

Apoptosis-like death as a feature of malaria infection in mosquitoes

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SUMMARY

Malaria parasites of the genus *Plasmodium* make a hazardous journey through their mosquito vectors. The majority die in the process, many as a result of the action of mosquito defence mechanisms. The mosquito too is not unscathed by the encounter with these parasites. Tissue damage occurs as a result of mid-gut invasion and reproductive fitness is lost when many developing ovarian follicles are resorbed. Here we discuss some of the mechanisms that are involved in killing the parasite and in the self-defence mechanisms employed by the mosquito to repair the mid-gut epithelium and to manipulate resources altering the trade-off position that balances reproduction and survival. In all cases, cells die by apoptotic-like mechanisms. In the midgut cells, apoptosis-induction pathways are being elucidated, the molecules involved in apoptosis are being recognised and *Drosophila* homologues sought. The death of ookinetes in the mosquito mid-gut lumen is associated with caspase-like activity and, although homologues of mammalian caspases are not present in the malaria genome, other cysteine proteases that are potential candidates have been discussed. In the ovary, apoptosis of patches of follicular epithelial cells is followed by resorption of the developing follicle and a subsequent loss of egg production in that follicle.

Key words: Apoptosis, *Plasmodium*, malaria, mosquito cysteine proteases.

INTRODUCTION

Mosquitoes play an essential role in the transmission and spread of malaria. If a female mosquito ingests blood containing *Plasmodium* gametocytes, the male and female gametes emerge from their erythrocytes within minutes of arriving in the mosquito midgut lumen and fertilization occurs within the first hour post-feeding. The zygote differentiates into a motile ookinete 8–24 hours later, moves out of the blood bolus and migrates across the peritrophic matrix, a chitin-containing layer secreted by the midgut epithelial cells in response to blood feeding. Ookinetes invade the single layer of columnar midgut epithelial cells, often travelling laterally through one or more adjacent cells, and finally exit on the basal side of the cells, coming to rest between the cell membrane and the basal lamina in the basal subepithelial space. Here they transform into a non-motile vegetative stage, the oocyst. The oocyst grows from about 5 to 50 μm in diameter whilst the nucleus divides approximately once a day to produce 2–8000 nuclei in 12–18 days. Following division of the cytoplasm, sporozoites develop at the edge of the oocyst and are eventually released into the haemolymph. A small proportion of these invade the lateral lobes of the

salivary glands. Eventually a small number of sporozoites are injected into a new host when the infected mosquito takes the next blood meal (Sinden, 2002; Baton and Ranford-Cartwright, 2005*b*).

Although several species of *Plasmodium* can readily be maintained in laboratory colonies of mosquitoes, several factors mitigate against the successful completion of the mosquito stage of the life cycle in the field. *Plasmodium* has a surprisingly long developmental period relative to the life expectation of a mosquito in the wild, and many infected mosquitoes will die before sporozoites have invaded the salivary glands. Furthermore, the few mosquito species that can act as vectors of *Plasmodium* have robust anti-parasite defence mechanisms; although these are more effective in some vector/parasite combinations than others. Mosquitoes thus exhibit varying degrees of refractoriness and this will determine prevalence and/or intensity of infection within a mosquito population (Ghosh, Edwards and Jacobs-Lorena, 2000; Sinden, Alavi and Raine, 2004). Our understanding of these defence mechanisms is expanding rapidly and has been the subject of many reviews including recent ones by Michel and Kafatos (2005) and Vlachou and Kafatos (2005).

The most significant loss of malaria parasites occurs in the first 24 h following an infective blood meal. This also appears to be a critical time for the infected mosquito. During this period, infection inflicts considerable fitness costs upon the mosquito because ookinete invasion of mosquito-midgut cells

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results in cell death and expulsion from the epithelial layer. The production of a batch of eggs that is taking place at this time is severely impaired due to the death of cells in the follicular epithelium of many ovarioles. It is clear that the complex molecular interactions between *Plasmodium* and its vector that occur at this time help to establish and maintain the infection and may ensure that the vector remains alive for long enough for sporozoites to develop and parasite transmission to occur. Evolutionary pressures will be operating upon the mosquito resulting in selection in favour of survival following infection. They will also be operating on the parasite to maximise its fitness without compromising vector survival; at least until transmission is ensured. We have proposed that death of parasite and vector cells plays a key role in this relationship (Hurd and Carter, 2004). In this review we discuss the mode of death of parasite and vector cells, potential triggers that induce this death and the role this death may have in the maintenance of the infection.

MOSQUITO DEFENCE RESPONSES AGAINST MALARIA

A complex anti-parasite response is mounted by the mosquito within 24 h of taking a *Plasmodium*-infected blood meal. Several defence molecules have already been implicated in this response, including antimicrobial peptides, host pattern recognition receptors (PRRs) such as peptidoglycan recognition proteins, Gram-negative binding proteins, thioester containing proteins (TEPs), scavenger receptors (SCRs), C-type lectins (CTLs) and molecules in the melanisation cascade (Michel and Kafatos, 2005). The genes encoding some of these molecules exhibit transcriptional activation or up-regulation in response to *Plasmodium* infection (Richman *et al.* 1997; Dimopoulos, 2003; Danielli *et al.* 2005) and a few have now been implicated in parasite-killing or protection from death, as demonstrated by the change in oocyst numbers when gene expression is altered (Blandin and Levashina, 2004; Osta *et al.* 2004b; Abraham *et al.* 2005).

Attention has been focused on the death of ookinetes as they traverse the midgut epithelium and exit beneath the basal lamina. Two processes have been reported to occur, namely, melanisation and 'lysis' of ookinetes. In a few strains of mosquito, specifically selected to be refractory, ookinetes are melanised as they emerge from epithelial cells and come into contact with molecules in the haemolymph (Collins *et al.* 1986; Zheng *et al.* 2003; Hurd *et al.* 2006). However, it would appear that, even in susceptible mosquitoes, many parasites disappear before oocyst formation. This disappearance mechanism has been termed 'lysis', though cell lysis is rarely visualized, and is likely to be mediated by the antagonistic action of the leucine rich-repeat

immune gene, *LRIM1* (Osta *et al.* 2004a). These parasites can be protected from death by a mechanism mediated by 2 members of the C-type lectin family (Osta *et al.* 2004a). Ookinetes undergoing lysis have been observed in mid-gut cells of a refractory line of *Aedes aegypti* (Vernick *et al.* 1995). However, this has not been observed in the midgut epithelium of anopheline mosquitoes and it is not clear whether disappearance of parasites in *Anopheles gambiae* occurs in the epithelial cells or, prior to invasion, in the mid-gut lumen.

THE MID-GUT LUMEN IS A HOSTILE ENVIRONMENT

In addition to the response mounted against the parasite by the mosquito, toxic components from the blood meal surround the developing ookinetes. Within the bolus of the blood meal, zygotes and ookinetes are directly exposed to white blood cell products and also serum and plasma factors. All of these reduce gametocyte infectivity (Lensen *et al.* 1998). A reactive oxygen-rich environment is created, partly as a result of the release of oxidative haem when haemoglobin is digested. The mid-gut environment may, in this respect, resemble the situation surrounding developing trophozoites in the vertebrate host (Atamna and Ginsburg, 1993). The cytotoxic effect of O_2^- upon *Plasmodium berghei* has been reported and O_2^- has been implicated in loss of gametocyte infectivity (Harada *et al.* 2001). In addition to the generation of O_2^- by haemoactivity within the mid-gut lumen, L-DOPA-dependent generation of O_2^- occurs in the mid-gut epithelium and it is suggested that parasite invasion may induce transportation of L-DOPA from the haemolymph into mid-gut epithelial cells (Lanz-Mendoza *et al.* 2002). The latter authors reported that *P. berghei* ookinetes differ in their susceptibility to O_2^- . Interestingly, the identification of peroxidase active organelles in the trophozoites of *P. falciparum* suggests the presence of antioxidant defensive mechanisms in *Plasmodium*. However, database searches of the *P. falciparum* genome failed to identify genes encoding peroxisomal enzymes or the classical peroxisomal import systems (McIntosh, Elliott and Joiner, 2005). In contrast, cytosolic redox systems that protect against oxidative insults resulting from the asexual stage's metabolism of haemoglobin are well documented and reviewed in Muller (2004) and a potential antioxidant, lipoic acid, that may be synthesised by the apicomplast could also be utilized as an antioxidant by the parasite (Toler, 2005).

NITRIC OXIDE

One molecule that has been particularly implicated in the killing of *Plasmodium* is nitric oxide (NO).

Inducible NO is synthesised during the oxidation of L-alanine to L-citrulline; catalysed by inducible NO-synthase enzymes (iNOS). NO rapidly forms reactive nitrogen intermediates (RNIs).

NO has long been known to be an anti-pathogen agent, although its pleiotrophic effects are dependent upon its concentration, location and reaction with superoxide. The complex relationship between NO and *P. falciparum* infections in the vertebrate host is still unresolved (Clark, Rockett and Burgner, 2003). It is likely that the bioavailability of NO in the vasculature of malaria-infected hosts is low, due to NO scavenging by haemoglobin and O_2^- , and there is evidence that NO does not have a killing effect on rodent-malaria (reviewed in Sobolewski *et al.* 2005) although this conclusion is controversial (Balmer *et al.* 2000). It appears that NO-derived products such as nitrosothiols, rather than NO itself, are toxic to the parasite (Rockett *et al.* 1991).

The situation in the insect midgut lumen may, however, be different compared with that in the vertebrate. Several workers have demonstrated that RNIs reduce malaria transmission by affecting gametocyte infectivity, and this inactivation has been associated with the presence of white blood cells (Motard *et al.* 1993; Naotunne *et al.* 1993). Furthermore the killing effect of NO on ookinetes has been demonstrated by trypan blue staining (Herrera-Ortiz *et al.* 2004).

In addition to NO generated within the blood meal, the expression of an inducible form of the *A. stephensi* NOS gene (*AsNOS*) has been shown to increase transiently in *P. berghei*-infected mosquitoes. Dietary provision of the NOS substrate, L-arginine, or a NOS inhibitor, L-NAME, reduced or increased oocyst numbers in infected mosquitoes, respectively; demonstrating that parasite numbers are limited by NO (Luckhart *et al.* 1998). *P. berghei*-infected blood meals also induce iNOS in the midguts of *A. pseudopunctipennis*, as does L-DOPA; indicating the participation of H_2O_2 (Herrera-Ortiz *et al.* 2004). *P. falciparum* has been shown to induce expression of *AsNOS* as early as 6 h post-infection (Crampton and Luckhart, 2001; Lim *et al.* 2005).

There is evidence to support the proposition that one inducer of *AsNOS* expression is an immunomodulatory cytokine from the host, namely mammalian transforming growth factor β 1 (TGF β 1). This factor plays a role in regulating inducible NOS in vertebrates. TGF β 1 is present in the blood in an inactive form and is activated in the midgut lumen, probably by haem or the redox product of NO, nitroxyl anion. It significantly reduces the number of *P. falciparum* oocysts developing on the midgut wall and induces NOS expression in an *A. stephensi* cell line (Luckhart *et al.* 2003). Interestingly, a member of the TGF β super-family, *As60A*, has been isolated from *A. stephensi* and is expressed in the midgut, brain, eyes, fat body and

ovaries. In the mosquito carcass, induction of *As60A* was detected 1 h after an infected blood meal, earlier than the induction seen at 3 h in the midgut. The expression of both *As60A* and *AsNOS* appears to be correlated with the intensity of parasite density, with high densities inducing expression and low densities suppressing it (Crampton and Luckhart, 2001).

There now appears to be a third source of NOS inducers: the parasite itself. Luckhart and colleagues have shown that the glycosylphosphatidylinositol (PfGPI) anchors of *P. falciparum* also induce *AsNOS* expression in midgut epithelial cells. Within the gut lumen PfGPIs could be derived from both sexual and asexual stages of *Plasmodium*. Merozoites act upon an *Anopheles stephensi* cell line to induce *NOS* expression in a dose-dependent manner, as do purified PfGPIs (Lim *et al.* 2005). This is in line with other studies showing that vertebrate-host proinflammatory cytokines and NO can be induced by parasite GPIs, and may be major factors that contribute to malaria pathogenesis (Tachado *et al.* 1996; Zhu, Krishnegowda and Gowda, 2005).

PARASITE DEATH

Oxygen radicals, nitric oxide and reactive nitrogen intermediates (RNIs) are known inducers of programmed cell death or apoptosis (Murphy, 1999; Bai *et al.* 2001) as discussed in Hurd and Carter (2004). There is evidence that H_2O_2 causes the death of *P. berghei* ookinetes (Herrera-Ortiz *et al.* 2004) but it is unclear whether this death equates to the apoptotic-like death observed in ookinetes *in vivo* and *in vitro* (Al-Olayan, Williams and Hurd, 2002). However, what is certain is that very few of the thousands of mosquito stages that begin their development in this hostile environment survive to become oocysts (Vaughan, Hensley and Beier, 1994a; Vaughan, Noden and Beier, 1994b; Alavi *et al.* 2003). Data for the reduction in numbers of *P. falciparum*, *P. yoelii* and *P. vivax* as they pass through different stages in development has been tabulated in Ghosh *et al.* (2000). These figures reflect large differences in the successful survival of the parasite in different malaria/mosquito species combinations, with losses ranging from 40- to 1223-fold at the gametocyte to ookinete stage and 69- to 660-fold from ookinetes to oocysts. This latter loss is usually detected by counting ookinetes in the midgut lumen of a cohort of mosquitoes and then examining the midguts of a further group of mosquitoes for the presence of oocysts several days later. Although it has been assumed that this loss occurs during the transit of the midgut epithelial layer (Whitten, Shiao and Levashina, 2006), there is evidence that a large proportion of ookinetes die whilst still in the midgut lumen, and that this death rate increases the longer they are exposed to the increasingly toxic

environment (Al-Olayan *et al.* 2002). It is feasible that NO and/or RNIs and O_2^- are the triggers that induce ookinete death. We propose that parasites die in the midgut lumen by a process of apoptosis and that this may be equivalent to the 'lysis' discussed by many authors.

INDICATORS OF APOPTOSIS-LIKE CELL DEATH IN *PLASMODIUM*

Apoptosis in *Plasmodium* spp. was first demonstrated when one marker of apoptosis, nuclear chromatin fragmentation, was detected in the erythrocytic stages of chloroquine-sensitive *P. falciparum* which had been exposed to the drug *in vitro* (Picot *et al.* 1997). More recent work has reported that apoptosis can be detected in both stressed cultures and untreated healthy cultures of erythrocytic stages (Deponte and Becker, 2004). However, there was significant increase in the *in situ* terminal-deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive apoptotic schizonts treated with anti-malarials and H_2O_2 , compared to the control (Deponte and Becker, 2004). Results of further experiments, conducted by these authors, to demonstrate externalization of phosphatidylserine from the inner to the outer leaflet of the parasite plasma membrane were confounded by the two additional membranes, the parasitophorous vacuole membrane and the erythrocyte membrane, that surround the parasite plasma membrane. Contamination of the parasite preparation with erythrocyte ghosts may have led to false-positive staining with annexin V.

Multiple features of apoptosis, such as chromatin condensation, detected with acridine orange labeling; DNA fragmentation, as detected by TUNEL labeling; phosphatidylserine translocated to the external leaflet of the plasma membrane, as detected by annexin labeling; and activation of a caspase-like enzyme activity (Fig. 1) have been observed in *P. berghei* ookinetes. Apoptosis occurs both *in vivo*, in the mosquito gut lumen, and *in vitro* (Al-Olayan *et al.* 2002). Apoptosis in *P. berghei* was inhibited by the general caspase inhibitors, z-VAD.fmk and Boc-ASP, and the caspase 3-specific inhibitor, z-DEVD.fmk, thus confirming the involvement of caspase-like activity in cell death in *Plasmodium*. *In vitro*, the presence of ookinetes exhibiting condensed chromatin was reduced from 80% to less than 10% by incubation with the caspase inhibitor z-DEVD.fmk and, *in vivo*, addition of z-VAD.fmk to the blood meal resulted in a reduction in the number of ookinetes exhibiting condensed chromatin from approximately 70% to 20%. Moreover, inclusion of a caspase inhibitor in the blood meal resulted in double the number of oocysts developing on the midgut wall *in vivo*, suggesting that apoptosis plays a major role in the mechanism that governs parasite

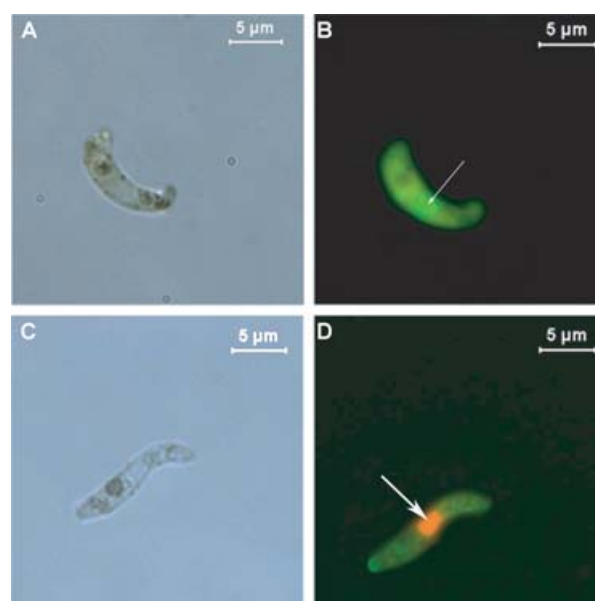


Fig. 1. *Plasmodium berghei* ookinetes exhibiting features of apoptosis-like cell death. A: ookinete viewed under phase contrast, B: the same ookinete viewed under conditions that show staining with acridine orange and containing a nucleus with condensed chromatin (thin arrow). C: ookinete viewed under phase contrast, D: the same ookinete viewed under conditions that show caspase-like activity in the cytoplasm after labelling with CaspaTagTM (Intergen Company) (green). The nucleus is stained with Hoechst stain (thick arrow).

numbers in the mosquito midgut lumen (Al-Olayan *et al.* 2002). The involvement of caspase-like activity must, however, be interpreted with caution, as Deponte and Becker (2004) pointed out, since nothing is known about uptake and stability of caspase inhibitors in *P. falciparum*.

CASPASE-LIKE ACTIVITY

In *P. berghei*, two different methods employing caspase-specific inhibitors have been used to detect a caspase-like enzyme in ookinetes: (1) whole cells were labeled with a fluorescently labeled caspase inhibitor (CaspaTag, a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor, FAM-VAD.fmk) (Fig. 1). This labeling correlates well with another marker for apoptosis, chromatin condensation (as visualized by acridine orange staining) (Fig. 1). (2) Ookinete cell extracts were incubated with a biotinylated caspase inhibitor (biotin-VAD.fmk) and the proteins which bound the inhibitor were subsequently visualized on a Western blot (Al-Olayan *et al.* 2002).

Therefore, *P. berghei* appears to possess a caspase-like activity that correlates with other markers of apoptosis. However, interrogation of the genome databases of *P. berghei* and the closely related *P. falciparum*, reveals that *Plasmodium* does not appear to possess a classical caspase enzyme.

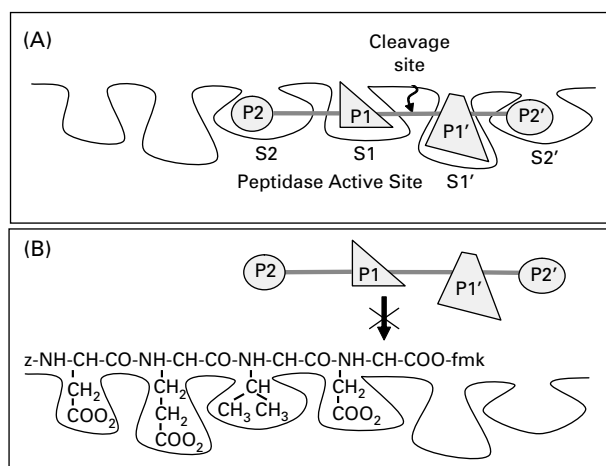


Fig. 2. Diagrammatic representation of the active site of a protease enzyme. (A) P1, P2, P1' and P2' refer to amino acid residues in the protein/peptide substrate relative to the cleavage site. S1, S2, S1' and S2' refer to the sub-sites within the active site of the enzyme. These bind amino acid residues in the protein or peptide substrate with different affinities and thus confer the specificity of the proteolysis. (B) protease inhibitors, such as z-DEVD-fmk, bind irreversibly into the active site and prevent binding of the substrate.

This calls into the question the true intracellular target of the 'caspase-specific' inhibitors.

Caspases are cysteine proteases belonging to the clan CD (Barrett and Rawlings, 2001). They possess a conserved caspase fold and a catalytic dyad, His and Cys, which are indispensable for proteolytic activity. One of the main limitations of working with caspase inhibitors is that their specificity is not as tight as once believed. These inhibitors do demonstrate remarkable specificity *in vitro* but this specificity is not maintained in cell-based assays (Rozman-Pungercar *et al.* 2003). This may be due to the irreversible nature of the inhibitor: non-optimal inhibitors may still bind weakly to the active site and, once bound, they will become covalently linked to the enzyme. The longer the incubation period, the greater chance there is that an inhibitor will become irreversibly attached to a non-optimal protease target. Moreover, many of these peptide-based inhibitors are not cell permeable and, to overcome this problem, ester-derivatives (where the aspartic acid of, for example, z-VAD, has been esterified) are commonly used in cell-based assays. Once in the cell, esterases are thought to remove the ester group, releasing the active inhibitor. However, the ester-derivatives lack the specificity of the free acid peptide inhibitors, thus confounding the situation further.

The peptide-based inhibitors act by mimicking the substrate and binding into the catalytic site (Fig. 2). Caspases have an absolute requirement for a single amino acid residue at P1 and exclusively hydrolyse the peptide bond following an aspartic acid

residue; the residue at the P4 position is also a critical determinant of specificity (Thornberry, 1997; Thornberry *et al.* 1997). However, many of the inhibitors designed to fulfill these criteria, for example z-VAD and z-DEVD, also fulfill the specificity determinants for cysteine proteases of a different clan, CA. Clan CA, which includes the lysosomal cathepsins and the calcium-dependent calpains, prefer a hydrophobic or large residue at P2 but have no preference for the P1 residue (Mottram *et al.* 2003). Hence, the alanine in z-VAD and the valine in z-DEVD, comply with clan CA requirements and these inhibitors have indeed been shown to inhibit cathepsin B, another clan CA protease (Rozman-Pungercar *et al.* 2003).

Metacaspase

So, if *Plasmodium* lacks true caspases, what other proteases could represent the target for the caspase inhibitors? One possible alternative is the recently discovered metacaspase proteins, another clan CD protease, which are distantly related to caspases. Metacaspases represent one of two families of ancient caspase-like proteins (paracaspases and metacaspases) which have been identified recently in metazoans (including humans), fungi and Protozoa (Uren *et al.* 2000). Based on evaluation of the *P. falciparum* genome sequence, this parasite appears to possess two metacaspases, although neither have been characterized or shown to be enzymatically active (Rosenthal, 2004). *P. berghei* has also been shown to possess two metacaspases genes to date (GenBank, accession numbers AJ555625 and AJ555626). Alignment of the *Plasmodium* metacaspases with caspases reveals that they retain the typical caspase fold and the catalytic dyad (His, Cys) required for catalytic activity but there is considerable sequence diversity in the vicinity of the active site cleft, which may indicate a difference in substrate specificity (Wu *et al.* 2003). Moreover, aspartic acid-directed processing sites in metacaspases cannot be predicted from homology with the caspases, implying that they are not processed in a similar manner (Mottram *et al.* 2003).

Phylogenetic analysis suggests that the metacaspases belong to a distinct clade which may represent the evolutionary progenitor of mammalian caspases (Mottram *et al.* 2003). In support of this theory is the fact that the yeast metacaspase has been shown to regulate programmed cell death in this unicellular organism (Madeo *et al.* 2002; Mazzoni *et al.* 2005). Induction of apoptosis is accompanied by an increase in a protease activity which cleaves caspase-specific substrates. Apoptosis is abrogated in metacaspase null mutants and is exacerbated in yeast which over-express the metacaspase. The yeast metacaspase can also be inhibited by z-VAD (Madeo *et al.* 2002).

Calpain

Another possible target for caspase inhibitors in *Plasmodium* is the calpains, cytosolic calcium-dependent cysteine proteases. Calpains are clan CA cysteine proteases but can be inhibited by caspase inhibitors such as z-VAD (Waterhouse *et al.* 1998). Calpains have been implicated in apoptosis in mammalian cells (Squier and Cohen, 1997) and are thought to be involved in both caspase-dependent (Nakagawa and Yuan, 2000; Blomgren *et al.* 2001) and caspase-independent pathways (Wolf *et al.* 1999). Moreover, calpain inhibitors have been shown to inhibit apoptosis in neuronal cells (Squier *et al.* 1994; Blomgren *et al.* 2001). Calpains may act in multiple ways: cleaving and activating effector caspases, cleaving caspase targets to potentiate their effects and disturbing calcium homeostasis and hence trigger apoptosis (Nakagawa and Yuan, 2000; Blomgren *et al.* 2001).

Database mining of the *P. falciparum* genome sequence has identified two putative calpain homologues in this parasite (Wu *et al.* 2003). They possess a catalytic domain with three active sites, which together constitute the catalytic cleft, but lack a calcium-binding domain, suggesting that the malarial calpains are calcium independent (Wu *et al.* 2003).

Cathepsins

Finally, the caspase inhibitors could be targeting lysosomal cysteine proteases, cathepsin L-like enzymes, of which *Plasmodium* has many, including the falcipains (or berghipains in *P. berghei*) and the SERAs (Wu *et al.* 2003; Rosenthal, 2004). In mammalian cells, lysosomal proteases have been implicated in apoptosis: some are thought to act indirectly by activating caspases (Turk *et al.* 2002); some are thought to act directly on apoptosis substrates (Stoka *et al.* 2001; Turk *et al.* 2002) and others have proposed that lysosomal proteases elicit programmed cell death through an alternative pathway, autophagy (Bursch, 2001).

The most characterized cathepsin L-like enzymes in *Plasmodium* are the falcipains. *P. falciparum* has four falcipain orthologues, falcipain 1, 2, 2' and 3 (Rosenthal, 2004). Falcipain 2 and 3 are involved in haemoglobin hydrolysis in the erythrocytic stages of the parasite (Rosenthal, 1995; Gamboa de Dominguez and Rosenthal, 1996; Rosenthal *et al.* 1996), but this does not preclude them having a distinct role in the insect stages. Falcipain 1 is thought to play a role in oocyst development (Eksi *et al.* 2004). Falcipain 1 null mutant parasites were viable and normal in the erythrocytic stages, gametocytes and gametes but produced 70–90% less oocysts when fed to mosquitoes. However, it is not clear when falcipain 1 acts during oocyst development: its

transcription escalates dramatically in gametocytes and gametes but falcipain 1 knockout mutants are morphologically normal at these stages (Eksi *et al.* 2004), raising the possibility that falcipain 1 acts during the intervening stage, the ookinete. Genes encoding falcipain homologues in other plasmodial species, including *P. berghei*, have been identified (Rosenthal *et al.* 2002). *P. berghei* has one falcipain 1 homologue and one falcipain 2/3 homologue. The falcipains are expressed to different extents in different life cycle stages of the malaria parasite (Le Roch *et al.* 2003) but no data exists for their expression in the ookinete or oocyst stages.

Another group of abundant cathepsin L-like cysteine proteases in *Plasmodium*, are the serine-repeat antigen or SERA proteins (Rosenthal, 2004). There are nine SERA protease genes, eight of which are located in an array on chromosome 2 and another single gene on chromosome 9 of *P. falciparum*. The SERA genes are expressed throughout the parasite's life cycle: in rings (SERA1), trophozoites (SERA2, 3 and 9), schizonts (SERA2-9), merozoites (apart from SERA8), gametocytes (SERA1, 2, 4, 6, 7 and 9) and sporozoites (apart from SERA3 and 5) (Miller *et al.* 2002; Bozdech *et al.* 2003; LeRoch *et al.* 2003). No data exists for the expression of SERA genes in ookinetes. In erythrocytic stages, despite all 9 genes being expressed, only SERAs4-7 could be detected at the protein level (Miller *et al.* 2002). SERA5 is the most abundant and has been implicated in parasite egress and/or erythrocyte invasion (Pang, Mitamura and Horii, 1999).

All SERA proteins contain a central domain with strong homology to papain-family cysteine proteases but several SERAs, including the abundant SERA5, have a serine residue in place of the active site cysteine (Kiefer *et al.* 1996; Gor *et al.* 1998; Hodder *et al.* 2003) which will undoubtedly influence the substrate specificity and inhibitor sensitivity. For example, SERA5 possesses a chymotrypsin-like protease activity, with a P1 specificity for an aromatic amino acid, which can be inhibited by a serine protease inhibitor (Hodder *et al.* 2003). Thus it is extremely unlikely that SERA5, and perhaps the other SERAs with active site serines, would be inhibited by the caspase inhibitors.

More detailed inhibitor studies are required, using inhibitors which can differentiate between the different potential candidates, to be able to ascertain which of these potential proteases could be the caspase-like enzyme in apoptotic ookinetes. The classical clan CA protease inhibitor, *L*-trans epoxy-succinyl-leucylamido-(4-guanidino)-butane or E64, will only inhibit the lysosomal cysteine proteases, although recent studies suggest that falcipain 1 may be insensitive to this inhibitor (Goh, Goh and Sim, 2005). Alternatively, tripeptide aldehyde inhibitors, such as antipain, could be used to inhibit the lysosomal cysteine proteases. Calpain inhibitors, such

as Z-LLY-FMK, also exist, and may be useful in eliminating these candidates.

Genome mining of the completed *P. falciparum* database unearths very few genes encoding for proteins involved in apoptosis. Not only are caspases missing but their downstream substrates, such as Bcl proteins, are also absent. Thus it appears that, although *Plasmodium* undergoes a form of cell death which resembles apoptosis, where the end results are conserved (chromatin condensation, DNA fragmentation etc.), the pathways are not the same as mammalian cells. Since *Plasmodium* possesses a metacaspase, which is thought to be the ancestral progenitor of the mammalian caspases (Wu *et al.* 2003), it suggests that higher eukaryotes have evolved and elaborated the caspase pathways in a very different manner from protozoa.

Timing of events

Markers of apoptosis are good indicators to study the onset of cell death and the sequence of events in the apoptotic pathway. Al-Olayan *et al.* (2002) studied the increase in the number of dying ookinetes in a population by detecting nuclear chromatin condensation. According to their results, apoptotic ookinetes were first observed at 12 h post-culture of gametocyaemic blood, and the proportion undergoing apoptosis increased up to 31% and 80% at 24 h and 36 h respectively. We studied the sequence of occurrence of two apoptotic markers, nuclear condensation and activation of caspase-like molecules, between 18 h to 26 h post-culture. A significant and increasing proportion of ookinetes become labelled with CaspaTag (FAM-VAD.fmk) from 18–26 h post culture of gametocyaemic blood onwards and this labelling correlates well with another marker for apoptosis, acridine orange labelling. Dead cells were detected by propidium iodide staining, identifying cells with compromised membranes, and were detected from 20 h onwards. The proportion of dead cells increased slowly with time but was significantly lower than ookinetes demonstrating markers of apoptosis (unpublished observations).

Temporal profiles of apoptosis-like cell death have yet to be performed *in vivo*. The observations made *in vitro* may not be comparable with the situation *in vivo*, where live ookinetes rapidly escape from their toxic environment as they invade the midgut, with the total numbers of ookinetes per midgut falling rapidly from 18–24 h post-feeding (Al-Olayan *et al.* 2002). At 18 h, a much higher proportion of ookinetes exhibit signs of apoptosis-like death *in vivo* than *in vitro*, suggesting that the triggers which induce apoptosis are more potent in the mosquito than in our culture conditions. By 24 h, however, the proportion of dying ookinetes in culture has reached similar levels to those in the midgut

lumen. This equalization of the proportion of dying cells may simply reflect the escape from the gut lumen of healthy parasites *in vivo*. We do not know how rapidly apoptosis-like cell death progresses in *P. berghei*. Ookinetes labelled with fluorochrome indicators of apoptosis appear normal when viewed under white light, their membrane permeability may be unaffected for several hours and they may even be capable of the gliding motility necessary for movement out of the blood bolus and cell invasion.

OOKINETE-INDUCED APOPTOSIS IN THE MID-GUT EPITHELIUM

During their passage out of the hostile environment of the mid-gut, ookinetes must traverse the monolayer of midgut epithelial cells. The route of their passage has been controversial. Observations of various malaria parasite/host combinations have generated hypotheses suggesting exclusively intracellular, exclusively intercellular or both routes. This debate has been reviewed recently (Baton and Ranford-Cartwright, 2004, 2005*a*) and will not be discussed in detail here, other than to observe that there now appears to be a consensus supporting the view that ookinetes do invade the epithelial cells rather than pass between them (Han *et al.* 2000; Zieler and Dvorak, 2000; Vlachou *et al.* 2004) though Levashina and co-workers (Whitten *et al.* 2006) report a much higher proportion of *P. berghei* ookinetes in extracellular spaces than intercellular.

What is also now clear is that, in several malaria parasite/vector permutations, transit through the cells causes damage. Invaded midgut epithelial cells rapidly exhibit morphological changes including loss of microvilli, nuclear pyknosis, surface blebbing and decrease in refractive index (reviewed in Hurd and Carter (2004) and Hurd, Carter and Nacer (2005). Invaded cells are extruded from the epithelial layer into the gut lumen.

The time-bomb theory

Ookinetes rapidly move laterally away from the toxic environment of the dying cell, often invading several cells before moving out of the epithelium and into the basal subepithelial space. This lateral invasion has been observed by video microscopy *in vivo* in *P. gallinaceum*-infected *A. aegypti* (Zieler and Dvorak, 2000) and by time confocal microscopy of *P. berghei* infection in both *A. stephensi* and *A. gambiae* (Vlachou *et al.* 2004). Han and co-workers coined the phrase 'time-bomb theory' to describe their hypothesis that the invading ookinetes trigger epithelial cell destruction (detonate a bomb) from which they must rapidly escape if they are to survive (Han *et al.* 2000; Han and Barillas-Mury, 2002; Baton and Ranford-Cartwright, 2004). They observed that 95% of the invading parasites escape

unharmful from the dying cells and suggested that the majority of parasite losses occur before the ookinete invades the epithelium or after the oocyst is formed. Invasion of additional cells may be dependent upon the speed at which invaded cells are extruded and Baton and Ranford-Cartwright (2005a) have proposed that rapid extrusion of invaded cells results in a cellular treadmill whereby ookinetes move from one cell to an adjacent one as the first cell is pushed out of the epithelial layer.

Vlachou *et al.* (2004) observed the cells surrounding the invaded cell undergoing extensive lamellipodia crawling to fill in the gap in the epithelial barrier caused by loss of the damaged cell. Invaded cells produced a 'hood' of lamellipodia that surrounds the ookinete as it exits the epithelial cell. These observations supplement the proposal of Han *et al.* that expulsion of invaded cells from the epithelial layer may be aided by a purse-string contraction of a basal actin ring in the damaged cell, although the method of cell extrusion may vary between different mosquito species (Gupta *et al.* 2005).

In addition to the GPI-anchored surface protein P28, ookinetes secrete a subtilisin-like serine protease, PbSub2, into the cytoplasm of invaded cells and this forms protein aggregates, often associated with the actin cytoskeleton (Han *et al.* 2000; Han and Barillas-Mury, 2002). The putative involvement of these parasite molecules in triggering up-regulation of midgut NOS and/or midgut cell apoptosis has yet to be investigated.

Apoptosis of mid-gut cells

Mid-gut epithelial cells that have been invaded by ookinetes are undergoing apoptosis as they protrude into the mid-gut lumen and are budded off. DNA fragmentation has been detected by TUNEL (Han *et al.* 2000). The apoptotic death of invaded cells was also revealed using Yo-PRO-1 staining as the dye passes selectively through the plasma membranes of apoptotic cells. In contrast, propidium iodide was excluded, ruling out necrotic cell death (Vlachou *et al.* 2004). Furthermore, caspase-like activity was detected in the invaded midgut cells of *P. gallinaceum* infected *Ae. aegypti* by preloading cells with the cell-permeable protease substrate PhilPhilLux-G₁D₂ prior to invasion (Zieler and Dvorak, 2000).

Biochemical events occurring in *P. berghei*-invaded mid-gut cells have been investigated by the group of Barillas-Mury (Han *et al.* 2000). Using immunofluorescence staining with antibodies raised against the ookinete surface molecule, P28, and a universal anti-NOS rabbit polyclonal, they were able to co-localise the parasite with NOS expressing mid-gut cells. They also demonstrated the *A. gambiae* transcription factor AgSTAT was expressed very weakly in the cytoplasm and not at

all in the nuclei of invaded cells. Epithelial cells protruding into the mid-gut lumen had lower levels of AgSTAT and their nuclei were TUNEL positive. This group propose that cell invasion causes physical damage and molecular changes that ultimately lead to death by apoptosis (Han *et al.* 2000).

Biochemical reactions that may initiate apoptosis

As discussed above, several authors have reported that, following *Plasmodium* infection, a generalized up-regulation of the NOS gene occurs in the gut that may be induced by a combination of mammalian, parasite and mosquito factors. Our understanding of the regulation of the mosquito inducible NOS and the damage that nitrosative stress may cause to vector tissues is still rudimentary. Interestingly, the recently identified mosquito peroxiredoxin enzyme, *A. stephensi* 2-Cys peroxiredoxin (AsPrx-47830) has been shown to protect against stresses induced by NO, nitroxyl, peroxynitrite and hydrogen peroxide (Peterson and Luckhart, 2006). Peroxynitrite, for instance, is involved in reactions that cause DNA damage and nitration of proteins (see discussion in Peterson and Luckhart, 2006). Feeding on an uninfected blood meal was shown to induce an increase in AsPrx-47830 midgut expression from 2–20 h post blood meal with a higher level of induction being observed in *P. berghei* infected-mosquito midguts from 12.5–48 h post blood meal (Peterson and Luckhart, 2006). It would thus appear that there may be measures in place to protect midgut cells from the potential cytotoxic effect of levels of nitric oxide generated as a result of the presence of an infected bloodmeal.

In addition to a general up-regulation of midgut NOS, levels of NOS expression that are detectable by immunostaining have been shown to specifically co-localise with invaded cells. This expression has been associated with the subsequent death by apoptosis of cells (Kumar *et al.* 2004). This apoptosis is thought to be initiated by nitric oxide, whose production is catalyzed by NOS activity in cells invaded by *P. falciparum* or *P. berghei* (Han *et al.* 2000; Luckhart *et al.* 1998). As discussed earlier, NO is one of the prime inducers of apoptosis and NO induced intracellular mechanisms result in the activation of caspases (Murphy, 1999).

Although titres of NO that are generated specifically in invaded cells could, in themselves, initiate the death of the cell, Kumar and colleagues (Kumar *et al.* 2004; Kumar and Barillas-Mury, 2005) propose that protein nitration may be involved in initiating apoptosis. They suggest that reactive nitrogen intermediates are produced that lead to protein nitration in some of the invaded cells, as detected by immunofluorescent staining of nitrotyrosine. Two different tyrosine nitration pathways have been proposed (Kumar *et al.* 2004; Kumar and

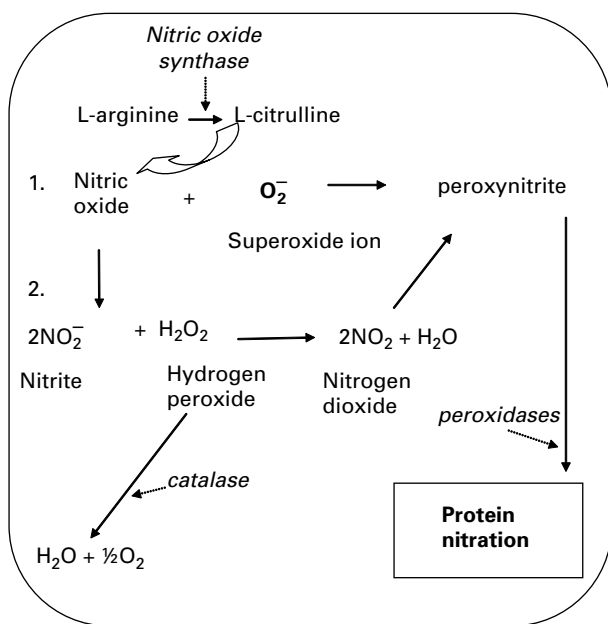


Fig. 3. Schematic of proposed reactions in a midgut cell invaded by an ookinete. Nitric oxide synthase catalyses the synthesis of nitric oxide. Superoxide ions are produced by the oxidation of oxygen. Peroxynitrite may be formed by one of two pathways (1, 2). Based on Kumar *et al.* (2004).

Barillas-Mury, 2005). In the classic pathway, NO reacts with superoxide ion to form peroxynitrite whilst the alternative pathway involves the initial conversion of NO to nitrite followed by its oxidation by peroxidases, in the presence of hydrogen peroxide, to nitrogen dioxide and eventual generation of peroxynitrite (Fig. 3). Inducible peroxidases were identified in *P. berghei*-infected *A. stephensi* that were gluteraldehyde, but not paraformaldehyde, resistant (Kumar *et al.* 2004) and high peroxidase activity was located in cells in advanced stages of apoptosis. Additionally, out of sixteen peroxidase genes, six were found to be differentially expressed in the midguts of *P. berghei*-infected *A. gambiae*, five were induced and the expression of the other one was reduced. Tyrosine nitration may inactivate proteins, particularly those with a tyrosine residue at the catalytic site (Alvarez and Radi, 2003), but a direct link between protein nitration and apoptosis in mosquito midgut epithelial cells has not been demonstrated. Although the work of Kumar and colleagues (Kumar *et al.* 2004) showed that peroxidase expression is associated with apoptosis in midgut cells, it did not demonstrate that protein nitration initiated cell death. Indeed, as nitration was only detected in cells in an advanced stage of apoptosis, it is unlikely to be a direct inducer of apoptosis. In addition, there is, as yet, no complete picture of the induction pathway for apoptosis. Neither all the intermediates in the classical nor the alternative pathways proposed by Kumar and

co-workers were demonstrated to be up-regulated specifically in invaded cells.

Currently, we have no understanding of the biochemical pathways involved in controlling apoptosis in the infected-mosquito midgut. Anacaspase-7 is one of three apoptosis-related molecules that has been identified in cDNA libraries enriched for sequences expressed immediately after midgut invasion (Abraham *et al.* 2004). It shares 40% identity and 60% homology with the *Drosophila* caspase DECAPY. Antibodies raised against Anacaspase-7 were used to recognise a putative proteolytic product that was only present in gut cells after invasion by large numbers of ookinetes, suggesting a dose-related activation of a caspase-related molecule (Abraham *et al.* 2004). Putative anti-apoptotic genes were expressed when oocysts were present (Srinivasan *et al.* 2004). Additionally, microarray analyses of the mosquito transcriptome at varying hours post infection have also detected upregulation of genes involved in apoptosis (Hall *et al.* 2005; Xu *et al.* 2005).

INFECTION-INDUCED APOPTOSIS IN MOSQUITO OVARIES

Plasmodium infection also results in apoptosis of cells in another epithelial layer, the follicular epithelium. These cells surround developing oocytes in the terminal follicle of each ovariole. Ingestion of a blood meal immediately initiates a gonotrophic cycle in anopheline mosquitoes. Yolk protein, or vitellogenin, is synthesised in the fat body and transported to the ovaries, where it is taken into developing oocytes by receptor mediated endocytosis. To gain access to the oolemma, vitellogenin must pass through the spaces that develop between the follicular epithelial cells. Provided the blood meal is large enough, all the terminal follicles in each ovary will produce an egg within about 50 hours. However, by 12 h post-feeding on an infected blood meal, patches of cells in the follicular epithelium of some of the ovarioles show characteristic signs of apoptosis. Staining with acridine orange and examination of ultrathin sections demonstrated nuclear condensation and DNA fragmentation was observed following a TUNEL assay (Hopwood *et al.* 2001). A comparison of ovaries from uninfected and *Plasmodium yoelii nigeriensis*-infected *A. stephensi*, at various times post-feeding, identified signs of apoptosis in 14–16% of follicles from infected females but never more than 4% in uninfected ones (Hopwood *et al.* 2001). In *A. gambiae* ovaries, *P. y. nigeriensis* infection was associated with the activation of a caspase-like molecule that could be detected with FAM-VAD.fmk in approximately 25% of the ovarian follicles. Injection of the immune elicitor lipopolysaccharide or sephadex beads that stimulate melanisation also produced a significant

increase in the number of follicles displaying caspase-like activity (Ahmed and Hurd, 2006). Follicle cell apoptosis resulted in the resorption of developing oocytes (Hopwood *et al.* 2001), thus smaller egg batches were produced by infected mosquitoes (reviewed in Hurd (2003)). Artificial induction of an immune response also caused fecundity reduction as a result of follicle resorption (Ahmed *et al.* 2002).

Apoptotic pathways in the ovary

Although we have identified caspase-like activity as important for the initiation of follicle resorption, we know nothing of the exact molecules involved in initiating or inhibiting apoptosis in the ovarian tissue of mosquitoes. In *Drosophila*, apoptosis occurs in response to both developmental and environmental signals. Whilst the death of nurse cells late in oogenesis is part of the normal developmental process, whole egg chambers, including follicle cells, undergo apoptosis in response to several triggers including a diet lacking protein or no transfer of male sex peptide during mating. The molecular genetics of this have been comprehensively summarised by McCall (McCall, 2004). Based on a variety of studies, a pathway for programmed cell death during mid-oogenesis was proposed that was initiated by insulin signalling-inhibited ecdysone signalling. It is suggested that a reduction in ecdysone signalling may act on the Apf-1 orthologue Dark, which activates an unknown initiator caspase. This, in turn, activates the effector caspase Dcp-1 which may be required to activate another effector caspase, Drice. Apoptosis activators such as *reaper* (*rpr*), *head involution defective* (*hid*) and (*grim*) are not required for germline apoptosis, however *rpr* and *hid*-induced apoptotic death in follicle cells results in death of follicles before maturity (Chao and Nagoshi, 1999). Cytochrome *c* is specifically expressed at late oogenesis and it is proposed that the initiator, Dredd, and other caspases may be involved at this stage (McCall, 2004).

Several caspases have been identified in the *A. gambiae* genome, and some of the *Drosophila* caspases have *Anopheles* orthologues (Christophides *et al.* 2002). These workers grouped the mosquito caspase S8 with the *Drosophila* S-prodomain caspase, *dcp1*, and *damm* was most similar to *Anopheles* S9 and S10. The caspases *dredd* and *dronc* also have orthologues, but *rpr* has not been identified in *A. gambiae*.

Peaks of expression of *A. stephensi* E517 caspase transcript and a bax-like inhibitor of apoptosis transcript occur in the midgut at 40 h and 14–20 d (Xu *et al.* 2005). In *A. gambiae* an analysis of transcriptome response to midgut invasion was examined by comparison with the transcriptome from mosquitoes fed a circumsporozoite and TRAP-related protein knockout (CTRPko) that develops in the

midgut lumen but is unable to invade the epithelium. Five inhibitor-of-apoptosis protein (IAPs) were up-regulated and one down-regulated during ookinete invasion. Three of these, together with viral IAP-associated factor, mapped to the major cytoskeleton clusters CL15 and CL12, together with the gene encoding the serpin SRPN10. There was also differential regulation of genes implicated in redox metabolism and detoxification included thioredoxin reductase, two thioredoxin peroxidases, a general peroxidase precursor and 3 glutathione-S-transferases (Vlachou *et al.* 2005).

Plasmodium does not come into direct contact with mosquito ovaries, so the induction of apoptosis and follicle resorption is not induced by invasion (as it is in the midgut) and must be triggered by external signals. Crampton and Luckhart noticed non-uniform expression of *As60A*, a member of the TGF β 1 super-family (see above), in the developing eggs of infected *A. stephensi* at 24 h post blood meal. It is possible that this expression could up-regulate NOS, but expression of NOS in ovarian follicles has, so far, not been reported. Elevated levels of NO or RNIs in the haemolymph are associated with infection and must be considered as candidate triggers. Finally, an infection-induced change in endocrine status may mirror events that lead to apoptosis in *Drosophila* follicles, though no examination of ecdysteroid or juvenile hormone titres have been reported in infected insects to date.

THE FUTURE

Our knowledge of interactions that occur between malaria parasites and their mosquito vectors has expanded enormously during the past decade, and is continuing to do so. This has been assisted by the publication of the full genome sequence of *A. gambiae*, a major malaria vector in Africa (Holt *et al.* 2002) and a human malaria parasite, *P. falciparum* (Gardner *et al.* 2002). The availability of associated bioinformatics resources (Kriventseva *et al.* 2005) is aiding the burgeoning application of genomics, transcriptomics and proteomics to mapping the sporogonic cycle of *Plasmodium* and the response of the mosquito to infection. It is to be hoped that this wealth of data will, eventually, assist in the unravelling of the molecular pathways controlling apoptosis in parasite and host, though its immediate use is limited as homologues or orthologues of most apoptosis related genes in mammalian, *C. elegans* or *Drosophila* pathways are absent.

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