The Interaction between *Drosophila* and its Microsporidian Parasite: From the Within-Host to the Evolutionary Scale

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Abstract

Resistance to parasites is advantageous for most organisms but investing limited resources into defence depends on the selection pressure involved and the nature and magnitude of costs involved. The evolutionary interactions between hosts and their parasites have received much attention but the effect of intracellular parasites in such systems is far less understood. Microsporidia are intracellular parasites of vertebrates and invertebrates that have gained prominence both as a pathogen and a potential biocontrol agent.

This thesis investigates evolution of resistance against intracellular parasites and the associated trade-offs in *Drosophila melanogaster* and *Tubulinosema kingi* system. Stage-specific susceptibility of the host to *T. kingi* infection and stage-specific within-host parasite proliferation; host tissue specificity and the impact of *T. kingi* on host sex ratio were also investigated in this system. Immune responses of *D. melanogaster* to *T. kingi* infection were examined. Increased haemocyte density and phenoloxidase activity was observed in infected flies and a negative effect of nitric oxide on parasite density was observed.

Five pairs of replicate *D. melanogaster* lines were used for experimental evolution of resistance to *T. kingi*. The within-host parasite density decreased significantly in selected lines indicating the evolution of resistance. The early fecundity and longevity of selected lines were significantly higher than of control lines when infected by *T. kingi*. Trade-offs associated with increased resistance against microsporidia was investigated. The evolved resistance was negatively correlated with fecundity both under normal and stressed conditions of the host; the selected lines were also poor larval competitors for scarce food resources. The haemocyte density and phenoloxidase activity in haemolymph of larvae from selected lines was higher than in control lines.

The implications of these results are discussed in relation to other host-parasite systems and the possibility of using the system as a model insect-microsporidia system to explore ecological and evolutionary interactions.

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Dedicated to my parents,

(Mrs. M.S. Indrani and Mr. D. Vijendra Varma)

Declaration

In chapter two section 2.2.3, the processing, sectioning and staining of microsporidian infected *Drosophila melanogaster* for light microscopy was done by Dr. Alastair McKinnon, University of Aberdeen and sectioning, staining and photography for electron microscopy was done by Dr. Alan Curry, University of Manchester.

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

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Prof. Donald Quicke (PhD Supervisor)

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Chapter One: General Introduction

1.1. Host-parasite interactions

Most organisms face threat from natural enemies such as predators, parasites and pathogens and interaction with these enemies is often inevitable. The evolutionary interactions between hosts and their parasites in particular have received a great deal of attention, since it presents a perfect scenario for understanding factors that drive co-evolutionary processes. Host-parasite interactions are highly complex involving a wide range of factors that greatly influence the ecology and evolution of both the host and its parasite (Anderson and May 1981). The host and their parasites can interact at different levels: individual, population and ecosystem. In this thesis I focus on the interactions between a host and its intracellular parasite which can be broadly classified into two levels. First, I explore crucial within-host interactions in the system including the immune responses of the host to its parasite. Second, I explore evolutionary interactions in terms of a host population evolving resistance against the parasite and further investigate the associated mechanisms and costs involved.

The interaction of a parasite with its host at an individual level involves finding a susceptible host or host stage followed by successful infection and within-host proliferation, and finally successful transmission to a new host. To ensure each of these is achieved a parasite has to overcome a range of obstacles put forward by its host. A host may primarily avoid the parasite behaviourally (Fauchald *et al.* 2007; Luong and Polak 2007), but once they are infected the host can only resist or tolerate the parasite (Boots and Bowers 1999; Miller *et al.* 2005). Most parasites are known to synchronise their lifecycle with that of their hosts to maximise their fitness (Kwiatkowski and Nowak 1991). Parasites further increase their ability to locate a susceptible host and ensure successful transmission to the next generation by evolving different modes of transmission; some use horizontal transmission involving environmentally stable forms, such as spores in the case of bacteria and fungi, while others use vertical transmission (Terry *et al.* 1997). However, this process is complex in certain parasites and may involve intermediate hosts or vectors and may use different modes of transmission at different stages.

To successfully infect and multiply in its host a parasite has to evade harsh within-host conditions such as gastric juices, physical barriers (gut wall) and host immune responses. Parasites are known to either neutralise such harsh host responses, may just evade it or may do both (Vacher *et al.* 2005). Successful infection and within-host establishment of the parasite usually leads to fitness reduction in the host (Altizer and Oberhauser 1999; Blaser and Schmid-Hempel 2005; Futerman *et al.* 2006), which could either be due to the cost incurred by the host in attempting to resist the parasite or may be due to the extensive damage that the parasite may have caused, in other words virulence of the parasite. Since parasite development mostly depends on the host's well-being there exists an important trade-off between the virulence exhibited by a parasite and the extent of damage caused to its host (Stewart *et al.* 2005). Theoretical studies consider mode of parasite transmission to play a key role in this trade-off and this has been demonstrated in a few experimental systems (Bull *et al.* 1991; Herre 1993; Stewart *et al.* 2005).

The host-parasite interactions at the individual level subsequently affect the populations of both the host and its parasites. The host can interact with its parasite either passively by "tolerance" or actively by "resistance" (Miller *et al.* 2005). In most literature "resistance" is broadly defined as mechanisms that inhibit or reduce infection, while "tolerance" is defined as mechanisms that negate the damage caused by the parasite, but does not limit the infection (Antonovics and Thrall 1994; Boots and Bowers 1999; Roy and Kirchner 2000; Miller *et al.* 2005; Miller *et al.* 2007). However, it is due to the selection pressure exerted by the parasite that host populations are selected for strategies that provide maximum fitness and this could either be avoidance, tolerance, resistance or a combination of all these towards the parasite (Boots and Bowers 1999). On the other hand parasites evolve counter-strategies that provide higher fitness to the parasite, such as better transmission (Terry *et al.* 1997), higher virulence (Levin 1996) and better evasion of host immune resistance (Kraaijeveld *et al.* 1998; Shelby *et al.* 2000; Nappi *et al.* 2004). Both the evolution of resistance in host population and the evolution of counter-defence strategies in parasite populations may involve costs (Lenski 1988; Kraaijeveld and Godfray 1997; Kraaijeveld *et al.* 1998; Webster and Woolhouse 1999; Green *et al.* 2000; Freitak *et al.* 2003; Lohse *et al.* 2006; Pennacchio and Strand 2006; Luong and Polak 2007). Another important interaction between hosts and their parasites has been reported. Parasites that are transmitted vertically have been reported to

skew the sex ratio of their host population to maximise their transmission success. The parasites achieve this by either exhibiting sex-specific virulence, feminising behaviour or by altering the sex-ratio of the host progeny (Dunn *et al.* 1993; Dunn *et al.* 1998; Hurst and Jiggins 2000; Montenegro *et al.* 2005; Bentley *et al.* 2007). At the ecosystem level parasites play an important role in structuring ecological communities by apparent competition and intraguild predation (Hatcher *et al.* 2006; van Veen *et al.* 2006).

In this chapter, I provide an introduction to the model study system used in this thesis: *Drosophila melanogaster* and its intracellular microsporidian parasite *Tubulinosema kingi*. I further briefly discuss the evolution of resistance in general and in particular refer to *Drosophila* and its parasites as a model system, and have included a brief summary of microsporidian biology.

1.2. Evolution of resistance

For an organism, defending itself from natural enemies within the community that are capable of reducing its fitness, such as parasites, pathogens and predators is crucial. To achieve this, an organism has to invest a significant part of available resources into defence functions (Schmid-Hempel 2003). Since resources are usually limited, the extent to which an organism invests in defence is mainly driven by factors such as the selection pressure applied by the parasite and the extent of costs involved (Rolff and Siva-Jothy 2003). Costs associated with resistance to parasites have been documented in a range of host-parasite systems: *Arabidopsis*-pathogen system (Burdon and Thrall 2003); bacteria-phage system (Lenski 1988); *Paramecium*-bacteria system (Lohse *et al.* 2006); *Plodia interpunctella*-virus system (Boots and Begon 1993); *Aedes aegypti*-*Plasmodium* system (Yan *et al.* 1997) and a snail-schistosome system (Webster and Woolhouse 1999). Apart from these, costs of resistance have also been reported in *Drosophila*-natural enemy systems, which are discussed later in this chapter. The costs associated with resistance can be broadly classified into the actual cost incurred by mounting a defence reaction against the parasite and the standing cost incurred by maintaining a defence reaction in anticipation of a parasite attack (Kraaijeveld *et al.* 2002).

In addition, the presence of additive genetic variation in traits associated with defence ability is essential for resistance to evolve and this has been commonly assumed in models for co-evolution in host-parasite systems (May and Anderson

1983; Schmid-Hempel 2003). Evidence for this has been demonstrated in plant-pathogen (Burdon 1980; Burdon and Thrall 2003) and plant-herbivore systems (Agrawal *et al.* 1999). Similar evidence has also been documented in a few animal-natural enemy systems, such as sheep-nematodes (Smith *et al.* 1999); snail-schistosome (Webster and Woolhouse 1998); *Daphnia*-bacteria (Ebert *et al.* 1998), *Daphnia*-midge (Spitze 1992); *Drosophila*-fungi (Tinsley *et al.* 2006) and *Drosophila*-parasitoid (reviewed by Kraaijeveld et al. (1998)).

As discussed earlier, evolving resistance to natural enemies has associated costs in terms of life-history traits; For example, the resistance in the snail system had a trade-off with fecundity (Webster and Woolhouse 1999), while in the *Plodia* system resistance was linked to development time and egg viability (Boots and Begon 1993). Apart from this it has also been suggested that evolved resistance to one natural enemy can be traded-off with resistance to a second natural enemy (Simms and Fritz 1990). However, this would be true only in cases where the genetic correlation between resistances to the two natural enemies is negative. In cases where correlations between the resistance to two natural enemies is positive, selection by one natural enemy will result in increased resistance not only to this species but also to the second natural enemy, even in its absence (Fellowes *et al.* 1999a). Therefore, the specificity of resistance can have complex implications for the structure and dynamics of natural communities (Fellowes and Kraaijeveld 1998c; Bohannan and Lenski 2000; Ferrari 2001; Poitrineau *et al.* 2003).

Selection experiments involving experimental evolution are valuable tools to investigate the evolution of resistance against a parasite followed by investigating the correlated responses to evolved resistance that might represent costs (Fry 2003). This technique is also advantageous since it allows replication of the treatment (Gibbs 1999; Harshman and Hoffmann 2000). However, selection experiments have drawbacks which include the unnatural selection regimes used in the experiments and unintentional selection (Partridge *et al.* 1999; Harshman and Hoffmann 2000). Other advantages and drawbacks associated with selection experiments will be discussed in chapter four along with the essential factors in designing selection experiments. In this thesis I report the use of an experimental evolution set-up to investigate the evolution of resistance and/or tolerance in a *Drosophila*-intracellular parasite system and then look for correlated responses to selection (see chapter four and five).

1.3. *Drosophila* and its parasites

The fruit fly, *Drosophila*, has been extensively used to investigate and understand ecological interactions and evolution of organisms. It has been used for the last 100 years by researchers from various fields as a model insect to test predictions and newly developed techniques. *Drosophila* cultures have been recently used in experiments to test the effect of space travel on immune system of an organism (Benguria *et al.* 1996; Marco *et al.* 1996), suggesting that *Drosophila* will continue to be an important model organism in the future (www. quest.arc.nasa.gov). The physiology and genetics of *Drosophila* is well understood (Schneider 2000) and can be easily manipulated (Ueda 2001). With the genome of *Drosophila* completely sequenced (Celniker 2000) and with new manipulative tools such as RNAi available (Ueda 2001), *Drosophila* is an excellent model organism. The recent reports on human homologues in *Drosophila* has provided researchers an opportunity to investigate a wide range of human diseases in the fruit fly for a better understanding of the host-pathogen/parasite interactions and disease causing mechanisms (Lemaitre *et al.* 1996; Bernards and Hariharan 2001; Mansfield *et al.* 2003; Shinzawa and Kanuka 2006; Jensen *et al.* 2007).

Drosophila is also a good model insect to study life-history related trade-offs involving the immune system; This is especially due to the wealth of information and wide range of techniques available in this model system (Ueda 2001; Tzou *et al.* 2002; Lemaitre and Hoffmann 2007). Since *Drosophila* have a short generation time and can be easily maintained as large populations in laboratories, they have been ideal systems in which specific traits could be artificially selected experimentally (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a; Partridge *et al.* 1999; Harshman and Hoffmann 2000; Fry 2003).

Drosophila is exposed to a range of ecto- and endoparasites such as parasitoids, mites, nematodes, fungi, bacteria and viruses, partially because they inhabit, feed and reproduce in decaying environments rich in microbes (D'Argenio *et al.* 2001; Mansfield *et al.* 2003). The *Drosophila* females are attracted to fermenting and decomposing substrates, upon which they lay their eggs. The larvae that hatch out feed on the decomposing food and moult through three instars prior to pupating. Within the pupae the larval tissue is restructured to form a fly which finally emerges from the pupal case. The duration of each life-stage and that of the lifecycle is species-specific and is dependent on a range of environmental

conditions such as temperature and day length. *D. melanogaster*, at 20ºC has a five day larval period, followed by seven to eight day pupal period then the emerging flies need a day to sexually mature. The different life stages of *Drosophila* are susceptible to different parasites (Kraaijeveld *et al.* in press). For example the parasitoid *Asobara tabida* parasitizes the larvae, while the parasitoid *Pachycrepoideus vindemiae* parasitizes the pupae and fungal parasite *Beauveria bassiana* attacks only the adult flies. However, *Drosophila* may be susceptible to other parasites irrespective of their life stages, for example *Drosophila* C virus (Gomarizzilber and Thomasorillard 1993). This stage-specific susceptibility is an important determinant of the host-parasite interactions (Law 1979; Briggs and Godfray 1995; Moerbeek and Vanden Bosch 1997).

Drosophila is parasitized by its endoparasites through one or more of the following routes: orally, through the cuticle and through the reproductive orifice. However, a few parasites use special organs for parasitizing their host, for example the ovipositor in parasitoids. In an attempt to resist the parasitic invasions *Drosophila* have developed a range of resistance mechanisms. These primarily involve physical barriers such as thicker cuticle and puparium, gut wall and mucosal lining, as well as innate immune mechanisms. The innate immunity in *Drosophila* comprises of humoral and cellular immune responses (Lemaitre and Hoffmann 2007). The *Drosophila* immunity is discussed later in chapter three.

The parasites infecting *Drosophila* vary in their objective: some utilize the host body (for feeding), others utilize the host metabolism, and a few others may use the host for transmission. Directly or indirectly, parasitism causes a reduction in *Drosophila* fitness such as reduced fecundity, reduced longevity or even death in some cases and this has a significant impact on *Drosophila* population dynamics. The selection pressure exerted by the parasites on the *Drosophila* population may result in the evolution of resistance against the parasite involved and this has been demonstrated previously using laboratory-based artificial selection experiments. Evolution of resistance against two larval parasitoids, *Asobara tabida* (Braconidae) and *Leptopilina boulardi* (Figitidae), in *D. melanogaster* population has been reported showing that there is considerable additive genetic variation in the *D. melanogaster* population for resisting parasitoids (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a). In a similar investigation Kraaijeveld and Godfray (subm.) demonstrate evolution of resistance against a fungal parasite *Beauveria bassiana* in the same *D. melanogaster* population. The

resistance evolved against these three parasites was found to involve costs associated with standing defence, although the nature and magnitude of these cost varied. The cost associated with resistance was weaker against fungi in comparison to that against the parasitoids (Kraaijeveld and Godfray, subm.). The *D. melanogaster* population selected for increased resistance to larval parasitoids *A. tabida* and *L. boulardi* was found to be poorer larval competitors in comparison to the control population suggesting that increased resistance was costly (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a). The *D. melanogaster* population selected for increased resistance against *B. bassiana* were found to have lower lifetime reproductive success compared to the control population suggesting a possible cost (Kraaijeveld and Godfray, subm.).

The evolutionary interactions investigated between *Drosophila* and its three endoparasites described in the previous paragraph show that resistance against parasites can evolve and involves costs, the nature and magnitude of which limits the evolution of resistance. Recent evidence suggests that *Drosophila* can similarly evolve behavioural resistance against an ectoparasitic mite *Macrocheles subbadius* and this again has a fitness cost in terms of host fecundity (Luong and Polak 2007). However, all the parasites investigated so far are extracellular in nature and it is not known if *Drosophila* can resist their intracellular parasites such as microsporidia (Kramer 1964a; Futerman *et al.* 2006) and *Wolbachia* (Ilinsky and Zakharov 2007). It is also possible that the immune reactions elicited against extracellular parasites may be infective against these parasites due to their intracellular nature. Can resistance to intracellular parasites evolve, and if so, what are the costs and mechanisms associated with it? Investigating these questions has potential implications on our understanding of host-parasite interactions.

1.4. Microsporidia - highly evolved parasites

The microsporidia are obligate intracellular parasites that are highly-derived fungi with greatly reduced morphology and genomes (Katinka *et al.* 2001; Keeling and Fast 2002; Keeling *et al.* 2005). Microsporidia are known to infect a wide range of vertebrates and invertebrate hosts including insects. This group of parasites have recently received considerable attention for two reasons; first, they have emerged as human pathogens that cause fatal infections in immuno-compromised patients (Hart *et al.* 2000; Weiss 2001; Visvesvara *et al.* 2005; Omalu *et al.* 2006; Dwivedi *et al.* 2007) and second, because of their potential to

be used for bio-control of arthropod pests and disease vectors (Sweeney and Becnel 1991; Hajek *et al.* 2007). The vital need to understand this newly emerged pathogen has led to the complete genome sequencing of the microsporidium *Encephalitozoon cuniculi*, which infects a range of mammals including man (Katinka *et al.* 2001).

The phylum Microspora, comprising more than 1200 species belonging to about 143 genera, was initially classified in the kingdom Protista. Recent studies using DNA techniques strongly suggest that the phylum should be classified in the kingdom Fungi or at least as a sister kingdom to Fungi (Hirt *et al.* 1999; Keeling *et al.* 2000; Bruns 2006; James *et al.* 2006; Hibbett *et al.* 2007) although the specifics are still currently debated. Taxonomic classification of microsporidia was traditionally based on their natural host and ultrastructural features such as size of mature and developing stages, arrangement of nucleus (mono- or diplokaryon), arrangement and number of polar filament coils (Omalu *et al.* 2006). Microsporidian classification may also consider other factors such as the interface of developing stages within the host cell (either directly in contact with host cell cytoplasm or within host cell derived vacuole) and the mode of cell and nuclear division (Omalu *et al.* 2006). Subsequently, molecular methods (Franzen and Muller 1999) have been utilized resulting in frequent revision and debate on class, order and families within the phylum Microspora (for example see Franzen *et al.* 2005).

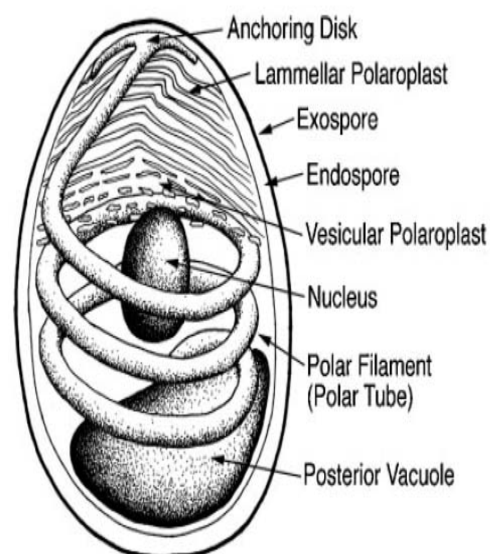

Fig. 1.1: General structure of a microsporidian spore, a diagrammatic representation (from Keeling and Fast 2002).

Microsporidia are exclusively intracellular and are characterised by their unique thick-walled, resistant transmission stage: the spore. The microsporidian spores are double walled, with an outer exospore and an inner endospore, which contains the sporoplasm and a posterior vacuole around which the polar filament coils, see figure 1.1 (Keeling and Fast 2002). Microsporidia have a unique mode of infecting their host cells. Resistant spores that are normally ingested by the host are triggered to extrude their polar filament (that acts as a combination of harpoon and hypodermic needle) towards the host cell, penetrating the host cell and inoculating it with the infective sporoplasm through the filament. Recently, it has been suggested that microsporidia gain access into the host cell alternatively by phagocytosis and the polar tube is used to escape from the maturing phagosomes and to infect the cytoplasm of host cells (Franzen 2004; Franzen 2005). Once within the host cytoplasm the microsporidium undergoes two characteristic developmental phases: a proliferative phase known as merogony, followed by a sporulation phase known as sporogony. Merogony results in the production of increased numbers of meronts (microsporidium development stage) within the cells, while sporogony results in the production of infective spores.

A few microsporidia produce two kinds of spores: the early and late spores. Early spores have thin exospore, a short polar tube and primarily infect neighbouring cells within the same host, while late spores have longer polar filaments, a thicker exospore and are mainly transmitted to new hosts. Microsporidia have been reported to use either one or both modes of transmission: horizontal transmission between unrelated hosts or vertical transmission between female hosts and their offspring (Dunn and Smith 2001; Didier *et al.* 2004). Horizontal transmission of microsporidia mainly occurs through an orofecal route, where hosts feed on spores released from faecal fluids (Chen *et al.* 2004) or infected cadavers (Becnel and Johnson 2000; Futerman *et al.* 2006). Microsporidia are transmitted vertically from infected females to their offspring transovarially (within their eggs) (Dunn *et al.* 2001) and microsporidia that are exclusively transmitted vertically have been shown to have serious implications on host sex ratio due to the manipulation of host reproduction (Dunn *et al.* 1993; Ironside *et al.* 2003). The life cycles of some microsporidia are simple and direct involving a single host and a single spore form, while in others it can be more complex involving multiple hosts and many spore forms transmitted by different modes at different stages in the life cycle (Johnson 1997; Dunn and Smith 2001; Vossbrinck *et al.* 2004; Futerman *et al.* 2006).

Ever since the first named microsporidian, *Nosema bombycis*, was isolated from the silk worm, *Bombyx mori*, a century ago, when the silk industry suffered severely because of a mysterious disease, other insect-infecting microsporidia have been regularly reported. Insect-infecting microsporidia have mainly been studied, either with a view to using them as a potential biocontrol agent for agricultural pests and disease vectors (Johnson 1997; Williams *et al.* 1998; Agnew and Koella 1999; Becnel and Johnson 2000; 2004), or as a parasite having adverse effects on beneficial insects (Schuld *et al.* 1999; Malone *et al.* 2001; Olsen and Hoy 2002). The effect of microsporidiosis in insect hosts varies greatly between being relatively benign in some (Weiss 2001) to being fatal before host maturation in others (Schuld *et al.* 1999). Microsporidia are also known to adversely affect a range of host life-history traits such as decreased fecundity (Schuld *et al.* 1999; Futerman *et al.* 2006), increased mortality (Wilson 1974), increased development period (Boohene *et al.* 2003) and reduced size (Agnew and Koella 1999; Futerman *et al.* 2006). These effects of microparasites on host fitness traits can have serious impact on host populations, including local extinction (Anderson and May 1981; Becnel and Johnson 2000; Kohler and Hoiland 2001).

1.5. Drosophila-microsporidia system

Drosophila have been extensively maintained as laboratory cultures in scientific institutions across the world and even in this artificial environment *Drosophila* have to face a range of enemies such as fungi, mites and microsporidia. There have been many reports of microsporidian infections in laboratory *Drosophila* populations, but the identity of the microsporidium has only been reported in three cases. First, *Octosporea muscaedomesticae* Flu, a microsporidium that has been found to infect a range of dipteran species including the families Drosophilidae, Muscidae, Sacrophagidae and Calliphoridae (Kramer 1973; Roxstrom-Lindquist *et al.* 2004) was observed in *D. busckii*, *D. confusa* and *D. melanogaster* (Kramer 1964b). Second, *Tubulinosema kingi* (Kramer) (formerly known as *Nosema kingi*) was found in laboratory cultures of *Drosophila willistoni* (Burnett and King 1962; Kramer 1964a), but was later known to infect a range of *Drosophila* species, including *D. melanogaster* and D*. subobscura* (Armstrong 1976; Armstrong *et al.* 1986; Armstrong and Bass 1989a; Armstrong and Bass 1989b; Franzen *et al.* 2006; Futerman *et al.* 2006). Finally, the recently described *Tubulinosema ratisbonensis* Franzen *et al.* was found in a single laboratory

culture of *D. melanogaster* (Franzen *et al.* 2005). I am not aware of the prevalence of microsporidium infections in field populations of *Drosophila*. The survey carried out by Futerman *et al.* (2006) in a university campus in southern England found only one microsporidium-infected fly, which they suggest could have escaped from the laboratory cultures (Futerman *et al.* 2006).

Tubulinosema kingi, the microsporidian parasite of *Drosophila* spp. used for investigating the host-parasite interactions in this thesis, was described four decades ago (Burnett and King 1962; Kramer 1964a). The *T. kingi* used for experimental inoculation in this thesis was from an infection that occurred in the *Drosophila/Asobara* culture system maintained in our laboratory for evolutionary studies (Kraaijeveld and Godfray 1997). The microsporidia were initially spotted in the parasitoids with physogastric (distended and pale) abdomens. Light microscopic observations of infected parasitoids showed microsporidian spores. The same spores were subsequently found in abdominal smears of *Drosophila* (Futerman *et al.* 2006). Detailed ultrastructure and molecular investigation of this infection identified the microsporidium parasite as *Tubulinosema kingi* (Franzen *et al.* 2006; Futerman *et al.* 2006).

Futerman *et al.* (2006) further investigated the effects of *T. kingi* infection on the fitness of its hosts *D. melanogaster, D. subobscura* and their parasitoid *Asobara tabida*. They also explored the routes of transmission of this microsporidian parasite within the *Drosophila*-parasitoid system. Amongst the different life history parameters that Futerman *et al.* (2006) compared in *T. kingi*-infected and uninfected *Drosophila*, they found the effect on early fecundity to be most severe. The *T. kingi*-infected flies had a 34-55% reduction in their early life fecundity (Futerman *et al.* 2006). The other traits that suffered due to microsporidian infection were increased developmental period and pupal mortality (Futerman *et al.* 2006). They found that *T. kingi* was mainly transmitted horizontally from dead cadavers to larvae, larvae to larvae and from contaminated food to larvae. However a very low level of vertical transmission from infected female flies to their offspring (approximately 10%) was also observed (Futerman *et al.* 2006). These results were in line with the previous studies investigating the effects of *T. kingi* on *Drosophila* (Armstrong 1976; Armstrong and Bass 1989a; Armstrong and Bass 1989b).

The *D. melanogaster* cultures used for the experiments described in this thesis were the same as those used by Kraaijeveld and Godfray (1997). This *D. melanogaster* population was originally derived from 250 wild flies captured near Leiden in The Netherlands and has been maintained as an outbred population for over a decade with non-overlapping generations. This *D. melanogaster* population was previously used to demonstrate evolution of resistance against the larval parasitoids *A. tabida* and *L. boulardi* (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a) and a fungal pathogen *Beauveria bassiana* (Kraaijeveld and Godfray, subm.) through artificial selection.

The investigations into host-parasite interactions required that the *D. melanogaster* base population used should be free of microsporidian infection. I examined the *D. melanogaster* base population by screening 250 randomly-selected flies from the population for *T. kingi spores* by observing their Giemsa stained abdominal smears (Pell and Canning 1993; Futerman 2005), see chapter two for method. It was necessary to maintain the base population free of microsporidium infection and hence the culturing of this population was carried out in sterile conditions. The equipment used was autoclaved and the work surface was swabbed with alcohol. The incubators and refrigerators used were periodically subjected to alcohol and bleach washes. The base population was maintained in sterile 300ml glass bottles containing a medium composed of 3.5% baker's yeast, 5% sugar, 0.5% Tartaric acid, 0.15% Potassium dihydrogen phosphate, 0.2% Ammonium sulphate, 0.05% Magnesium sulphate heptahydrate and 2% agar (hereafter referred to as yeast/sugar medium) along with a little live yeast. At periodic intervals the base population was examined for *T. kingi* infections by screening a random sample of flies from the population.

1.6. Thesis Objectives

There are many references to microsporidia infecting insect hosts. However most studies appear to concentrate on the identification and classification of the parasite with brief descriptions of their effects on their hosts but scarcely investigate the interactions between the host and its parasite. This work furthers the investigation carried out by Futerman (2005) on the *D. melanogaster*–*T. kingi* model system. Futerman examined the impact of a shared microsporidian parasite in a *Drosophila*-parasitoid system (Futerman *et al.* 2006). In this thesis I investigate the interactions between *Drosophila* and its microsporidian parasite. To the best of my knowledge this study is the first in which the evolution of

resistance against an intracellular microsporidian parasite has been investigated along with its associated costs and mechanisms. In addition, this system was useful in exploring a range of host-parasite interactions including insect immune response to microsporidia. The main body of this thesis is divided into four chapters (two to five), each describing a set of experiments investigating the interactions between *Drosophila* and its microsporidian parasite at either individual or population levels.

In chapter two I explore the within-host dynamics of *T. kingi*. I subjected different *Drosophila* life-stages to *T. kingi* infection and explored if they varied in their susceptibility, since this was unknown. I then investigated the proliferation of *T. kingi* spores within the host and identified the tissues targeted by *T. kingi* for better understanding of how the microsporidian infection spreads within the host. Among other tissues, the female reproductive organ was found to be infected by *T. kingi* and since low levels of vertical transmission had been previously observed in this system (Futerman *et al.* 2006) I explored the effect of *T .kingi* on the *Drosophila* sex ratio. In chapter three I explain how I further investigated the host-parasite interactions at an immunological level by subjecting *Drosophila* to *T. kingi* infections and looking for correlated cellular and humoral immune responses.

In chapter four I describe experimental evolution for increased resistance and/or tolerance to microsporidia in replicated populations of *D. melanogaster* along with bioassays of host life-history traits. In chapter five, I explore the costs and immune mechanisms in *Drosophila* that could be associated to the evolved resistance/tolerance against *T. kingi*.

Chapters two and three provided an insight into the interactions between *Drosophila* and *T. kingi* at an individual level, while chapters four and five explore these interactions at an evolutionary scale. In chapter six I summarise the results of the preceding chapters and examine the implications of these results on furthering our knowledge of host-parasite interactions, natural communities and biocontrol programs. I conclude this final chapter with suggestions on further investigations that could enhance our understanding of host-parasite interactions.

Chapter Two: Within-host dynamics of *Tubulinosema kingi* in *Drosophila melanogaster*

2.1. Introduction

Prior to exploring a prospective host-parasite system it is crucial to understand that factors such as dose, susceptible stages of host, parasite replication, host immune responses, parasite evasion of host resistance and mode of parasite transmission influence the interactions in such systems (Briggs and Godfray 1995; Moerbeek and Vanden Bosch 1997; Blaser and Schmid-Hempel 2005). Insect models investigating host-parasite interactions are valuable for understanding the factors involved in a disease such as: host resistance and its evolution (Kraaijeveld *et al.* 2002; Mansfield *et al.* 2003; Kanost *et al.* 2004; Schmid-Hempel 2005), pathogen virulence (Day 2002; Day 2003; Blaser and Schmid-Hempel 2005) and its modes of transmission (Day 2001; Ebert and Bull 2003), parasite-host population dynamics (Dunn and Hatcher 1997; Vizoso and Ebert 2004; Futerman *et al.* 2006) and biological pest management (Sweeney and Becnel 1991).

Very few studies have reported on the within-host dynamics of insect-infecting microsporidia (Milner 1973; Blaser and Schmid-Hempel 2005; Tokarev *et al.* 2007) even though these fundamental questions are vital in understanding the significance of microsporidia both as an opportunistic pathogen and as a plausible biocontrol agent. Thus this investigation was carried out in the *T. kingi* – *Drosophila* system, which provides for a potentially usable insect-microsporidia model. Former studies on this system (Kramer 1964a; Armstrong 1976; Futerman *et al.* 2006) have described modes of parasite transmission, fitness losses to the host and reliable methods of infecting *D. melanogaster* with *T. kingi.* However very little is known about the within-host interactions in this host-parasite system and fundamental questions such as the susceptibility of different host stages to *T. kingi* remains unanswered.

In nature insects are typically susceptible to a given parasite only for short periods of their life-cycle (Briggs and Godfray 1995). Theoretical studies have demonstrated the significance and consequences of stage specific susceptibility to the dynamics of insect-parasite systems (Briggs and Godfray 1995; Moerbeek and Vanden Bosch 1997). Microsporidia are known to depend on specific host

stages for infection, effective replication and transmission (Tanada and Kaya 1992). The change in host age or stage often corresponds to a change in host structure, behaviour, diet and probability of infection (Kennedy 1975). This type of stage- or age-specificity exhibited by a parasite for infection and transmission might be due to infection probability, mode of infection, vulnerability of the host, histology of the host stage, host stage specific immune mechanisms, temporal or spatial availability of the host, or its size and behaviour (Kennedy 1975; Elliot *et al.* 2002; Blaser and Schmid-Hempel 2005). Younger larval stages are usually reported to be more susceptible to microsporidia than the older larvae (Weiser 1969; Blaser and Schmid-Hempel 2005) but the adults are considered to be either resistant (Blaser and Schmid-Hempel 2005) or very slightly susceptible (Milner 1973). This phenomenon has been broadly termed ‘maturation immunity’ (Tanada and Kaya 1992).

Endo-parasites usually exhibit a degree of specificity to the tissues they infect and replicate within. The tissue-susceptibility to parasites depends on within-host factors like host behaviour, host and parasite genetics, natural and acquired resistance, factors associated with host age and host sex, inter- and intraspecific interactions and parasite density (Bush *et al.* 2001). However the portal of parasite entry also influences the within-host parasite establishment: parasites that infect through the oral route often either localize in the gut epithelium or cross beyond the gut wall to infect other tissues and organs, for example fat body. Parasites infecting the host through damaged body surfaces are commonly known to infect superficial tissues, while those infecting through the reproductive openings usually infect the reproductive organs and other tissues within the abdomen (Steinhaus 1949). This tissue-susceptibility is an important factor that determines the virulence of the parasite and the extent to which the host suffers (Tanada and Kaya 1992). Microsporidia infect a wide range of host tissues and *T. kingi* in particular has been reported from fat body, reproductive organs, and epithelial matrix of abdominal tracheae and alimentary tract of *D. williston* (Kramer 1964a; Armstrong 1976).

Parasites that are transmitted vertically are known frequently to skew the sex ratio of their host in order to increase their own fitness and transmission since both these factors depend on females and not males (Dunn *et al.* 1993; Dunn and Hatcher 1997; Terry *et al.* 1997; Dunn and Smith 2001; Terry *et al.* 2004). Such sex-specific virulence is achieved by parthenogenesis (Huigens *et al.* 2000),

male killing (Hurst *et al.* 1999; Bentley *et al.* 2007) and feminisation (Bouchon *et al.* 1998), and is considered to be an essential strategy for maintaining these parasites (Hurst *et al.* 1993; Dunn *et al.* 1998; Dunn *et al.* 2001; Charlat *et al.* 2003; Ironside *et al.* 2003). *Tubulinosema kingi* is transmitted both horizontally and vertically in the laboratory cultures of *Drosophila*. Transmission studies for this parasite have shown that horizontal transmission is much more prevalent than vertical transmission, but nothing is known of their relative rates in the field (Armstrong 1976; Futerman *et al.* 2006). Although sex ratio distortion has been reported in vertically transmitted parasites, it is not known if parasites with mixed modes of transmission use such strategies.

This chapter aims to address the following fundamental questions. First, to identify the *Drosophila* life stages susceptible to the *T. kingi* infection and establish whether infecting different host stages had an effect on parasite density within the host. Second, to examine how *T. kingi* proliferates during host development, and the duration after infection when mature spores are produced. Third, to identify the host tissues which are targeted by *T. king.* Finally to investigate, the possible influence of *T. kingi* on the sex ratio of *D. melanogaster* progeny.

2.2. Material and Methods

The large outbred population of *D. melanogaster* (introduced in Chapter one) reared under our standard laboratory conditions (20ºC in 16:8 light:dark regime and ambient humidity) formed the base stock for these experiments. The *T. kingi* used for experimental infections throughout this thesis was extracted from symptomatic parasitoids of *Drosophila*, *Asobara tabida*, using a method described in Futerman *et al.* (2006). The infected parasitoids were first surface sterilised by immersing them in 1% sodium hypochlorite containing traces of 0.1% SDS solution with brisk shaking for five minutes. The parasitoids were then thoroughly washed with distilled water and homogenised in 0.1% SDS and filtered through muslin cloth to remove tissue debris of the parasitoid. The spore suspension (filtrate) thus obtained was then quantified by haemocytometer. A standard spore dose of approximately 2.5×10^6 spores per 50 *D. melanogaster* larvae was used for most experiments, a dose recommended by Futerman *et al*. (2006). The spore suspensions were freshly prepared prior to inoculation for all the experiments.

2.2.1 Host susceptibility

To study the host life stages that are susceptible to *T. kingi* infection, 24 vials (80x22mm) containing yeast/sugar medium and live baker's yeast were set up. Each contained 50 *D. melanogaster* eggs which were collected by allowing flies to mate and oviposit overnight in sterile 300ml bottles with medium and live yeast. These vials were incubated at 20ºC and 16:8 light:dark regime. The *D. melanogaster* life stages investigated here were a) 1st, 2nd, early 3rd and late 3rd instar larval stages, determined by time since oviposition, b) pupae and darkened pupae just prior to emergence, determined by morphology, and c) just emerged flies and three day old flies, six hours and 72 hours after emergence respectively. The 24 vials were divided into eight batches of three vials, each of which were treated as replicates for each life stage. As the individuals within the vials developed and reached each of the above mentioned stages the three vials were inoculated with ~2.5×10^6 *T. kingi* spores per vial, the spore suspensions for each vial was prepared individually. 24 hours post inoculation the individuals within the vial were washed out and rinsed repeatedly with millipore water to remove any adhering spores (with the exception of the adult stages) before being transferred to sterile vials containing medium and live yeast, which were then incubated at 20°C. The date of inoculation was recorded for each of the eight life stages. On the 17th day after inoculation for each stage, ten individuals from each of the three replicate vials were randomly selected and smeared on glass slides and giemsa stained (Pell and Canning 1993). It was thus ensured that the infection status of each stage was examined after the same duration of infection. The following Giemsa staining procedure was used. Slides with smeared samples were fixed with 100% methanol for five minutes and then air-dried. The smears were then stained in 10% Gurr's improved R66 Giemsa stain in pH 7.2 phosphate buffer for 45 minutes (Futerman 2005). The slides were then rinsed in running water and gently blot-dried. The slides were examined under oil immersion at 1000x magnification.

The infection status of the host was determined semi-quantitatively (Futerman *et al.* 2006) by assigning the following scores to the microscope observations of spore density: slides with numerous spores spread all over were considered heavily infected and scored 4; slides with plenty of spores mostly in large groups were considered moderately infected and scored 3; slides with few spores found mostly in small groups were considered lightly infected and scored 2; slides with just a few spores found occasionally across the slide were considered very lightly

infected and scored 1; whilst slides that were free of any *T. kingi* spores were considered to be uninfected and scored 0.

2.2.2 *T. kingi* spore proliferation

To monitor the *T. kingi* spore proliferation within the host after infection, *D. melanogaster* adults were allowed to mate and oviposit overnight in sterile 300ml bottles with yeast/sugar medium and live baker's yeast. The eggs were collected the following day and transferred to a Petri plate (9cm) containing yeast/sugar medium with live yeast and the surface smeared with *T. kingi* spore suspension (~ $1x10^7$ spores). The plates were then incubated at 25ºC for 24 hours, after which the hatched larvae were washed from the plate and rinsed with sterile water. Twenty vials (80x22mm) were set up with fresh medium and live baker's yeast. Approximately 50 larvae were transferred into each vial and the vials were then incubated at 20ºC in a 16:8 light:dark regime. For the 20 following days, one vial per day was randomly selected. Ten randomly selected individuals from this vial were giemsa stained as described in section 2.2.1. The slides were then examined and semi-quantitatively scored as in section 2.2.1.

2.2.3 Host tissue specificity

To identify the tissues targeted and infected by *T. kingi*, *D. melanogaster* from the base population were allowed to oviposit in bottles containing medium and live yeast for six hours. 50 eggs were transferred into each of the three vials (80x22mm) containing medium and live yeast. 24 hours later, ~$2.5x10^6$ *T. kingi* spores in 0.1% SDS were added to each vial and incubated at 20ºC. 24 hours after the flies emerged, two adult flies of each sex per vial were sectioned and observed by light microscopy. The flies were fixed in 4% phosphate buffered formaldehyde (PBF) for 48 hours, the head, wings and legs of the specimens were cut off and the specimen dehydrated in 70% ethanol for one hour, 95% ethanol for three hours and thrice in 100% acetone for three hours. The specimens were then infiltrated (impregnated) overnight in 1:1 acetone:Spurr's mixture (an embedding resin), followed by 24 hours in 1:5 acetone:Spurr's mixture and finally in 100% Spurr's mixture for another 24 hours. All of the above processing was carried out at room temperature.

After infiltration, the specimens were transferred to fresh 100% Spurr's mixture and placed in a vacuum oven for 30 minutes. They were then transferred into capsules and the resin was allowed to polymerise at 60ºC. Semi-thin sections

(0.5 micron and 1micron) of the specimens were cut on a Reichert Ultracut microtome and 3 levels per slide (15micron between levels) were stained with 0.1% toluidine blue in 1% borax for one minute at room temperature. The slides were examined by light microscopy at 100X magnification. Qualitative data was collected on the tissues that harboured the spores and the representative tissues were photographed. In addition to this one fly of each sex embedded in Spurr's resin mixture was sent to the laboratory of Dr. Alan Curry at Manchester Royal Infirmary to be sectioned and stained for electron microscopy.

2.2.4 Sex ratio distortion

To investigate the influence of parasite infection on the sex ratio of the host offspring adult *D. melanogaster* from the base stock were allowed to lay eggs overnight in sterile 300ml bottles with yeast/sugar medium and live yeast. The eggs were washed and 20 vials (80x22mm) with medium and yeast, containing 50 eggs in each were set up. Ten of the 20 vials were then infected with ~$2.5x10^6$ *T. kingi* spores in 0.1% SDS per vial and rest of the vials were treated with equal volumes of sterile 0.1% SDS solution. The vials were incubated at 20ºC and 16:8 hour light:dark regime till the flies emerged, after which three randomly selected females per vial were transferred into three individual vials (80x22mm) containing medium and yeast. Two males from the same vial were added to each of the three vials to ensure mating. The set up therefore had two sets of 30 vials containing infected flies and uninfected control flies. The vials were then incubated for 24 hours to allow the flies to mature and mate. Following this the flies were transferred every 24 hours into fresh vials containing medium and live yeast and the previous day vials, containing eggs were incubated at 20ºC. This process of transferring the flies into fresh vials every day was stopped when the respective females died. Once the offspring flies from the incubated vials emerged they were transferred to plastic containers, labelled and frozen at -20ºC, to be counted and sexed later.

2.3. Results

2.3.1. Host susceptibility

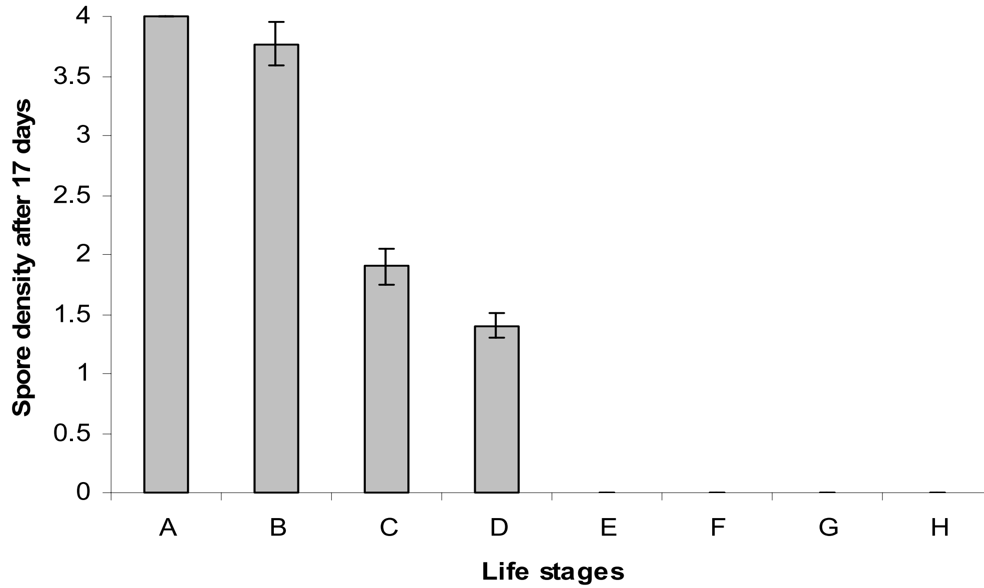

Fig. 2.1. Susceptibility of different *Drosophila* life stages to *T. kingi* (density ± S.E.). A- 1st instar; B- 2nd instar; C- early 3rd instar; D- late 3rd instar; E- pupae; F- darkened pupae; G- just emerged adult fly & H- 3 day old flies.

Tubulinosema kingi spores were only observed in smears of *D. melanogaster* infected as larvae; the smears of flies infected as pupae and adults showed no signs of infection. The mean parasite densities (n = 10) per replicate was used for analysis. A one-way ANOVA revealed significant differences in spore density between the larval stages ($F_{3,\ 8}$ = 101.11, p<0.001). The means and standard deviations are presented in Fig. 2.1. The Tukey HSD procedure revealed that not all the pairwise differences among means were significant. The P-values are presented in Table 2.1.

d.f. = 8				
	1st instar	2nd instar	Early 3rd instar	Late 3rd instar
1st instar		0.67	0.00014	0.00014
2nd instar			0.00014	0.00014
Early 3rd instar				0.070

Table 2.1. Tukey HSD test comparing the susceptibility of different *D. melanogaster* life stages to *T. kingi*.

2.3.2. *T. kingi* spore proliferation

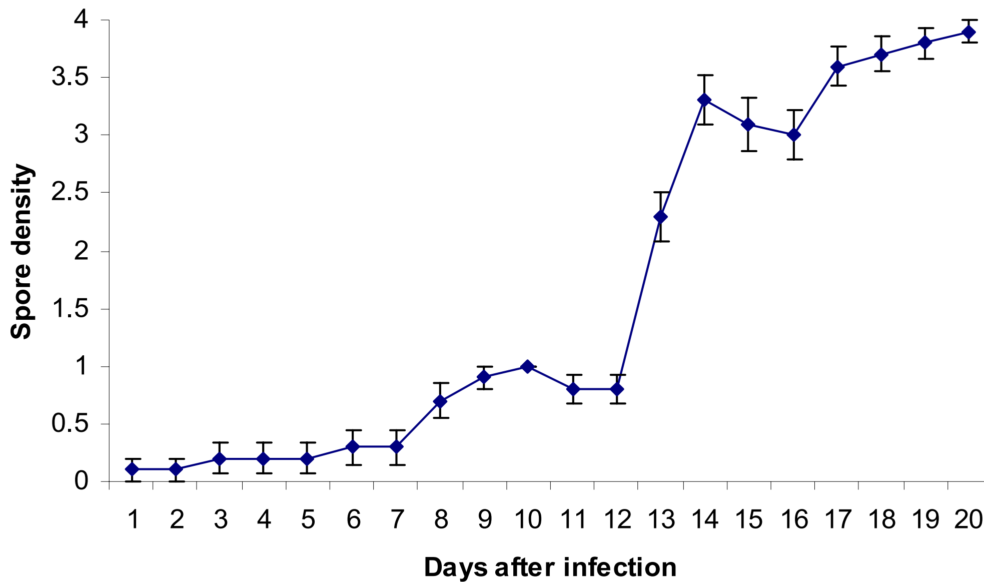

Fig. 2.2. Within-host proliferation of *T. kingi* across 20 days after infection (density ± S.E.). Stages corresponding to days are; larvae: day 1-5, pupae: day 6-12, adult: day 13-20.

The spore density of *T. kingi* was quantified across the *D. melanogaster* developmental period. The mean spore densities in larvae (mean=0.16 ± 0.05, n =5), pupae (mean = 0.67 ± 0.28, n =7) and adults (mean = 3.34 ± 0.53, n =8) was used for analysis. A one-way ANOVA showed that the spore density was significantly different between the different life-stages ($F_{2, 17} = 140.98$, $p<0.001$). The spore proliferation over the 20 days following infection is presented in Fig. 2.2. The Tukey HSD procedure revealed that the pairwise differences among means were significant only between adults and pre-adults. The p-values are presented in Table 2.2.

d.f. = 17			
	Larvae	Pupae	Adult
Larvae		0.083	0.00016
Pupae			0.00016

Table 2.2. Tukey HSD test comparing the *T. kingi* spore density in different *D. melanogaster* life stages.

2.3.3. Host tissue specificity

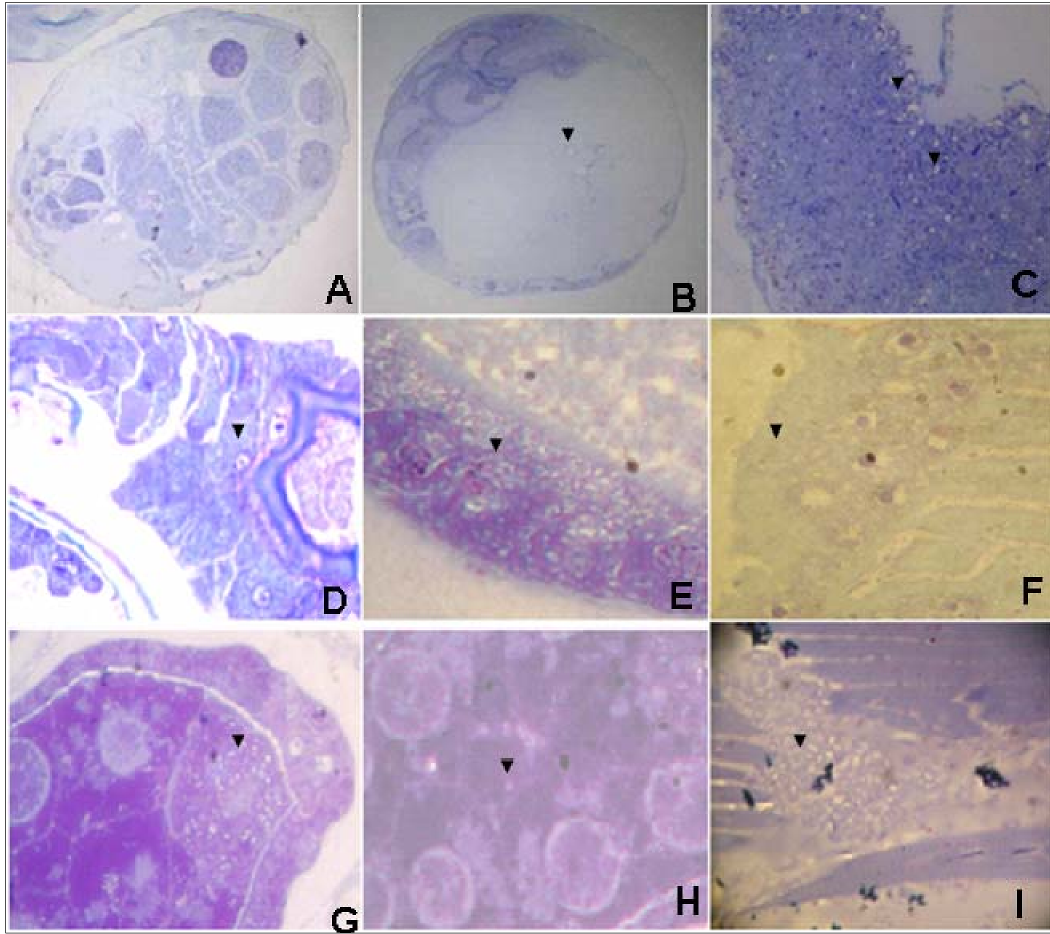

Fig. 2.3. Histological sections of *Drosophila melanogaster* infected with microsporidian parasite *Tubulinosema kingi* (indicated by black arrow heads) ; (A & B) Cross section through abdomen of infected female and male respectively. (C) Infected fat body tissue, (D, E & F) infected tissues of the alimentary canal; (G & H) infected female reproductive tissues, and (I) infected thoracic muscles.

In longitudinal sections of *D. melanogaster* of both sexes the parasite *T. kingi* was mostly observed in the abdominal region, but a few spores were observed in the thorax of males, where the muscles were observed to be infected (see I in Fig. 2.3). The cross sections of *T. kingi* infected males showed greater host tissue degeneration in contrast to the cross sections of infected females (see A & B in Fig.2.3). *Tubulinosema kingi* was found in the fat body (see C in Fig. 2.3) and in the epithelium of malpighian tubules and alimentary canal of both sexes (see D, E & F in Fig 2.3). The female reproductive tissue was observed to be lightly infected with a few spores found in the follicles (see G & H in Fig 2.3), I failed to examine or identify the male reproductive tissue since most abdominal tissues had degenerated following infection. The transmission electron

micrographs of *D. melanogaster* male and female showed the different developmental stages of *Tubulinosema kingi*. Presented below is a representative micrograph of *D. melanogaster* female showing the different developmental stages of *T. kingi* (see Fig. 2.4.).

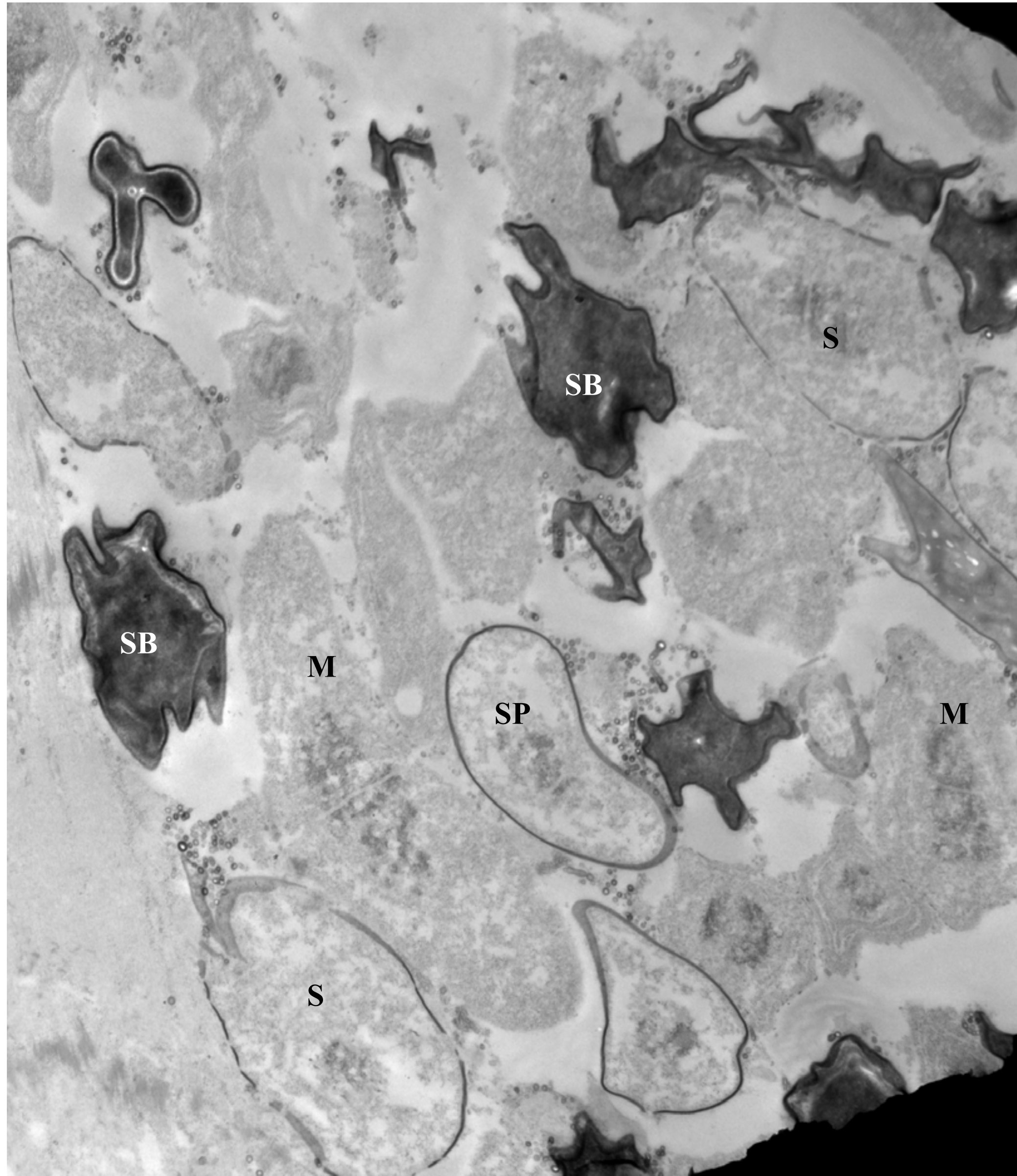

Fig. 2.4: Transmission electron micrograph of *Tubulinosema kingi* developmental stages (M-meronts, S-beginning of sporogony, SP-sporont and SB-sporoblast) in a *Drosophila melanogaster* female (Courtesy A. Curry).

2.3.4. Sex ratio distortion

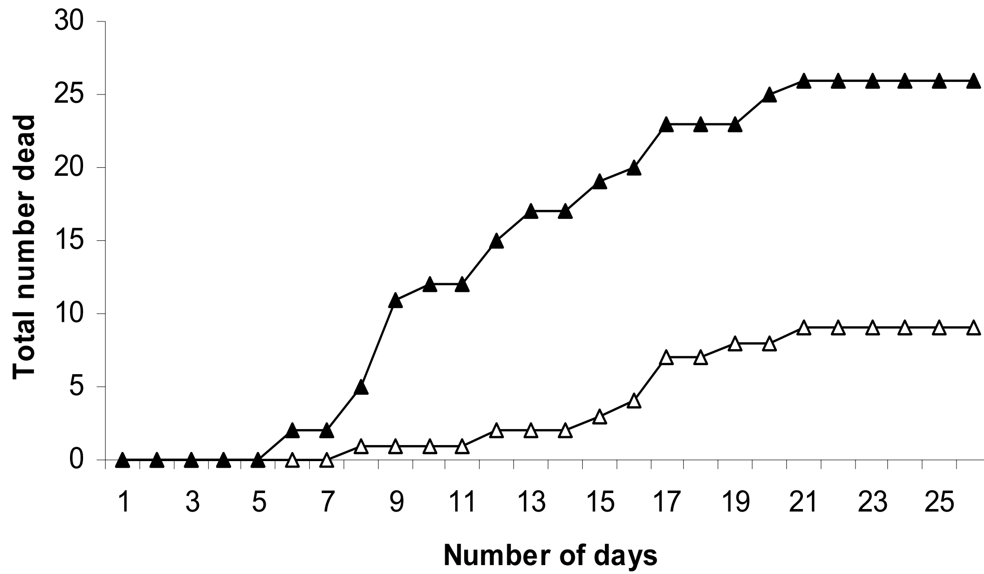

Fig. 2.5. Mortality of infected (filled triangles) and control (open triangles) females during the sex ratio assay.

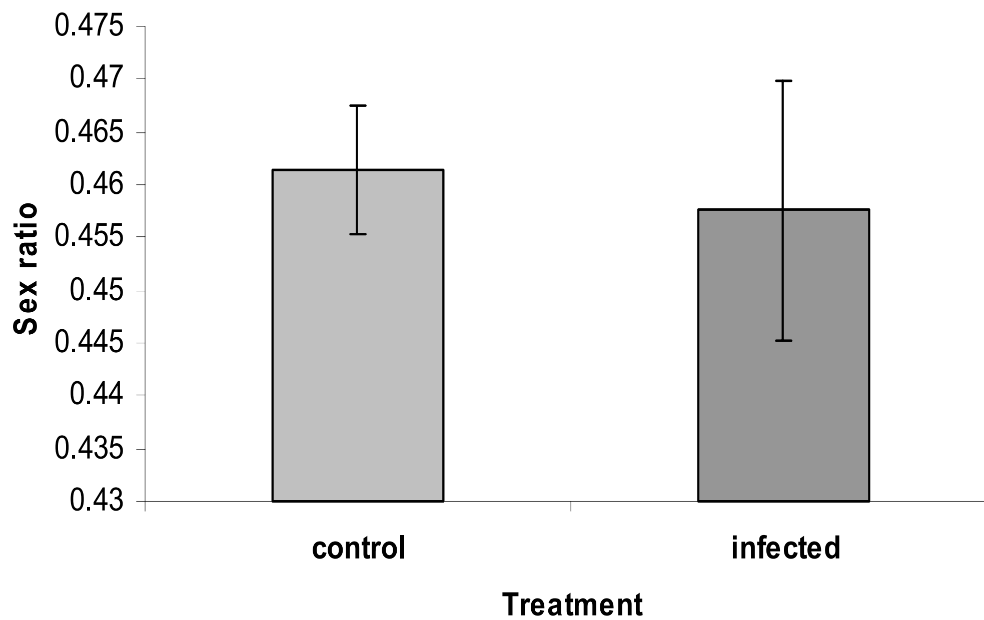

Fig. 2.6. The effect of microsporidian infection on offspring sex-ratio (mean ± S.E.) of *D. melanogaster*.

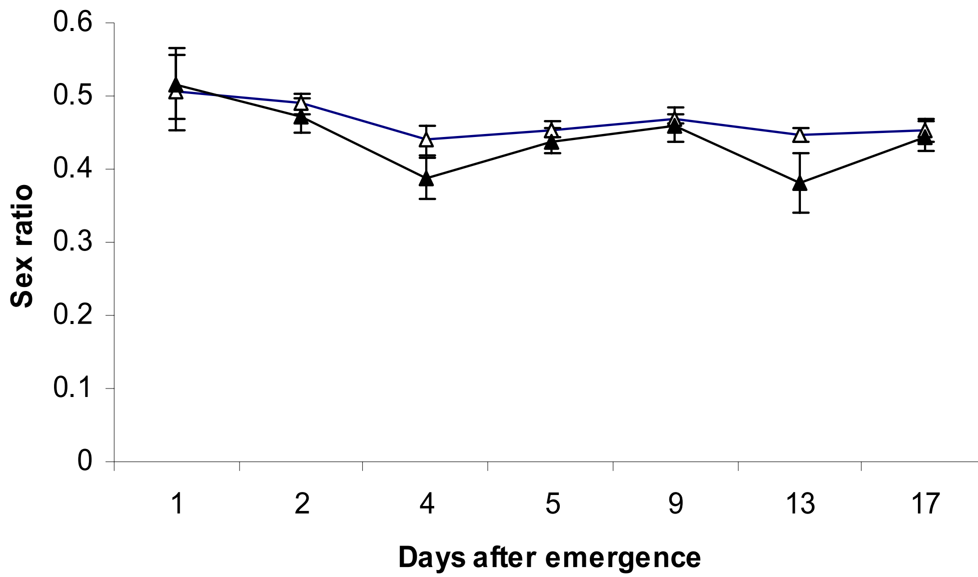

Fig. 2.7. The effect of duration since infection on sex ratio of *D. melanogaster***.** The sex ratio (male: female ± S.E.) of control flies (open triangles) and infected flies (filled triangle) are shown across seven days.

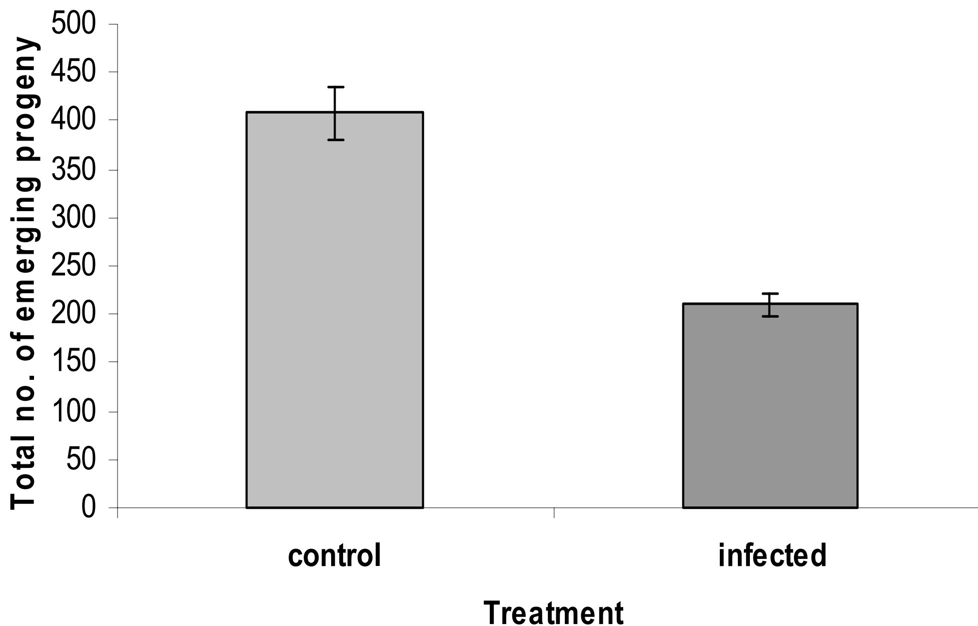

Fig. 2.8. The effect of microsporidian infection upon the number of emerging progeny (mean/treatment ± S.E.).

The experiment was stopped after 26 days, when all the 30 infected females from ten replicates had died. Mortality occurred early in infected females (see Fig. 2.5) with ~70% dead by day 17 which was significantly higher than in uninfected

females where only ~20% died (Fishers exact test, p=0); after day 17 the infected females that lived laid very few or no eggs while the uninfected females continued to produce large number of eggs. I counted and sexed the progeny of infected and uninfected females at days 1, 2, 4, 5, 9, 13 and 17 thus aiming at including days when most females were alive along with covering the entire period. The total number of males and females produced by the three females in each replicate were used to calculate the sex ratio for the replicate and for subsequent analysis.

The figure 2.7 shows the sex ratio of the offspring of *T. kingi* infected and uninfected females 1, 2, 4, 5, 9, 13 and 17 days after emergence and suggests that there might be a slight tendency for female biased sex ratios over time. However, a repeated-measures ANOVA on the (arcsine-transformed) sex ratios shows no difference in sex ratios between the offspring of females from the two treatments ($F_{7,4}$=0.83, p=0.61). This is confirmed by the lack of significant difference when comparing the overall sex ratios of the offspring of infected and uninfected females (Fig.2.6) (t_{18} = 0.68, p=0.05). However as expected a highly significant difference ($F_{1,18}$ = 46.334, p< 0.001) in the total number of emerging offspring of infected females and uninfected females (Fig. 2.8) was observed.

2.4. Discussion

The results of the experiments described in this chapter clearly indicate that different host life stages are crucial to microsporidia for infection, proliferation and transmission. This is most clearly seen here with *D. melanogaster* in which *T. kingi* infection and proliferation within the host and transmission between hosts strongly depended on host stage such that: a) only larval stages are susceptible (Fig. 2.1), b) microsporidia proliferate only in pupae and adult flies (Fig. 2.2) and c) mature spores are mainly observed within adult flies. The tissues targeted by *T. kingi* indicate that the parasite, after orally infecting its host, crosses the gut wall and infects the target organs that lie beyond. The microsporidia had no impact on the host sex ratio, although the number of offspring produced by infected flies was significantly lower than by uninfected flies.

The susceptibility of larvae and the decrease in susceptibility with larval age was found to be in agreement with earlier reports with other insect-microsporidia models (Milner 1973; Onstad and Maddox 1990; Altizer and Oberhauser 1999;

Maddox *et al.* 2000; Blaser and Schmid-Hempel 2005). The causes of larval susceptibility to microsporidia are not clear, but this may be due to physical factors such as histology of the larval gut. Blaser and Schmid-Hempel (2005), studying microsporidian infection in *Tribolium*, highlighted the role played by the gut wall in stage-specific infection. However, the parasite might have evolved to only target the larvae. In *Tribolium*, adults and larvae coexist in stored products therefore having the same probability of feeding on microsporidian spores but in *Drosophila*, adults and larvae differ in their feeding behaviour and hence in the probability of feeding microsporidian spores. The microsporidium might thus never encounter an adult since adults are in contact with food patches (potential source for infection) only for short durations: to feed, mate and oviposit thus suggesting that *T. kingi* might have evolved to target only the larvae. The time spent by larvae foraging is presumably higher than by adults suggesting that larval behaviour could also be responsible for its susceptibility. The decrease in susceptibility of older larvae (3rd instar) (Fig. 2.1) observed here could be due to the fact that younger larvae (2nd instars) forage usually on the surface of the medium where dead cadavers and free microsporidian spores are usually found in laboratory cultures, while the older larvae tend to burrow into the medium and are likely to have had reduced contacts with the spores. Other factors such as gut volume (Weiser 1969), gut content and histological reorganisation during pupation (Milner 1973) could also be responsible for this stage-specific susceptibility to the parasite.

Tubulinosema kingi has been reported in infected *Drosophila* flies while its presence in larvae and pupae has never been investigated (Kramer 1964a; Armstrong 1976; Franzen *et al.* 2006; Futerman *et al.* 2006). The microsporidial infection is most obvious in smears of adult stages due to the presence of distinguishable spores, while in larval and pupal smears only the developmental stages of microsporidia are observed which are difficult to distinguish. The difference in transmissible spore densities between the three host life stages sheds light on the within-host dynamics of the parasite. This host-stage correlated delay in production of transmissible spores might be associated with three main factors. Transmission studies of *T. kingi* in the laboratory have shown that the parasite is mainly transmitted by infected adult cadavers, in faeces and in or on eggs, suggesting that parasite transmission success thus depends on adult hosts (Armstrong 1976; Futerman *et al.* 2006). b) The parasite might have been selected for delayed proliferation to avoid detrimental effects on juvenile

stages, thus allowing subsequent emergence of spore carrying adult flies (Futerman *et al.* 2006).Finally the interaction of the parasite with its host's internal environment, such as insect immune responses and inter- and intra-specific competition among parasites may itself restrict parasite development and/or proliferation. Although scarcely reported and indecisive these within-host factors cannot be neglected while addressing such questions (David and Weiser 1994; Nassonova *et al.* 2001; Hoch *et al.* 2004; Futerman *et al.* 2006).

The adult *D. melanogaster* tissues targeted by *T. kingi* were similar to those targeted in *D. willistoni* (Kramer 1964a; Armstrong 1976). To reach the target organs *T. kingi* needs to cross the hostile insect gut, which it appears to breach effectively suggesting that proliferation route involved is complex and needs detailed investigation. The tissues of the alimentary canal and reproductive organs that were observed to be infected are usually targeted by parasites as a potential exit from the host (Tanada and Kaya 1992). These tissues are known to be targeted only during the final stages of infection to avoid premature host death by septicaemia due to other microbes present in the gut and reproductive tracts (Maddox *et al.* 2000). The other common tissues infected by microsporidia is fat bodies, which may be targeted for nutritional requirements (Hoch *et al.* 2002). The sections of infected males showed comparatively greater degeneration than those of infected females. However, due to the small sample size examined here, it would be inappropriate to class this as differential virulence.

Parasite strategies such as host feminisation and male killing are well known phenomena in parasitology and parasites that are mainly transmitted vertically have been reported to increase their transmission fitness by distorting the sex ratio of the host. Microsporidia are known to cause sex ratio distortion in invertebrates (Weiser 1976; Dunn *et al.* 1993; Ironside *et al.* 2003; Terry *et al.* 2004). No evidence for sex ratio distortion by *T. kingi* was observed suggesting that these strategies are applicable to parasites that are mainly transmitted vertically.

The within-host interactions demonstrated here between *T. kingi* and *D. melanogaster* were important in both designing the experiments and interpreting the results obtained in the following chapters. The information on susceptibility of the host would be useful in designing bio-control programs for pests and in eradication of parasites from host populations. Information on the within-host

parasite proliferation would also be of use in recovering maximum yield of a bio-pesticide, determining the modes of parasite transmission and in identifing the parasite during diagnosis. In conclusion, the study here highlights the need for further research on the parasite’s interactions with the internal environment of the host and suggests that information on the within-host dynamics of a parasite is crucial for understanding host-parasite interactions.

Chapter Three: Innate immune responses to *T. kingi* infection in *Drosophila melanogaster*

3.1. Introduction

In the previous chapter, I explored within-host interactions between the microsporidian parasite *Tubulinosema kingi* and its host *Drosophila melanogaster* in terms of host susceptibility, tissue specificity and parasite proliferation. In this chapter I shall continue investigating this host-parasite interaction at an immunological level. Insects are generally exposed to a wide range of natural parasites, from microorganisms such as viruses, bacteria and fungi, to macro-parasites such as mites, nematodes and parasitoids. To counteract such parasitic invasions insects provide their parasites with hostile environments, both in terms of physiological barriers, like the gut wall or cuticle, and effectors of innate immune responses, such as haemocytes and antimicrobial peptides. Insect survival in many cases depends on their ability to tolerate and/or resist these parasites. In insects, the mechanistic basis of such tolerance or resistance is through innate immunity (Lemaitre and Hoffmann 2007). The insect innate immune system generally responds to a parasite either after it is recognised as non-self, or as a result of damage caused by it, by mounting both cellular and humoral defence reactions (Hoch *et al.* 2004). The cellular immunity consists of different classes of haemocytes, or blood cells, that are involved in phagocytosis and encapsulation of foreign organisms, while the humoral immunity constitutes of a number of processes including phenoloxidase activation for melanisation and induction of antimicrobial peptides in response to parasitic infections (Lemaitre and Hoffmann 2007).

Very little is known about insect immune responses to microsporidian infections (Hoch *et al.* 2004). Recent studies have indicated that microsporidian infections can trigger both cellular and humoral immune responses in insects (Kurtz *et al.* 2000; Hoch *et al.* 2004). Laigo and Paschke (1966) observed a temporary decrease in the number of circulating haemocytes in cabbage looper, *Trichoplusia ni* when infected by microsporidia. Hoch *et al.* (2004) report a significant increase in haemocyte counts in *Lymantria dispar* when infected by the microsporidium *Vairimorpha disparis*, however the haemocyte density of *L. dispar* was found to decrease when infected with different host strains of *Vairimorpha*.

Phagocytosis of microsporidia by insect haemocytes has been reported (Laigo and Paschke 1966; Cali and Briggs 1967; Kellen and Lindegren 1972; Abe 1978; Nassonova *et al.* 2001; Hoch *et al.* 2004) suggesting that haemocytes contribute to host immune responses against microsporidian infections. However, observations of microsporidian stages within circulating haemocytes have suggested that insect phagocytosis is inefficient in killing the parasite and that instead of destroying these spores the haemocytes themselves get infected (Hazard and Fukuda 1974; Hazard *et al.* 1984; David and Weiser 1994; Kurtz *et al.* 2000). David and Weiser (1994) observed that haemocytes get infected by spores they phagocytose and suggested that these haemocytes facilitate the spread of microsporidian infection throughout the host body. Other studies on insect-infecting microsporidia have often identified haemocytes as sites of microsporidian infection, thus providing more support for David and Weiser's hypothsis (Larsson 1992; Sokolova and Lange 2002; Sokolova *et al.* 2003). Kurtz *et al.* (2000) confirmed phagocytosis of spores and suggest that if extrusion of polar filament could be determined with certainty this observation could support the view of David and Weiser (1994) that haemocytes support parasite multiplication and proliferation. Further evidence to support this was provided by Nassonova *et al.* (2001), who demonstrated that phagocytosis of microsporidian spores *in vivo* and *in vitro* by haemocytes lead to replication and proliferation of the parasite rather than its destruction. They also found evidence that microsporidian spores are able to prevent acidification of the phagosomes they reside in, thus preventing their destruction and ensuring further dissemination. Apart from phagocytosis of microsporidian spores, haemocytes have also been reported to encapsulate microsporidium-infected insect tissues (Hoch *et al.* 2004).

Microsporidian infection in insect hosts has been shown to elicit humoral immune responses such as nodule formation and melanin deposition resulting in the formation of atypically shaped spores (Tokarev *et al.* 2007). Decreased melanisation levels and reduced phenoloxidase activity has been reported in microsporidium-infected insects (Tokarev and Sokolova 2005). However, in other insect-microsporidium systems elevated rates of melanisation have been observed in response to infection (Hoch *et al.* 2004; Tokarev *et al.* 2007).

In *Drosophila*, the immune response can be broadly divided into three categories: a cellular response involving phagocytosis and encapsulation; a phenoloxidase cascade resulting in deposition of melanin on the target site (wound or foreign

object); and an antimicrobial peptide response (Lavine and Strand 2002; Tzou *et al.* 2002; Hoffmann 2003; Hultmark 2003; Meister and Lagueux 2003; Lemaitre and Hoffmann 2007).

The immune defence reactions of *Drosophila* reported so far has been mostly against extra cellular parasites such as fungi, bacteria and parasitoids (Lemaitre and Hoffmann 2007). In comparison very little is known about its immune responses against an intracellular parasite such as microsporidia. *Drosophila* uses both cellular and humoral responses against macro-parasites such as parasitoids and this involves encapsulation followed by melanisation. Similarly, *Drosophila* combats microparasites such as bacteria and fungi with specific antimicrobial peptides and phagocytosis (Lemaitre and Hoffmann 2007). Parasite recognition is considered to be the primary step in *Drosophila* immune response and this involves recognition of parasitic material as self and non-self prior to destroying them (Wang and Ligoxygakis 2006; Hultmark and Borge-Renberg 2007). Microsporidia, due to their intracellular nature, presents the *Drosophila* immune system with a unique challenge.

The cellular response is mainly observed in larvae and involves haemocytes that can be distinguished into three functional types: plasmatocytes that are involved in phagocytosis of invaders like bacteria, yeast and apoptotic bodies; lamellocytes that are involved with encapsulation of larger objects such as parasitoid eggs and infected tissues; and finally crystal cells that contain phenoloxidase and are considered to play a role in melanisation (Lavine and Strand 2002). The cellular response in insects to parasites has been previously quantified by determining the haemocyte counts in larval haemolymph (Kraaijeveld *et al.* 2001b; Silva *et al.* 2002; Hoch *et al.* 2004). Futerman (2005) investigated the effect of haemocytes on microsporidia using *D. melanogaster* lines that were previously selected for increased resistance against parasitoid wasps and which were known to vary in their total haemocyte densities (Kraaijeveld and Godfray 1997; Kraaijeveld *et al.* 2001b). It is important to stress here that the base population used for experiments here is same as that used by (Kraaijeveld and Godfray 1997) and hence have the same genetic background (see chapter one). Futerman (2005) infected these *Drosophila* lines with *T. kingi* and compared the fitness loss in terms of early fecundity. *Tubulinosema kingi* infection had previously shown to affect this particular life-history trait greatly (Futerman *et al.* 2006). Futerman found that higher haemocyte density did not

decrease the fitness loss in the host, suggesting that haemocytes do not play an important role in resisting microsporidia (Futerman 2005). Apart from this study, nothing is known about the cellular immune response of *Drosophila* to microsporidia.

The phenoloxidase system is an humoral immune response in *Drosophila*, where injuries and presence of non-self objects result in melanin deposition around the damaged tissue or intruding objects (Bidla *et al.* 2005). The enzyme phenoloxidase is present in insect haemolymph in the form of inactive prophenoloxidase and is activated by a serine protease after recognition of injury or intrusion. This active phenoloxidase catalyses the oxidation of phenols to quinones that further polymerize to melanin (Soderhall and Cerenius 1998; Cerenius and Soderhall 2004; Hoch *et al.* 2004). Determining the phenoloxidase activity in insect haemolymph has been often used as a measure of haemolymph melanisation (Tzou *et al.* 2002; Hoch *et al.* 2004; Schwarzenbach *et al.* 2005). Melanin and its biosynthetic byproducts, such as hydrogen peroxide and nitric oxide, are considered to be directly toxic to microorganisms (Evans *et al.* 2003). However, more recent evidence has suggested that phenoloxidase activation is not essential to combat microbial infections in *Drosophila* (Leclerc *et al.* 2006). Thus the role of phenoloxidase in resisting microparasites of *Drosophila* is ambiguous and the phenoloxidase response to its intracellular parasite *T. kingi* is not known.

Drosophila also relies on a battery of injury- or pathogen-induced antimicrobial peptides secreted by the fat body (Lemaitre and Hoffmann 2007). To date seven distinct peptides (plus isoforms) have been identified in *Drosophila*, upregulated in response to microbial infections (Lemaitre and Hoffmann 2007). The expression of these antimicrobial peptides is regulated through two immune pathways, the Toll pathway and the *imd* pathway (Lemaitre *et al.* 1997; Lemaitre and Hoffmann 2007). Differential induction of antimicrobial peptides in *Drosophila* by various classes of microbes has been reported (Lemaitre *et al.* 1997) but did not include microsporidia. Roxstrom-Lindquist *et al*. (2004) also investigated parasite-specific immune responses in *Drosophila* using a genomic approach, and included a microsporidian parasite *Octosporea muscaedomesticae* among the parasites investigated in this study. Antimicrobial peptides were not upregulated in response to microsporidian infection, however a range of lysozymes were found to be upregulated (Roxstrom-Lindquist *et al.* 2004).

Apart from the above mentioned three categories of *Drosophila* immune responses, nitric oxide (NO), a highly reactive molecule with an innate immune function of destroying invading microorganisms, has recently gained attention (Nappi *et al.* 2000; Rivero 2006). A number of studies provide evidence for the antiparasitic nature of NO (Foley and O'Farrell 2003; Faraldo *et al.* 2005; Krishnan *et al.* 2006). Within-host NO levels can be increased by including L-arginine in the diet, from which NO is synthesized by NO synthase (NOS) (Regulski and Tully 1995) and this has provided a method to determine the effect of NO on parasites (Nappi *et al.* 2000; Foley and O'Farrell 2003).

In this chapter, I quantify three parameters of *D. melanogaster* immune response to an intracellular microsporidian parasite *T. kingi*. First, to quantify the cellular immune response I determined the effect of *T. kingi* infection on larval haemocyte density. Second, to quantify the phenoloxidase response I determined the effect of *T. kingi* infection on phenoloxidase activity in larval haemolymph. Third, I quantified the effect of increased L-arginine intake on parasite density.

3.2. Materials and methods

3.2.1. Cellular immune response

To determine the effect of *T. kingi* infection on the larval haemocyte density *D. melanogaster* from the base stock were allowed to oviposit in sterile 300ml bottles with yeast/sugar medium and live yeast. The eggs were collected and distributed (~75 eggs per vial) into 60 glass rearing vials (80x22mm) with yeast/sugar medium and live yeast. The vials were incubated overnight at 25ºC and the following day ~$2.5x10^6$ *T. kingi* spores in 0.1% SDS was added to 30 vials and an equal volume of 0.1% SDS to the remaining 30 vials, which were thus designated as ‘infected’ and ‘control’ treatments respectively. The vials were then divided into three groups, each comprising ten infected and ten control vials. The vials were then incubated at 20ºC with a 16:8 hour light:dark regime. The three groups differed in the post-infection incubation period: 24 hours, 48 hours and 72 hours; after these time periods, the larvae in the vials will have reached second, early third and late third instar, respectively. After the respective incubation period, larvae from 9 infected and 9 control vials per group were washed and collected separately. A haemolymph sample was extracted from a single set of larvae per vial and the haemocyte count in it was determined twice. The mean haemocyte count per vial was calculated as the average of the two

counts. In the first group 20 second instar larvae from each vial were bled and 3µl of the pooled haemolymph was pipetted onto a haemocytometer to determine the haemocyte counts at 40X magnification under a light microscope. While for the second and third groups haemocyte counts were determined similarly but with pooled haemolymph from 15 larvae per vial rather than 20. The remaining pair of vials in each of the three groups was further incubated at 20ºC; on emergence of the adult flies, abdominal smears of 15 flies per vial were screened for *T. kingi* infection by Giemsa staining (as described in chapter one) to confirm the absence of infection in flies from control vials, and the presence of infection in flies from infected vials. All flies from the control vials from all three groups were uninfected while all flies from the infected vials from all three groups were infected. The mean haemocyte density per vial was analysed by a two-way ANOVA, with incubation period and treatment as the two factors.

3.2.2. Phenoloxidase activity

To determine the effect of *T. kingi* infection on phenoloxidase activity in larval haemolymph, *D. melanogaster* from the base stock were allowed to oviposit in sterile 300ml bottles with yeast/sugar medium and live yeast. The eggs were collected and distributed (50 eggs per vial) into 16 glass vials (80x22mm) with yeast/sugar medium and live yeast. The vials were incubated at 25ºC with a 16:8 hour light:dark regime for a day. After 24 hours approximately 2.5×10^{6} *T. kingi* spores in 0.1% SDS were added to eight vials and an equal volume of 0.1% SDS to the remaining eight vials, designated as 'infected' and 'control', respectively, and further incubated for 72 hours at 20ºC.

Phenoloxidase activity was measured in two haemolymph samples, extracted from two separate sets of larvae per vial. The phenoloxidase activity was determined using the method suggested in Tzou *et al*. (2002). For each sample, 3µl of haemolymph pooled from ten third instar larvae was added to 50µl of 10mM phosphate buffer (pH 5.9) containing 10mM L-DOPA in a 50µl-2000µl disposable cuvette (Eppendorf catalogue number: 952010069). The optical density was recorded at five minute intervals for 30 minutes at 470nm in a spectrophotometer (WPA, Lightwave, UK). The enzyme activity for each sample was measured as the slope (absorbance vs. time) of the reaction curve during the linear phase of the reaction. Any optical density readings that were recorded as greater than two were removed from the analysis, as these were values which the spectrophotometer failed to measure. The mean phenoloxidase activity per

vial was calculated as the average of the slopes of the two replicates measured during the linear phase and this was used in the analysis. The phenoloxidase activity in *T. kingi*-infected and uninfected larvae was compared using a t-test for unequal variance.

3.2.3. Effect of nitric oxide

To study the effect of increased L-arginine intake on parasite density, 40 glass rearing vials (80x22mm) with yeast/sugar media were set up and split into four groups of ten vials each. The four groups were supplemented with 0mM, 0.63mM, 6.3mM and 63mM (corresponding to 0mg/ml, 0.11mg/ml, 1.1mg/ml and 11mg/ml respectively) concentrations of L-arginine respectively. I used three different levels of L-arginine since the optimum concentration of L-arginine for such an experiment was not known. Uninfected *D. melanogaster* were allowed to oviposit in sterile culture bottles containing medium and live yeast at 25ºC for 6 hours. Subsequently, the eggs were collected and 50 eggs were added to each of the 40 vials. Approximately 2.5×10^6 *T. kingi* spores were then added to each of the 40 vials, which were incubated at 20ºC with a 16:8 hour light:dark regime, until the flies emerged. Three flies per vial were chosen at random to determine within-host parasite density. The abdomen of each fly was homogenised in 100µl of 0.1% SDS and the *T. kingi* spore density in the sample was determined using a haemocytometer at 40X magnification under a light microscope.

3.3. Results

3.3.1. Cellular immune response

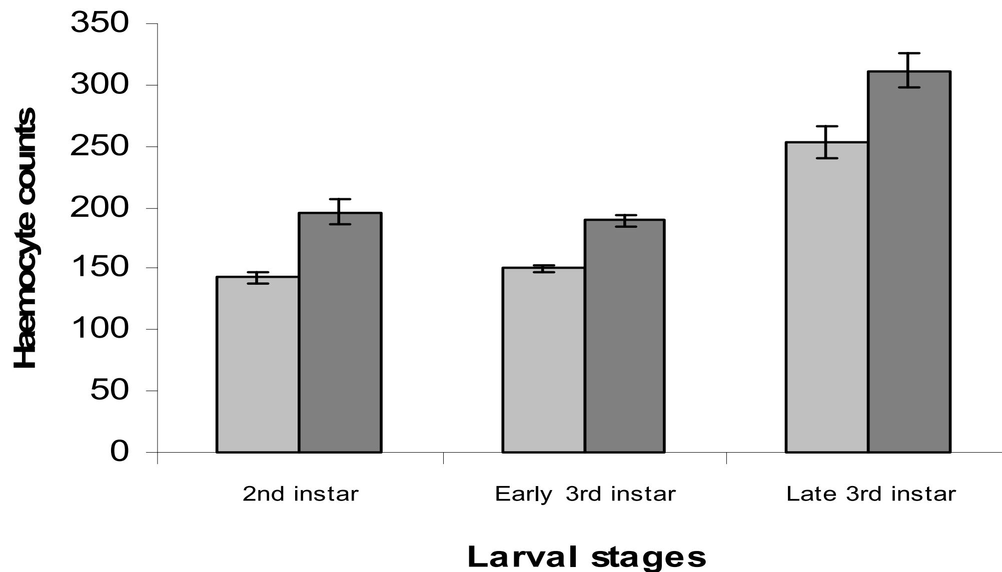

Fig. 3.1. The haemocyte density (count ± S.E.) of *D. melanogaster* larvae infected with *T. kingi* (dark bars) and control larvae (light bars) at different larval stages.

Haemocyte densities (Fig. 3.1) in *D. melanogaster* larvae infected with *T. kingi* were significantly higher than in control larvae ($F_{1,\ 48}$ = 47.3, p=0.0132). The haemocyte density across the larval stages was also found to differ significantly ($F_{2,\ 48}$ = 106.4, p=0.001). However, the interaction between larval stage and infection was not significant ($F_{2,\ 48}$ = 0.62, p= 0.5396), indicating that the larvae of different stages did not differ in their response to *T. kingi* infection and thus that the increase in haemocyte density following infection was consistent across the different larval stages.

3.3.2. Phenoloxidase activity

The phenoloxidase activity, measured as the slope of absorbance against time, in haemolymph extracted from *D. melanogaster* larvae infected with microsporidia was significantly higher than the phenoloxidase activity in haemolymph extracted from uninfected control larvae (Fig 3.2; $t_{7.399}$ = -5.3458,p =0.0008).

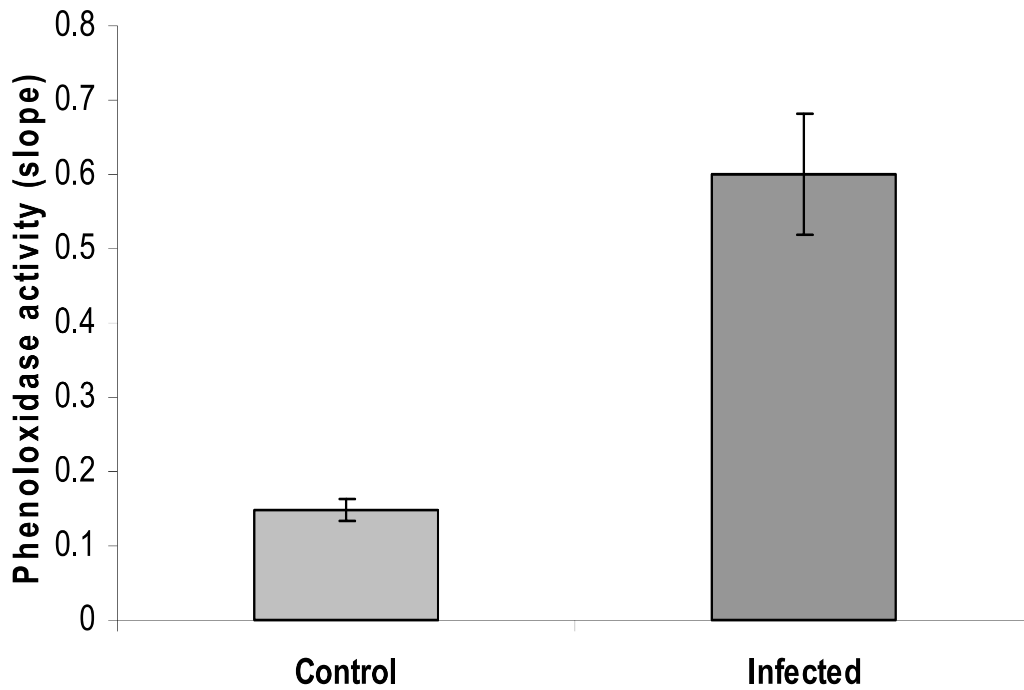

Fig. 3.2. The phenoloxidase activity (slope ± S.E.) in *D. melanogaster* larvae infected with *T. kingi* (dark bar) and in uninfected control larvae (light bar).

3.3.3. Effect of nitric oxide

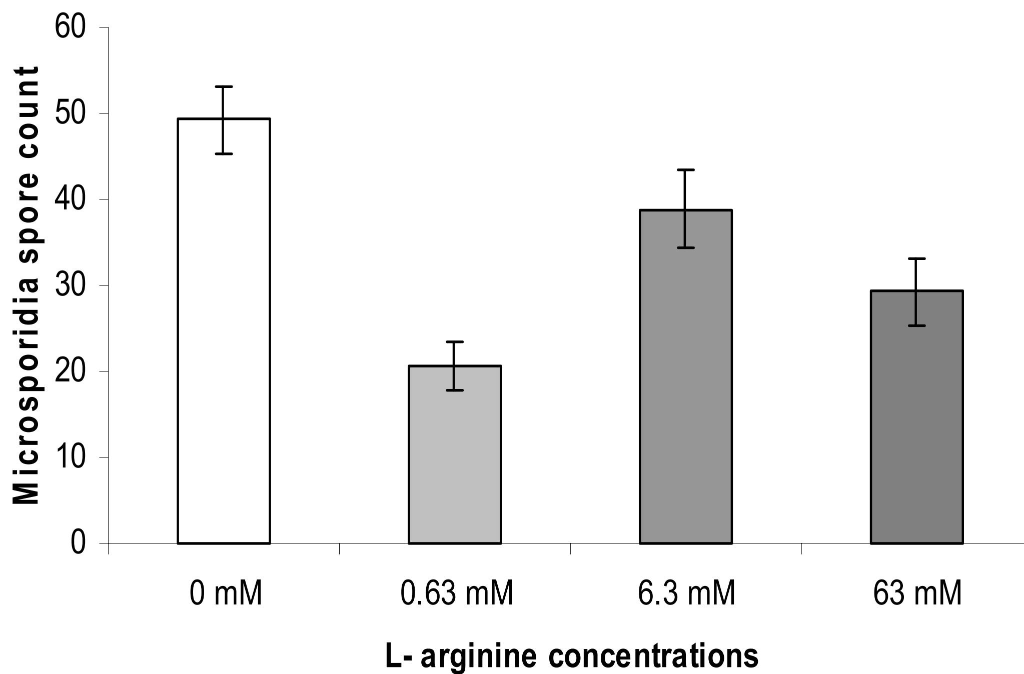

Fig.3.3. The spore density (count ± S.E.) of *T. kingi* in *D. melanogaster* reared on medium with different concentrations of L-arginine.

A one-way analysis of variance revealed significant differences in spore density between larvae reared on medium with different concentrations of L-arginine, ($F_{3, 36}$ = 10.439, p<0.001; Fig 3.4). However, the decrease in parasite density was non-linear with concentration of L-arginine. The Tukey HSD procedure revealed that not all the pairwise differences among means were significant (table 3.1).

d.f. = 36				
	Control	**0.63 mM**	**6.3 mM**	**63 mM**
Control		0.00003	0.233	0.0038
0.63 mM			0.009	0.3915
6.3 mM				0.3027

Table 3.1. Tukey HSD test (p-values) comparing the *T. kingi* spore density in *D. melanogaster* reared as larvae on diet supplemented with different concentrations of L-arginine.

3.4. Discussion

The results of the experiments described in this chapter clearly indicate that immune responses in *Drosophila* are upregulated when infected with the microsporidium *T. kingi*. This is plainly seen both in the cellular and phenoloxidase response. The number of circulating haemocytes in the larval haemolymph increased with larval development and the haemocyte density of microsporidian infected larvae was significantly higher in all the three stages investigated. The phenoloxidase activity in infected larvae was also significantly higher than in control larvae. Higher levels of within-host nitric oxide had a deleterious effect on parasite density, but this effect was ambiguous.

The total haemocyte density was significantly upregulated after *T. kingi* infection. The haemocytes in the haemolymph of infected *D. melanogaster* larvae were circulating freely and there was no aggregation of haemocytes, which would indicate nodule formation or encapsulation. This is consistent with the elevation in haemocyte density observed in *L. dispar* infected with the microsporidium *Vairimorpha disparis* (Hoch *et al.* 2004). Considering that haemocyte upregulation can also occur due to tissue damage (Ramet *et al.* 2002; Evans *et al.* 2003); microsporidian infection causes tissue damage (Hoch *et al.* 2004); and microsporidia are capable of altering the contents of phagocytic vacuoles and germinate within them (David and Weiser 1994; Nassonova *et al.* 2001), it would be unwise to conclude that the upregulation of haemocytes observed here is in response to microsporidia; nevertheless, it seems a possibility. Although Hoch *et*

al. (2004) observed haemocyte upregulation they concluded that this response was due to tissue damage, rather than the parasite, since these responses failed to prevent the establishment of an infection. Similarly although Kurtz *et al.* (2000) observed phagocytosis of microsporidian spores they agreed with (David and Weiser 1994) that this could be a propagation route for the parasite rather than a immune response against it. Futerman (2005) observed no effect of higher haemocyte numbers in improving host fitness when infected with *T. kingi*, suggesting that haemocytes are not involved in resisting microsporidia. However, he suggested that this could be due to the difference in the type of haemocytes involved in resisting microsporidia and parasitoids. I suggest that further investigation into the fate of microsporidian spores ingested by haemocytes is needed prior to drawing any conclusions.

Microsporidian infection in *D. melanogaster* larvae induced the activation of phenoloxidase enzyme and the levels were significantly higher than in uninfected larvae. Hoch *et al.* (2004) observed a similar increase in phenoloxidase activity in *L. dispar* haemolymph after microsporidian infection. They do not consider this increase in phenoloxidase activity to be induced by microsporidia, but by the damaging effects of heavy infections (Hoch *et al.* 2004). This higher phenoloxidase activity corresponds to higher melanin production, which could result in melanisation of either infected or damaged tissues. Considering the extensive damage microsporidia cause to a range of host tissues in *Drosophila* (see chapter two) I conclude that the measured alterations to phenoloxidase activity observed here is more likely due to the damage caused by the parasite rather than the parasite itself.

A significant decrease in microsporidia spore density was observed in *D. melanogaster* flies reared as larvae on diet supplemented with L-arginine. NO, a highly reactive molecule synthesised from L-arginine, is speculated to have caused the deleterious effect on the parasite. NO has been observed to play an important role in combating gram negative bacteria in *Drosophila* (Foley and O'Farrell 2003) and its antiparasitic nature in *Drosophila* and other invertebrates is currently being debated (Nappi *et al.* 2000; Rivero 2006; Lemaitre and Hoffmann 2007). The result here is a *prima-facie* evidence that NO is involved in combating microsporidia and thus benefiting the host. The effect of L-arginine on *T. kingi* observed here suggests that the increased intake of L-arginine could be boosting an immune defence reaction against microsporidia, however the

absence of a dose effect suggests L-arginine could have other, unknown effects on the microsporidian spores. Further investigation is required to improve our understanding of these effects.

In conclusion, the results obtained in this chapter confirm that microsporidian infection in *Drosophila* leads to immunological responses such as increased haemocyte density and phenoloxidase activity. The results also provide some preliminary evidence suggesting that NO mediates parasite suppression. However, these results do not provide evidence for the individual roles of these immune functions in combating microsporidian infection. I therefore suggest the following experiments to help determine the role and efficiency of these immune responses in this system.

To study the role of haemocytes upregulated following microsporidian infection, we could first determine and isolate the haemocyte subpopulations that are upregulated, presumably plasmatocytes, which are the phagocytic haemocytes. This could be easily carried out using plasmatocyte-specific antibodies and the Fluorescent Activated Cell Sorting (FACS) technique (Asha *et al.* 2003; Tirouvanziam *et al.* 2004; Vilmos *et al.* 2004). The isolated plasmatocytes and the contents of their phagosome could then be examined by transmission electron microscopy to determine the fate of phagocytosed spores. The presence of any developmental stages in the cytoplasm and spores with extruded polar filaments in the phagosomes would confirm their role in parasite propagation, while absence of developmental stages in the cytoplasm and the presence of digested remains of the spores could confirm their immune efficiency. This experiment would therefore provide us with greater insight into the efficiency of haemocytes as immune cells.

In order to confirm the role of phenoloxidase activity in combating microsporidia I suggest the use of replicated *Drosophila* lines artificially selected for increased phenoloxidase activity in a manner similar to experiments with yellow dung flies, *Scathophaga stercoraria* in (Schwarzenbach and Ward 2006). Though considerable development would be required to produce a workable selection regime, in principle infecting pairs of lines that vary in their phenoloxidase activity with a microsporidian parasite and then measuring host life-history parameters such as fecundity and survival (Futerman 2005) along with within-host parasite density would confirm the effect of phenoloxidase activity on microsporidia. If

indeed melanisation is an immune response against microsporidia, I would expect flies with higher phenoloxidase activity to have higher fitness and lower parasite density in comparison to flies with lower phenoloxidase activity.

To confirm the role of nitric oxide as an antiparasitic molecule, the nitric oxide synthase (NOS) enzyme in *Drosophila* could be chemically blocked using NOS-inhibitory arginine analog Nω-Nitro-L-Arginine-Methyl-Ester (L-NAME) (Foley and O'Farrell 2003). An inactive D-enantiomer (D-NAME) could be used as a control (Foley and O'Farrell 2003). Infecting *Drosophila* that is reared with or without the NOS-inhibitor with *T. kingi* and measuring life-history parameters and parasite densities similar to the previous experiment could explain the effect of L-arginine intake reported here. If NO functions as an antiparasitic molecule against microsporidia the NOS inhibited flies with low NO levels would have lower fitness and higher parasite load in comparison to the control flies.

Parasite-specific immunity in *Drosophila* has intrigued many researchers especially since *Drosophila* has shown fascinating mechanisms of defence against a wide range of natural enemies. However, research on *Drosophila* immune responses to microsporidia and other intracellular parasites is still at its infancy and needs extensive investigations.

Chapter Four: The Evolution of host tolerance/resistance in *Drosophila melanogaster –Tubulinosema kingi* system

4.1. Introduction

Parasitism is a common kind of association found among organisms, where one organism depends on the resources of another organism for both its maintenance and reproduction. In response to such parasitic infection, hosts have evolved a diverse array of defence mechanisms which involve either resisting or tolerating the parasite (Roy and Kirchner 2000; Miller *et al.* 2005). It has been established that parasites can significantly alter the host's evolutionary dynamics, when the hosts vary in their relative resistance to parasites (Boots and Bowers 1999; Boots and Haraguchi 1999). There have been theoretical studies investigating the evolution of resistance to parasites (Anderson and May 1981) and it is widely accepted that increased resistance to parasites can be a mixed blessing to the host, since it can be costly in terms of other life-history traits (Fellowes *et al.* 1998a; Boots and Haraguchi 1999; Kraaijeveld *et al.* 2002). Insect populations have been reported to evolve resistance after prolonged exposure to their parasites (Boots and Begon 1993; Kraaijeveld and Godfray 1997; Yan *et al.* 1997; Fellowes *et al.* 1998a; Luong and Polak 2007). Evolution of resistance in insects has been identified towards parasites as varied as viruses (Ignoffo and Allen 1972), bacteria (Janmaat and Myers 2003), fungi (Kraaijeveld and Godfray, subm.) and parasitoids (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a).

The host's resistance to microparasites has been functionally classified as avoidance, resistance and tolerance (Boots and Bowers 1999; Miller *et al.* 2005). The rate of and direction in which host resistance will evolve depends on the combination of selection pressure exerted by parasites and the nature and extent of the costs involved (Kraaijeveld *et al.* 2002). It is argued that the host may not evolve resistance under circumstances where the cost of defence is greater than the negative effect of the parasite on its host and that the spread of resistance genes in a population can be slowed down when evolved resistance has negative effect on other fitness parameters of the host (Kraaijeveld *et al.* 2002).

Drosophila melanogaster relies on multiple innate defence reactions to combat several natural enemies, from microparasites such as bacteria, fungi and viruses

to macroparasites such as mites, nematodes and parasitoids (Lemaitre *et al.* 1997; Tzou *et al.* 2002). *D. melanogaster* populations are known to vary in their ability to resist bacteria (Lazzaro *et al.* 2006), fungi (Tinsley *et al.* 2006) and parasitoids (Kraaijeveld and Godfray 1997). Parasites such as microsporidia have been shown to cause fitness reduction in *D. melanogaster* (Futerman et al. 2006). There is thus strong selection on the flies to evolve resistance to parasites (Miller *et al.* 2005). Kraaijeveld and Godfray (1997) selected a susceptible population of *D. melanogaster* for resistance to its parasitoid *Asobara tabida* and Fellowes *et al.* (1998) selected the same *D. melanogaster* population for resistance to another parasitoid *Leptopilina boulardi*.

Tubulinosema kingi is an intracellular parasite which is known to reduce *D. melanogaster* fitness. However, the fly succumbs only when severely infected suggesting either tolerance or resistance to the parasite (Kramer 1964a; Armstrong 1976; Armstrong and Bass 1989a; Futerman *et al.* 2006). The larvae of *D. melanogaster* are susceptible to *T. kingi* (see chapter 2) through both horizontal and vertical transmission (Futerman *et al.* 2006). In the previous chapter it was shown that haemocyte numbers and phenoloxidase activity increased in the *D. melanogaster* larval haemolymph in response to microsporidian infection. Although the role of these responses against microsporidia is unclear they are known to be important in combating several other natural enemies. The *T. kingi* spores have to withstand recognition and attack by the invertebrate immune system, in order to parasitize and establish within the host. The extent to which- and mechanisms by which- *D. melanogaster* resist *T. kingi* is still unclear, although there is limited evidence that the flies do have immune responses when attacked by microsporidia (see chapter 3). The maintenance cost of resistance has been demonstrated previously in the *D. melanogaster* model system through artificial selection for increased resistance against parasitoids. After selection, the correlated reductions in other fitness components were examined (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a; Kraaijeveld *et al.* 2002). Here I use the same approach to investigate if *D. melanogaster* populations can be selected for increased resistance or tolerance to intracellular parasites like microsporidia and identify the associated costs involved.

Selection experiments are important from an evolutionary perspective as they provide an opportunity to observe evolution as it occurs and to study correlated

responses to experimentally manipulated traits, for example increased resistance (Kraaijeveld and Godfray 1997) or higher phenoloxidase activity (Schwarzenbach and Ward 2006). Selection experiments are also useful since their experimental design includes replication of selected and control treatments and relatively high stability in experimental conditions (Gibbs 1999; Harshman and Hoffmann 2000). Despite these advantages, certain drawbacks have been highlighted in selection experiments by Harshman and Hoffman (2000). Their causes for concern were the heterogeneity of correlated responses in similar selection experiments and the effect of laboratory culture methods and unnatural selection regimes on the outcome of experiments. Similarly (Fuller *et al.* 2005) stress the critical role of selection intensity, experimental design and execution, these factors according to them could affect the results of a selection experiment profoundly. It has been suggested that design of selection experiments should especially consider avoiding unintentional selection (Partridge *et al.* 1999).

A carefully planned and executed experimental design can overcome most of the limitations listed above by considering a few essential factors. Long-established laboratory populations have been advocated for base population in selection experiments since they are well-adapted to laboratory conditions however it is recommended that they be started with multiple founders and maintained at a large size (Fry 2003). The population to be selected and the control population should be derived from the same base population and should be maintained in similar environments to detect correlated responses to selection (Fry 2003). To avoid inbreeding depression in selected and control populations it is essential to maintain them as moderate-to-large population sizes (Harshman and Hoffmann 2000; Fry 2003). Replication of selection regimes, including the controls, allows the effects of selection to be distinguished from those caused by random drift (Fry 2003). The fitness assays to determine if selected and control populations differ in their fitness must be carried out on samples of populations that were reared in common environment for at least one generation to avoid maternal effects (Fry 2003).

Sometimes populations fail to evolve resistance due to three main reasons: lack of genetic variation in the host population; the selection pressure or intensity being low; or the costs of resistance being too high initially (Kraaijeveld and Godfray subm.). Although the lack of genetic variation in the *D. melanogaster* base population is unlikely, since the same population was previously selected

for increased resistance against parasitoids (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a), it does not guarantee genetic variation in traits linked with resistance to microsporidia. Any failure to select for increased resistance or tolerance against microsporidia could be more likely due to low selection pressure and this need to be investigated before concluding that the host population cannot evolve resistance against the parasite.

In this chapter, I report the results of an experimental evolution in which the *D. melanogaster* population evolved resistance to *T. kingi*. The selection protocol was designed to allow for increased representation of progeny from resistant or tolerant females in the successive generation, i.e. resistant or tolerant flies would survive longer and lay more eggs than susceptible flies when infected with *T. kingi*. Therefore, when eggs were collected, the resistant females would have a higher probability of being represented due to both their longevity and higher fecundity.

Tubulinosema kingi reduces the early fecundity and adult survival of infected *D. melanogaster* among other fitness traits (Futerman *et al.* 2006). In the current selection experiment the early fecundity and adult survival of population from selection and control regimes were assayed. To exclude maternal effects the sample populations were reared in the absence of selection pressure for one full generation before the assays. The assays on selected and control lines were conducted both with and without *T. kingi* infection. It was expected that the selected lines would show increased performance over the controls when infected if resistance or tolerance against the parasite had evolved and the control lines would fare better than selected line in the absence of infection if a trade-off existed.

As mentioned previously, *D. melanogaster* could evolve to resist *T. kingi* or simply to tolerate it when artificially selected, however the former strategy does not rule out a tolerance component (Boots and Bowers 1999; Miller *et al.* 2005). It is therefore important to distinguish between tolerance and resistance (Miller *et al.* 2005). The parasite density within a host correlates with the type of strategy evolved. If tolerance has evolved, parasite density would be expected to remain constant in both selected and control individuals when infected. However, if resistance has evolved, tolerance cannot be ruled out, but then the selected

individuals should foster a significantly lower parasite density than the control individuals.

4.2. Materials and methods

4.2.1. The selection experiment

The large outbred population of *D.melanogaster* (described in chapter one) formed the base population for this experiment. This base population was first split into five subpopulations, each of which was further divided into control and selection lines. The resulting five pairs of lines were reared at 20°C in Perspex cages (20x20x20cm). The cages were designed to reduce cross infection by restricting the air flow in the cages through five sterilized cotton plugs (see Fig. 4.1.). Control and selection lines were maintained in exactly the same way except that the selection lines were routinely exposed to microsporidia. Each pair of lines was started from 500 eggs laid by flies from the uninfected *D. melanogaster* base population in culture bottles with *Drosophila* yeast/sugar medium and live yeast. The 500 eggs were divided into two batches of 250 eggs and placed in two separate sterile culture bottles containing medium and live yeast. To one batch ~1x10^{7} *T.kingi* spores in 0.1% SDS were added and to the second batch an equal volume of 0.1% sterile SDS was added. The bottles were then incubated at 20°C, with a 16:8h light:dark regime. When the flies emerged, they were released into a pair of identical Perspex cages (20cmx20cmx20cm) maintained at 20°C, with a 16:8h light:dark regime in a controlled-temperature room at ambient humidity, with constant access to honey and water.

The control and selection lines were maintained as cage cultures with overlapping generations. Twice a week the flies from control and selection lines were allowed to oviposit for 24 hours in a sterilized Petri dish (9cm) containing medium and yeast, 150 eggs per plate were collected and transferred to rearing bottles containing medium and live yeast. The eggs collected from the selection lines were treated with ~ 7.5x10^{6} *T.kingi* spores in 0.1% SDS while the eggs from the control lines were treated with equal volume of 0.1% SDS solution. The *T. kingi* spore dose used to infect the selection lines was increased ten fold during the second half (week 37 onwards) of the experiment due to reasons I discuss in detail a little later in the chapter. The rearing bottles with duly treated eggs were incubated at 20°C untill the flies emerged, when they were released into their respective cages.

The cadavers of dead flies in the cages were removed every second day, as they are potential source of infections which could interfere with the standard spore dose used in the selection regime. The water in the cages was changed every fortnight while the honey was replenished weekly. Back-up populations were maintained in addition to the cage cultures. Every two weeks approximately 250

Fig. 4.1. Population cages set up for the selection experiment, which were especially designed to prevent cross contamination.

eggs per line, were taken from the standard culture, treated appropriately with or without spores, they were cultured in bottles containing yeast/agar medium and live yeast. A day prior to collecting eggs for the backup population, ten flies randomly collected from the control cages were screened using Giemsa staining for cross-infections. Ten flies from the selection cages were also removed to avoid any bias. The back-up population was maintained to be used as a replacement in case a control cage got infected.

The selection for increased resistance or tolerance to microsporidia in five replicate populations of *D. melanogaster* was carried out for 73 weeks. Selection was suspended for 13 weeks between the 19th and 31st week due to unavoidable circumstances. During this 13 week period, the populations were maintained in

sterile culture bottles containing medium and live yeast as non-overlapping generations. The control populations remained uninfected throughout the selection regime: the Giemsa-stained abdominal smears of flies from the control lines examined prior to creating back-up populations at intervals of two weeks were all negative for *T. kingi* infection.

4.2.2. Bioassays

Fecundity and survival bioassays were conducted to investigate if selected lines had evolved to become tolerant or resistant to microsporidia. This was done twice during the selection period, after 34 and 60 weeks of selection. The selected line populations were subjected to curing process to eliminate *T. kingi* infection; while the control line populations were treated similarly to avoid treatment bias. The flies from both control and selection lines were cured by allowing them to lay eggs for a day in culture bottles containing medium and live yeast, ~300 eggs per line were collected and surface-sterilised with 0.6% NaOCl solution for five minutes and then washed several times before transferring these de-chorinated eggs into rearing bottles containing medium and live yeast. Once the flies emerged they were screened for *T. kingi* infection by examining Giemsa-stained abdomen smears of 15 randomly selected flies per line. On both occasions (first and second bioassays) all the flies examined for infection were uninfected. After confirming the absence of *T. kingi* in these lines, flies from both control and selected lines were allowed to lay eggs and cultured in bottles containing medium and yeast for an extra generation to remove any maternal effects.

The experimental design for the assays conducted after 34 and 60 weeks were identical, with the exception of the *T. kingi* spore dosage used to infect the flies. For the first fecundity and survival assay a spore dose of ~ 2.5×10^6 *T. kingi* spores was used. For the second set of assays the spore dose was increased ten-fold, to “mirror” the increased dosage used during the second half of the selection experiment.

4.2.2.1. Fecundity assay

The control and selected lines were allowed to oviposit in culture bottles containing medium and live yeast for six hours. Eight vials (80x22mm) per line containing medium and live yeast, each with 50 eggs collected from respective bottles were set up. Four of these vials were treated with *T. kingi* spores in 0.1%

SDS while the rest were treated with an equal volume of 0.1% SDS solution. The vials were incubated at 20ºC to facilitate development. On emergence, four females from each vial were placed in individual vials of the same size containing medium and live yeast along with two males from the same vial. The assay therefore comprised 32 vials per line (control or selection): 16 with *T. kingi* infection and 16 controls. The following day all the flies were placed in fresh laying vials containing medium and live yeast and the previous vials were discarded. For the next ten consecutive days the vials were replaced every 24 hours; and eggs laid during the previous 24 hours counted and recorded. Any dead males were removed from these vials and replaced. To ascertain that females of both lines that were not exposed to *T. kingi* were uninfected and those treated with *T. kingi* were infected, all the females used in the fecundity assay were screened for *T. kingi* after the ten day period by observing their Giemsa-stained abdomen smears.

For the females that died during the assay, the data for the remaining days were recorded as missing values for calculating the mean number of eggs laid per day. The fecundity of control and selected lines when infected demonstrate the response to selection for increased resistance while the fecundity of control and selection lines when uninfected demonstrates the potential trade off associated with increased resistance and analysing them together as an interaction between selection (line) and infection (treatment) using a two-way analysis of variance could obscure the significance of each. Therefore the analysis was split into two: control lines versus selected lines with infection and without infection.

4.2.2.2. Survival assay

Approximately 400 eggs per line were collected from control and selected lines cured of maternal effects in the same way as for the fecundity assay. The 400 eggs collected per line were distributed equally into two bottles containing medium and live yeast. To one bottle *T. kingi* spores in 0.1% SDS were added while to the other an equal volume of 0.1% SDS was added. The bottles were incubated at 20ºC untill the pupae within darkened. The darkened pupae were washed out of the bottle gently, 100 dark pupae per bottle were transferred to individual glass vials (50x12mm) containing a small amount of honey. The vials were closed with a cotton-wool plug soaked with water. The plugs were maintained wet through out the assay. The vials were incubated at 20ºC with a 16:8h light:dark regime. Survival of each fly was recorded from the day of its

emergence. The flies were observed daily to record which flies had died during the previous 24 hours. Once all the flies had died they were sexed to determine if survival differs between the sexes in selected and control lines.

Flies from the pupae that failed to emerge during the assay were not included in the analysis. As with the analysis of fecundity data (see section 4.2.2.), the survival data was analysed separately for control lines versus selected lines with infection and without infection. The analysis of sex specific survival in selected and control lines with or without infection was done using a three-way analysis of variance.

4.2.3. Spore dose effect

Prior to increasing the selection pressure (spore dose) in the selection experiment protocol from week 37, the effect of spore dose on within-host parasite density and host mortality was assayed. The *D. melanogaster* larvae were infected with different doses of *T. kingi* spores to investigate if increased dose had deleterious effects on *D. melanogaster*. Flies from the base population were allowed to oviposit in culture bottles containing medium and live yeast for six hours. The eggs laid in the bottles were collected and eight vials (80x22mm) containing medium, live yeast and 50 eggs in each were set up. The following *T. kingi* spore doses: a) ~2.5×10^6 spores, b) ~12.5×10^6 spores and c) ~25×0^6 spores in 0.1% SDS were added to three pairs of vials respectively. The last pair of vials was treated as controls and an equal volume of 0.1% SDS to that in spore dose 'c' was added to them. The vials were incubated at 20ºC until emergence. The number of flies emerging per vial was recorded. The parasite spore density per fly was determined by homogenising five flies per vial in 100µl of 0.1% SDS and counting the spores in 3µl of each sample using a haemocytometer under a light microscope at 40X magnification.

4.2.4. Tolerance versus resistance

The spore densities in infected control and selection line flies were determined to identify the cause (tolerance or resistance) of increased fitness in infected selection line flies. The selection and control line flies cured of *T. kingi* and maternal effects were allowed to oviposit in culture bottles containing medium and live yeast. Three hundred eggs were collected per line and distributed equally into six vials (80x22mm) containing medium and live yeast. On the following day ~2.5×10^7 *T. kingi* spores were added to each vial which was then

incubated at 20ºC. On emergence the spore density of five randomly chosen flies per vial was determined using a haemocytometer after each individual fly was homogenised in 100µl of 0.1% SDS,

4.3. Results

The results in this section have been presented chronologically in four sub-sections. First, I report the results of the first bioassays; second, the effects of increased *T. kingi* spore dose on its host; third, the results of the second bioassays; and finally the result which determines if the strategy evolved by the host against its parasite is tolerance or resistance.

4.3.1. First bioassays

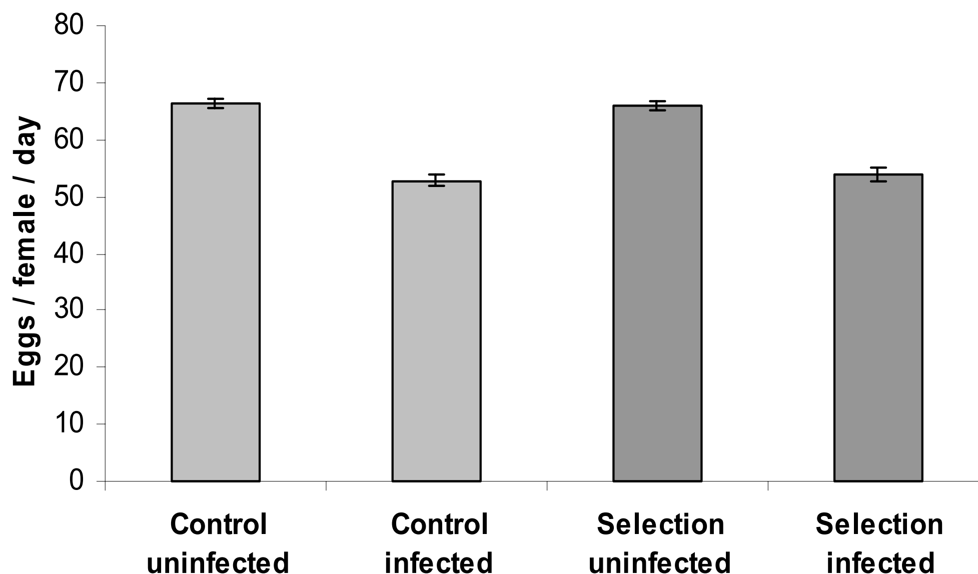

Fig. 4.2. The effect of selection for increased resistance to *T. kingi* upon early fecundity (mean ± S.E.) of *D. melanogaster* control lines (light bars) and selection lines (dark bars).

The early fecundity (Fig. 4.2.) of *T. kingi* infected *D. melanogaster* females selected for increased resistance (53.98 ± 1.26) was not significantly different from *T. kingi* infected control flies (52.80 ± 1.14), ($F_{1, 8}$ = 0.48, p = 0.5072). The early fecundity of uninfected females from selected lines (67.46 ± 0.93) was not different from uninfected females from control lines (66.42 ± 1.03) ($F_{1, 8}$ = 0.563, p = 0.4747).

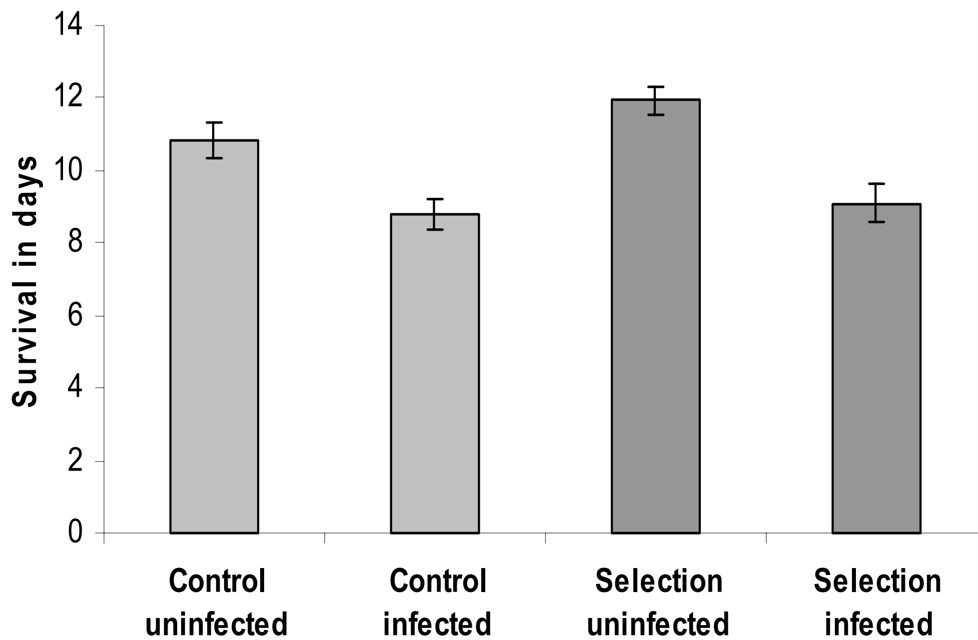

Fig. 4.3. The effect of selection for increased resistance to *T. kingi* upon adult survival (mean ± S.E.) of *D. melanogaster*: control lines (light bars) and selection lines (dark bars).

The survival (Fig. 4.3.) of *T. kingi* infected *D. melanogaster* females selected for increased resistance (9.09± 0.52) was not significantly different from *T. kingi* infected control flies (8.77± 0.42), ($F_{1, 8}$ = 0.218, p = 0.6529). The survival of males and females from control and selected lines, with or without *T. kingi* infection, did not differ significantly ($F_{1, 32}$ = 0.514, p = 0.4785). The interaction between sex and selection was not significant ($F_{1, 32}$ = 0.007, p = 0.9308). Similarly, the interaction between sex and *T. kingi* infection was also not significant ($F_{1,32}$ = 0.593, p = 0.8091).

4.3.2. Spore dose effect

The number of flies emerging (Fig. 4.4.) from vials treated with different *T. kingi* spore doses were not significantly different ($F_{3,4}$ = 3.75, p = 0.1171). A Tukey HSD comparing the means showed no significant difference in number of flies emerging from the vials subjected to different *T. kingi* spore doses. The mean spore density per fly (see Fig. 4.5.) in vials infected with the largest spore dose $25x10^6$ spores was significantly higher than in vials infected with regular spore dose of $2.5x10^6$ spores ($F_{1,2}$ =21.75, p =0.0430).

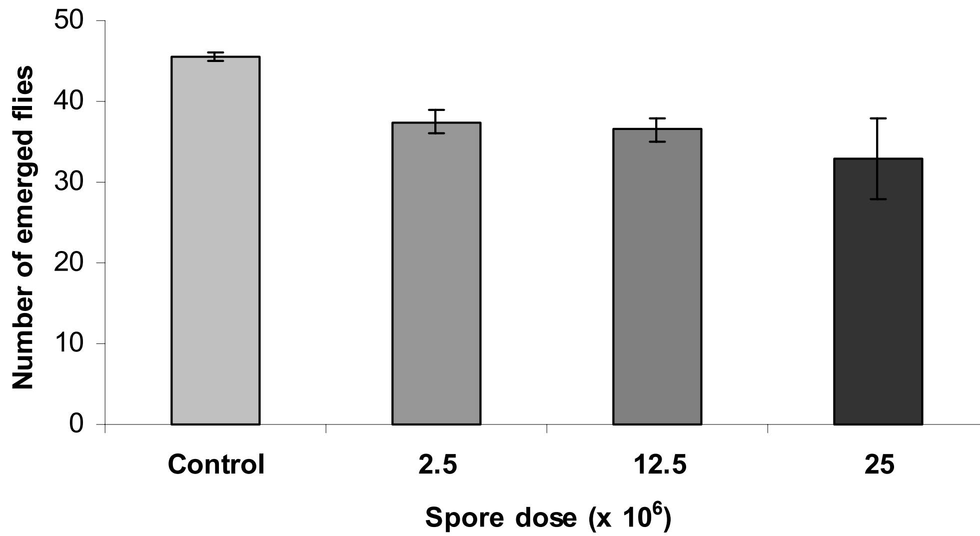

Fig. 4.4. The effect of increased *T. kingi* spore dose upon emergence (mean ± S.E.) of *D. melanogaster*:

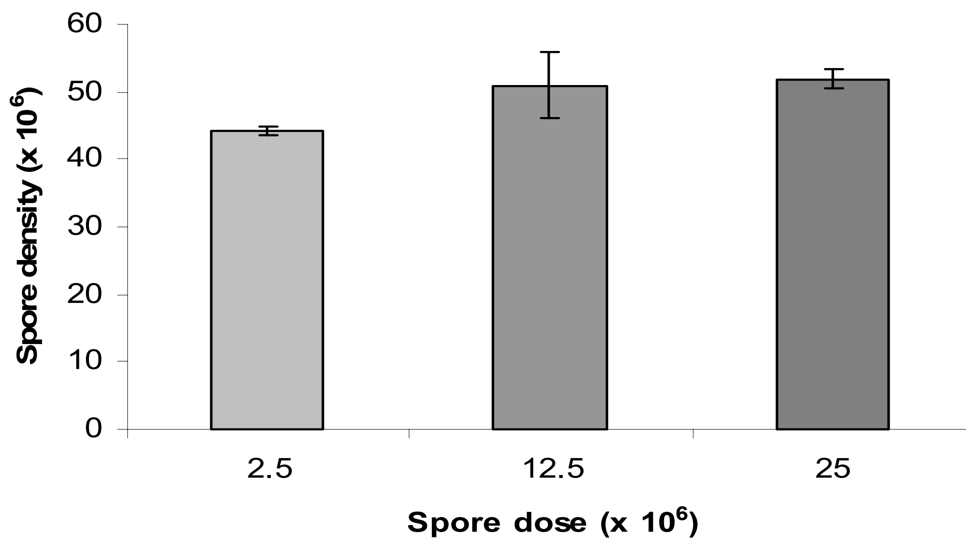

Fig 4.5. The effect of *T. kingi* spore dose upon within host parasite density (mean ± S.E.).

4.3.3. Second bioassays

4.3.3.1. Fecundity assay

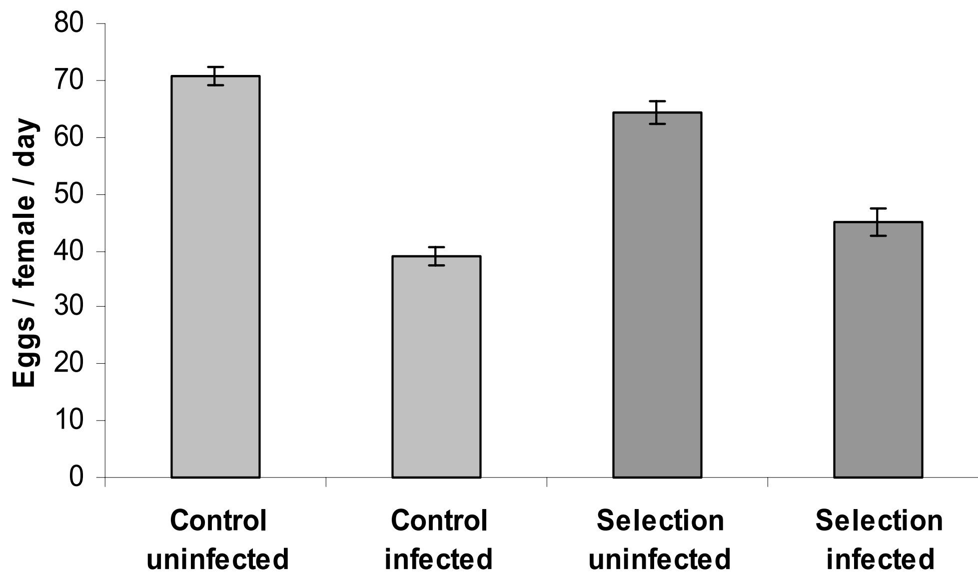

Fig. 4.6. The effect of selection for increased resistance to *T. kingi* upon early fecundity (mean ± S.E.) of *D. melanogaster*: control lines (light bars) and selection lines (dark bars).

A few females that did not lay any eggs during the ten day assay period were termed 'sterile' and were excluded from the analysis after it was confirmed by doing a Fisher's exact test (p= 0.1098) that the sterility occurred to the same extent in control and selected lines irrespective of *T. kingi* infection. The early fecundity (Fig. 4.6.) of *T. kingi* infected *D. melanogaster* females selected for increased resistance (45.11± 2.38) was significantly higher than *T. kingi* infected control flies (39.14± 1.65), ($F_{1, 8}$ = 5.376, p= 0.0490). The early fecundity (eggs/female/day ± S.E.) of uninfected females selected for increased resistance (64.35± 2.13) was significantly lower than uninfected control flies (70.70± 1.62), ($F_{1, 8}$ = 5.616, p= 0.0453).

4.3.3.2. Survival assay

The mean adult survival (Fig.4.7.) of *T. kingi* infected *D. melanogaster* selected for increased resistance to microsporidia was significantly higher than the *T. kingi* infected control flies ($F_{1, 8}$ =6.58, p = 0.0336), however the uninfected flies from the selected lines and control lines did not differ significantly in their adult survival ($F_{1, 8}$ =0.06, p = 0.8069). The survival of males and females from control and selected lines with or without *T. kingi* infection did not differ significantly ($F_{1, 32}$ =

0.136, $p = 0.7150$). The interaction between sex and selection was not significant ($F_{1, 32} = 0.532$, $p = 0.4712$) and similarly interaction between sex and *T. kingi* infection was also not significant ($F_{1, 32} = 0.024$, $p = 0.8786$).

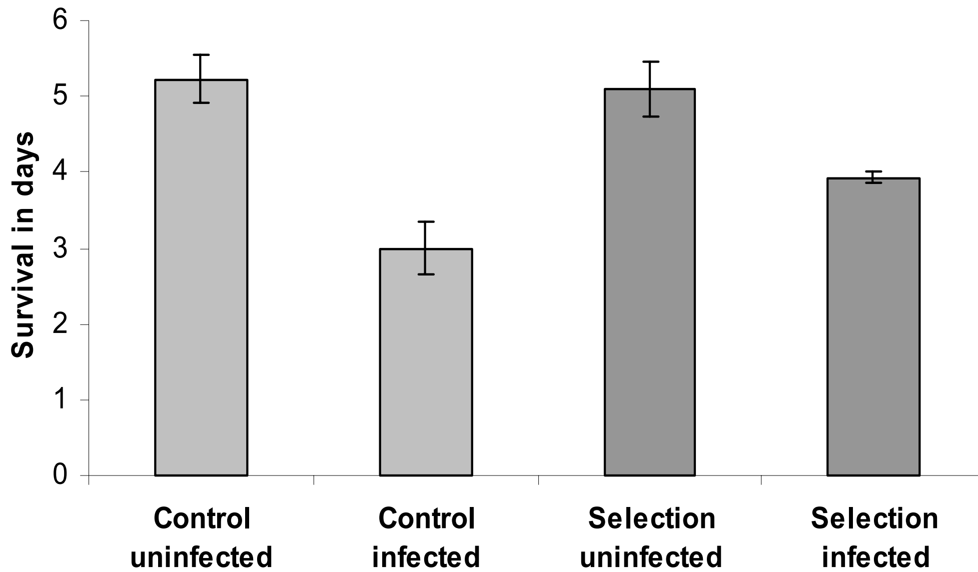

Fig. 4.7. The effect of selection for increased resistance to *T. kingi* upon adult survival (mean ± S.E.) of *D. melanogaster*: control lines (light bars) and selection lines (dark bars).

4.3.4. Tolerance versus resistance

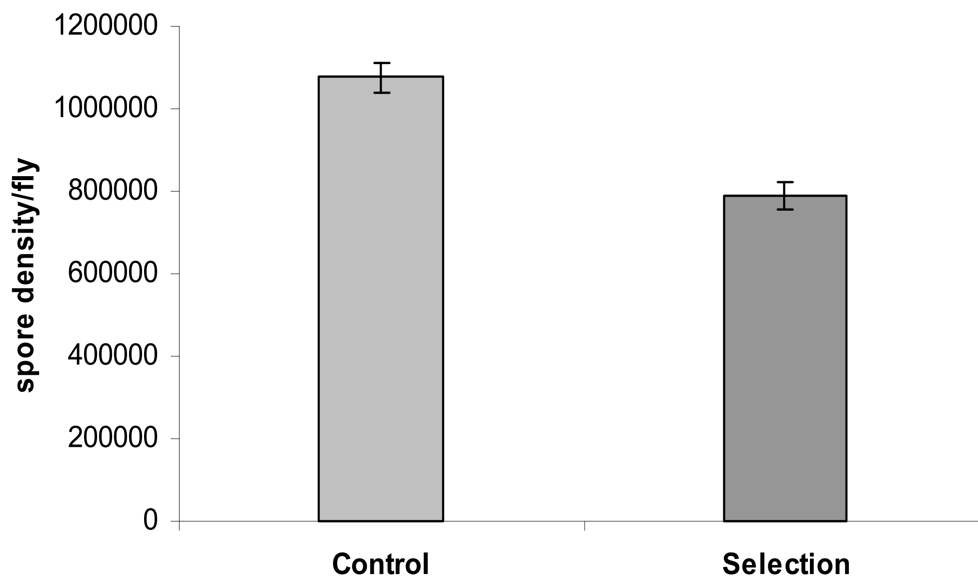

Fig. 4.8. The effect of selection for increased resistance to *T. kingi* upon within host parasite density (mean ± S.E.) in *D. melanogaster*: control lines (light bar) and selection lines (dark bar).

All the flies from the control and selected lines used to determine within-host spore density were infected with microsporidia. The mean spore density per fly (see Fig.4.8.) in the five selection lines was significantly lower than in the five control lines ($F_{1,8} = 31.726$, $p = 0.0004$).

4.4. Discussion

The experiments described in this chapter clearly indicate that *D. melanogaster* within a population vary in their ability to resist microsporidia and can evolve to become increasingly resistant to an intracellular parasite *Tubulinosema kingi*. The response to selection was similar across the five lines and in the analysis the lines were treated as replicates. Early fecundity and adult survival were the two traits compared between selected and control lines. No differences between control and selected lines were found in early fecundity and adult survival in the first bioassay.

The selected lines failed to evolve resistance or tolerance against microsporidia during the initial selection regime where each batch of larvae from these lines was fed ~7.5×10^6 *T. kingi* spores. This spore dose was similar to the dosage used to explore the immune responses of *D. melanogaster* to *T. kingi* infection (see chapter three) and by Futerman *et al*. (2006) to determine fitness effects. This lack of evolution was more likely due to low selection intensity rather than lack of genetic variation in the host population.

In order to increase the selection pressure it was necessary to determine if an increased spore dose would have other consequences on the host population, such as very high mortality. A five-fold and a ten-fold increase in the spore dose were evaluated and no difference in the number of flies emerging was found between the different doses. However, the flies treated with ten-fold increased spore dose had a significantly higher parasite density than the regularly used dosage, indicating a greater impact on the host. The selection regime was therefore modified with a ten-fold increase in the spore dose, to increase selection intensity, in the second half of the selection experiment.

The second bioassays, conducted after approximately 13 generations of selection with increased spore dose, clearly indicated that the selected populations of *D. melanogaster* had evolved resistance against *T. kingi*. The higher fecundity and adult survival in selected flies when infected by *T. kingi* in

comparison to infected control flies indicated that the selected flies suffered less fitness loss due to evolved mechanisms to resist the parasite.

Evolution of immunological resistance to the larval parasitoids *A. tabida* and *L. boulardi* in *D. melanogaster* (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a) and evolution of behavioural resistance to ectoparasitic mites *M. subbadius* in *Drosophila nigrospiracula* (Luong and Polak 2007) has been demonstrated using artificial selection experiments. A similar approach was used here to demonstrate the evolution of resistance against microsporidian parasite *T. kingi* in *D. melanogaster*. The mechanism of evolved resistance against parasitoids is not fully understood, but probably involves an increased haemocyte density (Kraaijeveld *et al.* 2001b). The evolved resistance against mites are behavioural in nature (Luong and Polak 2007). However, the precise mechanisms involved in resistance against microsporidia is not clear. The evolution of resistance against parasitoids was rapid and changes in survival were higher in comparison to resistance against microsporidia (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a). In fact, changes in resistance to microsporidia became apparent only when the microsporidian spore dose used to infect the population was increased ten-fold.

As mentioned earlier in this chapter, theoretical studies have broadly classified resistance into tolerance and control (Miller *et al.* 2005). It is evident that in this selection experiment the evolved resistance is a control strategy where the parasite density is lowered by host defence mechanisms, however, this does not rule out tolerance.

Evolution of resistance is considered costly: the increased resistance to parasites in a host involves resources redirected from other fitness traits and is therefore assumed to carry costs (Schmid-Hempel 2005). There are two types of costs involved while considering the costs of resistance: the costs involved in mounting the immune response and the costs involved in maintaining the immunity (Kraaijeveld *et al.* 2002). In *Drosophila* lower competitive ability in larvae has been identified as costs of immunological resistance against parasitoids (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a), while increased resistance to mites was negatively correlated to fecundity (Luong and Polak 2007). In my study I report the *prima-facie* evidence that the evolved resistance against *T. kingi* in *D. melanogaster* has a cost. The selected lines flies had lower

fecundity compared to flies from control lines, in the absence of *T. kingi* infection, indicating a trade-off between increased resistance and early fecundity. The following chapter further explores the potential costs involved in this increased resistance and the different mechanisms involved.

This is the first time that evolution of resistance and an associated cost has been demonstrated against microsporidia. Microsporidia, as mentioned earlier, are gaining prominence both as an opportunistic pathogen and a potential bio-control agent. In this study I demonstrate the possibility of insect populations evolving resistance against microsporidia and suggest that bio-control programs for the control of insects, both as disease vectors and as pests, should consider the implications of evolving resistance in field populations when designing them. The results reported in this chapter also highlight the role played by the intensity of selection pressure in evolving resistance, therefore suggesting that regulated use of bio-pesticides is equally important.

Chapter Five: Trade-offs and mechanisms associated with increased resistance to *Tubulinosema kingi* in *Drosophila melanogaster*

5.1. Introduction

Attacks from natural enemies, like predators and parasites, are inevitable for most, if not all, organisms, thus causing these organisms to evolve some sort of defence mechanisms against their enemies. However, mounting evidence has made it clear that such resistance against parasites and pathogens requires resources and is costly (Schmid-Hempel 2005). Costs associated with resistance are considered to maintain heritable genetic variation for resistance in field populations (Henter and Via 1995; Kraaijeveld *et al.* 1998; Rigby *et al.* 2002). Costs of resistance to natural enemies have been identified in a range of organisms; such as bacteria (Lenski 1988), plants (Bergelson and Purrington 1996; Siva-Jothy *et al.* 2001), invertebrates (Kraaijeveld and Godfray 1997; Webster and Woolhouse 1999; Luong and Polak 2007) and vertebrates (Sheldon and Verhulst 1996; Verhulst *et al.* 1999). The nature and magnitude of the costs of resistance, along with the selection pressure exerted by the natural enemies, has been considered to play a key role in the rate and direction of the evolution of resistance (Kraaijeveld *et al.* 2002). The costs of resistance can be distinguished into two types: those associated with mounting the actual defence and those associated with maintaining the standing defences (Kraaijeveld *et al.* 2002). The costs associated with the actual defence are incurred due to the energy and resources used for mounting the defence against parasites or pathogens, whilst the cost associated with maintaining standing defence involve resources redirected into the immune or other defence systems in anticipation of potential attacks (Kraaijeveld *et al.* 2002). Costs of standing defence is important evolutionarily since it could influence evolution of resistance (Kraaijeveld *et al.* 2002).

A range of costs involved in mounting actual defence has been identified in insect-parasite systems, including reduced adult size, longer development period, decreased fecundity, reduced survival, increased susceptibility to other parasites and lower reproductive and foraging activity (Boots and Begon 1993; Yan *et al.* 1997; Siva-Jothy *et al.* 1998; Doums and Schmid-Hempel 2000; Siva-Jothy *et al.*

2001). However, it is often difficult to distinguish the costs of actual defence from the negative effects of the parasite (Kraaijeveld *et al.* 2002).

The trade-offs between resistance and the other fitness parameters of a host indicate the costs involved in standing defence. Selection experiments and quantitative genetic estimation of trait covariance are powerful methods for detecting such costs (Reznick 1985; Fry 2003). Trade-offs associated with evolution of resistance have been demonstrated in a few systems. The evolution of resistance against a granulosis virus was found to be correlated with increased developmental time and reduced egg viability in the Indian meal moth, *Plodia interpunctella* (Boots and Begon 1993). In mosquitoes, *Aedes aegypti* resistance to the malarial parasite decreased adult body size, fecundity and longevity (Yan *et al.* 1997). In *D. melanogaster*, resistance to parasitoids decreased larval competitive ability (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a), while resistance to the ectoparasitic mite, *Macrocheles subbadius* reduced fecundity (Luong and Polak 2007). Selection for increased immune function (phenoloxidase activity) in yellow dung flies was found to be positively correlated with fertility and fecundity and negatively correlated with longevity under starvation (Schwarzenbach and Ward 2006).

The *Drosophila* system, with its wide range of parasites (see Introduction), is a valuable model system for investigating costs of resistance (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a; Luong and Polak 2007) because of its short generation time, relatively simple immune system (compared with vertebrates) and fully sequenced genome (Orr and Irving 1997; Fellowes *et al.* 1998a; Kraaijeveld *et al.* 2002). The evolution of resistance in *D. melanogaster* against the larval parasitoids *Asobara tabida* and *Leptopilina boulardi* involved increased encapsulation and was associated with a fitness cost in terms of reduced larval competitive ability in both cases (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a). The costs of mounting a successful defence against the larval parasitoids in *Drosophila* was reduced adult size and fecundity and increased susceptibility to pupal parasitoids (Carton and David 1983; Fellowes *et al.* 1998b; Fellowes *et al.* 1999b). The evolution of resistance in *Drosophila nigrospiracula* against ectoparasitic mites was costly in terms of reduced fecundity (Luong and Polak 2007). The costs of standing defence were obscured when the selected and control lines were compared for competitive ability under conditions of excess larval food, but became apparent when compared under conditions of

scarce larval food (Kraaijeveld and Godfray 1997), hence trade-offs are more likely to become obvious when organisms are under stress (Bergelson and Purrington 1996; Kraaijeveld *et al.* 2002). The level of resistance against the larval parasitoid, *A. tabida*, shown by different lines of *D. melanogaster* is positively correlated with the number of haemocytes circulating in the larval haemolymph. It has been suggested that this could be the basis of the improved resistance in the selected lines (Kraaijeveld *et al.* 2001b).

Among other parasites of *D. melanogaster*, the microsporidium *Tubulinosema kingi* provides a potential host-parasite system to investigate the evolution of resistance and its associated costs (Kramer 1964a; Armstrong 1976; Futerman *et al.* 2006). Infection by *T. kingi* in *D. melanogaster* has been shown to reduce early fecundity and adult body size (Futerman *et al.* 2006), but it is unclear if these fitness losses observed are due to the costs of actual defence or due to the negative effect of microsporidia on the host. Earlier, in chapter three, it was shown that in response to infection by *T. kingi*, a higher haemocyte density and increased phenoloxidase activity was observed in the haemolymph of *D. melanogaster* larvae. Although it is still unclear if these immune responses are effective against *T. kingi*, it has been suggested that these activities are likely to require resources and hence have an associated cost (Kraaijeveld *et al.* 2002).

In the previous chapter it was shown that a *D. melanogaster* population can be experimentally evolved to resist *T. kingi*. The evolved resistance in the uninfected selected flies was found to be correlated with decreased fecundity when compared to uninfected control flies. This chapter has two aims: to test whether there are costs associated with standing defence against microsporidia and to explore the immunological basis for the evolved resistance found in chapter four.

To detect the costs associated with increased resistance to microsporidia, I first investigated if the trade-off between resistance and fecundity observed in the previous chapter becomes more apparent under stressed condition. The early fecundity was previously measured under ideal conditions, with surplus resources. As discussed above, this could conceal or diminish the costs of standing defence and hence I subject the larvae to the stress of food scarcity. Second, I examined whether lines experimentally evolved to resist *T. kingi* had reduced larval competitive ability. Competition for food between larvae is a crucial trait in natural *Drosophila* populations (Atkinson 1979) and as mentioned

above reduction in larval competitive ability has been previously reported as a cost of resistance (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a).

In chapter three, I reported the up-regulation of both haemocyte density and phenoloxidase activity in *D. melanogaster* larval haemolymph when infected with *T. kingi*. To investigate the immunological basis for the evolved resistance, I compared the number of circulating haemocytes and phenoloxidase activity in the larval haemolymph of control and selection lines.

5.2. Materials and methods

The four experiments described below were conducted on the *D. melanogaster* lines experimentally evolved for increased resistance to *T. kingi* and their respective control lines (see chapter four). Prior to these experiments, the lines were cured of *T. kingi* infection and cultured without infection for a generation to remove any maternal effects. The curing protocol is described in section 4.3.2. Ten flies per line were screened for *T. kingi* infection by Giemsa-staining of the abdomen smear, to confirm that the lines were indeed infection free. No microsporidia were observed in the stained smears of the examined flies.

5.2.1. Fecundity under resource scarcity as larvae

When larvae of control and selected lines are subjected to stress in the form of food (resource) scarcity, the allocation of available resources to development of reproductive organs versus immune functions would be constrained, therefore affecting the adult fecundity. It has been reported that when 30 second instar larvae fed on 0.1ml of larval food (~0.003ml per larvae) their developmental period increases and their size on emergence was reduced (Kraaijeveld and Godfray 1997). In this experiment, allowing 50 first instar larvae to feed on 0.25ml of larval food (0.005ml per larvae) was expected to cause stress, through limited resources.

The control and selected lines were allowed to oviposit in culture bottles containing yeast/sugar medium and live bakers yeast for six hours at 25ºC. 200 eggs per line were distributed equally into four vials (80x22mm) lined with agar and 0.25ml of larval food (25g live bakers yeast per 100ml water). The vials were incubated at 20ºC for further development. When the flies emerged, four females and eight males, from each vial were randomly selected. Each female together with two males were placed in individual vials (80x22mm) containing

yeast/sugar medium and live yeast. The experiment therefore comprised 16 vials per line (control or selection). The following day, all the flies were transferred into fresh laying vials (80x22mm) containing medium and live yeast and the previous day's vials were discarded. For the next ten days the vials were replaced every 24 hours; and eggs laid during the previous 24 hours counted and recorded. Any dead males were removed and replaced. The data for females that died during the assay period were included as missing values for analysis. The mean number of eggs, per female, per day for each line was calculated and the lines were treated as replicates in the statistical analysis. The difference in the early fecundity of flies from selected and control lines, reared as larvae with limited food, was tested by a one-way ANOVA.

5.2.2. Larval competitive ability

To determine and compare the competitive ability of two phenotypically indistinguishable strains comparison of their respective performances against a mutant tester stock has been suggested (Santos *et al.* 1992). Here, I compare the relative performance of the *D. melanogaster* control lines with lines selected for increased resistance to *T. kingi* under strong or weak intra-specific competition regimes, by rearing them with larvae of *sparkling poliert 1* (tester flies) (Kraaijeveld and Godfray 1997). The tester flies are mutants with sparkling red eyes, while the experimental flies have normal red eyes and this identifiable phenotypic difference was used to determine the relative competitive ability of experimental lines.

The flies from the experimental lines and the tester stock were allowed to oviposit overnight in bottles containing medium and live yeast at 25ºC. The bottles were further incubated at 20ºC for 48 hours after which the larvae were washed out of the culture bottles. I transferred 15 second-instar larvae from either control or selection lines, together with 15 second-instar larvae from the tester stock, into Petri dishes (5cm) lined with agar and either 0.2ml or 0.1ml of larval food (25g live bakers yeast per 100ml water). These resource levels, as demonstrated by Kraaijeveld and Godfray (1997), represent weak and strong competition regimes respectively. I set up 15 replicates for each combination of line and resource level. The Petri dishes were incubated at 20ºC until the flies emerged. The number of experimental and tester flies that survived per plate was recorded. These survival data were analysed by calculating the competitive index, log (e/(t+1)), where *e* is the number of experimental and *t* is the number of tester

flies that survived in each replicate (Santos *et al.* 1992). The means of the competitive indices for the ten lines were calculated and the difference between the selected and control flies was tested using a t-test with unequal variances.

5.2.3. Haemocyte density

To compare the haemocyte density in the haemolymph of selected and control lines in the absence of *T. kingi* infection, flies from both lines were allowed to oviposit in culture bottles containing *Drosophila* medium and live yeast at 25ºC overnight. The eggs were washed out of the bottles and four vials (80x22mm) per line containing medium and live yeast were set up with 50 eggs in each. The vials were incubated at 20ºC for 96 hours. 15 third instar larvae from each vial were bled and their haemolymph pooled; 3μl of haemolymph was pipetted onto a haemocytometer and the total haemocytes in the samples were counted using a light microscope at 40X magnification. The counts were repeated twice for each haemolymph sample. The means of the haemocyte counts for the ten lines were calculated and the difference between the haemocyte density of the selected and control larvae was tested using a one-way ANOVA.

5.2.4. Phenoloxidase activity

The phenoloxidase activity in the haemolymph of *Drosophila* larvae selected for increased resistance to microsporidia was compared to that in larvae from control lines. The protocol outlined below was based on the suggestion in (Tzou *et al.* 2002). Flies from both selected and control lines were allowed to oviposit overnight in culture bottles containing *Drosophila* medium and live yeast at 25ºC. The eggs within these bottles were washed out and three vials per line (80x22mm) containing medium and live yeast were set up with 50 eggs in each. The vials were incubated at 20ºC for 96 hours. Ten third instar larvae per vial were bled and the haemolymph was pooled to determine phenoloxidase activity. 3μl of pooled haemolymph was added to 50μl of 10mM phosphate buffer (pH 5.9) containing 10mM L-DOPA in a 50μl-2000μl disposable cuvette (Eppendorf catalogue number:952010069). The optical density was recorded at five minute intervals for 30 minutes at 470nm in a spectrophotometer (WPA, Lightwave, UK). The enzyme activity for each sample was measured as the slope (absorbance vs. time) of the reaction curve during the linear phase of the reaction. Any optical density readings that were recorded as greater than two were removed from the analysis, since these were values which the spectrophotometer failed to measure. The mean phenoloxidase activity per vial was calculated as the average of the

slopes of the two replicates measured during the linear phase and this was used in the analysis. The selected and control lines were compared using a t-test with unequal variances.

5.3. Results

5.3.1. Fecundity under resource scarcity as larvae

The *D. melanogaster* females from selected and control lines were reared with limited food as larvae and their early fecundity under stress was determined. The early fecundity (Fig. 5.1) of females selected for increased resistance to *T. kingi* (37.73 ± 0.99) was significantly lower than control flies (60.39 ± 1.73), ($F_{1, 8} = 129.43$, $p < 0.001$).

5.3.2. Larval competitive ability

The relative competitive ability of *D. melanogaster* larvae from selected and control lines were determined at two levels of competitions. At a low level of competition (Fig.5.2) (0.2ml of larval food) survival was high (~80%) and there was no significant difference in competitive ability between the selected and control larvae (p=0.0866). However, at a high level of competition (0.1ml of larval food) survival was reduced (~50%) and the competitive ability of selected line larvae was significantly lower than larvae from control lines (p =0.0008).

5.3.3. Haemocyte density

The haemocyte density (Fig. 5.3.) of *D. melanogaster* larvae from lines selected for increased resistance to microsporidia (119.19 ± 2.17) was significantly higher than that in larvae from control lines (104.5 ± 3.31), ($F_{1, 8} = 15.12$, $p = 0.0046$).

5.3.4. Phenoloxidase activity

The phenoloxidase activity, measured as the slope (absorbance vs. time) was determined for haemolymph samples extracted from selected and control line larvae. The phenoloxidase activity (slope) (Fig.5.4) in haemolymph extracted from *D. melanogaster* larvae selected for increased resistance to microsporidia was significantly higher than the phenoloxidase activity (slope) in haemolymph extracted from control line larvae (p =0.0423).

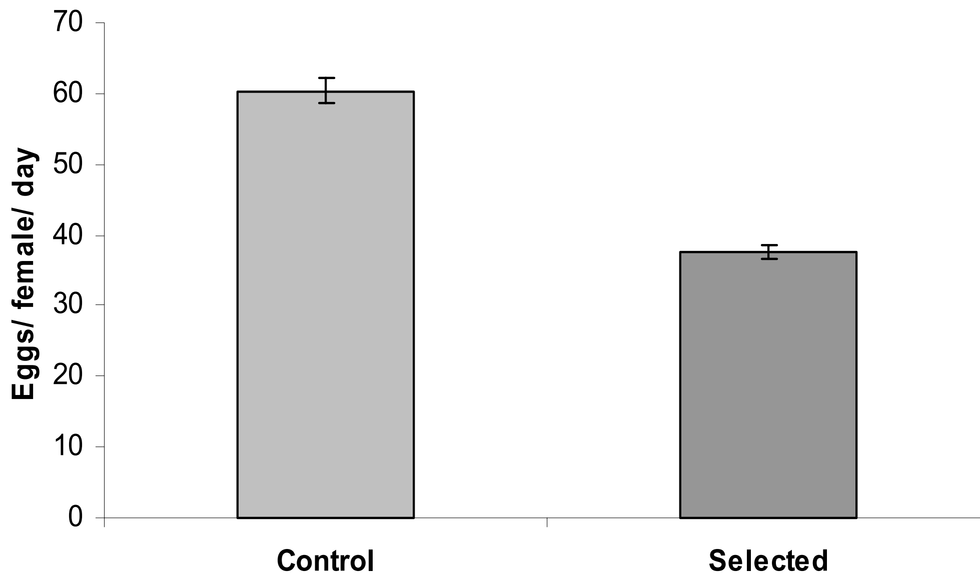

Fig. 5.1. The early fecundity (eggs/day/female ± S.E.) of *D. melanogaster* selected for increased resistance to *T. kingi* (dark bar) and control flies (light bar) under stressed conditions.

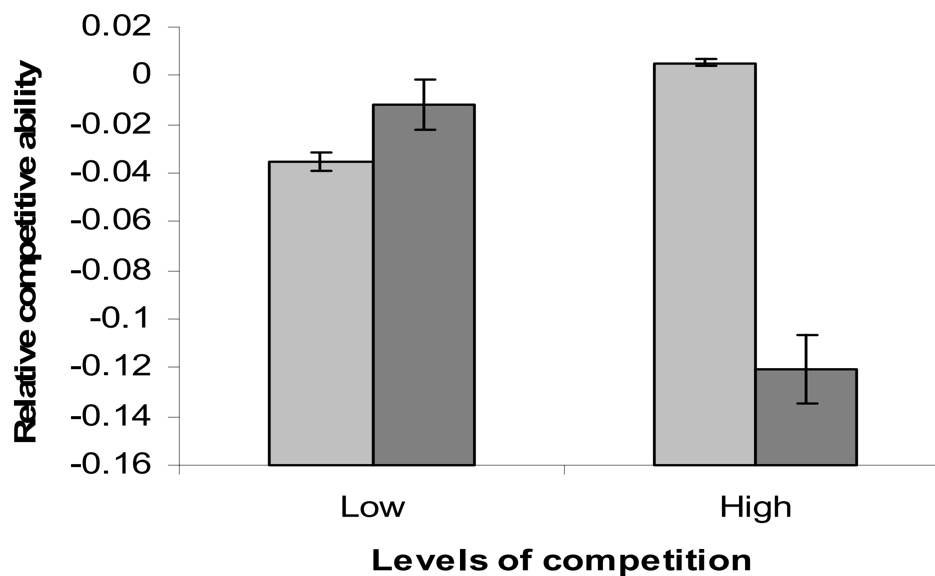

Fig. 5.2. The competitive ability (± S.E.) of *D. melanogaster* selected for increased resistance to *T. kingi* (dark bars) and their respective control lines (light bars) relative to a tester strain at low and high levels of larval competition.

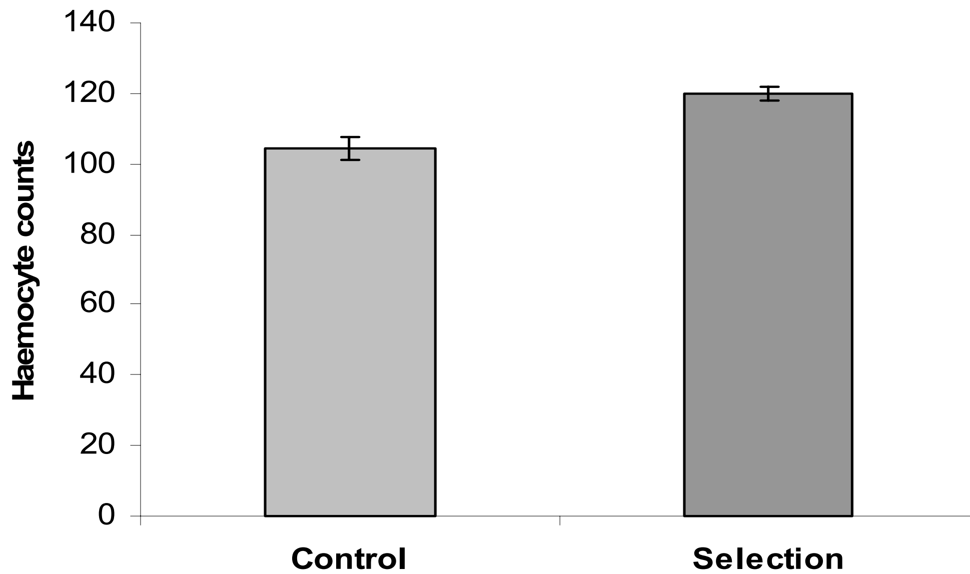

Fig. 5.3. The haemocyte density (count ± S.E.) of *D. melanogaster* larvae selected for increased resistance to *T. kingi* (dark bar) and control larvae (light bar).

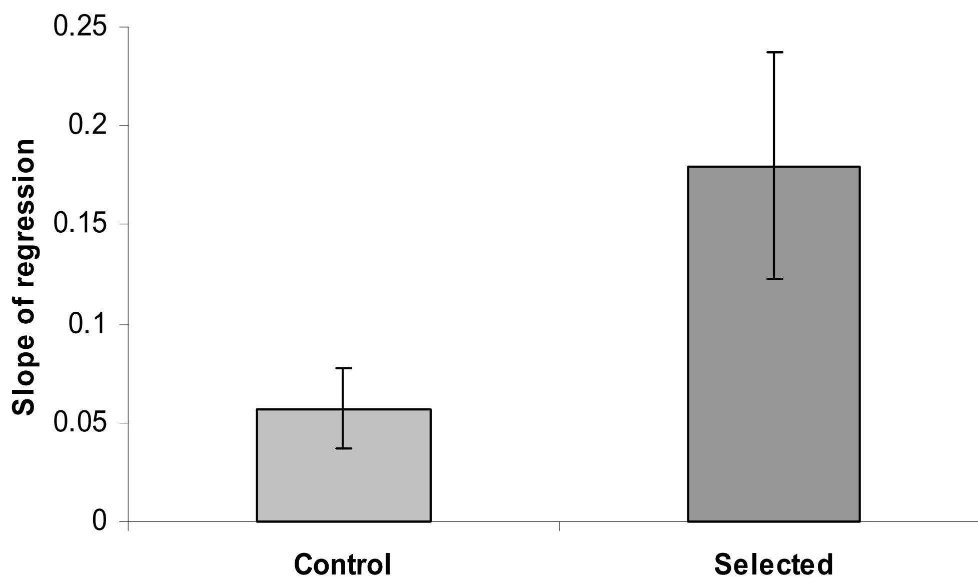

Fig. 5.4. The phenoloxidase activity (slope of regression on time ± S.E.) in *D. melanogaster* larvae selected for increased resistance to *T. kingi* (dark bar) and control larvae (light bar).

5.4. Discussion

It is evident from studies of insect-parasite systems in the past decade that resistance against natural enemies involves costs which can be distinguished into actual costs and standing costs (Boots and Begon 1993; Kraaijeveld and Godfray 1997; Yan *et al.* 1997; Fellowes *et al.* 1998a; Luong and Polak 2007). The nature and magnitude of these costs, along with the selection pressure applied on the host by the parasite, determines the rate and direction in which resistance evolves. Resource allocation to one fitness-relevant trait must be traded off against allocation to other fitness components and this is applicable to the evolution of resistance (Rolff and Siva-Jothy 2003) where allocation of resources into either actual or standing defence should trade-off against other fitness components.

Costs of actual defence are quite often difficult to distinguish from the pathogenic effects of the parasite. Delayed and reduced fecundity is observed in mosquitoes (*Armigeres subalbatus*) that successfully encapsulate their filarial parasite, but it is unclear if this is a cost of encapsulation, the effect of parasite, or both (Ferdig *et al.* 1993). Futerman (2006) similarly reported that *Drosophila* infected with microsporidian parasite *T. kingi* that are successful in emerging as adults have reduced body size and fecundity. However, it is unclear whether this is a cost of the increased haemocyte counts or phenoloxidase activity observed in larval haemolymph, or a pathogenic effect of the microsporidia, or a combination of both. It is important to stress here that although the effectiveness of these immunological responses is unclear; they are likely to require resources.

Selection experiments involving insect models that have short generation times and simple immune systems have provided an excellent tool for investigating potential genetic correlations between immune parameters and life-history traits (Reznick 1985; Fry 2003; Schwarzenbach and Ward 2006). In the previous chapter, replicate lines of *D. melanogaster* were selected for increased resistance to their microsporidian parasite, *T. kingi.* This chapter further investigated the potential trade-offs in life-history traits associated with increased resistance against *T. kingi.*

The early fecundity of flies selected for increased resistance significantly decreased compared to the control flies when stressed by scarce food during the larval stage indicating a trade-off between evolved resistance and host fecundity

under stressed conditions. This result is consistent with the reduced fecundity (~10%) of selected lines in comparison to control lines when kept under surplus food conditions reported in the previous chapter. However, under resource scarcity the fecundity of selected lines reduced drastically (~40%). This reduced fecundity in a scarce-resource environment is a correlated response to selection for improved defence against microsporidian infection in *D. melanogaster.* The flies selected for increased resistance were also found to have poorer larval competitive ability when compared to control flies under high level of competition for food. However, this difference in competitive ability disappeared at lower levels of competition for food. I thus demonstrate here yet another trade-off with increased microsporidium resistance: reduced larval competitive ability in *D. melanogaster.* I suspect that under scarce food conditions the selected larvae allocate more resources into resistance mechanisms against microsporidia resulting in them have poorly developed reproductive organs and a reduced ability to withstand competition. The conditions of food scarcity under which the reduced fecundity and competitive ability was observed in selected lines are realistic since *D. melanogaster* in the wild often occur under comparable levels of competition (Atkinson 1979). The result thus also highlights the widely accepted fact that costs are more obvious when organisms are under stress (Kraaijeveld and Godfray 1997).

The larval haemolymph from fly lines selected for increased resistance to *T. kingi* and larval haemolymph from control lines were compared for two immune parameters: number of circulating haemocytes and phenoloxidase activity. The number of haemocytes and the phenoloxidase activity in the haemolymph of larvae from selected lines were higher than in the haemolymph of larvae from control lines. *T. kingi* infection is known to cause an up-regulation of haemocyte density and phenoloxidase activity in *D. melanogaster* larvae (see chapter three). Phagocytosis of microsporidia has been reported previously (Weiser 1976; Kurtz *et al.* 2000), while encapsulation of infected tissue by haemocytes followed by melanisation has also been observed (Hoch *et al.* 2004), suggesting that the increase in haemocytes and the increase in phenoloxidase activity observed in the selected lines could be evolved resistance mechanisms to combat microsporidia. However, it has been argued that haemocytes are inefficient at combating microsporidia (David and Weiser 1994), which could imply that the higher haemocyte density might have unintentionally evolved in response to the extensive tissue damage that microsporidia are known to cause (Hoch *et al.*

2004). Futerman (2005) observed no difference in the effect of *T. kingi* on the early fecundity of *D. melanogaster* lines differing in their haemocyte density, demonstrating that haemocytes played an insignificant role in combating microsporidia. It is similarly unclear if the higher phenoloxidase activity in selected line larvae has evolved in response to *T. kingi* infection or to the tissue damage it causes. The increased immune mechanisms detected in the selected lines provide no evidence that these responses are either efficient or inefficient in combating microsporidia. Nevertheless, deeper investigation to distinguish between these alternative hypotheses is required since they have potential implications on the ecology and evolution of hosts and their parasites.

Kraaijeveld and Godfray (1997) reported similar trade-off in *D. melanogaster* between evolution of resistance against *A. tabida* and larval competitive ability. The increased resistance was due to the higher haemocyte density observed in the selected lines (Kraaijeveld *et al.* 2001b). Luong and Polaok (2007) have reported yet another similar trade-off in a *Drosophila-Macrocheles* system, between evolution of behavioural resistance and fecundity. The similarity in costs associated with increased resistance to parasitoids and microsporidia (reduced larval competitive ability) and with increased resistance to mites and microsporidia (reduced fecundity) in *Drosophila* suggests that parasite-mediated directional selection for increased resistance has conserved costs. However, though we find an up-regulation in haemocyte density in response to selection for increased resistance against both parasitoids and microsporidia, it would be inappropriate to suggest that the underlying immune mechanisms may be similar, since the role of haemocytes against parasitoids is known but against microsporidia is not clear.

Kraaijeveld *et al.* (2001) hypothesised that the basis of the trade-off observed between parasitoid resistance and larval competitive ability was due to reallocation of limiting resources from trophic to defensive functions. Considering that early fecundity and larval competitive ability are correlated with increased resistance against microsporidia, I believe that limiting resources are reallocated to defence functions from a range of physiological functions rather than just the trophic functions.

In conclusion, it is evident here that evolving resistance against a micro-parasite (microsporidia) is costly. The costs of resistance identified here are similar to

costs associated with increased resistance to macro-parasites, both endoparasites (parasitoids) and ectoparasites (mites). Two trade-offs were identified between microsporidia resistance and fecundity under stress and between microsporidium resistance and larval competitive ability. Larvae of selected lines have been shown to have higher haemocyte counts and higher phenoloxidase activity in comparison to control larvae, but it is not clear whether they evolved in response to parasitism or merely to tissue damage caused by the parasite. In either case it is most likely that up-regulating these traits need resources and hence involve costs.

Chapter Six: General Discussion

6.1. Introduction

The investigations presented in this thesis examine the interactions between a host and its intracellular parasite and is in succession to the work presented by Futerman *et al.* (2006). First, I investigated the within-host dynamics of the intracellular parasite and examined the immune responses of the host. Next, I explored if the host population can evolve tolerance or resistance against the intracellular parasite using an artificial selection experiment. Finally, I investigated the correlated responses to selection that might represent the associated costs and the involved mechanisms for increased resistance.

Each experimental chapter finished with a detailed discussion and therefore in this chapter I present a summary of results followed by a general discussion on the implications of these results on understanding host-parasite interactions.

6.2. Summary of results

Chapter two describes a set of four experiments examining the within-host dynamics of *T. kingi* in *D. melanogaster*. The first experiment explored the susceptibility of *D. melanogaster* to *T. king* where it was found that only the larval stages of the fruitfly were susceptible. The second experiment investigated the within-host proliferation of *T. kingi* where it was shown that the parasite spore proliferation occurred mainly after the fly emerged from its pupal-case. The within-host spore density was lowest in larvae, slightly higher in pupae and highest in the fly, suggesting that parasite proliferation in this system was specific to the life-stage of its host. The third experiment aimed at identifying the *Drosophila* tissues that were targeted by *T. kingi*. The *Drosophila* fat body, female reproductive organs and alimentary canal tissues were found to be targeted by the microsporidium. The final experiment in the chapter investigated if *T. kingi* caused sex-ratio distortion in host progeny. No evidence was found to suggest that *T. kingi* skews the host sex-ratio.

Chapter three describes three experiments that investigated the immune responses of *D. melanogaster* to *T. kingi* infection. The first experiment examined the cellular immune responses of *D. melanogaster* to *T. kingi* infection, where a significant upregulation of haemocyte numbers in haemolymph of infected

Drosophila larvae was found. The second experiment examined the humoral immune responses in terms of phenoloxidase activity. The phenoloxidase activity was found to be higher in the haemolymph of *T. kingi* infected larvae. The third experiment investigated the role of nitric oxide (NO) in resisting *T. kingi*. *Drosophila* that were fed on L-arginine supplement had a lower parasite density, suggesting that NO might play an important role in resisting microsporidia. Although *Drosophila* immune responses were found to be upregulated after *T. kingi* infection, it was not evident if the immune responses were targeted towards microsporidia.

Chapter four describes a selection experiment designed to explore if *D. melanogaster* population could evolve resistance or tolerance against its intracellular microsporidian parasite, *T. kingi*, under experimental conditions. The *D. melanogaster* selected and control lines did not differ in the correlated fitness traits examined (early fecundity and longevity) after the initial selection regime, suggesting that the population failed to evolve resistance. When the *T. kingi* spore dose was increased ten-fold in the selection regime, the *D. melanogaster* selected lines were found to resist *T. kingi* better than the control lines. The selected lines had a higher early fecundity and higher longevity than the control lines when infected with *T. kingi*. However, the selected lines had a lower fecundity compared to the control lines in the absence of infection, thus providing preliminary evidence that resisting microsporidia is costly.

Chapter five describes the investigations into the trade-offs associated with increased resistance in a *D. melanogaster* population and the immune mechanisms involved with the increased resistance. Larval competitive ability and early fecundity were measured under conditions of food scarcity in *D. melanogaster* selected and control lines to identify the trade-offs associated with increased resistance to microsporidia. The selected line larvae were poorer larval competitors than the control line larvae. The early fecundity of selected line flies that were stressed as larvae with scarce food was found to be lower than that of control line flies that were similarly stressed. To identify the immune mechanisms associated with increased resistance I compared the haemocyte density and phenoloxidase activity in the haemolymph of selected and control line *Drosophila* larvae. The haemolymph from selected line larvae was found to have higher haemocyte density and a higher phenoloxidase activity than in the control line larvae.

6.3. General conclusions

In conclusion, this thesis examines the interactions between an insect host and its intracellular microsporidian parasite, including evolution of resistance. Previous studies in other insect-microsporidian systems have argued both for and against the presence of successful innate immune defences against microsporidia (David and Weiser 1994; Kurtz *et al.* 2000; Hoch *et al.* 2004; Roxstrom-Lindquist *et al.* 2004; Tokarev *et al.* 2007). In the *Drosophila*-microsporidia system it had been previously shown that the host is moderately infected and has a reduced fitness (Futerman *et al.* 2006). The *Drosophila* immune responses towards *T. kingi*, observed in chapter three; the evolution of resistance demonstrated in chapter four; and the heightened immune responses of selected lines reported in chapter five suggest that *Drosophila* could have competent defences against microsporidia, although the mechanisms involved are yet to be confirmed. Innate immune responses in *Drosophila* towards microsporidia were shown to involve both cellular and humoral immunity. Preliminary evidence also suggests that nitric oxide may be involved in the *Drosophila* immune defence against microsporidia. Clearly, further work is needs to be undertaken to identify the immune pathways associated with microsporidian resistance, which is discussed later in this chapter.

This is also the first study of which I am aware that reports evolution of resistance in a host population towards a microsporidian parasite, including the trade-offs and potential immune mechanisms associated with the evolved resistance. Previous works have shown that *Drosophila* can evolve resistance to extracellular parasites such as parasitoids (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998a), fungus (Kraaijeveld and Godfray, subm.) and mites (Luong and Polak 2007). This study found that *Drosophila* can also evolve resistance against an intracellular microsporidian parasite and this involves costs in terms of fecundity and larval competitive ability. Similar costs are associated with increased resistance in Drosophila against its other enemies: resistance to fungus and mites have been shown to trade-off with fecundity, whilst resistance to parasitoids trades-off with competitive ability. As discussed in chapter five, costs of resistance can be of two forms: actual costs and standing costs, both of which can constrain evolution. The magnitude and nature of standing costs have the potential to prevent evolution of resistance, whilst the actual costs can only reduce its rate. Hypothetically, the lack of such trade-offs would have driven the genes associated with defence to become fixed throughout the host population,

resulting in a parasite-free world (Sheldon and Verhulst 1996; Gemmill and Read 1998).

Phagocytosis and melanisation have been suggested as the mechanistic basis of the increased immunity to microsporidia. The haemocytes involved in phagocytosis (plasmatocytes) are also known to differentiate into lamellocytes involved in encapsulation of parasitoid eggs. Evolution is more likely to favour immune defences that are effective against multiple enemies rather than defences effective against a specific enemy (Boots and Haraguchi 1999; Poitrineau *et al.* 2003). This study hence suggests that mechanisms involved in evolution and maintenance of resistance against multiple enemies may be conserved.

The *Drosophila* population evolved resistance only when subjected to a strong selection pressure of increased microsporidian spore dose. Futerman *et al.* (2006) investigated the fitness effect of *T. kingi* infection on its hosts *D. melanogaster*, *D. subobscura* and *Asobara tabida*. Though the microsporidian spore dose used to infect was kept constant, the parasitoid *A. tabida* was found to become heavily infected by microsporidia and suffer a greater reduction in fitness in comparison to the *Drosophila* hosts that were moderately infected. These results imply that spore dose is a crucial factor that needs to be considered when parasites such as microsporidia are used as bio-pesticides for insect pests and vectors, since they can determine both the evolution of resistance in target insects and their effect on other non-target insects.

Previous work by Futerman *et al.* (2006) had determined that *T. kingi* was mainly transmitted horizontally and to a lesser extent vertically within the *Drosophila* population. This study has furthered our understanding of *Drosophila*-*T. kingi* model system, by demonstrating that stage-specific susceptibility of the host and stage-specific proliferation of the parasite exists in this system. Figure 6.1, presents a diagrammatic representation of the synchrony between the host and parasite lifecycles in *Drosophila*-*T. kingi* model system. This study also re-confirmes previous reports (Kramer 1964a; Armstrong 1976) on *Drosophila* tissues targeted by *T. kingi* and found that though the reproductive tissues were infected, *T. kingi* had no detrimental effects on the host sex-ratio. The work on *Drosophila* immune responses to its microsporidian parasite, *T. kingi*, reported in

Futerman (2005) and in this thesis, suggest that further investigations in this front is essential.

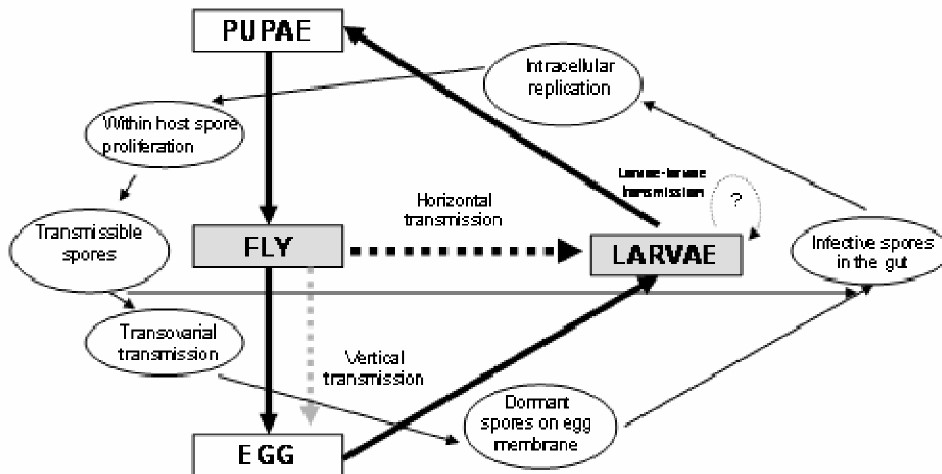

Fig. 6.1. Diagrammatic representation of *Drosophila* (dark arrows) and *Tubulinosema kingi* (light arrows) life-stages and the synchrony in between them.

As mentioned earlier, Futerman *et al.* (2006) demonstrated differential virulence of *T. kingi* infection in *Drosophila* and its parasitoid, *A. tabida*. The effects of microsporidiosis in the parasitoid were severe and this suggests that the parasitoids are under a higher selection pressure to evolve resistance than the flies. The parasitoid population is hence expected to evolve resistance towards *T. kingi* more rapidly than the *Drosophila* population. The evolution of resistance against a shared parasite in either the *Drosophila* or parasitoid population has serious implications on the ecological and evolutionary dynamics of the host-parasitoid system. Assuming that the evolution of resistance in the parasitoid population is also likely to involve costs, it would be interesting to compare the costs (actual and standing) associated with resisting the same parasite in hosts that differ trophically. This information could provide more insight into the dynamics of parasites with vector mediated transmission.

Further investigations in this model system are essential for a deeper understanding of host-parasite interactions and its evolution. I conclude this thesis with examining a few future directions suggested by this work in the following three sections.

6.4. Microsporidia and intraguild predation

Intraguild predation is broadly defined as a phenomenon where two species share a common prey (or host) and concurrently engage in a prey-predator (or host-parasitoid) relationship with each other (Polis *et al.* 1989). The two species that engage in intraguild predation are commonly termed intraguild predator and intraguild prey (Polis *et al.* 1989). The intraguild predation theory assumes that intraguild predator and intraguild prey compete for the shared resource (host or prey) and that this resource competition is an important factor that determines their interactions (Holt and Polis 1997). The theory also assumes that the intraguild predator kills both the primary and intraguild prey species (Holt and Polis 1997). However, there are some systems where neither this resource competition between the intraguild prey and intraguild predator is present nor does the intraguild predator kill the primary and intraguild prey species, for example the *Drosophila*-parasitoid-microsporidia systems (Futerman 2005; Futerman *et al.* 2006). In this system, the presence of microsporidian infection in *Drosophila* does not deter the parasitoids from parasitisation, nor does parasitism of *Drosophila* by the parasitoid subsequently exclude the microsporidia (Futerman *et al.* 2006). Futerman (2005) suggested that the current intraguild predation framework is perhaps inadequate for investigating such systems and that further expansion of it to include shared pathogens as a separate case was essential. In this section, I discuss the role of microsporidia as an intraguild predator in *Drosophila*-parasitoid system.

Parasitoids are a unique group of animals that share the features of parasites and predators; they develop on their hosts like any other parasite but invariably cause death of their hosts. They are considered to be one of the most species rich groups among animals. The hosts for parasitoids are usually other insects on, or in whose bodies they feed (Godfray 1994). The host-parasitoid interactions have received considerable attention, especially by ecologists and evolutionary biologists and this is especially because parasitoids are important regulators of their insect hosts (Godfray and Shimada 1999). Theoretical models of host-

parasitoid populations have been demonstrated to be inherently unstable and most work in this field has concentrated on identifying factors which promote the stability in natural host-parasitoid populations (Hassell 2000). Among other factors that could have an effect on the dynamics of host-parasitoid interactions, the addition of other species to the simple host-parasitoid system is stated to be significant (Hassell 2000). The impact of including higher order parasitoids and predators into host-parasitoids systems is unpredictable, though they may stabilise the interactions (Rosenheim *et al.* 1995; Rosenheim 1998). However, pathogens can also affect the host-parasitoid systems in similar ways and this has been investigated both theoretically and empirically, although most models tend to assume that parasitoids and pathogens are competing for resources (the hosts) (Hochberg *et al.* 1990). The *Drosophila*-parasitoid-microsporidia model system (Futerman 2005; Futerman *et al.* 2006) is one such example, but as mentioned earlier, the parasitoid and pathogen in this system are not really in competition.

The impact that shared micro-parasites may have on the dynamics of the host-parasitoid system is influenced by the relative degree to which the host and parasitoid are affected by them. It is hypothesised that if the host suffers a greater loss in its fitness in comparison to the parasitoid, when infected by a shared parasite, then the host population suffers since the effects of parasitoid and parasite is expected to be additive. In a reversed situation, where the effect of parasites is greater on the parasitoids than their hosts, it is expected that the host population thrives since the parasitoid control on them is relaxed. The *Drosophila*-*T. kingi* system explored in this thesis, along with well-studied *Drosophila*-parasitoid systems such as those established by Kraaijeveld and Godfray (1997), can be used as an ideal model for experimental investigation into the effects of a shared pathogen on the dynamics of a host-parasitoid system.

Tubulinosema kingi, the microsporidian parasite, and *Asobara tabida*, the larval parasitoid, share a common host, *Drosophila*, and also engage in a host-parasite interaction of their own. Futerman *et al.* (2006), have shown that the effect *T. kingi* has on *A. tabida* is greater than its effect on *Drosophila*, thus suggesting that *T. kingi* might lead to reduced parasite-mediated host suppression in *Drosophila* populations. Experimental manipulation in his intraguild system is possible, with the potential for tightly controlled population experiments (for

example see Green *et al*. (2000) and the selection experiment in this thesis). Futerman (2005) had proposed a population cage experiment with two treatments involving *Drosophila*-*A. tabida* systems maintained either with or without *T. kingi*, to determine if the presence of a shared parasite reduces the parasitoid's ability to regulate host populations.

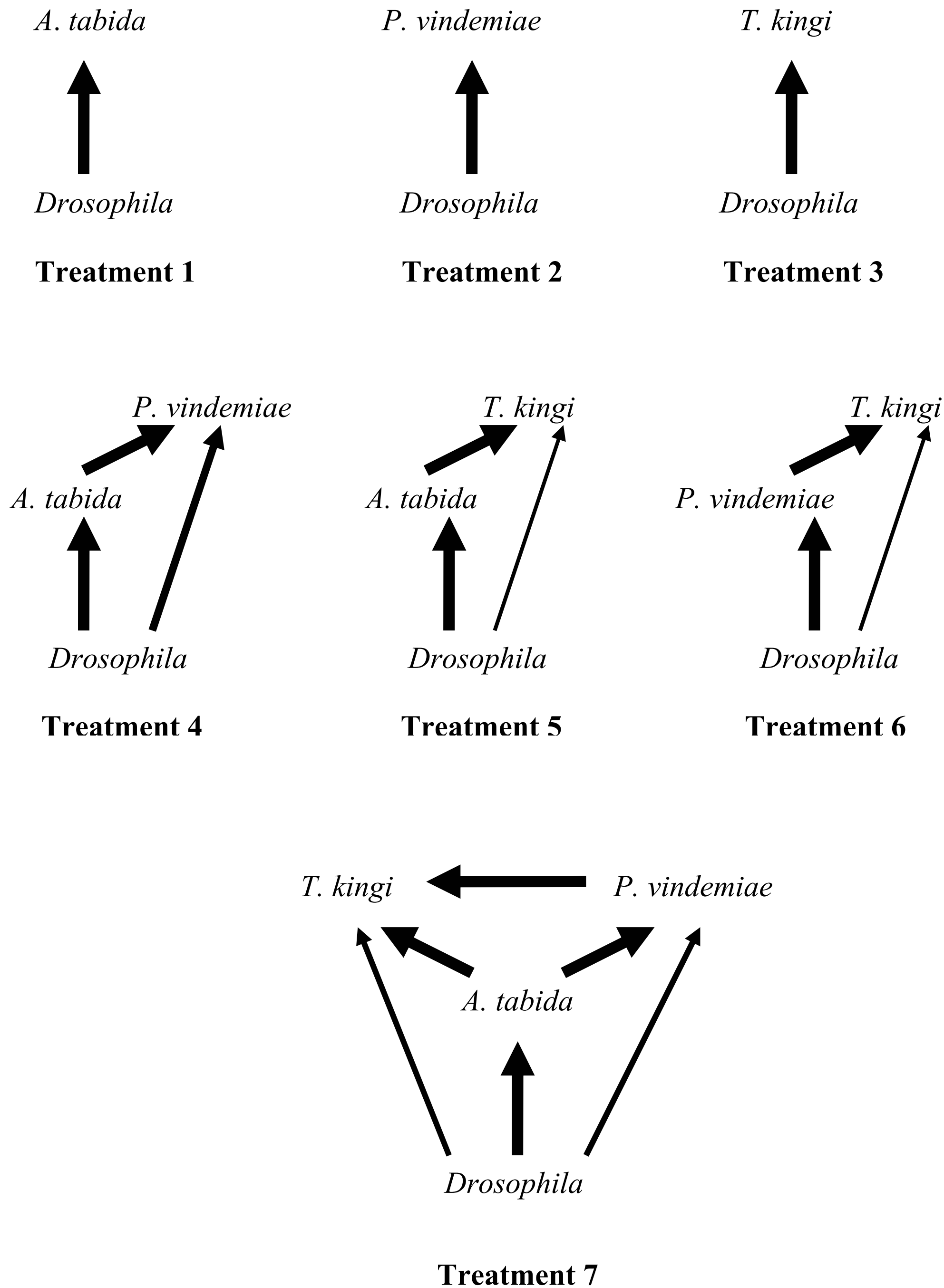

Fig. 6.2. Proposed population cage experiment to determine the impact of *T. kingi* on the dynamics of *Drosophila*--*A. tabida*--*P. vindemiae* system. Treatments 1 & 5, were previously proposed by Futerman (2005).

This *Drosophila*-parasitoid-microsporidia system can be further investigated by the inclusion of a hyper-parasitoid, *Pachycrepoideus vindemiae*. This hyper-parasitoid is known to attack the *Drosophila* pupae, including those that are already parasitised by *A. tabida* (Fellowes *et al.* 1998b), hence it is in itself an intraguild predator. Therefore parasitism by *P. vindemiae* results in the equal and efficient suppression of both *Drosophila* and *A. tabida* populations. However, when *P. vindemiae* parasitises *T. kingi* infected-pupae, it has been found that the emerging hyper-parasitoid is also infected (A.R. Kraaijeveld pers. comm.). Therefore microsporidia can act as an Intraguild predator of *Drosophila-A. tabida-P. vindemiae* system. Although considerable thought would be required in designing a workable experiment, in principle the study would involve replicate population cages with treatments as in Fig 6.2 above, could be very informative.

The hypothesis to be tested would be as follows: in simple host-parasite/parasitoid treatments involving *Drosophila*-*T. kingi*, *Drosophila*-*A. tabida* and *Drosophila*-*P. vindemiae*, strong suppression mediated by parasite or parasitoids is expected and *Drosophila* populations will therefore be maintained at low densities. In treatments involving a single intraguild prey and intraguild predator species, the outcome may depend on the effect of the intraguild predator on its primary and intraguild prey. In *Drosophila*-*A. tabida*-*P. vindemiae* treatment I expect no change in host densities, since the effect of *P. vindemiae* on *Drosophila* and *A. tabida* is equal. In *Drosophila*-*A. tabida*-*T. kingi* and *Drosophila*-*P. vindemiae*-*T. kingi* treatment, I expect the *Drosophila* populations to be maintained at a higher mean density than the simple two species treatment because I expect *T. kingi* to cause a reduction in host suppression. If my hypothesis is correct, the four species treatment with two levels of intraguild predation would lead to a reduction in host suppression, though the magnitude of this is unpredictable.

6.5. Parasite transmission and evolution of virulence

Among the different factors influencing the evolution of virulence, the mode of transmission is believed to play an important role in determining pathogen virulence over evolutionary time (Lipsitch *et al.* 1996). Vertically transmitted parasites are likely to be less virulent to their hosts in comparison to horizontally transmitted parasites, since host reproduction and hence its survival translates very directly to the fitness (reproduction) of the parasite (Lipsitch *et al.* 1996). The

trade-off associated with evolution of virulence and mode of transmission has been demonstrated in *Escherichia coli*-bacteriophage system (Bull *et al.* 1991). Observations in fig wasps have also demonstrated a close correlation between nematode virulence and the degree of horizontal transmission relative to vertical transmission (Herre 1993). A similar trade-off has also been demonstrated in a plant (barley) and virus (barley stripe mosaic-virus) system (Stewart *et al.* 2005). The *Drosophila*-*T. kingi* model system investigated in this thesis involves a parasite that is known to spread within the host population by both horizontal and vertical transmission (Futerman *et al.* 2006). This system thus provides an opportunity to explore the effects of the differential selection pressures exerted by the two modes of transmission on the evolution of microsporidian virulence.

To explore the correlation between transmission and evolution of virulence I suggest an experimental evolution set-up where the mode of parasite transmission could be restricted to exclusively vertical in one line and mainly horizontal in the other line. A large number of *T. kingi* infected *D. melanogaster* females in each line would be allowed to mate with uninfected males from the base population and oviposit in individual vials containing yeast/sugar media for a day. In one line, the females would be individually homogenised and fed to its offspring, whilst in the second line all the females would be homogenised together and then fed equally to all the offspring. The idea of feeding the mother (containing infective spores) to its offspring is to ensure vertical transmission while feeding spores mostly from unrelated females ensures horizontal transmission. The offspring from each line are then pooled together before randomly selecting the females for the next generation. The same protocol, if repeated over a number of generations, is expected to result in selecting the microsporidia in the first line to be less virulent than the microsporidia in the second line. The virulence of microsporidia can be assayed by measuring the effect it has on host life history traits.

6.6. Insect immune responses and the microsporidia

Three experiments were suggested in the discussion of chapter three, that could determine the effectiveness of the *Drosophila* immune responses towards its microsporidian parasite. In addition, I present below two more possible experiments that could be carried out in this model system, to further our understanding of insect immunity and host-parasite interactions.

Drosophila immune responses to parasites and pathogens are considered to be regulated broadly by two immune pathways: the Toll and *imd* pathways (Tanji *et al.* 2007). The Toll pathway is considered to be mainly activated in response to infections by fungi and Gram-positive bacteria whilst the *imd*-pathway in response to mainly Gram-negative bacteria. The *Drosophila* cellular immunity involving phagocytosis, encapsulation and melanisation is regulated by the Toll-pathway, which also activates an antifungal peptide *Drosomycin*. The *imd*-pathway on the other hand activates other antimicrobial-peptides: *Diptericin*, *Attacin*, *Drosocin*, *Cecropin*, *Defensin* and *Metchnikowin* (Lemaitre and Hoffmann 2007). The antimicrobial-peptide genes for *Drosomycin* and *Diptericin* are used as read-out genes for the Toll and *imd* pathways respectively (Tanji *et al.* 2007).

The *Drosophila* immune pathways and defences have been investigated extensively against parasitoids, bacteria, fungi and viruses (Lemaitre and Hoffmann 2007), but nothing is known about the immune pathways associated with defence against intracellular microsporidia. Roxtrom-Lindquist *et al.* (2004) analysed the *Drosophila* gene expression and though 59 genes were uniquely expressed in response to infection by *Octosporea muscaedomesticae*, most of these genes were of unknown function and included no antimicrobial peptides. We cannot be sure that microsporidia do not induce antimicrobial peptides, since Roxtrom-Lindquist *et al.* (2004) measured gene expression after feeding the microsporidian spores to adult flies and in case of *T. kingi* infections, only larvae were found to be susceptible (see chapter two). Hence, I suggest the following experiment to determine the immune pathway associated with resisting microsporidia in *Drosophila*.

Gene expression of *Drosomycin* and *Diptericin*, the read-out genes for the Toll and *imd* pathways respectively (Tanji *et al.* 2007) can be quantified in RNA extracted from *Drosophila* larvae at different time points after being fed with microsporidian spores. An efficient method to measure expression could be reverse transcription-polymerase chain reaction (RT-PCR). This molecular technique involves preparation of cDNA from the extracted RNA samples, followed by amplifying the target genes using specific primers and suitable probes such as SYBERGREEN. It is essential for the quantification process that a housekeeping gene (for example, Actin) be included in the assay, along with using RNA samples from control larvae. The increased expression of either *Drosomycin* or *Diptericin* in the microsporidia infected larvae would clearly

indicate the immune pathway involved in resisting these intracellular parasites. It is very likely that the Toll-pathway would be associated with resisting microsporidia since they are highly evolved fungi and have been shown to elicit cellular immune responses (see chapters three and five). Apart from the possibility that the *imd*-pathway is associated with microsporidian resistance, suppression of immune pathways may be observed due to microsporidian manipulation, since suppression of insect immune responses has also been claimed in parasitic infections, such as microsporidia (Lozinskaya *et al.* 2004) and *Spiroplasma* (Hurst *et al.* 2003). Assuming that either Toll or *imd* pathway is associated with microsporidian resistance, it would be interesting to determine if the costs (actual and standing) associated with resisting different parasites is pathway specific. I presume it is very likely that resisting different parasites through an analogous immune-pathway would have identical costs and hence be favoured to evolve.

Tubulinosema kingi successfully pass through the larval gut wall and infect the tissues present beyond, despite the presence of defences such as lysozymes and locally produced antimicrobial peptides (Lemaitre and Hoffmann 2007). In addition, the guts of insects are also lined by an extracellular matrix known as peritropic membrane that consists of proteins, chitin and proteoglycans. This functions as a hostile physical barrier to parasites (Lehane 1997). The *Drosophila*-microsporidia model system thus presents an opportunity to investigate the host-gut immune defences to parasite invasion through the alimentary canal, including the strategies that parasites use to evade them. The non-susceptibility of *Drosophila* flies to *T. kingi* observed in chapter two could possibly be due to the adult-gut immunity, which could either be more effective or just different to that in susceptible larval stages. This hypothesis could further explain why Roxtrom-Lindquist *et al.* (2004) observed elevated expression of lysozymes, but no antimicrobial peptides, in response to feeding *O. muscaedomesticae* spores to *Drosophila* flies. The interaction between *T. kingi* and *Drosophila* gut epithelia of both larval and adult stages can be explored using the range of mutant flies and live imaging techniques currently available.

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