Interactions between the bumblebee, *Bombus terrestris*, and its trypanosome parasite, *Crithidia bombi*

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

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University of Leicester

September 2009

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Abstract

Theory predicts that hosts should evolve highly specific immune defences when there are significant fitness costs associated with parasitic infection. Historically, mechanistic studies have defined invertebrate immunity as innate and non-specific. However, recent evidence from ecological studies challenges this view by finding a high degree of specificity between host and parasite and evidence of immune priming, that are indicative of a more complex system. Critics of the ecological perspective assert that without mechanistic evidence, there is no sound reason to assume that these phenomena are generated by innate immunity. To begin bridging the gap between mechanistic and ecological fields of innate immunity, I have examined the molecular basis of specificity and priming in the model *Bombus*-*Crithidia* host-parasite system.

My studies show that immune gene expression mirrors the interaction effect found with indirect, ecological measures of immunity, providing unequivocal evidence of innate immune specificity in invertebrates. A similar examination of immune priming suggested that the genes I analysed were not involved in this phenomenom and is most likely to be indicative of the relative importance of different arms of immunity in the primed immune response. Finally, I examined more general aspects of the immune response to *Crithidia* by characterising the temporal dynamics of immune gene expression throughout infection for the first time in this model host-parasite system. I also confirmed that the assumed link between virulence and intensity of *Crithidia* infection is valid. To conclude, my studies have shown that integration of molecular knowledge into natural host-parasite systems can only serve to enrich our understanding of the wider capabilities of invertebrate innate immunity.

Acknowledgements

I'd like to say a huge "thank-you" to my supervisor, Dr. Eamonn Mallon, who has been immensely supportive during my PhD. My interest in social insects and parasitology began during an undergraduate project in his lab and I am grateful for the inspiration and opportunities he has provided throughout my PhD to continue to explore these interests. Many people in the Biology department at Leicester have helped me. Thanks especially to Donna Betts, Sally Adams and Ceinwen Tilley for all their technical support and patience with my endless questions. Everything I know about molecular biology was learnt from them and their advice was invaluable to my research. The SSH technique was demystified with the kind help of Dr. Seirian Sumner during a short stay working at ZSL, London, UK. Thanks to Akram and Cris from my lab who helped with the bees. I would also like to acknowledge my sponsors, BBSRC, for funding my research for the last three years.

Finally, thank you to my mum, sister and all my friends, old and new, for their wise words, encouragement and advice; they have been an enormous source of support and I am grateful to each and every one of them for their input over the last four years.

Collaborator contributions

A number of people contributed help with various aspects of my PhD:

- Eamonn Mallon (PhD supervisor) provided practical assistance infecting bees and collecting samples for experiments in Chapters 3, 5 and 6. He also assisted with experimental design and statistical analysis of the data in all my studies.
- Sally Adams (Biology Department, Leicester) contributed help and advice with all the molecular work, analysis of raw qPCR data and also provided the actin housekeeping primers used in the SSH PCRs. She contributed advice to the design of the specificity experiment in Chapter 2 and preparation of the results for publication.
- Paul Schmid-Hempel (ETH, Zurich) collaborated on the specificity experiment in Chapter 4 and provided help in the design of the experiment. PSH also helped write up the study for publication.
- Seirian Sumner (ZSL, London) collaborated with me to provide assistance and advice with the SSH technique in Chapter 5. She has also been involved in writing the results of the temporal study up for publication.

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1. Main introduction

1.1. Ecological and evolutionary immunology

The immune system is the last line of defence against pathogens when behavioural strategies and physical barriers fail to prevent an infection (Schmid-Hempel and Ebert, 2003). However, despite the obvious benefits of a robust immune response that can eradicate infection, invertebrates remain susceptible to disease and individuals of a species vary in their response to a pathogen. Evidently, the host's ecology will shape the structure and complexity of immune defences, and evolutionary ecology seeks to understand how these factors maintain immune variation and what the consequences are for the host. A variety of factors can contribute to variation in immune defence, such as the environment-dependent (biotic and abiotic) expression of immune traits (Lazzaro et al., 2008, Wolinska and King, 2009); intraspecific conflicts such as sexual selection (Hamilton and Zuk 1982), cost-based trade-off of immune defence (Sheldon and Verhulst, 1996); and population-level selective processes (Boots et al., 2009). This thesis is interested in another cause of variation in immune defence namely the interactions between the host and its parasites.

Parasite advantage

Where there are high fitness costs associated with parasitism, hosts are under large selective pressures to counteract the parasite. For example, severe selective pressures are imposed on adult water fleas (*Daphnia pulex*) infected with the bacteria *Pasteuria ramosa* because the parasite sterilises its host (Carius et al., 2001) and the growth, survival and reproduction of *Drosophila* can be severely reduced by larval parasitoid

infection (Fellowes and Godfray, 2000). However due to their generally short generation times, large population sizes, and high mutation rates, pathogens can rapidly evolve adaptations to evade immune defences (Schmid-Hempel, 2008). The most exceptional examples are the human immunodeficiency virus (HIV) and influenza-A virus, which still evade effective immunisation because of their rapid evolution through high rates of replication, mutation and recombination (Goudsmit et al., 1991), but similar evasion, through antigen variation, is found in *Trypanosoma cruzi* (Mello et al., 1996) and *Plasmodium* species (Beeson and Brown, 2002). Given these two counteracting forces co-evolution between host and parasite should be expected.

Host-parasite co-evolution

The host-parasite co-evolution theory suggests that, under selection pressures from parasitism, host immunity (susceptibility) and parasite infectivity will co-evolve in a reciprocal manner through natural selection. In effect, the parasite repeatedly evolves adaptations to evade immune defences that are resisted by counter-adaptation in the host. The Red Queen hypothesis predicts that co-evolution can evolve from negative frequency-dependent selection as parasites adapt to the most common host genotype (Van Valen, 1973, Hamilton et al., 1990, Lively and Dybdahl, 2000, Dybdahl and Lively, 1995). Thus, rare host genotypes gain a selective advantage by retaining resistance to the most prevalent parasite genotype. After a short period, host and parasite genotype frequencies begin to fluctuate in a time-lagged fashion as common genotypes are counteracted by antagonistic co-evolution, leading to maintenance of variation in both parasite infectivity and host immunity (Dybdahl and Lively, 1998) (Figure 1.1).

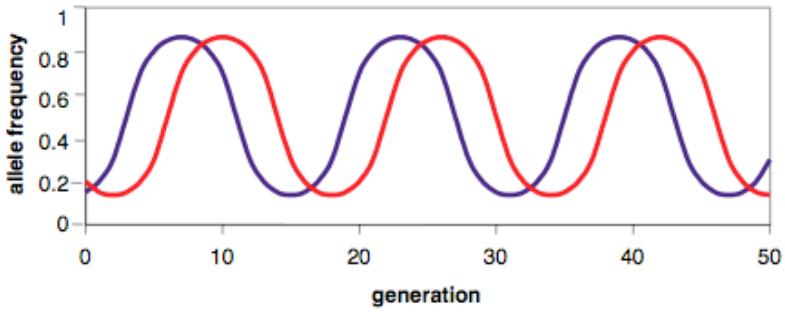

Figure 1.1 Time-lagged negative frequency-dependent co-evolution between host and parasite. Host (black) and parasite (grey) genotypes both contain variation in immunity and infectivity alleles respectively. Parasite adaptation tracks the most common host genotype in a time-lagged fashion, causing negative frequency-dependent selection on the host. Selection is non-directional meaning that host genotypes can be maintained at low frequency until selection eases. From (Woolhouse et al., 2002)

It has been suggested that the requirement for the host to keep up with the rapid evolution of the parasite could be one of the driving forces for sexual reproduction in hosts because meiosis can generate offspring with the genetic diversity needed to continue counteracting parasite adaptation (reviewed in (Hamilton et al., 1990). Studies have found evidence in support of co-evolution in natural host-parasite populations. For example, parasites have shown: local adaptation, infecting sympatric host populations more easily than allopatric populations (Lively et al., 1990, Refardt and Ebert, 2007), negative frequency-dependent selection on the common host genotype, causing a decrease in its frequency (Wolinska and Spaak, 2009) and the rapid induction of Red Queen co-evolutionary dynamics in the host (Decaestecker et al., 2007).

Natural variation in host immunity and parasite infectivity

A number of assumptions must hold for frequency-dependent selection to stimulate co-evolution. Firstly, both host and parasite populations must show natural genetic variation in immune defence and infectivity respectively, which selection can act on. A number of studies have validated these criteria and found variation in susceptibility to specific pathogens in individual bumblebee patrilines (Baer and Schmid-Hempel,

2003), in water flea, *Daphnia pulex* (Little and Ebert, 1999) and pea aphid, *Acyrthosiphon pisum* (Ferrari et al., 2001) clones, and within populations of *Drosophila melanogaster* (Tinsley et al., 2006) and Soay sheep, *Ovis aries* (Smith et al., 1999). Although less extensively studied, examples of variation in parasite infectivity have also been found, for example in the tapeworm, *Schistocephalus solidus* to the three-spined stickleback, *Gasterosteus aculeatus* (Hammerschmidt and Kurtz, 2005) and the parasitoid, *Asobara tabida* to *Drosophila* (Kraaijeveld and Nicole N, 1994).

Specificity in the host-parasite interaction

Secondly, to create a selective advantage for rare genotypes the interaction between host and parasite must be genotype-specific, such that the outcome of infection cannot be predicted from the host-line and infecting strain of parasite. That is to say, that high infectivity of a parasite strain or resistance of a host line cannot be universal but will instead be specific to a proportion of the respective host or parasite genotype interactions. Explicitly, hosts from genetic line A are more susceptible to parasite strain I, than to strain II, whereas host line B are more susceptible to strain II and less so to strain I.

Numerous examples of highly specific interactions have been found in natural host-parasite systems of invertebrates and plants. For example, individual clones of *Daphnia pulex* have varying susceptibility to different strains of the bacterium *Pasteuria ramosa* (Carius et al., 2001, Little et al., 2006, Little and Ebert, 2000), bumblebees maintain different levels of the trypanosomatid *Crithidia bombi* in their gut according to the infecting strain present (Schmid-Hempel, 2001), and the intensity of infection with the oomycete *Hyaloperonospora arabidopsis* is highly variable across individual

Arabidopsis lines (Salvaudon et al., 2007). Similar trends are seen in the interactions of *Anopheles gambiae* with the trypanosome *Plasmodium falciparum* (Lambrechts et al., 2006) and between snails and schistosome trematode worms (Webster et al., 2004). Experimental co-evolution of the bacterium *Pseudomonas fluorescens* with its DNA phage Φ2 can also induce the rapid development of specific genotype-genotype interactions as evidenced by variation in the growth rate of bacteria after infection (Poullain et al., 2008). Theoretical models describing the genetics of co-evolution also utilise the genotype-genotype interaction to generate specificity. For example, the 'matching-alleles' models assumes that a parasite can only evade a host immune response if its allele matches the host's at a specific locus, similar to the familiar self/non-self recognition process in the immune system.

Innate immune specificity

The importance of genotype-genotype interactions in natural host-parasite systems strongly implies that co-evolution will favour immune defences with greater specificity toward their parasite. In vertebrates this is accomplished with adaptive immunity, using specialised immune cells and antibody production to differentiate between pathogens and rapidly form immune memory of past infections (Kurtz, 2004). However, numerous examples of ecological immune specificity are found in invertebrates where adaptive vertebrate-like immunity is absent and defences, comprised of a small suite of cellular and humoral defences, are instead innate and considered broad-acting and generalised. There is therefore a dichotomy between wide spread ecological evidence for specificity and molecular evidence for a generalised response.

Critics of invertebrate immune specificity suggest that the ecological measures used to represent immunity such as host survival, fecundity or growth rate do not necessarily reflect the host's resistance to infection, but may be the result of any number of interactions between host and parasite, not just the immune system. These interactions may include behavioural modification, reproductive isolation or a switch in life history strategies and therefore conclusions of immune specificity are premature (Hauton and Smith, 2007) (Figure 1.2).

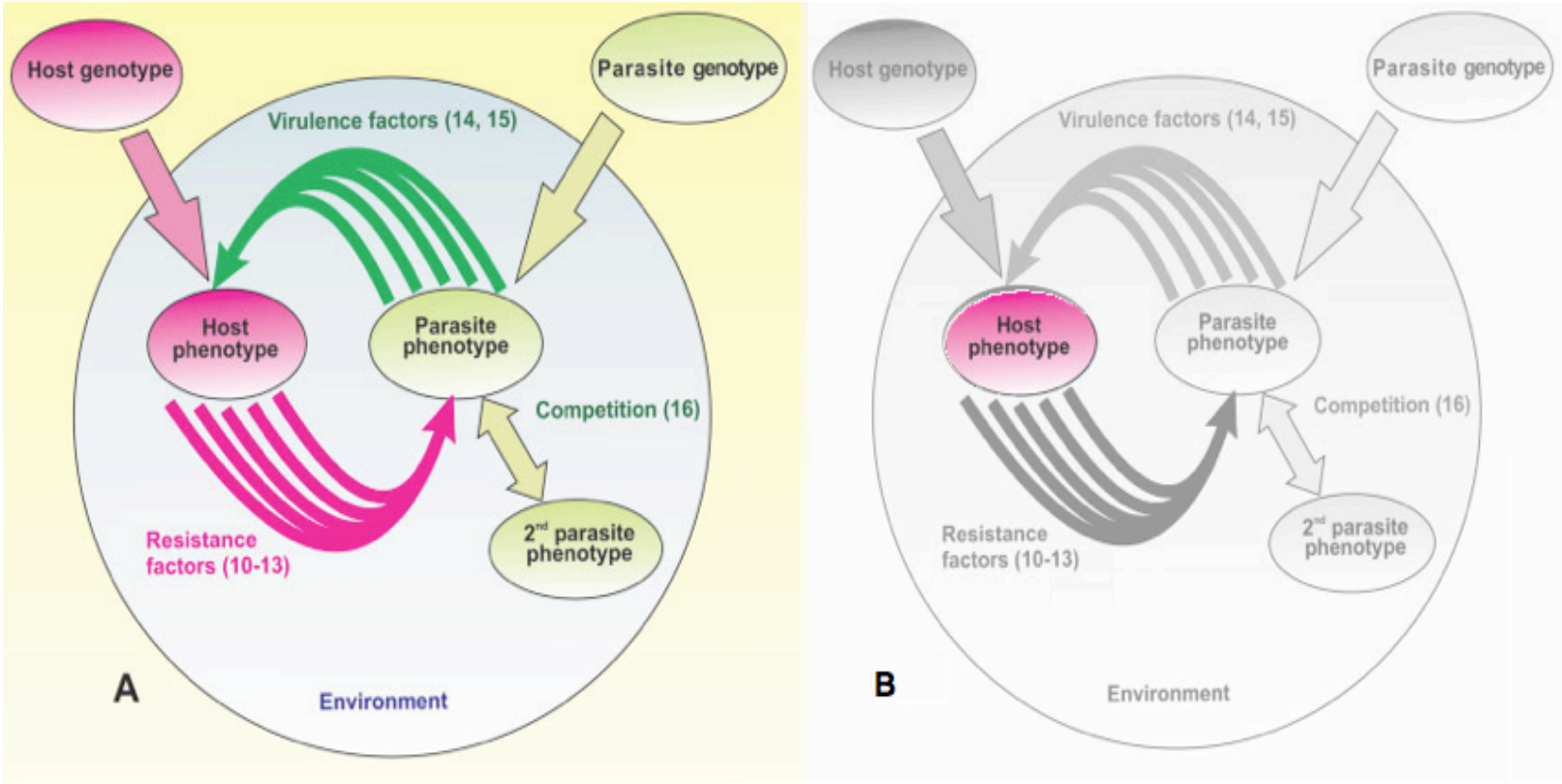

Figure 1.2 A. Components of the host-parasite interaction. From (Hauton and Smith, 2007). Host and parasite genotypes interact and their phenotypes are expressed within an environment. Immune and non-immune adaptations can all manifest themselves as increased host resistance. Parasite virulence is also the product of a number of factors. B. The focus of ecological immunology. Critics suggest that conclusions of immune specificity are premature, arguing that ecological immunologists study the host phenotype in isolation and do not consider other factors that may explain the phenomenon. Bracketed numbers can be ignored.

However, it is difficult to accept this argument in light of mounting ecological evidence of additional immune phenomena being found in invertebrates. Specific immune priming, i.e. the ability to respond more strongly and specifically to secondary infection with the same pathogen, is one such example. Though mechanistic models would not have predicted immune priming in insects, evidence has been found in ecological studies of cockroaches (Faulhaber and Karp, 1992), fruit flies (Pham et al., 2007), bumblebees (Sadd and Schmid-Hempel, 2006), flour beetles (Roth et al., 2008) and copepods (Kurtz and Franz, 2003) with other studies finding the priming effect can be passed on to offspring and improve their resistance to the same pathogen in bumblebees (Sadd et al., 2005) and copepods (Little, 2003). Priming is not a new phenomenon either; transplantation studies with the cockroach in the 1980's showed that a second cuticle graft was more strongly rejected by the recipient if both grafts originated from a genetically-distinct donor (Pham and Schneider, 2008).

Evidently, there is need for an approach that treats the mechanisms of innate immunity as a black box and makes no prior assumptions about its capabilities, because this has enabled the identification of new immune phenomena (Little et al., 2005). However, the gap between mechanistic and ecological models of innate immunity still stands and empirical evidence is needed to prove that the specificity and priming found in these host-parasite systems is generated by the immune response. That is to say that evidence must be given that cannot be attributed to anything other than an active response on behalf of the host. This issue is addressed by firstly reviewing the main features of innate immunity using examples from *Drosophila* and the mosquito, *Anopheles gambiae* to highlight more recent advances in our understanding.

1.2. Invertebrate innate immunity

Once a host is infected by a parasite, humoral and cell-mediated immune responses need to be deployed to control or eradicate the pathogen. These responses are classically defined by mechanistic studies as either adaptive or innate. Historically, adaptive immunity is only recognised in the vertebrates and through recombination-activating genes (RAG) and the actions of T and B lymphocytes (specialised immune cells) generates both specificity and immunological memory after initial exposure to a novel pathogen (Kurtz, 2004). Here, specificity enhances immune-recognition and the speed and strength of the immune response towards the novel pathogen. Specificity is represented by molecules such as antibodies and memory T cells that persist beyond the duration of the initial infection and immune response, in effect creating an immunological memory. In this form, the host is conferred with long-lasting protection to the pathogen since secondary infection will be met by an enhanced anticipatory immune response (Kurtz, 2004). By comparison, innate immunity is considered non-adaptive, with low-level specificity to broad pathogen classes such as fungi, and gram positive or negative bacteria (Hauton and Smith, 2007). Innate defences are the ancestral form of immunity and conserved in all plants and animals. Many vertebrate homologues of innate immunity have been identified in invertebrates, but similar conservation of adaptive immune components is not found (Hoffmann et al., 1999) and invertebrate immunity is exclusively innate. However, although relatively much simpler than adaptive immunity, innate immunity is still an effective defence against infection (Figure 1.3).

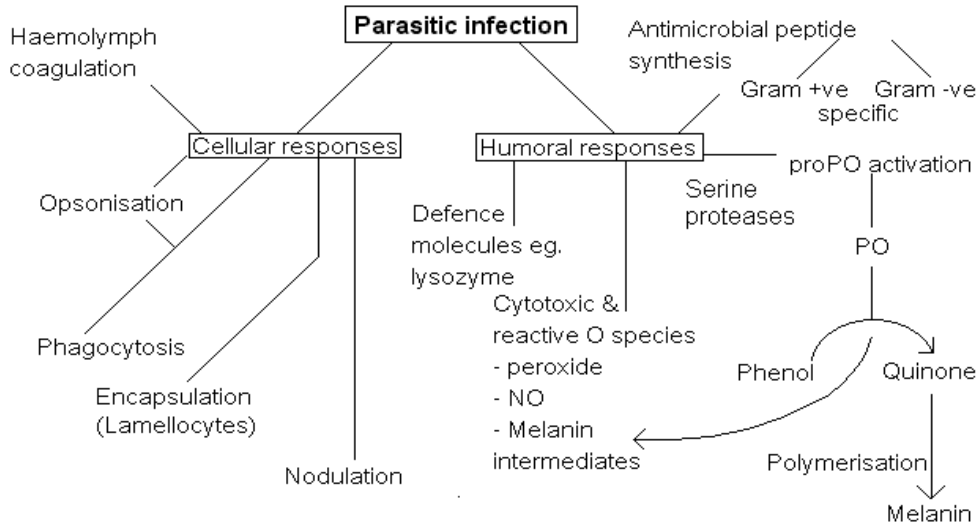

Figure 1.3. Main features of invertebrate innate immune response. Adapted from text: (Schmid-Hempel, 2005a)

Innate immunity has both cellular and humoral arms (Figure 1.3) that can be deployed systemically, in the haemolymph (equivalent to the blood system), or locally at barrier epithelia such as the gut or the malpigian tubules. Insects have an open circulation system that allows immune molecules and phagocytic cells to rapidly move to the site of infection during a systemic immune response. The insect fat-body, equivalent to the mammalian liver, is an important tissue for the synthesis of immune molecules and these are directly secreted into the haemolymph. The systemic immune response is activated after cuticular wounding or damage to barrier epithelia, but local responses at these sites are equally vital to try to prevent initial infection. Barrier epithelia, such as those lining the trachea, reproductive system and gut, frequently encounter pathogens and are equipped with humoral defences, and may signal to other tissues to initiate further responses.

Some defences are constitutive and rapidly activated upon infection, such as the prophenoloxidase system and phagocytosis. However, the synthesis of a vast number of

immune molecules is only induced after infection and therefore forms part of a slow-acting response. All immune defences have three basic elements: pathogen recognition, signalling via molecular pathways and enzyme cascades to modulate and amplify the response, and an effector that acts against the pathogen. The following section provides an overview of innate immune defence in insects since this group is of greatest relevance to my research.

1.2.1. Humoral effectors

Humoral immune responses form an important part of immunity and use constitutive and inducible elements. A substantial number of molecules are often found to be up-regulated during an infection and particular arrays of genes can be associated with specific pathogens (Dimopoulos, 2002, Ursic-Bedoya and Lowenberger, 2007) and immune tissues (Dimopoulos et al., 1998). These genes may be related to recognition, signalling or effectors, or have indirect roles in the response and it is clear that these gene expression is under tight transcriptional control and regulated by complex signalling pathways (Dimopoulos, 2002). The main elements of humoral immune defence and their regulation are now discussed.

Anti-microbial peptides

AMPs are ancient defence molecules that are widespread throughout plants (Broekaert et al., 1995) and animals (Zasloff, 2002), and form a major part of the innate immune response. They are active against broad classes of pathogen such as fungi and gram positive and negative bacteria. In excess of 170 insect AMPs have been discovered (Bulet et al., 1999) with as many as 20 AMPs utilised in *Drosophila* alone (Lemaitre

and Hoffmann, 2007). AMPs are small, cationic molecules, each with distinct properties, and are highly effective at controlling microbial infections. Some, such as the defensins, are highly conserved, reflecting their importance in innate immunity (Bulet et al., 1999). During the systemic immune response, AMPs are synthesised and secreted into the haemolymph from the fat body (Hoffmann and Reichhart, 2002), haemocytes (Dimarcq et al., 1997) and gut (Tzou et al., 2000, Boulanger et al., 2002b, Lehane et al., 1997). In *Drosophila*, restoring expression of a single AMP in an immune-compromised mutant fly is sufficient to rescue the phenotype and confer resistance to some pathogens (Tzou et al., 2002). Structurally, the AMPs fall into three major groups that delineate whether they primarily target gram-positive bacteria, gram-negative bacteria or fungi. Some, such as the cecropins and defensins, are broad-acting while others act specifically against a pathogen class, for example attacin (Bulet et al., 1999). Wounding alone can initiate non-specific AMP induction, but this is transient and moderate compared to septic injury where specific AMP repertoires are induced in response to particular assaults (Lemaitre, 1997).

AMPs disrupt the function and proliferation of bacteria and fungi, leading to cell death. The actual mechanisms of antimicrobial activity are poorly characterised but it is clear that many AMPs can increase the permeability of the cell membrane (Lehrer et al., 1989) by creating pores (Yang et al., 2000) or ion channels (Brogden, 2005) to cause ionic imbalances in the cell and reduce the activity of its biosynthetic pathways. Similar mechanisms may also be effective against eukaryotic cells since loss of membrane integrity has been found in trypanosomes after treatment with AMPs (McGwire et al., 2003). Some AMPs also appear to shut down cells by crossing the cell membrane and inhibiting intracellular targets (Kragol et al., 2001, Brogden, 2005). Indirect evidence

for an anti-parasitic role of AMPs has been found towards flagellate protozoa such as *Leishmania major* (Boulanger et al., 2004) and *Plasmodium berghei* (Dimopoulos et al., 1997). Shahabuddin et al. (1998) showed that injecting mosquitoes, *Aedes aegypti*, with Defensin after *per os Plasmodium* infection reduced the number of parasite oocysts (spores) successfully surviving in the haemolymph of their host (Shahabuddin et al., 1998).

The dynamics of AMP expression vary considerably according to factors such as the type of infecting pathogen, the AMP being analysed, the route of infection and dose-dependent effects. In *Drosophila,* AMPs can appear in the haemolymph as early as 1h after septic injury and peak after three to 24h (Lemaitre, 1997) but transcriptional expression has been found to be prolonged for as long as one week after infection, and peptides can circulate in the haemolymph for two to three weeks (Uttenweiler-Joseph et al., 1998). Stimulating the insect immune response with a natural pathogen or infection route alters AMP induction and expression kinetics quite dramatically. Most insects will normally encounter pathogens and micro-parasites through activities such as foraging, feeding and grooming and not via cuticular wounding, yet a systemic response is still frequently under these circumstances (Boulanger et al., 2001). For example, *Drosophila* naturally ingests bacteria while eating rotten fruit, including the bacteria *Erwinia carotovora,* which develops in the fly's gut and occasionally invades the haemolymph (Basset et al., 2000). Systemic expression of the AMP Drosomycin was three times lower when *E.carotovora* was fed to *Drosophila* larvae than when injected directly into the haemolymph (Basset et al., 2000). Similarly, a 490-fold increase of Defensin after intra-thoracic injection of *E.carotovora* suggests that *P.duboscqi* reacts aggressively to the pathogen, but natural *per os* infection induces a smaller, though still significant, 32-

fold increase (Boulanger et al., 2004). The dynamics of AMP expression during natural infection correlate with specific infection phases for example, when the mid-gut epithelium is breached or when the parasite matures to its next developmental stage. AMP expression in the sandfly, *Phlebotomus duboscqi* does not appear in response to *Leishmania major* infection until day 10 when the parasite has developed into its insect-specific promastigote form and is in abundance in the fly's gut (Boulanger et al., 2004). The regulation and induction of AMP expression falls under the control of two major signalling pathways that are discussed in a subsequent section.

The prophenoloxidase (proPO) system

Dark pigmentation often forms at the site of cuticular wounding or on parasites that invade the haemolymph and is caused by the melanisation reaction (Söderhäll and Cerenius, 1998). Deposition of melanin creates a physical barrier to prevent further infection at a wound site and also kills pathogens that are too large to be removed by phagocytosis (Gillespie et al., 1997, Cerenius et al., 2008). Melanisation is a rapid, non-specific reaction caused by activation of the constitutive prophenoloxidase (proPO) system, and can increase host susceptibility if inhibited during infection (Volz et al., 2006). Phenoloxidase (PO) catalyses the oxidation of phenols into quinones that then polymerise to melanin (Söderhäll and Cerenius, 1998). Related molecules, such as the oxygen carrier, haemocyanin, may also have PO properties (Cerenius and Soderhall, 2004). Melanin and its intermediates, such as reactive oxygen species (ROS), semi quinones and quinone methides, all have cytotoxic properties (Nappi and Ottaviani, 2000, Söderhäll and Cerenius, 1998) and activation of PO is tightly controlled in the absence of infection to prevent inappropriate activation. PO is synthesised as an inactive pro-enzyme (proPO) by subsets of haemocytes. In *Drosophila* larvae, crystal cells

rupture during an immune response and release proPO into the haemolymph for activation (Bidla et al., 2007). Regulation of cell rupture is directly controlled by signalling pathways and not via transcriptional responses (Bidla et al., 2007), providing an instant source of proPO during immune invasion. Similar mechanisms are predicted in other insects (Cerenius and Soderhall, 2004). Proteolytic cleavage of ProPO into its active form is carried out by proPO-activating-enzyme (PPAE) that is itself activated in the terminal step of a serine protease cascade initiated after pathogen detection (Söderhäll and Cerenius, 1998). In *Drosophila,* serine-protease inhibitors (Serpins) prevent excessive or prolonged activity of the cascade to limit host self-damage and must be degraded to initiate the pathway (De Gregorio et al., 2002, Ligoxygakis et al., 2002b).

1.2.2. Cellular effectors

Insect haemocytes (blood cells) exist in both free-floating and sessile forms in the haemolymph and perform a variety of immune functions during infection (Lanot et al., 2001). In *Drosophila,* haemocyte differentiation and proliferation occurs in the lymph gland during larval haematopoiesis but this tissue disintegrates prior to pupation, and haematopoietic tissue has not yet been found in adult flies (Lanot et al., 2001). It is therefore assumed that the adult haemocyte population is finite. 95% of haemocytes are granulocytes (Lepidoptera) or plasmatocytes (Diptera) (Ribeiro and Brehelin, 2006), which carry out phagocytosis, engulfing microbes and small particles into an internal phagolysosome where digestive and lytic enzymes are released to kill the pathogen (Stuart and Ezekowitz, 2008). The response is initiated by receptor-bound molecules on the phagocyte but can be enhanced by signalling pathways (Agaisse and Perrimon, 2004) and circulating pathogen-binding molecules such as opsonins and other

complement-like proteins (Boman and Hultmark, 1987). Larger organisms, such as parasitoid eggs and synthetic latex beads, trigger the encapsulation response which activates large, flat haemocytes known as lamellocytes (Diptera) to adhere to the parasite, forming a capsule formed from layers of cells. Encapsulation frequently occurs alongside crystal cell rupture to melanise and eventually kill the parasite (Carton et al., 2008). In *Drosophila* larvae, lamellocytes only differentiate from prohaemocytes after infection and are absent in pupae and adults, as are crystal cells (Lanot et al., 2001). In addition to synthesising proPO, haemocytes synthesise a number of proteins required in clot formation (coagulation) and wound healing (Ratcliffe et al., 1984), as well as antimicrobial peptides (Dimarcq et al., 1997) and opsonins that enhance haemocyte binding.

1.2.3. Conserved signalling pathways

An abundance of molecules are expressed in response to infection, and the majority are regulated at the transcriptional level by signalling pathways. The evolutionarily ancient Toll and Imd (Immune-deficiency) pathways control the synthesis of many effectors by targeting mammalian – like NF-κB (nuclear-factor-κB) binding domains in the promoter region of immune genes, including the AMPs (Figure 1.4). In *Drosophila*, three members of the NF-κB-like inducible transactivator family of Rel proteins, Dorsal, Dif (dorsal-related immune-factor) and Relish bind these sites in the terminal step of signalling. Toll regulates Dorsal and Dif activity; Imd regulates Relish. Imd and Toll have been extensively studied over the last 20 years but other signalling pathways, including JNK and JAK/STAT, also play important roles in regulating and mediating the immune response.

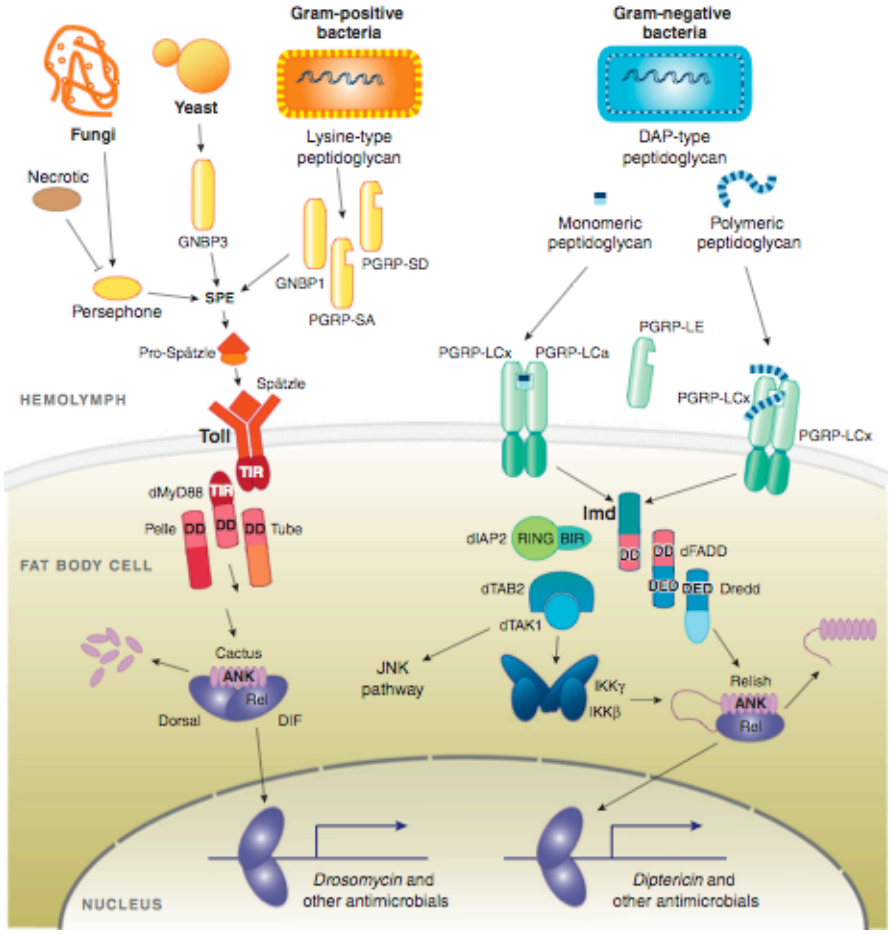

Figure 1.4 The Toll and Imd pathways in *Drosophila*, from (Lemaitre and Hoffmann, 2007). Pro-Spätzle is cleaved after pathogen detection, and the activated ligand binds the Toll receptor, initiating a cascade of intracellular interactions to mediate degradation of the inhibitor Cactus and translocation of Dif to the nucleus. Imd is activated intracellularly via PGRP, a pathogen-recognition receptor, and terminates in a) the cleavage of the Rel protein Relish to permit translocation into the nucleus to initiate immune gene expression and b) activation of a secondary pathway, JNK, that has roles in haemocyte activation and AMP synthesis (Lemaitre and Hoffmann, 2007).

Toll pathway

The Toll pathway was first characterised for its role in dorsal-ventral patterning of the *Drosophila* embryo (Belvin and Anderson, 1996), and many of its components share similarities to the mammalian IL-1R (Interleukin-1 receptor) signalling cascade that regulates the vertebrate inflammatory response (Khush et al., 2001). The transmembrane receptor Toll initiates signalling once bound to the extracellular cytokine-like ligand Spätzle and does not bind pathogen-associated molecular patterns (PAMPs) directly, in contrast to vertebrate Toll-like receptors (Hoffmann and Reichhart, 2002). Spätzle circulates in the haemolymph as a pro-protein and is cleaved in the terminal step of an infection-induced proteolytic enzyme cascade, before binding the Toll receptor and initiating signalling (Lemaitre and Hoffmann, 2007) (Figure 1.4). Some larval Toll components are not required for AMP induction in adults, such as Dorsal (Meng et al., 1999, Gross et al., 1996).

Imd pathway

The Imd (immune deficiency) pathway has homology to the mammalian tumour-necrosis-factor-receptor (TNFR) pathway (Hoffmann et al., 1999, Khush et al., 2001) that is used extensively in the vertebrates, specifically in adaptive immunity to co-ordinate lymphocyte responses and regulate cell death (Locksley et al., 2001). The Imd pathway is entirely intracellular and activated by signalling from the membrane-bound PGRP-LC receptor, a pathogen recognition protein (Figure 1.4). Mutations in the *Drosophila imd* gene confer susceptibility to gram-negative bacterial infection but not fungi, which correlates with the Imd-independent expression of the anti-fungal AMP Drosomycin (Lemaitre et al., 1995). Secondary to its targeted activation of Relish,

bifurcation of the Imd pathway at TAK1 activates the JNK (c-Jun N-terminal kinase) signalling cascade (Silverman, 2003).

Immune-gene induction is complex and multi-layered, since it involves many pathways and their differential stimulation by specific pathogens. However, clear trends in AMP expression are found that correspond with Toll or Imd activation, or a specific class of pathogen. Imd pathway activation alone is adequate for Diptericin expression, (Meng et al., 1999, Hedengren et al., 1999) whereas the anti-fungal Drosomycin is Toll-dependent, and unaffected by mutation of the *imd* path (Lemaitre, 1997). Flies with Toll pathway mutations are susceptible to fungal and gram-positive infections but remain resistant to gram-negative infections (Lemaitre et al., 1996, Rutschmann et al., 2002). It is likely that there is cross-talk between Toll and Imd while other factors contribute to regulating spatial expression as is apparent with the AMPs (Uvell and Engström, 2007). Full induction of the antibacterial Cecropin, and to some extent Defensin and Attacin require activation of both Imd and Toll; mutations in either pathway reduce or eradicate expression (Lemaitre et al., 1996, Lemaitre et al., 1995, Tanji et al., 2007).

JNK pathway

Aside from its major developmental roles JNK (c-Jun N-terminal kinase) is a classic stress-signalling pathway activated in response to multiple factors such as LPS (a bacterial cell-wall constituent) and septic injury with bacteria (Boutros et al., 2002), as well as through signalling molecules such as the endogenous TNF (tumour necrosis factor)-cytokine-homologue, Eiger (Igaki et al., 2002). Eiger mutants are susceptible to extra-cellular bacterial infections (Schneider et al., 2007). Many *Drosophila* JNK components have mammalian homologues (Goberdhan and Wilson, 1998). The

pathway mediates cytoskeletal processes (Boutros et al., 2002), wound closure (Ramet, 2002), NF-kB-independent AMP synthesis (Kallio et al., 2005) and the release of prophenoloxidase from crystal cells (Bidla et al., 2007).

JAK/STAT pathway

The JAK/STAT (Janus kinase/Signal transducer and activator of transcription) pathway mediates cytokine signal transduction and in mammals, has connections with immune signalling (Shuai and Liu, 2003). In *Drosophila*, the path is composed of the ligand - Unpaired (Upd), the cytokine receptor - Domeless (Dome), JAK (Janus Kinase) enzyme - Hopscotch (Hop), and STAT transcription factor (STAT92E/Marelle) (Agaisse and Perrimon, 2004). Nuclear localisation of STAT in fat-body cells occurs after septic-injury in the mosquito *Anopheles gambiae* (Barillas-Mury et al., 1999) and in *Drosophila* can be mediated by inputs from Imd pathway signalling (Agaisse et al., 2003). JAK/STAT targets are implicated in humoral (haemolymph-based) and cellular immunity (Agaisse and Perrimon, 2004), and signalling between haemocytes and tissues may also be mediated by JAK/STAT (Agaisse et al., 2003). The exact role of JAK/STAT in immune responses is uncertain as mutants show wild-type resistance to infection (Lemaitre and Hoffmann, 2007).

1.2.4. Pathogen recognition

Innate immune recognition is carried out by pattern recognition receptors (PRRs) that target conserved pathogen associated molecular patterns (PAMPs) on the infecting organism. These are molecules or structural motifs that are unique to pathogens and are not expressed on host self cells. Typically, the PAMPs are highly conserved structures

since they are essential to pathogen survival and cannot rapidly evolve to evade detection. What is more, PAMPs can be used by innate immunity to differentiate between different classes of pathogen such as fungi and bacteria, giving the response a degree of specificity (Medzhitov and Janeway, 2000). The PRRs can circulate in the haemolymph, or be cell-based as surface or intracellular receptors, and activate a range of responses including phagocytosis, gene induction, enzymatic responses and opsonisation of pathogens.

Recognition of gram-positive bacteria

Gram-positive bacteria possess lysine peptidoglycan (Lys-PGN) that is targeted by the secreted PRRs PGRP-SA (peptidoglycan receptor protein – SA), PGRP-SD and GNBP-1 (gram-negative-binding protein-1) (Ferrandon et al., 2007). GNBP-1 can also be membrane-bound (Kim, 2000). GNBP-1 degrades Lys-PGN for detection by PGRP-SA (Filipe et al., 2005) and the two PRRs act synergistically (Gobert, 2003, Bischoff et al., 2004). For example, GNBP-1 interaction with PGRP-SA is enhanced in the presence of peptidoglycan (Wang et al., 2006). PRR activation triggers a proteolytic cascade ending in the cleavage and activation of Spätzle, the ligand for Toll pathway induction (Lemaitre and Hoffmann, 2007) although there is evidence that other pathways utilise PGRP-SA and GNBP-1 for their activation. For example, fly mortality and *in vivo* bacterial growth from *Staphylococcus aureus* infection is greater in PGRP-SD mutants than Toll pathway (Dif) mutants (Bischoff et al., 2004). A single study in the beetle *Tenebrio molitor* has documented the interaction of a modular serine protease with the PGRP-SA-GNBP-1-Lys-PGN complex that activates the prophenoloxidase pathway (Park et al., 2007).

Fungal recognition

Fungi activate the Toll pathway and the expression of fungi-specific AMPs, such as Drosomycin (Lemaitre, 1997). Fungal detection is relatively less well-studied but a recent paper suggests a dual detection system is utilised in *Drosophila* (Gottar et al., 2006). Recognition is mediated through GNBP3, which is conserved amongst insects (Jiang et al., 2004, Ochiai and Ashida, 1988) and has greatest binding affinity for β-(1,3) glucans, a cell wall constituent of fungi (Lemaitre and Hoffmann, 2007). Binding is specific to fungi (Gottar et al., 2006) and whilst GNBP3 works independently of PGRP-SA and GNBP-1 it still converges onto the proteolytic cascade upstream of the Toll pathway (Lemaitre and Hoffmann, 2007). A second detection system operates in parallel to GNBP3 in *Drosophila* that exploits the activity of serine proteases secreted by entomopathogenic fungi during infection. The fungal serine protease activates, Persephone (Psh), another serine protease that circulates in the haemolymph, which consequently initiates cleavage of Spätzle and Toll signalling (Ligoxygakis et al., 2002a). Transgenic flies expressing fungal serine proteases have high levels of Drosomycin that is abolished in Psh mutants (Gottar et al., 2006). Another PGRP-like molecule that also binds β-(1,3) glucans can initiate prophenoloxidase activation in *Holotrichia diomphalia*, suggesting additional pathways and effectors can be involved the anti-fungal response (Lee, 2003).

Gram-negative bacterial detection

Gram-negative bacteria and some gram-positive bacteria possess diaminopimelic acid peptidoglycan (DAP-PGN). It is detected in its intact form or as the DAP-PGN fragment, tracheal cytotoxin (TCT) by PGRP-LE and PGRP-LC (Leulier et al., 2003, Stenbak et al., 2004, Kaneko et al., 2004). Injection of purified DAP-PGN, gram-

negative *Escherichia coli* or gram-positive *Bacillus subtilis* induces expression of Diptericin, an AMP tightly regulated by the Imd pathway (Leulier et al., 2003). PGRP-LE and –LC act synergistically to detect gram-negative bacteria but PGRP-LC is indispensable for Imd pathway activation (Gottar et al., 2002). Alternative splicing generates three isoforms of PGRP-LC (LCx, LCy and LCa) with distinct extra-cellular domains in an otherwise identical protein, permitting PGRP-LC isoform dimerisation (Werner, 2003). PGRP-LCx homodimer detect DAP-PGN while PGRP-LCx-PGRP-LCa heterodimers detect TCT (Kaneko et al., 2004) and dimerisation is enhanced in the presence of DAP-PGN (Mellroth et al., 2005). Similar to the mammalian PRR CD14, PGRP-LE functions both intra-and extra-cellularly to detect DAP-PGN (Kaneko et al., 2006). Synergistic interactions between PRGP-LE and PGRP-LC's enhances DAP-PGN detection (Takehana et al., 2002). *In vitro*, PGRP-LE may facilitate the delivery of monomeric TCT to PGRP-LC at the cell surface, whereas intracellular PGRP-LE detects TCT that has crossed the cell membrane (Kaneko et al., 2006). Imd signaling induces synthesis of the extracellular scavenger receptor PGRP-LB, which degrades DAP-PGN, and therefore provides negative feedback to modulate the immune response and prevent over-activity of the Imd pathway (Stenbak et al., 2004).

Regulation of phagocytosis

A variety of cell-surface receptors have been found to play a role in activating and regulating phagocytosis. One group are the scavenger receptors that have broad specificity and can individual receptors can bind multiple ligands such as bacteria, apoptotic cells and synthetic proteins (Krieger et al., 1993). Notable examples are the *Drosophila* class B scavenger receptors, Croquemort and Peste. Croquemort mediates phagocytic clearance of apoptotic cells in larvae but targets pathogens in adult flies and

is homologous to the CD36 receptor on mammalian macrophages (Stuart and Ezekowitz, 2008). Reducing Peste expression *in vitro* in *Drosophila* cells prevents uptake of mycobacteria and this specificity is preserved in the mammalian homologue (Philips et al., 2005). Receptors containing EGF (epidermal-growth-factor) – like repeats also play a major role in phagocytosis. *Drosophila* haemocytes express the EGF-containing receptor Eater that has a highly variable pathogen-binding region able to recognise multiple ligands. Mutant flies and cell lines lacking Eater expression have impaired phagocytic activity toward both gram positive and negative bacteria (Kocks et al., 2005). Circulating opsonins can enhance phagocytosis further. In vertebrates, complement proteins bind (opsonise) PAMPs to label the item as foreign and speed up its recognition and removal by haemocytes. A number of proteins with complement-like opsonin activity are apparent in invertebrates. These include the immunoglobulin-containing protein Dscam (Watson et al., 2005), lectins that recognise particular carbohydrate residues on pathogens (Wormald and Sharon, 2004) and the thioester-containing proteins (TEPs) that have similarity to mammalian alpha-2 macroglobulin and complement protein C3. TEPs have recently been identified in *Drosophila* and *Anopheles* where there are six and nineteen TEPs respectively (Blandin and Levashina, 2004). Some mosquito TEPs show a high degree of polymorphism that is thought to be reflective of co-evolution with parasites (Obbard et al., 2008). Synthesis and secretion of TEP from haemocytes can be up-regulated in response to bacterial (Lagueux, 2000) and *Plasmodium* (protozoan) infection (Blandin and Levashina, 2004). Similar to membrane-bound receptors, binding can be highly specific since different TEP forms can bind and enhance the phagocytosis of particular bacteria, for example differentiating between *E.coli* (gram-negative bacteria) and *S.aureus* (gram-positive bacteria) (Stroschein-Stevenson et al., 2006). In some invertebrates TEP seems to play a

critical role in resistance to infection as knock-down of TEP expression can cause refractory *Anopheles* mosquitoes to become susceptible to *Plasmodium berghei* infection (Blandin et al., 2004). It is therefore clear that a number of proteins can regulate phagocytosis with varying degrees of specificity.

In conclusion, a variety of immune defences can be activated in response to infection, but their capacity to differentiate between pathogens appears crude and at best responds with low-level specificity to broad pathogen classes such as fungi or gram positive/negative bacteria. None of these defences appear to generate highly specific immune responses. Therefore a search began for a molecular basis to ecological immune phenomena using the model host-parasite system of bumblebees (*Bombus terrestris* and their parasite *Crithidia bombi* that is introduced in the next section.

1.3. The bumblebee-*Crithidia bombi* host-parasite system

Bumblebees (*Bombus spp*.) are annual, eusocial insects of temperate climates and are host to a variety of parasites. In particular, the interaction between *Bombus* and its trypanosome *Crithidia bombi* (Trypanosomatidae, Zoomastigophorea, Lipa and Triggiani, 1988 (Lipa and Triggiani, 1988)) has been well studied since the parasite is highly prevalent in host populations (up to 80%) (Shykoff and Schmid-Hempel, 1991b) and affects various stages of the bumblebee's colony life cycle (Schmid-Hempel, 2001). The typical seasonal cycle starts for bumblebees when a mated queen emerges from hibernation in spring and founds a new colony. The queen produces sterile female workers that will forage and tend to the worker eggs she continually lays. The colony's worker population continues to grow until mid-summer when the queen switches to producing reproductive sexuals i.e. queens/gynes (females) and drones (males) that

leave the nest to mate. Inseminated queens then go into hibernation until the following spring whereas males and the rest of the colony die at the end of the summer (Schmid-Hempel, 2001).

C.bombi is a flagellated parasite that attaches to the bee's mid and hindgut. Controlled experiments show that the parasite rapidly multiples and begins to release infective cells into the faeces 2-3 days after infecting a host (Schmid-Hempel and Schmid-Hempel, 1993). Cell numbers then typically show a steady increase for 7-10 days before reaching a plateau phase (see Figure 1.5) (Schmid-Hempel and Schmid-Hempel, 1993). In naturally infected bees, i.e. those that have acquired infection in the natal colony, *C.bombi* infection lasts for at least 2 weeks. During its decline, faecal cell numbers can oscillate and vary considerably between individual bees (Otterstatter and Thomson, 2006).

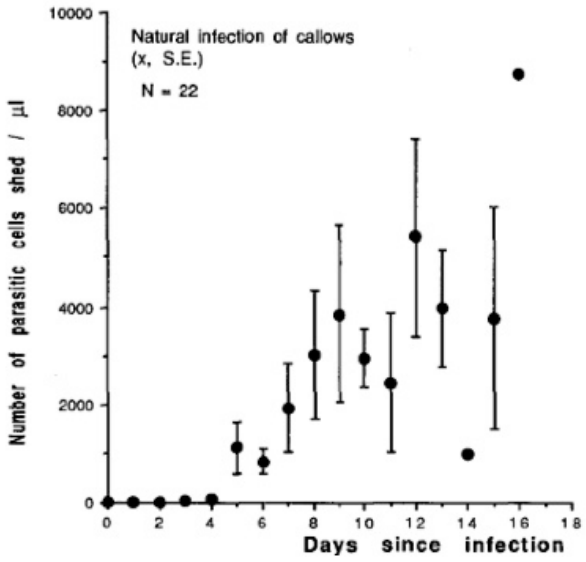

Figure 1.5 From (Schmid-Hempel and Schmid-Hempel, 1993). Average +/-SE number of *C.bombi* shed each day after infection. Cell numbers appear in measurable numbers after 2-3 days (small number, not visible on graph) and steadily rise until 7-10 days where numbers level off.

Little is known about *C.bombi*, but studies have suggested that they are diploid and reproduce clonally (Schmid-Hempel, 2001). Sexual reproduction is likely to be highly infrequent and rare (Schmid-Hempel, 2001, Schmid-Hempel and Funk, 2004). Unlike other *Crithidia* species *C.bombi* does not harbour endosymbionts (Bouquet, 2004). The parasite is transmitted when non-infected host ingests parasite cells from contaminated material such as nectar, pollen or nesting materials. Within the colony infection can be horizontal, between nest mates, but can also occur vertically from a queen who contracted *C.bombi* infection in her natal colony (Schmid-Hempel, 2001). Infection passes efficiently between colonies when workers feed at flowers that have been visited by infected bees (Durrer and Schmid-Hempel, 1994) and the rate of inter-colony transmission appears to increase as the season progresses (Imhoof and Schmid-Hempel, 1999). Infection by *C.bombi* does not appear to correlate with infestations of other parasite species (Shykoff and Schmid-Hempel, 1991b, Imhoof and Schmid-Hempel, 1999). *C.bombi* has subtle effects on the colony cycle of its host under favourable conditions (i.e., adequate food and good climatic conditions). For example, queens have reduced success in colony founding (Brown et al., 2003b), colonies have smaller worker populations and produce fewer sexual offspring (Brown et al., 2000), and the ability to learn floral cues is impaired in infected workers (Gegear et al., 2006). Although not every individual may contract *C.bombi*, infection reduces the overall fitness of the colony (Brown et al., 2003b). The virulence (i.e., parasite induced host-death) of the parasite is condition-dependent and under stressful conditions, such as starvation, can increase host mortality by as much as 50%, revealing that the host is compensating for infection under favourable conditions (Brown et al., 2000).

This effect is reflected in the physiological changes seen during *C.bombi* infestation. In older, non-infected colonies workers can become reproductive once a queen loses her dominance and no longer releases the pheromones that suppress worker ovary development (Schmid-Hempel, 2001). In *C.bombi*-infected workers, ovaries remain under-developed for longer and worker-reproduction occurs later relative to non-infected colonies (Shykoff and Schmid-Hempel, 1991a). These changes appear to be caused by the infection-induced re-allocation of resources from the reproductive system to the fat-body (Brown et al., 2000). It is possible that reallocation of resources to this immunologically important tissue may be a host response to improve its ability to deal with and control infection during *C.bombi* infestation (Brown et al., 2000). Few studies have investigated the immune response to *C.bombi*, although it is clear that infection can increase standing levels of pro-phenoloxidase (proPO) in the haemolymph (Brown et al., 2003a) that seems to correlate positively with the intensity of infection in the gut (Otterstatter and Thomson, 2006). *C.bombi* has never been found to invade the haemolymph, and a systemic increase in haemolymph proPO suggests that the gut may be signalling to other tissues to heighten the level of immune-responsiveness in case of subsequent infections (Brown et al., 2003a) as is found in flour beetles (Moret and Siva-Jothy, 2003). Similar suggestions of signalling have been made in the response of *Drosophila* to *Crithidia* infection (Boulanger et al., 2001).

Although *C.bombi* seems to be clonal, microsatellite analysis indicates that there is a high level of diversity within populations, even when sampled from the same geographical region (Schmid-Hempel and Funk, 2004). Schmid-Hempel and Funk found that co-infection of individual *B.terrestris* workers with more two or more *C.bombi* genotypes was low (around 16%) whereas half of all colonies harboured more

than one genotype. What is more, the *C.bombi* population was highly structured: specific parasite genotypes associated with particular bumblebee genotypes (represented by individual colonies). This study strongly suggests that *C.bombi* population structure results from the specific interaction between host and parasite genotypes (Schmid-Hempel and Funk, 2004).

Studies in support of strong host-parasite genotype interactions are inferred from a number of other studies of the *Bombus*-*Crithidia* system. Firstly, transmission and infection success of *C.bombi* from a bee that has imbibed a multi-strain (genotype) inoculum is highly dependent on the genotype of the recipient (Schmid-Hempel et al., 1999). Specifically, there is differential distribution of parasite strains from the original multi-strain inoculum across the recipient genotypes. In effect, individual colonies "filter out" specific *C.bombi* strains that most successfully infect the host. Secondly, Schmid-Hempel and Schmid–Hempel find that specific *C.bombi* strains are adapted for transmission within certain time frames but infection success (cell numbers attained in new host) also depends on host genotype (Schmid-Hempel and Schmid-Hempel, 1993). Finally, Imhoof and Schmid-Hempel present evidence of local adaptation in *C.bombi* populations (Imhoof and Schmid-Hempel, 1998). In conclusion, the interaction between *Bombus* and *C.bombi* genotypes is highly specific and implies that the bumblebee's immune response is differentiating between parasite strains much more specifically than is currently thought possible in current mechanistic models of innate immunity.

Aims

The main aims of my PhD have been to:

- Address the gap in our knowledge between mechanistic and ecological perspectives on innate immunity.
- Investigate how *Bombus* responds to *C. bombi* infection
- Investigate the molecular basis of the interaction between *Critihidia bombi* and its host, *Bombus terrestris;* the *Bombus*-*Crithidia* system
- Test whether there is any proof for immune specificity and immune priming in the immune response of bees.

Thesis outline

In Chapter 2, I have outlined all the methodology. Some experimental techniques are outlined in their relevant chapter where necessary. Chapter 3 discusses my initial explorations in the *Bombus*-*C.bombi* system as I investigated the validity of the assumed link between *C.bombi* virulence and infection intensity. I confirmed that there is a positive correlation between these two factors. In Chapter 4, I present the results of my investigation into immune specificity where I found empirical evidence of specificity in the innate immune response to *C.bombi*, measured in terms of AMP expression. I next began a search for novel immune genes used in response to *C.bombi* infection. This experiment is outlined in Chapter 5, as well as an experiment I carried out looking at the temporal expression of immune genes throughout *C.bombi* infection. In Chapter 6, I outline my final experiment that studied the expression of antimicrobial peptides during immune priming to test their possible role in this phenomenon. I conclude my thesis in Chapter 7 by discussing the major findings of my experimental work and their significance to the field of evolutionary immunology.

2. Materials & Methods

2.1. *Bombus terrestris* and *Crithidia bombi*

2.1.1. Bumblebees: sources and maintenance

Parasite-free bumblebee (*Bombus terrestris*) colonies were sourced either from a commercial supplier (Koppert; Haverhill, UK) or by growing colonies on from wild caught queens collected in the University of Leicester Botanical Gardens, Oadby in the spring of 2006, 2007 and 2008. After capture, wild queens were screened for parasites (*Nosema bombi*, *Crithidia bombi* and *Sphaerularia bombi* by microscopic examination of faecal sample and mites e.g. *Parasitus fucorum* attached to the thorax) and maintained in a 8:16hlight:dark cycle, 27°C, 60% humidity on cat litter with a 50% diluted glucose/fructose apiary solution (Meliose – Roquette, France). All apiary syrup solutions contained Niapagine fungicide at (1.8g/L). Pollen mixed with 50% apiary syrup and rounded into a small ball was provided on a small Petri lid to encourage queens to lay eggs. Extra pollen was provided every other day. Once the first 5-6 workers had emerged and brood was present, the colony was transferred to colony boxes. All colonies were maintained in constant darkness under red-light conditions at 28°C and 60% humidity on a diet of 50% v/v apiary solution and pollen *ad libitum* (Percie du sert, France).

2.1.2. *C. bombi*: strains & maintenance

C. bombi strains were collected from faecal samples of wild *Bombus terrestris* queens (see 2.1.1) and maintained in the lab by weekly inoculation into naïve, disease-free bees from Koppert (UK). Each week, *C.bombi* strains were collected from infected bees by

placing individuals into a small pot before gently shaking them to encourage defecation – a natural defensive behaviour used by bumblebees. Faeces were collected with a glass micropipette and diluted in apiary syrup solution in a 1.5ml eppendorf tube. *C.bombi* cells were counted using a haemocytometer (Neubauer improved). An inoculum of 1000 cells μl^{-1} was prepared with 50% apiary syrup solution. Individual naïve bees were separated and each fed 20µl of a single *C.bombi* inoculum. After imbibing the solution, infected bees were kept in *C.bombi* strain-specific groups, separate to the colony, on 25% (v/v) apiary syrup with pollen *ad libitum* at 26°C, 60% humidity in constant red light.

All experiments were carried out on disease-free *B.terrestris* colonies once there was a minimum of 30 workers. To control for possible age effects on the immune response, newly hatched (1-2h post-eclosion) workers were collected and kept separate from the colony for 5-8 days before infection. Experimental bees were maintained under standard conditions as mentioned above.

2.2. Molecular biology - overview

2.2.1. General notes

Unless otherwise stated standard lab techniques were carried out as follows:

2.2.2. Centrifugations and incubations

All centrifugations were carried out in a Progen GenFuge 24D bench-top centrifuge at maximum speed (13,000rpm/16,000xg unless otherwise stated) for room temperature centrifugations (16-22°C) and an Eppendorf Centrifuge 5415R with a F45-24-11 fixed rotor at 11,000rpm at 4°C for all RNA centrifugations. Reactions were pipette mixed. Incubations using a thermal cycler were carried out in a 96 well Biometra T1 Thermal Cycler with the heated lid 20°C above the incubation temperature.

2.2.3. Sample storage

RNA samples and glycerol stocks of clones were stored at -80°C; DNA and plasmid preps were stored at -20°C in a non-frost-free freezer.

2.2.4. Agarose gel electrophoresis

Electrophoresis gels were prepared by dissolving 0.8 – 2% standard electrophoresis agarose (Melford, UK) in 1xTAE (EDTA; acetic acid; ddH_20) at 95°C and swirling the solution until the agarose had melted. Gels were cooled to hand-hot (approximately 65°C) before adding 1% ethidium bromide (Fisher Scientific, UK) swirling to mix, and pouring the solution into a gel tray with comb. Gels were left for 30 min to set in a fume hood. DNA and RNA were run on electrophoresis gels in 1xTAE at 80 -120V for 30 – 60min hr alongside DNA size standards (Q-Step 1; YorkBio, UK and Hyperladder 1; Bioline, UK) where appropriate. Gels were visualised on a GeneFlash (Syngene, UK) transilluminator gel doc system.

2.2.5. Antibiotic, X-gal and IPTG: stocks and working concentrations

Ampicillin and Kanamycin were stored in stock solutions of 100mg/ml at -20°C for long-term storage and at 4°C for up to one month. Ampicillin was used at a working concentration of 100μg/ml and Kanamycin at 50μg/ml concentration. X-gal, for blue/white selection, was stored as a 20mg/ml stock at -20°C and used at 40μg/ml.

2.3. Total and mRNA extractions

2.3.1. Total RNA extraction

Total RNA was sampled from the abdomens of experimental bees. Individual abdomens were ground using a mortar and pestle. During this process samples were kept frozen with liquid nitrogen to prevent RNA degradation. 100mg of ground tissue was placed in an RNase-free 1.5ml eppendorf tube before adding and gently mixing in 1ml of Tri reagent (Sigma-Aldrich, UK). Samples were left to stand at room temperature for 5 min to allow nucleoprotein complexes to dissociate from the RNA. 100μl of chloroform was added before shaking the mixture vigorously for 15sec and leaving to stand for 15 min at room temperature. The RNA-Tri reagent mix was centrifuged for 15 min, and the upper aqueous phase, containing RNA, removed to a fresh RNase-free 1.5ml eppendorf tube. 500μl of isopropanol was added to the aqueous phase, mixed, and left to stand for 10 min at room temperature to allow the RNA to precipitate out of solution. The sample was centrifuged for 10 min and the supernatant carefully aspirated off the RNA pellet. 1ml 80% ethanol was added to the tube, and centrifuged for 5 min. The supernatant was aspirated off the pellet before briefly centrifuging for 3-4sec and aspirating again. The pellet was left to air dry at room temperature for 5-10 min and re-suspended in 50μl of DEPC-treated ddH_2O. To remove residual genomic DNA, total RNA samples were

treated with DNase (Sigma-Aldrich, UK). 5µl of 10x reaction buffer (Sigma, UK), and 5µl of DNase were added to the 50µl sample, mixed and incubated at room temperature for 15 min. 5µl of stop solution (Sigma, UK) was added, mixed and heated at 70°C for 10 min to inactivate the DNase. To remove enzyme inhibitors and contaminants, total RNA samples were cleaned using an RNeasy mini kit (Qiagen, UK) according to manufacturer's instructions for RNA clean-up and eluted into 100µl of DEPC-treated ddH_2O. Total RNA was analysed by gel electrophoresis to check for degradation and quantified by spectrophotometry.

2.3.2. mRNA extraction

mRNA was extracted from total RNA samples using the GenElute mRNA Miniprep Kit (Sigma-Aldrich, UK) according to manufacturer's instructions into 100µl of elution solution. In preparation for SSH, mRNA was concentrated by addition of 1/10 volume 3M sodium acetate (NaOAc) and 2.5 volumes 96% ethanol, and thoroughly mixed. Samples were incubated for 40 min - 1 hr at -80°C and centrifuged at 4°C for 30 min at full speed (13,200 rpm). The precipitation supernatant was carefully aspirated off the pellet. 1ml 80% ethanol was added over the pellet and centrifuged for 10 min at 4°C and maximum speed (15,000rpm). All traces of ethanol were removed from the mRNA pellet by double aspiration. The supernatant was carefully aspirated off the pellet and briefly re-spun for 3-4 sec, and aspirated again to remove all traces of ethanol. The pellet was left to air dry at room temperature for 5-10 min before re-suspension in 20µl of DEPC-treated ddH_2O. 2µl was used to quantify the mRNA (Section 2.3.3).

2.3.3. RNA quantification

RNA was quantified in a spectrophotometer by measuring the absorbance at 260nm (OD_{260}). Samples were diluted (total RNA; mRNA 1:100) in 200µl ddH_2O and calibrated to ultra-pure ddH_2O.

2.4. cDNA synthesis

2.4.1. First-strand synthesis

First strand synthesis was carried out using 2µg of RNA. 1µl of oligo dT_{23} primer (1µg/µl) was added to 2µg of RNA in a total volume of 15µl DEPC-treated ddH_2O, and heated at 70°C for 5 min to melt secondary structures. The primer-RNA mix was immediately placed on ice and left to cool for 5 min and spun briefly for 1-2sec in a Spectrafuge mini centrifuge (Labnet, UK). 5µl of 5x reaction buffer (Promega, UK), 1.25µl of 10mM dNTP and 1µl (200 units) of Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) (Promega, UK) were added to the RNA-primer mix. DEPC-treated ddH_2O was added to a total volume of 25µl and mixed carefully. The reaction was incubated at 42°C for 60 min then placed on ice.

2.4.2. Second strand cDNA synthesis

Second strand cDNA synthesis was carried out according to the PCR-select cDNA subtraction kit (Clontech, UK) protocol. 8µl of ligase buffer (NEB, UK), 8µl of 10x salt solution (500mM NaCl, 100mM $(NH_4)_2SO_4$, 1M KCl) and 1.6µl of 10mM dNTP were added to the first strand reaction. A 20x enzyme cocktail of DNA polymerase I (6 units/µl), RHase H (0.25 units/µl) and *E.coli* DNA ligase (1.2 units/µl) was prepared in ligase buffer. All enzymes and buffers were sourced from NEB, UK. 4µl of the

enzyme cocktail was added and 33.4µl of ddH_2O to a final volume of 80µl. The reaction was carefully mixed and incubated for 2hin a thermalcycler at 16°C. 2µl of T4 DNA polymerase was added, mixed and incubated for a further 30 min. 4µl of 20X EDTA/glycogen mix (0.2M EDTA; 1mg/ml glycogen) was added and mixed, to stop the reaction.

Cleaning the cDNA

cDNA was cleaned by phenol:chloroform extraction. 100µl of phenol:chloroform: isoamyl alcohol (25:24:1) was added to the cDNA, mixed and centrifuged for 10 min. The top aqueous layer was removed to a fresh 1.5ml eppendorf tube. An equal volume of water was added to the spent phenol solution, vortexed and centrifuged for 10 min to optimise DNA recovery. The upper aqueous layer was collected and pooled with the first aqueous sample. An equal volume of chloroform:isoamyl alcohol (24:1) was added, vortexed and spun for 10 min. The upper aqueous layer was removed to a fresh 1.5ml eppendorf tube. 1/10 volume 3M sodium acetate (NaOAc) and 2.5 volumes 96% ethanol was added and thoroughly mixed to precipitate the cDNA. Samples were centrifuged for 20 min at room temperature to pellet the cDNA and the supernatant was removed. 1ml 80% ethanol was added and centrifuged for 10 min. All traces of ethanol were removed from the cDNA pellet by double aspiration (see above) and air-drying the pellet for 5-10 min at room temperature. The pellet was re-suspended in 50µl of double distilled autoclaved H_2O (ddH_2O).

2.5. SSH-specific procedures

2.5.1. RsaI digestion

cDNA of non-infected, infected and control (provided by kit manufacturer) samples was digested with RsaI to generate shorter, blunt-ended fragments suitable for subtractive hybridisation and produce blunt ends for ligation of adaptors respectively. RsaI endonuclease and buffers were sourced from NEB, UK. 2.3µl of ddH_2O, 41µl of double-stranded cDNA, 5µl of 10x buffer and 0.2µl of acetylated BSA were mixed well before adding 1.5µl of RsaI (10U/µl) in a total volume of 50µl, and was incubated at 37°C for 1 hr 30 min in a thermal cycler. A 5µl aliquot of this digest was analysed on a 1.2% agarose gel, and compared with un-digested DNA. The former had a smaller average size, and had different bands, which indicated that digestion was successful. After stopping the reaction with 2.5µl of EDTA/Glycogen mix, the cDNA was cleaned (Section 2.1.12.1) above and re-suspended in 5.5µl of ddH_2O in preparation for adaptor ligation. Aliquots of these digests would be used later as driver cDNA during subtractive hybridisation whereas another aliquot of digest was ligated with adaptors (Section 2.6), forming the tester cDNA.

2.5.2. Adaptor ligation

Adaptors were ligated to tester (infected and non-infected) cDNA to permit the amplification of differentially expressed sequences during PCR. Adaptors were not ligated to driver cDNA as it is purely used to sequester homologous sequences from the tester sample and prevent their amplification during PCR. 1µl of Rsa-digested tester cDNA was diluted with 5µl of ddH_2O. The manufacturer's control was prepared for ligation by adding HaeIII digest to the control tester cDNA sample to simulate up-

regulated transcripts. 5µl of HaeIII digest (150ng/ml) was mixed with 1µl of control skeletal muscle cDNA. Half of each diluted tester cDNA sample (2µl) was ligated to adaptor-1 and the other to adaptor-2R. 2µl of adaptor (10µM), 2µl of 5x ligation buffer (NEB, UK) and 3µl of ddH_2O were added to 2µl of dilute tester cDNA to a final volume of 10µl, mixed carefully and briefly spun (1-2 sec). An additional mix was made for each tester group, combining 2µl from each adaptor (adaptor-1 and 2R) reaction mix, collectively forming the unsubtracted tester controls as a positive control for ligation and a negative control for subtraction. All reactions were incubated at 16°C overnight in a thermal-cycler. To inactivate the ligase 1µl of EDTA was added, carefully mixed and heated at 72°C for 5 min. After briefly spinning the reaction, 1µl was removed from each unsubtracted tester control reaction and diluted in 1ml of ddH_2O for testing during suppression PCR.

2.5.3. Ligation analysis

To check that adaptor ligation had occurred on at least 25% of the cDNA strands, ligation analysis was carried out. In essence, two PCRs were performed on each ligation reaction; one using a housekeeping primer plus an adaptor-specific primer and a second using both house-keeping primers. The bands from these two PCRs should be of similar intensity, indicating that adaptors have been ligated to a high proportion of sequences, and if the intensity differs by greater than 4-fold the ligation was very inefficient and needs repeating. PCR was carried out on the adaptor-1 and adaptor-2R-ligated samples from infected and non-infected testers using the actin housekeeping gene (provided by Sally Adams, University of Leicester, UK) whereas the manufacturer supplied primers for testing the control. 25µl of PCR mix containing 12.5µl of 2x cDNA polymerase mix (Clontech, USA), 9.5µl of ddH_2O and 1µl of each primer (10µM) was added to 1µl of

tester cDNA, mixed well and incubated at 75°C to extend the adaptors before commencing PCR for 20 cycles of the following protocol: 95°C, 30 min, 59°C, 30 min, 72°C, 1 min. 5µl of PCR products were run on a 1.2% agarose gel.

2.5.4. Hybridisation

Hybridisation 1

Each tester population was denatured and mixed with excess driver to allow sequences homologous to the two samples to hybridise, thus enriching for differentially expressed genes that remain as single-stranded cDNA (ss cDNA) molecules. In a second round of hybridisation, adaptor-1-ligated and adaptor-2R-ligated tester cDNA was now mixed together with fresh driver cDNA to allow the ss cDNA molecules to hybridise to their homologous sequence in the other adaptor-ligated sample. Three separate subtractions could now take place (1) using infected bee cDNA as tester and non-infected cDNA as driver to identify genes up-regulated during infection (2) using non-infected bee cDNA as tester and infected cDNA as driver effectively identifying genes down-regulated during infection and (3) the control subtraction supplied by the SSH kit manufacturer (Clontech, UK). For both control and experimental samples, 1.5µl of RsaI-digested driver cDNA was mixed with either 1.5 µl of adaptor-1 or 1.5µl of adaptor-2R-ligated tester cDNA and 1µl of 4x hybridisation buffer, before laying a drop of mineral oil over the sample to prevent evaporation. Samples were incubated in the thermal cycler at 98°C to denature the cDNA before reducing the temperature to 68°C for 8h to facilitate hybridisation.

2.5.5. Hybridisation 2

To facilitate the hybridisation between ss cDNA molecules from adaptor-1-ligated and adaptor-2R-ligated tester samples, the two were mixed in the presence of fresh, denatured driver cDNA to further enrich for these differentially expressed sequences. In preparation, 1µl of driver cDNA was mixed with 1µl of 4x hybridisation buffer and 2µl of ddH_2O overlaid with a drop of mineral oil and heated at 98°C in a thermal cycler to denature the molecules. The entire adaptor-2R-ligated tester sample was then drawn up into a 20µl pipette tip before drawing up the driver cDNA, leaving a small air space between the two samples. The samples were then transferred into the adaptor-1-ligated tester cDNA tube, and gently mixed before incubating the mix overnight at 68°C in the thermal cycler to allow hybridisation of ss cDNA from each tester population. Thus, only differentially expressed sequences acquire two different adaptors (one from each DNA strand). After incubation, 200µl of dilution buffer (Clontech, UK) was added to each subtraction (forward, reverse and control), mixed and heated at 68°C for 7 min in the thermal cycler.

2.5.6. Suppression PCR

Suppression PCR selectively targets differentially expressed sequences for amplification, as these molecules possess different adaptors at each end of the sequence to permit their exponential amplification. Though non-differentially expressed sequences will still be present in the mixture, these molecules will lack one or both adaptors (i.e. primer site) and will only amplify linearly or not at all respectively. Others will possess homologous adaptors that hybridise to one another during PCR to form a

pan-like structure thus preventing access by primers and polymerase and suppressing exponential amplification of the molecule.

Eight PCR reactions were run: 1) forward-subtracted infected cDNA; 2) unsubtracted infected tester control; 3) reverse-subtracted non-infected cDNA; 4) unsubtracted non-infected tester control; 5) subtracted control cDNA; 6) unsubtracted control cDNA; 7) PCR control-subtracted cDNA (a positive control for the manufacturer's control); 8) a positive PCR control provided with the PCR polymerase mix (Clontech, UK). A PCR master mix was prepared corresponding to 2.5µl of 10x PCR reaction buffer, 0.5µl 50x PCR polymerase mix, 1µl of PCR primer 1 (10µM) and 0.5µl of dNTPs (10µM) (all from Clontech, UK) and 19.5µl of ddH_2O for each reaction. A separate reaction was set-up for the PCR control using the same reaction mix except with 1µl of a primer mix (10µM) provided by the manufacturer. 1µl of the respective dilute cDNA was added to the 24µl of the reaction mix to a total volume of 25µl. The reactions were mixed and briefly spun down, and overlaid with a drop of mineral oil before incubation in a thermal cycler at 75°C for 5 min to extend the adaptors and provide the sequences with their second primer site. PCR commenced immediately after incubation with 31 cycles of the following protocol: 30sec at 94°C, 30sec at 66°C, and 90sec at 72°C. 5µl of the reaction was run out on a 2% gel to look for crude differences in the intensity and banding between unsubtracted and subtracted samples.

2.5.7. Nested PCR

To further enrich for the subtracted sequences, the products of suppression PCR were amplified using nested PCR primers. 3µl of each suppression PCR reaction (except PCR positive control) was diluted in 27µl of ddH_2O. A master mix was prepared

corresponding to 2.5µl of 10x buffer, 1µl of nested primer-1 (10µM), 1µl of nested primer-2R (10µM), 0.5µl of dNTPs (10µM) 0.5µl of 50x polymerase mix plus 18.5µl of ddH_2O in each reaction. 1µl of each dilute PCR reaction was added to 24µl of reaction mix, mixed well and briefly spun down. One drop of mineral oil was added to each reaction and PCR commenced in a thermal cycler for 11 cycles of the following protocol: 94°C for 30 sec, 68°C for 30 sec, and 72°C for 90 sec. 5µl of the PCR reactions were run out on a 2% gel to compare the subtracted and unsubtracted reactions.

2.5.8. Subtraction efficiency

To test how efficiently subtraction removed non-differentially expressed sequences, semi-quantitative PCR was carried out on unsubtracted and subtracted nested PCR products to compare levels of the actin housekeeping gene in each sample. A PCR master mix was prepared corresponding to 1.2µl of actin-F (10µM) and 1.2µl of actin-R (10µM), 15µl of a 2x PCR ready mix (Sigma, UK) and 11.6µl of ddH_2O in each reaction. A separate mix was prepared for the control reaction, instead using the manufacturers G3PDH primers. Nested PCR products were diluted 1 in 10 in ddH_2O and 1µl of these were added to 29µl of PCR reaction mix, and briefly spun down. A drop of mineral oil was laid over each reaction and PCR commenced in the thermal cycler immediately with 34 cycles of the following protocol: 30sec at 94°C, 30sec at 58°C and 120sec at 72°C plus a final extension period of 90sec at 72°C. A 5µl sample was taken of each reaction after 18 cycles, and compared with the 34 cycle PCR products on a 2% gel. If the subtraction had worked well, actin would appear later (or not at all) in subtracted samples, relative to the unsubtracted PCR.

2.5.9. Cloning and transformation of subtracted sequences

1µl of each secondary PCR reaction (less than one day old) was cloned into pCR2.1 vector using the TA cloning kit (Invitrogen, UK) and protocols. Clones were transformed into high efficiency (1-3 x 10^9cfu/µg pUC19 DNA) DH5α competent *E.coli* (NEB, UK). Competent *E.coli* were thawed out on ice and 50µl mixed with 1µl of plasmid DNA, by gently flicking the tube 4 times, before incubation on ice for 30 min. The cell-plasmid mix was heat-shocked in a 42°C water bath for exactly 30sec and chilled on ice for 5 min. 1ml of sterile luria broth (LB) was added, mixed gently by pipette and incubated at 37°C for 60 min in a shaking incubator (250rpm). 50µl of the transformed *E.coli* were pipetted and spread onto luria agar (LA) plates containing Ampicillin and Kanamycin (to select for transformants), and X-gal and IPTG (to perform blue-white selection for colonies containing inserts). Plates were left to soak for 30 min before incubation overnight at 37°C. White colonies were checked for an insert by colony PCR and grown overnight in 5ml LB medium, containing Ampicillin and Kanamycin, at 37°C overnight in a shaking incubator (250rpm). Overnight cultures of colonies were stored in glycerol (1:1 50% sterile glycerol:overnight culture) in 96 well cell culture plates (Nunclon, Denmark) at -80°C.

2.5.10. Differential screening

Although the SSH process enriches for differentially expressed sequences, highly expressed sequences that are common to both experimental cDNA populations can still appear in the subtracted library, and are referred to as false positives. Differential screening was therefore used to identify and remove these false positives from further analyses. Here, subtracted libraries were screened with radiolabelled DNA probes synthesised from tester and driver, i.e. infected and non-infected, cDNA. Truly

differentially expressed genes will not be present in both forward and reverse subtracted libraries yet highly expressed false positives will, allowing the latter type to be identified and removed from further analyses.

2.5.11. Colony blot preparation

Colonies were spotted from glycerol stocks onto Hybond membrane using a multi-channel pipette in a sterile flow hood. Duplicates of each array were made and the blot orientation marked on the membrane. Membranes were placed on LA plates containing Ampicillin and Kanamycin and incubated overnight in a 37°C air incubator. Colonies growth was prevented from becoming too large to avoid neighbouring colonies merging on the blot.

Blots were treated with a series of buffers to liberate and denature DNA from cells before UV cross-linking to the membrane. The blot was placed, colony side-up, on filter paper soaked in denaturation buffer (1.5M NaCl, 500mM NaOH) for exactly 4 min to prevent colonies merging. The membrane was briefly placed onto blotting paper to remove excess buffer before placing it flush with filter paper soaked in neutralisation buffer (1.5mM NaCl, 500mM Tris base) for 4 min. The blot was dried at room temperature for 30 min on blotting paper before UV cross-linking (conditions) the DNA to fix it to the membrane. Blots were stored on blotting paper wrapped in cling-film and stored at 4°C or used immediately in hybridisation.

2.5.12. Probe synthesis using random primer labelling

To remove secondary structures, 25ng of cDNA was heated at 100°C in a sterile screw-top cryotube for 5 min and rapidly chilled on ice. 2µl each of dATP, dGTP, dTTP, 15µl of random primer buffer mix (Invitrogen, UK), 26µl of ddH_2O, and 5µl [α^{32}P] dCTP was added to the total RNA, mixed well and incubated at 25°C for 1h. The reaction was terminated with 5µl of stop solution.

2.5.13. Southern blot analysis

Hybridisation solutions and tubes were pre-warmed to 65°C before probe hybridisation. Blots were placed into a glass hybridisation tube before adding 10ml of hybridisation buffer, ensuring there were no bubbles under the membrane. Initial experiments used Church buffer (7% SDS, 1mM EDTA, 1% BSA, 0.5M $NaHPO_4$ (NaPi) buffer, pH 7.2) but later ones used a modified buffer (without BSA) for practical reasons. After incubating the blot in a rotary oven for 30 min at 65°C, the probe (Section 2.13.2) was heated for 5 min at 100°C to denature the strands and added directly to the hybridisation solution before incubation with the blot overnight at 65°C in the rotary oven. To remove un-hybridised probe and reduce background, the blots were rinsed in 50-100ml of a series of stringency washes: 1 brief rinse of 2x SSC 0.1% SDS at room temperature; 5-10 min in 2xSSC, 0.1% SDS at 65°C; 5-10 min 1xSSC, 0.1% SDS; 5-10 min 0.2x SSC, 0.1% SDS. A second method using two, briefer, 5-minute washes with 3x SSC 0.1% SDS (one at room temperature, one at 65°C) was also tried in separate hybridisations. Excess wash was removed using filter paper and the blot was wrapped in clingfilm before exposing it to x-ray film (Fuji medical x-ray film, UK) in an autoradiography cassette with intensifying screen for between 30 min to >8 hr (overnight). Exposure was

carried out at -80°C to improve the signal. Blots could be re-probed after stripping the first probe off the membrane. Stripping was done by pouring a boiling solution of 0.1% (w/v) SDS onto the blot that was then left to cool before washing the membrane twice in 2x SSC and leaving it to dry. The success of stripping was checked by autoradiography overnight.

Note on Southern blots and probe synthesis.

The Southern blot technique was initially optimised using cloned DNA and showed that probe synthesis and hybridisation to the blot worked well. Binding of the probe to non-specific targets was low whilst hybridisation to the membrane-bound target DNA was strong, as indicated by autoradiography. However, the technique did not work well with the subtracted libraries since probe binding was very weak and binding patterns were variable between screens of a single blot. For this reason, Southern blot-based techniques (Sections 2.5.11-2.5.13) were not used to perform differential screening of the subtracted libraries derived from SSH, which were instead screened using qPCR, as detailed in Section 2.6.

2.5.14. Sequencing of clones

In a sterile flow hood clones were picked from blue/white selection plates using a pipette tip and then grown overnight in LB/Ampicillin/Kanamycin medium in a shaking incubator (250rpm, 37°C). Plasmid preps were made from these overnight cultures using the GenElute plasmid miniprep kit (Sigma, UK) and eluted into 50µl of ddH_2O. Plasmids were digested with the restriction endonuclease EcoR1 (Sigma, UK) and run out on a gel to check for an insert. 2µl of plasmid was mixed with 0.5µl of EcoR1, 10x buffer (Sigma, UK) and 6.5µl ddH_2O, and incubated at 37°C in a thermal cycler for 1h.

Digests were analysed by gel electrophoresis and plasmid preps sent in ddH_2O to the John Innes Centre Genome sequencing lab (Norwich, UK) to be sequenced with the M13 reverse primer for pCR2.1 vector (Invitrogen, UK).

2.6. Standard PCR and reverse-transcription quantitative PCR (RT-qPCR)

2.6.1. Standard (non-quantitative) PCR

0.5-2µl of DNA was mixed with 0.5µl of forward and reverse primers (10mM each), 5µl of 2x PCR mix (YorkBio, UK) containing 10mM of each dNTP, Taq Polymerase (0.1u/µl) and $MgCl_2$ (3mM). Reaction mixes were made up to 10µl with ddH_20. Master mixes were prepared when performing PCR on multiple samples, to minimise reaction variation. All standard PCRs were carried out in a 96 well Biometra T1 Thermal Cycler with heated lid (100°C), using a protocol of: 30sec 95°C denaturation, 30sec annealing and 30-120sec 72°C extension steps, for 30-34 cycles with standard cycler settings. Annealing temperatures varied according to the primers used (Section 2.15.4). Colony PCRs included an additional 5 minute cell lysis step at 95°C prior to cycling. Samples were kept at 4°C once the PCR was complete.

2.6.2. RT-qPCR

2µg of total RNA was reverse transcribed (Section 2.1.11) and the cDNA diluted 1:9 with ddH_20. No-RT mixes, containing all reagents except reverse transcriptase, were also made to check for gDNA contamination in the RNA and reaction mix. 5µl of dilute cDNA was added to a qPCR reaction mix containing 10µl 2x SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, UK) 7.6µl ddH_20 and 0.4µl of the forward and reverse primer mix (5µM each). Two technical replicates were run per sample. Negative controls (cDNA replaced with ddH_20) were run for each primer set to check for gDNA contamination in the reaction reagents. Multiple sample reaction mixes were prepared, and cDNA pipetted into thermoscientific plates using a 96 well PCR plates for qPCR (ThermoScientific, UK). All reaction master mixes and cDNA were pipetted into PCR

plates using a Corbett robotics machine and software. Wells were sealed with optically clear caps (ThermoScientific,UK).

qPCR was carried out on a PTC-200 MJ research machine thermal cycler with a Chromo 4 continuous fluorescence detector, using standard settings, and Opticon software v4.7.97.A for Windows 2000 Professional. Cycle conditions were as follows: 95°C for 5 min, followed by 35-41 cycles of a 30 second 95°C denaturation, 30 second 61°C annealing and 30 second 72°C extension protocol. Well fluorescence was measured at the end of each cycle. Immediately after qPCR completion, melting curve analysis was performed on amplicons to check for non-specific products e.g. primer dimers. Samples were heated in 1°C steps from 50 to 95.1°C, measuring fluorescence after each heat graduation, and plotting -dF/dT against temperature, using the Opticon qPCR analysis software, to check for a single peak in the melting range.

2.6.3. Calculating fold changes from qPCR data

C_T values were measured during the exponential phase of gene amplification. Samples were only included in further analysis if their technical replicates were within 0.5 of one another. QPCR was repeated for any samples with replicates outside this margin. An average of the 2 technical replicates was taken so that each biological sample was represented by an average C_T value. Sample ΔC_T was calculated by deducting each C_T value, in control and treatment groups, from the lowest C_T value in the control (naive) samples in both housekeeping genes and the gene under investigation ($C_{T\ control}$ - $C_{T\ sample}$). The other control values were used during statistical analysis to confirm that a gene had been significantly up-regulated.

Fold change in expression was calculated with the $2^{(-\Delta\Delta CT)}$ approximation method (Livak and Schmittgen, 2001), using the housekeeping gene's C_T data to normalise the immune gene C_T value. Using this method, expression between non-infected and infected bees could be compared. Ribosomal protein S5 (RPS5) was used as the housekeeping gene in all the qPCR experiments as it shows moderate expression in honeybees (Evans and Wheeler, 2001) and is consistently used as a control gene in studies of the honeybee's transcriptional immune response to infection (Evans and Pettis, 2005a, Evans, 2004).

2.6.4. Primers

All primers, except M13, are suitable for qPCR and are outlined in the following table.

Primer	Forward primer	Reverse primer	Annealing temp.
Bombus Actin	5'-ACACATGTACCCCGGTATTGC-3'	5'-CTTGATCTTGATGGTCGATGG-3'	60-62°C
M13 (cloning vector)	5'-GTAAAACGACGGCCAG-3'	5'-CAGGAAACAGCTATGAC-3'	50°C
Bombus RPS5	5'-GAGAAGATTCCACGCGTATTGG-3'	5'-TCGTCGTAACGGAGAAACATCC-3'	60-62°C
Fat-spondin	5'-TGGATCTCTGTTTTCGGATAATCG-3'	5'-CAACTGTTTTCCGATTCTTTGACC-3'	60-62°C
Peroxidase	5'-CGATACGCTACTCTGTGGGAAACT-3'	5'-TCGGAAACAACCTACACCTTCAGT-3'	60-62°C
Vitellogenin	5'-GTCGGGAATTATCATTTGCATTGT-3'	5'-CAAGGCACGATTATTGCATTACAG-3'	60-62°C
Plexin A	5'-CTTCCATGTGTGAACAACTTCAGG-3'	5'-GTTTGACTTTATGGACGACCAAGC-3'	60-62°C
Tamo	5'-TTGGAAGCGTCTGACTACCATGT-3'	5'-CACGATGCATCTTCAGAAAGTCA-3'	60-62°C
Serpin-like	5'-CGAGAGTGTCATCCTTCGTATCCT-3'	5'-GTCTTCCGGTTGTTCTTCAGTTTC-3'	60-62°C
Cct5	5'-TATAGAGCGTTGCGTGAATACGAA-3'	5'-TAGCTTCATCATCAAATCCCCATT-3'	60-62°C
IK2	5'-GGTGCAGTTTTTCAAGGTGTGAAT-3'	5'-GCATATGACTGAGCTGATTGAACG-3'	60-62°C
Trypsin-like endopeptidase	5'-CGTGTGACGTCTCATCTTAATTGG-3'	5'-ACTTCGACTTCCAGTTCACGTTTC-3'	60-62°C

Calcineurin	5’-AACATCAGGCAGTTCAATTGTTCA-3’	5’-TTTCACCTACAAATGGCAAAGACC-3’	60-62°C
Defensin	5’-AACTGTCTCAGCATGGGCAAAG-3’	5’-AGAGATCCTTGAAGTTGGTCTTGC-3’	60-62°C
HDLBP	5’-ACGGTCCCTTTTCGTCCTATTATC-3’	5’-GGAAGCTGAACGTCAAGATAGAGC-3’	60-62°C
Abaecin	5’-ATGAAGGCAGTAATGTTTATTTTC-3’	5’-GGAAAGGTTGGAAACGGTTTAGAT-3’	60-62°C
Hymenoptaecin	5’-CCTTGTTATCGATGGAAAGAAACC-3’	5’-GTTGATGATAATCGACGTCCAAGG-3’	60-62°C
Sarcophaga/ Cathespin-L-like	5’AACAGGCGTTATTGATTGTTCAT-3’	5’-CATCTTCAGTAGGCAAACCTCCAT-3’	60-62°C

2.7. Primer design

Primers were ordered from Sigma-Aldrich, UK

2.7.1. Clones

Primers for cloned sequences, i.e. derived from SSH, were designed using the Invitrogen web-based Oligoperfect primer designer program: https://tools.invitrogen.com/content.cfm?pageid=9716. Primers were designed to be around 24bp long, with a GC content of 40-60% and a melting temperature of 63°C. Primer sets returned with a GC clamp on the 3' end were preferentially selected, and checked for absence of primer dimers and secondary structures using the Sigma web-based online primer DNA calculator tool: http://www.sigma-genosys.com/calc/DNACalc.asp. Primers were tested using RT-PCR and checked for specificity using melting curve analysis after qPCR.

2.7.2. Candidate genes

Primers were initially designed to conserved regions of candidate genes from a closely related species of *B.terrestris*. PCR products from these initial primers and *B.terrestris* cDNA were sequenced and used to design qPCR-specific primers with higher sequence-specificity.

Candidate genes, specifically mRNA-derived cDNA sequences, were first found in a close relative of *Bombus terrestris;* preferably *Bombus ignitus* or *Bombus pratorum* (or *Apis mellifera* if these *Bombus* species were not available) by searching the NCBI database (http://www.ncbi.nlm.nih.gov). Nucleotide sequences highly similar to the close relative were then identified using the initial sequence in BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and searching the "other nucleotide" database

(with default settings). With preference for insect species, 2-4 separate species hits with a returned e-value of >10e-10^3 were aligned using the CLC sequence viewer programme (CLC version 4.6.1. for Macintosh, default settings) to identify conserved regions. Primers were designed to the conserved regions with a melting temperature of around 55°C, 20-22bp long, with a GC clamp at the 3' end and GC content of 40-60%. The product was 300-500bp long. Primers were checked for absence of primer dimers and secondary structures using the Sigma DNA calculator tool as above.

Primers were tested and optimised (varying annealing temperature, magnesium concentration, cDNA concentration and cycle number) in RT-PCR on *B.terrestris* cDNA from infected and non-infected mRNA samples. To prepare the PCR product for sequencing, PCR reactions were cleaned up using the Minelute PCR purification kit (Qiagen, UK). Where products were extracted after gel electrophoresis, the desired band was cut from the gel on the UV transilluminator (minimising UV exposure to prevent DNA degradation) and cleaned using the Wizard SV Gel and PCR clean-up system (Promega, UK). The PCR product was sent to the John Innes Centre Genome sequencing lab (Norwich, UK) to be sequenced with its reverse primer, and the returned sequences were put through BLASTn ("other" nucleotide databases; default settings) to check that the correct sequence had been amplified. QPCR primers were designed on the returned sequences in the same way as for clones in Section 2.7.1.

3. A trade-off between host and parasite fitness in the bumblebee-trypanosome model system.

3.1. Introduction

Parasites and their hosts both share the same resource pool, so intuitively their fitness should be traded-off against one another. Although this relationship is frequently observed, numerous counter examples exist that challenge such a generalised view. Recent studies pointed out that a trade-off does not take consideration of potential genotype by genotype interactions between the host and parasite that could also affect the outcome of infection. Such interactions are known to occur between the bumblebee and its gut parasite, *Crithidia bombi.* The aim of this chapter was thus to clarify the relationship between host and parasite fitness in this system and assess the relative importance of trade-offs and genotype by genotype interactions on the outcome of infection.

Parasite transmission is directly related to its fecundity. All else being equal, a parasite should increase its transmission rate to maximise fitness. However, as the host and parasite share the same resource pool, increasing transmission rate will have costs for the host, such as infection-related damage. If these costs are too great the host will die, thereby decreasing the parasite's lifespan. Therefore, for any given parasite-host population there is an optimal trade-off between the parasite's fecundity (its transmission rate) and the parasite's life span controlled by the parasite-induced death of the host i.e., the parasite's virulence. One major assumption of this modern theory of virulence evolution (Anderson and May, 1991) is that the relative virulence of pathogen strains is stable across a range of host types (Grech et al., 2006).

Recently it has been pointed out that this parasite-centred view of the evolution of virulence is incongruent to the large body of data that have found genotype-genotype interactions between host and parasites (Grech et al., 2006) that may also drive virulence evolution (Woolhouse et al., 2002). Studies in plants (Salvaudon et al., 2007) and invertebrates (Carius et al., 2001) find that parasite virulence varies in different host backgrounds due to the specifics of the parasite – host interaction, throwing the generality of a trade-off model, that ignores such interactions, into question.

In the *Plasmodium chabaudi*–mouse system although host-parasite genotype interactions are present, they explained little of the variation in virulence when compared to the host or parasite effects (Grech et al., 2006). Another study using *Arabidopsis thaliana* and its fungus parasite *Hyaloperonospora arabidopsis* found the opposite result (Salvaudon et al., 2007). In this system, host-parasite interactions were the most important factor in explaining the variation in virulence: and the expected trade-off between parasite transmission and host fitness was only found in one host line (Salvaudon et al., 2007).

Given that the trade-off model may apply in one system but not in another, before any further predictions can be made for any given system, it should first be established whether a trade-off takes place or not. In the bumblebee (*Bombus terrestris*), the trypanosomatid *Crithidia bombi* has been shown to have multiple effects on its bumblebee host (Brown et al., 2000, Brown et al., 2003a, Gegear et al., 2006). Most importantly for the present study, worker mortality under starved conditions increases 50% in infected bees compared to non-infected ones (Brown et al., 2000). This is important, as mortality under starvation is used as a measure of virulence in this

experiment. With such a large increase in mortality due to infection it is likely that most of the variation in mortality across the colonies is due to differences in the colonies' responses to *C.bombi* and not simply due to a non-pathogenic effect such as variation in a colony's ability to withstand starvation. Previous studies of *C.bombi* frequently used faecal or gut cell counts as a measure of susceptibility, with high cell numbers indicating higher susceptibility (Mallon and Schmid-Hempel, 2004). However more properly, this is a measure of parasite transmission, according to the trade-off model, an increase in which leads to an increase in parasite virulence. Here, the relationship between transmission and virulence in the *Bombus*-*C.bombi* system was investigated.

If the trade-off theory is applicable to the *Crithidia*-*Bombus* model, it can be predicted that higher levels of infection will be met by a corresponding decrease in host survival because of the mounting infection-related costs incurred by the host. To test this hypothesis, faecal cell counts were measured every day from the second day after infection as an indicator of *C.bombi* transmission (Otterstatter and Thomson, 2006). The link between parasite transmission and replication (cell counts) is theoretical and has never been explicitly tested in the *Bombus*-*Crithidia* system. However, studies in related parasite systems, such as the malaria, *Plasmodium chabaudi*,-mouse model show that the two are strongly correlated (Mackinnon and Read, 1999). The survival of individual workers was also measured throughout infection. Using nine colonies and four individual parasite strains a cross-infection experiment was also performed to examine which factors (i.e., host, parasite strain or host-parasite interactions) have the strongest effect on parasite transmission.

3.2. Methods

A sample of workers from each of nine commercially reared bumblebee colonies (representing different host lines) were collected and infected with a combined inoculum of faeces from two wild infected colonies (181 bees in total). To prepare *C. bombi* strains, faeces were collected from workers of naturally infected colonies, and mixed with 50% diluted Meliose to create a standardized dose of 500 *Crithidia* cells per μl of inoculum. The two strains were then combined to form a single inoculum and 20μl was fed to each worker. Workers were four days old at the time of infection and were kept in colony groups separate from the colony once infected. Faecal cell counts and survival were then tested on sub-groups of infected bees:

- *Faecal cell counts*

A sample of workers from each of nine bumblebee colonies (representing different host lines) were infected with a combined inoculum of faeces from two wild infected colonies (181 bees in total) and maintained under standard conditions. *Crithidia* begin to be released into the faeces (Schmid-Hempel and Schmid-Hempel, 1993). The faecal cell count of *Crithidia* was measured in each infected bee on a single day 2 to 8 days after initial infection. *Crithidia* were counted using a Neubauer haemocytometer. A mean of 2.9 +/-1.0 bees were infected and measured per day, per colony.

- *Survival experiment*

A separate group of bees of known age (ten bees per colony, except colony 10 where there were eight) were infected as above. Four days after infection their food source was taken away. Every hour afterwards (beginning at 11 a.m.) the bees were checked, and dead bees (i.e., bee did not move when touched) were counted and removed.

- *Cross infection experiment*

The cross infections were as above except bees from each of nine colonies were infected with each of the four strains separately (mean number = 5.8 bees per interaction, 209 bees in total). The faecal cell count measure was taken between days five and seven. Different colonies and strains were used for the survival and cross-infection experiments.

- *Statistical analysis*

Cell counts in the survival experiment were analysed with a repeated measures ANOVA, with day as the repeated measure. Survival curves for each colony were calculated using the Kaplan-Meier method. All analyses were carried out using Intercooled Stata 8.2 for Mac.

3.3. Results

In total, 181 bees were infected. A mean of 2.9 +/-1.0 bees were collected per colony per day. The day that bees were infected on significantly affected cell counts ($F_{6,119}$ = 9.30, $p < 0.00001$), see Figure 3.1, whereas there was no significant effect of colony ($F_{8,119}$ = 2.01, p = 0.0512). There was a significant interaction between colony and days ($F_{47,119}$ = 2.06, p = 0.0009) (Figure 3.2). Cell counts for day six were the only day that showed significant differences between colonies ($F_{8,20}$ = 2.59, p = 0.04). The survival data were plotted as Kaplan-Meier survival estimates for each colony (Figure 3.3). Colony had a significant effect on survival of starved bees (Cox proportional hazards model: z =2.93, n = 88, p = 0.003). Pearson's correlation reported a significant correlation between day six mean cell counts and the mean latency (mean survivorship) during the survival assay of each colony (r = -0.7919, n = 9, ρ = 0.0192) (Figure 3.4). If Bonferroni's correction is applied to account for multiple testing of each day the result is non-significant.

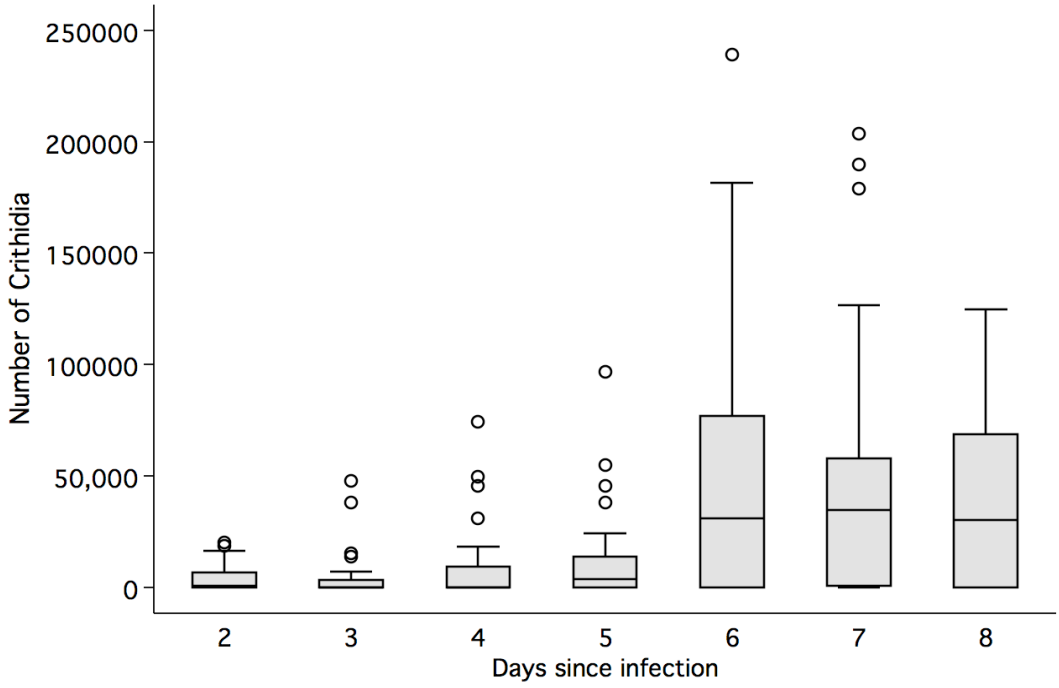

Figure 3.1 Faecal cell counts of *C.bombi* infection (cells per µl) over eight days, pooled from 9 colonies. 181 bees from 9 colonies were infected. Levels of *Crithdia* in the faeces were measured once in each bee on one day from 2-8 days after infection. A mean of 2.9 +/-1.0 bees were collected per day, per colony. Box length represents inter quartile range with top and bottom bars of each box representing upper (75%) and lower (25%) quartiles respectively and middle bar representing median value. Whiskers indicate minimum and maximum data value in with suspected outliers shown as °.

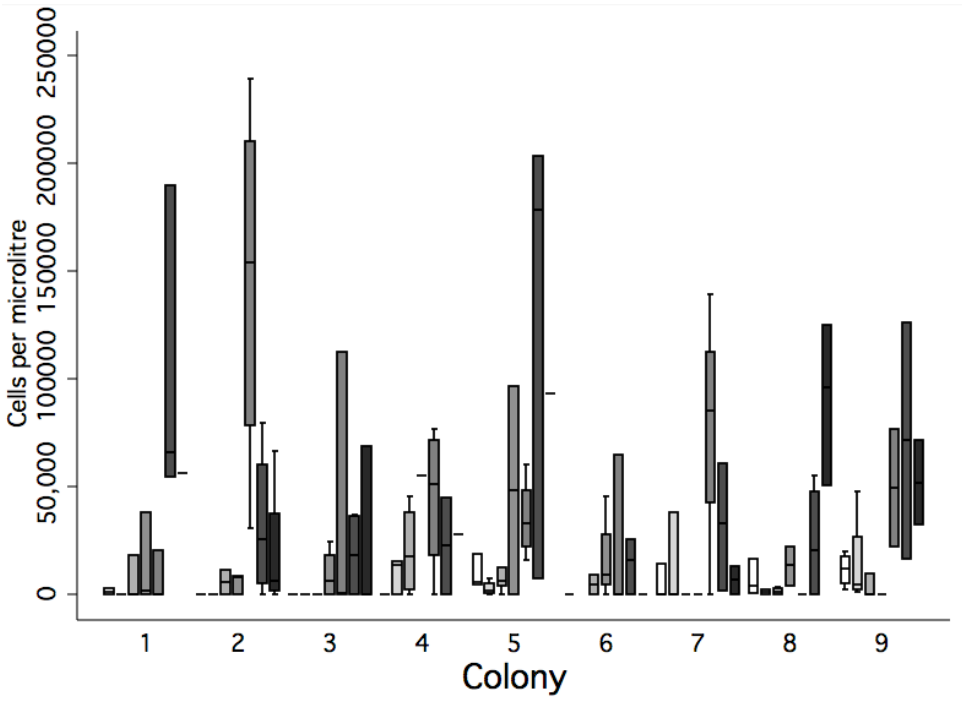

Figure 3.2 Box plot showing breakdown of faecal cell counts in each colony. 181 bees from 9 colonies were infected. Bars represent mean cell count of 2.9 +/-1.0 bees measured on one day from 2-8 days after infection. Days are graded from white (day 2) to black (day 8) for each of the 9 colonies. Box length represents inter quartile range with top and bottom bars of each box representing upper (75%) and lower (25%) quartiles respectively and middle bar representing median value. Whiskers indicate minimum and maximum data value in with suspected outliers shown as °.

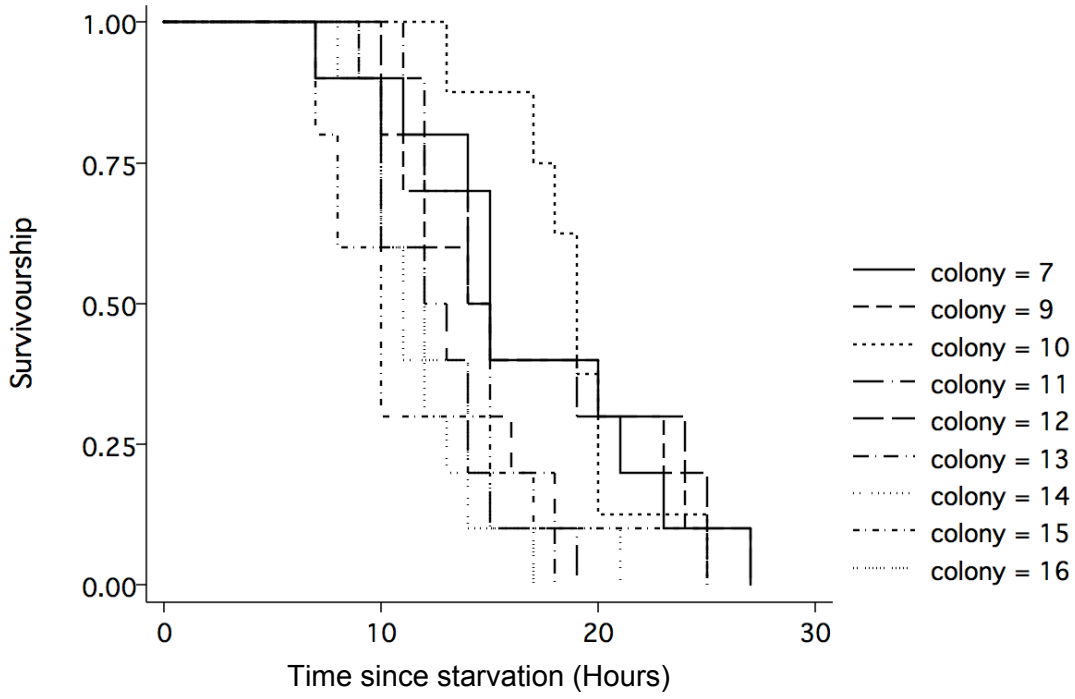

Figure 3.3 Kaplan-Meier survivor estimates by colony. 4 day old workers from 9 colonies were starved four days after oral infection with a mixed-strain inoculum of *C.bombi* to calculate survival rate of individual colonies after infection. A total of 209 bees were sampled with a mean of 5.8 bees per interaction.

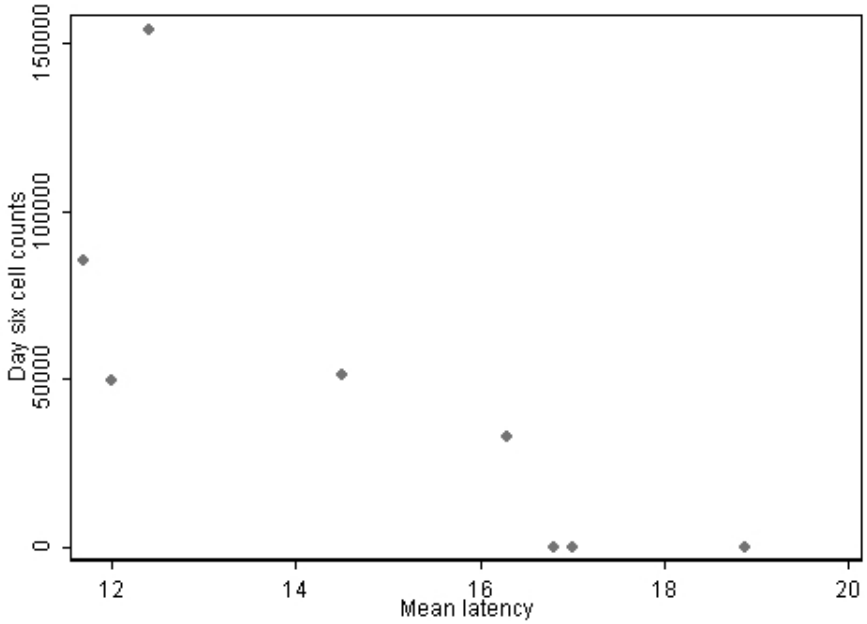

Figure 3.4 The correlation between mean latency (host survival) and parasite transmission. Bees were infected with a mixed-strain inoculum of *C.bombi*. Faecal cell counts were collected from bees between 2-8 days after infection. In total, 181 bees were infected that originated from 2 colonies. A significant correlation was found between cell counts on day 6 of infection and survival rate in each colony. If Bonferroni's correction is applied to account for multiple testing of each day the result is non-significant.

In the cross infection experiment (Figure 3.5), it was clear that individual *C.bombi* strains produced greater numbers of cells, regardless of the host genotype they were infecting ($F_{3,208}$ = 16.61, p < 0.00001). Likewise, some colonies were generally more susceptible to *Crithidia* than others, regardless of the *Crithidia* strain they were infected with ($F_{24,208}$ = 1.62, p = 0.04). The level of infection was also significantly affected by the interaction between colony (host) and parasite strain i.e., the level of infection in the host was dependent on the specific combination of host and parasite genotype ($F_{24,208}$ = 14.60, p = 0.04). This interaction accounted for 13.6% of the variation in faecal cell counts (η^2= 0.136).

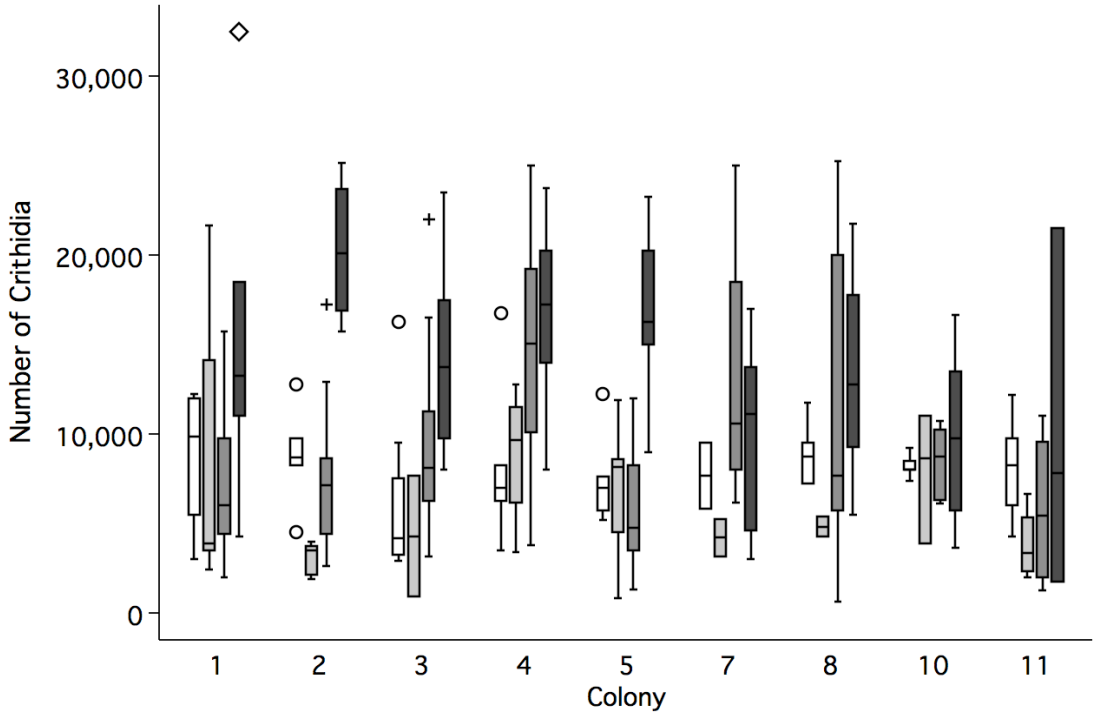

Figure 3.5 Faecal *Crithidia* levels (cells per µl) of the different colonies used in the cross-infection experiment. The four colour boxes (white, light grey, grey and charcoal) represent the values of the infections by the four different strains. Box length represents inter quartile range with top and bottom bars of each box representing upper (75%) and lower (25%) quartiles respectively and middle bar representing median value. Whiskers indicate minimum and maximum data value in with suspected outliers shown as ° or +.

3.4. Discussion

The link between virulence (survival) and transmission (faecal cell counts) was investigated between the bumblebee and its gut parasite *Crithidia bombi*. The results showed that some colonies had better survival than others when infected. Worker survival correlated with the faecal levels of *C.bombi* six days after infection. A separate cross-infection experiment found that the faecal cell count depended on the genotype of both the host and parasite, i.e. that there was an interaction between host and parasite genotype.

If the trade-off model holds in this system, virulence and transmission should be correlated. In agreement, the experiment here found a correlation between parasite virulence and cell counts at day six. Why does the trade-off model apply in the *Bombus*-*Crithidia* system, but not in others? Grech et al.'s study (2006) provides one suggestion. They too found that host-parasite interactions were present, but were unimportant in explaining the variation in virulence and transmission, or their trade-off, in the *Plasmodium*-mouse model (Grech et al., 2006). Their findings suggest that the importance of the host-parasite interaction in a given system defines whether a trade-off is detectable. The experiment here found that the interaction between the host and parasite in the cross infection experiment explained 13.6% of the total variation in faecal cell counts, compared to 4.2% in the *Plasmodium*-mouse model examined by Grech et al. (2006). It seems that the interaction term between the bumblebee hosts and their parasite strains reflects an important part of the overall disease biology. However, it has to be accepted that if the Bonferroni correction is applied then it cannot be assumed that there is any significant correlation between parasite transmission and host survival.

An alternative explanation for the trade-off result found in this chapter is that workers from less robust colonies die quicker and are easier for the parasite to exploit. Thus, the correlation could be a function of colony robustness, rather than the relationship between virulence and transmission initially hypothesised. Thus, this could be a classic example of the general problem with correlational analyses: they do not show causal relationships.

In agreement with Salvaudon et al.'s work (2007), the results showed that there was variation between the host and parasite genotypes in their respective resistance or infection phenotype. However, more importantly the combination of host and parasite genotype affected the outcome of infection and such interactions could play an important role, particularly with respect to the maintenance of variation in parasite infectivity and host resistance to infection. As Salvaudon et al. point out, if parasite infection success is specific to particular host genotypes and vice versa, then the selective landscape is frequently changing as new host-parasite combinations come together and successful combinations are broken up. In their study, this was evident in the fact that *Hyaloperonospora arabidopsis* (parasite) lines collected from the same region had different infection phenotypes in the same host genotype.

If a host and parasite share the host's limited resources, logically there must be a trade-off. Frequently this is not the case though and signals of a trade-off are weak or non-existent (Ebert and Bull, 2003). Salvaudon et al. (2007) also found little evidence for a trade-off and hypothesise that its appearance depends on the compatibility between the host and a given parasite (Salvaudon et al., 2007). Compatibility here refers to the quantitative adequacy of the host-parasite association for each individual (Salvaudon et

al., 2007). Interactions that are compatible, for example that do not induce a strong, costly immune response in the host, can share the entire resource pool (Figure 3.4b). Conversely, a highly incompatible host and parasite use up the resources fighting each other, thereby altering the trade-off, which still takes place but on a much smaller resource pool (Figure 3.4c). Variation in compatibility will therefore mask any trade-off (Salvaudon et al., 2007). This argument is precisely equivalent to that which is used to explain the lack of a negative correlation in trade-offs of various life history traits within an organism. There, it is hypothesised that genetic variation exists not only in resource allocation, analogous to the trade-off between host and parasite fitness, but also in resource acquisition, analogous to compatibility (Reznick et al., 2000). If there is more variation in allocation and less in acquisition, a negative correlation between any two life history traits is expected. Vice versa, a positive correlation would be expected if there were more variation in acquisition, just as increasing compatibility increases the resource pool for both host and parasite (Van Noordwijk and Dejong, 1986) (see Figure 3.4).

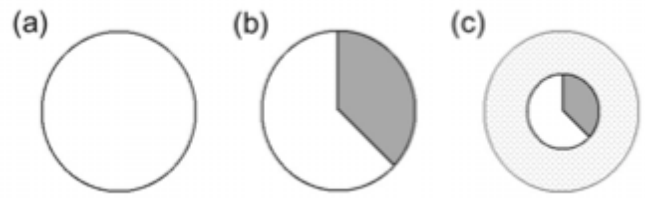

Figure 3.4 Representation of resource allocation in a host-parasite system, from (Salvaudon et al., 2007). A) The circle represents the absolute resource pool provided via the host. B) Host (white area) and a fully compatible parasite (grey area) each diverts host resources to reproduction. C) Here, the outer portion (light grey) represents resources lost when a host is infected with a partially incompatible parasite, for example through costly activation of the immune response. Though the relative amounts of resources sequestered by host and parasite remain constant, the absolute resource pool available for reproduction is lower.

Why, given that the results have shown a host-parasite interaction in the *Bombus*-*C.bombi* system, is the predicted trade-off still found? Salvaudon et al. (2007) suggest that a trade-off should be seen most clearly in hosts that are highly susceptible to all given parasite strains, i.e. they are highly compatible. The cross-infection data show in the experiment clearly shows that different hosts have different infection intensities in response to different parasite strains. However, there are two aspects to infection; initial parasite infection of the host and parasite multiplication within the host (Imhoof and Schmid-Hempel, 1998). In the cross infection experiment all bees (209) developed an infection. Inoculated bees sometimes do not develop an infection (personal observation), and it has never been investigated if infection success is controlled by host or parasite genotype or some other variable. There is at least the possibility that all host lines in the wild are susceptible to a *C.bombi* infection, and that the cell counts of *C.bombi* infections actually represent within host multiplication. If this is the case then Salvaudon et al.'s model stands, with the bumblebee-*C.bombi* system being an example of a highly susceptible host.

Normally, when searching for a trade-off between host and parasite, it is the parasite strain that is varied and the host genotype kept constant (de Roode et al., 2005). This experiment did the reverse and still found a negative relationship between transmission and virulence. The trade-off theory takes no account of the interaction between the host and parasite, but recent work has shown that this interaction is vital to understanding the evolution of virulence (Lambrechts et al., 2006, Woolhouse et al., 2002). The data here, with its use of multiple hosts, confirms the importance of testing the interaction between a host and a given parasite before making predictions about the relationship between virulence (host survival) and parasite transmission (faecal cell counts).

4. Specificity in the immune response of *B. terrestris* during *C.bombi* infection.

4.1. Introduction

In agreement with ecological studies in invertebrates, the initial cross-infection experiment in Chapter 3 showed that the outcome of an infection is dependent on the interaction between the host and pathogen genotypes, and is specific (Schmid-Hempel, 2005b). Such specificity contrasts with the long-held mechanistic view of innate immunity as a generalised, broad-spectrum recognition system and is instead indicative of greater complexity underlying the invertebrate immune response. However, critics of ecological immune-specificity assert that measures such as host survival, reproductive rate, or in the case of the bumblebees, faecal cell counts, are affected by any number of interactions between host and parasite, not just the immune system (Hauton and Smith, 2007). These interactions may include behavioural modification, reproductive isolation or a switch in life history strategies. That is, the specificity found in ecological studies may not be the direct result of an immune response and so interpretations of invertebrate immune-specificity are therefore premature.

A dichotomy regarding invertebrate immunity's true capabilities therefore exists, created by the direction from which we study it. Although traditional mechanistic models cannot predict or explain ecological immune phenomena, candidate immune molecules theoretically capable of generating ecological-level specificity have recently been discovered in a number of invertebrates (Schulenburg et al., 2007). Yet whilst such mechanistic studies suggest a basis of specificity, it has yet to be proven that they can mediate differential activation of immune effectors against specific parasite strains, which is central to the argument for innate immune specificity. To address the

dichotomy, i.e., whether the invertebrate innate immune response is truly specific, evidence is needed of host-parasite genotype-genotype interactions that cannot be attributed to anything other than an active immune response by the host.

To begin looking for this evidence, repeated the cross-infection protocol used in Chapter 3 was repeated and this time directly measured the bumblebee immune response during infection with specific strains of its parasite *C. bombi*. Flagellates such as *Leishmania*, *Trypanosoma sp.* and *Crithidia* develop exclusively in their insect host's gut and do not migrate into the haemolymph (Boulanger et al., 2006). Local immune responses in the gut epithelium, including antimicrobial peptide (AMP) production (Ryu et al., 2006, Liehl et al., 2006), are therefore likely to be important in controlling these infections (Tzou et al., 2000). In the sand fly *Phlebotomus duboscqi*, Defensin is induced in the gut epithelia and systemically in the fat-body during *Leishmania major* infection (Boulanger et al., 2004), and similar AMP induction is cited in the insect host's immune response to *Trypanosoma brucei* (Boulanger et al., 2002a) and *Crithidia sp.* (Boulanger et al., 2001). Although they primarily target bacteria and fungi, invertebrate AMPs may have direct anti-parasitic activity as suggested by studies of the protozoan *Leishmania major* (Boulanger et al., 2004), *Plasmodium berghei* (Dimopoulos et al., 1997) and *Plasmodium gallinaceum* (Shahabuddin et al., 1998).

Based on this literature, expression levels of three AMP genes, *Abaecin*, *Defensin* and *Hymenoptaecin* were measured as a signal of differential immune responses toward specific pathogens. Abaecin and Hymenoptaecin are general, broad-acting anti-bacterial peptides where as Defensin is most active against gram-positive bacteria (Casteels et al., 1990, Casteels et al., 1993, Bulet et al., 1999). To test the specificity of AMP

expression, *B.terrestris* workers from four host lines (as defined by colony identity) were naturally infected by feeding them one of four *C.bombi* isolates. QPCR was used to measure expression levels of the three *B.terrestris* AMPs after 24 and 48h post-infection. The expression dynamics of AMP expression in response to *C.bombi* have not been elucidated, so the 24h time-point was selected based on Boulanger et al.'s study of AMP expression in *Crithidia* infected *Drosophila* (Boulanger et al., 2001). An additional 48h time-point was also taken as AMP expression may be sustained during infection (Lowenberger et al., 1999). A total of 93 individuals were analysed, including non-infected controls for each host-line.

4.2. Methods

Experiments were carried out on two commercially reared bumblebee colonies from Koppert Biological Systems U.K. and two colonies from wild caught queens. All parasite isolates used originated from wild queens collected in the Spring of 2007 and 2008 in the botanical gardens, University of Leicester. Experiments began when the colonies had a minimum of thirty workers, approximately four weeks old. Between observations, colonies were fed *ad libitum* with pollen (Percie du sert, France) and 50% diluted glucose/fructose mix (Meliose – Roquette, France). Before and during the experiments colonies were kept at 26°C and 60% humidity in constant red light.

4.2.1. Infections

To prepare *C. bombi* isolates, faeces was collected from workers of naturally infected colonies, and mixed with 50% diluted Meliose to create a standardized dose of 500 *C.bombi* cells per μl of inoculum. Previous studies had shown that such inocula,

prepared from different colonies, are genotypically different (Schmid-Hempel and Funk, 2004) and generate specific responses in novel hosts (Schmid-Hempel et al., 1999). Workers from each of four bumblebee colonies (representing different host lines) were infected by feeding individuals with an inoculum of faeces from one of four *C.bombi* strains. Bees were four days old at the time of infection. 83 workers were infected in total, of which 2-3 workers were infected and sampled for each time-point (24 or 48h) and *Crithidia* strain combination in each of the 4 colonies. A total of 10 non-infected controls were collected, with two workers from colonies 1 and 2 and three workers from colonies 3 and 4. After infection bees were kept in colony x strain groups and fed *ad libitum*. 24h or 48h post-infection the bees were sacrificed by freezing in liquid nitrogen. Gene expression was sampled at 24 or 48h, well within the 8-10 day growth phase of *C.bombi* infection when cell numbers are steadily increasing and not oscillating as is found at later stages (Schmid-Hempel and Schmid-Hempel, 1993). Non-infected, 4-day-old control bees that had not received an inoculum were also sacrificed at the 24h time-point to provide a baseline level of each AMP's expression. Sacrificed bees were stored at -80^{o}C.

4.2.2. Primer sets

Identification of Antimicrobial Peptide homologues in *Bombus terrestris*

In order to identify *Defensin*, *Hymenoptaecin* and RPS5 (ribosomal protein S5) homologues in *Bombus terrestris* regions of conservation in these genes were first identified in other Hymenoptera and other insects. *Defensin*, *Hymenoptaecin* and RPS5 genes were identified in honeybees or bumblebees (*Defensin*: *B.ignitus* AY23050 ; *Hymenoptaecin*: *B.ignitus* EU411043; RPS5 *Apis mellifera* XM_393226.3) and used as

the subject in searches of the translated nucleotide NCBI database (http://www.ncbi.nlm.nih.gov). For each gene, 2 to 3 sequences (*Defensin*: *A.mellifera* AY333923 & NM_001011616; *Hymenoptaecin*: *B.ignitus* EU411044 & *A.mellifera* NM_001011615; RPS5 *Nasonia vitripennis* XM_001599025.1 & *Diaphorina citri* DQ673389.1) showing the highest degree of homology were aligned using the CLC sequence viewer programme (CLC version 4.6.1. for Macintosh) to identify conserved regions within the sequence. Primers were then designed within these regions of conservation using the perfect-primer software (Invitrogen, UK) and used to amplify sequences from *Bombus terrestris* cDNA from infected bees.

The *Abaecin* primer was designed slightly differently since few *Abaecin* sequences were available in the NCBI database at the time and homologues in *A.mellifera* did not have adequate sequence conservation to *B.ignitus* to design primers to. In addition, the sole *B.ignitus* sequence available was 177bp long, making it difficult to obtain an initial *B.terrestris* sequence on which to design qPCR primers. Instead, qPCR primers were designed straight from the *B.ignitus* sequence (GenBank accession AY423049.1), using a previously published forward primer for *Abaecin* (Choi et al., 2008) and a reverse primer designed by eye, and checked using the Sigma DNA calculator (www.**sigma**-genosys.com/calc/**DNA**Calc.asp) to target an 88bp fragment. These primers were run in an RT-PCR to check for a single product as below but using an annealing temperature of 60°C. Since designing these primers, a study by Xu et al. (2009) (Xu et al., 2009) has identified multiple copies of the *Abaecin* transcript in the Asiatic honeybee *Apis cerana* that show high homology and sequence conservation (90% nucleotide homology) with the *B.ignitus* sequence used to design the primers.

Primer testing

Primer sets were tested with standard PCR (Section 2.6.1) using 0.5µl of infected *B.terrestris* cDNA. To optimise the reaction and produce a single amplicon, primers were run using the following protocol: 94°C for 3 min then 30-34 cycles of 30 second 94°C denaturation, 30 second 50-55°C annealing step and 60sec 72°C extension step. PCR reactions were run on a gel, and recovered amplicons were sequenced (John Innes Centre, Norwich, UK) (Section 2.5.14). The identity of these primary amplicons was checked using BLASTn, and all showed high homology to the desired antimicrobial genes (>70% homology) in *Apis sp.*, *Bombus sp.* and *N.vitripennis.* RPS5 was highly homologous to many insect RPS5 genes, including *A.mellifera, Bombus sp.* and species of butterfly (*Aporia crataegi*) and wasp (*Lysiphlebus testaceipes*) with >70% nucleotide sequence homology. Primers were then designed within the *Bombus* sequence to produce an amplicon of around 100bp, suitable for qPCR analysis. Primers were synthesised by Sigma-Aldrich, UK.

GenBank submissions

Both *B.terrestris* AMP sequences and RPS5 were submitted to the GenBank database – accession numbers as follows: RPS5: FJ931041; *Defensin*: FJ839454 and *Hymenoptaecin*: FJ839453.

4.2.3. RNA extraction, cDNA synthesis and qPCR analysis.

Total RNA was extracted from individual homogenised abdomens and cleaned before treatment with DNase to remove residual genomic DNA (gDNA). After synthesising first-strand cDNA (Section 2.4.1), each sample was diluted 10 fold with nuclease-free water. Each qPCR reaction contained 5µl of dilute infected or control cDNA. Each sample was tested with the housekeeping gene RPS5 (Evans and Pettis, 2005b) and all

three AMPs, and two technical replicates were run per reaction. Reactions for qPCR were performed using the following program: 95°C for 5 min, followed by 42 cycles of a 30sec; 95°C denaturation, 30sec; 61°C annealing and 30sec, 72°C extension (Section 2.6.2).

C_T values were taken at a threshold fluorescence value of 0.02, during the exponential phase of gene amplification. Samples were only included in further analysis if their technical replicates were within 0.5 of one another. QPCR was repeated for any samples with replicates outside this margin. An average of the 2 technical replicates was taken so that each biological sample was represented by an average C_T value. Sample ΔC_T was calculated by deducting each C_T value, in control and treatment groups, from the lowest C_T value in the control (naive) samples in both housekeeping genes and the gene under investigation ($C_{T\ control}$ - $C_{T\ sample}$). Fold change in expression was calculated with the $2^{(_{\Delta}-_{\Delta}CT)}$ approximation method, using the housekeeping gene's C_T data to normalise the immune gene C_T value.

4.2.4. Statistical analysis

Fold changes in *Abaecin* and *Defensin* expression were box-transformed, and *Hymenoptaecin* zero-skewness log-transformed to fit the data to a normal distribution. Fold change values were transformed to fit the data to a normal distribution (*Hymenoptaecin*: zero skew log transformed as: ln (fold change in *Hymenoptaecin* expression – 0.1952191); *Abaecin*: box-cox transformed: fold change$^{-1.0302212}$/-0.0302212; *Defensin*: box-cox transformed: fold change$^{-0.955746}$/0.044254) before analysis. A MANOVA was first performed comparing expression of the 3 AMPs in infected and non-infected samples. A separate analysis examined the possible effects of

colony (host), parasite strain or their interaction on AMP expression in all the infected workers. In this respect control data, from non-infected workers, is irrelevant and for this reason was omitted from the analysis. Other studies do not include non-infected samples in analyses of this type of experimental data (Salvaudon et al., 2007). Fold data for all three AMPs was first analysed from all infected workers using a MANOVA, and if significant effects were found, separate ANOVAs were carried out for each AMP. All data analyses were performed using Intercooled STATA 8.2 for Macintosh.

4.3. Results

Primary analysis of the qPCR data suggested that overall, AMPs were not significantly up-regulated over baseline levels in infected bees (overall MANOVA: $F_{3,89} = 0.87$, $P = 0.4591$; Wilks' $\lambda = 0.3530$ with status (i.e., infected/ non-infected) as explanatory variable). To investigate this further, separate ANOVAs were carried out for each AMP and these tests also suggested that infection did not cause an increase in AMP expression (*Abaecin:* $F_{1,91} = 0.92$; $P = 0.3397$; *Defensin*: $F_{1,91} = 0.05$; $P = 0.81$; *Hymenoptaecin*: $F_{1,91} = 0.02$; $P = 0.90$). Finally, to establish whether a specific strain caused an increase in AMP expression relative to controls post-hoc tests were run on the transformed data. Significant *Abaecin* up-regulation was found in bees infected with *Crithidia* strain 4 (Fisher-Hayter post-hoc test: $F_{4,91} = 3.701$; $P = 0.05$). Variance was equal between infected and non-infected bees for all three AMPs (Levene's robust test for unequal variance: *Abaecin:* $W50_{1,91} = 1.5980699$, $P = 0.209$; *Defensin*: $W50_{1,91} = 0.00098843$, $P = 0.975$; *Hymenoptaecin*: $W50_{1,91} = 0.11169488$, $P = 0.739$

Analysis of the cross-infection data, which omitted non-infected controls, clearly showed that the level of AMP expression seen in samples was dependent on the identity of the host line (i.e., colony) (MANOVA with the three AMPs as responses; overall model: $F_{9,155.9} = 2.28$, $P = 0.02$; Wilks' $\lambda = 0.7405$), especially for the expression of *Hymenoptaecin*: (ANOVA $F_{3,66} = 5.19$; $P = 0.0028$). Some parasite strains also elicited stronger AMP expression overall (overall MANOVA: $F_{9,155.9} = 9.25$, $P = <0.00001$; Wilks' $\lambda = 0.3530$), which was highly significant with respect to *Abaecin* (ANOVA: $F_{3,66} = 13.76$; $P = < 0.00001$) and *Hymenoptaecin* (ANOVA $F_{3,66} = 4.31$; $P = 0.0078$). *Defensin* expression also varied significantly according to the time of sample collection at either 24 or 48h (overall MANOVA not significant; *Defensin* expression: ANOVA $F_{1,66} = 4.34$; $P = 0.041$). Most importantly, and in line with the hypothesis, AMP expression levels varied highly significantly according to the interaction between colony and parasite isolate, i.e. AMP expression varied according to the specific combination of host line and *Crithidia* strain (MANOVA: $F_{27,187.6} = 2.30$, $P = 0.0006$; Wilks' $\lambda = 0.4332$), particularly with respect to *Defensin* (ANOVA: $F_{9,66} = 2.12$; $P = 0.0396$, Figure 4.3) and *Hymenoptaecin* (ANOVA: $F_{9,66} = 2.14$; $P = 0.0380$, Figure 4.1). *Abaecin* did not have a significant interaction between colony and strain (ANOVA: $F_{9,66} = 1.6$; $P = 0.1328$, see Figure 4.2).

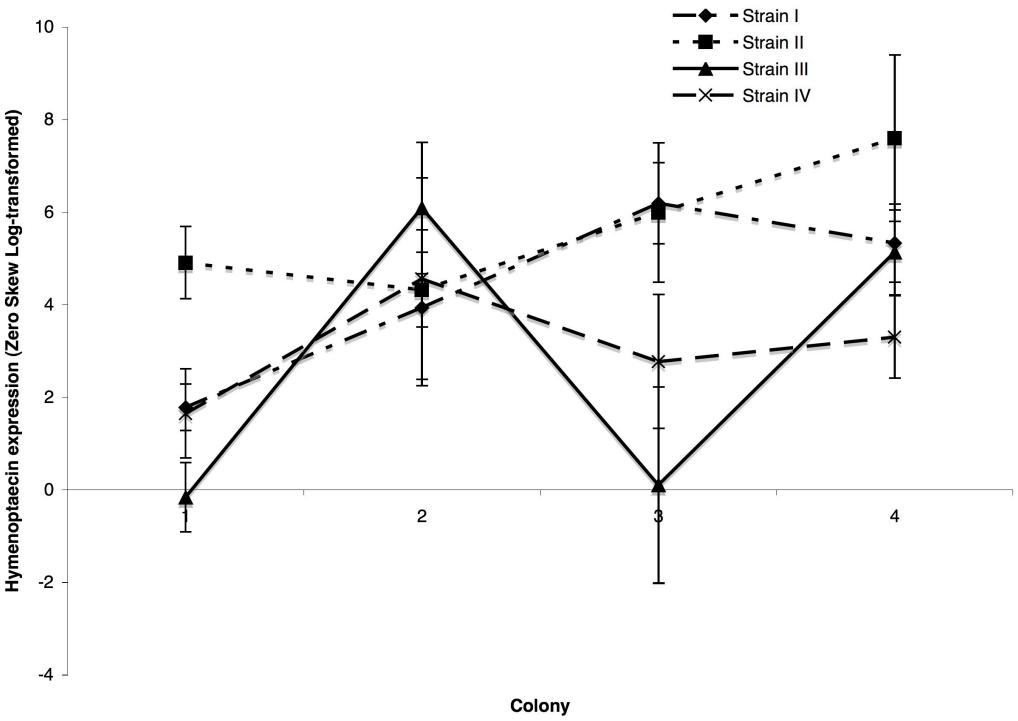

Figure 4.1 Relative expression of *Hymenoptaecin* across four *B.terrestris* colonies (host lines) in response to four different *Crithidia* isolates (see in-graph legend). Zero-Skew log-transformed AMP expression data is plotted. Each point is averaged from 24 and 48h time-points (n=5.2 +/-0.8). 2-3 biological replicates were collected at both 24 and 48hfor each parasite isolate-host (colony) combination. A total of 83 worker bees were infected. Bars represent standard error.

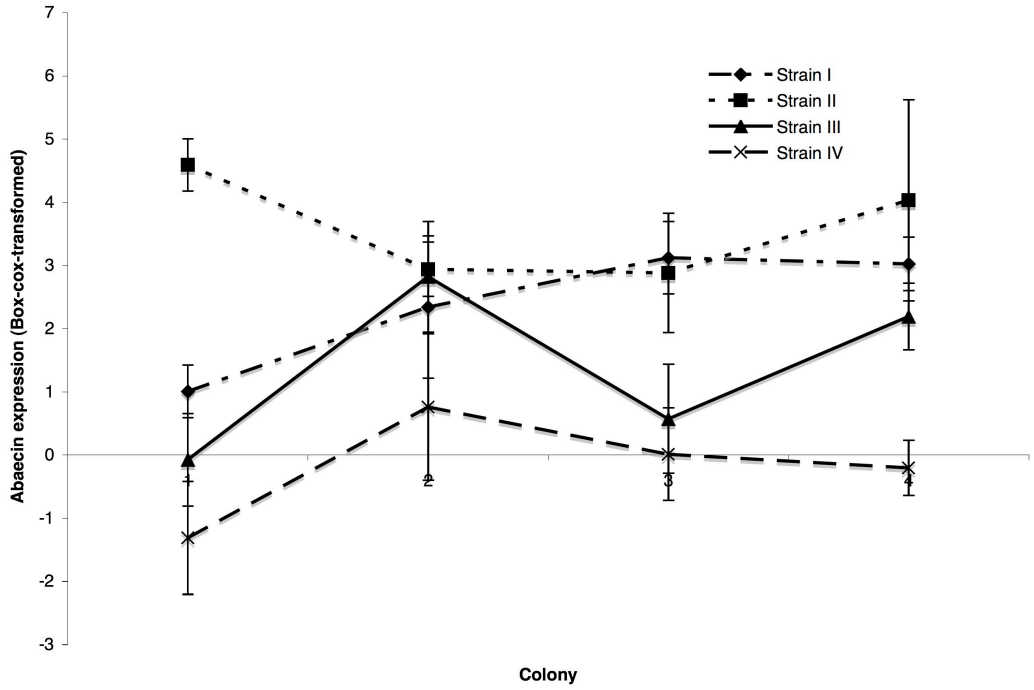

Figure 4.2 Relative expression of *Abaecin* across four *B.terrestris* colonies (host-lines) in response to four different *C.bombi* strains (see in-graph legend). Box-cox transformed AMP expression data is plotted. Each point is averaged from 24 and 48h time-points (n=5.2 +/-0.8). 2-3 biological replicates were collected at both 24 and 48hfor each parasite isolate-host (colony) combination. A total of 83 worker bees were infected. Bars represent standard error.

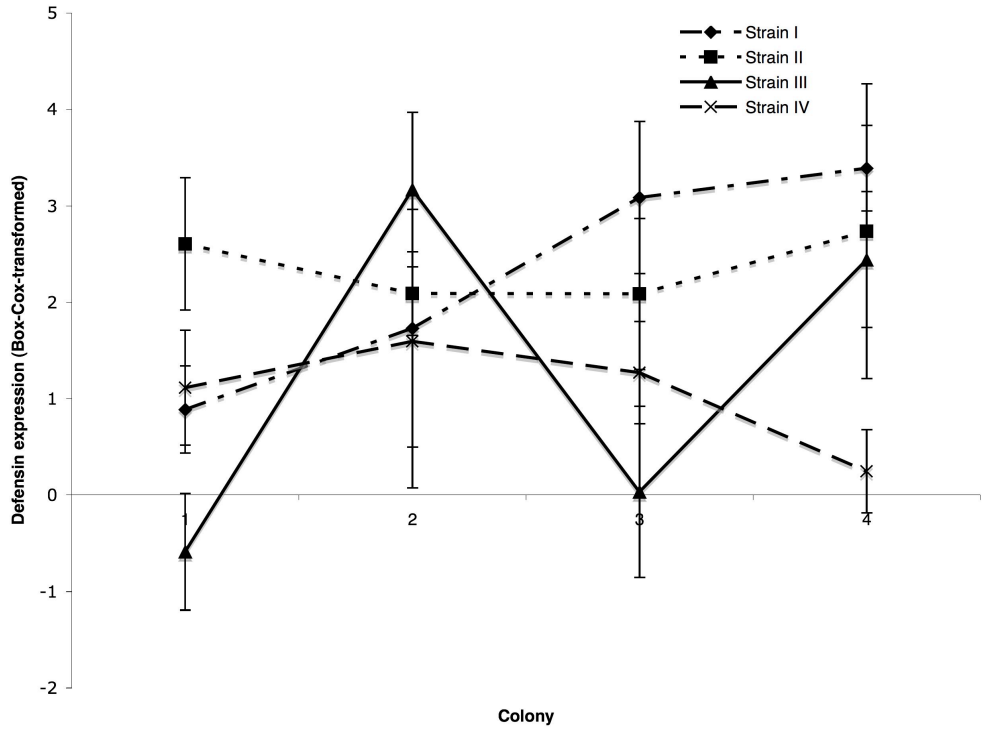

Figure 4.3 Relative expression of *Defensin* across four *B.terrestris* colonies (host-lines) in response to four different *C.bombi* strains (see in-graph legend). Box-cox transformed AMP expression data is plotted. Each point is averaged from 24 and 48h time-points (n=5.2 +/-0.8). 2-3 biological replicates were collected at both 24 and 48hfor each parasite isolate-host (colony) combination. A total of 83 worker bees were infected. Bars represent standard error.

4.4. Discussion

The results suggest that AMPs were not significantly up-regulated in response to *C.bombi*. There are a number of possible explanations for this finding. Firstly, the control group sample size is very small relative to the infected pool: 10 control versus 83 infected bees and this consequently reduces statistical power. The experiment in Chapter 5 found that AMP expression varies by as much as 1000-fold in non-infected bees. This level is of variation is also found in honeybees (Evans and Pettis, 2005b). Therefore it is possible that there was too much variation in the control data for the analysis to detect any significant difference between non-infected and infected samples. Evidently a repeat of the experiment is needed with a larger sample size for the control group. A second point to make is that since cross-infection experiment is testing for the presence of variation in immunity to *Crithidia*, there is no reason to assume that every host-parasite combination will induce a strong response. It is therefore possible that some host-parasite combinations will have responded weakly to some parasite strains, which combined with high variance in AMP expression, would be hard to detect above control AMP levels. It is likely that individual colonies (genotypes) did not respond strongly to all the *C.bombi* strains. The cross-infection was testing the response of individual host genotypes to individual parasite strains. In support of this, the post-hoc test carried out on *Abaecin* expression did find that strain 4 caused significant up-regulation in bees. Finally, the results cannot account for the possibility that individual host lines or host-parasite combinations respond to *Crithidia* along different time courses and that measuring AMP levels at 24 and 48h has missed peaks in expression elsewhere.

Otherwise, as expected, there were both colony and strain effects on AMP expression. That is some colonies were more capable of responding to *C.bombi* infection, and some strains of *C.bombi* were more able to induce a response in their host. It is already known that some strains of *C.bombi* are particularly effective at establishing an infection in *B.terrestris* (Schmid-Hempel and Schmid-Hempel, 1993). The cross-infection data show that AMP expression varies according to the interaction between host and parasite genotypes, and is specific. Each of the three AMPs showed similar patterns of expression between different colony-strain pairings (Figures 4.1-4.3), which can be expected since all three are controlled by the Imd pathway to some extent (Lemaitre et al., 1995, Schluns and Crozier, 2007). Previous indirect, ecological measures of immunity could not suggest how such specificity operates mechanistically. In addition, a variety of candidate molecules and mechanisms have been proposed to play a part in innate immunity to generate greater differentiation between pathogens but cannot infer the effect of this specificity on the active immune response. These issues have been addressed by directly measuring AMP expression within an ecological context and for the first time demonstrated specificity in the expression of these effectors during the immune response. Therefore, these results mirror previous ecological studies that have used life history traits such as survival or fecundity to represent invertebrate immunity, and provides further evidence to suggest that specificity has an immunological basis and is not solely an artefact of uncontrolled factors outside innate immunity.

My findings suggest that differential up-regulation of immune effectors i.e., dosage effects, may contribute to these specific host-parasite interactions. Although invertebrate AMPs can display considerable genetic diversity (Lazzaro and Clark, 2001, Xu et al., 2009), there is little evidence that variation in these effectors alone can

improve resistance to specific pathogens (Lazzaro et al., 2006, Lazzaro et al., 2004), nor that anti-microbial peptides need to continually evolve since the bacterial membrane is under functional constraints that limit its capacity to evolve and evade anti-bacterial molecules (Zasloff, 2002). Thus, the results do not make any predictions about the mechanism(s) generating such responses, which could be the result of particular receptors, regulatory pathways or a combination of these and their synergistic or epistatic interactions (Schmid-Hempel, 2005a, Lazzaro et al., 2004, Schulenburg et al., 2007).

4.4.1. The specificity gap

Current mechanistic models of innate immunity would not have predicted such fine-scale specificity, emphasising the importance of using natural host-parasite systems to discover and research new phenomena in invertebrate immunity. The gap in our mechanistic understanding of specificity may be partially explained by the conventional use of artificial immunological challenges and immune-elicitors that insects do not normally encounter (Boulanger et al., 2001). For example, the insect immune response is frequently challenged by septic injury i.e., piercing of the cuticle with a needle coated in generalist/opportunistic bacteria such as *Escherichia coli* or *Staphylococcus aureus*, which are favoured representatives of gram-negative and gram-positive bacteria respectively. These methods have proven invaluable to elucidate the molecular basis of innate immunity, such as the major regulatory pathways of antimicrobial peptide synthesis and pathogen-recognition-protein-receptors (Lemaitre, 2004, Lemaitre and Hoffmann, 2007), but are not representative of natural infection routes nor the specialist pathogens that use specific hosts, such as *Plasmodium* that specifically targets *Anopheles* mosquitoes as a vector. It also ignores parasite strategies designed to evade

immune detection such as the *Leishmania* trypanosome that uses antigenic variation to undermine sand fly immune defences (Sacks et al., 1995). These strategies are not uncommon, having evolved several times in parasites (Schmid-Hempel, 2005b), making invertebrate immune specificity highly beneficial, if not indispensable, to track changes in the parasite population and it is unsurprising that artificial methods with a generalist, ubiquitous pathogen invoke a broad-acting immune response.

Insects acquire the vast majority of pathogenic infections during activities such as foraging, feeding and grooming and not via cuticular wounding, with some exceptions such as specific fungi. For example, *Drosophila* is naturally infected with the gram-negative bacteria *Erwinia carotovora* whilst feeding on rotten plant matter, and the infection proliferates primarily in the fly's gut. Systemic expression of the AMP Drosomycin is 3 times lower when Ecc-15 (an *E.carotovora* strain) is fed to *Drosophila* larvae than when injected directly into the haemolymph (Basset et al., 2000). A much larger, 418-fold, difference was seen in *P.duboscqi* AMP expression in response to *E.carotovora* (Boulanger et al., 2004). In contrast to acute AMP expression after septic injury, natural infections induce temporal expression profiles that correlate with characteristic infection phases, such as when the mid-gut epithelium is breached or when the parasite matures to its next developmental stage. For example, *L.major* infections induce Defensin synthesis in *P.duboscqi* at day 10 when the parasite develops into its insect-specific promastigote form and is in abundance in the fly's gut (Boulanger et al., 2004).

Therefore, it is adavantageous to stimulate immunity with a parasite that the host has co-evolved since they have adapted to one another over time, and the response is more

reflective of the host's reaction to infection. Ecological immunologists have therefore simply exploited these natural, co-evolved associations and tested *how specifically* the host is differentiating between strains of the parasite by measuring variation in life-history traits.

4.4.2. Validating the use of life-history traits as measures of immunity

Critics have argued that life-history measures are not a valid representation of the immune response because their variation could result from any number of interactions between the host and parasite, not just the immune system, and therefore could not be interpreted as immune specificity (Hauton and Smith, 2007). The next section discusses whether the narrow mechanistic definition of immunity is justified. By its very nature, immunity is a whole-organism phenomenon encompassing all biological defences that prevent infection or minimise its consequences (Little et al., 2008), and behavioural modifications or life-history strategy alterations are one way to achieve this. For example, the nematode *Caenorhabditis elegans* detects and physically avoids substances produced by its bacterial pathogen *Serratia marcesens* (Pradel et al., 2007), mosquitoes can decrease pupation time in response to microsporidia infections to reduce the parasite's virulence (Koella et al., 1998), reproduction is initiated earlier in snails when infected with a castrating parasite (Lafferty, 1993) and beetles accelerate their development after a non-pathogenic immune challenge (Roth and Kurtz, 2008). However, one factor that unites all these prophylactic measures with the innate immune system is that their use incurs a cost met by diverting resources from other physiological functions, and these trade-offs may in turn cause a reduction in host fitness (Sheldon and Verhulst, 1996). For example, infection with *C.bombi* reduces the bumblebee's ability to learn colour and odour cues (Gegear et al., 2006) that is replicated with a non-

pathogenic immune elicitor, indicating a direct effect of the immune response (Mallon et al., 2003, Riddell and Mallon, 2006, Alghamdi et al., 2008). Chapter 1 established that bumblebee survival during *C.bombi* infection correlates with the intensity of the parasite infection. Numerous other studies have similar findings. For example, the larvae of *Drosophila melanogaster* lines artificially selected for resistance to an endoparasitoid show retarded growth and a lower survival rate when competing with wild-type larvae for food (Kraaijeveld and Godfray, 1997) and in honeybees there is a negative correlation between levels of worker Abaecin expression and colony productivity (Evans and Pettis, 2005b). Evidence of a cost can be context-dependent, such as under starvation conditions, because the host may otherwise compensate. In bumblebees, compensatory resource intake masks the effect of the immune response on workers (Moret and Schmid-Hempel, 2000, Riddell and Mallon, 2006). Conversely, greater investment in other functions can negatively affect immunity. Crickets (*Gryllus texensis*) show reduced immune function and increased disease susceptibility in response to multiple stressors (Adamo and Parsons, 2006), male damselflies (*Calopteryx splendens xanthostoma*) show a weak phenoloxidase response if they have invested heavily in melanin-based sexual signals (Siva-Jothy, 2000) and bumblebees (*Bombus terrestris*) react more strongly to a novel immune elicitor if prevented from foraging (König and Schmid-Hempel, 1995). The costs can be compounded by the indiscriminate action of cytotoxic molecules released during the immune response that cause self-damage to host cells and tissues and require resources for repair and regeneration. For example, stimulating a cellular immune response in the beetle, *Tenebrio molitor,* causes melanisation of both the "foreign" nylon implant and the surrounding Malpighian tubules (the insect equivalent of the kidney) and impairs the latter's function (Sadd and Siva-Jothy, 2006).

Conclusions

To summarise, this experiment has demonstrated that the active immune response is specific and dependent on the interaction between host and parasite genotype. This result mirrors the findings of previous ecological studies that have used indirect measures of the immune response such as cell counts or host survival. However, the underlying mechanisms regulating phenomenon have yet to be fully understood and their role in other innate immune phenomena such as immune memory is still uncertain.

5. Temporal dynamics of *B.terrestris* immune gene expression in response to infection with *C.bombi*

5.1. Introduction

In Chapter 4, it was established that AMP expression in response to *C.bombi* was specific and defined by the interaction between host and parasite genotypes. Compared to the extensive ecological understanding there is of the *Bombus*-*Crithidia* system, very little is known about how the bumblebee responds to the parasite immunologically and nothing is known about the dynamics of their interaction at the molecular level. It is known that standing levels of prophenoloxidase increase upon infection with *Crithidia* (Brown et al., 2003a) and that several quantitative trait loci are linked to immune defence against this parasite (Wilfert et al., 2007). Some clues to the immune response of *Bombus* may come from other insect/trypanosomatid models. *Drosophila* expresses anti-microbial peptides (AMPs) systemically in response to natural, *per os* infection with *Crithidia* (Boulanger et al., 2001) as do *Glossina* (Tsetse flies) in response to trypanosomes (Hu and Aksoy, 2006). Reactive oxygen intermediates from *Anopheles* may exert cytotoxic effects on *Plasmodium* (Kumar, 2003). Lectins could also play a major role; in *Rhodnius prolixus* they recognise distinct carbohydrate moieties expressed on the surface of *Trypanosoma cruzi* (Mello et al., 1996), and in the mid-gut of *Glossina* (sp.) they aid parasite eradication (Welburn et al., 1994), and they are induced after *Plasmodium* infection in *Anopheles* (Dimopoulos et al., 1997). Melanisation and encapsulation are also important for the removal of parasites from the haemolymph (review, (Richman and Kafatos, 1996)) but their role against *C.bombi* is questionable, as this flagellate does not migrate out of the gut (Brown et al., 2003a).

To begin investigating the molecular basis of the immune response to *C.bombi*, a search was first carried out to identify genes expressed as part of the immune response to the parasite using subtractive suppression hybridisation (SSH). This is a powerful technique designed to compare the transcriptional profiles (represented as cDNA) of two experimental populations and identify sequences that are differentially expressed in each population (Diatchenko et al., 1996). One cDNA population, containing differentially expressed transcripts (tester population), is modified with adaptors before hybridisation with the second (driver), cDNA population, to allow homologous sequences from the two samples (i.e., non-differentially expressed cDNA) to hybridise to one another, thus sequestering them from the process. The remaining unhybridised molecules from each tester group, representing up-regulated genes, undergo a second hybridisation to acquire a second adaptor sequence. Subsequently, PCR is used to enrich for these differentially expressed sequences and suppress the amplification of any remaining non-differentially expressed sequences that were not removed by the initial subtractive hybridisation. In this experiment, the expression of SSH-derived genes, plus some additional candidates selected from the literature, was then determined throughout the early stages of *C.bombi* infection using quantitative PCR (qPCR).

5.2. Methods

All molecular protocols relating to SSH and qPCR analysis are described briefly here and outlined in greater detail in Chapter 2.

5.2.1. Subtractive suppression hybridisation – SSH

See Figure 5.1 for outline of the SSH process.

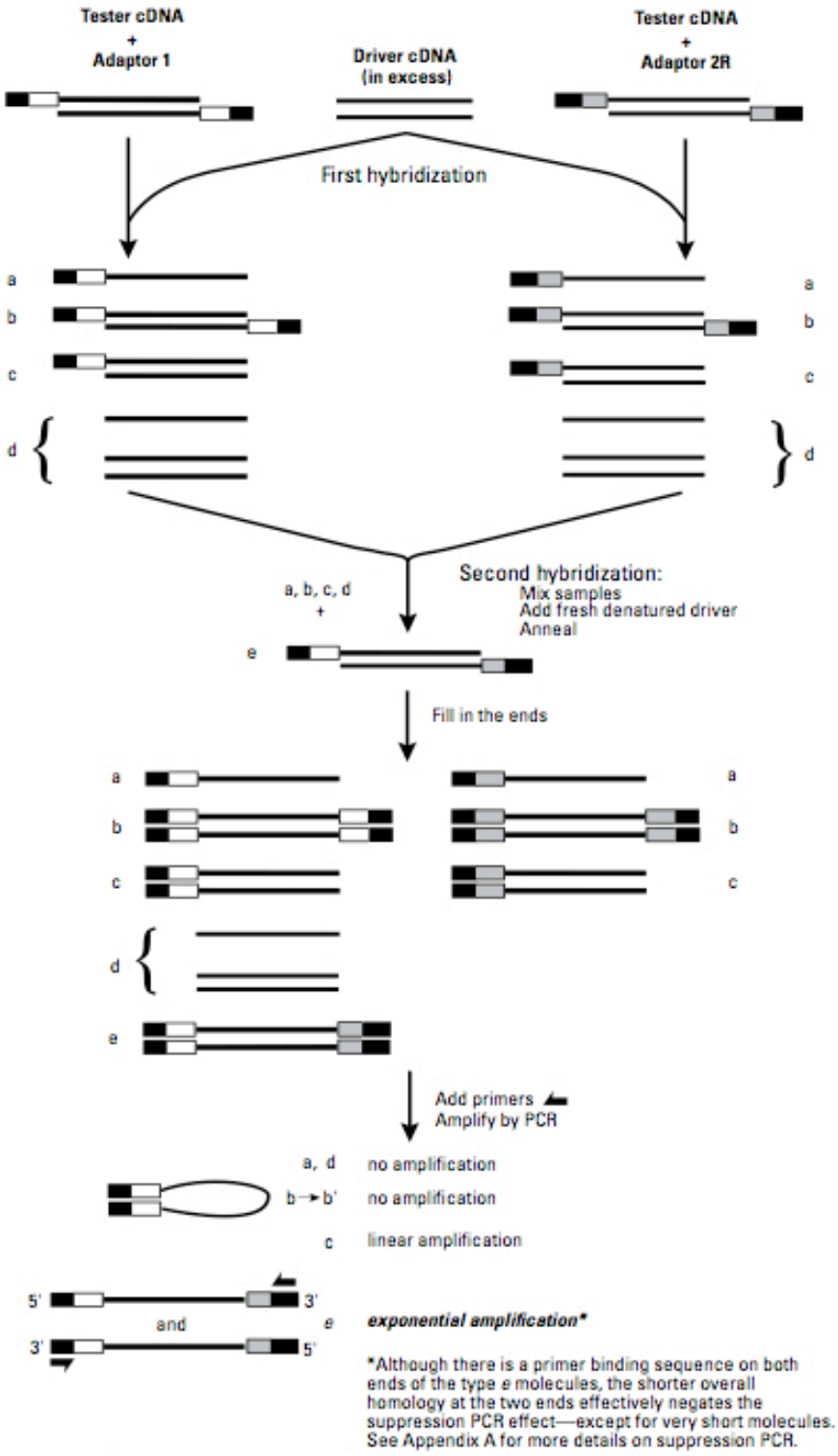

Figure 5.1 The SSH process (from Clontech SSH kit manual, 1997). During the first hybridisation, the two types of adaptor-linked tester cDNA are mixed with driver cDNA. Homologous (i.e. non-differentially expressed) sequences bind (c), enriching the single-stranded pool for differentially expressed cDNA molecules (a). Other sequence pairs or forms can also persist in the mix (b&d). The 2 tester pools are mixed together allowing single-stranded molecules to hybridise and obtain the second adaptor sequence (e). During 2 successive rounds of PCR, e-type molecules will amplify exponentially whilst molecules with 2 identical adaptors will form secondary structures that inhibit amplification (b-b') and remaining single-adaptor-linked cDNA will amplify linearly. PCR thus enriches and normalises for differentially expressed sequences in the tester cDNA population.

Infection protocol

Age-controlled immune naïve bees from a single commercially-reared colony were fed a mixed strain inoculum of two *C.bombi* strains collected from the faeces of infected workers. Both strains were equally represented in the final inoculum to produce an overall concentration of 1000 cells/µl in 50% apiary syrup, and 20µl was fed to each bee. Those bees that did not imbibe the solution were not used. After infection, bees were maintained under standard conditions as before. Infected and non-infected bees were sacrificed at 1,2,3,4,5,24,26 and 48h post-infection by freezing in liquid nitrogen, before storage at -80°C to preserve RNA. Studies in *Drosophila* with a natural gut-exclusive pathogen suggest that the majority of transcriptional responses occur within 24h of infection (Vodovar et al., 2005). Samples were collected during the growth phase of infection where cell numbers are steadily increasing and not oscillating as seen later in infection (Schmid-Hempel and Schmid-Hempel, 1993). This increased the chance that bees were responding to similar levels of infection throughout the time course. 4 to 5 biological replicates were taken at each time point in infected and non-infected groups.

Total RNA and mRNA extraction

Total RNA was collected from the abdomens of each bee and therefore examined AMP expression in the haemolymph and tissues such as the gut and fat body. Abdomens from individuals at each time-point were pooled together and total RNA was extracted from 100mg of the mixed homogenised tissue. The total RNA sample was used to extract mRNA, i.e. the transcriptome of expressed genes, in preparation for SSH using the PCR-select cDNA subtraction kit from Clontech (UK). An mRNA sample of human

skeletal muscle was supplied with the Clontech SSH kit for use as a positive control throughout the process.

cDNA synthesis

Although the initial cDNA synthesis using the kit was successful, subsequent technical errors in the downstream ligation step required that the entire process be repeated with a fresh extract of RNA from the pooled tissue sample (and kit control mRNA). Consequently, the reagents and enzymes used in cDNA synthesis, RsaI digestion and adaptor ligation were replaced and sourced from other companies (as described in Section 2.4.1), as they were unavailable from the SSH-kit manufacturer. All steps were still prepared and carried out according to the original Clontech protocols and once at the hybridisation stage the SSH process was completed using the reagents supplied with the Clontech SSH kit.

First-strand synthesis of all samples was carried out with M-MLV reverse transcriptase (Promega, UK) as outlined in Section 2.4.1. For second strand cDNA synthesis, buffers and enzyme cocktails were sourced from NEB (UK) (Section 2.4.2). The resultant cDNA population represented genes transcribed during infection, including constitutively expressed genes that were not differentially expressed in response to infection.

Rsa I digestion of cDNA

Infected, non-infected and control cDNA samples were digested with the restriction endonuclease Rsa I (NEB, UK) to create shorter, blunt-ended fragments that were suitable for efficient hybridisation and adaptor ligation (Section 2.5.1). An aliquot of

these samples was reserved for use as driver during SSH, with the remainder earmarked for use as tester cDNA after ligation of adaptors to the blunt ends.

Adaptor ligation

Each *tester* population i.e. infected (E), non-infected (N) and control samples, was ligated to 1 of 2 adaptors (Ad1 and Ad2, provided by Clontech) using DNA ligase (NEB, UK), to create two sub-populations of adaptor-ligated cDNA for each sample: E-Ad1, E-Ad2; N-Ad1, N-Ad2; and C-Ad1, C-Ad2 (Figure 5.2; Section 2.5.2). Adaptors are simply small oligonucleotide sequences containing 2 primer sites and inverted terminal repeats to implement amplification and suppression respectively during PCR, and the targeted enrichment of differentially expressed sequences.

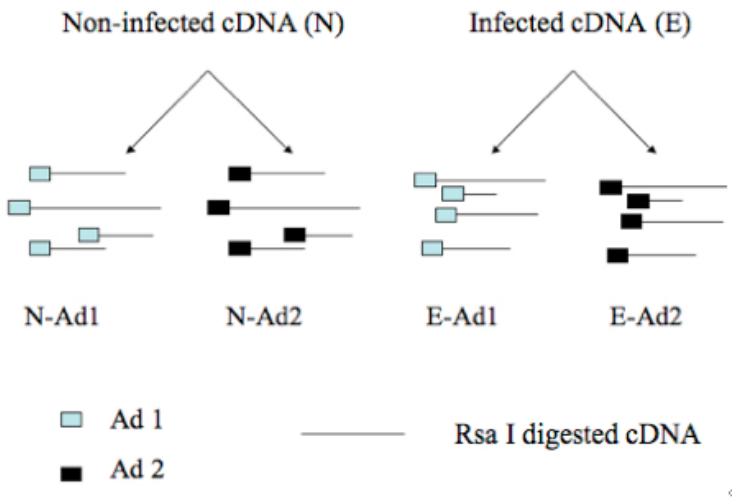

Figure 5.2 Adaptor ligation to infected (E) and non-infected (N) cDNA populations. Both cDNA samples were divided into 2 and ligated to either Adaptor 1 (Ad1) or Adaptor 2 (Ad2).

Ligation efficiency analysis was assessed using the actin house-keeping primers for *Bombus terrestris* samples, according to the manufacturer's (Clontech, UK) instructions (Section 2.5.3). Essentially, two PCRs were performed – one, using an adaptor-specific primer (Clontech, UK) and a housekeeping primer, and a second using both the

housekeeping gene's primers. The products of both PCRs should be of similar quantity, indicating that adaptors have been ligated to a high percentage of cDNA strands.

SSH hybridisations

Hybridisations were performed with the Clontech subtraction kit protocols and reagents. Initially, each tester population was mixed with excess driver (non-infected cDNA) to sequester single stranded (ss) non-differentially expressed sequences from the tester (infected) cDNA population by hybridisation to its homologous sequence. This process enriched for heterologous, i.e. differentially expressed, sequences in the sample. Primary hybridisations for each experimental sample were then mixed together, now allowing *differentially expressed* ss cDNAs to hybridise with their homologous sequence and acquire the second primer site needed for exponential amplification during PCR. Whilst held under annealing conditions, fresh driver (non-infected cDNA) was also added to further enrich for differentially expressed sequences. Three subtractions were performed; a forward subtraction using infected cDNA as the tester and non-infected cDNA as driver, a reverse subtraction using non-infected tester-cDNA as tester and infected cDNA as driver, and the control subtraction. In effect, transcripts from the forward subtraction would represent genes up-regulated in response to *C.bombi*. Sequences from the reverse-subtraction would primarily be used to screen the forward subtracted cDNA library and identify false positives, i.e. sequences that were not truly up-regulated in response to *C.bombi* infection. This process is detailed fully in Section 2.5.4. and 2.5.5.

SSH PCRs & creation of a subtracted cDNA library

To enrich for differentially expressed molecules and normalise the levels of individual transcripts in the sample, hybridised sequences were amplified in 2 rounds of PCR using the Advantage cDNA Polymerase Mix (Clontech, UK) and cDNA subtraction kit protocols (Clontech, UK). Before PCR commenced, reactions were incubated at 75°C to extend the adaptors and complete the primer binding sites. Differentially expressed molecules were selectively amplified exponentially by a suppression-PCR effect on non-target sequences with identical adaptors (non-differentially expressed sequences) and linear amplification of sequences with only one adaptor (Section 2.5.6). A secondary PCR using nested primer sites was performed to enrich further for differentially expressed sequences and reduce background (Section 2.5.7).

The secondary PCR products were cloned and transformed into DH5α competent *E.coli*. Individual clones containing an insert were grown overnight and stored in glycerol at -80ºC, creating a subtracted cDNA library of differentially expressed sequences from infected and non-infected bees.

SSH subtraction efficiency analysis

Subtraction analysis tested for the presence of non-differentially expressed genes in the subtracted PCR product, using a housekeeping gene (*Actin*) (Section 2.5.8). Subtraction analysis used semi-quantitative PCR to measure the level of this housekeeping gene in the subtracted PCR sample. If the subtraction efficiency was good, amplification would occur in the later cycles of the subtracted cDNA PCR, relative to unsubtracted (pre-SSH) cDNA samples.

Differential screening

Although the SSH process greatly enriches for differentially expressed genes, false-positives (i.e. sequences common to both infected and non-infected samples) can still appear if particular genes are constitutively expressed at high levels, or if there is minimal differential expression between the two cDNA populations. Differential screening was therefore needed to identify any false positives and remove them before further analysis.

To begin screening the entire forward-subtracted library was sequenced to identify immune-related sequences that were up-regulated after infection (Section 2.13). The reverse-subtracted library itself is primarily used for probe-based differential screening, but this technique had been abandoned after preliminary tests did not work (Section 2.5.13). For this reason the reverse-subtracted library was not sequenced. Fourteen sequences were randomly selected after screening the forward-subtracted library and analysed (genes marked with an asterisk in Table 1) using qPCR to compare their expression levels in infected and non-infected bees and therefore identify truly up-regulated genes. This approach to screening has been successfully used in other SSH studies (He et al., 2004). Again, cDNA was created from the same, pooled tissue homogenates used for SSH, and qPCR primers were designed on the sequenced clone. Three technical replicates were run for every gene analysed on non-infected and infected cDNA, and C_T values were normalised with the housekeeping gene RPS5.

5.2.2. Temporal dynamics

Immune genes showing differential expression between infected and non-infected samples from SSH were studied further to elucidate their temporal expression throughout the early stages of *C.bombi* infection.

Infection protocol

Experiments were carried out on two commercially-reared colonies (Koppert Biological Systems, UK). Ideally, gene expression would have been compared between infected and control bees from the same colony, but this was not possible at the time and therefore two different colonies were used to collect control and infected samples. In one colony, age-controlled, 4-5 day old naïve bees were fed a mixed strain inoculum of four *C.bombi* strains collected from the faeces of infected workers. Each strain was equally represented in the final inoculum to produce an overall concentration of 1000 cells/µl in 50% apiary syrup. 15µl of the inoculum was fed to individual bees, and those that did not imbibe the solution were not used. Bees were always infected around 1pm each day to control for any effect of time of day on gene expression. Samples were collected during the growth phase of infection where cell numbers are steadily increasing and not oscillating as seen later in infection (Schmid-Hempel and Schmid-Hempel, 1993). This increased the chance that bees were responding to similar levels of infection throughout the time course. After infection, bees were maintained under standard conditions as before. As stated previously, uinfected age-controlled bees were collected from a separate colony to provide a baseline for gene expression. These controls were also 4-5 days old and were maintained under standard conditions until their sacrifice at the appropriate time-point. Infected and non-infected (control) bees were collected and sacrificed at 1,2,4,8,12,24,36 and 48h post-infection, by freezing in liquid nitrogen, before storage at -80°C to preserve RNA. Bees were all infected around

1pm each day to control for time effects on gene expression. Time-points for sample collection were adapted from Korner and Schmid-Hempel's temporal study of immune activity in response to *C.bombi* (Korner and Schmid-Hempel, 2004b) to give more balanced coverage of the first 24h, since it is suggested that the majority of transcriptional responses occur within this time-frame (Roxström-Lindquist, 2004).

qPCR

Total RNA was extracted and prepared from individual homogenised abdomens before synthesising first-strand cDNA (Section 2.3.1). cDNA samples were then diluted 10-fold in ddH_2O, and 5µl of the diluted cDNA was used in each 25µl qPCR reaction. Each sample was tested with the 5 immune genes identified by SSH and the house-keeping gene RPS5 (Evans and Pettis, 2005b) (Section 2.6.2). The expression of *Abaecin*, *Defensin* and *Hymenoptaecin* (see Chapter 4 for primer design) and *Galectin* (A lectin-see Chapter 5 for primer design) was also tested since these genes have been found to be up-regulated during protozoan infection (Dimopoulos, 1996, Boulanger et al., 2004, Dimopoulos et al., 1997).

Genes were first tested on samples from 1 to 24h post-infection in order to run all time-points in one qPCR and minimise the introduction of variation from differing reaction efficiency between qPCR runs. This approach allowed the identification of genes showing differential expression (greater than two fold) between infected and non-infected bees. Studies have shown that pathogens generally begin to induce changes in the expression of immune-related genes within 24h of host infection (Roxström-Lindquist, 2004, Vodovar et al., 2005). This point was incorporated into the experiment by ensuring that 75% of time-points covered the 24h period. Therefore, only those

genes showing differential expression across the first 24h were also analysed at the 36 and 48h time-points since this would help identify when expression began to decrease or plateau. Genes without any clear suggestion of differential expression were only analysed over 1-24h post-infection.

For each gene, two technical replicates were run per sample to yield an average C_T value. C_T values were measured during the exponential phase of gene amplification. Samples were only included in further analysis if their technical replicates were within 0.5 of one another. QPCR was repeated for any samples with replicates outside this margin. An average of the 2 technical replicates was taken so that each biological sample was represented by an average C_T value. Sample ΔC_T was calculated by deducting each C_T value, in control and treatment groups, from the lowest C_T value in the control (naive) samples in both housekeeping genes and the gene under investigation ($C_{T\ control} - C_{T\ sample}$). Fold change in expression was calculated with the $2^{(-\Delta CT)}$ approximation method, using the housekeeping gene's C_T data to normalise the immune gene C_T value on account of varying starting levels of cDNA amongst samples. Using this method, expression between non-infected and infected bees could be compared. Ribosomal protein S5 (RPS5) was used as the housekeeping gene in all the qPCR experiments as it shows moderate expression in honeybees (Evans and Wheeler, 2001) and is consistently used as a control gene in studies of the honeybee's transcriptional immune response to infection (Evans and Pettis, 2005a, Evans, 2004). The resulting fold-change values were log-transformed to fit the data to a normal distribution prior to statistical analysis. As individual bees were sampled at each time-point, a test incorporating repeated measures was not needed, and ANOVAs were carried out on each immune gene with fold-change as the dependent variable and time

(h post-infection) and infection status (non-infected or infected) as independent variables.

Galectin primer design

The *Galectin* primers were designed in the same way as detailed for the AMPs in Chapter 4. To identify conserved regions within the *Galectin* sequence that could be used as a basis for primer design, the gene transcript was first identified in the honeybee *Apis mellifera* (accession number XM_392379) from the NCBI database, and used as the subject of a search for highly similar sequences in other insect species using BLASTn. Sequences were identified in *Anopheles gambiae*, *Anopheles stephensi* and *Haliotis discus* (accession numbers XM_309359, AY162251 and EF392832 respectively) and aligned with the *Apis* sequence using CLC sequence viewer 4 for Macintosh. Primers were designed to highly conserved nucleotide sequences and yielded the predicted 600bp product from the cDNA of an infected bee (0.5µl cDNA, PCR: 95ºC 3min, 34 cycles of 1min at 94ºC; 1min 50ºC; 30sec 72ºC and final 5min at 72ºC). The amplicon was sequenced (GenBank accession number: GQ281705) and its identity checked using BLASTn, before designing qPCR primers targeting a 100bp *Galectin* fragment.

5.3. Results

5.3.1. SSH

Subtraction analysis indicated that PCR amplification of the actin housekeeping gene was only successful from unsubtracted cDNA, indicating that SSH had efficiently removed non-differentially expressed sequences. A total of 82 clones were sequenced from the forward subtracted library (Figure 5.3), 75% of which had homology to known sequences within the NCBI database, identified using BLASTx and tBLASTx. Also, 29% of these sequences had previously been shown to be up-regulated during infection in other invertebrate species these genes and were categorised as having potential links with immunity (Table 5.1). Some transcripts (17%) were represented in more than one clone. Signalling molecules were included in the immune category if the gene was part of a larger super-family with diverse physiological roles. Sequences of ribosomal origins (8%) were excluded from further analysis because these are dedicated genes that code for the transcriptional machinery.

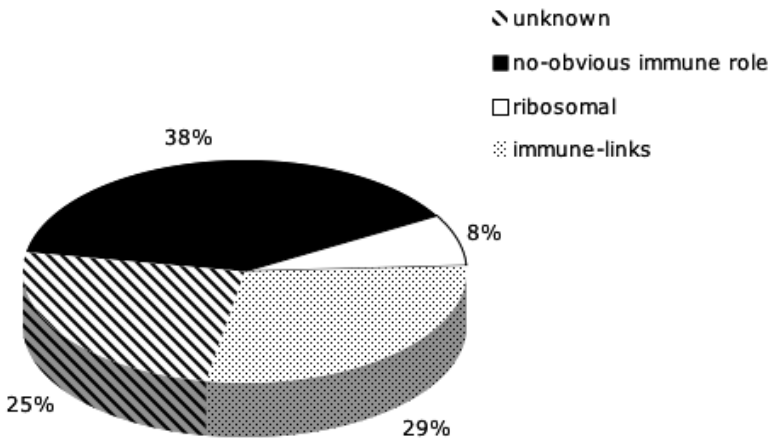

Figure 5.3 The proportion of clones from the forward SSH with links to immunity. Genes with potential immune links were identified by searching previous gene expression studies. SSH compared the gene expression between non-infected and infected bees pooled from 1,2,3,4,5,24,26 and 48h after infection with *C.bombi.* 4-5 bees were collected at each time-point in non-infected and infected groups.

Table 5.1 Summary of sequences from forward SSH with links to immunity, and their potential roles. Sequences were identified using tBLASTx and BLASTx and only considered if the % identity at a given section of sequence was greater than 25%. Asterisks denote sequences that were screened by qPCR for differential expression between non-infected and infected bees4-5 bees were collected for each time point in infected and non-infected bees.

Identified clone	Species, accession number, % identity	Function and proposed immune role	Examples
Windbeutel-like	*Apis mellifera*, XM_001120162, 90%	Primarily regulates embryonic dorsal-ventral patterning via Toll, but similar sequence found up-regulated during the immune response in *Drosophila*	(Apidianakis et al., 2005)
RhoGAP	*Apis mellifera,* XM_392788.3, 64%	Signalling and cell migration	(Tcherkezian and Lamarche-Vane, 2007){
Cathespin-like molecule *	*Periplanta americana*, BAA86911,	Peptidase-activity. Some forms secreted by haemocytes after immune challenge in the flesh fly *Sarcophaga*	(Natori et al., 1999)
Peroxidase precursor *	*Apis mellifera*, XM_623937.2, 54%	Implicated in proPO pathway activation and encapsulation response in crayfish.	(Cerenius and Soderhall, 2004)
Hypothetical; Exchange factor for Rho/Rac/Cdc42-like GTPases	*Nasonia vitripennis*, XM_001605937.1, 52%	Involved in many physiological processes by mediating signal transduction. Can mediate actin polymerisation and cytoskeletal changes, has been targeted by pathogen virulence factors	(Tcherkezian and Lamarche-Vane, 2007, Colinet et al., 2007)
Vitellogenin *	*Apis mellifera*, AJ517411.1, 54%	An anti-oxidant. Promotes longevity in queens and workers (honeybees), also implicated in immune defence regulation	(Amdam et al., 2004)
Calcineurin *	*Apis mellifera*, XM_394519.3, >97%	A protein phosphatase. Linked to the T-cell response in vertebrates. Promotes induction of the immune response and can mediate NO production in *Drosophila*	(Dijkers and Ofarrell, 2007)

Identified clone	Species, accession number, % identity	Function and proposed immune role	Examples
Paxillin	*Apis mellifera*, XM_624305, 98%	A focal adhesion protein in vertebrates. Some links to Toll-mediated processes in rat macrophages. Can regulate local cytoskeletal changes	(Hazeki et al., 2003, Schaller, 2001)
IK2 *	*Apis mellifera,* XM_396937.3, 95%	Intracellular signalling molecule with kinase activity. Liberates NF-κB proteins from inhibitors	(Hacker and Karin, 2006)
HDLBP *	*Apis mellifera,* XM_001122778.1, 97%	A possible stress-responsive molecule but also found to be functionally homologous to GNBP in the white shrimp *Penaeus vannamei*. May bind bacterial constituents such as LPS	(Yepiz-Plascencia et al., 1998, Netea et al., 1998)
Serpin-like molecule*	*Bombus ignitus*, DQ489309, 95%	Large family of serine-protease inhibitors (Serpins). Some with a role in *Drosophila* immunity have been found to be evolving under positive selection	(Cerenius and Soderhall, 2004, Jiggins and Kim, 2007)
Ran-type (Ras-related molecule in the nucleus) molecule	*Apis mellifera,* XM_001120684, 89%	Signalling molecule. Has been found up-regulated after immune challenge in the shrimp *Penaeus japonicus*	(He et al., 2004)
Sur-8: Ras signalling element*	*Apis mellifera*, XM_396017.2, 100%	Regulator of Ras signalling. Stabilises protein complexes in the mitogen-activated protein kinase (MAPK) pathway; a major signal transduction pathway with some putative immune roles. The functional significance of many Ras proteins is unclear.	(Lemaitre and Hoffmann, 2007)

Identified clone	Species, accession number, % identity	Function and proposed immune role	Examples
CCT5: T-complex chaperonin 5-like molecule*	*Apis mellifera*. XM_393315.3, 97%	A molecular chaperone protein. Found in gene expression screen after infection in shrimp *Penaeus japonicus*, and is implicated in actin and tubulin interactions, possibly in phagocyte activity	(Loseva and Engstrom, 2004, He et al., 2004)
Conserved zinc finger binding motif in Ran-type molecules including Tamo*	*Apis mellifera*, XP_395588.2, 38% Tamo: *Drosophila melanogaster* NM_138045.2, 57% (2009)	Tamo regulates nuclear import of the Rel protein Dorsal, and accumulates in cells after immune response in *Drosophila*	(Minakhina et al., 2003)
Fat-spondin-like*	*Apis mellifera*, XR_014987.1, 92%	Serine-type endopeptidase inhibitor (serpin), and regulator of cell adhesion. Has been found up-regulated in response to infection in *Drosophila*	(De Gregorio et al., 2001)
Aconitase *	*Apis mellifera,* XM_391994, 94%	A component of respiratory pathway that can be depleted after NO activity – up-regulated to replenish protein?	(Nappi and Ottaviani, 2000)
Plexin A-like*	*Apis mellifera*, XM_394261, *Drosophila melanogaster,* NM_166806, 87%	Secreted and membrane-associated proteins with diverse physiological roles. Initially identified for major role in neuronal guidance, but also have regulatory roles in vertebrate adaptive immunity.	(Takegahara et al., 2006, Suzuki et al., 2008, Yamamoto et al., 2008)
Activator of Heat-shock protein-90 domain*	*Anopheles gambiae,* XP_314487, 100%	Stress response	(Wallin et al., 2002)

Identified clone	Species, accession number, % identity	Function and proposed immune role	Examples
Trypsin-like serine-endopeptidase / kallikrein-like molecule.	*Apis mellifera*, XM_624810.2, 70%	In mammals kallikrein responds to septic shock and initiates inflammation response. A similar role is postulated in *Drosophila*	(Irving et al., 2001)

5.3.2. Differential screen using qPCR

Using qPCR, expression of 14 immune genes isolated by SSH was analysed in non-infected and *C.bombi*-infected bees from a single colony. Of these genes 5 showed 3-8-fold up-regulation, representing the accumulative expression at a range of times from one to 48h after *C.bombi* infection (Table 5.2). The other eight genes did not show any up-regulation, suggesting that these were likely to be false positives.

Table 5.1 Immune genes showing up-regulation in infected bees relative to non-infected controls. Samples were pooled over 1,2,3,4,5,24,26, and 48hpost-infection.

Gene	Fold-change in expression
Vitellogenin	None
HDLBP	None
Fat-spondin	None
Cathespin	None
Serpin	None
Aconitase	None
Sur8	None
Hsp90	None
Ran-type molecule	None
IK2	+3
Peroxidase	+3
Calcineurin	+8
Tamo-like	+7
Plexin A	+6

5.3.3. Temporal expression of immune genes

Temporal expression of the five genes identified by SSH, plus three AMPs (*Abaecin*, *Defensin* and *Hymenoptaecin*) and a lectin (*Galectin*) were examined at 1,2,4,8,12,24,36 and 48h after *C.bombi* infection. A total of 44-45 individuals were analysed per gene over 1-48h after *C.bombi* infection, 50% of which were non-infected controls from a separate colony. 2-3 bees were collected per time-point, per colony, for infected and non-infected treatment groups. Fold change-values were transformed before statistical analysis to fit the data to a normal distribution. Though some temporal expression patterns seemed apparent in *Tamo-like, IK2, Calcineurin, Cathespin*, *Galectin* and *Plexin A* (Figures 5.6 - 5.8), there were no significant effects (ANOVA: $P = >0.05$) of time, infection status (except *Tamo-like*) or time*status interactions on their expression in the ANOVA.

Time had a significant effect on AMP expression (*Abaecin*: $F_{7,44} = 3.81$, $P = 0.0048$; *Hymenoptaecin*: $F_{7,43} = 4.46$, $P = 0.0019$; *Defensin*: $F_{7,44} = 4.85$, $P = 0.0010$) as did infection status (i.e. infected or non-infected) on Tamo (Tamo-like: $F_{1,44} = 5.07$, $P = 0.0320$). *Abaecin* and *Defensin* expression also showed significant time*infection status interactions (*Abaecin*: $F_{7,44} = 3.88$, $P = 0.0043$; *Defensin*: $F_{7,44,} = 2.52$, $P = 0.0372$) as did *Peroxidase* ($F_{5,33} = 2.71$, $P = 0.0470$) (see Figures 5.4 and 5.5). This interaction indicates that AMPs and *Peroxidase* expression do differ over time according to the infection status of the bee. Statistically, *Hymenoptaecin* was not significantly up-regulated throughout *C.bombi* infection ($F_{7,44} = 2.17$, $P = 0.0689$), even though a graphical plot of the expression data suggests that it is strongly induced after 12h (Figure 5.5), similar to *Defensin* and *Abaecin*, and that it is more strongly induced relative to these two genes (Figure 5.4).

Abaecin, *Defensin* and *Hymenoptaecin* all showed a high degree of variability in their expression, and were found to differ by >1000-fold between individual bees in both treatment (infected or non-infected) groups, despite controlling for age and time of infection. Variance was equally high between non-infected and infected bees was not significantly different (Brown-Forsythe test for unequal variance, *Defensin*: $F_{23,20}$ = 1.059, P = 0.8959; *Abaecin*: $F_{23,20}$ = 0.759, P = 0.5291; *Hymenoptaecin*: $F_{23,19}$ = 1.719, P = 0.2250) (Figure 5.3).

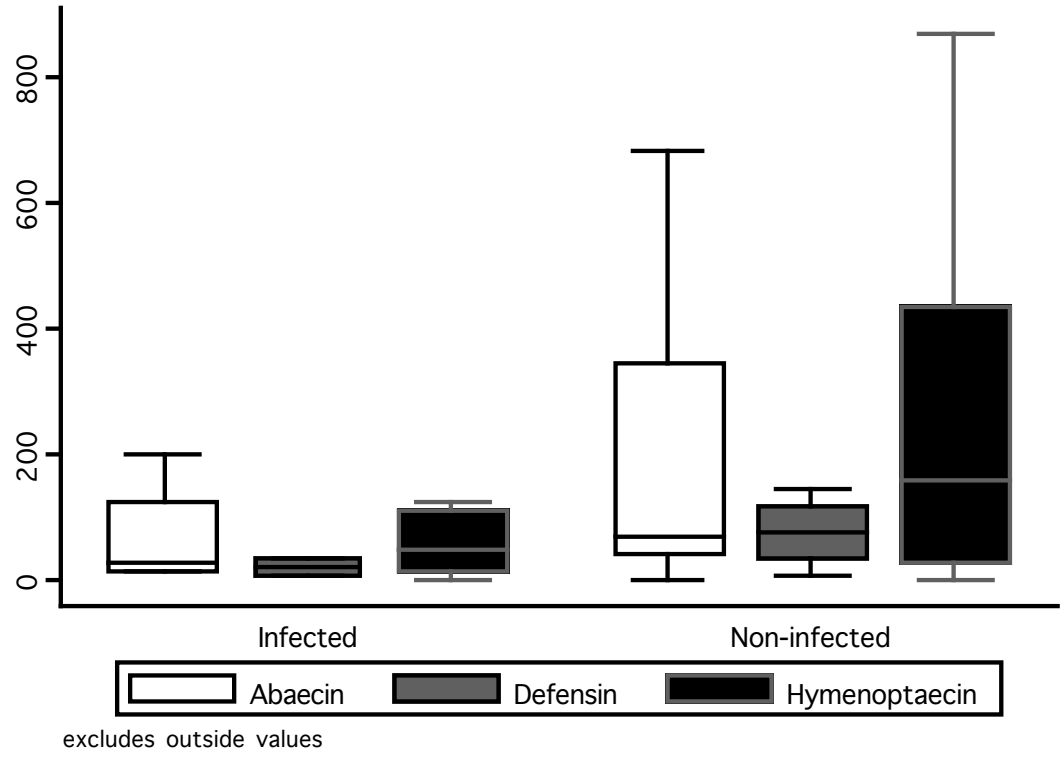

Figure 5.3 Box plot to illustrate the spread of AMP expression data in non-infected and infected bees. Data were pooled across all time-points. Total infected n= 24; non-infected n=21. Top and bottom bars of box represent upper (75%) and lower (25%) quartiles respectively and median denoted by middle bar. Total bar length represents middle 50% of data. Minimum and maximum data values represented by whiskers, excluding outliers. Potential outliers are omitted to prevent compression of graph. All 3 AMPs showed large variation in expression in both treatment groups but there was no significant difference in variance between infected and non-infected bees (Brown-Forsythe test for unequal variance, *Defensin*: $F_{23,20} = 1.059$, $P = 0.8959$; *Abaecin*: $F_{23,20} = 0.759$, $P = 0.5291$; *Hymenoptaecin*: $F_{23,19} = 1.719$, $P = 0.2250$).

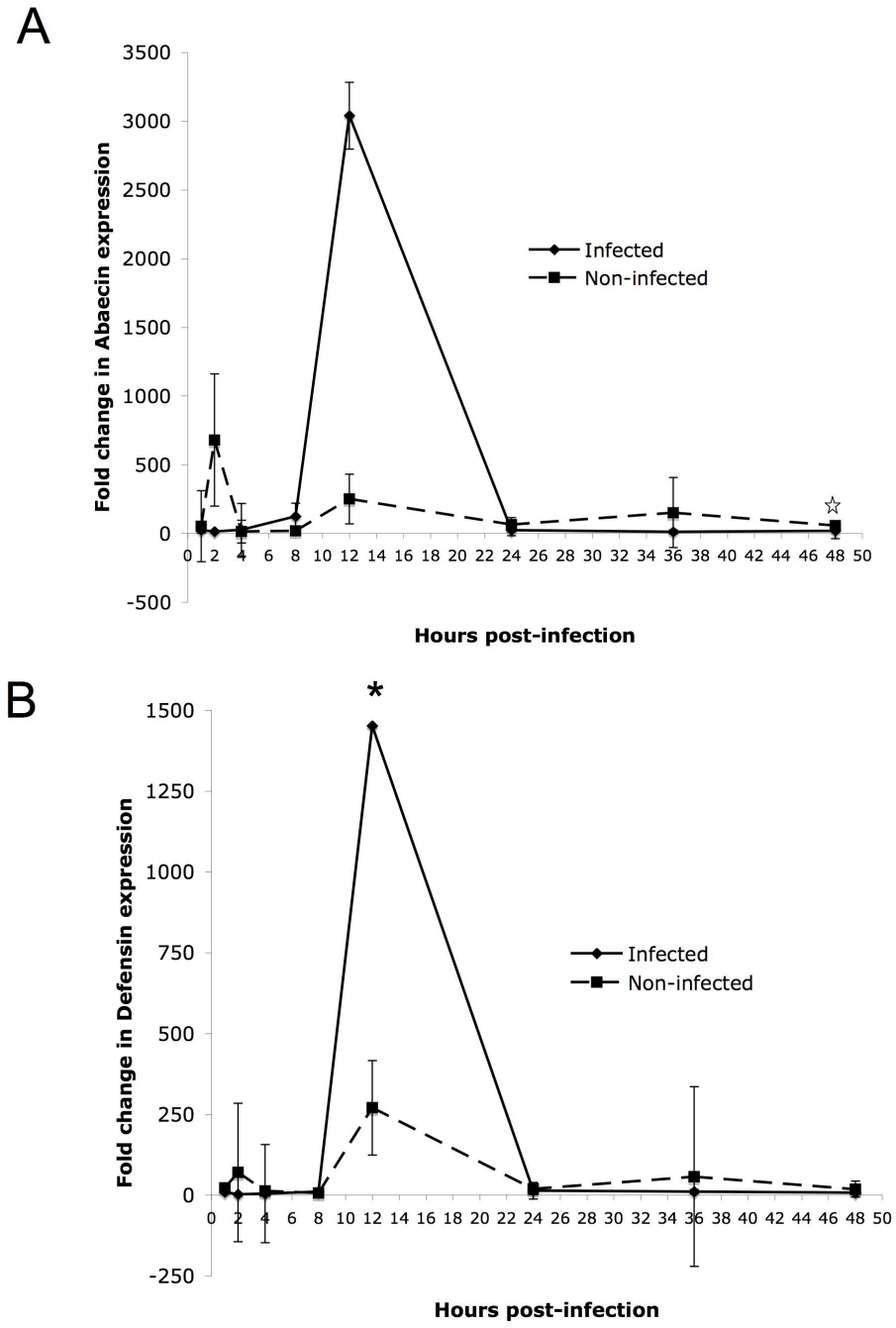

Figure 5.4 Temporal expression of Abaecin (A) and Defensin (B) during *Crithidia* infection. Gene expression was sampled in workers from 1-48h after imbibing a mixed-strain *C.bombi* infection. Each point represents median fold change of 2-3 infected workers. Non-infected and infected workers were from different colonies. Samples that amplified poorly were omitted from analysis. (A) used a total of n=25 infected and n=22 non-infected workers and (B) n=25 infected and n=22 non-infected workers. Error bars represent standard deviation (SD). Large SDs (*) are omitted to prevent compression of the graph. Stars denote controls with one sample. The second sample at these time-points was used to normalise the Ct values.

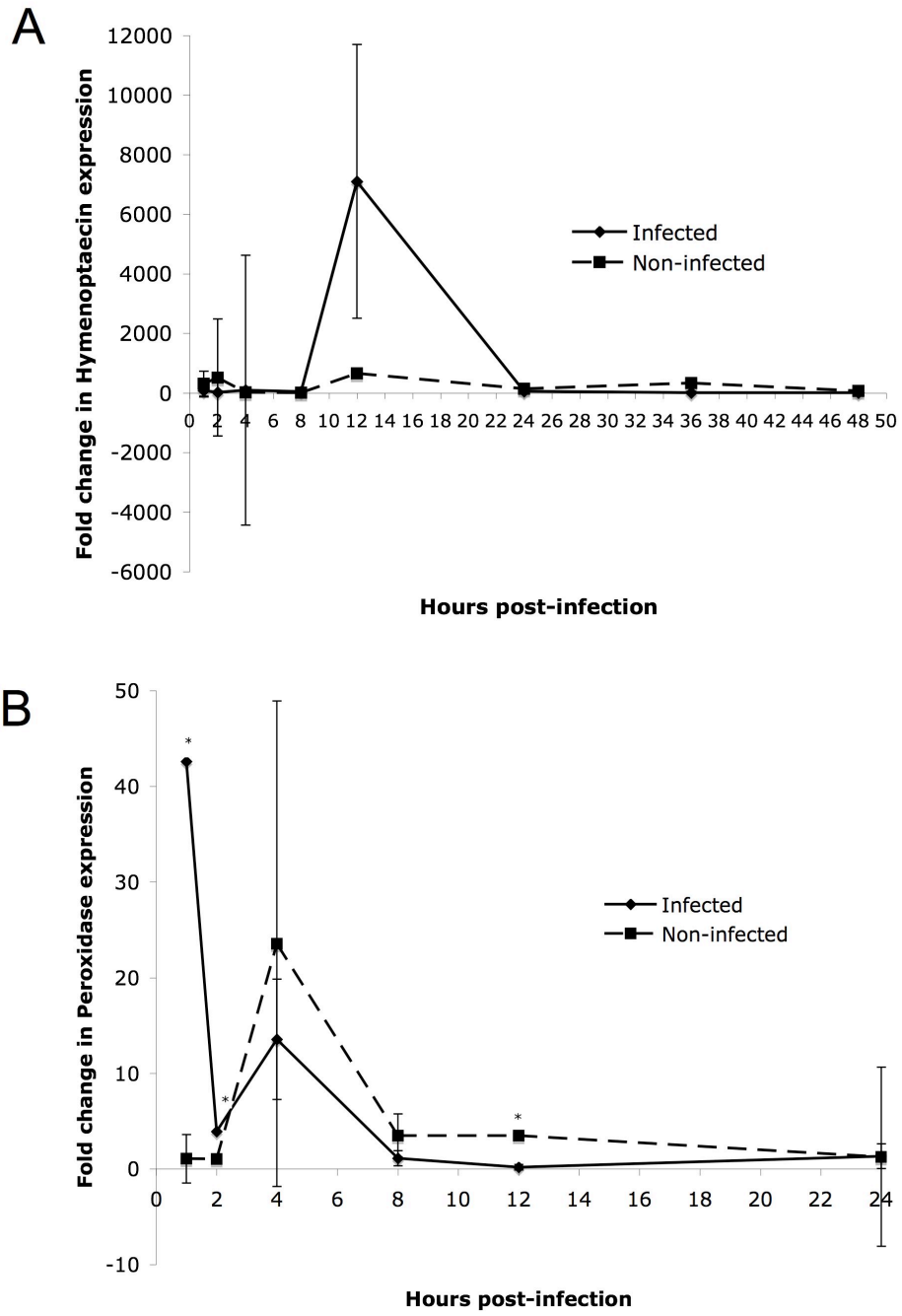

Figure 5.5 Temporal expression of Hymenoptaecin (A) and Peroxidase (B) during *Crithidia* infection. Gene expression was sampled in workers from 1-24h after imbibing a mixed-strain *C.bombi* infection. Each point represents median fold change of 2-3 infected workers. Non-infected and infected workers were from different colonies. Samples that amplified poorly were omitted from analysis. (A) used a total of n=25 infected and n=20 non-infected workers and (B) n=19 infected and n=17 non-infected workers. Error bars represent standard deviation (SD). Large SDs (*) are omitted to prevent compression of the graph.

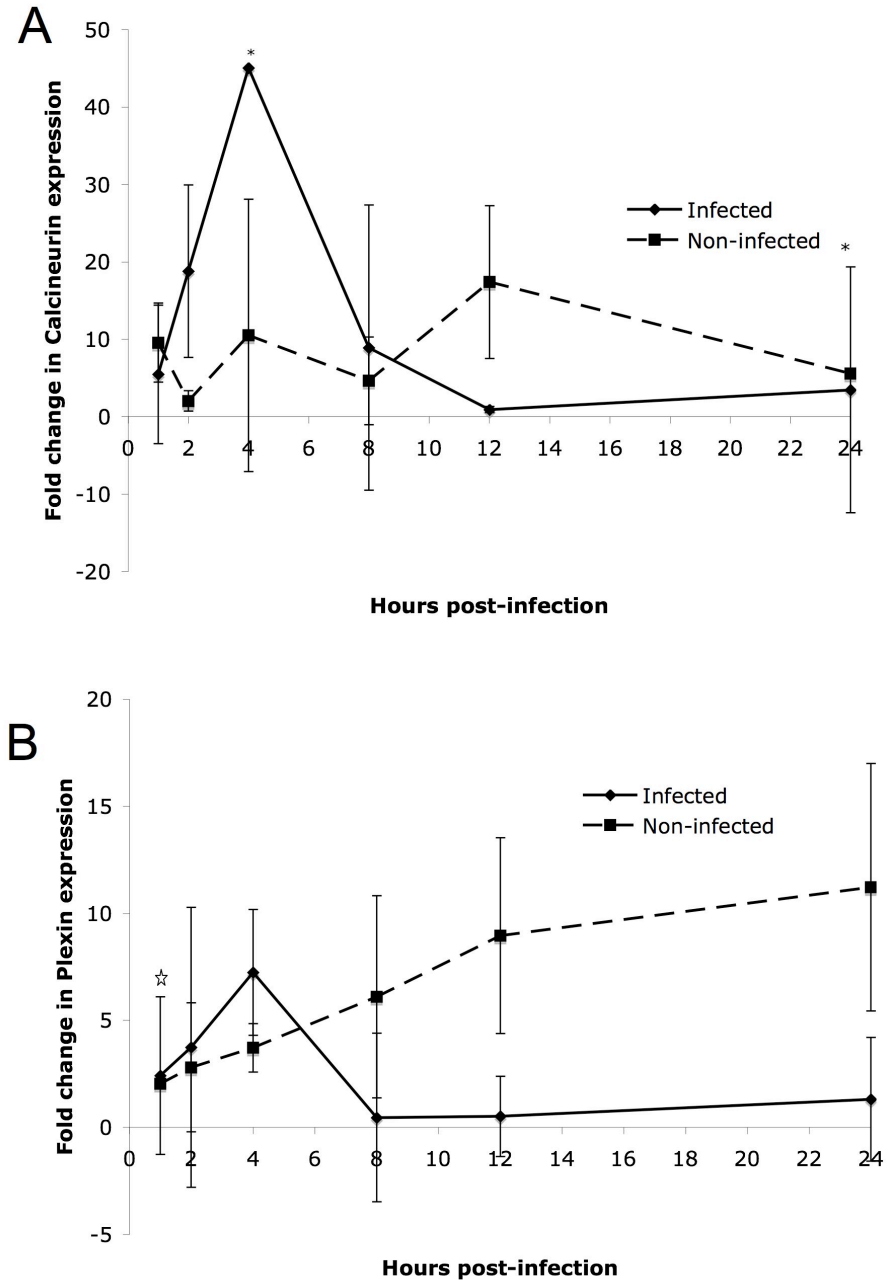

Figure 5.6 Temporal expression of Peroxidase (A) and Calcineurin (B) during *Crithidia* infection. Gene expression was sampled in workers from 1-24h after imbibing a mixed-strain *C.bombi* infection. Each point represents median fold change of 2-3 infected workers. Non-infected and infected workers were from different colonies. Samples that amplified poorly were omitted from analysis. (A) used a total of n=19 infected and n=17 non-infected workers and (B) n=19 infected and n=17 non-infected workers. Error bars represent standard deviation (SD). Large SDs (*) are omitted to prevent compression of the graph. Stars denote controls with one sample. The second sample at these time-points was used to normalise the Ct values.

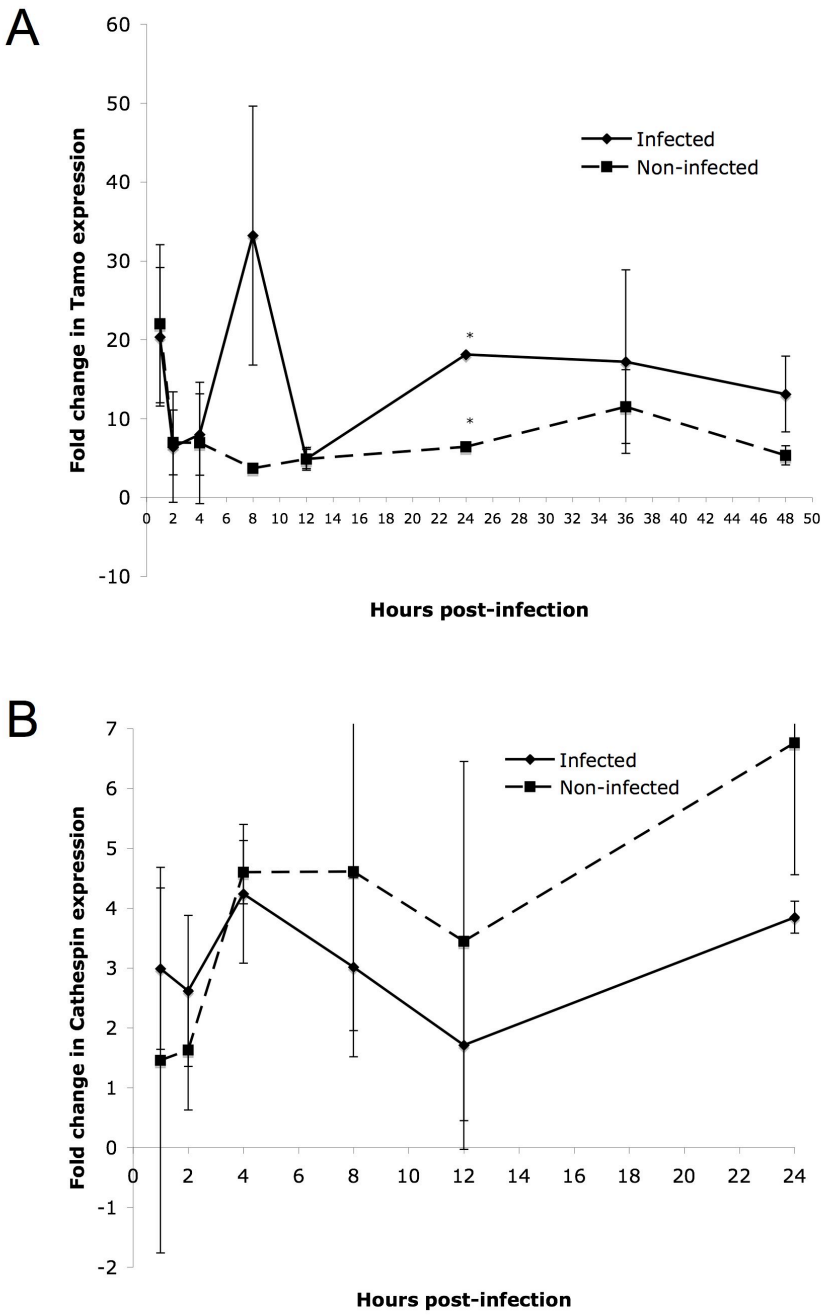

Figure 5.7 Temporal expression of Tamo (A) and Cathespin (B) during *Crithidia* infection. Gene expression was sampled in workers at 1-24h after imbibing a mixed-strain *C.bombi* infection. Each point represents median fold change of 2-3 infected workers. Non-infected and infected workers were from different colonies. Samples that amplified poorly were omitted from analysis. (A) used a total of n=24 infected and n=22 non-infected workers and (B) n=17 infected and n=17 non-infected workers. Error bars represent standard deviation (SD). Large SDs (*) are omitted to prevent compression of the graph.

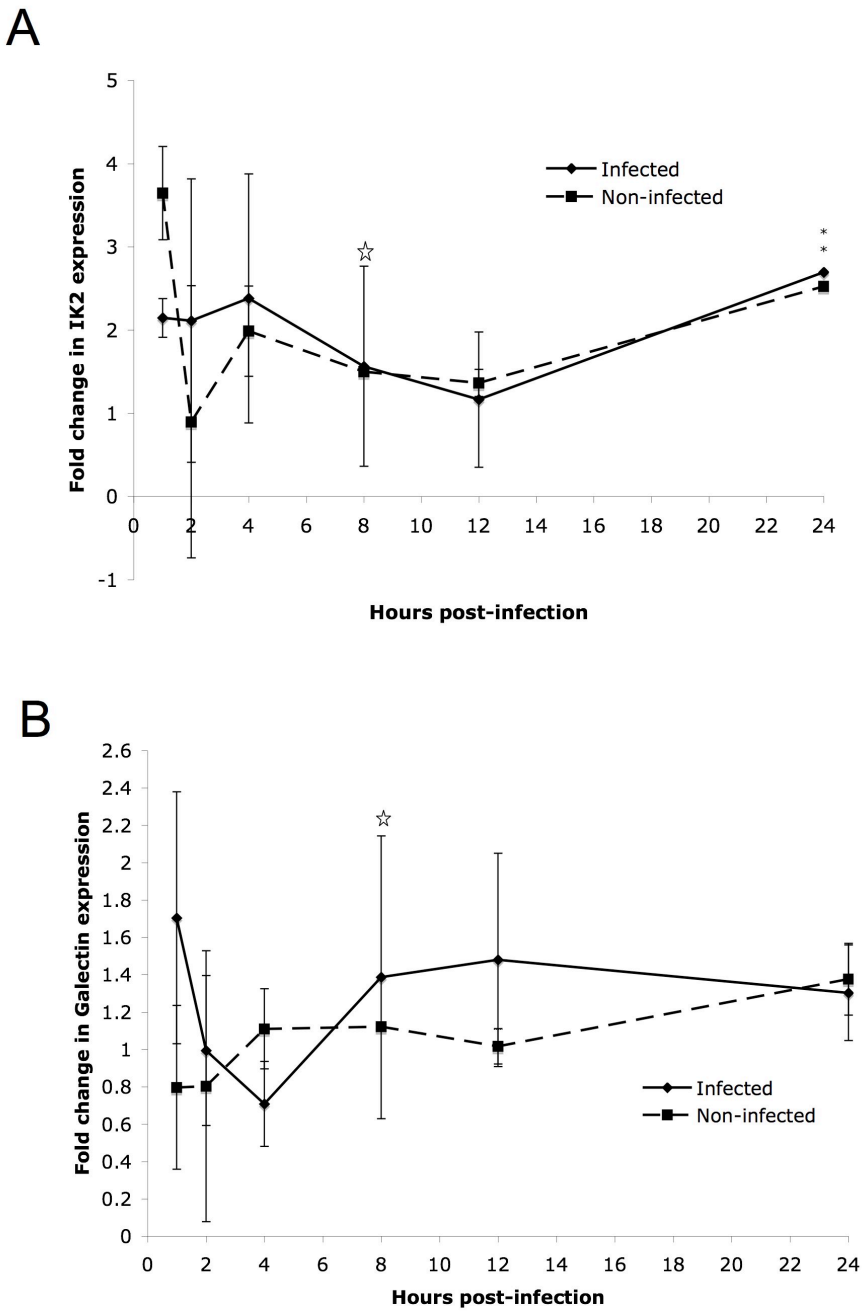

Figure 5.8 Temporal expression of IK2 (A) and Galectin (B) during *Crithidia* infection. Gene expression was sampled in workers at set time-points from 1-24h after imbibing a mixed-strain *C.bombi* infection. Each point represents median fold change of 2-3 infected workers. Non-infected and infected workers were from different colonies. Samples that amplified poorly were omitted from analysis. (A) used a total of n=19 infected and n=17 non-infected workers and (B) n=19 infected and n=17 non-infected workers. Error bars represent standard deviation (SD). Large SDs (*) are omitted to prevent compression of the graph. Stars denote controls with one sample. The second sample at these time-points was used to normalise the Ct values.

5.4. Discussion

5.4.1. SSH and differential screening

SSH

SSH is a powerful technique to identify differentially expressed sequences (Diatchenko et al., 1996) and was used to compare the transcriptional profiles of non-infected workers with bees infected with *C.bombi*. The forward subtraction targeted sequences that were unique to the infected bee transcriptome i.e., genes that were up regulated in response to *C.bombi* infection.

25% of the clones had no known homology to DNA sequences in the NCBI database, and most likely correspond to genes of unknown function. Sequences of this type have been reported in studies using SSH (Pereboom et al., 2005)). 75% of the returned sequences from the subtraction had high homology to known genes in the NCBI database. Of these, 29% were immune-related and had links to genes with enzymatic activity (*Cathespin, Peroxidase*), cytoskeletal movements and cell adhesion (Rho/Rac/Cdc42-like molecule, *Cct5*, *Paxillin*), respiration (*Aconitase*) and the stress response (*Hsp90*), and could potentially play a role in immune regulation (see Table 1). Interestingly, the vast majority of sequences were related to signal transduction or modulation such as *IK2*, *Serpin*-like sequences and the nuclear protein-import regulator *Tamo*. Sequences related to the Ras signalling element Sur-8 and Ran (Ras-related protein in the nucleus) were also of interest since they are part of the mitogen-activated-protein-kinase (MAPK) pathway, a large signal transduction network that can feed into

other signalling networks (such as the immune-related JAK-STAT pathway) and cellular immune responses (Lemaitre and Hoffmann, 2007). A similar signalling-biased suite of genes have been reported from an SSH carried out on shrimp (*Penaeus japonicus*) haemocytes after immune challenge (He et al., 2004), suggesting that the subtraction had been successful. One possible reason for the low number of positive clones is that the SSH hybridisations were excessively stringent. In effect, the longer the first round of hybridisation proceeds, the more stringent the subtraction becomes as it increases the chance that a "tester" single-stranded DNA molecule will find a sequence to hybridise to in the "driver" cDNA population, even if the former is differentially expressed. These molecules are thus effectively removed from the SSH process and will not appear in the subtracted library. Low abundance sequences are most susceptible to this process, which biases the final subtracted library towards strongly up-regulated genes. Stringency could therefore provide an explanation for the low number of differentially expressed sequences from the SSH as the times recommended by the manufacturer were used, which are optimised for mammalian systems. However, SSH is suitable for detecting genes showing around and upwards of a 5-fold difference in expression between tester and driver cDNA, and those immune-genes that were differentially expressed during qPCR are well within the minimal range, from 3 to a maximum of 8-fold up-regulation, suggesting that the SSH was sensitive enough to detect smaller transcriptional changes.

Differential screening

A qPCR-based screen of fourteen of these immune-related sequences found only five genes that were up-regulated in *C.bombi*-infected bees, suggesting that the other 9

sequences were false-positives, i.e. transcribed in both non-infected and infected bees and therefore not differentially expressed. This result is surprising as the subtraction efficiency analysis was carried out and showed that non-differentially expressed sequences had been removed, since PCR amplification of a housekeeping gene was only possible from unsubtracted cDNA and not subtracted cDNA. An explanation for the low number of positive clones could be that relatively few sequences were differentially expressed between the non-infected and infected cDNA populations. Under this scenario, there is a greater chance that non-differentially expressed sequences will persist through subtractive hybridisation, and thus result in a higher background of false-positive clones.

5.4.2. Temporal dynamics of immune gene expression

SSH-derived immune genes

Importantly, five sequences were found to be differentially expressed throughout *C.bombi* infection: IK2, *Calcineurin*, *Peroxidase*, *Plexin A* and *Tamo*-like, and their levels were measured at various time-points after infection to establish their temporal expression pattern in response to *C.bombi*. Four of the five genes identified by SSH showed no significant change in expression after bees were infected with *C.bombi*, despite suggestions of interesting patterns for genes such as *Tamo*-like and *Plexin A* (figures 5.6 and 5.7). This is perhaps unsurprising when it is considered that these genes had only shown between 3-8-fold up-regulation in the initial qPCR screen, a signal that would be difficult to detect over background from natural variation in immune gene expression (Decanini et al., 2007, Evans and Pettis, 2005b).

Individual bees did show much greater variation in gene expression than would have been predicted from the qPCR screen, but their range in infected and non-infected bees did not differ significantly. For example, expression of the *Tamo*-like sequence varied by around 50-fold in both non-infected (4 to 56-fold) and infected (4 to 52-fold) bees throughout the 48h infection period, and could be as low as 2-6-fold in some genes (*Cathespin*), but the variance was consistently similar between the two experimental groups or colonies (Figure 5.3). The obvious solution to reduce the effect of any variation in gene expression is to increase the sample sizes and use a single colony to collect both non-infected and infected bees, but this is limited by the number of female workers produced by a colony.

Temporal analysis of AMP and *Peroxidase* expression

In contrast to the majority of SSH-derived genes, *Peroxidase* did show differential expression in the first hour after infection. Conversely, *Abaecin* and *Defensin* showed strong induction after 12h. These results indicate that *C.bombi* does activate an immune response within the first 48h after infecting the bee.

AMPs

Finding strong up-regulation of the AMPs during *C.bombi* infection was surprising and was probably not found by SSH because the 12h time-point was not included. It is uncertain how the dynamics of AMP induction relate to the progression of *C.bombi* infection, although it is likely that the response is triggered by particular stages of the parasite's development, as is found for Tsetse fly (*Glossina*) (Hao et al., 2001, Boulanger et al., 2006) and mosquito (*Anopheles gambiae*) (Dimopoulos et al., 1998) AMP expression after trypanosome infection. It is clear that AMP induction is not

sustained throughout *C.bombi* infection and it would be interesting to further investigate the expression dynamics around 12h post-infection.

Crithidia sp. exist in two forms throughout their lifecycle, firstly as a non-motile, infective amastigote stage before developing into the characteristic flagellated choanomastigote that has both motile (leptomonad form) and non-motile (haptomonad) forms that adhere to the gut wall and reproduce clonally (Wallace, 1979). Trypanosomatids are coated in a dense coat of glycoproteins that vary in their expression during specific developmental stages to evade host immune detection, mediate adhesion/detachment to host epithelia and other flagellates, provide protection against host defences, or act as virulence factors (de Souza, 1995). These molecules include sialoglycoconjugates (dos Santos et al., 2002, Silva et al., 2009, Chava et al., 2004) and the zinc metalloprotease, gp63 (Jaffe and Dwyer, 2003, Pereira et al., 2009, Santos et al., 2006). Some molecules are exclusive to particular trypanosomatid genera such as lipophosphoglycan (LPG), the core surface constituent of *Leishmania*, that undergoes conformational changes during the parasite's transformation into an infective stage (Sacks et al., 1995). Such alterations to molecular composition also occur in the lower trypanosomatids such as *Herpetomonas samuelpessoai*, which expresses distinct sialoglycoconjugates after chemical-induced parasite differentiation (dos Santos et al., 2002) and mid-gut binding is prevented if these molecules are abnormal (do Valle Matta et al., 1999). Gene expression was sampled over the first 48h of *C.bombi* infection, within the 8-10 day growth phase. It is therefore possible that *C.bombi* begin to express particular surface molecules during the growth stage that are detected by the host before initiating AMP synthesis. Stage-specific AMP expression occurs in *Phlebotomus duboscqi* after *L. major* infection and corresponds with development of

the parasite into its insect-specific promastigote form and subsequent proliferation in the fly's gut (Boulanger et al., 2004).

One possibility is that AMP up-regulation is not a functional response against *C.bombi*, but rather a response to physical damage (Schaub, 1994) since *Crithidia* forms a specialised attachment (hemidesmosome) to the mid-gut epithelia via its flagella (Brooker, 1971) that can damage the gut wall (Schaub, 1994). *In vitro* studies with *Crithidia fasciculata* find that substrate adhesion is growth-phase dependent, and flagellates can begin to reproduce before anchoring themselves to the substrate (Scolaro et al., 2005). This could explain the 12h lag preceding strong AMP induction if the bee was responding to a vast population of *C.bombi* adhering to the mid-gut. Tissue damage frequently elicits AMP induction in *Anopheles* when *Plasmodium* migrates through the mid-gut epithelia and enters the haemolymph around 24h after infection (Vizioli et al., 2001), but such major disruption to the intestinal wall is unlikely to occur in monoxenous trypanosomatids such as *Crithidia* because these parasites do not migrate away from the gut (Brown et al., 2003a), most likely because they are transmitted in the faeces. *Crithidia* also naturally infect the gut of *Drosophila* and do not invade the haemocoel (Boulanger et al., 2001). After *per os* infection *Drospohila* up-regulates AMP expression in the haemolymph and not locally in the gut epithelia (Boulanger et al., 2001), suggesting that damage is minimal and that some form of signal is released from the site of infection that stimulates a systemic immune response, for example in the fat-body. Cytokines are one such signalling candidate and Boulanger et al. (2001) have extracted a number of unidentified molecules from the haemolymph of *Crithidia*-infected *Drosophila* that may perform this signalling role (Boulanger et al., 2001).

Similar systemic responses have been found in *Glossina* infected with *Trypanosoma brucei brucei* that also does not breach the mid-gut barrier (Boulanger et al., 2002a).

In contrast to the result here, a study by Brown et al. (2003) found no evidence of increased anti-microbial activity in bumblebees after *C.bombi* infection (Brown et al., 2003a). However, the experiment here measured anti-microbial activity after seven and fourteen days, at much later stages of the infection. The discrepancy in anti-microbial activity therefore most likely relates to the dynamics of AMP degradation. For example, in *Drosophila* it has been shown that haemolymph levels of AMPs peak around 24h after a septic injury and then begin to fall as the proteins degrade (Uttenweiler-Joseph et al., 1998). It is likely that a similar event occurs in the *Bombus* immune response to *C.bombi*, whereby a brief burst of AMP synthesis appears shortly after infection around 12h, and the efficacy of anti-microbial peptide activity is gradually lost as the peptides naturally degrade, for example through proteolytic cleavage (Uttenweiler-Joseph et al., 1998).

Increased AMP expression has frequently been found in the immune response of insects to trypanosomatids but the functional role of these molecules during infection is poorly understood (Boulanger et al., 2006). AMPs are primarily active against bacteria and fungi, but studies suggest that they can directly interact with flagellate parasites. For example, *in vitro* application of exogenous AMPs to *Leishmania braziliensis* or *Trypanosoma cruzi* kills the parasites and appears to be most effective on specific developmental stages (Löfgren et al., 2008). Studies with the tsetse fly, *Glossina mortisans mortisans*, show that inhibition of AMP expression with RNAi increases the fly's susceptibility to *Trypanosoma brucei* and permits the parasite to achieve higher

infection intensities in its host (Hu and Aksoy, 2006). Supplementing the blood meal of freshly-infected flies with recombinant tsetse Defensin reduces infection levels by as much as 60% (Hu and Aksoy, 2005). A third study finds that Defensin damages the plasma membrane of *Trypanosoma brucei brucei* that is consistent with cell lysis (Yamage et al., 2009), providing compelling evidence that AMPs mediate killing by direct interaction with the parasite.

One final point to consider about the AMP expression data is the effect of time in both infected and naïve bees. AMP expression clearly varied over the course of the experiment, even in bees that were not infected. For example, there were clear peaks in *Abaecin* and *Defensin* expression at 2 and 36h post-infection in non-infected bees whilst both treatment groups showed up-regulation after 12h. Indeed, the 12h AMP peak of *Abaecin* and *Defensin* could be an enhancement of an underlying expression pattern. Similarly, after up-regulation in the first hour, *Peroxidase* expression in infected bees rapidly decreased to non-infected levels and from there on both treatment groups showed similar expression profiles with a peak at 4h. Distinct expression profiles are also seen in genes where no significant up-regulation was found, such as *IK2* or *Cathespin*. Clearly, other factors affect the expression of these genes.

I am also comparing immune gene expression between two different colonies, and therefore genetic backgrounds, which confounds any effect of infection on expression levels. Clearly, this makes interpretation of the data more complex. However, if colony-level effects were responsible for any differences in gene expression, this would be reflected in the ANOVA and show a significant effect of infection status, i.e. infected or non-infected. Such an effect was only found for a single gene (*Tamo*-like), suggesting

that inter-colony variation was not responsible for the differences seen in gene expression. Using this argument opens up the possibility that the analysis has simply tested for the effects of "time of day" that were then exaggerated by the immune response in infected bees.

One possible explanation for the fluctuations in AMP expression is that gene expression naturally fluctuates under circadian regulation as has been found in *Drosophila* (McDonald and Rosbash, 2001). The expression of a number of immune elements such as AMPs, signal transducers and recognition molecules are linked to the activity of the transcription factor CLOCK, which is itself part of the central circadian network and promotes activation of downstream gene networks during the circadian cycle (McDonald and Rosbash, 2001). The benefits of such cycling are debated but there are suggestions that it may serve to control resource allocation to physiological processes throughout the day (Lee and Edery, 2008). Immunity is metabolically demanding and incurs costs both in terms of its maintenance and usage (Sheldon and Verhulst, 1996) and thus can be predicted to be traded-off with other costly activities throughout the day such as foraging. For example, Lee and Edery (2008) find that *Drosophila* is most able to resist microbial infection if infected during the night and that this response is regulated by CLOCK, suggesting that the circadian network permits greater allocation of resources to immunity at times when the metabolic demands from other biological processes are lowered (Lee and Edery, 2008). With respect to this experiment, the largest fluctuation in AMP expression occurs at 12h and similar fluctuations are not seen at 36 or 48h, if a respective 12 or 24h cycles are presumed. It is therefore unclear whether there is circadian cycling of these genes.

Another possible explanation for the expression patterns is that handling the bees, during inoculation or separation from the colony, could have caused the activation of stress-related signalling that terminated in the up-regulation of immune genes. Both neural and endocrine systems can modulate the activity of immunity according to the physiological needs of the animal (Rolff and Reynolds, 2009). For example, physical stress is frequently found to cause acute stress-induced immunosuppression, and an increase in disease susceptibility (Adamo and Parsons, 2006). However, a recent study with the wax moth (*Galleria mellonella*) found the opposite by showing that physically shaking the larvae caused an increase in transcription of the anti-microbial peptide, *Galiomicin*, as well as increased numbers of circulating haemocytes (Mowlds et al., 2008). Evidently, stress can induce changes in the regulation and sensitivity of immune responses, although the direction and magnitude of the change is not necessarily predictable. However, the effect of handling in the current experiment remains unclear. Non-infected bees were only handled after hatching to transfer them into a separate box and briefly before sacrifice. Conversely, treated bees were additionally handled for inoculation, 4-5 days after hatching and were fed the inoculum. Ideally, non-infected would be handled and fed a sugar solution to control for this.

Whether caused by handling, circadian rhythms or some other element, the results of this experiment suggest that other factors have an input into the modulation and regulation of immune gene expression. However, the timing of the AMP expression peak in infected bees is along a similar timescale to those seen in *Drosophila* after oral infection with a gut bacteria. Vodovar et al. (2005) quite clearly show that *Pseudomonas entomophila* initiates an acute immune response (within 6h) if injected into flies, but if fed it induces a much slower response that peaks around 24h (Vodovar

et al., 2005). Therefore, AMP induction does seem to form part of an acute response to enteric infection and instead increases many hours after infection. In this regard, the peaks in *Abaecin* and *Defensin* that were observed here would seem to be within the normal time-frame for natural infection with gut pathogens.

Peroxidase

Peroxidase up-regulation within the first hour of during infection fits with the role of this effector during the immune response. Proteins containing the Peroxidase domain are implicated in the production of cytotoxic reactive oxygen species (ROS), which are rapidly synthesised by barrier epithelia and phagocytes in the early stages of bacterial and protozoan infections (Nappi and Ottaviani, 2000, Kumar, 2003, Lemaitre and Hoffmann, 2007). One such protein in *Drosophila*, dual oxidase (Duox), plays a major role in the induction of ROS synthesis in the gut epithelia to control microbial proliferation. These activities also appear to take place independently of NF-κB-regulated AMP synthesis (Ha, 2005). Some studies indicate that peroxidases can have an anti-oxidant role during the immune response to minimise the damage of ROS (Ha et al., 2005), such as in the fly *Glossina mortisans mortisans* (Munks et al., 2005). Therefore, *Peroxidase* may have been up-regulated to increase the production of ROS or replace the peptide as it is degraded.

5.4.3. Conclusions

In summary, these experiments have identified four genes that are induced in response to *C.bombi.* Three of these genes (*Abaecin*, *Defensin* and *Hymenoptaecin*) did not appear in the SSH, and were found to be differentially expressed after including 12h in

the temporal dynamics experiment. Genes that were identified from the SSH were mainly signalling molecules, showing marginal up-regulation and all but one did not show any significant temporal change in expression in response to *C.bombi* infection. It seems logical that signalling molecules are the least likely component of the immune pathways to be up-regulated, since they are already constitutively expressed and regulated before immune challenge. Their role is purely to consolidate and transmit pathogen-binding receptor signals and mediate induction of the appropriate effectors. In contrast, many effectors can potentially damage the host or are costly to produce and so are induced and synthesised as needed, and their up-regulation is therefore much more pronounced. It is possible that the differential expression of the signalling genes identified by SSH was colony (i.e., genotype) specific, providing a possible reason why they were not confirmed to be differentially expressed in the temporal experiment. As discussed in the thesis introduction, host genotype has a strong effect on the immune response. Similar conclusions have been drawn from quantitative trait loci (QTL) analysis of *C.bombi* resistance in *Bombus terrestris* males from three separate colonies, where considerable variation in the position of QTL and the interacting loci was high between the populations and a number of non-homologous linkage groups were apparent (Wilfert et al., 2007).

The experiment here has established a temporal expression pattern for AMP induction during the bumblebee immune response to *C.bombi* infection. So far, these molecules have been described as anti-bacterial or anti-fungal in their actions, but studies consistently find that AMPs are up-regulated in invertebrates during protozoan infection (reviewed by Boulanger (2006)) and affect the parasite's viability (Rodriguez et al., 1995, Gwadz et al., 1989, Chalk et al., 1995, Löfgren et al., 2008, Shahabuddin et al.,

1998). In tsetse flies, knockdown of Attacin expression increases trypanosome infection load (Hu and Aksoy, 2006). *C.bombi* is confined to the gut of its host (Brown et al., 2003a), and it is likely that the mid-gut epithelia induces a local immune response to control the infection (Lehane et al., 2003, Tzou et al., 2000). However, concomitant systemic AMP induction in the fat-body and haemolymph is also well-documented in a number of insects such as Tsetse (Hao et al., 2001), *Glossina* (Boulanger et al., 2002a), *Anopheles* (Dimopoulos et al., 1997), *Drosophila* (Boulanger et al., 2001) and *Phlebotomus* (Boulanger et al., 2004) where the flagellate does not cross the gut epithelia into the haemolymph. Evidence is accumulating that signalling occurs between the gut and fat-body (Lopez, 2003, Boulanger et al., 2001) perhaps by the release of signalling molecules, such as cytokine-like molecules (Beschin et al., 2001) or nitric oxide (Foley, 2003), from the site of infection that raise the general level of immune defence in case of further infections. The increase in standing levels of prophenoloxidase in the haemolymph of *C.bombi*-infected bees already suggests that signalling may play a part in *Bombus* immune responses (Brown et al., 2003a) and future studies would therefore be beneficial to clarify the spatial expression pattern with regard to AMPs.

6. Investigating the molecular basis of specific immune priming

6.1. Introduction

Immunological priming is the ability to produce an enhanced immune response after initial exposure to a pathogen (Pham et al., 2007). In vertebrates, adaptive immunity fulfils this role, using T and B cells and antibodies to create a highly specific "immune memory" of a past exposure, thus ensuring more efficient and faster removal of a secondary infection (Kurtz, 2004). Despite its relative simplicity and lack of a dedicated cellular system, there is now conclusive evidence that invertebrate innate immunity also exhibits adaptive characteristics that confer the host with greater protection against secondary pathogen infection. Recently, studies have shown that this immune priming can also be highly specific and enhanced towards the particular species or strain of pathogen initially encountered (Roth et al., 2008).

In its simplest form, invertebrate immune priming causes the immune system to remain in a heightened state and react in a generalised manner against subsequent infection. For example, stimulating the immune system of the mealworm beetle, *Tenebrio molitor*, by injection of a non-pathogenic bacterial cell wall constituent induced a long-lasting response that reduced mortality during fungal infection(Moret and Siva-Jothy, 2003). However, recent studies have determined that the primed immune response can differentiate much more specifically between pathogens.

Specific immune priming is demonstrated by first inoculating an animal with a pathogen, followed by a time delay to allow for induction of response and allow the host to recover. Then, the host is challenged a second time inoculation, with either the

same (homologous) or a different (heterologous) pathogen. The reciprocal sets of heterologous infections can also be carried out to ensure that any change in immune response is not due to one pathogen simply eliciting a stronger, but not specific, immune response than the other. Immune priming is evident when there is a clear difference between homologous-challenged and heterologous-challenged animals in some aspect of the response to the secondary pathogen, such as re-infection success (Little et al., 2005).

Specific immune priming has been demonstrated in the copepod, *Macrocyclops albidus* response to its natural parasite, the tapeworm, *Schistocephalus solidus*. Re-infection success after three days was higher when the tapeworm larvae were only distantly related to those in the primary infection; and un-related parasites also achieved more intense infections (Kurtz and Franz, 2003). Roth and co-workers (2008) also carried out an extensive reciprocal infection study in the flour beetle, *Tribolium castaneum*, and found that beetles receiving homologous infections had higher survival rates than those receiving heterologous infections. Priming was highly specific and differentiated between three *Bacillus* species and between bacterial strains of the same species (Roth et al., 2008).

One inherent problem with studies that use non-immunological measures of immunity, such as survival, is that they cannot explicitly test the which factors are improving host resistance. For example, greater resistance could be due to an enhanced immune response that is controlling/clearing infection more efficiently or simply that the host has greater tolerance to secondary infection. Recently, Sadd and Schmid-Hempel (2006) have provided an unambiguous example of specific immune priming in the bumblebee,

Bombus terrestris by showing that homologous infections are cleared more quickly and cause lower host mortality. Bumblebees were able to differentiate between bacterial species belonging to the same genus. Bees injected with the honeybee bacterial (gram-positive) parasite, *Paenibacillus alvei*, showed improved clearance of bacteria when eight days previously they had been injected with a priming dose of the same bacteria but not when they had been injected with *P. larvae,* a closely related species causing a similar disease (Sadd and Schmid-Hempel, 2006). The reciprocal was also true; bees injected with *P. larvae* cleared a second dose of *P. larvae* more quickly than those who had first been injected with *P. alvei.* After a twenty-two day lag between injections, the difference in pathogen clearance was still seen but in addition, host survival following homologous-pathogen exposure was now notably greater than heterologous mortality, the latter being comparable to that after injection of a naïve bee. These exciting findings confirm that immune priming can provide long-lasting specific protection for the host (Sadd and Schmid-Hempel, 2006).

The mechanisms underlying specific immune priming in bumblebees have yet to be studied, but it is likely that the end-result is the biased-activation and mobilisation of effectors that are most suited to eradicating the parasite. Sadd and Schmid-Hempel (2006) could not find any increase in anti-microbial activity towards *Paenibacillus* during immune priming. However, their study used the zone of inhibition assay, an *in vitro* technique which would be unable to detect smaller differences that may still be biologically significant and improve pathogen removal. To address this issue, a mechanistic analysis of Sadd and Schmid-Hempel's 8-day result was carried out by measuring AMP (anti-microbial peptide) transcription after the different combinations of immune challenge.

6.2. Methods

Experiments were carried out on two commercially reared bumblebee colonies from Koppert Biological Systems U.K.

6.2.1. Injections

Bees either received (1) a control to test for the effect of different bacteria and treatment regimes on AMP expression i.e. Ringer-*P.alvei;* Ringer- *P.larvae;* a single injection of ringer, *P.alvei* or *P.larvae*; Ringer-Ringer; *P.alvei*-Ringer or *P.larvae*-Ringer (2) heterologous injections, i.e., *P.larvae-P.alvei* or *P.alvei-P.larvae* or (3) homologous injections, i.e., *P.larvae- P.larvae* or *P.alvei- P.alvei* (see Figure 6.1). Hetereologous and homologous treatments tested the specificity of immune priming. That is, whether a primary injection caused the immune response to increase its activity in a generalised manner to any subsequent infection (heterologous treatment) or if immunity only became primed towards a previously encountered pathogen (homologous treatment). There was an 8 day period between injections. Bees were sacrificed 24h after the final injection. All three AMPs have previously been shown to be strongly expressed 24h after injection of *P.larvae* in the honeybee, *Apis mellifera* (Evans et al., 2006), and are active against gram –negative bacteria (Choi et al., 2008). AMP transcription should return to base levels by three days post-infection (Lemaitre and Hoffmann, 2007), thus any transcriptional change recorded after the second challenge in the study here should reflect an active immune response.

Paenibacillus larvae (NCIMB 11201) and *P. alvei* (NCIMB 9371) were grown in the appropriate media (www.ncimb.co.uk). Bees were five to seven days old at the time of first injection. Bees were injected between the first and second abdominal tergite with

2μl of either a Ringer solution or a 5 X 10^4 cells/ml solution of one of the bacteria. This concentration of bacteria was shown to be non-lethal and clearable by bumblebee workers (Sadd and Schmid-Hempel, 2006). 24h later (on bees 6-8 days old), a sample of these bees (see Figure 6.1 for sample numbers) were sacrificed by freezing in liquid nitrogen. These samples were collected to test for the effect of the type of injection administered i.e., *P.larvae, P.alvei* or ringer. Non-infected, age-specific control bees were also sacrificed to provide a baseline of AMP expression. Sacrificed bees were stored at -80^{o}C. The rest of the bees were left for 8 days post-injection in colony and treatment specific groups. These were then injected with a 1.5 X 10^6 cells/ml solution of either *Paenibacillus larvae, P. alvei* or ringer solution of the same volume (see figure 6.1 for sample numbers). This higher concentration was used to insure a strong immune response and to mirror the protocol used by Sadd and Schmid-Hempel when studying the effects on bacterial clearance (Sadd and Schmid-Hempel, 2006). 24h later these bees were sacrificed at 14-16 days old. Due to experimental error, bees for the *P.alvei* – ringer injection combination were not collected. See figure 6.1 for full experimental design.

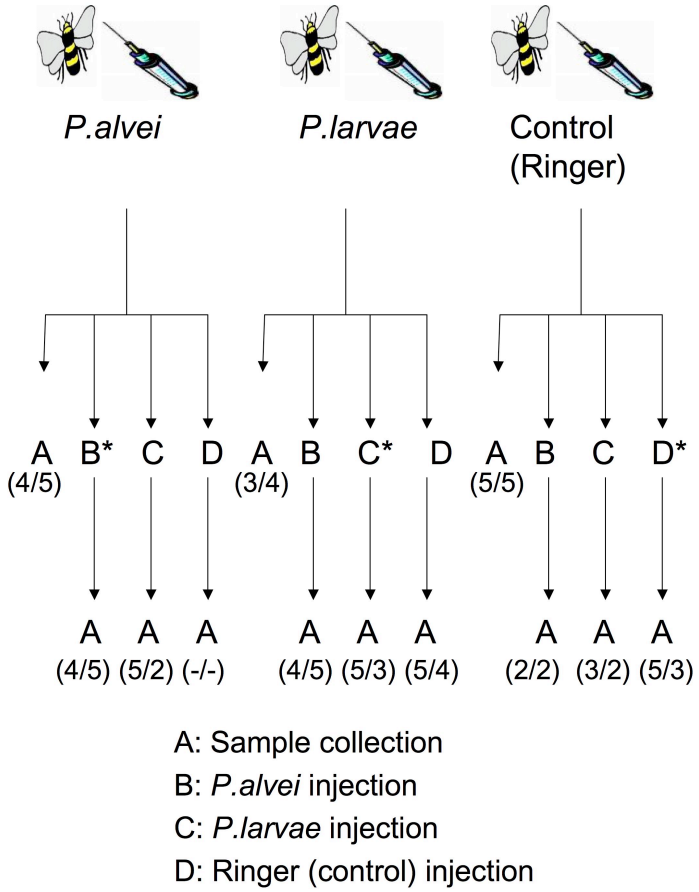

Figure 6.1. Set-up of priming experiment. 5-7 day-old workers were injected with an inoculum of *Paenibacillus alvei, P. larvae*, or a control injection of ringer solution (insect saline). 24h after injection, a sample from each group of bees were sacrificed whilst the remainder were re-injected after 8 days with either *P. alvei, P. larvae*, or Ringer solution. 24h after the second injection, bees were sacrificed. Asterisks (*) denote homologous injection combinations whilst all others were heterologous. This protocol was carried out twice with 2 different colonies, with a minimum of 2 biological replicates per treatment regime. Final number of samples analysed in qPCR are represented in brackets as (colony 1/ colony 2). Samples for *P. alvei*-ringer combination were not collected for either colony.

6.2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from individual homogenised abdomens, cleaned, and treated with DNase to remove genomic DNA (Section 2.3.1). First strand cDNA synthesis was carried out by reverse transcription of 2μg of total RNA (Section 2.4.1). First strand cDNA samples and controls were diluted 10 fold with nuclease-free water (Section 2.6.2). Each qPCR reaction contained 5μl of dilute cDNA or control, 1x SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, UK) and gene specific primers (final concentration 0.1μM). Each sample was tested with the housekeeping gene RPS5 (Evans and Pettis, 2005b) and all 3 AMPs. Two technical replicates were run per qPCR reaction on the following program: 95°c for 5 min, followed by 42 cycles of a 30 second 95°C denaturation, 30 second 61°C annealing and 30 second 72°C extension steps. Fold-changes in gene expression were calculated as outlined in (Section 2.6.3).

6.2.3. Statistical analysis

All fold changes in gene expression were box-transformed to fit the data to a normal distribution. Fold data for all three AMPs were first analysed using a MANOVA, and if significant effects were apparent then separate ANOVAs were carried out for each AMP. All data analyses were performed using Intercooled STATA 8.2 for Macintosh.

6.3. Results

Final sample numbers for each injection combination are outlined in Figure 6.1 to aid clarity. In bees that had received a single injection, (n=84) AMP expression was significantly different between *P.larvae*, *P.alvei* or ringer injected bees (ANOVA:

Abaecin: $F_{2,20} = 3.62$, p = 0.0456, *Defensin*: $F_{2,20} = 5.48$, p = 0.0127, *Hymenoptaecin*: $F_{2,20} = 4.88$, p = 0.0188)(Figures 6.2-6.4. There were also significant colony-specific patterns of AMP expression across treatment groups regardless of the type of injection that bees had received (ANOVA: *Abaecin*: $F_{1,20} = 66.46$, p < 0.00001, *Defensin*: $F_{1,20} = 24.38$, p = 0.0001, *Hymenoptaecin*: $F_{1,20} = 13.19$, p = 0.0017) (Figures 6.3 and 6.4). The treatment effect seems to come mainly from the differences in AMP expression between ringer injection and the two bacterial strains (*Defensin*: Fisher – Hayter post-hoc test show significant differences between Pa vs. Ringer ($F_{1,20} = 3.3762$, p <0.05) and Pl versus ringer ($F_{1,20} = 3.6571$, p <0.05), *Hymenoptaecin*: Fisher – Hayter post-hoc test show significant differences between Pa vs. Ringer ($F_{1,20} = 3.2559$, p <0.05) but not Pl vs. ringer ($F_{1,20} = 2.7943$, p >0.05, although the critical value is only slightly higher at 2.95, *Abaecin* no significant post-hoc tests). Transformed data were used for this post-hoc analysis and met the requirements of normality and equal variances required by the Fisher-Hayter test.

In those bees that received two injections, the level of AMP induction did not differ according to the combination of injections (i.e., ringer then bacteria, homologous or heterologous injections) that the bee received (MANOVA: $F_{2,36} = 1.47$, $P = 0.2024$; Wilks' $\lambda = 0.7838$). In these bees colony strongly affected AMP expression (ANOVA: *Abaecin* $F_{1,36} = 101.20$, p < 0.00001, *Defensin* $F_{1,36} = 21.06$, p = 0.0001, *Hymenoptaecin* $F_{1,36} = 6.71$, p = 0.0138).

In bees that had received a primary injection of ringer solution there were no differences in AMP expression in response to a secondary injection of either *P.alvei*, *P.larvae*, or ringer solution (MANOVA: $F_{2,36} = 1.18$, $P = 0.3255$; Wilks' $\lambda = 0.8677$). Again, only

colony-specific patterns of AMP expression were apparent (MANOVA: $F_{1,36} = 59.28$, $P < 0.00001$; Wilks' $\lambda = 0.2125$, ANOVA: *Abaecin* $F_{1,51} = 162.87$, $p < 0.00001$, *Defensin* $F_{1,51} = 32.87$, $p < 0.00001$, *Hymenoptaecin* $F_{1,51} = 16.55$, $p = 0.0002$).

When testing for any time effect created between bees sacrificed after either primary or secondary injection, again, there was no effect (day 6-8 or day 14-16) on the response of bees to the different injections (MANOVA: $F_{1,77} = 0.37$, $P = 0.7733$; Wilks' $\lambda = 0.9853$).

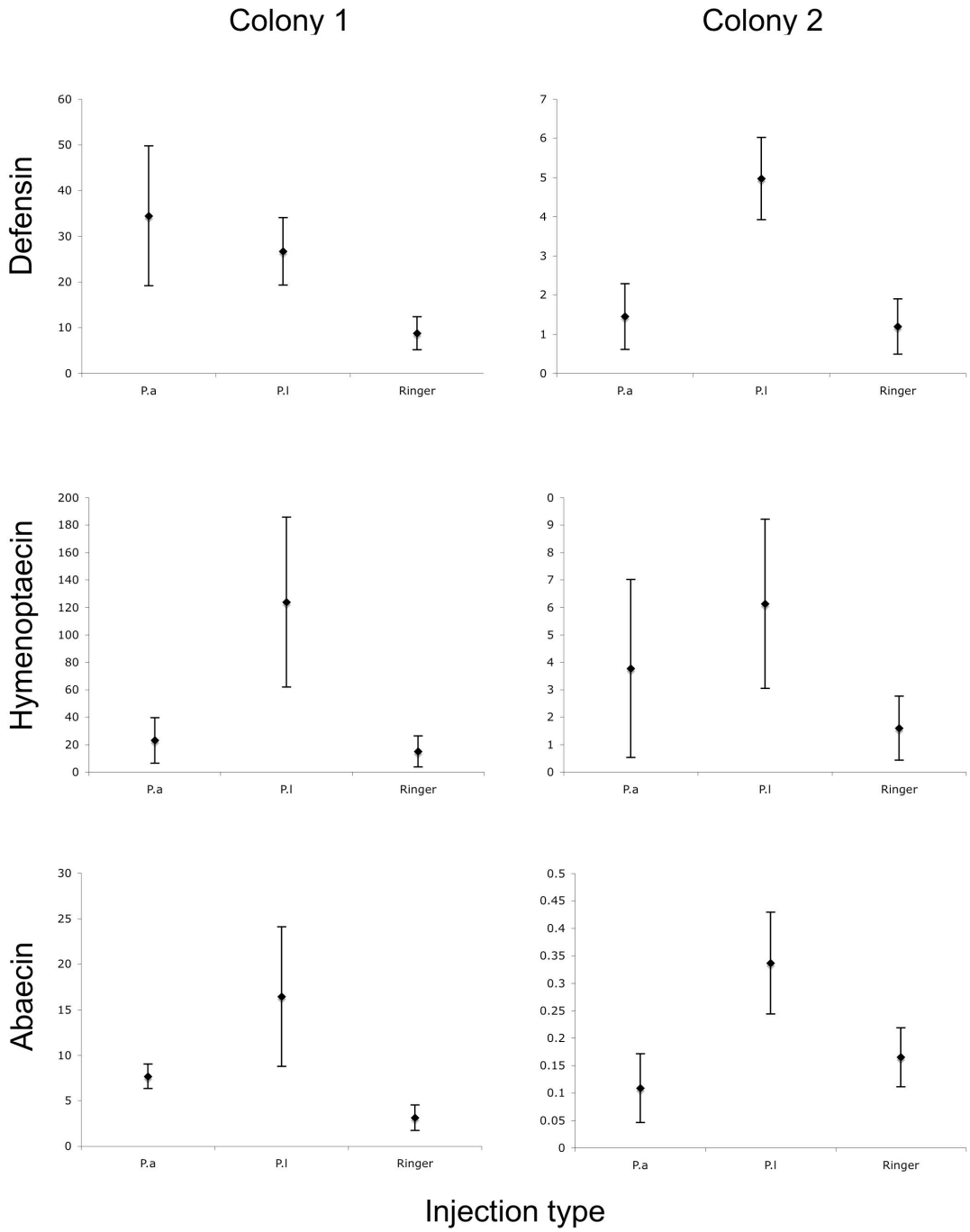

Figure 6.2 The variable effect of a single injection on AMP expression. The y axis represents fold change in gene expression relative to a non-infected control. Significant differences in AMP-up-regulation were apparent between bees that had received an injection of *P. larvae*, *P. alvei*, or a ringer control, specifically between *P. alvei* and ringer and *P. larvae* – ringer injected bees. AMP expression was measured 24h after injection. Sample numbers for each injection combination are outlined in Figure 6.1 Dots represent the mean and error bars the standard error.

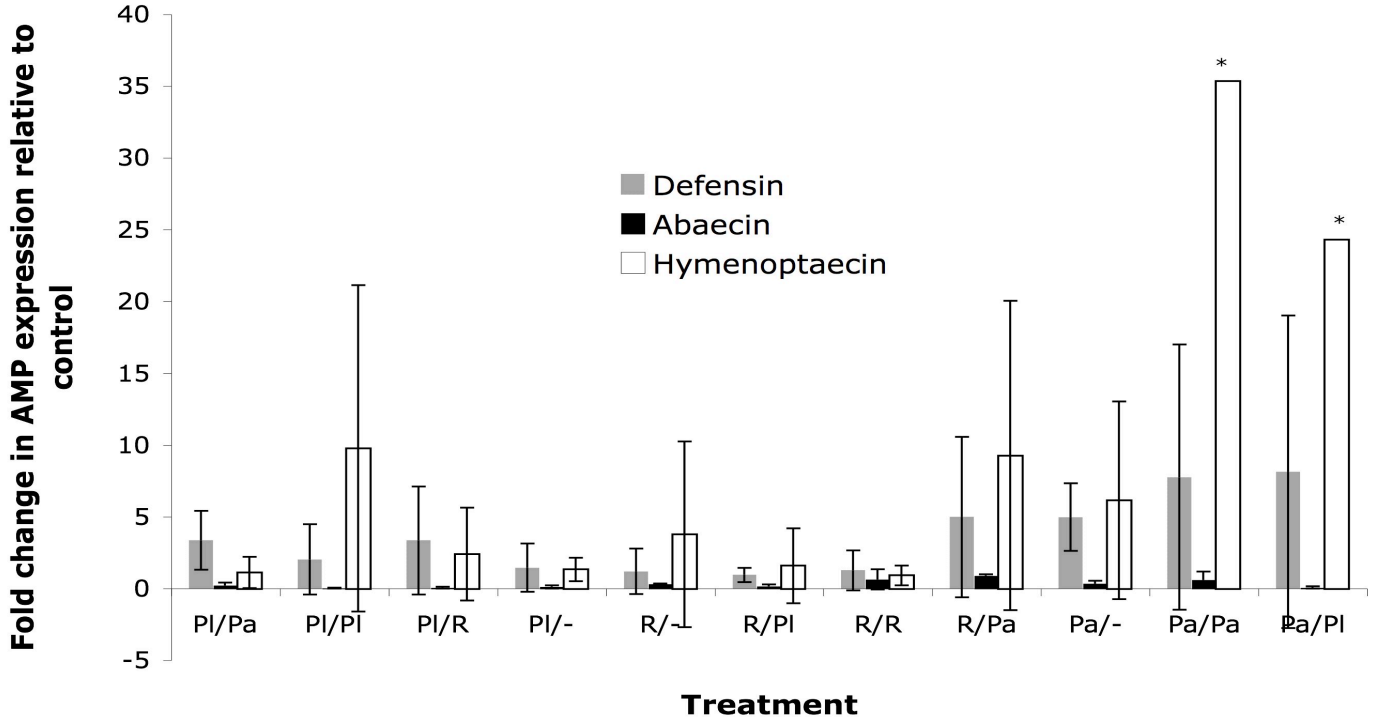

Figure 6.3 The effect of different injection regimes on expression of the AMPs Defensin, Abaecin and Hymenoptaecin of colony 1. A sample of 5-7 day old workers received a single injection of *Paenibacillus larvae* (Pl/-), *Paenibacillus alvei* (Pa/-) or ringer solution (R/-)(control) before being sacrificed 24h later. Another group of bees received a second injection 8 days after the primary injection. Bars represent median values of AMP expression for each injection combination. Bees were sacrificed 24h after secondary infection. Error bars represent standard deviation (SD) and large SDs are omitted for bars marked with * to prevent compression of the graph. Clear colony-specific trends in AMP expression were apparent, regardless of the injection regime bees had been subjected to. There were significant differences in AMP expression between bees that received a single injection of ringer solution (control) and those injected with either *P. alvei* or *P. larvae*. AMP expression was not significantly different between bees that had received either heterologous or homologous injections.

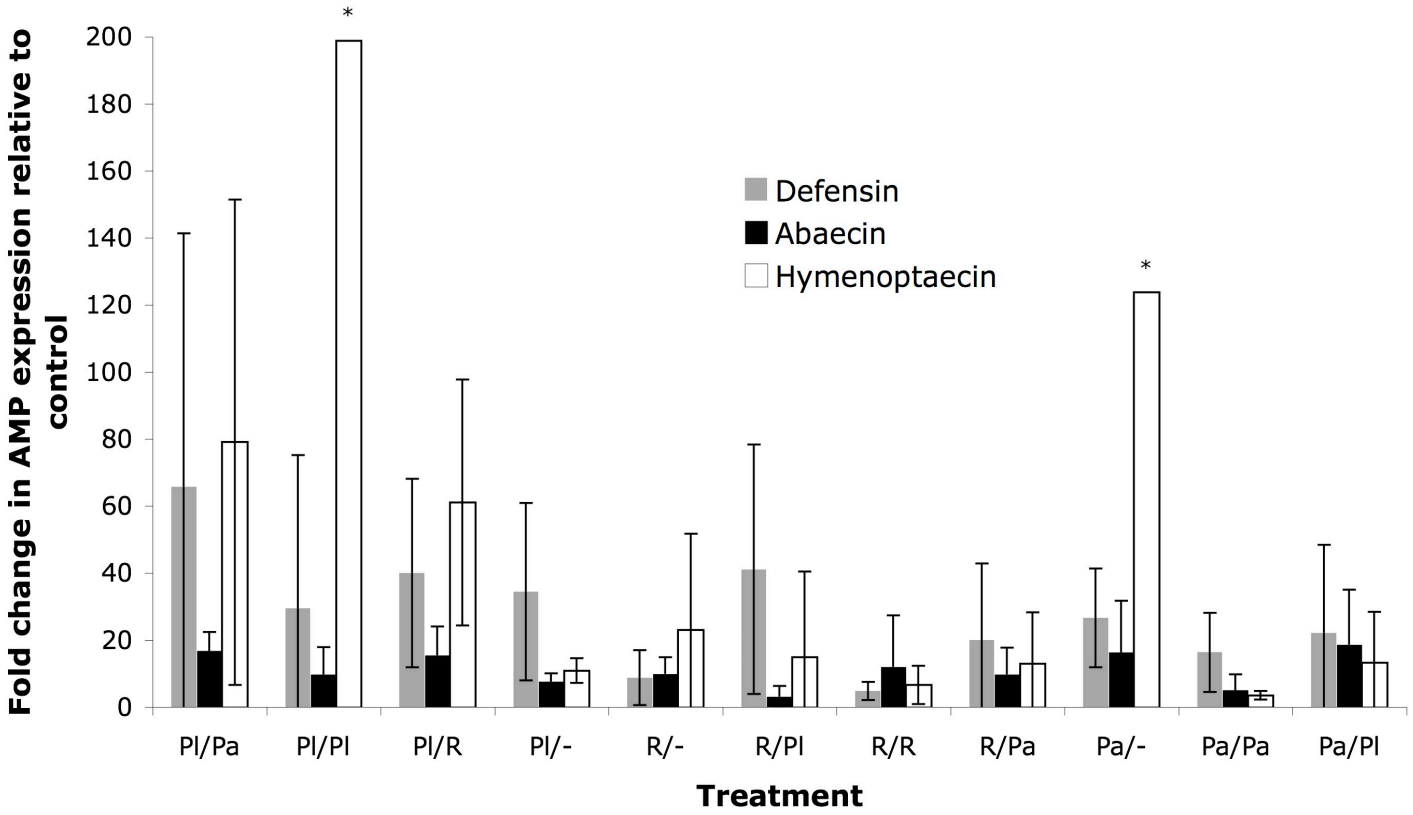

Figure 6.4 The effect of different injection regimes on expression of the AMPs Defensin, Abaecin and Hymenoptaecin of colony 2. A sample of 5-7 day old workers received a single injection of *Paenibacillus larvae* (Pl/-), *Paenibacillus alvei* (Pa/-) or ringer solution (R/-)(control) before being sacrificed 24h later. Another group of bees received a second injection 8 days after the primary injection. Bars represent median values of AMP expression for each injection combination. Bees were sacrificed 24h after secondary infection. Error bars represent standard deviation (SD) and are large SDs are omitted for bars marked with * to prevent compression of the graph. Clear colony-specific trends in AMP expression were apparent, regardless of the injection regime bees had been subjected to. There were significant differences in AMP expression between bees that received a single injection of ringer solution (control) and those injected with either either *P. alvei* or *P. larvae*. AMP expression was not significantly different between bees that had received either heterologous or homologous injections.

6.4. Discussion

This experiment tested whether AMPs were involved in specific immune priming previously demonstrated in bumblebees towards species of the gram-positive bacteria *Paenibacillus* (Sadd and Schmid-Hempel, 2006). The results showed that primary bacterial injection increased transcription of AMPs. However, AMP expression in bees that had received homologous injections was no greater than AMP expression in bees that received heterologous treatments. There were clear differences between the colonies in the levels of AMP expression.

As immunity is costly (Sheldon and Verhulst, 1996), an age related decrease in disease resistance, known as immunosenescence, has been predicted and found in numerous vertebrates and invertebrates, including bumblebees (Moret and Schmid-Hempel, 2009). Bees that received a secondary injection were 8-10 days older than bees that were sacrificed after primary injection. The MANOVA indicated that there was no effect of time on AMP expression, suggesting that there was not any effect of age on the transcription of AMPs after an insult. Gene expression levels were the same after the first injection as their equivalents after the second injection. Bumblebee worker life span in the wild is between four and six weeks (Goulson, 2003). Workers used in this experiment were well within this lifespan, being between six and sixteen days old, and it is therefore likely that immune senescence was minimal. This lack of immunosenescence agrees with a recent finding that found that AMP production did not decrease during the life of a worker (Moret and Schmid-Hempel, 2009). One possible explanation is that as AMPs are inducible they are less costly than constitutive aspects

of immunity (such as phenoloxidase activity) and there is therefore less selective pressure for them to decline with age.

The 2 colonies studied had a 10-fold difference in their response to the same bacterial insult (figure 6.1), suggesting that colonies vary in their response to *Paenibacillus* infections. This is unsurprising: analysis of AMP expression in colonies of singly-mated honeybee (*Apis mellifera*) queens is known to differ by as much as 1000-fold (Evans and Pettis, 2005b) and these expression differences are heritable (Decanini et al., 2007). Bumblebees also arise from a singly-mated queen (Schmid-Hempel and Schmid-Hempel, 2000) and are likely to that likely harbour polymorphisms in the immune gene pathways and affect the reactivity and magnitude of the immune response (Lazzaro et al., 2004) (Lazzaro et al., 2006).

The results do show that, as in honeybee workers (Evans et al., 2006), bumblebee workers also increase transcription of *Abaecin*, *Defensin* and *Hymenoptaecin* after a single injection of *Paenibacillus* bacteria (figure 6.1). It is puzzling that a similar, or larger, increase in anti-microbial peptides was not seen after secondary injection of bacteria, relative to bees receiving a secondary injection of ringer solution. One possible answer is that there were still anti-microbial peptides circulating in the haemolymph eight days after primary injection that provided enough anti-microbial activity to be effective against any secondary infection. General antibacterial activity in the haemolymph has been found to remain raised for as long as 2 weeks in bees injected with non-pathogenic bacterial constituents (Korner and Schmid-Hempel, 2004a). In *Drosophila* AMPs can increase in the haemolymph for the first 24h of infection but can

persist much longer and gradually degrade over 3 weeks (Uttenweiler-Joseph et al., 1998). There is therefore a chance that AMPs degrade over a similar time-frame in bees. However, the possibility remains that AMPs do not play a major role during *Paenibacillus* infection, which could explain the relatively low levels of AMP induction found in this experiment. Sadd and Schmid-Hempel (2006) clearly showed that bees that had received a single injection of *P.larvae* or *P.alvei*, did not have increased anti-bacterial activity in their haemolymph and this experiment complements their finding. In addition, a study in honeybee found that AMPs were induced at similar levels within 24h of a saline (i.e., control), *E.coli* or *P.larvae* injection (Evans and Pettis, 2005b), indicating that AMPs can form part of a more broad response to injury and do not necessarily show differential expression to individual immune challenges.

Sadd and Schmid-Hempel (2006) also found evidence for more a generalised protective response early after infection. Though bacterial clearance was greater in homologously-infected than heterologous-infected bees after eight days, there was no difference in the two group's survival. Both groups had greater survival than naïve-infected bees suggesting that a more generalised response occurs early after infection that provides broad cross-protection against un-related infections. Later into the infection, after twenty-two days, this cross-reactivity disappeared and was replaced by more specific protection. A similar cross-protective response has been noted in the first few days after priming doses of bacteria in the cockroach, *Periplaneta americana*, before development of a more specific response (Faulhaber and Karp, 1992). Thus, molecules such as the AMPs may contribute to this more generalised immune response early on after a primary infection since although they are induced in both heterologously and

homologously-infected bees after an 8 day interval, the level of expression between the two groups does not differ significantly.

This experiment did not look at AMP expression after a 22-day exposure lag because preliminary tests with the bees showed that the survival rate up to the 22 day point was very low, making it impossible to complete the experiment fully. The difference in survival between studies is most likely down to genotypic differences between colonies (Moret and Schmid-Hempel, 2000): where Sadd and Schmid-Hemepl (2006) used native, wild-caught bumblebee colonies, this experiment used bees sourced from a commercial breeder. As there were still clear differences in bacterial clearance rates between heterologous and homologously infected bees after 8 days it was conceivable that differences in AMP expression might also be present, so this time-point was investigated instead. Therefore, the results do rule out the possibility that there is differential expression of AMPs after a 22-day lag between exposures.

As there was no change in AMP expression between heterologous and homologously-infected bees after 8 days, this asks us to question which mechanisms and effectors *are* used to improve the bee's response toward *Paenibacillus* bacteria. Some clues come from studies in *Drospohila*. In agreement with the result of this experiment, Pham et al. conclude that AMPs are highly unlikely to mediate the priming effect in *Drosophila* (Pham et al., 2007). As in bumblebees (Sadd and Schmid-Hempel, 2006), Pham and co-workers found that a priming dose of *Streptococcus pneumoniae* specifically protected flies against a second, lethal dose of the same bacteria and enhanced its clearance from the haemolymph, lasting for the lifetime of the fly (Pham et al., 2007). Importantly, they established that phagocytosis was crucial to control and eradicate *S.pneumoniae*

infections and if blocked could prevent the primed effect, thus implicating cellular immunity in priming for the first time. Therefore it seems that the cellular immune response is likely to play a more important role in the phenomena of immune priming than humoral responses such as the *de novo* synthesis of anti-microbial peptides.

7. General discussion

Recent ecological studies with invertebrates have indicated that the interaction between host and parasite is highly specific and affects the outcome of infection (Little et al., 2008). These findings contrast with mechanistic studies that define invertebrate innate immunity as non-specific and broad acting (Lemaitre and Hoffmann, 2007). An important point needing validation across all these ecological host-parasite systems is confirmation that specificity is being generated by variation in the host innate immune response and is not the result of other interactions between host and parasite genotypes. This thesis has examined this question with regard to the specificity found between *Bombus terrestris* and its parasite *Crithidia bombi* and found strong evidence that there is a molecular basis to this phenomenon.

In Chapter 4, the experiment provided the first data to show that *Bombus* AMP expression during *C.bombi* infection mirrors the interaction effect previously represented by indirect measures of immunity (such as cell counts) (Schmid-Hempel et al., 1999). The result clearly indicated that the level of AMP expression depends on the specific combination of host line and parasite strain. However, although they are highly effective against specific pathogen classes, it seems unlikely that AMPs are the source of specificity because they can still act generally against other microorganisms (Bulet et al., 1999), and polymorphisms in the AMPs do not correlate with variation in disease resistance (Lazzaro et al., 2004). Differential expression of AMPs is therefore *associated* with immune specificity generated elsewhere in the immune response pathways.

The experiment here does not rule out the possibility that the specificity found in AMP expression is caused by parasite-mediated damage to the host's gut epithelia. The pathology of *C.bombi* infection is poorly understood but it is possible that parasite attachment damages the gut epithelia, which initiates further immune responses, perhaps by signalling to distant immune tissues or after leakage of bacteria into the haemolymph. This therefore raises the possibility that particular strains of *C.bombi* damage the gut cells of some host lines more easily than others and this interaction would not necessarily require immune specificity. The simplest way to differentiate between these possibilities would be to find functional evidence that AMPs are effective against *C.bombi.* The ultimate test would be to use RNAi to knock-down AMP expression in the bee and look at the effect this has on *C.bombi* infection. Assuming that AMPs have an anti-parasite effect, it can be predicted that their absence during infection would allow increased numbers of the parasite to develop in the gut. RNAi-based interference has already demonstrated that AMPs regulate the intensity of trypanosome infection in the tsetse fly, *Glossina mortisans mortisans* where the parasite is confined to the mid-gut and salivary glands (Hu and Aksoy, 2006).

7.1.1. The molecular basis of specificity

In summary, there are adequate empirical data to justify the use of life-history traits as an indirect measure of the immune response. However, the inevitable drawback is that these measures cannot further our mechanistic understanding of immune specificity. Thus, ecological immunology has now begun to shift its focus to elucidating the molecular basis of this phenomenon. In the honeybee, it is has already been demonstrated that expression of the anti-microbial gene *Abaecin* is extremely variable

and genotype-specific, and although higher *Abaecin* expression is associated with better disease resistance, overall productivity of these colonies is lower, thus linking the activation of the immune response to life-history variation (Evans and Pettis, 2005b). Further, a large-scale study on natural populations of *Drosophila melanogaster* indicates that variation in the disease resistance of individual genetic lines correlates significantly with genetic polymorphism within immune genes, especially in recognition and regulatory elements. It also notes the major contribution of epistatic effects to immunocompetence (Lazzaro et al., 2004). Lazzaro et al. subsequently found that particular polymorphisms had broad benefits against a number of pathogens whilst others were most effective against a subset of bacteria, and no one genome was resistant to all 4 bacterial species (Lazzaro et al., 2006). However, most of the individual polymorphisms associated with resistance to a particular pathogen could only account for 15% or less of the overall phenotypic (i.e. immunocompetence) variance of a genetic line (Lazzaro et al., 2004, Lazzaro et al., 2006), indicating that other factors contribute to the disease resistance phenotype and its variation, such as genotype*environment interactions (Lazzaro, 2008). Such immune gene diversity has been identified in a number of recognition molecules such as TEP (Obbard et al., 2008) and scavenger receptors (Lazzaro, 2005) and is possibly maintained to counteract parasite manipulation/suppression of host immune defences, in turn stimulating host-parasite co-evolution (Jiggins and Kim, 2007). These molecules have diversified and are under positive selection, perhaps driven by pathogen evolution (Sackton et al., 2007). For example, the scavenger receptors show particularly high levels of polymorphism in *Drosophila*, and have undergone duplication events (Lazzaro, 2005, Sackton et al., 2007). TEP1 contains a hypervariable region that could play a role in pathogen interactions and mosquitoes are more susceptible to *Plasmodium* infection if

the gene's expression is suppressed (Blandin and Levashina, 2004). However, studies into two invertebrate proteins from the immunoglobulin superfamily (IgSF), Down syndrome adhesion molecule (Dscam) and lectin-like fibrinogen-related proteins (FREPs), provide tantalising evidence that mechanisms more functionally akin to vertebrate adaptive immunity may also contribute to invertebrate immune specificity. In mammals, immunoglobulins are responsible for a large proportion of the specificity of surface recognition events and antigen binding/presentation seen in lymphocytes, phagocytes and antibodies (Alberts et al., 2002), and these molecules may confer similar specificity in invertebrates as discussed below.

Dscam

Down syndrome adhesion molecule (Dscam) is a highly conserved trans-membrane receptor found in nematodes, arthropods and vertebrates (Crayton et al., 2006) and was initially characterised in *Drosophila* for its major role in developmental neuronal guidance (Schmucker et al., 2000). Subsequent studies have found Dscam expressed on insect fat-body cells, haemocytes and gut epithelia, and circulating in the haemolymph (Watson et al., 2005, Dong et al., 2006b). The Dscam molecule consists of ten immunoglobulin-like (Ig) domains, six fibronectin domains and single transmembrane and cytoplasmic domains (Schmucker et al., 2000). Four exons within the Dscam molecule have multiple variants, arranged in tandem arrays that are selected by alternative splicing of the pre-RNA transcript in a mutually exclusive manner. All but one of these variable exons codes for a region in three separate extracellular Ig domains (the fourth is within the transmembrane domain) and in combination with constant exons can potentially produce in excess of 18,000 different immune-specific Dscam

isoforms in *Drosophila*, each with different binding affinities and specificities (Schmucker et al., 2000, Watson et al., 2005). Individual tissues, such as the brain or fat body, express specific isoform subsets (Watson et al., 2005). Examples of Dscam have been found in the beetle *Tribolium castaneum* and moth *Bombyx mori* (Watson et al., 2005). In the honeybee, *Apis mellifera*, and water flea, *Daphnia magna*, the potential number of Dscam isoforms is in the range of 12,000 to 13,000 as these species have fewer splice alternatives at each variable exon (Graveley et al., 2004, Brites et al., 2008) (Figure 7.1)

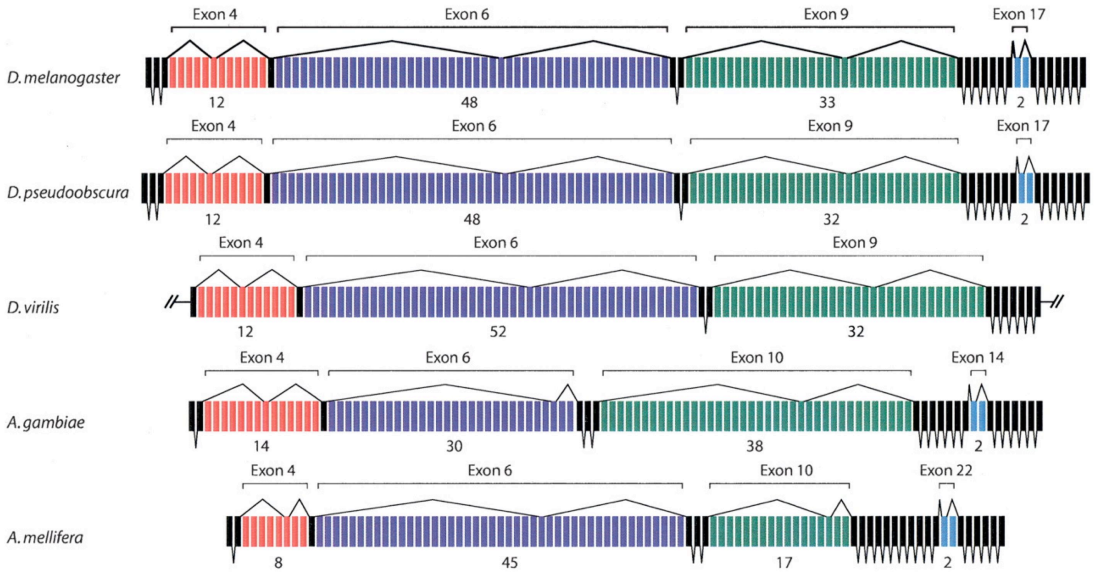

Figure 7.1 The Dscam gene in representative Dipteran and Hymenopteran species (From Graveley et al. 2004). Exons 4,6,9/10 and 17 represent variable exons that undergo alternative splicing and constant exons are represented in black. Exon 17 is contained in the transmembrane domain. Variation in variable exon number is apparent between the 5 species.

The Dscam molecule has undergone modular evolution (Crayton et al., 2006), reflective of varying functional constraints imposed by its dual role in mediating both homophilic (i.e., Dscam-Dscam) and heterophilic (i.e., Dscam-protein/carbohydrate) interactions through two separate protein epitopes (Meijers et al., 2007). Notably, it is the variable

exons within the heterophilic-binding domain that exhibit considerable diversity between species and have more species-specific exon variants, whereas the homophilic-domain-specific exon (4 in *D.melanogaster*) has more orthologs throughout insect species. These findings are consistent with a putative role for the heterophilic domain as an immune receptor for evolving pathogen-associated-molecular-patterns (PAMPs) (Graveley et al., 2004, Meijers et al., 2007). RNAi studies support this theory, finding that knockdown of *Anopheles gambiae* Dscam (AgDscam) expression in adult mosquitoes causes an increase in basal opportunistic microbe levels and mortality rates after infection (Dong et al., 2006b).

Unchallenged *Drosophila* S2 cells express multiple Dscam isoforms (Neves et al., 2004), but infection appears to stimulate a change in composition to improve pathogen detection. *Anopheles* Sua5B cells (a haemocyte-derived cell line) challenged with bacteria, fungi or PAMPs induce targeted alternative splicing of AgDscam, producing specific repertoires of splice variants that impart the receptor with a greater binding affinity to the class of infecting pathogen (Dong et al., 2006b), indicating that splicing is regulated and non-random. Silencing these specific exon variants and repeating the immune challenge markedly reduces AgDscam-pathogen associations, illustrating the discriminatory capability conferred by this response. A similar splicing bias occurs in mid-gut AgDscam in response to two *Plasmodium* species that is impaired by specific variant exon silencing (Dong et al., 2006b). Coupled with its proven ability to bind to a range of pathogen classes and enhance their phagocytic removal in both *Drosophila* (Watson et al., 2005) and *Anopheles* (Dong et al., 2006b), the hypervariable Dscam receptor is likely to play an prominent role in mediating fine-scale pathogen discrimination.

Fibrinogen-related proteins (FREPs)

Invertebrate FREPs are highly related to the mammalian clotting factor fibrinogen, on account of possessing a conserved homophilic fibrinogen-like (FBG) domain in addition to one or two IgSF domains (Léonard et al., 2001, Zhang et al., 2001). In humans, the FREP ficolin displays lectin-like activity towards pathogens and initiates their destruction by enhancing phagocytosis and activating the complement system (Lu et al., 2002). These activities are mediated through binding of specific pathogen-associated carbohydrate moieties such as N-acetyl-D-glucosamine (GlcNAc) (Matsushita et al., 1996). FREPs with similar binding properties and activities have been identified in invertebrates, such as sialic acid-binding lectin, expressed in the epidermis and mucosa of the slug, *Limax flavus* (Kurachi et al.) and ficolin-like molecules in the ascidian *Halocynthia roretzi* that bind N-acetylglucosamine (Kenjo et al., 2001). Tachylectins agglutinate bacteria and enhance big defensin (an AMP) activity in the horseshoe crab *Tachypleus tridentatus*, (Gokudan et al., 1999), and aslectin in the mosquito, *Armigeres subalbatus*, is up-regulated after bacterial infection and binds the elicitor (Wang et al., 2004). FREPs from the snail *Biomphalara glabrata* appear to have a specific role against digenetic trematodes such as *Echinostoma paraensei* since they are upregulated in the hemolymph after infection and can bind and agglutinate trematode sporocysts (a developmental parasite stage) and the secretory-excretory products (SEPs) they release (Adema et al., 1997). Though bacteria can be bound by *B.glabrata* FREPs (Zhang et al., 2008), the agglutination response is absent during microbial insults and thus appears specific to trematode infection in this model (Adema et al., 1999). However, Zhang et al. indicate in the scallop *Argopecten irradians* that FREP-mediated agglutination is still utilised against microbial infections

(Zhang et al., 2009). Thus, the functional properties of FREPs are highly varied and diverse between species, but generally appear to act as lectin-type molecules, with opsonin-like properties to attract immune cells to a pathogen and activate complement-like cascades, or limit the growth of infection.

The most interesting aspect of FREPs is their extensive diversity, generated at the genomic and transcriptional level, which may confer individual molecules with specific binding properties and functional roles. For example, there are a notable lack of 1:1:1 orthologs between the FREP genes of *A.gambiae*, *D.melanogaster* and the mosquito *Aedes aegypti*, but there are vast numbers of species-specific forms, contrasting with 1:1:1 orthology of most Imd pathway elements (Waterhouse et al., 2007). Multi-gene FREP families are reported in many species, with 14-47 genes across *Drosophila* species (Middha and Wang, 2008), 53 in the mosquito *A. gambiae* (Wang et al., 2005) and 13 subfamilies in *B.glabrata* (Léonard et al., 2001, Zhang and Loker, 2003), and many have proposed carbohydrate binding capabilities and roles in innate immunity. FREP diversity is extended further by alternative exon splicing (Zhang and Loker, 2003), gene conversion and hypermutation (Zhang et al., 2004) in somatic cells of *B.glabrata*, and may increase FREP diversity in a similar manner in *Drosophila melanogaster* (Wang et al., 2005). To illustrate, Zhang et al. identified extremely high diversity in the IgSF1 domain of the FREP3 gene in *B.glabrata*, finding only one common sequence amongst a total of 82 unique sequences after comparing the genomic IgSF1 composition of two snails (Zhang et al., 2004), but the FBG domain is much more conserved (Zhang et al., 2001). By contrast the FBG domain of *A.gambiae* FREPs shows sequence conservation at core residues that conserve secondary structures, but has diverged outside these regions, which may alter its carbohydrate binding properties

(Dong and Dimopoulos, 2009). Though the consequence of these mechanisms on FREP binding specificity has yet to be directly tested, molecular studies studying multi-gene diversity provide some attractive suggestions.

Studies in *B.glabrata* imply that FREP variation may confer differential binding specificities that are selectively up-regulated in response to infection. A *Schistosoma mansoni*-resistant strain of *B.glabrata* (BS-90) shows nearly a 60 fold increase in FREP2 expression after infection with the digenetic trematode, whereas a susceptible snail strain (M) shows no transcriptional response (Hertel et al., 2005). Both snail strains are susceptible to a second digenetic trematode, *Echinostoma paraensi*, despite up-regulating a suite of FREPs in response to infection (Zhang et al., 2008). Zhang et al. established that plasma from snails pre-exposed to bacteria, *E.paraensi* or *S.mansoni* contained different pools of FREPs, by mixing the individual plasma extracts with *E.paraensi* SEPs and sporocysts, and purifying the proteins that bound the pathogen. "M-line", susceptible snails responded to all three challenges by releasing a similar broad suite of FREPs that could bind *E.paraensi*. In contrast, FREPs from "BS-90", resistant plasma only bound *E.paraensi* if the plasma originated from snails pre-exposed to the same parasite. Binding by *S.mansoni*-induced FREPs was weak, indicating that *B.glabrata* can discriminate between infections and respond by up-regulating specific suites of FREPs with particular binding properties (Zhang et al., 2008). By contrast, knocking-down expression of *A.gambiae* FREP39 with RNAi causes a notable increase in permissiveness to *Plasmodium falciparum* infections, and seems specific to this pathogen (Dong et al., 2006a) and other RNAi studies targeting multiple FREP genes indicate that synergism and complementarity between FREP subfamilies is also employed to co-localise and interact with *Plasmodium* in the mid-gut epithelium (Dong

and Dimopoulos, 2009). Evidently, FREPs are capable of responding to broad classes of pathogens by releasing specific suites of FREPs with greater binding affinity. However, the full extent of their discriminatory abilities have yet to be tested and it remains to be seen whether fine-scale pathogen differentiation, as is needed to produce immune-specificity, can be generated by the underlying mechanisms and genetic diversity found within this extensive gene family.

7.1.2. Possible mechanism of immune priming

Sadd and Schmid-Hempel (2006) showed that the level of AMP induction 24h after a secondary infection is no greater than 24h after a primary insult with the same pathogen, suggesting that these molecules are not involved in the primed immune response. Although 24h post-injection is a suitable point to look for a change in AMP expression (Lemaitre, 1997), there is the possibility that peak in "primed" AMP induction occurs earlier and was therefore missed in this experiment and this can only be checked by repeating the experiment with more time points. Otherwise, if the the result of this experiment is accepted, intuitively, a stronger AMP up-regulation after secondary challenge could be expected because the primed response should increase the rate of microbial death in the haemolymph. This does not appear to be the case in *Bombus*. Pham et al. (2007) have demonstrated in *Drosophila* that phagocytosis is central to the primed immune response whilst haemolymph antibacterial activity remained unchanged (Pham et al., 2007).

A cell-based priming mechanism seems highly plausible and would fit very well with recent work implicating this Dscam in immunological priming. One hypothesis is that a

primary infection induces alternative splicing of Dscam, producing variants that recognize and bind the particular pathogen most effectively. These Dscam receptors can act as free-floating opsonins in the haemolymph in addition to their bound forms on cells such as the haemocytes (Dong et al., 2006b). During a homologous secondary infection, these receptors would then bind to the previously encountered pathogen, and more rapidly initiate cell-based responses such as phagocytosis (Pham et al., 2007) through interaction with cells exhibiting membrane-bound Dscam receptors with a homologous exon representation (Dong et al., 2006b). Although the binding specificity of Dscam splice-variants has yet to be determined, the enhancement to phagocytosis that it can provide is evocative of the improved bacterial clearance found in both bumblebees (Sadd and Schmid-Hempel, 2006) and *Drosophila* (Pham et al., 2007) during specific immune priming, suggesting that Dscam is likely to play an important role in this phenomenon.

In hindsight, that phagocytosis is used to create a primed response is unsurprising since they are constitutive and begin to engulf bacteria within minutes of infection (Hillyer et al., 2003). By comparison, priming of inducible defences, such as AMP synthesis, would be more difficult because gene expression and *de novo* protein synthesis is a slow and relatively inflexible process that limits its ability to respond more quickly, even if recognition has been improved by priming. Using *Drosophila*, Lemaitre et al (1997) show that transcription of AMPs increases around 1h after injection of bacteria or fungi into the haemolymph and does not peak until 2-4h later (Lemaitre, 1997). Thus, it would be difficult to imagine how the speed of this process would be able to increase. A study by Haine et al. suggests that this slow rise in antibacterial activity is beneficial and serves to "mop-up" bacteria that have survived constitutive responses such as

phagocytosis. Using the flour beetle (*Tenebrio molitor*) the showed that microbial survivors of constitutive defences, such as phagocytosis, are more resistant to host immunity. The delayed rise in antibacterial activity may therefore help to slow the build-up of these resistant bacteria in the haemolymph (Haine et al., 2008).

In a wider context, it is clear that recognition and regulatory pathways regulate large sections of innate immune defences. Many immune proteins participate in synergistic interactions that increase their functional diversity beyond that which is contained genetically (Schulenburg et al., 2007). Thus, variation in these molecules could potentially have effects on the regulation of many downstream targets and therefore bring about larger changes in the dynamics of the immune response to a particular pathogen (Lazzaro et al., 2004, Lazzaro et al., 2006). This experiment supports this idea by demonstrating that individual host genotypes show differential expression of AMPs to specific strains of *C.bombi.*

In Chapter 5, *Bombus* immune genes were identified that were up-regulated in response to *C.bombi* and their temporal expression was characterised during infection. This experiment is the first to investigate gene expression in this system. One criticism of the experiment is that it used different colonies to compare expression between infected and non-infected bees and this "colony effect" cannot be separated from the effects of infection. Therefore the experiment needs to be repeated in a single colony to verify the result. Also, it is unclear why non-infected bees showed some fluctuations and peaks in AMP expression that were apparent in infected bees. For example, there was a large peak in AMP expression in infected bees at 12h, but similarly there was clearly a smaller peak in non-infected bees as well. It seems unlikely that this pattern is an effect

of handling since non-infected bees were not handled or fed a control solution when the infected group were inoculated, so they effectively remained undisturbed until they were sacrificed. As discussed in detail in Chapter 5, it is possible that underlying circadian patterns regulate the level of AMP expression throughout the day that might create "windows" in AMP regulation where regulation promotes higher standing levels of immune gene expression and stronger induction of the stronger immune response. Other studies have also found that host susceptibility to infection varied according to the time of day that infection was administered (McDonald and Rosbash, 2001, Lazzaro et al., 2004, Lee and Edery, 2008). Even if circadian cycling of immune gene expression was responsible for the fluctuations in AMP expression, the results still suggest that the bee is responding to *C.bombi* infection, which has not been demonstrated before. Finally to improve the experiment further, greater sampling of time-points around the peak of AMP expression would also be useful to understand the dynamics of AMPs in greater detail. What is unclear from this experiment is how changes in gene expression relate to the development of *C.bombi* in the bee. Any repeat of this temporal experiment should therefore also examine the within-host dynamics of *C.bombi* infection to clarify this point.

In conclusion, this thesis has provided molecular evidence for the ecologically-based phenomenon of immune specificity. This research reiterates the importance of using natural host-parasite systems when testing the specificity and adaptive nature of invertebrate immunity. Approaching studies of immunity by integration of molecular knowledge into natural host-parasite systems in this way can only serve to enrich our understanding of the higher capabilities and regulation of invertebrate innate immunity.

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