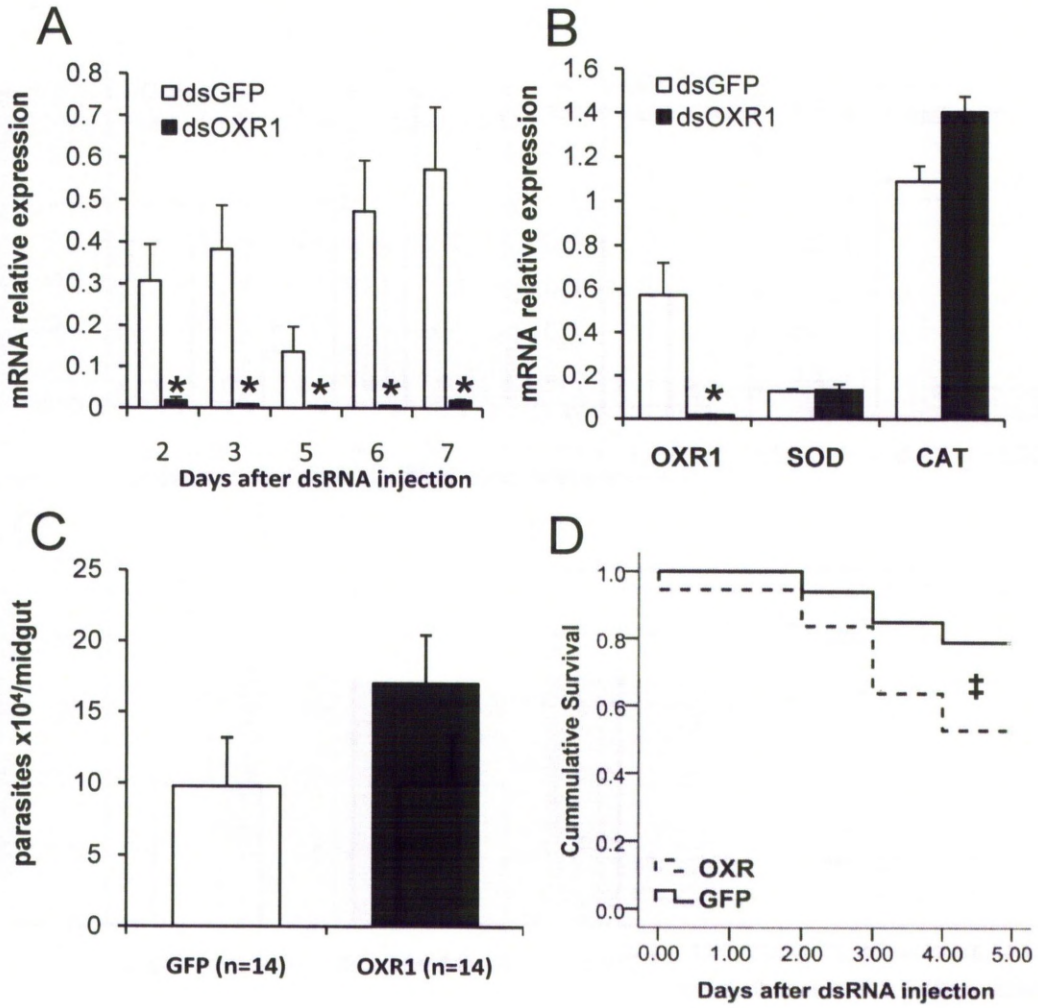


Figure 5.8: dsRNA-mediated knock down of the OXR1 gene in female *Lu. longipalpis*. *A*, relative expression of OXR1 mRNA in whole fly homogenates from OXR1 dsRNA-injected and "mock-injected" sand flies up to seven days after injection (N=15). *B*, relative expression of OXR1, SOD and CAT in whole fly homogenates from OXR1 dsRNA-injected and "mock-injected" sand flies on day 7 after injections (n=3). *C*, effect of dsRNA-mediated OXR1 silencing on *Leishmania* infection. Individual midguts were dissected and sampled for *L. mexicana* at 96 h after infection. *D*, cumulative survival of dsRNA-injected and "mock-injected" sand flies up to five days after injections (N=50). Bar charts represent mean ± SEM of combined samples from at least 1 independent experiment. Asterisk indicates statistical difference at $p<0.05$. Survival curves were compared using the Kaplan-Meier Log Rank χ^2 test and significance at $p<0.05$ is denoted by ‡ symbol.

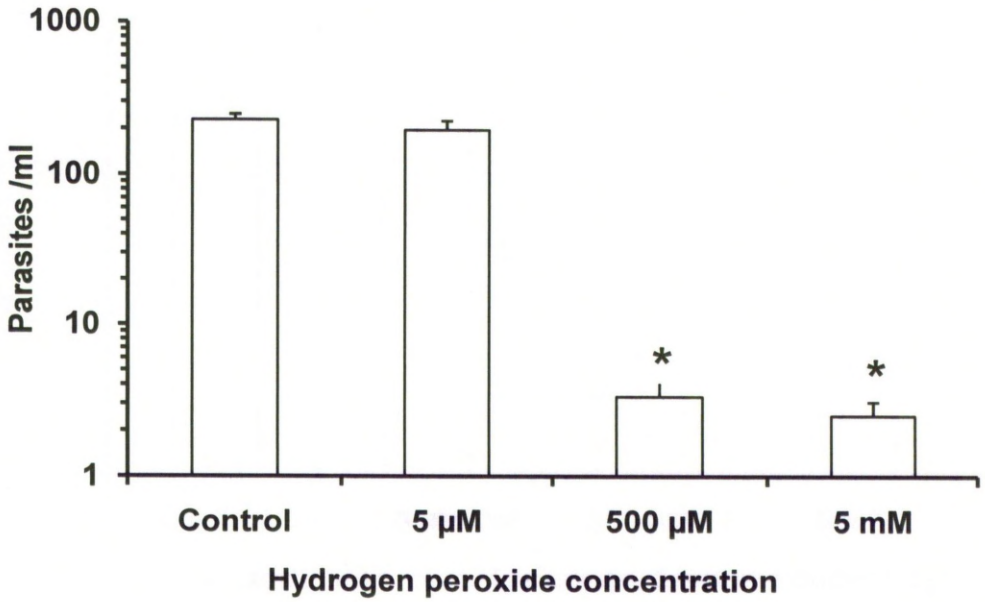


Figure 5.9: Effect of hydrogen peroxide on *Leishmania mexicana*. Promastigotes were incubated in different concentrations of hydrogen peroxide-supplemented M199 medium for two hours, washed and incubated in regular medium for 24 h before parasite count using a haemocytometer. Bar chart represents mean ± SEM of combined samples from 2 independent experiments. Asterisk indicates statistical difference at $p<0.01$.

5.3. Discussion

The results presented in this chapter suggest that ROS-regulatory gene expression changes throughout digestion and that gene expression of CAT and SOD is modulated in the sand fly midgut after infection with the flagellate protozoan *L. mexicana* or *per os* inoculation with the insect pathogenic gram-negative bacterium *S. marcescens*. The catalase enzymatic activity was lower in the *Leishmania*-colonised midgut and it was concluded that ROS regulation influences the sand fly host-*Leishmania* parasite-gut microbiota interaction. It was demonstrated that changes in midgut ROS activities after oral administration of uric acid (an exogenous ROS scavenger) alters the dynamics of sand fly midgut homeostasis, favouring the growth of commensal sand fly gut bacteria.

5.3.1. Gene expression

CAT was differentially expressed during blood feeding in *Lu. longipalpis* (Fig 5.1A) in an expression profile that correlates with the process of bloodmeal digestion. Expression of blood-induced digestive trypsin enzyme in *Lu. longipalpis* is detectable from 2 to 72h PBF, reaching its peak at 12 h (Telleria *et al.* 2007). A similar expression pattern of ROS-detoxifying enzymes should occur, since ROS are largely produced during blood digestion (Graca-Souza *et al.* 2006). In different blood-sucking insects, catalase is expressed upon blood ingestion (Paes *et al.* 2001; Sanders *et al.* 2003; Munks *et al.* 2005; Jochim *et al.* 2008; Magalhaes *et al.* 2008; Molina-Cruz *et al.* 2008). It has been postulated that this enzyme plays an important role detoxifying ROS derived from haemoglobin breakdown (Graca-Souza *et al.* 2005; Graca-Souza *et al.* 2006). An increasing expression of catalase could reflect the use of this enzyme during digestion. In *Drosophila*, differences in catalase mRNA expression and protein accumulation profiles suggest that catalase expression might be affected by transcriptional and post-transcriptional regulation (Radyuk *et al.* 2000). Catalase could be present as an inactive storage proform for fast release to avoid ROS-induced damage during blood digestion.

Previous studies in *Anopheles gambiae* have shown an increase in systemic expression of ROS detoxification enzymes at 24 and 48 h after blood feeding.

During *Plasmodium* infection, catalase was downregulated 24 h post-infection and it was suggested that such suppression might be a response against the parasite that would ultimately lead to higher intracellular levels of H_2O_2, protein nitration and finally, apoptosis of infected cells (Molina-Cruz *et al.* 2008). Interestingly, *Leishmania*-infected sand flies showed a significant reduction in catalase expression in comparison to bloodfed controls up to 96h post infection. Such a reduction in catalase expression could reflect a midgut-specific physiological reaction in response to *Leishmania* infection. As *Leishmania* development is restricted to the sand fly midgut (Bates 2007), any reduction in catalase expression would result in increased oxidative stress, ultimately leading to an impact on *Leishmania* survival within the sand fly midgut. Although it is possible that a reduction in catalase expression in *Leishmania*-infected midguts represents a response against the parasite (as suggested in *Plasmodium*-infected Anopheline mosquitoes), this would not explain how *Lu. longipalpis* is able to harbour heavy *Leishmania* infections in experimental and field conditions (Deane and Deane 1954; Freitas *et al.* 2002; Killick-Kendrick and Rioux 2002). However, H_2O_2 measurements in *Leishmania*-infected midguts were not significantly higher compared to non-infected controls (Fig 5.2). As we measured the H_2O_2 steady-state levels, it is possible that the activity of the enzymes that generate ROS (midgut NADPH oxidases) could be diminished. If we consider the killing effect of some bacteria on *Leishmania* parasites (Moraes *et al.* 2009), we can speculate that parasites would not elicit a complete reduction of ROS levels in the sand fly midgut, as this would favour potential competitor microorganisms.

There were no significant changes in OXR1 expression in the midgut of *Lu. longipalpis* during blood digestion and *Leishmania* or *Serratia* infections. In *An. gambiae*, OXR1 regulates the expression of ROS-detoxifying enzymes, specifically catalase and glutathione peroxidase. In our study, primers were also designed to cover the carboxyl terminal TLD_C domain, the most highly conserved region of the gene. However, gene expression between *Lu longipalpis* OXR1 and catalase was not related and there was no indication that OXR1 expression controls catalase

expression in a similar way previously described in *A. gambiae* (Jaramillo-Gutierrez *et al.* 2011).

5.3.2. ROS production-bacteria

The H_2O_2 concentration was significantly higher in *Serratia*-infected flies two days after blood feeding (Fig 5.2). Additionally, *in vivo* detection of superoxide confirmed high levels of this species from 1 to 72 h after bacterial challenge (Fig 5.4A). It is possible that the increase in ROS was due to epithelial cell death caused by pathogen proliferation (Buchon *et al.* 2009). Alternatively, this increase could be part of the oxidative burst against pathogenic bacteria that has been observed in *Drosophila* (Ha *et al.* 2005b). A similar inverse correlation between bacterial growth and ROS levels has been shown recently in *Aedes aegypti* (Oliveira *et al.* 2011). In chapter 4 we showed that feeding sand flies with the ROS-scavenger ascorbic acid was detrimental to survival (Diaz-Albiter *et al.* 2011) and suggested that mortality could be caused by bacterial infections due to ROS reduction in the midgut. In the present study oral administration of a ROS-scavenger also decreased survival in *Serratia*-infected flies continuously fed on uric acid compared to *Serratia*-infected flies fed on sucrose solution. It is very unlikely that differences in mortality were caused by uric acid toxicity since flies fed on this antioxidant did not show any differences in survival compared to control. The choice of uric acid instead of ascorbic acid in this chapter was due to the fact that ascorbic acid has been shown to inhibit phenoloxidase (PO) activity (Ballarin *et al.* 1998). This inhibition of PO could have had an undesired effect on midgut bacterial populations with concomitant misleading results.

The addition of dietary uric acid increased resident gut microbiota in both *Serratia*-infected and non-infected flies but had an opposite effect on the *Serratia* population. In similar experiments performed in *Aedes* with *Enterobacter asburiae*, the addition of dietary ROS inhibitors increased both endogenous microbiota and *Enterobacter* (Oliveira *et al.* 2011). In our work, midguts were dissected 48 hours after infection, when mortality reached ~80%. It is possible that survivors sampled at that time point were more resistant to infection and harboured a

lower *Serratia* load. Another possibility is that the increase in the *Serratia* population was impeded by resident microbes, which were indeed significantly higher in *Serratia*-infected flies supplemented with uric acid.

5.3.3. ROS production-*Leishmania*

We have shown that hydrogen peroxide can kill *Leishmania in vitro* (Fig 5.9) and that oral administration of H_2O_2 to infected flies is detrimental to *Leishmania* survival within the sand fly gut. RNAi mediated gene silencing of the H_2O_2-detoxifying enzyme catalase led to lower *Leishmania* population in the gut providing further confirmation that *Leishmania* parasites are sensitive to ROS generation. An estimated reduction of ~50% in catalase expression was achieved in catalase dsRNA-injected sand flies in comparison to "mock-injected" controls. Catalase knockdown sand flies exhibited a reduction in parasite numbers when compared to dsGFP-injected insects. Since only fully engorged females were selected for the experiments, it is unlikely that the size of the bloodmeal could account for the difference in parasite numbers. It was quite intriguing to find a lack of ROS activity in *Leishmania*-infected sand flies in comparison to *Anopheles* and *Plasmodium* (Molina-Cruz *et al.* 2008). The fact that two different ROS ($O_2^{\bullet}$ and H_2O_2) and their associated enzymes (superoxide dismutase and catalase) did not exhibit induction in either expression profiles or biochemical assays in sand fly midguts raises the possibility that *Leishmania* could "evade" the oxidative burst by an unknown mechanism or avoid eliciting a ROS-based response to ensure survival within the gut.

One potential scenario would be detoxification of ROS by the *Leishmania* during blood meal digestion using the protozoan's antioxidant enzymes, especially if we consider the reduction in catalase enzymatic activity in *Leishmania*-colonised midguts (Fig 5.3). Antioxidant enzymes are crucial for *Leishmania* parasites during infections inside the macrophage. It has been shown that virulence in *Leishmania* correlates with antioxidant enzyme expression in the parasite (Krieger *et al.* 2000; Steenkamp 2002), as well as with its resistance to hydrogen peroxide toxicity (Goyal *et al.* 1996; Acestor *et al.* 2006; Pal *et al.* 2010). Moreover, in an

interspecies microarray performed in *L. mexicana*, one of the most upregulated genes in promastigotes compared to lesion amastigotes was peroxidoxin (Holzer *et al.* 2006) which when expressed as a protein was able to break down ROS in protozoa (Levick *et al.* 1998; McGonigle *et al.* 1998; Barr and Gedamu 2001). The hypothesis of a *Leishmania*-mediated ROS detoxification during bloodmeal digestion is currently being studied in our laboratory.

5.3.4. OXR1 knockdown

Although the OXR1 gene was successfully silenced in female sand flies as shown by a consistent and prolonged reduction in expression as well as a decrease in sand fly survival, *Leishmania*-infected sand flies did not exhibit a concomitant significant reduction in parasite population after gene knock down. Results from similar experiments performed in the malaria vector *Anopheles gambiae* showed that OXR1 protects mosquitoes against oxidative stress and that silencing of this gene also reduces the expression of catalase and glutathione peroxidase and negatively affects the development of *Plasmodium berghei* (Brandt *et al.* 2008; Jaramillo-Gutierrez *et al.* 2011). The OXR1 gene is highly conserved from yeast to humans (Stowers *et al.* 1999a; Volkert *et al.* 2000; Fischer *et al.* 2001; Elliott and Volkert 2004), and has been found to provide protection against DNA damage from endogenous and exogenous oxidants (Volkert *et al.* 2000). OXR1-silenced *Lu. longipalpis* displayed a change in phenotype as mortality was higher in experimental flies. It is possible that such mortality was due to DNA damage. *OXR1* is the homologue of *Drosophila L82*, a gene that belongs to a novel family involved in metamorphosis and controlled by both ecdysone-dependent and ecdysone-independent regulatory mechanisms. Mutations in *L82* in led to developmental arrest and death upon eclosion of *Drosophila* (Stowers *et al.* 1999b). Although gene knockdown was performed in adult specimens, it is possible that OXR1 could be involved in an unknown process and negatively affect survival when silenced in fully developed flies.

As the body of sand fly sequence data increases (Dillon *et al.* 2006; Ramalho-Ortigão *et al.* 2007; Jochim *et al.* 2008; Dostalova *et al.* 2011) we can begin to

infer that the sand fly gut immune response is similar in overall organisation to that of the more extensively studied dipterans; mosquitoes and *Drosophila*. In these insects there is a reliance on the innate immune response mainly via the two types of effectors; ROS and AMPs (antimicrobial peptides). Manipulating the sand fly ROS system revealed the potential complexity underlying immune homeostasis in the gut. The challenge for the fly is to regulate ROS production within the gut to attempt suppression of potential pathogens whilst allowing development of potentially beneficial microorganisms. Our results suggest that the ROS is harmful to the Leishmania and that experimental activation of the ROS system in the sand fly results in a reduced *Leishmania* population. But there is an apparent tolerance of the *Leishmania* by its sand fly host allowing the development of large populations. Addition of antioxidant to the gut and subsequent effects on co-habiting bacterial species provide us with a glimpse into the fine 'tuning' between ROS levels, bacterial communities and the sand fly vector of *Leishmania.*

Chapter 6

Development of software for analysing *Leishmania* morphometrics

6.1. Introduction

It has been 113 years since the publication of the first description of amastigotes of *Leishmania* by the Russian surgeon Peter Borovsky from smears of an infected patient (Borovsky 1898; Hoare 1938). Today, *Leishmania* are still stained and analysed under the microscope as they used to over a century ago. The present chapter charts the development of a software- image analysis program for *Leishmania;* we compare morphometric data obtained manually from stained smears of cultured parasites with data produced using the same samples from software using a computer program developed by collaborators from the Department of Physics, University of Liverpool.

The *Leishmania* genus spans over 30 different species, 20 of which are of medical and veterinary relevance (Bates 2007; Antinori *et al.* 2011). The life cycle of *Leishmania* involves two major stages: non-motile, amastigote forms that occur inside macrophages of mammals, and motile flagellated promastigote forms that develop inside the invertebrate sand fly host from amastigotes ingested after blood feeding from the infected vertebrate (Fig 6.1). Subsequent developmental changes in amastigotes produce different promastigote morphological forms (Killick-Kendrick 1979). The development of *Leishmania* inside its insect host is restricted to the midgut in two distinctive subgenera-specific colonization patterns. The subgenus *Leishmania* are suprapylarian (*i.e.*, that develop in the midgut and foregut), while subgenus *Viannia* are peripylarian (*i.e.*, that also colonise the hindgut) (Lainson and Shaw 1987). Regardless of colonisation preferences in the midgut, *Leishmania* development ultimately leads to metacyclic promastigotes (Bates 2007). These are the flagellated forms that

bridge the transfer of the *Leishmania* between vector and mammalian host during the bite of the sand fly (Bates and Rogers 2004).

Because of its medical importance, the amastigote stage of *Leishmania* has received more attention compared to the flagellated stage (Handman and Bullen 2002). Different promastigote forms were described inside the phlebotomine host two decades ago (Lawyer *et al.* 1987; Killick-Kendrick 1990; Walters 1993), but there was a lack of general agreement in the scientific community as reflected by the fact that flagellated forms other than metacyclics were collectively denominated as procyclic promastigotes in some references (Pinto-Da-Silva *et al.* 2002; Soares *et al.* 2002). The publication of two seminal papers using *Leishmania (Leishmania) mexicana* and *Leishmania* (*Leishmania*) *infantum* (syn. *Leishmania chagasi*) as models (Rogers *et al.* 2002; Gossage *et al.* 2003) provided evidence robust enough to define six types of flagellated subpopulations, namely procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, haptomonad promastigotes, paramastigotes and metacyclic promastigotes. The authors also included morphometric information, such as body width, body length and flagellum length for the first time on four of the flagellated forms (Table 6.1).

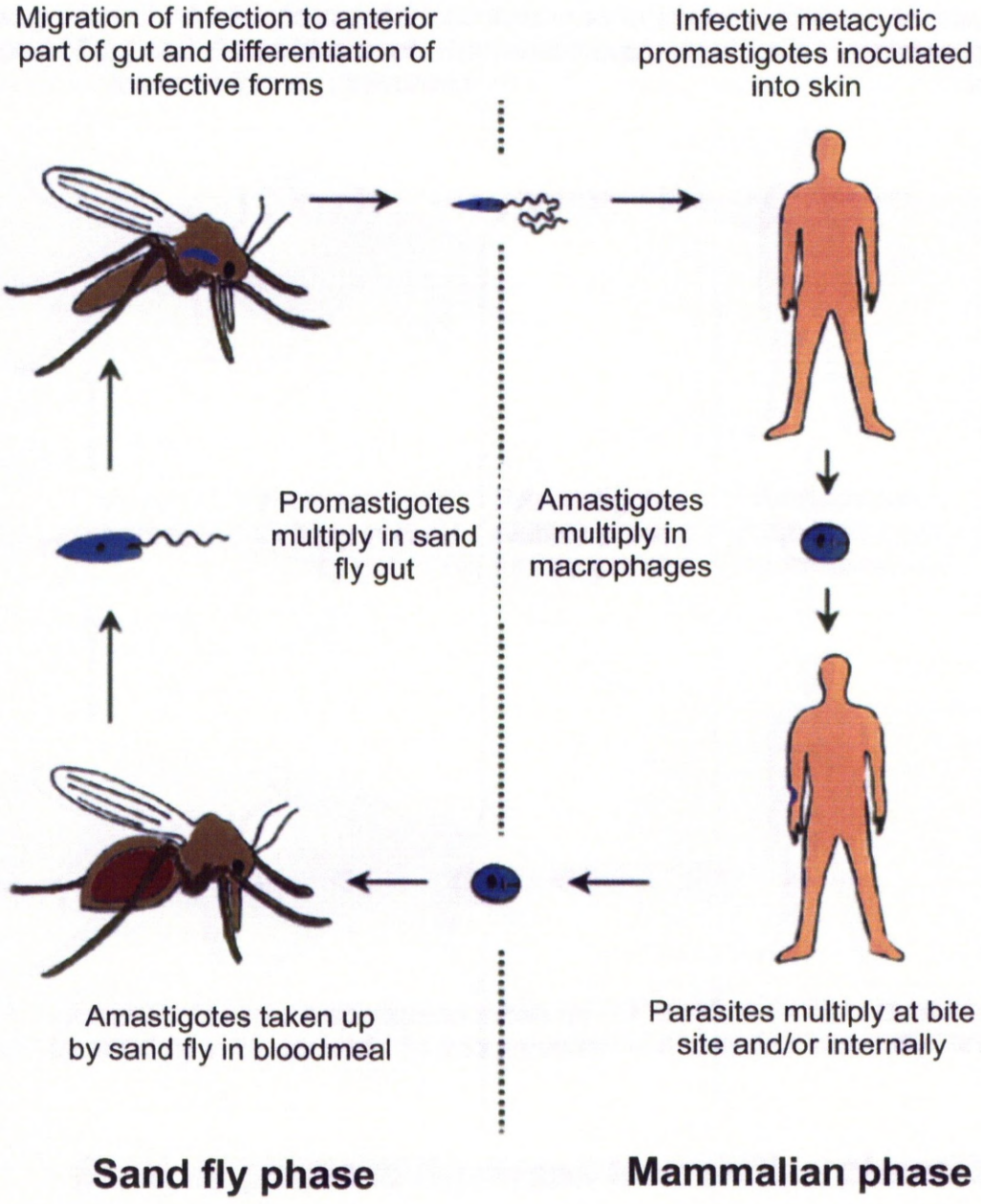


Figure 6.1: Overview of the *Leishmania* cycle. Adapted from Bates and Rogers (2004).

Table 6.1. Summary of attributes, morphometrics and graphic examples of subpopulations of *Leishmania mexicana*. Modified from (Rogers *et al.* 2002; Bates and Rogers 2004)

Morphological subpopulation	Description	Morphometrics	Illustration
Amastigote	Taken up by sand flies in the bloodmeal, may undergo limited division but mainly transform to procyclic promastigotes. First experience a period of trypsin sensitivity as surface membrane is remodelled (Pimenta *et al.* 1997).	Ovoid body form, no flagellum protruding from flagellar pocket	
Procyclic promastigote	Develop from amastigotes taken up in the bloodmeal in 24-48 hours dividing forms responsible for initial expansion of parasite population in the abdominal midgut (bloodmeal phase).	Body length 6·5 – 11·5 µm, flagellum<body length (body width variable)	
Nectomonad promastigote	Develop from procyclic promastigotes non-dividing migratory forms responsible for anterior spread and establishment of the infection in the thoracic midgut (Killick-Kendrick *et al.* 1974).	Body length ≥12 µm, (body width and flagellar length variable)	
Leptomonad promastigote	Develop from nectomonad promastigotes , dividing forms responsible for further expansion of the population in the thoracic midgut and foregut produce Promastigote Secretory Gel (PSG) a gel-like substance important in transmission (Rogers *et al.* 2002).	Body length 6·5 – 11·5 µm, flagellum ≥ body	
Haptomonad promastigote	Developmental origin uncertain, probably from leptomonad promastigotes attached forms that possess an expanded flagellar tip and bind to cuticular surfaces via hemidesmososme-like attachment plaques (Killick-Kendrick *et al.* 1988).	Disc-like expansion of flagellar tip (body form and flagellar length variable)	
Metacyclic promastigote	Mammalian-infective stage, pre-adapted for survival in the mammalian host, they become complement resistant, express stage-specific genes and are biochemically part-way to becoming amastigotes (Sacks 1989).	Body length≤8 µm, body width<1·0 µm, flagellum> body length	
Paramastigote	Not a promastigote *sensu stricto*, developmental role and origin uncertain.	Kinetoplast adjacent to nucleus, external flagellum present	

The applied use of quantitative information derived from morphological features in organisms has a long history. It seems that Robert E. Blackith coined the term 'morphometrics' from his research on morphological changes in the grasshopper's carapace and its association with the development of the swarming phase of locusts (Blackith 1957; Reyment 2010). Morphometrics is a vast field and probably the most up-to-date definition is that of Elewa who devised it as "quantification and visualization of shape to solve numerous problems related to wide ranges of scientific research" (Elewa 2010). Current applications cover a wide variety of subjects, from shape variation of butterfly

wings and evolutionary ecology (Breuker *et al.* 2010) to typology of ceramic materials and archaeology (Martínez-Carrillo *et al.* 2010).

The development of computer-assisted image analysis has also played an important role in the evolution of morphometric data analysis with many lines of research currently using image analysis software (Brown *et al.* 2011; Drake 2011; Lee *et al.* 2011; Markiewicz 2011; Mosher *et al.* 2011). Although software capacity for shape recognition is presently far behind that of the human brain, it is also true that computers have proven far superior and more reliable in performing certain carefully designed monotonous and repetitive tasks much more rapidly than the human operator. Quantitative examination of microscopic biological samples such as bacteria, yeast and blood cells used to be a laborious process when performed manually under the microscope. This task has become more efficient with the aid of different devices such as coulter counters and modern flow cytometers (Andersson and Berg 2004). The latter are able to measure complex parameters such as volume, nucleic acid content, enzymatic activity, apoptosis, oxidative burst among others (Rieseberg *et al.* 2001). However, the variation in length of the flagellum as a morphometric character for *Leishmania* subpopulation classification makes difficult the use of flow cytometry for subpopulation analysis.

Perhaps the most successful example of automated morphometric recognition and classification of a flagellated form comes from the field of reproductive biology. Sperm morphology has been associated with *in vivo* fertility (Rodriguez-Martinez 2006); manual sperm morphological classification has been found to be subjective and highly variable between technician and laboratories (Saacke 1982). To tackle this problem, a computer-assisted sperm head morphometry analysis (ASMA) was developed in the 1980s (Katz *et al.* 1986) and improved up to a point where it was able to detect changes in sperm morphology otherwise undetectable by manual assessment (Davis *et al.* 1993). ASMA has been used in sperm from different animal species such as horses (Ball and Mohammed 1995) ,

boar (Hirai *et al.* 2001), cattle (Gravance *et al.* 1996) and humans (Kruger *et al.* 1996) and recently in llama (Casaretto *et al.* 2011).

An accumulating body of work exploring *Leishmania* transmission by its phlebotomine host has recorded differences in parasite numbers as *de facto* indicative of changes inside the sand fly environment that might affect the parasite. Although differences in parasite load inside the phlebotomine are useful in such experiments, it is possible that changes in parasite subpopulations inside the sand fly midgut could be happening in experiments where differences in parasite numbers were not significant between experimental and control groups. Practical limitations mean that most studies measure the relative size of the promastigote population as an important parameter of the 'success' of the *Leishmania* in establishing within the sand fly vector. However a more accurate parameter of 'success' would be the rate of appearance of the mammalian infective metacyclic promastigotes. For example one experiment may contain a sand fly cohort where 100% are infected with *Leishmania* but only 5% contain metacyclics; the majority of sand flies are therefore unlikely to transmit an infection if they were to blood feed. The rate of development of the metacyclic population is therefore a very important, but under researched, aspect that would have important implications for understanding the epidemiology of the disease. The program would also be useful for in vitro analyses of Leishmania development and other related kinetoplastid such as *Trypanosoma cruzi* and *Trypanosoma brucei* in their respective vectors.

One of the major reasons why samples are not more routinely analysed by classification of parasite subpopulations is because it is very labour-intensive and slow and also requires advance microscopy skills compared to perform direct counts of all parasite forms present in the midgut. An accurate, fast, non-operator biased computer driven tool to analyse parasite subpopulations of *Leishmania* would provide us with an additional measurement of the development of *Leishmania* populations both *in vivo* to investigate sand fly–*Leishmania* interactions and also *in vitro* examining growth parameters on

promastigotes. This was an interdisciplinary project with Prof Steve Barrett from the Department of Physics of the University of Liverpool who modified his pre-existent image analysis software (Image SXM, (Barrett 2008)) to measure and discriminate different *Leishmania* subpopulations from photographic images. This promastigote project formed part of the Microscopy Image Analysis Software for Medical Applications (MIASMA) project (http://www.liv.ac.uk/~sdb/MIASMA). This modified version of the Image SXM was called ParaMorph.

6.2. Results and Discussion

6.2.1. Software Development of Image SXM ParaMorph

Image SXM was originally written by S. D. Barrett (Barrett 2008) and further developed into ParaMorph by students P. Mulligan (V. 3.0) and H. Delemare (V. 3.1) to identify subpopulations of parasites from photographs of microscopic slide preparations. Although the main differences between both versions are overviewed in this section, both student projects have been included as Appendices at the end of this chapter for further details. Broadly speaking and regardless of the version, ParaMorph has six main steps (Fig 6.2):

Calibration. The algorithm assumes that a folder full of images to be analysed contains an image of a 50 μm calibration grid. This is used to define an image scale (pixels/μm) that is applied to all subsequent images.

Colour and Background. The green channel of the RGB (red-green-blue) colour image is selected as having the greatest contrast between parasites and the background. Variations in the background intensity across the image field of view are removed to improve the effectiveness of later analysis.

Thresholding. Images are thresholded (*i.e.*, all pixels darker than a threshold value are 'kept' and set to black; all pixels lighter are 'thrown away' and set to white). This results in an image with black objects on a white background. The optimum value for threshold is determined from the distribution of the pixel intensities

throughout the image. One threshold value shows the bodies and flagella; a higher (darker) threshold value shows only the bodies.

Separating Bodies and Flagella. The two images – one showing the bodies and flagella, the other showing only the bodies – can then be processed. Taking the difference of these two images gives a third image showing only the flagella. Flagella that are not attached to any bodies are eliminated at this point.

Measuring Bodies and Flagella. Once an image has been reduced to just black and white pixels (black = the objects of interest, white = everything else) then each black object can be measured to give its length and width. This is done for the image of bodies and again for the image of flagella. Objects that are not actually parasite bodies are rejected on the basis of their size and/or shape.

Parasite Type. Definitions of the various developmental stages of the parasites are used to determine which type best characterises each parasite body/flagellum pairing.

6.2.2. Differences between versions

Version 3.0 was firstly developed by Mulligan and it was initially tested with a batch of photographs and compared against manual measurements following the same protocol as in the present chapter. Thresholding in version 3.0 was calculated from observations and trial an error from the original photograph. One of the major weaknesses of version 3.0 was that there was a high error in measuring flagella length; this had a major impact on parasite detection and classification. The algorithms that Delemare (V 3.1) explored differed from version 3.0 in the details of how to determine the threshold level, which allows the bodies or flagella to be differentiated from the background. This is a function of the distribution of the pixel intensity values for the image. In Version 3.1 different filters were added in an attempt to make the parasites stand out clearly from the background, without influencing the required metrics of parasite size/shape.

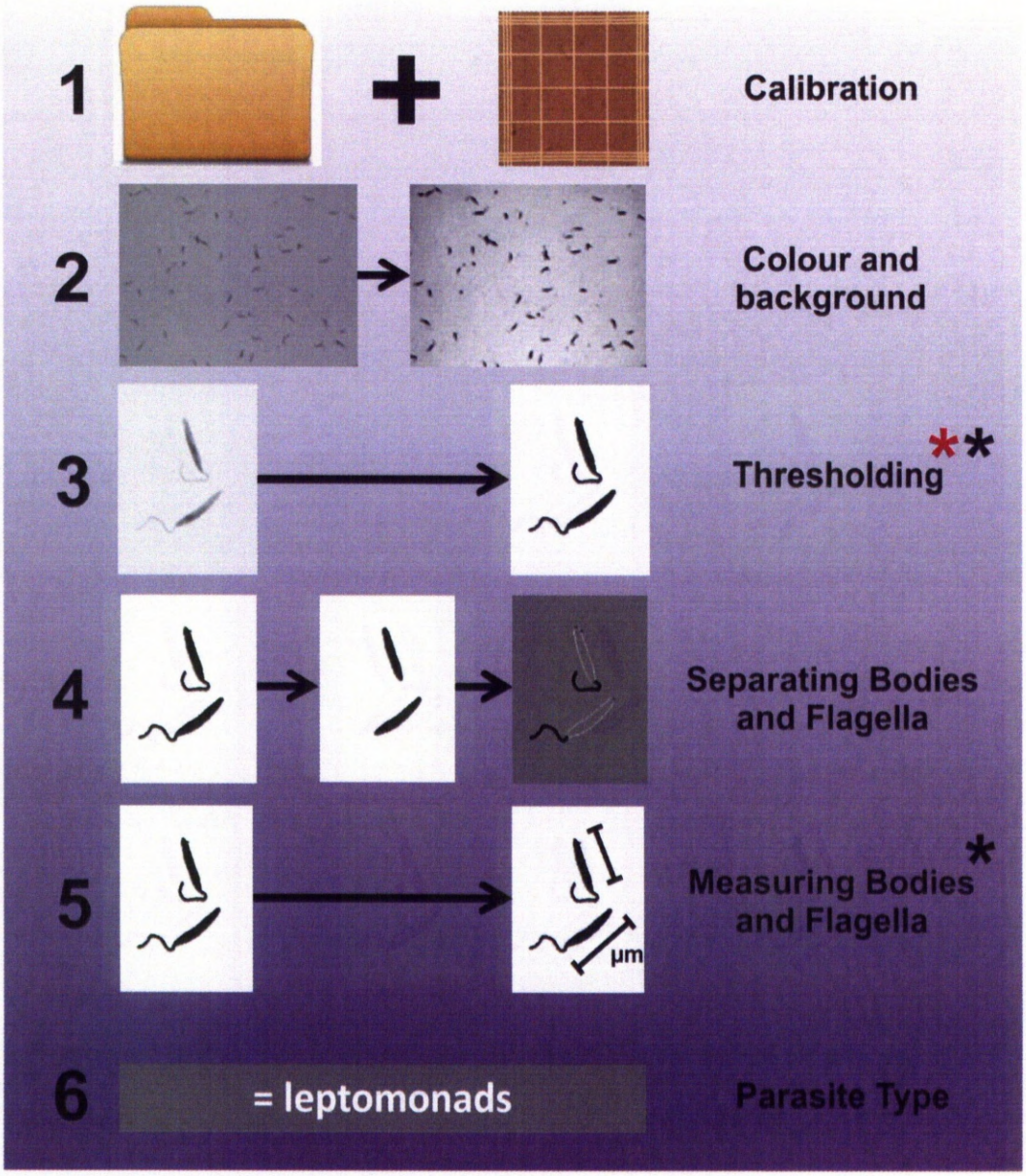


Figure 6.2: Outline of Image SXM ParaMorph. The software has six main steps which start with an image folder which includes a photograph of haemocytometer grid to calibrate the measurements from pixels to micrometers and culminates with file containing parasite subpopulations relative frequencies, total numbers and position in the original photograph. Red asterisk indicates programming modifications from version 3.0 to 3.1. Black asterisk indicates steps with major difficulties on parasite morphology analysis.

6.2.3. Parasite subpopulation clustering

To find out whether parasite subpopulations could be identified as discrete clusters, morphometric data from manual and computer-assisted measurements (body length, body width and flagellum length) were plotted in a three-dimensional XYZ scatter plot. Parasite subpopulations did not cluster in evident discrete units when body and flagellum measurements were plotted in an XYZ 3D graph, neither in manually obtained measurements nor in software-based analysis (Figs 6.3 to 6.5). Moreover, measurements seem to follow a normal distribution in XY (body length-body width-[green]), ZX (body length-flagellum length [magenta]) and YZ (body width-flagellum length [blue]) panes from data obtained after analysis of images by ParaMorph V. 3.0 (Fig. 6.4).

Data from V. 3.1 seem to follow the same pattern behaviour, with slight positive skewing in body length vs. body width (green, Fig. 6.5). It is not possible to conclude the same for manual measurements since observations were not as numerous as in data from ParaMorph. The apparent lack of clustering in the promastigote forms reveals, for the first time, that the morphological changes apparently follow a normal distribution and are continuous; there is no sudden switch in morphology from *e.g.* a procyclic to a nectomonad. As such the definition of the present classification system is based, at least in part, on a 'perception' of differences due to human observation. The development of a reliable ParaMorph version using thousands of measurements would enable an unbiased examination of the present classification system. Perhaps the only exception of data clustering was the case of amastigotes which were assigned a flagellum length of zero. Amastigotes can be distinguished from the cloud of data in all scatter plots (arrows and circles, Figs 6.3-6.5).

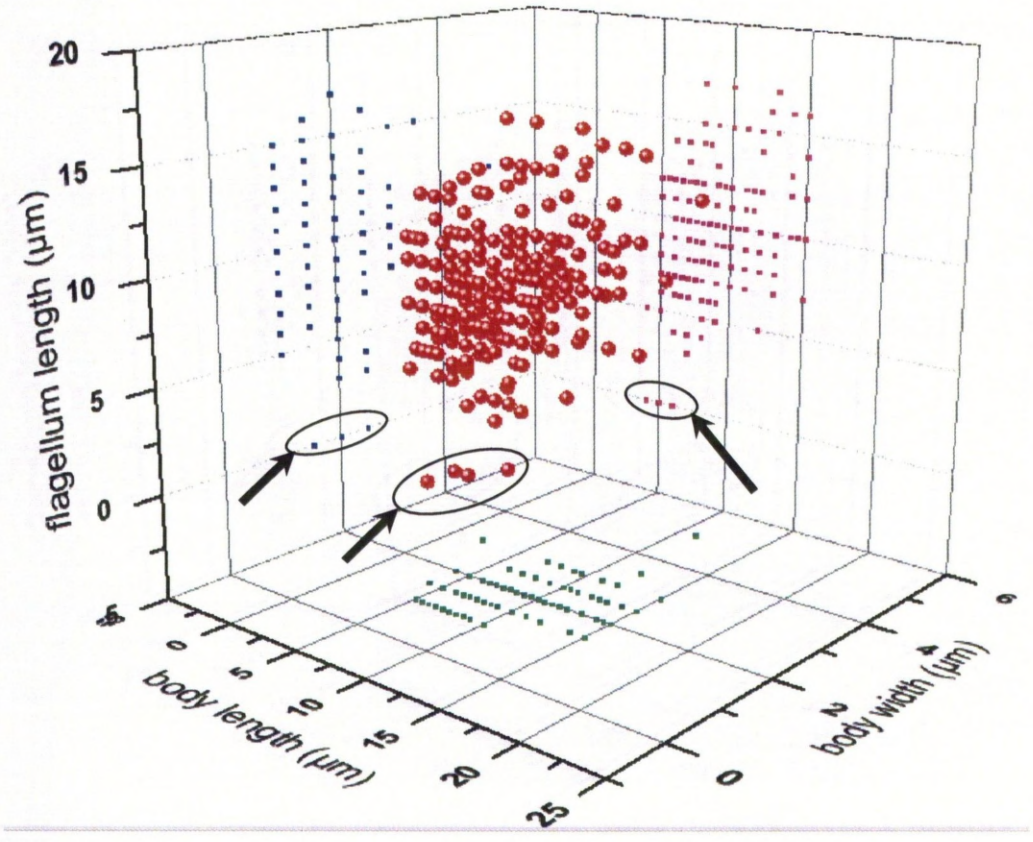


Figure 6.3: Three-dimensional scatter plot of all parasite subpopulations from 24 h, 48, h, 7d and 8 d post-metacyclogenesis from manual counts. X-axis represents parasite length, Y-axis represents parasite width and Z-axis represents flagellum length. Projections of each point are plotted against the XY pane (green), ZX pane (magenta) and YZ pane (blue). Amastigote subpopulation is indicated by arrows and circles. N= 600 parasites.

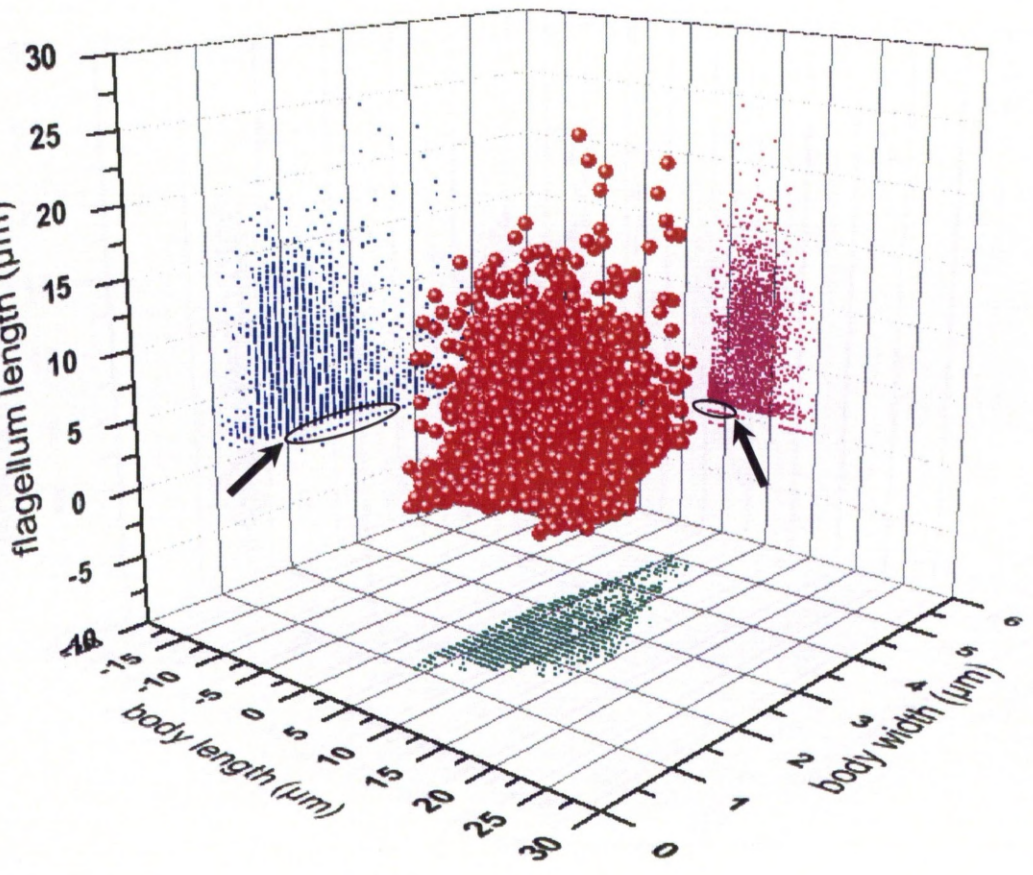


Figure 6.4: Three-dimensional scatter plot of all parasite subpopulations from 24 h, 48, h, 7d and 8 d post-metacyclogenesis from photographs analysed with ParaMorph V. 3.0. X-axis represents parasite length, X-axis represents parasite length, Y-axis represents parasite width and Z-axis represents flagellum length. Projections of each point are plotted against the XY pane (green), ZX pane (magenta) and YZ pane (blue). Amastigote subpopulation is indicated by arrows and circles. N= 2575 parasites.

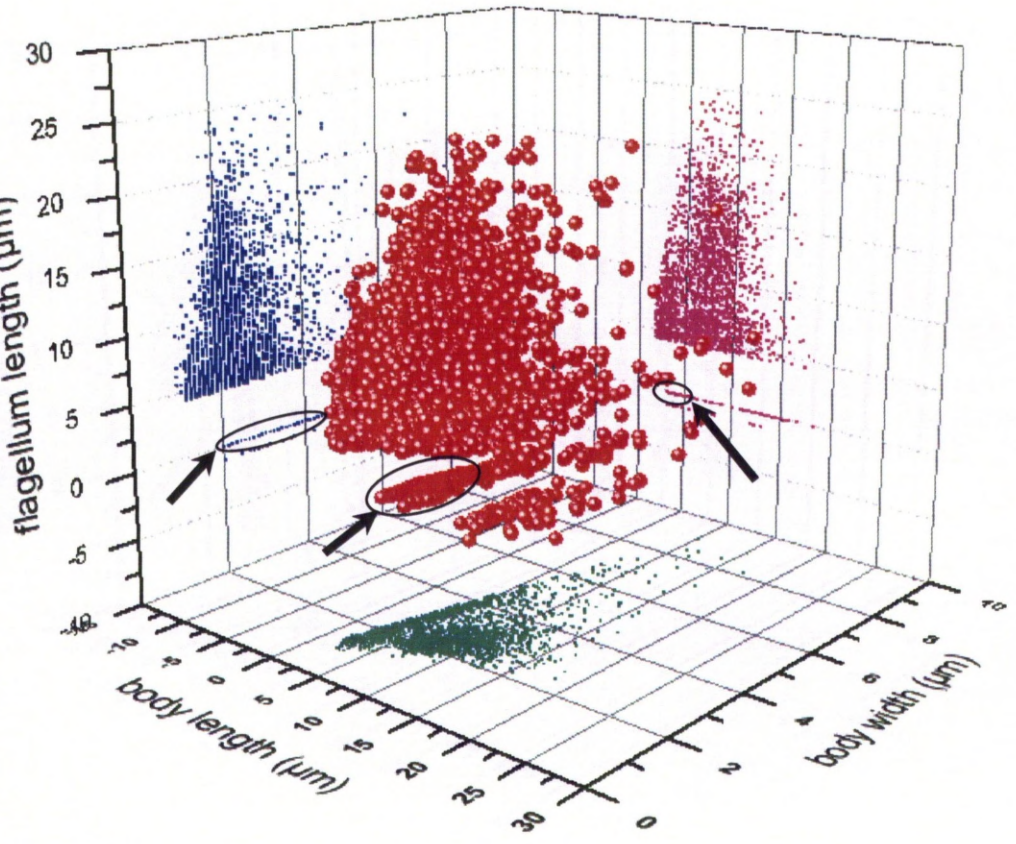


Figure 6.5: Three-dimensional scatter plot of all parasite subpopulations from 24 h, 48, h, 7d and 8 d post-metacyclogenesis from photographs analysed with ParaMorph V. 3.1. X-axis represents parasite length, Y-axis represents parasite width and Z-axis represents flagellum length. Projections of each point are plotted against the XY pane (green), ZX pane (magenta) and YZ pane (blue). Amastigote subpopulation is indicated by arrows and circles. N= 3048 parasites.

There are no similar morphometric studies on flagellated protozoa to compare with the present work. However, in studies with mammalian sperm performed with boar ejaculates using computer-assisted morphological analysis, Fourier coefficients (*i.e.*, how closely head shape is related to a series of mathematical shapes relative to a fundamental circular component) were determined from sperm and the three morphometric subpopulations were plotted together with no obvious discrete clusters (Thurston *et al.* 2001). However, in another study performed on boar sperm, plotting two major morphometric features produced two different clustering patterns in spermatozoa (classified as 'good freezer' or 'bad' freezer) extracted from boars that differed in their ability to produce sperm able to withstand freezing-thawing (Pena *et al.* 2005).

6.2.4. Validation

To validate software-based parasite subpopulation classification, two different approaches were used with samples from each time point (24 h, 48, h, 7d and 8 d post-metacyclogenesis induction). Relative frequencies of parasite subpopulations were obtained manually from microscopic slides and compared against relative frequencies reported from ParaMorph. Also one photograph from each time point set was randomly selected and parasites in the image were compared against measurements and location in photograph as provided by ParaMorph. Relative frequencies of parasite subpopulations of manual vs. software were statistically different in all time points. No amastigotes were detected from manual observations in samples from 24 h and 48 h post-metacyclogenesis (PMG). However, both versions of ParaMorph report the presence of this form (Fig. 6.6 A and B). Samples from 7 and 8 d PMG were the most heterogeneous, showing all 5 forms of parasites and although ParaMorph relative frequencies were statistically different from Manual measurements, Version 3.1 shows a closer profile to manual measurements than V 3.0 (Fig 6.6 D). Relative frequencies from all time points pooled together show significant differences between the three groups (Fig. 6.7). However, it is noticeable that ParaMorph V 3.1 displays a subpopulation pattern with fewer procyclics, more

leptomonad promastigotes and more metacyclic promastigotes compared to ParaMorph V 3.0. Also, this pattern is closer to that obtained from manual assessment. It is interesting to note that nectomonad promastigotes show the closest relative frequency between methods. It is possible that given the considerable longer body length of this subpopulation (Rogers *et al.* 2002) is easier for ParaMorph software to classify this parasite form.

Accuracy of ParaMorph varied between versions and was higher in version 3.1., it was found that relative frequencies were significantly different among the three methods (Figs 6.6 and 6.7). But how many parasites were correctly classified? When parasites were manually inspected in randomly selected photographs and compared to data from ParaMorph, over 40 percent of the parasites clear to the human eye were not found by ParaMorph 3.0, but this value this was lower in ParaMorph V 3.1 (Fig 6.8). Also some parasites were found in the image but unclassified (13 and 14%, V 3.0 and 3.1, respectively) or found but classified incorrectly (26 and 19 %, V 3.0 and 3.1, respectively). Finally, parasites that were correctly found and classified accounted for 19% in V 3.0 and 36% in V 3.1. ParaMorph 3.1 was 17% more accurate that V 3.0.

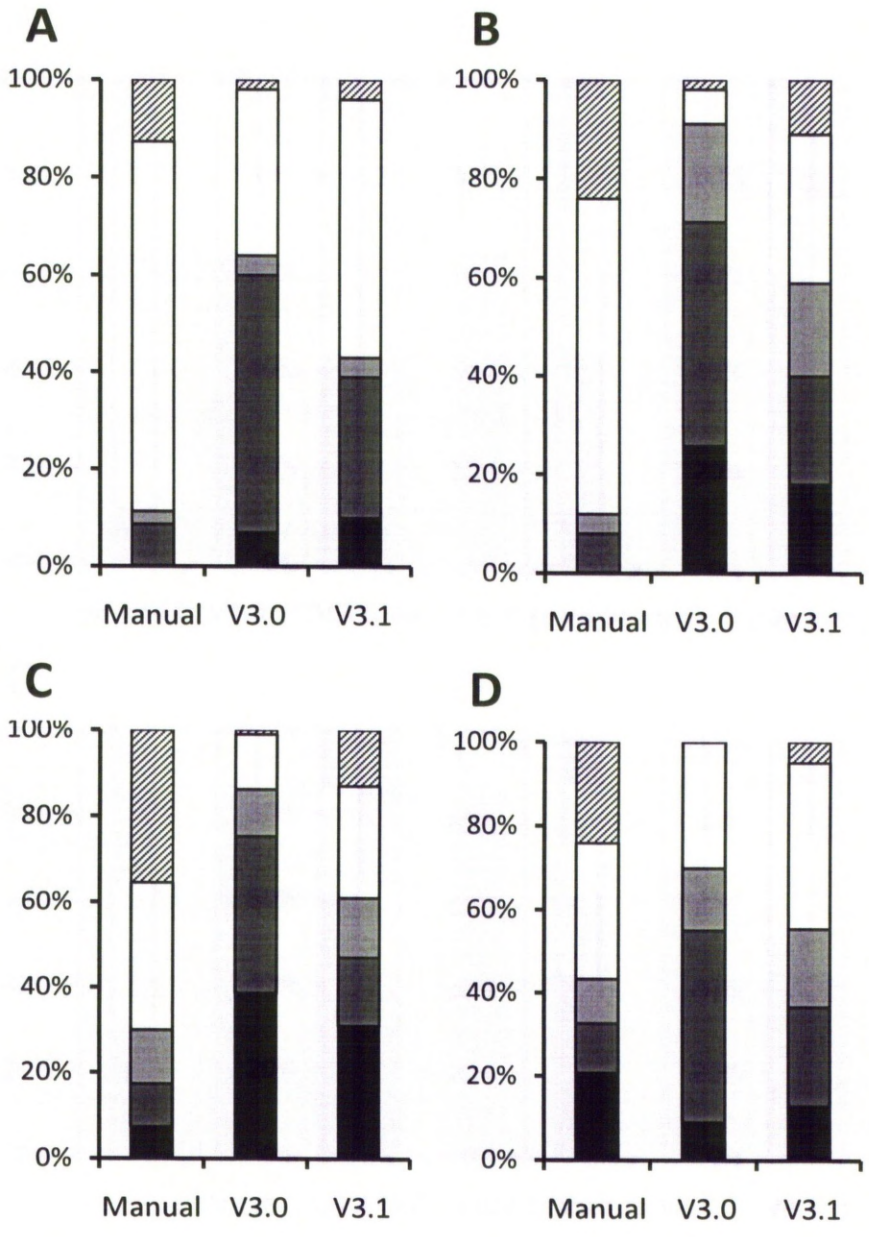


Figure 6.6: Comparison of relative frequencies of subpopulations of *Leishmania mexicana* obtained manually and from image analysis by ParaMorph software V. 3.0 and V.3.1. Cultures were sampled at 24 h, 48 h, 7 d and 8 d (A, B, C and D, respectively) after induction of metacyclogenesis. Manual classification was performed from 50 randomly selected parasites per slide and three smears were performed per time point (n=150 parasites). For *in silico* analysis, 20 photographs were randomly taken per time point. Relative frequencies of subpopulations are represented by different colours in bars. Amastigotes=black, procyclics=dark grey, nectomonads=light grey, leptomonads white and metacyclics=dashed pattern. Relative frequencies are statistically significant between the three groups ($p<0.001$, chi-square)

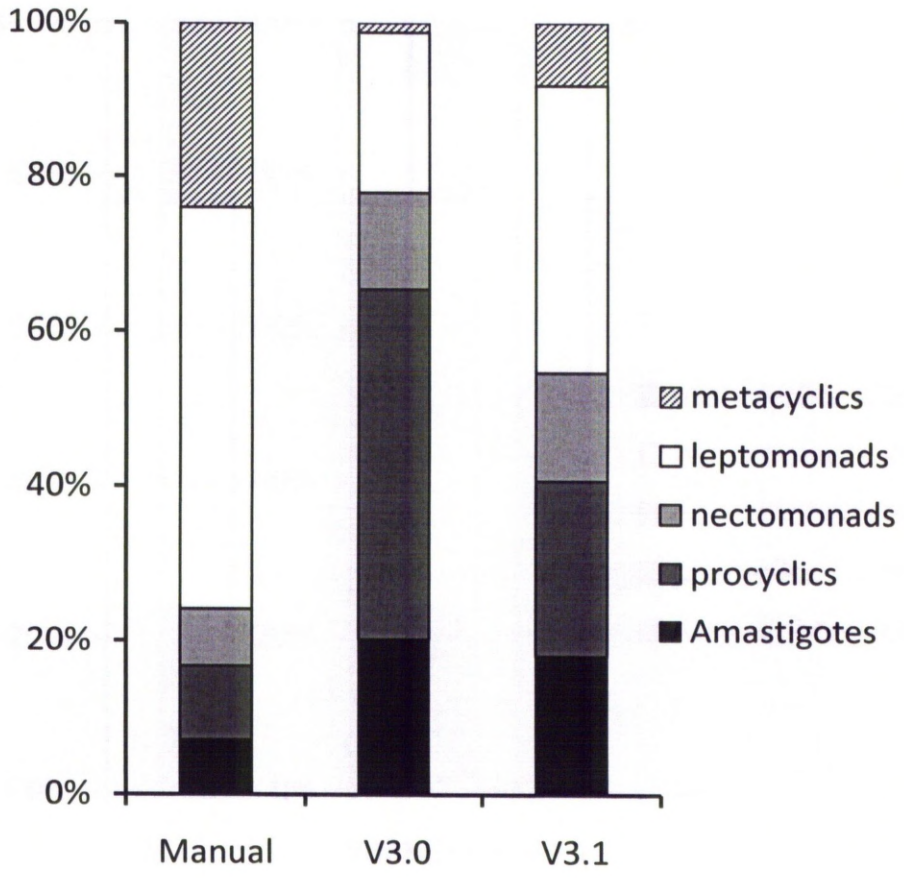
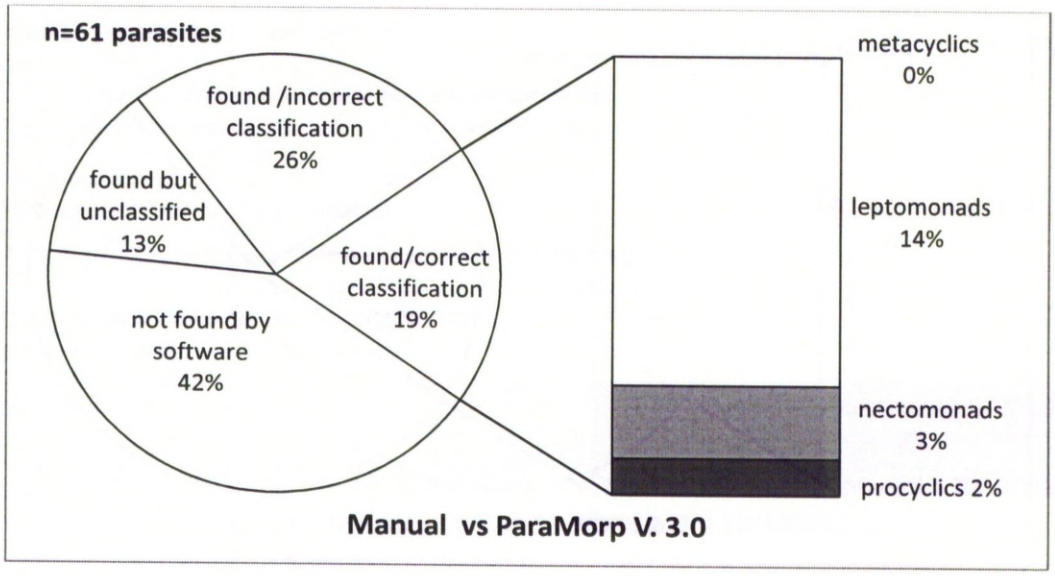


Figure 6.7: Comparison of relative frequencies of subpopulations of *Leishmania mexicana* obtained manually and from image analysis by ParaMorph software V. 3.0 and V. 3.1. Stacked graph represents combined results of all time points from Fig. 6.6. Cultures were sampled at 24 h, 48h, 7 d and 8d after induction of metacyclogenesis. Manual classification was performed from 50 randomly selected parasites per slide and three smears were performed per time point. For *in silico* analysis, 20 photographs were randomly taken per time point. Relative frequencies of subpopulations are represented by different colours in bars. Amastigotes=black, procyclics=dark grey, nectomonads=light grey, leptomonads white and metacyclics=dashed pattern. Relative frequencies are statistically significant between the three groups ($p<0.001$, chi-square) .



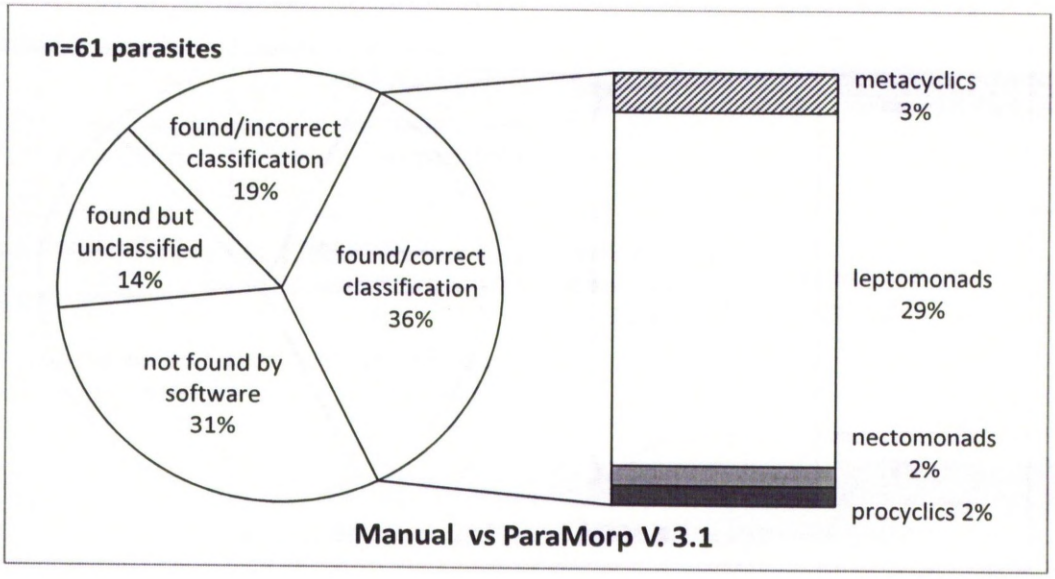


Figure 6.8: Confirmation of parasites classification reported from image analysis by ParaMorph software. One photograph per time point was randomly selected (n=4). Photographs were inspected manually and distinguishable parasites that would have been selected in regular manual classification were considered and compared against data from the software. Parasites from all time points were pooled together (n=61 parasites). Evaluation included the ability of the software to accurately find the parasite in the photograph, to accurately find and classify the parasite, and to accurately find and place the parasite into the correct subpopulation. Pie chart represents relative frequencies.

6.2.5. Limitations of the software

The principal difficulties associated with automating the analysis of parasite morphology in microscope images are two-fold:

Recognition

Identification of the parasites and differentiation from the 'background clutter' within the image (*i.e.,* features that could be easily mistaken for parasites). This is why most of the programming modifications have been performed in the thresholding step. In both versions of the software, the threshold value has to be chosen by the operator based on the native photograph and deciding which threshold value removes most of the background without removing parasite features. The software also is unable to recognise overlapping parasites and overlapping flagella. Also when a flagellum convolutes (overlaps itself), ParaMorph will measure the flagellum from the body junction until the point where it convolutes, reporting a lower length.

Segmentation

For an object identified as a parasite, it is necessary to identify the body and flagellum so that the lengths and widths of both can be measured separately. The challenge in this case is to ensure that the lengths and widths measured for a body are correlated with the length of the right flagellum (*i.e.*, the one that is attached to that body). In practice, this means a bit of image processing and some careful bookkeeping of the data, especially in the situations where more than one flagellum appears to be attached to one body.

6.2.6. Advantages of the software

Although ParaMorph V 3.1 has shown an accuracy of 36%, more development is required for the software to arrive at a version powerful enough to be used in research as a tool to find differences in subpopulation profiles. However, it is interesting to note that despite Paramorph's V. 3.1 inaccuracy of 64 percent in correct classification of parasite subpopulations, it seems that the software is more precise than the human eye for parasite measurement. It is possible that

human observations are underestimating parasite size; especially flagellum length (compare Fig 6.3 vs. 6.4 and 6.5). Both versions of the software report flagella as long as 25 µm, whereas none of the parasites measured manually reached flagella lengths greater than 17 µm. Also, manual mean flagellum length was significantly smaller ($p<0.05$) compared to those calculated by ParaMorph V 3.0 and 3.1 (table 6.2). This is not surprising if we consider how manual measurements are performed under the microscope and the major limitation of this approach (Fig 6.9). Another major advantage of the software approach is the increase in efficiency as well as in number of observations per experiment. It took a few days to manually measure and classify the parasites from all smears in this experiment with a total of 600 parasites Fig (6.3). It took a few hours to take all the photographs and minutes to run them through both versions of the software with thousands of observations (Fig. 6.4 and 6.5).

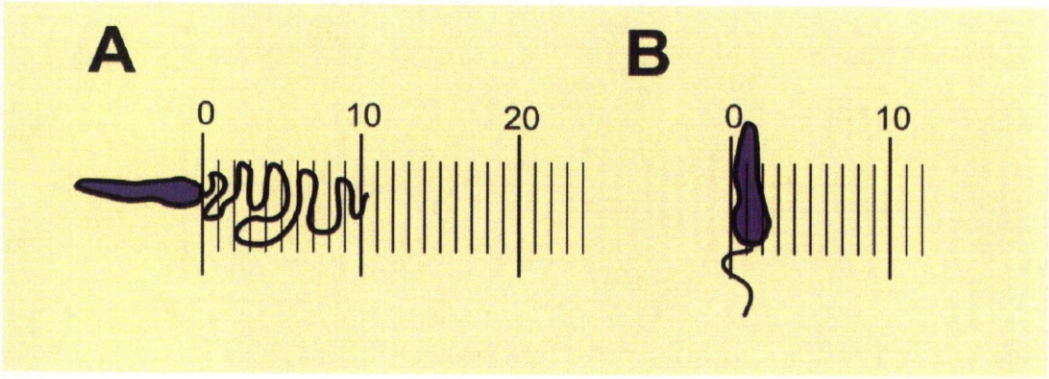


Fig 6.9: Major limitations of manual measurements of *Leishmania* with an eyepiece graticule. A, *sinuous flagellum*. The flagellum is rarely arranged over a straight line making accurate length measurement difficult. *B, graticule resolution limit*. The eyepiece unit has a resolution up to 1 µm. In the parasite depicted, the operator has to decide whether its body width should be considered as 2 or 3 µm.

Table 6.2 Mean values of different morphometric parameters from manual method vs. software (ParaMorph V. 3.0 and V. 3.1). Characters in bold and asterisk represent significant differences at $p<0.05$ compared to manual measurements.

	Body length Mean (µm)			Body width Mean (µm)			Flagellum length Mean (µm)		
	Manual	V 3.0	V 3.1	Manual	V 3.0	V 3.1	Manual	V 3.0	V 3.1
Amastigote	4.43	**3.88***	**4.08***	1.99	**2.92***	**3.06***	-------	-------	-------
Procyclic	9.23	9.06	8.88	1.94	2.41	1.98	7.84	**5.86***	**5.41***
Nectomonad	13.00	12.56	12.38	2.13	2.28	2.00	11.24	8.39	7.18
Leptomonad	8.81	8.56	8.59	1.84	2.47	1.99	10.05	**12.34***	**12.23***
Metacyclic	7.10	**5.31***	**5.05***	1.21	**1.37***	**1.36***	10.10	10.87	9.04

6.2.7. Further development

During the first attempts to perform morphometrics with sperm the cells were contoured manually from images projected in a monitor from a microscope using x100 oil immersion bright field lenses. Once morphometric data were obtained, sperm head/width radio from infertile and fertile human males were found to show significant differences (Katz *et al.* 1986). In another study, human sperm from healthy donors was stained and 283 sperm were selected as prototypic examples of the 10 morphology classes using the teams' pre-existent classification system. Different measurements from spermatozoa were obtained from the stereotypes and used to classify a sperm into either a 'normal' or 'abnormal' category with 95% accuracy and a correct classification of 86% into one of the 10 shape classes (Moruzzi *et al.* 1988).

Perhaps a similar approach should be performed in the future development of ParaMorph; use images of selected parasite subpopulations of *Leishmania mexicana* to get a more reliable morphometric profile of each form, instead of using mixed populations. It is possible to culture a pure population of axenic amastigotes (Bates *et al.* 1992). Metacyclic promastigotes can be separated out from a mixed culture using the biochemical characteristics of the different promastigote forms. One approach would be to separate based on changes in the lipophosphopglycan (LPG) profile of flagella using D-galactose-binding lectins (Sacks *et al.* 1985) perhaps by attachment on sepharose beads. This would provide an enriched sample of promastigotes based on their lectin binding profile. Metacyclics can also be recovered from a mixed culture by density gradient

centrifugation (Spath and Beverley 2001). Other parasite forms might be more difficult to isolate in culture, but leptomonad procyclics are present in large numbers in PSG plugs of infected flies followed by metacyclics (Rogers *et al.* 2002), so it is possible to isolate at least those two parasite forms, prepare slides and capture images from them. Computer-assisted measurements from these pre-selected subpopulations might be more precise than human ones and perhaps, challenge the existing parameters of morphometric data of *Leishmania mexicana*.

CHAPTER 7

CONCLUSIONS AND FURTHER RESEARCH

The purpose of this study was to characterise and analyze the gene expression of different antioxidant enzymes throughout blood digestion in the midgut of the phlebotomine sand fly *Lutzomyia longipalpis* and to examine the role of ROS and antioxidant enzymes in immunity against the kinetoplastid protozoan *Leishmania mexicana* and the Gram-negative enteric bacteria *Serratia marcescens*. The following paragraphs present some key conclusions and suggestions for further research:

Antioxidant genes were differentially expressed throughout blood digestion

Different sequences for putative antioxidant enzymes were found in a whole body cDNA library of *Lu. longipalpis*. However only putative antioxidants, also present in midgut-specific libraries, were selected for this research; namely catalase, superoxide dismutase and peroxiredoxin. Oxidation resistance protein 1 was also considered since evidence in other nematocerans suggested a possible role of this enzyme in antioxidant regulation. Overall, the expression profile of the majority of antioxidants exhibited an increment that peaked at 48 hours and a subsequent reduction up to 96 hours after bloodfeeding. This pattern matched that of hydrolytic digestive enzymes during blood digestion reported in previous studies. This boost of antioxidant gene expression has been found in other haematophagous insects and the data add to the cumulative evidence underpinning the role of antioxidant enzymes in protecting the midgut from toxic blood-derived reactive oxygen species. Although informative, quantification of gene expression by reverse-transcription of mRNA provides a pre-translational picture that could be improved. The next step forward would be to include the use of protein-profiling. There are no *Lu. longipalpis* antibodies commercially available but antibodies from close-related species (*i.e. Anopheles* or *Drosophila*)

might cross-react with *Lu. longipalpis* proteins to analyse changes in protein levels during blood digestion through Western-blotting.

Antioxidant genes were well conserved throughout different insect species

Most of *Lu. longipalpis* predicted antioxidant genes sequences displayed a high degree of conservation at active and catalytic sites compared with different insect species. One exception was the Oxidation Resistance Protein 1 —which only showed conservation for the TLDc domain with *Glossina* and *Drosophila*.

The group that shared most amino acid sequence similarities with *Lu. longipalpis* was Nematocera. This is an order which includes *Aedes aegypti* and *Anopheles gambiae*. These mosquito species, just like phlebotomine sand flies, are sugar feeders that will take blood only a few times throughout their lifetime and are also vectors of infectious diseases. There are several studies published on vector-microbe interactions for mosquitoes, especially *Anopheles*, which lay the ground work to understand the dynamics of parasite-vector interactions in the context of insect immunity. The fact that sand flies share many features at genomic and ecological levels with their anopheline counterparts suggests the exploitation of anopheline data as an experimental compass. Some of the most relevant findings in *Anopheles-Plasmodium* relationships should be experimentally examined in the *sand fly-Leishmania* model.

Gene profiling was different in RT-PCR and microarrays

One of the major reasons antioxidants were selected for this study was that the sequences were found to be differentially expressed in a *Leishmania*-infected vs. non-infected whole body *Lu. longipalpis* microarray (Dillon, unpublished). The method of choice for midgut-specific gene expression profiling/microarray validation was semiquantitative RT-PCR. Genes from both profiles were differentially expressed but expression patterns were not similar between techniques. This was expected as the microarray was developed from whole bodies whereas the RT-PCR was midgut-specific and antioxidants are expressed in other tissues. RT-PCR profiling evaluates gene expression semi-quantitatively

after image analysis of band intensity. In experienced hands this technique yields very useful results, useful in providing evidence towards differential expression. But it is also true that technology for gene profiling has evolved over the past few years since this research started. Perhaps the best candidate for future gene profiling would be real-time RT-PCR (qRT-PCR). Although more expensive, this procedure not only allows a quantitative analysis of expression, it also provides more robust results since multiple housekeeping genes can be included in the experiments. Although not following the same patterns, antioxidant enzymes exhibited changes in expression after bloodfeeding in both microarray and RT-PCR analysis. Interestingly, differences in expression in RT-PCR were more dramatic compared to those derived from the microarray. These results are encouraging since even though no putative genes exhibited large differences in up or down regulation from microarray data of *Leishmania*-infected vs. non-infected sand flies, perhaps moderate ones will prove larger when profiled by qRT-PCR or if explored using a proteomic approach.

Sand fly fecundity and longevity was affected by redox balance

Data from gene profiling analysis suggest that catalase can be quite relevant throughout blood digestion and *Leishmania*-interactions and hence a good candidate for gene knockdown in the context of *Leishmania*-sand fly interactions. RNAi-mediated silencing of catalase led to an interesting change in phenotype with sand flies that exhibited less eggs compared to negative controls but similar to those from older flies. The finding that antioxidant supplementation conferred an increase in eggs whilst there was an accumulation of catalase in the developing egg (as reported in *Anopheles* by others) highlighted the importance of oxidative stress in fecundity. Catalase silencing also led to a significant reduction in longevity, a change in phenotype previously observed in genetically antioxidant-depleted *Drosophila melanogaster* which (among other findings) fuelled the oxidative stress theory of ageing during the 1990s. Oral supplementation of flies with vitamin C as a ROS sequestrator was used to explore redox balance and longevity. Contrary to what was initially expected, dietary addition of an exogenous antioxidant had the opposite effect on longevity.

A higher phenoloxidase activity in the vitamin C-supplemented group of flies suggested that such an increase in mortality could be associated with bacterial infections. These findings encouraged the exploration of the role of ROS in regulating resident microbial populations in the sand fly gut.

Antioxidant genes were differentially expressed in *Leishmania* and *Serratia* colonised guts

L. mexicana-colonised sand flies exhibited an overall reduction in relative expression of antioxidant enzymes during blood digestion while *S. marcescens*-infected guts displayed an opposite trend. The genes that showed the highest difference in expression upon *Leishmania* and *Serratia* challenge were catalase and superoxide dismutase. Higher expression of antioxidant enzymes during bacterial infections was associated with an observed increase in oxidative stress in the midgut. Preliminary experiments suggest that *Serratia* is able to produce ROS *in vitro*, specifically hydrogen peroxide (Sant'Anna, Diaz-Albiter, Dillon unpublished). Future experiments towards understanding *Serratia* entomopathogenicity should address whether ROS released by this bacterium are a virulence factor and also how does the sand fly host react to it.

The downregulation of catalase by *L. mexicana* was particularly striking and worth exploring through alternative avenues. Is *Leishmania* increasing the oxidative stress inside the midgut by reducing catalase expression? It is very unlikely. The present study suggested that ROS were not significantly higher in *Leishmania*-colonised midguts and that catalase depletion was detrimental to the protozoan. Future experiments should focus on whether reduction in catalase is a) a result of negative feedback from a *Leishmania*-derived ROS sequestration or b) a consequence of reduction in blood-derived ROS release via a decrease in proteolytic activity by *Leishmania*. In the first hypothesis it should be considered that *Leishmania* is able to detoxify hydrogen peroxide *in vitro* (as shown here and by others) and this can perhaps be achieved via peroxiredoxin activation. One could envision a case in which *Leishmania* employs its own set of antioxidant enzymes to detoxify blood-derived ROS during digestion and avoid detrimental

oxidative stress. Lower ROS levels might result in a reduction in sand fly-expressed antioxidant enzymes that would be redundant. Experimental approaches to answer this could include infections with *L. mexicana* mutants unable to express functional peroxiredoxins. This hypothetical protection of the midgut by the protozoan could potentially challenge the current status of *Leishmania* as a sand fly 'parasite' and make it more of a commensal.

The second hypothesis is based on the fact that *Leishmania* reduces the expression of proteolytic enzymes during blood digestion through an unknown mechanism. A reduction in digestive enzymes activity might reduce the release of blood-derived ROS. Future experiments should also explore the mechanism through which *Leishmania* modifies proteolysis in the gut, perhaps by looking at differences in expression of genes regulating digestion upstream.

Pathogenic bacteria and not *Leishmania* increased midgut ROS

Infection of sand flies with *Serratia* resulted in significantly higher concentration of hydrogen peroxide at 48 h post-bloodfeeding, as well as of oxidized compounds of dihydroethidium (DHE) from 24 to 72 h post-bloodfeeding. DHE can be oxidised by compounds other than the ROS superoxide therefore it should be addressed in further experiments whether fluorescence in *Serratia*-infected midguts was due to the presence of superoxide. However, similar assays performed in *Aedes aegypti* have incriminated superoxide as the major oxidative agent (Oliveira *et al.* 2011). One of the major disadvantages of using biochemical assays to understand ROS and midgut immunity is that assays are not specific enough to discriminate whether the increment in ROS is caused by an active release by the host as a defence mechanism against the pathogen or produced as consequence of cell-death (as in apoptosis). *Drosophila* is able to fight enteric pathogens by regulating ROS and this is orchestrated in the gut epithelium by two main actors: the dual oxidase Duox and an Immune regulated Catalse (IRC). Unfortunately it was not possible to find any Duox or IRC homologues in any of the cDNA libraries available during this study. However, a preliminary genome assembly obtained with our Jacobina strain of *Lu. longipalpis* has been published

online by the Baylor College of Medicine. Recent BLAST search of *Drosophila* Duox homologues has retrieved promising candidate sequences (Dillon pers. comm.). The future steps to find out if *Lu. longipalpis* is able to mount a ROS-mediated immune response regulated by a similar mechanism as *Drosophila* include performing RNAi-mediated silencing of Duox in sand flies followed by a challenge with ROS-sensitive bacteria. Duox expression of *Leishmania*-colonised sand flies could also provide new data to confirm that *Leishmania* does not elicit a ROS mediated immune response as suggested by this study.

Pathogenic and resident bacteria are affected by the redox balance in the gut

Abrogation of endogenous antioxidants such as catalase had a negative effect on sand fly survival. However, dietary administration of antioxidants such as vitamin C had the same effect. This was intriguing at the time but made sense from the point of view of ROS-mediated gut microbe regulation in other dipterans such as *Drosophila*, *Anopheles* and *Aedes*. In the present work it was shown that mortality and phenoloxidase activity was higher in antioxidant-supplemented sand flies. To discount the fact that mortality was due to a possible toxicity of vitamin C and to incriminate bacterial infection as the cause of mortality, the experiment was repeated with a different antioxidant and flies were infected with *Serratia marcescens*. Infected sand flies fed on uric acid-supplemented sugar meal displayed the same mortality trend exhibited in previous experiments with vitamin C. However, contrary to our initial hypothesis, *Serratia* CFU numbers turned out to be significantly lower in antioxidant supplemented sand flies. Furthermore, resident microbiota growth behaved in the exact opposite way. This was further confirmed in CFU counts from insects not infected with *Serratia*.

It is still intriguing that *Serratia* numbers were lower in the group with the highest mortality, it was speculated that this was be due to sampling bias since survivors collected 48 after inoculation might have been more resistant to infection. *Serratia* is a very efficient insect pathogen which is not only able to detoxify ROS via catalase expression, but also to secrete hydrogen peroxide (Sant'Anna,

unpublished). Future experiments exploring ROS-mediated microbe regulation in the gut are already being performed with less pathogenic bacteria and sampling should avoid bias of survivors. A good candidate might be *Asaia.* Species of this Gram-negative acetic acid bacteria were discovered in wild *Lu. longipalpis* (Dillon, unpublished), and preliminary experimental infections in sand flies have shown lower sand fly mortality associated with the presence of this potential symbiont. DHE-staining of the midgut (a strong indicative or ROS activity) suggest that the sand fly response to *Asaia* is similar to that of the *Leishmania*; no significant difference in superoxide production as detected by staining for DHE (Sant'Anna, Diaz-Albiter, Al Salem ,Dillon unpublished). A further intriguing twist in this story is that *Asaia* produces significantly higher amounts of its own ROS; perhaps ten times higher than other bacteria such as *Serratia*. Furthermore, *Asaia* was shown to prevent colonisation of the sand fly gut by *Leishmania* (Sant'Anna, Diaz-Albiter, Genta, Dillon, unpublished) The recent demonstration that the anti-plasmodial effect of a commensal bacterium in the anopheline insect gut may be due to bacterial generation of ROS (Cirimotich *et al.* 2011) further emphasises the complexity of the ROS mediated interactions and their importance in gut immune homeostasis in insect vectors of medically important parasites.

Morphometric analysis of *Leishmania mexicana* subpopulations could be achieved by software-based image analysis

All *Leishmania* infections performed in the present study were quantified by direct counts of microorganisms in haemocytometers. Significant differences in total number of *Leishmania* are of course relevant in experiments that aim to lower vectorial capacity of sand flies. But what if an experimental condition changes parasite subpopulation profiles that are likely to be missed by total counts? Throughout the duration of this work, a collaborative work with colleagues from the Department of Physics, University of Liverpool, resulted in the development of ParaMorph, a computer program designed to differentiate and count *Leishmania* subpopulations from photographs of microscopic slides by analysis of morphometric data. ParaMorph was tested against observations from

a human operator. The latest version (V. 3.1) was able to correctly categorize 36 percent of parasite forms, which was an improvement of 17 % over previous ParaMorph V. 3.0. Comparison of manual vs. ParaMorph data also suggested that the current morphometric data available for *L. mexicana* might be underestimated, especially flagellum length. The ultimate aim of developing a working version of ParaMorph is now in sight but awaits a further version trained with separated parasite subpopulations (metacyclics, leptomonads) to obtain morphometric data directly from separated *L. mexicana* subpopulation preparations.

Placing *Leishmania* in a microbial ecology context

Any study of the interactions of *Leishmania* with its vector should be placed into the context of the microbial ecology of the sand fly and its response to the myriad forms of microbes encountered during its life; firstly as a larva in composted animal droppings through to the encounters as an adult with mammals and plants. This study of antioxidant genes and ROS production in *Lu. longipalpis* has opened a new area of study of the interactions between sand flies, *Leishmania* and other microorganisms. Studies on transmission and vectorial capacity have become multidimensional only in the last few years in other species such as *Anopheles* and *Aedes*. As we uncover more aspects of the response and counter – response of insects and their microbes and medically important parasites we become acutely aware that there are still important pieces of the puzzle remaining to be discovered. Research which includes the study of gut microbes will provide us with context and more coherent, efficient approaches for developing transmission blocking strategies and vector control. This has already started in *Anopheles* and *Aedes*. Leishmaniases are neglected diseases; it is about time we stop neglecting sand fly research as well.

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