Ageing and the cellular immune response in adult *Drosophila melanogaster*

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Abstract

Senescence is the age-related progressive deterioration of physiological processes leading to an increased likelihood of death and is a phenomenon that occurs nearly universally throughout all the world's organisms. This thesis initially investigated the impact of ageing on the adult *Drosophila melanogaster* cellular immune response and demonstrated that the cellular immune response in *D. melanogaster* adults did experience an age-dependent decline in function. There was a striking reduction in haemocyte ability to phagocytose foreign particles with up to 30% less phagocytosis occurring in four week old flies compared to one week olds. Haemocyte number also declined in female flies by up to 32% across these ages.

An exploration into the mechanisms that could underlie these observed senescent declines in haemocyte number and function revealed that the age-dependent reduction in the circulating haemocyte population occurred regardless of whether flies were unharmed, wounded or infected. The loss of phagocytosis ability in haemocytes in ageing flies was shown to be a cell autonomous process; there was an equal age-dependent decline (~13%) in haemocyte phagocytic activity in both *in vivo* and *ex vivo* assays. However, an attempt to identify phagocytic receptor systems that drove senescence in haemocyte function was unsuccessful. The contribution of the cellular immune response in determining survival following a fungal infection was not conclusively demonstrated, however flies with reduced *Dif* expression had significantly increased pathogen susceptibility.

Although pathogen resistance can decline due to immune senescence, disease defence may also be enhanced as an animal's life progresses through the formation of immunological memories of prior microbial encounters. This thesis revealed that the cellular immune response in *D. melanogaster* provides a strong, broadly specific and

relatively long-lasting immunological priming response. Haemocytes phagocytosed up to 33% more microbes per cell during a secondary encounter, and up to 50% more if flies had received two homologous primes. This was not general immune upregulation as a heterologous microbial encounter caused a reduction in the phagocytic ability of haemocytes compared to controls. The level of enhancement in the phagocytic ability of haemocytes also declined with the age of the fly, meaning that the ability to develop a primed response senesced. These results are unprecedented in *Drosophila* and challenge our conventional interpretation of immune senescence because individual immune history has been shown to shape later cellular immune responses.

Ageing is a complex and variable process. Some of the differences observed in ageing rates between populations can be due to different selection pressures. Natural selection acts on genetic variation within a population to increase fitness whereas host-parasite interactions predominantly influence genes related to immune parameters. Many genes have pleiotropic effects as well as there being potential trade-offs between investment in longevity, reproduction and immunity. To explore potential genetic variation in immune and life history traits and whether variation in immune parameters negatively influenced other life history traits related to ageing, a panel of outcrossed genotypes of *D. melanogaster* were assessed. As the flies were derived from individuals originally sourced from a natural population, the results suggest that a striking amount of genetic variation in immune and life history traits is present in wild populations. However no significant correlations between genetic variation in ageing and genetic variation in investment in immunity were identified.

Though, perhaps not surprisingly, no key biomarker of ageing in *D. melanogaster* was identified; this thesis has contributed some significant findings on the effects of ageing on adult *D. melanogaster* especially relating to their cellular immune response.

Declaration of authorship

I, Danielle Kyle Mackenzie, declare that this thesis has been composed by myself and that it embodies the results of my own research. Where appropriate, I have acknowledged the nature and extent of work carried out in collaboration with others included in the thesis.

Signed …….

Date ……

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Chapter 1: General Introduction

Ageing, or senescence, is a complex process that causes the progressive degeneration of physiological capacity, resulting in a greater probability of death (Kirkwood & Melov, 2011). Senescence can either be observed in the decline of an organism's functional capabilities as it ages (functional senescence) or in an age-related increase in the chance of mortality (demographic senescence). Interventions that alleviate the detrimental effects of ageing have great value, thus identifying the mechanisms behind ageing has been the key aim of many current scientific studies (Chou *et al*., 2013; Pace *et al*., 2013; Rodella *et al*., 2013).

Although the discovery of means to increase human 'health-span' and lifespan is a principal driver for many researchers, ageing is not a phenomenon restricted to humans, but occurs throughout most of the world's organisms. Much research is devoted to exploring the mechanisms of ageing in order to understand the evolutionary processes behind it. Medawar wrote an essay in 1952 entitled "An unsolved problem in biology", focussed on the paradox that as senescence involved the decline of fitness, why had natural selection not acted to ameliorate the effects of senescence from populations? The German theorist August Weismann had discussed the topic prior to Medawar (Weismann, 1889), but Medawar was the first to formalise what theorists before him had proposed: the reason senescence existed was due to the declining force of natural selection as populations aged (Fisher, 1930; Haldane, 1941; Medawar, 1952). From then until now, theories explaining the evolution of ageing continue to arise and develop.

1.1. Evolutionary theories of ageing

A review in 1990 classified 300 theories of ageing (Medvedev, 1990), some of which have been relegated due to a lack of experimental support, and many of which have been merged and altered as advances in our understanding of physiological processes are made. It is widely acknowledged that selection acts powerfully to prevent ageing occurring before an organism reaches the age of first reproduction (Kirkwood, 2008); however, the evolutionary mechanisms of ageing after this age are fiercely debated.

The main evolutionary theories of ageing can be broadly categorised into two classes. Either they suggest that senescence occurs because alleles causing late-life fitness-deterioration are selectively neutral and have therefore not been purged by selection; alternatively selection may favour genes which have deleterious late-life consequences if they provide an overall benefit due to trade-offs between fitness early and late in life. The mutation accumulation theory of ageing (Medawar, 1952) falls into the first category, whereas antagonistic pleiotropy (Williams, 1957) and the disposable soma theory (Kirkwood, 1977) fall into the latter.

1.1.1. Antagonistic pleiotropy

One main theory related to the weakening of the force of natural selection in older individuals is that of antagonistic pleiotropy (Williams, 1957). The premise is that some alleles exist within the genome due to their beneficial fitness effects in early-life, though they may have detrimental effects in late-life (Williams, 1957). Empirical evidence for these genes must demonstrate a causative link between early-life activity and late-life activity. A range of such genes have been identified, especially those involved in the target of rapamycin (TOR) signalling pathway (reviewed by Stipp, 2013). The TOR pathway initially drives developmental growth but then senescence and age-related diseases in later life (Jia *et al*., 2004; Partridge *et al*., 2011).

Negative correlations between reproduction and longevity are a commonly-cited example of antagonistic pleiotropy and have been identified in multiple species: *Caenorhabditis elegans* (Kenyon, 2010); *Drosophila* (Rose & Charlesworth, 1980); and recently demonstrated in Asian elephants (Robinson *et al*., 2012). Although a recent selection experiment with *Drosophila* did not find a negative correlation between longevity and reproduction (Wit *et al*., 2013a).

1.1.2. Mutation accumulation

Medawar proposed the theory of mutation accumulation to explain the reason why senescence occurs (Medawar, 1952). Mutations occur constantly within populations and many have negative implications for fitness and viability (Medawar, 1952). Natural selection acts strongly on detrimental mutations that have fitness effects in early life. However, because the force of selection declines with age, mutations that have negative fitness effects only on older organisms are not eliminated efficiently by selection (Medawar, 1952). The mutation accumulation theory proposed that over time such mutations with late-life-specific fitness effects would accumulate in populations and that this would be manifested as senescence. It has been predicted that if a large number of genes in the genome only have late-life negative effects then this would mean that additive genetic variation for fitness-related traits would increase when these were assayed at older ages, compared to younger individuals (Edney and Gill, 1968).

A complicating factor in testing whether additive genetic variance increases in later-life is that additive genetic variance may be inflated in early-life via antagonistic pleiotropy. Under this premise, antagonistic pleiotropy does not influence late-life traits and so is not an evolutionary mechanism of ageing, but it does increase the additive variance present in early-life (Rose, 1982). Although a significant number of conserved age-

specific gene expression patterns have been identified across a range of species (McCarroll *et al*., 2004), studies on *Drosophila* age-specific genetic variance are generally dated and do not exclusively support the mutation accumulation theory of ageing (Charlesworth & Hughes, 1996; Promislow *et al*., 1996; Snoke & Promislow, 2003).

1.1.3. Disposable soma

The 'disposable soma theory' (DST) postulates that the accumulation of molecular damage within an individual causes ageing because repair is costly and resources are limited (Kirkwood, 1977). Organisms have groups of physiological activities that metabolic resources must be allocated to, such as reproduction, growth, maintenance and repair (Kirkwood, 2008). If resources are limited then investment in one activity may restrict the resources available for another. This may lead to a fundamental trade-off between reproduction and ageing, because the mechanisms for maintenance and repair that are necessary to protect against ageing are resource-limited.

Studies have demonstrated that genes which control metabolism impact longevity. For example, *Drosophila melanogaster* females with a mutated insulin-like receptor (InR) gene have an 85% increase in lifespan (Tatar *et al*., 2001); similarly genes related to the target of rapamycin (TOR) signalling pathway in *C. elegans* (Jia *et al*., 2004), *Drosophila* (Partridge *et al*., 2011), mice (Neff *et al*., 2013), and humans (Paastoors *et al*., 2013).

1.1.4. Hyperfunction theory

Some evolution of ageing theorists attribute greater importance to metabolism rates being the source of ageing, rather than wider trade-offs between different physiological processes. The argument is that when resources are not limited somatic damage still

accumulates; conversely, when resources are limited (calorific restriction) ageing decelerates and lifespan increases (Blagosklonny, 2010). A separate theory arose termed the 'hyperfunction theory' (Blagosklonny, 2010; Gems & de la Guardia, 2012; Gems & Partridge, 2013). This postulates that molecular pathways intrinsically linked to an organism's development though beneficial in early-life becomes harmful in later-life. This theory is predominantly focused on the target of rapamycin (TOR) signalling pathway mentioned previously (Stipp, 2012). Calorific restriction interferes with metabolic signalling including TOR which slows senescence in older individuals and increases lifespan (reviewed by Stipp, 2012). Calorific restriction memetics, including the drug rapamycin, dramatically alter ageing and longevity in mice (Harrison *et al*., 2009; Neff *et al*., 2013; Wilkinson *et al*., 2012), but they do not stop ageing entirely.

1.2. Mechanisms of ageing

In the same way that there is no consensus on a theory of the evolution of ageing, the principal mechanisms of ageing are equally disputed. Identifying the factors causing age-related damage may offer promise for how ageing can be most effectively prevented or at least moderated.

1.2.1. Oxidative damage

The 'free radical hypothesis' is still a developing theory. The initial premise was that the generation of oxygen free radicals formed in mitochondrial electron transport chains are toxic and cause ageing (Harman, 1956). This theory merged with the 'oxidative stress theory', which focused more on the balance between reactive oxygen/nitrogen species (ROS) pro-oxidants and the antioxidants required to mitigate ROS-mediated oxidative damage (Sohal & Allan, 1990). However, recent work has revised our understanding of the role of ROS, emphasising it as important in cell signalling and other features (Forman *et al*., 2010; review by Sohal & Orr, 2012). The

expectation that higher levels of antioxidants would alleviate functional senescence and increase lifespan has not been supported experimentally (Gems & Doonan, 2009; Gems & de la Guardia, 2012; Mockett & Sohal, 2010). Although the balance between pro-oxidants and antioxidants alters protein activity in aged organisms, the link between ROS and ageing is still being explored (Clancy & Birdsall, 2013; Sohal & Orr, 2012).

1.2.2. Telomeres

Telomeres are DNA repeats (TTAGGG) at the end of chromosomes which progressively shorten following replication leading to cellular senescence or apoptosis (Counter *et al*., 1992). The enzyme telomerase can elongate telomeres leading to cell-lineage immortality in tumour cells; however its expression has also been intensively studied as an anti-ageing therapy (reviewed by Martinez & Blasco, 2011). In addition to telomere DNA, the associated shelterin protein complex also has a role in cell maintenance as mutant mice with normal length telomeres but lacking shelterin proteins displayed accelerated ageing phenotypes (Donate & Blasco, 2011). Telomere shortening is the mechanism behind the Hayflick limit: a naturally evolved cellular clock which limits the number of cellular divisions thus leading to cellular senescence and ageing, but with the benefit of providing an efficient tumour suppression mechanism (Hayflick, 1961).

However, this mechanism of ageing can be interrupted as a few studies in mice have shown. Cancer resistant individuals which overexpressed telomerase reverse transcriptase (TERT) experienced reduced telomere damage with age, delayed ageing characteristics and a 40% increase in median lifespan (Tomas-Loba *et al*., 2008). Additionally, a specific increase in later-life expression of telomerase delayed ageing and increased longevity by 24% and 13% respectively, when activated in 1 and 2 year

old mice without a corresponding increase in the presence of cancers (Bernardes de Jesus *et al*., 2012). This type of anti-ageing therapy therefore could be effective in adults and older individuals.

1.2.3. Genetic control of ageing

An individual increases in fitness the longer they are reproductively viable, therefore ageing itself does not provide an evolutionary advantage (Kirkwood & Melov, 2011). Whilst selection does not act to limit lifespan, a wide variety of genes have been identified that are pro-ageing and which reduce lifespan. The pro-ageing effects of the insulin-like receptor (InR) gene in *D. melanogaster*, mentioned earlier, are one example (Tatar *et al*., 2001). Variation in *methuselah* gene expression in *Drosophila* correlates with variation in lifespan and senescence (Schmidt *et al*., 2000). However, although a natural population of *D. melanogaster* in Australia demonstrated variation in *methuselah* gene expression, this was not correlated with variation in lifespan (Sgrò *et al*., 2013). This suggested that though *methuselah* expression can influence lifespan, variation in longevity in the wild is more strongly determined by other factors.

1.2.4. p53

The tumour suppressing gene *p53* has received a great deal of attention as a possible 'anti-ageing' gene (reviewed by Rufini *et al*., 2013). Its activity is demonstrated to maintain cell homeostasis and its expression declines in ageing humans (Liang *et al*., 2013). Altered *p53* expression produced sex-specific pleiotropic effects on adult lifespan in *Drosophila* (Wasker *et al*., 2009). However, enhanced *p53* expression in mice reduced lifespan and individuals demonstrated characteristics of accelerated ageing (Tyner *et al*., 2002). In a later study, mice with an additional activator of *p53* ($p19^{ARF}$) alongside heightened *p53* expression had improved regulation of *p53* expression and demonstrated an increase in longevity, reduced ageing rates and

maintained cancer resistance (Matheu *et al*., 2007). The precise mechanisms that link *p53* expression with ageing and lifespan are not fully understood, but due to this gene's key role in tumour suppression and association with longevity and senescence it continues to be intensively studied (Rufini *et al*., 2013).

1.3. Senescence

Senescence is the age-dependent progressive deterioration of physiological processes and the greater probability of death. Extensive work has been done on the effect of senescence on humans, demonstrating that as humans age their functional status declines (such as reduced mobility and brain function and increased susceptibility to disease (reviewed by Naumova *et al*., 2009)). Much of the work has often been based on related studies in model organisms such as *D. melanogaster*, which experience the same aspects of functional senescence (Leips, 2009).

For example, an age-dependent reduction in motor function occurs in both *Drosophila* (Gargano *et al*., 2005) and humans (Jang & Van Remmen, 2011). Some of the molecular pathways between the two are conserved. This has led to the use of *Drosophila* as a model for understanding the pathologies of, and developing therapies for, age-associated motor diseases such as Huntington's and Parkinson's disease (Guo, 2010; Zala *et al*., 2013).

Large-scale genetic screens in *Drosophila* have identified candidate ageing or lifespan determining genes (Pletcher *et al*., 2002; Wilson *et al*., 2013; Wit *et al*., 2013b); sometimes these are conserved across species, an example being the TOR signalling pathway described earlier. This makes *Drosophila* an invaluable model for understanding the processes of ageing, not just in insects generally, but also with the benefit of understanding similar processes in other species, including humans.

1.3.1. Senescence in the wild

Some of the original theories on the evolution of ageing suggested that senescence did not occur in natural populations, but only in the abnormal protected environments which humans and captive animals experience (Kirkwood, 1977; Medawar, 1952). The basis for this supposition was that organisms in the wild rarely reach an age when senescence would begin to take effect, due to early mortality caused by extrinsic factors such as predation, disease, starvation or cold (Kirkwood, 2008).

However, abundant evidence has since accumulated irrefutably demonstrating that senescence does occur in wild populations (reviewed by Nussey *et al*., 2013). Importantly, senescence is not just the senility or extreme frailty of old age, but can be a gradual deterioration of physiological process that begins from the age of first reproduction (Hamilton, 1966; Williams, 1957). Longitudinal studies on wild populations have demonstrated age-related increased mortality and declining reproduction in multiple species such as great tits (Bouwhuis *et al*., 2009), guillemots (Reed *et al*., 2008), mute swans (Charmantier *et al*., 2006), red deer (Nussey *et al*., 2006), and even insects that live only a few weeks such as male antler flies (*Protopiophila litigata*) (Bonduriansky & Brassil, 2002).

1.3.2. *Immunosenescence*

Immunosenescence is the deterioration of the immune system in ageing organisms, and is another aspect of functional senescence. In elderly humans, the effectiveness of the adaptive immune response becomes much reduced, lowering the efficacy of vaccination (Chen *et al*., 2009; Lang *et al*., 2011; Lang *et al*., 2012) and increasing individuals' susceptibility to pathogens (Chou & Effros, 2013; Dewan *et al*., 2012; Ma & Fang, 2013). Immunosenescence is multifaceted, for example there is a reduction in lymphocyte (B and T cell) number during ageing (Aberle *et al*., 2013); furthermore

there is a decrease in macrophage phagocytic activity and their ability to present antigens to T cells (Solana *et al*., 2012). However, though the adaptive immune response demonstrates a decline in function, loss of regulation of the innate immune system in ageing individuals often leads to heightened immune activity in the elderly; 'inflamm-ageing' (Franceschi *et al*., 2000). This leads to the increased prevalence of autoimmune diseases such as Type II diabetes and rheumatoid arthritis (Boots *et al*., 2013; Pietropaolo *et al*., 2000), and overly active inflammatory responses in the elderly (Pereira *et al*., 2013).

Due to the age-related decline in the adaptive immune response in vertebrates and their greater reliance on the innate immune response in later life to combat disease, understanding the age-related change in the innate immune response has become the focus of many studies. Invertebrates do not possess the adaptive immune system present in vertebrates; they rely exclusively on their innate immune response to defend against infection. This comparative simplicity and the many conserved elements of immunity between vertebrates and invertebrates make invertebrates ideal for addressing questions related to ageing and immunity that are difficult to examine in vertebrate systems (Leips, 2009; Schneider, 2000).

Drosophila have always played a key role in these investigations (reviewed by Pandey & Nichols, 2011), due to their status as a model organism and the conserved innate immune traits between them and vertebrates (Eleftherianos & Castillo, 2012; Ramet, 2012). Examples of this conservation include the roles of Toll proteins and NF-κB transcription factors in the immune responses of both *Drosophila* and mammals (Leulier & Lemaitre, 2008). Furthermore, the use of *Drosophila* as a model for studying mammalian phagocytic processes was first validated when the scavenger receptor known as Croquemort, which mediates binding of *Drosophila* phagocytic immune cells to apoptotic cells, was found to be homologous with class B scavenger receptors from

the CD36 superfamily that mediate the engulfment of apoptotic cells in mammals (Franc *et al*., 1996).

Just as ageing humans suffer increased morbidity and mortality from pathogen infections, so too do invertebrates. As *Drosophila* age they contain higher bacterial loads (Ren *et al*., 2007), and increased infection-induced mortality (Ramsden *et al*., 2008). Pathogen susceptibility of the worm *Caenorhabditis elegans* increases with age (Laws *et al*., 2004), and mosquitoes (*Aedes aegypti*) have been demonstrated to have a declining haemocyte population during adulthood, associated with increased pathogen susceptibility (Hillyer *et al*., 2005).

1.4. *Drosophila* immunity

The *Drosophila* immune response is comprised of the integrated response of humoral and cellular mechanisms cooperating together to combat pathogen attack. If the fly's primary defences are breached (its chitinous cuticle and the barrier epithelia of the gut, trachea and reproductive tract), this activates a wide array of proteolytic cascades which initially trigger melanisation, coagulation and encapsulation (Lemaitre & Hoffmann, 2007). These responses are mediated by signals from receptor molecules such as peptidoglycan recognition proteins (PGRPs) and gram-negative binding proteins (GNBPs) which circulate in the fly's haemolymph (Ferrandon *et al*., 2007). PGRPs were originally identified in the silkworm (*Bombyx mori*) as activators for the prophenoloxidase (proPO) cascade (Yoshida *et al*., 1996).

There have been thirteen PGRP family members identified in *Drosophila*, all of which have been found to possess a C-terminal PGRP-domain that is conserved between insects and mammals (Leclerc & Reichhart, 2004). Both the PGRPs and GNBPs perform vital roles in activating the fat body (analogous to the mammalian liver) to

produce antimicrobial peptides (AMPs), which are secreted directly into the haemolymph to kill invading microorganisms (Tzou *et al*., 2002). AMPs are also produced, though in smaller quantities, by barrier epithelia (Pinheiro & Ellar, 2006). Eight classes of AMPs have currently been identified, which have different specificities against microbial immune challenges (Hultmark, 2003). AMP expression is principally regulated by two main signalling pathways (activated in part by PGRPs and GNBPs): the Toll pathway and the immune deficiency pathway (IMD).

1.4.1. Humoral immune response

The Toll pathway is predominantly activated by fungal infection and gram-positive bacteria, while the IMD pathway is preferentially activated by gram-negative bacteria (Lemaitre *et al*., 1997). These two pathways control the expression of AMP genes through NF-κB-like transcription factors (Pinheiro & Ellar, 2006) and have close homology to two pathways found in mammalian immune defence: the TLR/interleukin-1 receptor (IL-1R) and tumour necrosis factor (TNF)-α pathways (Tzou *et al*., 2002).

A third signal transduction pathway is the Hop pathway, also known as the JAK/STAT pathway (Agaisse *et al*., 2003). It is activated in response to septic injury and again triggers production of effector molecules by the fat body (Lemaitre & Hoffmann, 2007). It was also recently demonstrated to mediate anti-viral defences against specific viruses (Kemp *et al*., 2013). Interestingly, this pathway has been shown to control the expression of the protein TEP1 which belongs to the family of thioester-containing proteins with strikingly comparable features to the complement C3/α2-macroglobulin superfamily found in mammals (Lagueux *et al*., 2000). TEP1 encourages phagocytosis by functioning as an opsonin in mosquito cells (Levashina *et al*., 2001).

A septic injury also promotes the activation of the phenoloxidase cascade, where the inactive proenzyme proPO is cleaved into active phenoloxidase (PO) by the serine protease proPO-activating enzyme (PPAE) (Leclerc *et al*., 2006). The oxidation of tyrosine-derived phenols to form quinones is catalysed by the active PO; quinones are necessary for melanin synthesis (Ling & Yu, 2005). Quinones and melanin are toxic with antimicrobial properties and are generated at sites of infection. Melanin promotes wound healing and has been observed to form layers around foreign particles which assist in killing encapsulated microbes and parasites (Leclerc *et al*., 2006).

1.4.2. Cellular immune response

In larvae of *Drosophila* there are three types of cells found in the haemolymph, these are: plasmatocytes, crystal cells and lamellocytes (collectively referred to as haemocytes) (Cherry & Silverman, 2006). Plasmatocytes are principally phagocytic cells, crystal cells store the enzyme proPO for melanin synthesis and lamellocytes are involved in specialised immune responses against macroparasites (Williams, 2007). There are two phases of haemopoiesis during fly development, involving the production and differentiation of haemocytes (Lanot *et al*., 2001). In the first phase plasmatocytes are produced for the clearance of apoptotic cells during embryogenesis. Additionally a population of crystal cells is produced which then localises to the anterior of the gut (Meister, 2004). Lymph glands develop along the dorsal vessel and act as a haemopoietic organ in larvae (Sorrentino *et al*., 2002). The second haemopoietic wave occurs at the beginning of metamorphosis; huge numbers of plasmatocytes are released from the lymph glands and are important in the remodelling of larvae to adults, after which the lymph glands degenerate (Lanot *et al*., 2001). Though there are three types of haemocytes in larvae, only plasmatocytes have been identified in adults (Lanot *et al*., 2001).

The principal responses haemocytes participate in are phagocytosis and encapsulation (reviewed by Cherry & Silverman, 2006). Phagocytosis involves the attachment of a phagocyte to a targeted particle, followed by its internalisation and destruction by degradative enzymes (Stuart & Ezekowitz, 2008). Phagocytosis in *Drosophila* can either be for the destruction of invading microorganisms or to clear apoptotic cells during development (Williams, 2007). Encapsulation of foreign material is especially relevant in the defence against parasitic wasps (Márkus *et al*., 2009). Female wasps lay their eggs inside *Drosophila* larvae, these are then encountered by plasmatocytes which adhere to the egg, but are unable to phagocytose it due to its size. Shortly afterwards a massive increase in the number of circulating proPO-containing crystal cells and the differentiation of lamellocytes is seen (Meister and Lagueux, 2003). Lamellocytes are cells that specifically respond to this type of immune challenge and form layers around the foreign body, which then blacken due to melanisation and so the parasite is suffocated and killed (Meister, 2004). These responses however are only observed in *Drosophila* larvae as adults are said not to possess crystal cells or lamellocytes (Lanot *et al*., 2001).

1.4.2a. Mechanisms of phagocytosis by plasmatocytes

Plasmatocytes recognise self and non-self molecules via an array of receptors (reviewed by Stuart & Ezekowitz, 2008). There are four main classes of these: complement-like opsonins, scavenger receptors, epidermal growth factor (EGF)-like-repeat-containing receptors, and the Down syndrome cell-adhesion molecule (Dscam).

In *D. melanogaster* the opsonins that have been studied the most are the thioester-containing proteins (TEPs). TEPs have been shown to be involved in a wide range of immune responses: TEPII enhances *Escherichia coli* phagocytosis, TEPIII increases *Staphylococcus aureus* phagocytosis, and TEPVI (also known as macroglobulin-

related protein (Mcr)) binds and increases phagocytosis of the fungus *Candida albicans* (Stroschein-Stevenson *et al*., 2005).

Scavenger receptors bind polyanionic ligands and function as important pattern recognition receptors (PRRs) in mammals as well as invertebrates (Gordon, 2002). One example of a class B scavenger receptor is Croquemort. Although initially identified as a receptor for apoptotic cells in flies (Franc *et al*., 1996), it was later discovered to have the additional function as a receptor for *S. aureus* (Stuart *et al*., 2005). Its mammalian homologue CD36 has the same functions in humans, and also interacts with Toll-like receptor signalling to stimulate the innate immune response (Stuart *et al*., 2005). It is unknown as yet whether Croquemort also coordinates with Toll signalling in *Drosophila*. One of the class C scavenger receptors that is specific to insects is SR-CI (Pearson *et al*., 1995). Exposure to bacteria causes increased expression of this receptor in *Drosophila* larvae (Irving *et al*., 2005). SR-CI displays naturally occurring sequence variation in wild *D. melanogaster* populations (Lazzaro, 2005) with potential links to variation in disease resistance.

Of the third class of receptors, EGF-like-repeat-containing receptors, three have been shown to have crucial roles in disease susceptibility. Eater is a PRR that binds directly to bacteria, facilitating their internalisation by plasmatocytes in *D. melanogaster* (Kocks *et al*., 2005). If Eater expression is silenced in fly plasmatocytes, their ability to phagocytose bacteria is compromised, resulting in increased susceptibility to some infections (Kocks *et al*., 2005). Nimrod C1 acts a phagocytic receptor and has a potential role as an opsonin (Kurucz *et al*., 2007; Zsamboki, 2013). Equally, Draper is a crucial phagocytic receptor in *D. melanogaster* plasmatocytes. In the same way as Croquemort, this receptor was initially believed to only have a role in the removal of apoptotic cells (Awasaki *et al*., 2006), however it was later shown that silencing this receptor increased susceptibility to *S. aureus* infection (Hashimoto *et al*., 2009).

The final class of phagocytosis receptors are the isoforms of Dscam. Dscam was originally identified through its role in neuronal development in *D. melanogaster* (Schmucker *et al*., 2000). However, it has rapidly become the key candidate mechanism behind receptor specificity in invertebrates (Watson *et al*., 2005). Dscam has been demonstrated to act as a phagocytic receptor and as an opsonin (Watson *et al*., 2005). Different immune stimuli appear to influence *Dscam* expression to generate a 'cloud' of variable receptors rather than a single highly specific immune molecule (Dong *et al*., 2012).

After pathogen recognition, phagocytic cells must undergo significant plasma membrane changes leading to substantial restructuring for engulfment to take place. A great deal is unknown about the mechanisms behind membrane restructuring, vesicle trafficking and the destinies of phagocytic cells once particle internalisation has occurred (Ulvila *et al*., 2011). Much of what has been learned is based on genetic screens using RNA interference (RNAi) techniques on *Drosophila* S2 cells (cultured embryonic cells physiologically closest to plasmatocytes). Three key mechanisms behind membrane restructuring have been identified: clathrin-mediated endocytic uptake (Veiga & Cossart, 2006), the coat-protein complex I (COPI) and also COPII (Ramet *et al*., 2002). Clathrin stabilises the budding membrane and also has a role in the internalisation of particular pathogens (Veiga & Cossart, 2006). COPI and COPII are associated with the *D. melanogaster* phagosome, but it is not certain what their exact function is (Stuart *et al*., 2007). It is suggested that they may help in the curvature of the membrane during reshaping to enclose a particle, or they may have a role when endosomes and lysosomes fuse to the phagosome to form the phagolysosome (Botelho *et al*., 2000).

Following target internalisation and destruction, the exocytosis of degraded waste products has been shown to amplify the systemic immune response in *Drosophila*

(Brennan *et al*., 2007). This is particularly through the increased presence of the lysosomal protein Psidin (Brennan *et al*., 2007). Mammals possess a homologue of this protein, but as yet a similar link between phagosome activity and the systemic immune response in mammals has not been identified (Stuart & Ezekowitz, 2008).

1.4.3. Immunological memory

Although invertebrates do not possess the vertebrate form of an adaptive immune response, they are able to demonstrate specificity and a version of immune memory; termed 'immunological priming' (Kvell *et al*., 2007; Ziauddin & Schneider, 2012). Immune priming in invertebrates received greater attention after mealworm beetles (*Tenebrio molitor*) were demonstrated to have enhanced survival when challenged with the fungus *Metarhizium anisopliae* if individuals had previously received an injection of lipopolysaccharides (LPS) compared to a Ringer pre-injection (Moret & Siva-Jothy, 2003). Other studies have revealed that this priming effect can be very specific in insects. The priming response in *Bombus terrestris* workers was shown to differentiate between two species of bacteria: *Paenibacillus alvei* and *Paenibacillus larvae* (Sadd & Schmid-Hempel, 2006). Bees were first injected with one or other bacterium, then later they were challenged with a potentially lethal dose, if the second challenge was homologous to the first there was enhanced survival, but this benefit of previous exposure was reduced for the heterologous regime. Demonstrating a similar level of specificity in red flour beetles (*Tribolium castaneum*), individuals that were primed and then infected with homologous compared to heterologous strains of the bacterium *Bacillus thuringiensis* had higher survival rates (Roth *et al*., 2009).

There appear to be limits to the efficacy of this priming response. In the Roth *et al*., (2009) red flour beetle study, whilst a great level of specific protection was demonstrated for *B. thuringiensis*, in the same experiment a pre-injection with *E. coli*

had no positive effect on survival following a pathogenic second exposure (Roth *et al*., 2009). Similarly, in priming studies on *D. melanogaster*, flies were able to form a protective primed response only for *Beauveria bassiana* or *Streptococcus pneumoniae*, and not for three other pathogens that were tested (Pham *et al*., 2007).

Importantly, the mechanism(s) behind immunological priming have yet to be determined. A link between plasmatocyte activity and the priming response has been identified but much of the detail remains unclear (Pham *et al*., 2007; Rodrigues *et al*., 2010). *D. melanogaster* did not develop a protective priming response against *S. pneumoniae* if the phagocytic cellular immune response was inhibited by a pre-injection of polystyrene beads (Pham *et al*., 2007). Additionally, when flies were pre-injected with *S. pneumoniae* there was no evidence of an increase in phagocytosis activity during a subsequent challenge with *E. coli*; this suggests that priming response specificity may relate to plasmatocyte activity (Pham *et al*., 2007). A study on mosquitoes (*Anopheles gambiae*) produced similar results, demonstrating that individuals with a disrupted phagocytic response from injection with Sephadex beads did not mount a priming response, compared to individuals that had not received a bead injection (Rodrigues *et al*., 2010). Interestingly, the priming response could be transferred to naïve individuals through the transfer of haemocytes from challenged mosquitoes, again suggestive of a key role for haemocytes in the priming response (Rodrigues *et al*., 2010).

1.5. Evolutionary interactions between hosts and parasites

The evolutionary relationship between hosts and parasites can be characterised by rapid antagonistic coevolutionary interactions (Haldane, 1941). These can lead to rapid adaptive divergence of immune genes between closely related species, as has been demonstrated in humans, chimpanzees and mice (Patil *et al*., 2004), *Daphnia*

(McTaggart *et al*., 2012a), and *Drosophila* (Obbard *et al*., 2009). Furthermore, these selective processes can also drive exceptionally fast evolutionary change on ecological timescales (Wilfert & Jiggins, 2013).

The dynamic nature of immune defence traits in host populations may have important evolutionary and ecological consequences for ageing. Immune defence is generally considered to be a costly trait (Bashir-Tanoli & Tinsley, 2014), potentially mediated by physiological trade-offs and limited resources. *Drosophila* lines that were selected to be strongly resistant to parasitoid wasps demonstrated lower competitive ability compared to non-selected controls in a low nutrient environment (Kraaijeveld *et al*., 1998). The cost of immunity was also shown when in starvation conditions immune deficient flies survived for longer than controls (Valtonen *et al*., 2010).

Referring back to reactive oxygen species (ROS) as a symptom of ageing, ROS are produced as part of the immune response of plasmatocytes and during the prophenoloxidase cascade in *Drosophila* (Nappi *et al*., 2009). Although this is a potent immune defence it also causes damage to its host and though its production is strictly controlled, this regulation lessens with increasing age (Cho *et al*., 2011). A trade-off between immune activation and the characteristics of ageing appears to exist in this case, and has been proposed to be a significant constraint in life-history evolution (Dowling & Simmons, 2009). If the link between immune defence and ageing is a general one, then host-parasite coevolutionary interactions may have the potential to drive correlated changes in senescence and have a key role in lifespan evolution.

Thesis Aims

1. **Establish whether the phenomenon of senescence occurs in the cellular immune response of adult *Drosophila melanogaster*.**

In ageing humans macrophages decline in number, phagocytic function and efficacy of antigen presentation (Solana *et al*., 2012). Plasmatocyte haemocytes in *Drosophila* have closest similarity to the monocyte lineage that gives rise to macrophages in vertebrates (Williams, 2007). Studies on other invertebrates, such as scorpion flies (*Panorpa vulgaris*) (Kurtz, 2002) and mosquitoes (*Aedes aegypti*) (Hillyer *et al*., 2005), have demonstrated an age-associated change in haemocyte number and phagocytic function. The first chapter of this thesis investigates whether there is a similar decline in plasmatocyte number and phagocytic function with age in *D. melanogaster* and whether the rate of age-dependent change is affected by the type or size of particle plasmatocytes are challenged with.

2. **Explore mechanisms that may underlie the observed senescent declines in plasmatocyte number and function.**

Senescence occurs in the cellular immune response of *D. melanogaster* (Chapter 2; Mackenzie *et al*., 2011). This thesis progressed to explore the potential mechanisms underlying the age-dependent changes observed. Initially, I tested whether a non-pathogenic infection influenced the size of the circulating haemocyte population, as apoptosis of these cells has been demonstrated to be part of the immune response in other invertebrate systems (Hsu *et al*., 2005; Sokolova, 2009; Xian *et al*., 2013). Secondly, plasmatocyte function was tested in the absence of haemolymph to determine whether components in the haemolymph influenced senescence in cell

function, or whether this was a cell autonomous process. Additionally, the phagocytic activity of plasmatocytes from flies with compromised phagocytic receptor systems was investigated to identify candidate receptor systems that could underlie the observed senescent decline in plasmatocyte phagocytic function in wildtype flies.

Latex bead injection compromises plasmatocyte phagocytic ability (Nehme *et al*., 2011), and the impaired expression of the Toll pathway gene *Dif* reduces the humoral immune response through the decreased production of key antimicrobial peptides (Nehme *et al*., 2011). I used these methods to explore the relative contributions of senescence in the cellular and humoral immune responses to increasing pathogen susceptibility as flies age.

3. Determine whether plasmatocytes play a role in immunological priming in *D. melanogaster* and whether this mechanism senesces.

Although invertebrates do not possess the same machinery behind immunological memory in vertebrates, the formation of immunological memory has been demonstrated in a number of invertebrates; termed ‘immunological priming’ (Pham *et al*., 2007; Powell *et al*., 2011; Roth *et al*., 2009; Sadd & Schmid-Hempel, 2006). A link between plasmatocyte activity and immunological priming has been suggested, including some indirect evidence in *Drosophila*. This thesis explored the role of *D. melanogaster* plasmatocytes in immunological priming and studied whether this effect experienced senescence.

4. **Establish the extent of genetic variation in life history and immune parameters in a panel of outcrossed genotypes of *D. melanogaster*; then determine whether pleiotropic links exist between investment in immunity and ageing.**

This thesis predominantly studied the impact of ageing on immune function. However, it is possible that immune function also has direct effects on ageing. The final section of my work investigated genetic variation in a range of life history, ageing and immunity traits in a panel of wild-type *D. melanogaster* lines derived from a natural population. I tested whether the level of investment in immune traits displayed by genotypes was associated with their lifespan and rate of senescence.

Chapter 2: Senescence of the cellular immune response in *Drosophila melanoagster*

This chapter has been published as:

Mackenzie, D. K., Bussiere, L. F., Tinsley, M. C. (2011) Senescence of the cellular immune response in *Drosophila melanogaster*. *Experimental Gerontology, 46*(11), 853-859.

2.1. Abstract

Immune system effectiveness generally declines as animals age, compromising disease resistance. In *Drosophila*, expression of a variety of immune-related genes elevates during ageing; however how this is linked to increasing pathogen susceptibility in older flies has remained unclear. We investigated whether changes in the *Drosophila* cellular immune response might contribute to immunosenescence. Experiments studied fly cohorts of different ages and compared the numbers and activity of the circulating haemocytes involved in pathogen defence. In female wildtype Samarkand and Oregon R flies the haemocyte population fell by 31.8% and 10.2% respectively during the first four weeks of adulthood. Interestingly we detected no such decline in male flies. The impact of ageing on the phagocytic activity of haemocytes was investigated by injecting flies with fluorescently labelled microbes or latex beads and assessing the ability of haemocytes to engulf them. For all immune challenges the proportion of actively phagocytosing haemocytes decreased as flies aged. Whilst 24.3% ± 1.15% of haemocytes in one-week-old flies phagocytosed *Escherichia coli* bacteria or *Beauveria bassiana* fungal spores, this decreased to 16.7% ± 0.99% in four-week-old flies. This clear senescence of the *Drosophila* cellular immune response may underpin increased disease susceptibility in older flies.

2.2. Introduction

Ageing has a profound effect on immune system performance in many organisms (Nikolich-Žugich & Čičin-Šain, 2010). Both the cellular basis and clinical relevance of immunosenescence are increasingly well characterised in humans (Weiskopf *et al*., 2009). In contrast, for invertebrates the mechanisms underpinning immunosenescence are poorly understood, even for the best-characterised invertebrate model, *Drosophila melanogaster.* Although invertebrates lack the acquired immune responses that are the hallmark of vertebrate immune defence, many innate immunity genes and signalling pathways are functionally conserved between invertebrates and vertebrates (Flajnik & Du Pasquier, 2004). Unravelling the processes which degenerate in the senescence of the *D. melanogaster* immune system is important to allow exploitation of *D. melanogaster* as a model for human ageing conditions.

For humans, ageing is accompanied by major alterations in both acquired and innate immune defence. Decreasing abundance of naïve B cells and an altered T cell repertoire result in a declining ability to form and retrieve immunological memories, leading to poor clinical responses to vaccination and infection late in life (Chen *et al*., 2009). The major ageing-dependent pattern in innate immune defence is one of increasing dysregulation; one consequence may be heightened inflammatory responses, termed 'inflamm-aging' (Franceschi *et al*., 2000). Chronic inflammatory states underlie many degenerative diseases of the elderly: arthritis, dementia and type II diabetes have all been linked to inflamm-aging (Bucci *et al*., 2009). In *Drosophila*, patterns of increasing disease susceptibility with age largely mirror those found in vertebrates (Ramsden *et al*., 2008). However, the immune system changes that cause this remain unclear.

The *Drosophila* immune system is made up of multiple defences (Cherry & Silverman, 2006). Pathogens entering the fly are detected as 'non-self' by pattern recognition receptors that bind to conserved microbial molecules, stimulating a coalition of immune defences (Hoffmann & Reichhart, 2002). Pathogen detection is followed by cellular and humoral responses. The principal component of the humoral immune response is the systemic and local production of antimicrobial peptides (AMPs) (Lemaitre & Hoffmann, 2007). Production of melanin by the enzyme phenoloxidase also contributes to the humoral response: melanin and its reactive synthesis intermediates are toxic to microorganisms and play a role in wound healing (Lemaitre & Hoffmann, 2007). An additional immune defence component is RNA interference (RNAi) which defends against RNA virus infection (Cherry & Silverman, 2006).

Phagocytosis is the main component of the *Drosophila* adult cellular immune response and is carried out by plasmatocytes (Williams, 2007). Two other haemocyte classes are present in *Drosophila*: lamellocytes encapsulate foreign objects (such as parasitoid eggs) and crystal cells store phenoloxidase for release following immune activation (Meister, 2004). However, lamellocytes and crystal cells are found only in larvae (Lanot *et al*., 2001), and are therefore not relevant to immune system ageing in adults. *Drosophila* haemocytes either circulate freely throughout the haemolymph or are sessile (Kocks *et al*., 2005). Phagocytosis is initiated by binding of pathogen recognition receptors such as Eater (Kocks *et al*., 2005) and PGRP-LC (Ramet *et al*., 2002), and may be mediated by opsonin molecules that target microbes for engulfment (Lemaitre & Hoffmann, 2007). Once haemocytes encounter a microbe the cytoskeleton is remodelled to enable endocytosis, before it is destroyed by lysosomal enzymes and reactive oxygen species (Meister, 2004).

Although the *Drosophila* immune system is well characterised, relatively little is known about how ageing affects its function. As is the case for vertebrates, mortality following

infection elevates in older flies (Ramsden *et al*., 2008), but we have limited knowledge of the senescent mechanisms underpinning these changes. A range of immune-related genes becomes upregulated as flies age (Landis *et al*., 2004; Pletcher *et al*., 2002; Seroude *et al*., 2002), which perhaps mirrors vertebrate inflamm-aging. However, the bacterial burden associated with flies also increases with age (Ren *et al*., 2007), therefore whether this elevated transcriptional activity reflects immune senescence directly or if it results from heightened activation is not known.

A small number of studies in *Drosophila* have investigated the mechanisms underlying age-related changes in immune defence. Ramsden *et al.,* (2008) suggested that increases in pathogen susceptibility during ageing were not due to impaired ability to clear infection, but perhaps resulted from reduced tolerance of infection pathology. Similarly, Lesser *et al.,* (2006) investigated how the ability of 25 *D. melanogaster* genotypes to clear *Escherichia coli* infection changed with age and found considerable variation in both the direction and magnitude of age-dependent changes. Zerosky and colleagues (2005) demonstrated that following infection expression of the AMP *diptericin* was higher and more prolonged in older compared to younger flies; however the significance of this result to overall immune competence is unclear.

We know more about invertebrate immune senescence from studies in non-model organisms. Ageing was associated with increased pathogen susceptibility and reduced phenoloxidase levels in the cricket *Gryllus texensis* (Adamo *et al.,* 2001). In scorpion flies (*Panorpa vulgaris*), whilst haemocyte numbers remained unchanged during ageing, the phagocytic activity of those cells declined (Kurtz, 2002). Hillyer *et al.,* (2005) showed that older mosquitoes (*Aedes aegypti*) had reduced survival following infection with *E. coli*, a lower ability to clear the infection, and fewer phagocytic haemocytes compared with younger mosquitoes. However they only assayed mosquitoes across the first five days of life, so it is questionable whether these trends

truly represent senescence or some other life history shift. Doums *et al.,* (2002) found older bumblebees (*Bombus terrestris*) had impaired ability to encapsulate foreign implants, but haemocyte numbers were unaffected by ageing. Similarly Whitehorn and colleagues (2011) found that in the wild older bumblebees (*Bombus muscorum*) had lower phenoloxidase titres. These studies each assayed varying suites of immune effectors. Whether differences between species in the immune system components shown to senesce represent true species-specific variation in the effects of ageing or whether this simply reflects differences in experimental power and design has not been determined.

Here we investigate the senescent mechanisms underpinning increased pathogen susceptibility in aged *D. melanogaster* and focus on changes in the cellular immune response. We test whether the number of circulating haemocytes declines with age and also whether the haemocytes of older individuals have impaired phagocytic activity. Vertebrate macrophages are less efficient at phagocytosing larger particles (Champion *et al*., 2008). With the expectation that the same would be true for *D. melanogaster* haemocytes, we investigate whether the rate at which phagocytosis ability senesces is faster for larger, more challenging targets.

2.3. Materials and Methods

2.3.1. Fly culturing

We used Oregon R and Samarkand wildtype genotypes (obtained from Bloomington Stock Centre). Haemocyte number was measured in both genotypes, but only Samarkand flies were used to determine the impact of ageing on haemocyte phagocytic activity. Flies were reared on Lewis medium (Lewis, 1960) at 25 °C, 70% RH, 12hr L/D. Both genotypes were initially bred at low density for two generations (five females oviposited in each bottle for three days). Cultures of both genotypes were set up weekly in bottles. To keep larval density constant eggs were collected on apple juice agar plates seeded with yeast, then washed and 13µl of eggs transferred to each bottle using a pipette (Clancy & Kennington, 2001). When offspring eclosed they were transferred to 11 litre demography cages (~400 mixed-sex flies per cage). Demography cages were sealed plastic tanks with a single 12cm diameter circular hole in one side. This was covered with a fabric sleeve which allowed access to the cage for the placement of food and the removal of flies for experiments. Flies were fed Petri dishes of Lewis medium every other day and left to age for between one and four weeks. Cages were set up every week so that flies of all ages were simultaneously available for experiments. A total of 16 independent demography cages were represented in the experiment, two cages per age per genotype. Using an insect pooter, flies were collected from each cage and sorted into single sex five-fly batches within which flies originated from the same cage and therefore were the same age. These vials were randomly allocated to treatments while maintaining an equal representation of each age class, gender and demography cage per treatment. Flies removed for experiments or that died were counted, and constant density was maintained by topping up cages from separate, similarly aged, populations maintained for that purpose.

2.3.2. Fluorescent material for assessing phagocytosis

Escherichia coli (strain BB4) was grown overnight in LB broth at 37°C. 1 ml of culture was washed four times by centrifuging and resuspending in 1ml of sodium carbonate buffer (0.1M $NaHCO_3$, pH 9). The final suspension was mixed with its own volume of Fluorescein isothiocyanate (FITC) (Sigma) (1mg ml^{-1} in $NaHCO_3$ buffer) and incubated for one hour at room temperature in the dark with continual shaking. Bacteria were then washed six times in the same manner, then resuspended in 1ml of PBS (pH 7) and diluted to 5 x 10^6 cells ml^{-1}. *Beauveria bassiana* spores originated from a strain used previously (Tinsley *et al*., 2006). Fungal material was grown for two weeks on potato dextrose agar with chloramphenicol (5 x 10^{-5}g ml^{-1}), then left to sporulate and dry at room temperature. Spores were FITC-labelled as for *E. coli* and diluted to the same concentration. We also assessed haemocyte activity using Fluoresbrite® Fluorescein labelled carboxylate-modified latex microspheres (Polysciences) of a range of sizes (0.5, 1.0, 2.0, 3.0 and 4.5µm) suspended in PBS at 5 x 10^6 beads ml^{-1}.

2.3.3. The influence of fly age on haemocyte phagocytic activity

A Drummond Scientific oocyte microinjection pipette was used to inject one to four-week old Samarkand flies with 0.05µl of the fluorescent microbe or bead suspensions prepared above. Flies were injected into their lateral thorax in single sex five-fly batches, then incubated at 25^{o}C for 30 min. The combined haemolymph of five flies was bled into a pool of 20µl Hoechst anticoagulant buffer (0.01mg ml^{-1} Hoechst (Invitrogen) in 10 mM sodium cacodylate, 10mM $CaCl_2$, 280mM sucrose, pH 7.4). The haemolymph mixture was transferred to a 1.5 ml microcentrifuge tube, and a further 20µl cacodylate buffer added, then left for 5 min to allow fat droplets to separate. The bottom 20µl was then transferred to a polylysine-coated well on a 96-well plate. Plates were centrifuged at 30.7 *g* for 3 min, then 10µl of 11.1% formaldehyde in PBS and a drop of VectaShield® containing DAPI (Vector Laboratories) were added. Plates were

viewed on an Axiovert 135 epifluorescence microscope; 100 cells were inspected in each well and the number of fluorescent included particles per cell recorded. 187 biological replicates (wells containing pools of five flies) were used for this experiment.

2.3.4. The effect of age on numbers of circulating haemocytes

One to four-week-old Samarkand and Oregon R flies were anesthetised on ice in single sex batches of three flies and haemolymph was extracted from each batch using a standardised pulled-glass capillary needle inserted into the thorax. The length of haemolymph in the capillary was measured using an eyepiece graticule before adding the haemolymph to a 1.5 ml microcentrifuge tube containing 25µl of cacodylate buffer (as described above). The cell density was then counted using a Neubuer haemocytometer. In total 263 biological replicates (three fly batches) were counted. The order in which fly batches were bled was random with respect to age and sex. The calibration between the fill-length of capillaries and the haemolymph volume they contained was calculated by filling 72 pulled needles to a range of lengths with a 2.0µm bead suspension of standard concentration. This fluid was mixed with buffer as before, beads counted on a haemocytometer and the corresponding capillary volume calculated. A fourth order polynomial curve was fitted through the data ($R^2 = 0.973$), which was then used to calculate the haemolymph volume extracted from each fly batch above.

2.3.5. Statistical Methods

Data were analysed using the Lme4 package (Bates *et al*., 2008) for R statistical software, version 2.11.1 (R Core Team, 2010). Linear mixed-effects models were constructed which included the random effects of the ‘cage’ in which flies were housed whilst ageing, and the ‘day’ on which experiments were carried out. Our fixed effects included fly ‘age’, ‘sex’, ‘time’ of day for the assay, and immune ‘treatment’ (injections

of beads, bacteria or fungal spores) in addition to the two-way interaction between ‘age’ and ‘treatment’ as well that between ‘age’ and ‘sex’. ‘Mass’ was also included as a fixed effect covariate in order to determine whether fly weight influenced haemocyte phagocytic activity. Potentially, as flies age their weight might decrease. If this decreased mass resulted in smaller haemolymph volume then the ratio of the volume of fluorescent particles to haemolymph volume in older flies would be greater than that present in younger flies. This could possibly lead to a larger amount of phagocytic activity of plasmatocytes in four week old flies compared to one week olds. To determine how these factors influenced the mean number of particles that haemocytes phagocytosed, the total number of inclusions per well were divided by the number of haemocytes assessed per well (100). A histogram of this response variable followed a normal distribution so models with Gaussian errors were used. Binomial errors were used for assessing the proportion of active haemocytes employing the cbind function in R to analyse a two-vector response including the number of active and inactive cells. For analyses of the haemocyte numbers in flies of each age, we used the same model structure including fly genotype as a fixed factor and the log10 transformation of cell number as response.

Models were sequentially simplified by selecting terms for which the parameter estimate was closest to zero and using Akaike’s Information Criterion (AIC) these factors were eliminated if their presence did not improve the explanatory power of the model by 2 AIC. Model simplification used AIC since though model fit generally improves as the number of explanatory parameters increases AIC is a useful measure of model fit as it penalises surplus parameters (Crawley, 2009). The cut-off of 2 AIC units difference between simplified and more complex models was selected to allow the conservative removal of parameters which lacked explanatory power while retaining parameters that more strongly influenced the data (see Burham & Anderson,

2002). Significance of terms was determined using likelihood ratio tests. The results were not sensitive to the order in which the variables were entered into the model as they were not determined by an ANOVA table. Instead a likelihood ratio test was conducted between a model containing the term in question and a model where the term was absent so the placement order of terms in the complete model did not influence the results. Means are given ± their standard errors throughout.

2.4. Results

2.4.1. The proportion and activity of phagocytically active cells declined with age

To test whether haemocyte phagocytic activity is affected by age, Samarkand flies aged 1, 2, 3 and 4 weeks post-eclosion were injected with fluorescently labelled bacteria (*Escherichia coli*) or fungal spores (*Beauveria bassiana*). As flies aged there was a reduction in the proportion of haemocytes that phagocytosed these microbes (χ^2_1 = 10.88, P = 0.001) (Table 1a). There was a ~30% decline during the first four weeks of life in the fraction of cells that were phagocytically active in this assay. This trend occurred both for fungal spores and bacteria (Fig 1A); there was no difference between these microbes in the rate of phagocytosis decline with age (age x microbe interaction: χ^2_1 = 0.03, P = 0.861). However, when other fixed effects were taken into account, there was a significant difference between the two microbes in the proportion of active haemocytes: slightly more haemocytes phagocytosed bacteria than fungal spores (χ^2_1 = 6.38, P = 0.012).

Marginally more of the cells in males phagocytosed microbes than in females (χ^2_1 = 3.68; P = 0.055); however both genders had the same rate of decline in haemocyte activity as they aged (age x sex interaction: χ^2_1 = 0.67, P = 0.413). The proportion of active haemocytes increased with fly mass (χ^2_1 = 6.52, P = 0.011) and flies injected later in the day had more active haemocytes than those injected earlier (χ^2_1 = 7.35, P = 0.007).

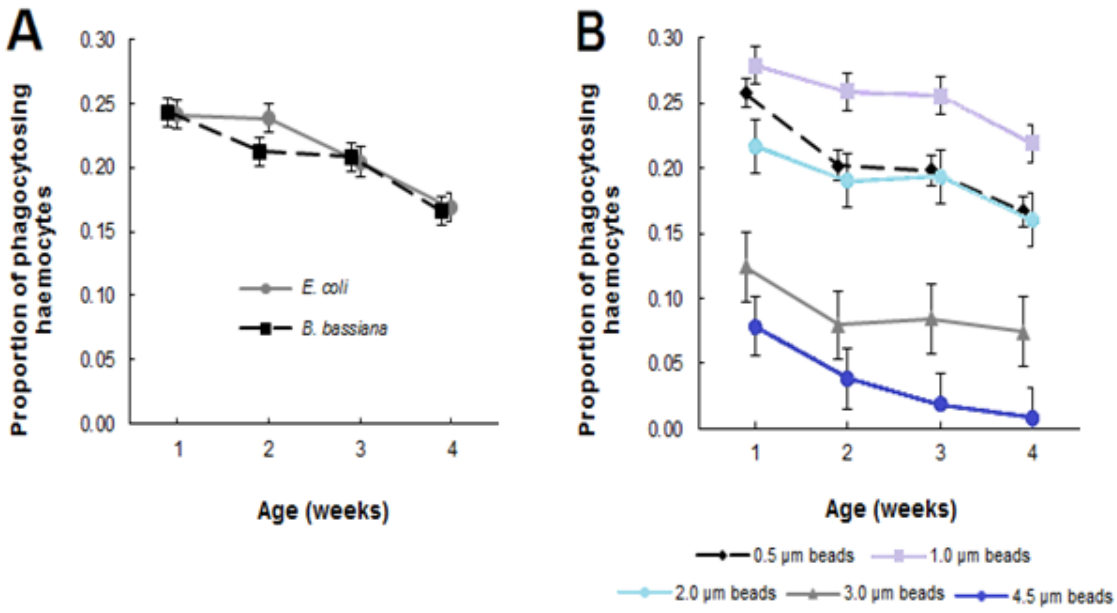

Figure 1: Changes in the proportion of actively phagocytosing haemocytes in adult *Drosophila melanogaster* between one and four weeks of age. (A) Flies were injected with fluorescently-labelled *Escherichia coli* or *Beauveria bassiana* spores. (B) Flies were injected with one of five sizes of fluorescent beads (0.5µm, 1.0µm, 2.0µm, 3.0µm, 4.5µm). Although investigated, no significant difference was detected between the sexes (see text). Points are staggered slightly along the x-axis for clarity. Bars represent standard errors.

After comparing the proportion of haemocytes that were phagocytically active in flies of different ages, we next investigated cellular immune senescence by assessing how the number of included particles per cell changed as flies aged. When Samarkand flies were injected with *E. coli* or *B. bassiana*, the mean number of inclusions per circulating haemocyte decreased significantly as flies aged ($\chi^2_1 = 8.52$, $P = 0.004$) (Table 1b); in one-week-old flies each haemocyte phagocytosed a mean of 0.30 particles (± 0.03), whereas this fell by 33.33% to 0.21 particles (± 0.02) by week four.

Table 1: Factors influencing the phagocytic activity of haemocytes challenged with microbes during ageing.

(a) Data analysed as the proportion of active haemocytes.

Factors	Parameter Estimates	SE	χ^2	P-value
Age (weeks)	- 0.105	0.029	10.88	0.001
Mass (mg)	0.086	0.015	6.52	0.011
Microbe (*B. bassiana* spores)	- 0.167	0.067	6.38	0.012
Sex (male)	0.124	0.065	3.68	0.055
Time of day (hrs)	0.12	0.021	7.35	0.007

Output of terms from the model analysing the proportion of phagocytosing haemocytes in adult *D. melanogaster* between one and four weeks of age. Flies were injected with fluorescently labelled *E. coli* or *B. bassiana* spores. Parameter estimates with reference to female flies injected with *E. coli*.

(b) Data analysed as the mean number of inclusions per haemocyte.

Factors	Parameter Estimates	SE	χ^2	P-value
Age (weeks)	- 0.018	0.006	8.52	0.004
Mass (mg)	0.025	0.003	11.42	0.001
Microbe (*B. bassiana* spores)	- 0.027	0.014	7.18	0.007
Sex (male)	0.038	0.014	10.14	0.001
Time of day (hrs)	0.031	0.004	10.56	0.001

Output of terms from the model analysing the mean number of included particles per haemocyte in adult *D. melanogaster* between one and four weeks of age. Flies were injected with fluorescently labelled *E. coli* or *B. bassiana* spores. Parameter estimates with reference to female flies injected with *E. coli*.

Furthermore, cells in flies challenged with *E. coli* had 4.5% more inclusions compared those receiving *B. bassiana* ($\chi^2_1 = 7.18$, $P = 0.007$). Cells in heavier flies phagocytosed

more particles (χ^2_1 = 11.42, P = 0.001). Those in males had a greater number of inclusions (χ^2_1 = 10.14, P = 0.001), and cells of flies injected later in the day phagocytosed more (χ^2_1 = 10.56, P = 0.001). Again, the gradient of age-related decline was the same between microbes and between sexes (age x microbe interaction: χ^2_1 = 0.87, P = 0.352; age x sex interaction: χ^2_1 = 1.36, P = 0.243).

2.4.2. Phagocytic ability of a subset of haemocytes is unaffected by ageing

Finally, we analysed the data for how the number of included microbes per cell changed as flies aged considering only those cells that were phagocytically active in our assay, ignoring those which had not engulfed particles. There was no age-related change in the number of inclusions for these active cells (χ^2_1 = 0.40, P = 0.526); the mean for one week old flies was 1.33 inclusions per cell (± 0.05), whereas that for four week old flies was 1.39 (± 0.06). In these analyses of active haemocytes, cells phagocytosed slightly more fungal spores than they did bacteria (χ^2_1 = 6.15, P = 0.013 (Table 2a)).

Table 2: Factors influencing the number of included particles per phagocytically active haemocyte during ageing.

(a) Flies challenged with microbes.

Factors	Parameter Estimates	SE	χ^2	P-value
Microbe (*B. bassiana* spores)	0.096	0.038	6.15	0.013

Output of terms from the model analysing the mean number of included particles per phagocytically active haemocyte in adult *D. melanogaster* between one and four weeks of age. Flies were injected with fluorescently labelled *E. coli* or *B. bassiana* spores. Parameter estimates with reference to flies injected with *E. coli*.

(b) Flies challenged with latex beads.

Factors	Parameter Estimates	SE	χ^2	P-value
Bead size (µm)	- 0.046	0.01	17.35	<0.001
Time of day (hrs)	- 0.016	0.007	4.86	0.027

Output of terms from the model analysing the mean number of included particles per phagocytically active haemocyte in adult *D. melanogaster* between one and four weeks of age. Flies were injected with one of five sizes of fluorescent beads (0.5µm, 1.0µm, 2.0µm, 3.0µm, 4.5µm).

2.4.3. The rate that phagocytic ability senesces was unaffected by particle size

We predicted that the ability of cells to phagocytose would be reduced for larger particles and that the rate of age-dependent decline might be faster for these more challenging targets. Samarkand flies from one to four weeks old were challenged with fluorescent beads ranging from 0.5µm to 4.5µm in size (Fig 1B). Significant differences existed in the ability of haemocytes to phagocytose different sized beads (χ^2_1 = 91.84, P < 0.001) (Table 3a): fewer than half as many cells were able to phagocytose the large beads (4.5µm) as were able to phagocytose the smaller beads (0.5-2.0µm) (Fig 1B). As before, there was a marked senescent decline in the proportion of phagocytosing haemocytes (χ^2_1 = 14.60, P < 0.001). However, our hypothesis that the rate of senescence in phagocytosis ability might be faster for more challenging phagocytosis targets was not supported: the age-dependent decrease in phagocytosis ability was consistent for the five beads sizes (age x bead size interaction: χ^2_1 = 0.72, P = 0.398). Again, more cells phagocytosed beads in flies injected later in the day (χ^2_1 =1.53, P = 0.040). There was no influence of fly mass (χ^2_1 = 0.02, P = 0.216), and no difference between males and females (χ^2_1 = 0.02, P = 0.888). Cell activity senesced at similar rates in both sexes (age x sex interaction: χ^2_1 = 0.12, P = 0.731).

Table 3: Factors influencing the phagocytic activity of haemocytes challenged with latex beads during ageing.

(a) Data analysed as the proportion of active haemocytes.

Factors	Parameter Estimates	SE	χ^2	P-value
Age (weeks)	- 0.145	0.025	14.6	<0.001
Bead size (µm)	- 0.227	0.024	91.84	<0.001
Time of day (hrs)	0.038	0.018	4.21	0.04

Output of terms from the model analysing the proportion of phagocytosing haemocytes in adult *D. melanogaster* between one and four weeks of age. Flies were injected with one of five sizes of fluorescent beads (0.5µm, 1.0µm, 2.0µm, 3.0µm, 4.5µm).

(b) Data analysed as the mean number of inclusions per haemocyte.

Factors	Parameter Estimates	SE	χ^2	P-value
Age (weeks)	- 0.029	0.006	11.7	0.001
Bead size (µm)	- 0.044	0.004	76.95	<0.001
Mass (mg)	- 0.009	0.004	5.27	0.022
Time of day (hrs)	0.009	0.003	6.7	0.01

Output of terms from the model analysing the mean number of included particles per haemocyte in adult *D. melanogaster* between one and four weeks of age. Flies were injected with one of five sizes of fluorescent beads (0.5µm, 1.0µm, 2.0µm, 3.0µm, 4.5µm).

2.4.4. Haemocyte number declined as females aged

The efficacy of the cellular immune system may be influenced by the number of cells as well as by their activity. We investigated how the density of cells circulating in the haemolymph changed with age. In this case we compared two wildtype fly genotypes, Oregon R and Samarkand. Cell number declined significantly as flies aged ($\chi^2_1 = 8.53$, $P = 0.0035$) (Table 4a) and the rate of decline was consistent for the two genotypes (age x genotype interaction: $\chi^2_1 = 2.59$, $P = 0.107$) (Fig 2). However, the age-

dependent trend in cell number was very different for the two sexes (age x sex interaction: $\chi^2_1 = 6.85$, $P = 0.009$). For both Samarkand and Oregon R, whilst cell number in females fell markedly with age ($\chi^2_1 = 11.18$, $P = 0.001$) (Table 4b), no such change occurred for males ($\chi^2_1 = 0.36$, $P = 0.547$). Female Samarkand flies had a 31.8% decline in cell number between one and four weeks of age; respectively for Oregon R female cell number fell by10.2%. In contrast, Samarkand male haemocyte number remained approximately constant across the ages. The same was true for Oregon R males. Males had on average 25.9% more haemocytes than females ($\chi^2_1 = 31.47$, $P < 0.0001$). Oregon R flies had slightly higher cell density than Samarkand (Fig 2), however this difference was not significant ($\chi^2_1 = 3.32$, $P = 0.069$). Cell number was not affected by time of day ($\chi^2_1 = 0.98$, $P = 0.322$).

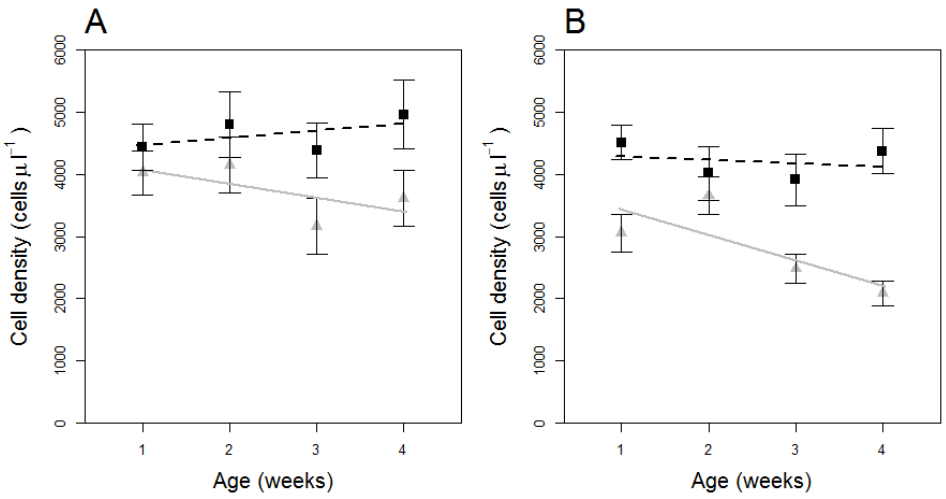

Figure 2: Age-dependent variation in the number of haemocytes circulating within the haemolymph for two fly genotypes: Oregon R (A) and Samarkand (B); divided into males (squares, black dashed line) and females (triangles, grey solid line). Bars represent standard errors.

Table 4: Factors influencing the number of haemocytes circulating within the haemolymph during ageing.

(a) Both sexes.

Factors	Parameter Estimates	SE	χ^2	P-value
Age (weeks)	- 0.063	0.016	8.53	0.004
Genotype (Samarkand)	- 0.101	0.053	3.32	0.069
Sex (male)	0.007	0.058	31.47	<0.001
Age x Sex Interaction	0.055	0.021	6.85	0.009

Output of terms from the model analysing the number of haemocytes circulating within the haemolymph in adult *D. melanogaster* for two genotypes: Oregon R and Samarkand, between one and four weeks of age. Parameter estimates with reference to Oregon R females.

(b) Female flies.

Factors	Parameter Estimates	SE	χ^2	P-value
Age (weeks)	- 0.052	0.016	11.18	0.001
Genotype (Samarkand)	- 0.116	0.041	4.88	0.027

Output of terms from the model analysing the number of haemocytes circulating within the haemolymph in adult female *D. melanogaster* for two genotypes: Oregon R and Samarkand, between one and four weeks of age. Parameter estimates with reference to Oregon R flies.

(c) Male flies.

Factors	Parameter Estimates	SE	χ^2	P-value
Genotype (Samarkand)	-0.997	0.045	4.18	0.041

Output of terms from the model analysing the number of haemocytes circulating within the haemolymph in adult male *D. melanogaster* for two genotypes: Oregon R and Samarkand, between one and four weeks of age. Parameter estimates with reference to Oregon R flies.

2.5. Discussion

For the first time in *Drosophila melanogaster* we demonstrate that the cellular immune response undergoes senescence. Age-dependent degeneration occurred in two aspects of cellular immunity. Firstly the haemocyte population, which is responsible for clearing microbes from the haemocoel, became less able to phagocytose microbes: fewer cells were phagocytically active in older flies. Secondly, in female flies the number of haemocytes circulating in the haemolymph declined during ageing.

By four weeks of age, only 70% of the haemocytes that had been active in young flies were still able to phagocytose microbes. In addition, whilst cell numbers in males did not change, in females there was an average fall of about 21.0% in haemocyte density between week one and week four. These combined effects mean that, at least for females, the phagocytic capacity of the haemocyte population reduced by ~46% over the first four weeks of life. These immunological changes must be due to senescent deterioration in individual flies, because the average total mortality in our fly cages between one and four weeks of age was too low (7.2% ± 0.8%) for selective mortality in the cage populations to explain this decline. We verified this by taking the week one dataset and simulating the extreme case where all the individuals which died during the experiment were those with the 7% highest phagocytosis data points. After removing the 7% most active data points the mean proportion of phagocytosing cells fell by only 11.5%, compared to the 30% decline from one to four weeks in our experiments. We therefore conclude that non-random mortality at the population level does not explain this decline. Our phagocytosis assay investigated the ability of cells to clear particles from the haemolymph within 30 minutes; if we had left flies for longer more particles would have been engulfed. However, the rate of activity of the immune response is important: whether and when an insect dies from infection may be determined by the relative rates at which a pathogen can grow and at which it is

cleared. Previous work indicated that flies in which phagocytosis was impaired by a null mutation in the phagocytosis receptor *Eater* succumbed to infection with *Serratia marcescens* two days before wild-type flies (Kocks *et al*., 2005). Our assay investigated changes in the free haemocyte population which circulates within the haemolymph. A proportion of haemocytes are sessile and bound to tissues (Elrod-Erickson *et al*., 2000; Lanot *et al*., 2001); further work will be required to investigate how these sessile cells are affected by ageing.

Haemocytes in older flies on average phagocytosed fewer microbes and fluorescent beads than those in young flies. This was due to a decline in the proportion of cells which were phagocytically active, rather than a decline in phagocytosis rate in all cells. Considering only those cells that were active (containing at least one phagocytic inclusion), there was no change in the number of included particles between one and four weeks of age. This suggests that there may be heterogeneity in the haemocyte population. Either haemocytes have specialised roles and the relative frequency of cells specialising in phagocytosis declines, or senescence may affect the phagocytic activity of some cells but not others. We used microbes and beads labelled with FITC to assess phagocytosis; FITC can modulate the efficacy of phagocytosis (Weingart *et al*., 1999) meaning our estimates may not directly equate with the ability of cells to engulf pathogens. However, labelling was consistent across age classes and particle types, and therefore could not have influenced our comparisons. The cellular response is important for pathogen defence: both domino mutant larvae (which lack haemocytes) and *Drosophila* adults in which phagocytosis has been blocked have compromised immunity (Braun *et al*., 1998; Elrod-Erickson *et al*., 2000). A quantitative comparison of humoral and cellular responses revealed that phagocytosis contributes a considerable amount to overall pathogen defence (Nehme *et al*., 2011). This leads to the prediction that as flies age and the cellular immune response declines their ability to clear microbial infection should decrease. However, this contrasts with the results of

Ramsden *et al*., (2008) who found that although mortality following infection increased in older flies, their ability to suppress *E. coli* growth was unchanged. Ramsden *et al*., (2008) hypothesised that age-dependent increases in infection mortality resulted from decreased tolerance to infection rather than decreased resistance to microbial replication.

In addition to phagocytosis, haemocytes have other immune defence roles. They produce hemolectin and other factors involved in blood clotting (Goto *et al*., 2003), and synthesise small quantities of AMPs besides those produced by the fat body and epithelia (Irving *et al*., 2005). Haemocytes also produce opsonins, such as thioester-containing proteins (TEPs), which promote phagocytosis (Lagueux *et al*., 2000). Furthermore, these cells do not act in isolation, but produce signalling molecules that interact with other parts of the immune system, such as the Toll ligand Spätzle (Irving *et al*., 2005; Shia *et al*., 2009) and Upd3 which triggers JAK/STAT pathway activity (Agaisse *et al*., 2003). Thus declining haemocyte numbers during senescence may have several immunological consequences. Whilst circulating haemocyte numbers declined strongly with age in female flies, those in males did not. Functional senescence of some behavioural and physiological traits is known to differ between the sexes and can vary according to genetic background (Grotewiel *et al*., 2005). Notably, flies in the current study were aged in mixed-sex populations and therefore mated throughout their lives. The immune systems of both males and females are influenced by mating (McKean & Nunney, 2001; Short & Lazzaro, 2010). It is therefore also possible that sexually antagonistic interactions or adaptive post-mating responses may have contributed to the decline in female cell density. As females experience copulatory wounding (Kamimura, 2007), haemocytes may be sequestered for wound repair and so removed from general circulation. In contrast to these trends in cell number, the age-dependent decline in phagocytic activity of cells occurred at similar rates in both males and females.

The phagocytic ability of mammalian macrophages is impaired for larger phagocytic targets (Champion *et al*., 2008). Here we demonstrate that the same phenomenon occurs in *D. melanogaster.* Flies were injected with beads ranging from 0.5µm to 4.5µm in diameter; above 2µm diameter the proportion of the haemocyte population that achieved phagocytosis decreased by around half. We hypothesised that if engulfing larger particles is more challenging, then the rate of phagocytosis senescence might be faster for larger targets. This was not the case; the rate of senescence was consistent for all five bead sizes, and indeed was not different for the two microbes tested (*E. coli* and *B. bassiana*). Although an element of pathogen specificity exists for some phagocytosis receptors (Philips *et al*., 2005; Watson *et al*., 2005), the consistency of these age-dependent declines suggests that phagocytosis senescence results from changes in general aspects of the phagocytic machinery. Phagocytosis may be modulated by opsonins and requires recognition and attachment of the haemocyte to the foreign body; remodelling of the cytoskeleton and particle engulfment then follows (Meister, 2004). Senescence of phagocytic ability could result from any of these processes being compromised. The age-dependent decline in haemocyte numbers could be due to increased apoptosis rates in the haemocyte population of older flies, as occurs in other tissues (Zheng *et al*., 2005). Alternatively, if haemocytes are lost when they clear microbes from the haemolymph, cumulative exposure during a fly's life may result in a reduced cell population.

This study demonstrates clear senescent declines in the cellular immune response. Previous ageing studies focussing on the expression of humoral immune response genes have shown transcript levels to increase, indicating heightened activity (Landis *et al*., 2004; Pletcher *et al*., 2002; Seroude *et al*., 2002; Zerofsky *et al*., 2005). Our findings suggest that age-dependent increases in humoral immunity may relate to the observed senescence of the cellular immune response. A compromised cellular response could result in increased microbial susceptibility thus intensifying stimulation

of the humoral response. Increased humoral activity can offset reduced cellular activity; when phagocytosis is experimentally inhibited overexpression of the AMP *Defensin* restored pathogen resistance (Nehme *et al*., 2011). A higher proportion of cells phagocytosed particles in those flies injected later in the day. This could reflect circadian rhythms in the immune response as has been shown in previous studies (Lazzaro *et al*., 2004; Lee & Edery, 2008), however in the absence of a controlled experiment to investigate this, we refrain from drawing strong conclusions. Little information about how long *Drosophila* lives in the field exists to put the age-dependent immunological changes we have observed into ecological context. However, it seems unlikely that many flies naturally survive to the oldest ages studied here. Nevertheless, senescence of phagocytosis ability was roughly linear from one through to four weeks of age. Thus, cellular immune senescence begins at an early age and may influence pathogen defence from week one onwards, an age relevant to wild populations.

Finally we note that plasmatocyte haemocytes in adult *Drosophila* have closest similarity to the monocyte lineage that gives rise to macrophages in vertebrates (Williams, 2007). Vertebrate macrophages have a plethora of roles including antibacterial defences, chemotaxis and wound repair but they specialise in phagocytosis; most of these functions decline with age in humans, mice and rats (Sebastián *et al*., 2009). Other vertebrate phagocytic cells such as dendritic cells and granulocytes also suffer impaired function in aged individuals (Agrawal *et al*., 2009; Linton & Dorshkind, 2004). The age-related changes we observed in *D. melanogaster* haemocyte phagocytosis and abundance mirror senescent changes known from vertebrate cellular immunity. That the phenomena underlying immunosenescence, if not the mechanisms themselves, may be conserved between flies and humans opens up novel possibilities to address questions of clinical relevance.

Chapter 3: Exploring mechanisms that could underlie the age-dependent decline in the cellular immune response

3.1. Abstract

The cellular immune response in *Drosophila* has been shown to senesce but the mechanisms underlying the observed age-dependent declines in haemocyte number and phagocytic function have not yet been identified. Potentially, the number of circulating haemocytes might be depleted as a result of immune encounters thoughout life. I investigated whether injecting flies with a dose of live *Escherichia coli* influenced the total number of circulating haemocytes 24hrs or 1 week post immune challenge. However, haemocyte number was unaffected by this immune challenge, suggesting that microbial encounters do not influence the size of the circulating haemocyte population.

Two approaches were used to investigate the mechanisms underlying senescent changes in plasmatocyte phagocytic function. First, I tested whether phagocytosis declines resulted from changes in the cells themselves by comparing age-dependent phagocytic declines when measured using *in vivo* and *ex vivo* assays. Phagocytosis declined by ~12% from one to four weeks of age in both assays, demonstrating that this was a cell autonomous process. The second approach attempted to explore the roles of a panel of phagocytosis receptors and their associated molecular pathways in the senescence of plasmatocyte function but the results were inconclusive.

To explore the relative contributions of cellular and humoral immune response changes to pathogen defence senescence I investigated the rates of age-dependent change in pathogen susceptibility in wildtype control flies, in flies with a compromised humoral immune response (RNAi knockdown of *Dif*) and in flies with compromised

phagocytosis (micro-bead injection). Interpretation of these experiments was hampered because the bead injection did not influence disease susceptibility and the control wildtype genotype did not suffer pathogen resistance senescence.

3.2. Introduction

Functional senescence is the deterioration of physiological systems through ageing. Immunosenescence focuses on the deleterious effects of ageing on the immune system. Although immunosenescence research typically characterises how immune function becomes impaired in the elderly, the processes are more complicated than just the reduction in activity of immune traits. A loss of ability to regulate immune responses in ageing organisms can result in heightened immune activity in older individuals. This is termed 'inflamm-ageing' (Franceschi *et al*., 2000). In *Drosophila melanogaster* antimicrobial peptide (AMP) transcription increases with age (Landis *et al*., 2004; Seroude *et al*., 2002; Zerofsky *et al*., 2005), several studies suggest that flies' ability to clear bacterial infection remains constant or improves with age (Felix *et al*., 2012; Khan & Prasad, 2013; Lesser *et al*., 2006; Ramsden *et al*., 2008). However, it was recently shown that the second arm of the *D. melanogaster* immune system does senesce in the conventional sense: the efficacy of the cellular immune response declines as flies age (Mackenzie *et al*., 2011).

Cellular immune defence in *Drosophila* adults relies on plasmatocytes: phagocytic haemocytes. Although other cell types exist in larvae these have not been identified in adults (Lanot *et al*., 2001). Importantly, there is a finite number of plasmatocytes within adult flies; cell proliferation has not been shown to occur and adults do not possess a hematopoietic organ (Lanot *et al*., 2001). Mackenzie *et al*., (2011) (Chapter 2) demonstrated that as flies aged the proportion of phagocytically active cells fell, and the number of circulating haemocytes also declined in female flies. The aim of this next chapter was to examine mechanisms that underlie these age-dependent declines in cellular immune function.

Many insect species demonstrate a change in circulating haemocyte numbers during ageing or following an immune challenge (King & Hillyer, 2013; Kurtz, 2002; Hillyer *et al*., 2005; Márkus *et al*., 2009). Potentially, the decline in cell numbers in adult *Drosophila* may have been due to cells being sequestered from circulation for wound healing. Females flies experience copulatory wounding (Kamimura, 2007), and haemocytes are known to play a role in wound repair (Fauvarque & Williams, 2011). Alternatively, cell number may decline if cells die, perhaps due to apoptosis.

Apoptosis is important for maintaining tissue homeostasis; including the removal of damaged or dysfunctional cells (reviewed by Favaloro *et al*., 2012). Apoptosis rates increase with age and are a key factor in the physiological deterioration experienced during ageing (reviewed by Zhang & Herman, 2002). A study on *Drosophila* demonstrated that apoptosis rates rose in many tissues as flies aged (Zheng *et al*., 2005), but *Drosophila* haemocytes were not investigated. The apoptosis of haemocytes in ageing honey bees (*Apis mellifera*) leads to the age-dependent transition of bees from nurse tasks to foragers (Amdam *et al*., 2005). In this case, the aged phenotype of a forager can be reversed through the proliferation of new cells (Amdam *et al*., 2005).

During phagocytosis internalised particles are degraded and processed within the phagolysosome (reviewed by Stuart & Ezekowitz, 2008). However the destinies of phagocytic cells once internalisation of a particle has occurred are unclear (Ulvila *et al*., 2011). Perhaps phagocytosis of microbes could lead to cell death; potentially the cell remodelling machinery necessary for continued phagocytic activity might become compromised once a number of particles had been engulfed. In order to continue internalising particles after phagocytosis, the cell membrane of phagocytic cells must be rapidly replenished. The melding of enzyme containing endosomes, lysosomes and the phagosome can achieve this recycling (Stuart *et al*., 2007); endoplasmic reticulum

components are potentially also involved (Gagnon *et al*., 2002). Mackenzie *et al*., (2011) showed that phagocytosis efficiency declines as flies age; if age-dependent impairment of phagocytosis machinery triggers apoptosis, then this could explain declining haemocyte densities.

In addition to its role in ageing, apoptosis is also linked to immunity. Some pathogens specifically prevent programmed cell death in order to maintain an infection. Baculoviruses are of commercial and clinical interest as they solely infect insects and are a useful biological pest control; their interference with apoptosis makes them of possible clinical use as a potential anti-cancer agent (reviewed by Clem, 2001). Apoptosis is a known immune defence in many invertebrate systems; including molluscs, crustaceans and several species of Lepidoptera (Clem, 2005; Hsu *et al*., 2005; Sokolova, 2009; Xian *et al*., 2013). Autophagy also has a role in immunity and is crucial in *D. melanogaster* survival following *Listeria monocytogenes* infection (Yano *et al*., 2008). There is also some evidence of cross-talk between these two degradative systems in response to a parasitic infection of the commercial silkworm *Bombyx mori* (Pradeep *et al*., 2013).

Another potential fate for plasmatocytes after they have phagocytosed microbes is that they may attach to tissues and become part of the sessile haemocyte population. The haemocyte population in *Drosophila* consists of two groups: sessile haemocytes and non-adherent (circulating) haemocytes (Elrod-Erickson *et al*., 2000; Lanot *et al*., 2001). Sessile haemocytes are also phagocytically active (Elrod-Erickson *et al*., 2000) but their number and functional activity in ageing *Drosophila* has not been explored. Potentially, non-adherent haemocytes might transfer into the sessile haemocyte population as flies age; then the age-dependent decline observed by Mackenzie *et al*., (2011) might not be a reduction in number but a change in location.

Considering the array of possibilities as to why the circulating haemocyte population declines during ageing, in this chapter I addressed a simple question: do circulating haemocyte numbers fall following an infection in female flies? *Drosophila* accumulate immune insults during their lifetime (Ren *et al*., 2007), perhaps it is due to these insults that non-adherent plasmatocytes diminish in number during ageing via one or more of the mechanisms described above.

The next part of this chapter focussed on potential mechanisms driving the 'loss-of-function' of haemocyte ability to phagocytose microbes in older flies. There were two questions considered in this section. Firstly, is the decline in the efficacy of phagocytosis a cell autonomous process, or do changing components in the haemolymph alter plasmatocyte function in aged flies? Secondly, is senescence in phagocytosis ability due to an age-dependent deterioration in the effectiveness of phagocytic receptor systems?

The *Drosophila* cellular immune response does not operate in isolation. Plasmatocytes produce signalling molecules which interact with other elements of the immune system; including the Toll ligand Spätzle (Irving *et al*., 2005), and Upd3 which triggers JAK/STAT pathway activity (Agaisse *et al*., 2003). Perhaps there is cross-talk between other parts of the immune response which can also influence cell function? Opsonin molecules act to increase phagocytic activity (Lagueux *et al*., 2000). Therefore, cell activity can be influenced by factors external to the cells themselves; it could be these factors that deteriorate during senescence. Alternatively, cell autonomous processes could be responsible for reduced plasmatocyte phagocytic ability in older flies. Plasmatocytes recognise self and non-self molecules via an array of cell receptors (reviewed by Stuart & Ezekowitz, 2008). Furthermore, complex processes must take place to achieve microbial engulfment and degradation (Stuart & Ezekowitz, 2008).

Alterations in any of these phagocytosis systems during a fly's life could influence the efficacy of phagocytosis.

To determine whether the age-related decline in phagocytic ability was a cell autonomous process or due to changes in soluble haemolymph factors I compared the rate of senescence in phagocytosis assays, carried out both *ex vivo* and *in vivo*, in flies of one to four weeks of age. The second approach was to investigate whether senescence in the phagocytic response of *D. melanogaster* is driven by deterioration in the efficacy of receptor systems. For this I compared how phagocytic ability declined with age in flies that had the expression of various genes coding for phagocytic receptors knocked-down through RNA interference (RNAi): *PGRP-LC, SR-CI*, *croquemort* and *eater*.

RNAi is a commonly used gene silencing technique which uses the antiviral immune defences of *Drosophila* to target its own mRNA and so silence the expression of specific genes (Dietzl *et al*., 2007). RNAi can be ubiquitously expressed (Dietzl *et al*., 2007), or specifically expressed within certain cells or tissues (Zettervall *et al*., 2004). Although a library of *Drosophila* stocks is available allowing for the silencing of many *Drosophila* genes (Dietzl *et al*., 2007), RNAi does not work for every gene and because of the pleiotropic effects of many genes, the full repercussions of all the available RNAi genetic lines have not yet been determined.

PGRP-LC is a peptidoglycan-recognition protein receptor that is known to facilitate the phagocytosis of *E. coli* (Ramet *et al*., 2002), and has been shown to activate the IMD pathway to induce the production of antimicrobial peptides (AMPs) (Choe *et al*., 2002; Gottar *et al*., 2002). The phagocytic receptors SR-CI and Croquemort are both scavenger receptors (Class C and Class B respectively) (Gordon, 2002). SR-CI is specific to insects (Pearson *et al*., 1995), and there is a potential link between disease resistance in *Drosophila* and naturally occurring variation in this receptor's expression

(Lazzaro *et al*., 2004; Lazzaro *et al*., 2006). Although Croquemort was initially identified as a receptor for apoptotic cells in flies (Franc *et al*., 1996), it was later discovered to have an additional function in disease resistance as a receptor for S*taphylococcus aureus* (Stuart *et al*., 2005). Finally, Eater is a member of the third class of receptors: epidermal growth factor (EGF)-like-repeat-containing receptors. Eater is a pattern recognition receptor that binds directly to bacteria, facilitating their internalisation by plasmatocytes in *D. melanogaster* (Kocks *et al*., 2005).

These genes were chosen to represent a breadth of receptor classes and were also selected due to their known association with disease resistance in *Drosophila*. The RNAi knockdown of *eater* expression was particularly expected to demonstrate a change in the efficacy of phagocytosis as this has been demonstrated before (Kocks *et al*., 2005). I predicted that if phagocytosis senescence occurred predominantly because of deterioration in the efficacy of one of these receptor systems, when that receptor's expression was knocked-down by RNAi the age-dependent decline in phagocytosis efficiency would slow or stop. I predicted that this would occur in addition to the overall negative impact of knock-down on phagocytic efficacy. This would indicate that in a wildtype genotype the receptor system in question was deteriorating as flies aged; thus highlighting a mechanism underlying the observed loss-of-function of plasmatocyte phagocytosis in ageing flies.

Both the humoral and the cellular immune responses are important for pathogen defence (Lemaitre *et al*., 1996; Nehme et al., 2011). Mutations in humoral response genes leave flies susceptible to pathogens (Yagi *et al*., 2013). Impairing phagocytosis through the injection of polystyrene beads also reduces disease resistance (Elrod-Erickson *et al*., 2000; Nehme et al., 2011; Pham *et al*., 2007). Some studies have investigated the relative importance of these two immune system wings in defending against different pathogens (Charroux & Royet, 2009; Nehme et al., 2011). Although

each arm of the immune system is important in pathogen defence, systemic AMP production can occur in adult *Drosophila* in the absence of plasmatocytes (Charroux & Royet, 2009), and upregulation of AMP gene expression can compensate for a compromised cellular immune response (Nehme *et al*., 2011).

This thesis focusses predominantly on the cellular immune response. However, the fact that phagocytosis efficacy declines during ageing (Mackenzie *et al*., 2011; Chapter 2) does not necessarily demonstrate that cellular immune senescence drives elevated pathogen susceptibility in aged flies. I sought to investigate the relative roles of age-dependent changes in the cellular and humoral immune responses in underpinning elevated pathogen susceptibility in older flies. To do this I compared three types of fly: those with a wildtype immune response, those that had received a bead injection to interfere with their cellular immune response and those in which I suppressed expression of the Toll pathway gene *Dif* by RNAi to impair the humoral immune response. For flies in each of these treatments I assessed the rate of age-dependent change in survival following infection with the entomopathogenic fungus *Beauveria bassiana*. I predicted that pathogen induced mortality would elevate in older flies and that if either the cellular or humoral immune response underlay this, then impairing its function would reduce the rate of age-dependent change in mortality.

As a brief summary, this chapter breaks down into four key questions. Are circulating haemocyte numbers influenced by infection? Is the age-related decline in the efficacy of phagocytosis a cell autonomous process, or driven by changes in soluble factors within the haemolymph? Do changes in pathogen receptor systems underlie senescence in plasmatocyte phagocytosis ability? Lastly, what are the relative roles of changes in the cellular and humoral immune responses in driving age-related changes in disease resistance?

3.3. Materials and Methods

3.3.1. Fly culturing

Every fly genotype described in this chapter was reared and maintained on standard Lewis medium (Lewis, 1960) at 25 ^{o}C, 70% RH, 12hr L/D. All lines were initially bred at low density for two generations and larval density controlled following Clancy and Kennington's (2001) method of pipetting 13µl of eggs suspended in saline into each bottle. Cultures were set up weekly in bottles to provide a continuous supply of flies of different ages.

To investigate the impact of immune challenge on haemocyte number, only one week old females from the wildtype genotype Samarkand were used. Flies from low density cultures were removed directly from bottles one week after eclosion: flies were not maintained in a demography cage. A total of 7 independent bottles were represented in this experiment.

Samarkand flies were also used to compare the impact of ageing on the phagocytosis ability of plasmatocytes in *ex vivo* and *in vivo* assays. Flies were reared as described, adults were placed in 11 litre demography cages (~400 mixed-sex flies per cage) and aged for between one and four weeks. Flies were fed Petri dishes of Lewis medium every other day and maintained until they were the age necessary for the experiment. Cages were set up every week so that flies of all ages were simultaneously available for experiments. Fly density was maintained in each cage by replacing any dead individuals with flies from separate, similarly aged, populations kept for that purpose. A total of 8 independent cages were represented in this experiment, two cages per age. Using an insect pooter, flies were collected from each cage and sorted into single sex five-fly batches within which flies originated from the same cage and therefore were

the same age. These vials were randomly allocated to treatments while maintaining an equal representation of each age class, gender and demography cage per treatment.

To investigate the potential role of defective phagocytic receptor systems in the senescence of haemocyte phagocytic ability RNAi strains were obtained from the Vienna Drosophila RNAi Centre: UAS-*PGRP-LC*-IR (transformant ID 101636), UAS-*dSR-CI*-IR (transformant ID 49964), UAS-*eater*-IR (transformant ID 4301) and UAS-*Cqr*-IR (transformant ID 45883). RNAi construct expression was triggered by a ubiquitously expressed *Actin-5C* driver (Act5C-Gal4/CyO*,* Bloomington stock #4414). Virgin Act5C-Gal4/CyO females were crossed with males from each RNAi strain to produce the active knowndown genotypes: Act5C-Gal4/UAS-*PGRP-LC*; Act5C-Gal4/UAS-*dSR-CI*; Act5C-Gal4/UAS-*eater*; Act5C-Gal4/UAS-*Cqr*. As a control, the background genotype into which the RNAi constructs had been inserted (w1118, Bloomington stock #6326) was also crossed with the driver line to generate the genotype Act5C-Gal4/+. Offspring from these crosses were reared in bottles, then the resulting adults were transferred to 11 litre mixed-sex demography cages and aged in a density controlled manner as described before. As only the w1118 control genotype, *croquemort* and *eater* knockdowns reached adulthood (see results) a total of 24 independent cages were represented in the experiment, two cages per age, per genotype. Cages were set up every week so that flies of all ages were simultaneously available for experiments. All flies in each cage were CO_2 anesthetised and the females sorted into five-fly batches within which flies originated from the same cage and therefore were the same age. All flies were allocated to the same treatment (see section **3.3.4.**).

The final experiment explored the effect of age on the relative roles of the cellular and humoral immune responses in disease resistance senescence. Control flies with a

wildtype immune response (generated as before: Act5C-Gal4/+) were compared to flies in which the Toll pathway transcription factor Dorsal-like immunity factor (*Dif*) was knocked down by RNAi (Act5-Gal4/UAS-*Dif*: UAS-*Dif*-IR, transformant ID 30579) generated following the methods described above. Adults were placed in 11 litre mixed-sex demography cages and aged for between one and four weeks. Cages were set up every week so that flies of all ages were simultaneously available for experiments and two batches of cages were set up. Fly density was maintained as before. All flies in each cage were CO_2 anesthetised and sorted into single sex ten-fly batches within which flies originated from the same cage and therefore were the same age. These vials were randomly allocated to treatments while maintaining an equal representation of each age class, gender and demography cage per treatment. There were a total of 48 independent demography cages represented in this experiment, two cages per age, per genotype, per batch.

3.3.2. Assessing haemocyte numbers following immune stimulation

To investigate whether the number of circulating haemocytes in the haemolymph was influenced by a live bacterial infection *Escherichia coli* (strain BB4) was grown overnight in LB broth at 37°C to form a saturated culture. One week old adult female Samarkand flies were separated into batches of 3-flies per vial then injected using a Drummond Scientific oocyte microinjection pipette with either 0.05µl of sterile LB broth (wounded controls) or the live *E. coli* culture. A third of the flies in the experiment were not injected (unharmed controls) but were anesthetised with CO_2 and handled before being returned to their vials. Flies were maintained in vials for either 24hrs or 1 week before their haemocytes were counted to explore temporal effects on cell numbers (1 week flies were transferred to fresh vials after three days).

The combined haemolymph of each 3-fly batch was then extracted using a standardised pulled glass capillary needle and the volume removed calculated following Mackenzie *et al*., (2011). Haemolymph was processed using standard methods (Mackenzie *et al*., 2011) before the haemocyte density was counted using an improved Neubauer haemocytometer. The mean from both grids was calculated. 190 slides, each containing the pooled haemolymph from three flies, were used for this experiment.

3.3.3. Investigating whether senescence of plasmatocyte phagocytosis ability is a cell autonomous process

Male and female Samarkand flies from four age classes (one, two, three and four weeks old) were separated into single-sex batches of 5-flies per vial. They were either subjected to an *in vivo* assay, adapted from methods used in Chapter 2, or an *ex vivo* assay was performed. For the *in vivo* assay, flies were injected with 0.05µl of either FITC labelled *B. bassiana* spores, or *E. coli*, or 2µm latex microspheres suspended in PBS (all at 5 x 10^6 microbes/beads ml^{-1}) (following methods in Mackenzie, *et al*., 2011). Flies were incubated at 25^{o}C for 30min before their haemolymph was collected (as described in **3.3.2.**). This was then added to a microcentrifuge tube containing 20µl ice-cold Schneider's *Drosophila* medium (Invitrogen). This was left foil-wrapped at room temperature for 5 min to allow fat droplets to separate. The bottom 18µl was then transferred to a well on an ice-cold 96-well plate already containing 100µl of Schneider's *Drosophila* medium. Plates were centrifuged at 30.7 *g* for 3 min, then the top 80µl was carefully removed and cells fixed using formaldehyde in PBS (working strength: 3.7%). When plates were ready to be viewed, 60µl of crystal violet (0.4% in PBS) was added per well and incubated for 10mins in order the quench any un-engulfed particles before plates were viewed on an Axiovert 135 epifluorescence

microscope. 100 cells were inspected in each well and the number of fluorescent included particles per cell recorded.

For the *ex vivo* assay, haemolymph was extracted from flies that had not previously received an injection, then processed and placed in a well containing 20µl Schneider's medium as described above. 0.05µl of one of the previously prepared fluorescent suspensions was added to each well. Plates were then incubated at 25^{o}C for 30 min for phagocytosis to take place, and then centrifuged and the cells fixed and quenched as described above. The assays were performed concurrently. A total of 208 biological replicates (wells containing the pooled haemolymph of five flies) were used for this experiment.

3.3.4. Investigating whether alterations in phagocytic receptor systems underpin age-related changes in phagocytosis

The RNAi crosses for two of the four phagocytosis receptors (PGRP-LC and dSR-CI) proved 100% lethal (see results). Therefore, for the study of whether senescence in haemocyte phagocytic ability resulted from the impaired function of the molecular pathways associated with phagocytic receptors, I focussed on Eater and Croquemort. Female flies from four age classes (one, two, three and four weeks old) of the genotypes: Act5C-Gal4/+; Act5C-Gal4/UAS-*eater*; and Act5C-Gal4/UAS-*Cqr* were separated into five-fly batches. Flies were injected as described above with 0.05µl of FITC-labelled *E. coli* (prepared as per previous experiments), incubated at 25^{o}C for 30 min and their haemolymph extracted and processed following the *in vivo* assay method described earlier (**3.3.3.**). In this case all cells in each well were counted and the number of fluorescent particles per cell recorded. There were 159 biological replicates (wells containing the pooled haemolymph of five flies) used in this experiment.

3.3.5. Investigating the roles of age-related changes in the cellular and humoral immune responses in senescence of disease resistance

To test the relative importance of age-dependent deterioration in the cellular and humoral immune responses for fungal pathogen resistance I experimentally impaired either phagocytosis or antimicrobial peptide transcription. The cellular immune response was compromised through the injection of 0.2µm carboxylate-modified blue fluorescent polystyrene microspheres (Invitrogen) (following Elrod-Erickson *et al*., 2000). Adult flies were injected as described above with 0.05µl of 0.2µm beads (5 x 10^9 beads ml^{-1}) 24hrs before the infection assay. The humoral immune response was inhibited by RNAi knocking down expression of the Toll pathway transcription factor *Dif*.

Beauveria bassiana spores originated from a strain used previously (Tinsley *et al*., 2006). Fungal material was grown following methods in Mackenzie *et al*., (2011) and diluted to 3 x 10^7 spores ml^{-1}. Cultures were vortexed and agitated briefly using a probe sonicator prior to use. Male and female flies from four age classes (one, two, three and four weeks old) were separated into single-sex batches of 10-flies per vial. Flies were kept on standard food medium (Lewis, 1960) throughout the experiment except that the anti-fungal agent 'Nipagen' that is normally added was omitted from food in experimental vials as it interferes with the growth of *B. bassiana* in flies. Half the flies with wildtype immune responses (Act5-Gal4/+) were injected with 0.2µm polystyrene beads to impair phagocytosis ability; the RNAi-*Dif* knockdown flies did not receive beads; all flies from both genotypes were then kept in vials for 24hrs before receiving the next treatment. Flies were then injected using a tungsten needle (ethanol sterilised) dipped in either blank oil (87.5% Shellsol T, 12.5% Ondina EL) (controls) or the live suspension of *B. bassiana* spores. Flies were maintained in single-sex vials

and checked every second day for mortality; corpses were recorded and the living flies were transferred to a new vial with fresh food.

3.3.6. Statistical Analysis

Data were analysed using the Lme4 package (Bates *et al*., 2013) for R statistical software, version 3.0.0 (R Core Team, 2013). Linear mixed-effects models were constructed for each dataset. The model for the data comparing haemocyte number following infection included the following random effects: the 'bottle' flies originated from, the 'vial' flies were housed in during the experiment and the 'day' on which experiments were performed. Fixed effects were the experimental 'treatment' received, the time post-treatment haemolymph was extracted (24hrs, or 1 week), the 'volume' of haemolymph extracted and the 'time' of day for the assay. Additionally, the interaction between 'treatment' and the post-treatment time was included in the model. The haemocyte count response variable was log10 transformed and a Gaussian error distribution was used.

The same model structure was used to analyse the dataset investigating whether haemocyte phagocytosis senescence was cell autonomous (**3.3.3**). The response variable was of the mean number of particles haemocytes phagocytosed. The random effects were the 'cage' flies were housed in and experimental 'day'. The fixed effects included fly 'age', 'sex', immune 'treatment' (injection of beads, bacteria or fungal spores), 'assay' type (*in vivo* or *ex vivo*), 'time' of day and haemolymph 'volume' extracted, in addition to the two-way interaction between 'age' and 'assay'. Analysis of the mean number of inclusions per active haemocyte (analysing only those cells that had successfully phagocytosed a microbe), used the same model structure. To calculate the proportion of active haemocytes, data were analysed with each cell as a replicate. Binary errors were used with a combined response variable of active or

inactive cells; sample was also included in the model as a random effect. This model generated the probability of cell activity and hence the proportion of active cells within the haemolymph.

The model testing whether age-related changes in phagocytosis receptor systems influenced plasmatocyte phagocytosis senescence contained the same random effects as above: ‘cage’ and ‘day’. The fixed effects included fly ‘age’, ‘genotype’,’ time’ of day and ‘volume’ of haemolymph and a two-way interaction between ‘age’ and ‘genotype’. Models were used to ascertain how these factors influenced the mean number of bacteria haemocytes phagocytosed. This model structure was repeated for the analyses of total haemocyte number, and other analyses addressing haemocyte function. Data were analysed as described above for calculating the proportion of active haemocytes.

The final dataset investigated the causes of senescence in disease resistance. The random effects were ‘cage’ and ‘batch’, as multiple batches of flies had been used. The fixed effects included ‘age’, ‘sex’, ‘infection’ (injection with fungal spores or control oil). For the test of whether impairing phagocytosis by bead treatment injection influenced senescence, data were analysed including bead ‘treatment’ as a fixed effect. Two two-way interactions were included: between ‘infection’ and ‘treatment’, and between ‘infection’ and ‘age’; as well as a three-way interaction between ‘age’, ‘infection’ and ‘treatment’. For the test of whether humoral immune knockdown influenced phagocytosis senescence, the term ‘treatment’ was not included (as flies did not receive bead injections), but this was replaced by the term ‘genotype’ in the same model structure to assess differences in trends between flies with wildtype immunity and *Dif* knockdowns.

Binomial errors were used for assessing the proportion of dead flies 6 days post infection employing the cbind function in R to analyse a two-vector response including the number of dead and alive flies at that time. Fly mortality during the first 24hrs post infection was excluded from the analysis as such early mortality would not have been due to fungal infection. Data from vials which contained fewer than 6 live flies 24hrs post infection were also removed as such high early mortality suggested that flies were dying of something other than *B. bassiana* (5% of vials were excluded (26/504)).

For all the analysis described, models were sequentially simplified by selecting terms for which the parameter estimate was closest to zero and eliminating them if their presence did not improve the explanatory power of the model by 2 AIC units. Significance of terms was determined using likelihood ratio tests. Means are given ± their standard errors throughout.

3.4. Results

3.4.1. Haemocyte number is unaffected by a bacterial challenge

One week old adult female Samarkand flies were divided into either unharmed controls, wounded controls, or injected with live *Escherichia coli*. The aim was to investigate whether bacterial infection changed the number of circulating haemocytes. If haemocyte numbers were depleted following an immune challenge this would offer a potential explanation for the previously observed age-related decline in cell number in female flies (Chapter 2; Mackenzie *et al*., 2011).

However, there was no difference between the control, sterile injection and septic injection treatments in the number of circulating haemocytes in the fly haemocoel ($\chi^2_{(2)}$ = 1.65; P = 0.438; Fig 1). Similarly, wounding did not affect haemocyte number, as unharmed flies contained the same number of haemocytes in their haemolymph as flies that had received an injection ($\chi^2_{(1)}$ = 0.16; P = 0.90). However, flies experienced a 15% decline in haemocyte numbers between extractions 24 hours post-treatment and those performed a week later. Numbers fell by the same amount regardless of which treatment flies had received (there was no treatment x extraction time interaction: $\chi^2_{(2)}$ = 2.37; P = 3.06). The time of day had no significant effect on cell number ($\chi^2_{(1)}$ = 0.25; P = 0.618), though the more haemolymph extracted the fewer cells were observed ($\chi^2_{(1)}$ = 311.8; P < 0.0001).

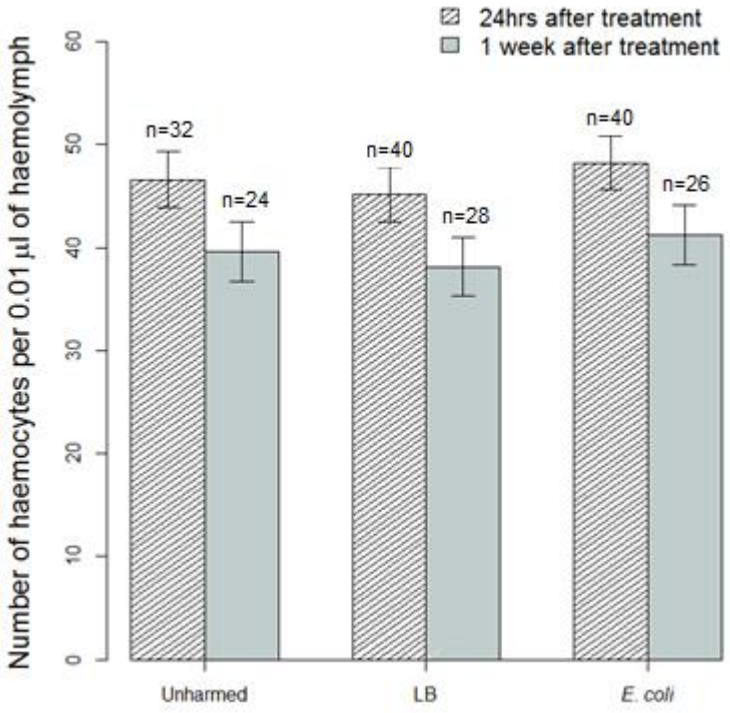

Figure 1: Impact of immune challenge on the number of circulating haemocytes 24hrs and one week after treatment. One week old adult female *Drosophila melanogaster* were separated into three-fly batches and handled but unharmed (controls) or injected with either 0.05µl of sterile LB broth (LB) (wounded controls) or 0.05µl of a saturated culture of live *Escherichia coli*. Haemocyte numbers were counted 24hrs or one week after treatment. Number of replicates shown and bars represent standard errors.

3.4.2. Haemocytes *ex vivo* demonstrate an age-dependent decline in phagocytosis ability

I investigated whether haemocytes from four classes of aged flies (one, two, three and four weeks old) demonstrated an age-dependent decline in phagocytic ability when phagocytosis occurred independently of opsonins and other soluble haemolymph factors. To test this, the phagocytic ability of haemocytes *in vivo*, was compared to the phagocytic ability of haemocytes that had been removed from flies before fluorescent particles were introduced (*ex vivo* phagocytosis).

The phagocytic ability of haemocytes declined with age in both the *in vivo* and *ex vivo* assays ($\chi^2_{(1)} = 26.4$; $P < 0.0001$) (Fig 2). In the *in vivo* experiment the proportion of haemocytes which phagocytosed a particle declined by 12% from one week to four weeks of age and this was mirrored for the *ex vivo* experiment with a 14% drop. Although *ex vivo* haemocytes demonstrated a 2% steeper decline in phagocytosis ability with age, this difference was not statistically significant (there was no age x assay interaction: $\chi^2_{(1)} = 0.95$; $P = 0.33$). There was no significant difference in the proportion of haemocytes phagocytosing *B. bassiana*, *E. coli* or the 2µm microspheres ($\chi^2_{(1)} = 0.05$; $P = 0.98$). The proportion of active haemocytes in both cases did not vary between males and females ($\chi^2_{(1)} = 0.27$; $P = 0.60$). Neither the time the assay was conducted nor the volume of haemolymph extracted influenced phagocytosis ($P > 0.05$).

As an alternative measure of the efficacy of phagocytosis in plasmatocytes, the mean number of particles phagocytosed per cell was also investigated. Again, there was no difference in this metric between the *in vivo* and *ex vivo* assays in their rate of age-related decline in phagocytosis ability (there was no age x assay interaction: $\chi^2_{(1)} = 1.66$; $P = 0.20$). There were fewer phagocytosed particles per haemocyte in older flies compared to younger ones ($\chi^2_{(1)} = 20.1$; $P < 0.0001$). For this metric, the mean number of inclusions per haemocyte varied between assay types ($\chi^2_{(1)} = 34.5$; $P < 0.0001$): haemocytes *in vivo* phagocytosed 13% fewer particles than haemocytes *ex vivo*. In one week old flies haemocytes phagocytosed an average of 0.44 ± 0.03 particles per cell *in vivo*, and an average of 0.53 ± 0.03 particles per cell *ex vivo*. In both assays haemocytes phagocytosed a greater number of bacteria than *B. bassiana* spores or 2µm microspheres ($\chi^2_{(2)} = 74.6$; $P < 0.0001$).

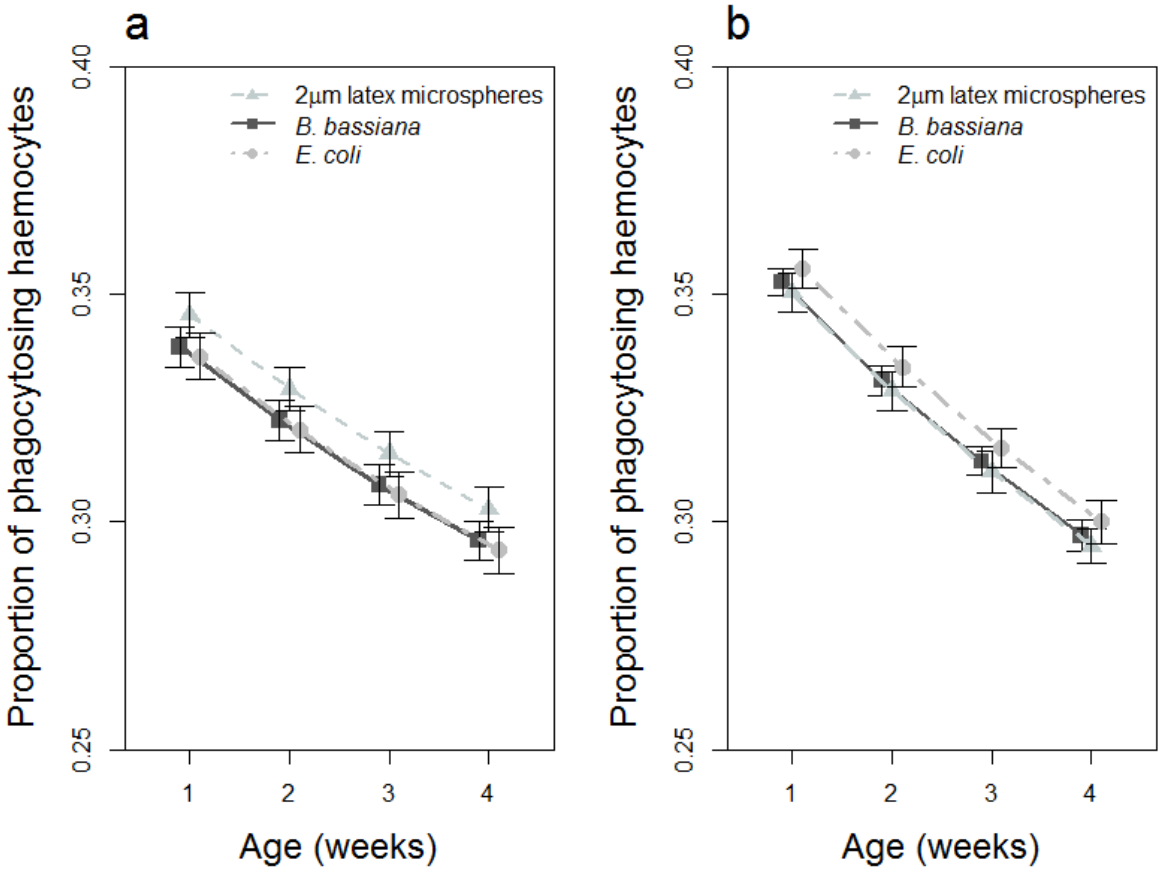

Figure 2: Changes in the proportion of actively phagocytosing haemocytes in adult *Drosophila melanogaster* between one and four weeks of age. Fluorescently labelled latex microspheres, *Beauveria bassiana* or *Escherichia coli* were either injected into flies directly for assays of *in vivo* phagocytosis (a) (n = 48, 23, and 23 replicates per treatment respectively), or these particles were introduced to haemocytes extracted from flies to assay *ex vivo* phagocytosis (b) (n = 48, 40, and 32 replicates per treatment respectively). Bars represent standard errors.

The same number of engulfed particles were found in both males and females ($\chi^2_{(1)}$ = 0.74; P = 0.39) and though the time of the assay and the volume of extracted haemolymph were fitted in the models, these did not impact the mean number of particles phagocytosed per haemocyte (P > 0.05).

In the final analyses, when only haemocytes that successfully phagocytosed a particle were analysed, there was no age-dependent effect; haemocytes phagocytosed an equal number of particles regardless of the age of the fly ($\chi^2_{(1)} = 1.66$; $P = 0.20$). Haemocytes phagocytosed significantly more bacteria than spores or microspheres ($\chi^2_{(1)} = 73.8$; $P < 0.0001$), and haemocytes *in vivo* phagocytosed fewer particles than haemocytes *ex vivo*: approximately 10% less (an average of 1.45 ± 0.08 particles per active cell *in vivo* compared to an average of 1.62 ± 0.05 *ex vivo*) ($\chi^2_{(1)} = 24.2$; $P < 0.0001$). There was no difference in haemocyte activity between males and females ($\chi^2_{(1)} = 2.41$; $P = 0.12$), and though time of the assay and the volume of extracted haemolymph were included as factors in the model these were not significant ($P > 0.05$).

3.4.3. Knockdown of *eater* and *croquemort* expression failed to find evidence that deterioration of phagocytosis receptor systems underpins senescence of plasmatocyte phagocytic ability

This experiment investigated whether senescence in phagocytic ability resulted from impaired function of the molecular pathways associated with key phagocytosis receptors. The aim was to see whether the rate of phagocytosis senescence slowed when the expression of phagocytosis receptor genes was knocked down by RNAi, evidence which would implicate these receptor pathways in the age-dependent decline in phagocytosis which takes place in wildtype flies.

Four phagocytosis receptors were identified for investigation. However, for the RNAi line crosses which elicited the knockdown of *PGRP-LC* and *SR-CI* no offspring without the balancer chromosome (those in which RNAi occurred) reached adulthood: these lines were therefore removed from the experiment. The RNAi knockdown genotypes *eater* and *croquemort*, as well as the immunologically wildtype control cross (hereafter

referred to as w1118) did reach adulthood. The proportion of haemocytes which phagocytosed *E. coli* did not differ significantly between these three genotypes ($\chi^2_{(2)}$ = 1.85; P = 0.40) (Fig 3).

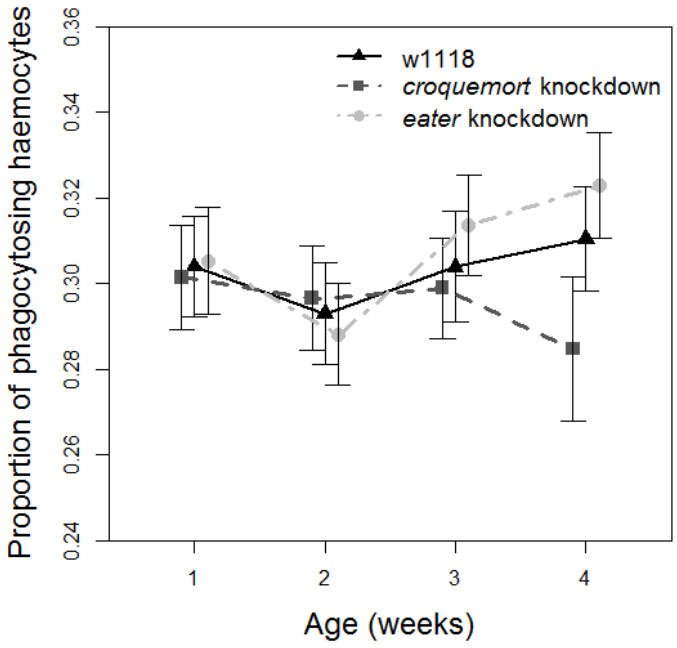

Figure 3: Comparison of age-related changes in plasmatocyte phagocytic function between immunologically wildtype w1118 flies and flies with the expression of phagocytosis receptor genes *croquemort* and *eater* knocked down by RNAi. Fluorescently labelled *Escherichia coli* were injected into flies directly for *in vivo* phagocytosis (n = 54, 47, and 56 replicates respectively per genotype). Bars represent standard errors.

Although Figure 3 suggests the proportion of phagocytically active cells may diverge between the three genotypes in four week old flies, this was not statistically significant (effect of genotype in four week old flies: $\chi^2_{(2)}$ = 1.78; P = 0.41). Additionally, the proportion of active haemocytes did not vary with fly age across the three genotypes ($\chi^2_{(1)}$ = 1.16; P = 0.28). Roughly 30% of cells successfully phagocytosed a bacterium in each genotype at all ages. Testing each genotype individually, there was no significant

age-dependent change in plasmatocyte phagocytosis ability for any genotype (w1118: $\chi^2_{(1)} = 0.53$; $P = 0.47$; *croquemort* knockdown: $\chi^2_{(1)} = 0.11$; $P = 0.74$; *eater* knockdown: $\chi^2_{(1)} = 3.01$; $P = 0.08$). Therefore, senescence was not detected in the phagocytic ability of plasmatocytes in any genotype in this experiment. The time of day of the assay and the volume of haemolymph extracted per sample were included in the model but neither were significant factors ($P > 0.05$).

The second set of analyses investigated changes in the mean number of bacteria phagocytosed per haemocyte. Again, no functional senescence in the cellular immune response was detected; there was no difference in phagocytosis ability between genotypes ($\chi^2_{(2)} = 0.29$; $P = 0.87$) or ages ($\chi^2_{(1)} = 1.44$; $P = 0.23$). Haemocytes from all the genotypes phagocytosed a mean of 0.49 ± 0.04 bacteria per cell at each age. In further analyses, considering only haemocytes that had successfully phagocytosed a bacterium, the mean number of included bacteria increased to 1.92 ± 0.07 per cell, but again there was no difference in phagocytosis ability between genotypes ($\chi^2_{(2)} = 0.62$; $P = 0.73$) or ages ($\chi^2_{(1)} = 1.30$; $P = 0.25$).

An alternative measure of phagocytic ability was to compare the total number of *E. coli* phagocytosed per sample (the combined haemolymph of five flies) providing an overall measure of the efficacy of phagocytosis in these flies. In this case there was a significant difference between genotypes ($\chi^2_{(1)} = 8.57$; $P = 0.01$). Haemocytes from *croquemort* knockdown flies phagocytosed the greatest number of bacteria (171.4 ± 19.6 bacteria per sample in one week old flies); 17% more than w1118 flies (142.8 ± 17.4) and 18% more than *eater* knockdown flies (141.3 ± 18.5) of the same age. There was a non-significant trend of declining total phagocytosis with age across the genotypes ($\chi^2_{(1)} = 3.24$; $P = 0.07$) (bacteria per sample in 4 week old w1118 flies: 112.5 ± 18.0; *croquemort* knockdowns: 143.7 ± 18.5; *eater* knockdowns: 154.7 ± 17.9). However, pooling across the whole dataset the rate of age-related decline appeared to

be equal for each genotype (there was no age x genotype interaction: $\chi^2_{(1)} = 1.62$; $P = 0.45$). Again, the time of day of the assay and the volume of haemolymph extracted per sample were included in the model, but neither were significant factors ($P > 0.05$).

Importantly, the previous analyses have already demonstrated that this difference in total phagocytosis ability between genotypes was not a per-cell effect. The reason for the elevated total phagocytic activity in *croquemort* knockdown flies was a significant difference in the total number of circulating haemocytes recorded per sample ($\chi^2_{(2)} = 11.9$; $P = 0.003$) (Fig 4). *Croquemort* knockdown flies contained 18% more haemocytes than w1118 flies and 14% more than *eater* knockdown flies. Although this variation between genotypes was significant, all genotypes retained the same number of circulating haemocytes as they aged ($\chi^2_{(1)} = 0.03$; $P = 0.87$).

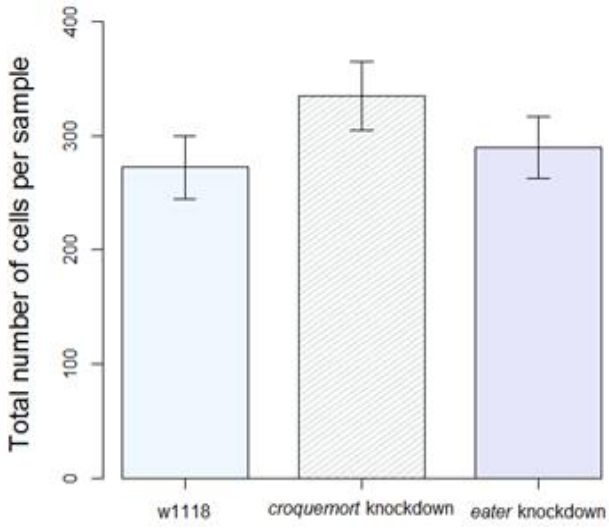

Figure 4: Variation in the total number of haemocytes per five-fly batch. Adult female *Drosophila melanogaster* from three different genotypes: immunologically wildtype w1118, and *croquemort* and *eater* RNAi knockdowns (n = 54, 47, and 56 replicates per genotype respectively). Bars represent standard errors.

3.4.4. *Dif* knockdowns demonstrated increased pathogen susceptibility, whereas flies injected with polystyrene beads did not

The final section of this chapter investigated the relative roles of age-dependent variation in the cellular and humoral immune responses in driving age-related changes in disease resistance. Flies with a wildtype immune response (non-bead w1118 control cross flies), were compared to flies with compromised humoral immunity through the inhibition of *Dif* expression by RNAi, and flies with a compromised cellular immune response through the injection of polystyrene beads (w1118 control cross with beads). Before dissecting the relative importance of these two systems, I initially investigated whether the fungal pathogen injection killed flies and found that fungal infection always negatively influenced survival (w1118 (no beads): $\chi^2_{(1)} = 12.32$; $P < 0.001$; w1118 (with beads); $\chi^2_{(1)} = 51.69$; $P < 0.0001$; *Dif* knockdowns: $\chi^2_{(1)} = 590.4$; $P < 0.0001$). Pooling all the data, survivorship in *B. bassiana* flies was on average 32% lower than in controls (Fig 5).

Next I tested to see whether the *Dif*-knockdown experienced increased pathogen susceptibility. *Dif* knockdown was successful in immuno-compromising flies; there was a significant reduction in disease resistance in *Dif* knockdown flies (considering only non-bead w1118 and *Dif* knockdown flies, infection x genotype interaction: $\chi^2_{(1)} = 38.15$; $P < 0.0001$). In immunologically wildtype flies, infected individuals suffered 26% greater mortality than controls; this increased to 42% pathogen-induced mortality in *Dif* knockdown flies. Potentially, *Dif* knockdown flies may have suffered greater mortality compared to w1118 flies following injection in the absence of infection however this was not the case ($\chi^2_{(1)} = 0.42$; $P = 0.517$).

I also investigated whether bead injection increased pathogen susceptibility. Bead injection did have an overall negative effect on fly survival (w1118 flies: $\chi^2_{(1)} = 26.2$; $P < 0.0001$), but this treatment did not increase fly pathogen susceptibility (there was no

treatment x infection interaction: considering only w1118 flies: $\chi^2_{(1)} = 1.54$; $P = 0.22$) (Fig 5). At one week of age, the non-bead w1118 mortality was 24% greater in pathogen injected than control flies, whereas this figure was 23% for the flies receiving bead injections.

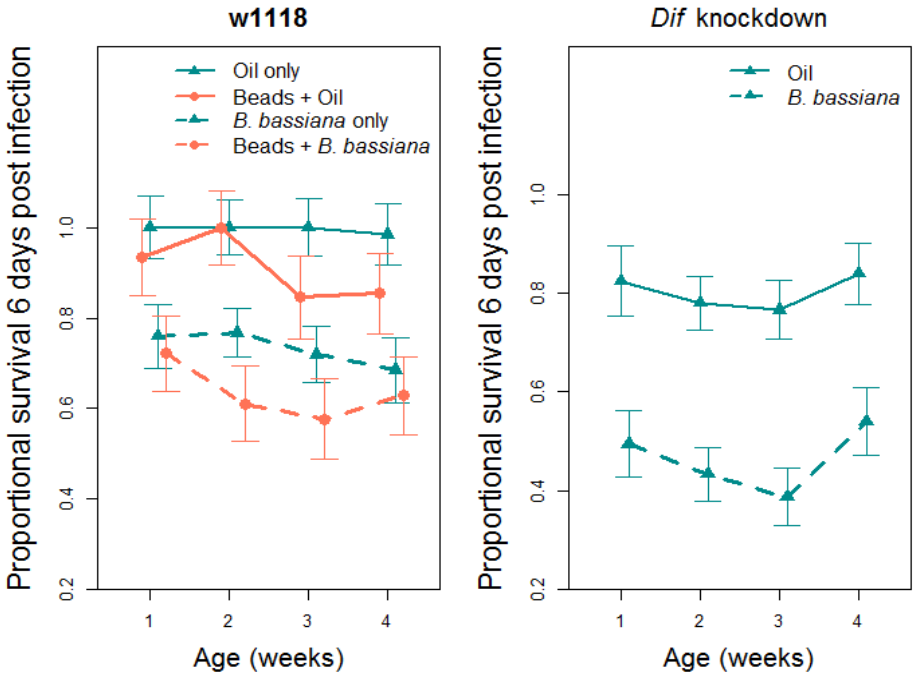

Figure 5: Age-related variation in disease resistance of flies following immune manipulations. Adult *Drosophila melanogaster* were from four age classes (one to four weeks old) and from two genotypes (an immunologically wildtype genotype w1118 and a line with expression of the gene *Dif* knocked down by RNAi). Flies from the w1118 genotype were either injected with 0.2µm polystyrene beads (orange lines) or did not receive a pre-injection (blue lines). 24hrs later, all flies either received a control injection (solid lines) or injection with the fungal pathogen *Beauveria bassiana* (dashed lines). The *Dif* knockdown was designed to compromise the humoral immune response (Toll pathway), whereas the bead injection aimed to impair the phagocytic cellular response. Bars represent standard errors.

Finally, I looked at whether immunologically wildtype flies (non-bead w1118) suffered disease resistance senescence. Averaging across the infected and uninfected flies,

the survivorship of non-bead w1118 flies declined as they aged ($\chi^2_{(1)}$ = 5.83; P = 0.02) (Fig 5). To study pathogen resistance senescence I investigated whether older flies suffered elevated *B. bassiana*-induced mortality. This experiment did not detect a difference between control and infected flies in the rate at which mortality increased with age in the immunologically wildtype flies (considering only non-bead w1118 flies there was no age x infection interaction: $\chi^2_{(1)}$ = 1.00; P = 0.32). Therefore, senescence in pathogen susceptibility was not detected in non-bead w1118 flies.

The initial aim of this experiment was hampered because I did not detect evidence of an influence of bead injection on fungal pathogen susceptibility, nor did I detect clear evidence of pathogen resistance senescence in immune-competent flies. Nevertheless, I had predicted that if humoral or cellular immunity underpinned pathogen resistance senescence, then when this system was impaired, this would affect the relative difference between the rate of age dependent mortality change in fungal infected and control flies. I investigated this by testing for a three-way interaction between age, infection and immune treatment. For the cellular immunity experiment, the relative difference in the rate of age-dependent mortality change between pathogen infected and control flies was not influenced by bead injection (w1118 flies only: age x infection x treatment: $\chi^2_{(1)}$ = 1.02; P = 0.31). However, for the humoral immunity experiment there was a strong difference in the relative pattern of age-related change in infected and uninfected flies between non-bead w1118 flies and *Dif* knockdowns (age x infection x genotype: $\chi^2_{(1)}$ = 23.0; P < 0.0001). This interaction resulted because whilst the mortality rates in pathogen infected w1118 flies elevated slightly relative to uninfected flies as they aged, this did not occur when the gene *Dif* was knocked down by RNAi ($\chi^2_{(1)}$ = 0.12; P = 0.73) (Fig 5). However, when *Dif* was knocked down, the extent of fungal-induced mortality actually declined as flies aged (age x infection interaction: $\chi^2_{(1)}$ = 4.26; P = 0.04), which contributes to the strength of the three-way interaction between age, infection and genotype.

3.5. Discussion

The aim of this chapter was to examine mechanisms that potentially underlie the observed age-dependent decline in the cellular immune response of *Drosophila melanogaster* (Chapter 2; Mackenzie *et al*., 2011). This work demonstrates that the decline in haemocyte numbers in female flies is unlikely to result from cells being lost after mounting an immune response against systemic bacterial infections. I show that senescence of plasmatocyte phagocytic function is a cell autonomous process and occurs independent of soluble factors in the haemolymph. There was no evidence that alteration in the molecular pathways associated with the phagocytic receptors investigated in this chapter have a role in phagocytosis senescence. Finally, my attempt to identify whether changes in the cellular or humoral immune responses underpin senescence of fungal pathogen resistance was relatively inconclusive.

The density of circulating haemocytes in female flies was unaffected by whether flies had been injected with live *Escherichia coli*, handled but unharmed, or injected with LB broth. Interestingly, there was a uniform 15% drop in circulating haemocyte numbers 1 week post-treatment in all treatments. When the circulating haemocyte population in female Samarkand flies were compared across one to four weeks of age in Chapter 2 (Mackenzie *et al*., 2011), cell numbers declined by 31.8%. This one-third reduction in cell number was not entirely linear and predominately occurred when flies were between 2-3 weeks of age. However, in the current study there was a 15% decline in the circulating haemocyte population when flies were between 1-2 weeks of age, which suggests that these changes may begin earlier.

Although the sampling technique was the same for both experiments, flies were maintained in mixed-sex cohorts in Chapter 2 but were kept in single-sex groups for this study. The fact that cell numbers declined one week post-treatment in the absence

of mating argues against the suggestion of Mackenzie *et al*., (2011) that the female-specific fall in circulating haemocyte numbers is the result of sexual antagonism and copulatory wounding. Instead, perhaps the contrast between males and females in their trends in age-related cell density is an example of immune investment varying between the sexes, as occurs in other traits (Hill-Burns, *et al*., 2009; Winterhalter *et al*., 2009).

Potentially, there could have been a confounding effect of wounding alongside the immune challenge with live *E. coli*. However this was not the case, as unharmed flies did not demonstrate an alteration in haemocyte number either. Although studies have demonstrated that elements of the immune system can be upregulated purely by a sterile injection (Márkus *et al*., 2005), recent work showed that fly metabolism rates were not influenced by wounding (Bashir & Tinsley, 2014), suggesting that a sterile injection does not always stimulate a metabolically costly immune response. In this experiment, neither wounding nor a live microbial challenge influenced the size of the circulating haemocyte population.

The fly haemocyte population is split between a sessile and a circulating fraction (Elrod-Erickson *et al*., 2000; Lanot *et al*., 2001). Non-adherent haemocytes may migrate into the sessile haemocyte population as female flies age, therefore it is not possible to definitively conclude that changing haemocyte numbers reflects a change in the total population. If the decline in non-adherent haemocytes occurs because increasing numbers of cells undergo apoptosis as female flies grow older, TUNEL staining, or other techniques, could be used to detect alterations in apoptosis rates.

The current study clearly demonstrated that challenging the cellular immune response with a non-pathogenic bacterial infection did not influence the total number of circulating haemocytes. Infections with truly pathogenic bacteria, rather than *E. coli*,

might provoke a more vigorous cellular immune response. However, these data suggest that the experience of repeated immune challenges throughout a fly's life is unlikely to be the factor that drives declining cell numbers in ageing female flies.

Apart from an age-related decline in haemocyte number, the ability of plasmatocytes to phagocytose objects reduced as flies aged (Chapter 2; Mackenzie *et al*., 2011). In the previous study, combining the results for the different particle types haemocytes were challenged with, the proportion of phagocytically active cells dropped by a marked 30% from one to four weeks of age (Mackenzie *et al*., 2011). In this chapter, flies were only challenged with *E. coli*, *Beauveria bassiana* and 2µm microspheres and demonstrated a less pronounced, but still significant, decline in haemocyte phagocytosis ability of haemocytes *in vivo*: by four weeks of age the proportion of phagocytically active haemocytes had declined by 12%. Importantly, haemocytes *ex vivo* demonstrated an almost identical rate of senescence in phagocytosis ability, thus showing that in the absence of soluble factors in the haemolymph the function of plasmatocytes was still compromised in older flies.

These results suggest that although factors in the haemolymph are known to alter plasmatocyte activity, the senescence of plasmatocyte ability to phagocytose particles is a cell autonomous process. As the decline in cell function occurred at the same rate in and out of the haemolymph, this suggests that the rate of senescence observed *in vivo* may be solely due to alterations in the cells themselves and not influenced by changes in the composition of haemolymph components. If the efficacy of opsonisation and processes of intercellular signalling do change as flies grow older, these are not the cause of plasmatocytes' phagocytic senescence.

Roughly 13% fewer particles were phagocytosed *in vivo* compared to *ex vivo* conditions. Within the fly there are multiple immune defences including epithelial cell

defences, the humoral immune response and blood clotting (Lemaitre & Hoffmann, 2007). Potentially due to the cooperative effect of these immune responses, fewer particles were present to be detected and engulfed by circulating plasmatocytes *in vivo* as opposed to these cells being the only immune effectors present in the *ex vivo* assay. The technique used to extract plasmatocytes collects only the circulating population of cells and does not account for any of those adhering to tissues that may have internalised a proportion of the particles in the *in vivo* assay. Also, in the *ex vivo* assay cells and particles were spun down on top of each other in a centrifuge, which may have contributed to the higher phagocytosis rates recorded there. Regardless of differences in the absolute level of plasmatocyte phagocytic ability, which may vary as a result of assay conditions, the relative rate of age-dependent decline in function was the same.

A cell autonomous age-dependent decline in phagocytosis ability could occur through various means. The detection of apoptotic, dead cells or pathogenic intruders is dependent on a number of receptors, some of which have been described in this chapter, but many are involved (reviewed by Ulvila *et al*., 2011). Age-dependent alterations in receptor expression could influence plasmatocyte phagocytic ability. *Drosophila* plasmatocytes have closest similarity to macrophages in vertebrates (Fauvarque & Williams, 2011). The expression of a subset of pattern recognition receptors in macrophages: Toll-like receptors (TLRs) have been shown to decline during ageing (Renshaw *et al*., 2002) and have a direct link with increased mortality following *Streptococcus pneumoniae* infection in aged mice (Boyd *et al*., 2012). Plasmatocyte internal mechanisms may also become compromised during ageing; inefficient membrane recycling or the compromised formation and melding of enzyme containing endosomes, lysosomes and the phagosome would all impact plasmatocyte phagocytosis ability.

Although it is clear that senescence in plasmatocyte function is a cell autonomous process, I was not able to demonstrate a link with either of the phagocytosis receptors investigated in this chapter. I reasoned that if RNAi knockdown of receptor expression had slowed the rate at which phagocytosis ability senesced, this would indicate that age-dependent changes in that receptor (or other parts of its molecular pathway) contributed to the natural degeneration of plasmatocyte function with age. However, haemocyte phagocytosis senescence was, surprisingly, not observed for the control genotype (immunologically wildtype w1118) in this experiment. I also observed no age-dependent decline in plasmatocyte function in flies with receptor expression knocked down (RNAi-*eater* and RNAi-*croquemort*). However, in the absence of a decline in control flies, it is not possible to conclude this reflects involvement of either Eater or Croquemort in plasmatocyte senescence. It is unclear why I did not observe phagocytosis senescence in this experiment. Mackenzie *et al*., (2011) observed it in Samarkand flies and Chapter 5 reports universal senescence in 18 outcrossed genotypes. Whether this reflects genuine genetic variation for the effect of age on phagocytic ability remains to be determined.

Whilst it is difficult to conclude anything about plasmatocyte senescence from this experiment, it is noteworthy that in flies from the RNAi crosses lower *eater* and *croquemort* expression did not exhibit impaired phagocytosis ability. It is important to state that the RNAi knockdowns used in this experiment were not assessed to demonstrate that the RNAi crosses effectively influenced receptor abundance. I did not assess the levels of receptor mRNA or protein in any of the flies; RT-PCR could have determined that the relevant genes were knocked down in the fly lines used. The PGRP-LC and dSR-CI knockdowns resulted in a lethal effect. It may be that these were the only crosses in which the knockdown was successfully induced. Sometimes the activation of the RNAi system can have off-target effects which may have led to the

lethality of these crosses (Mohr & Perrimon, 2012). Alternatively, these genes may have had pleiotropic effects meaning that their expression may have been crucial for successful morphogenesis as well as having a role in plasmatocyte phagocytic function during an immune challenge.

Nevertheless, haemocyte abundance was on average 16% higher in the *croquemort* knockdown flies, suggesting that the RNAi did create a distinct phenotype. The cell number increase in these flies could be related to Croquemort's role as a receptor for apoptotic cells (Franc *et al*., 1996). Low Croquemort receptor abundance might result in the accumulation of dying cells in the haemolymph. However, in the *croquemort* knockdown flies, these additional cells were phagocytically active: the cell population phagocytosed a greater number of bacteria per sample compared to the other lines (17% more than w1118 flies and 18% more than *eater* knockdown flies). This then suggests that the cells that are present in *croquemort* knockdown flies are not dying but still phagocytically active. It would be interesting to test whether these flies also contain more sessile haemocytes and whether the presence of more circulating haemocytes makes flies more resistant to pathogens.

The *eater* knockdown genotype used in this chapter was different from that used in other studies; in contrast to this work these showed that Eater influences phagocytosis activity. I used the UAS-*eater*-IR genotype supplied by the Vienna stock centre (transformant ID 4301). Kocks *et al.,* (2005) used deficiency lines obtained from the Bloomington Stock Centre (#823 and #1911). The only other *in vivo* experiment recently conducted using an *eater* knockdown used one gifted from Christine Kocks (Christofi and Apidianakis, 2013). It is possible that the Vienna line is not comparable.

Additional fly lines could have been included in the experiment to verify the effects of the RNAi; alternative RNAi lines with the same phenotype, and negative controls (Niwa

& Slack, 2008). Another alteration that could be considered in the future would be to use a haemocyte specific driver line: *Hemese-GAL4* (Zettervall *et al*., 2004), *Hml-GAL4* (Goto *et al*., 2003) or *srp-GAL4* (Waltzer *et al*., 2002). This would limit the inhibition of receptor gene expression to just the haemocytes and may have mitigated any confounding factors that systemic inhibition may have had on the flies.

The final section of this chapter explored the contributions of age-dependent changes in the cellular and humoral immune responses to declining *B. bassiana* fungal pathogen resistance as flies grew older. I used two different immune interventions: RNAi to knockdown expression of *Dif* which is a key component of the Toll-mediated humoral response (Pinheiro & Ellar, 2006), and bead injection which inhibits the cellular phagocytic response (Elrod-Erickson *et al*., 2000). I predicted that if either humoral or cellular immunity were the principal driver of pathogen resistance senescence, then when that system was compromised the rate at which pathogen susceptibility elevated with age would decrease. I investigated this by testing a three-way statistical interaction between age, infection and immune intervention treatment. This three-way interaction was highly significant for the humoral immune intervention (RNAi knockdown of *Dif*) but not for the cellular immune intervention (bead injection). Naïvely this might suggest that senescence of pathogen resistance primarily results from changes in the humoral rather than the cellular immune response.

Unfortunately, the conclusion that humoral changes drive pathogen susceptibility senescence is complicated by several factors. Whilst bead injection had a detrimental effect on the overall survival of w1118 flies it impacted control and infected fly survival equally, so there was no evidence that the bead treatment used in this study influenced susceptibility to fungal infection. Previous studies have demonstrated that bead injection compromises phagocytosis (Elrod-Erickson *et al*., 2000; Nehme *et al*., 2011; Pham *et al*., 2007). Additionally, bead injection lowered survival following

infections with *Micrococcus luteus*, *Enterococcus faecalis*, *Staphylococcus aureus*, (Nehme *et al*., 2011), and *Streptococcus pneumoniae* (Pham *et al*., 2007). The impact of functionally inhibited plasmatocytes on survivorship following infection could be unique to bacterial challenges; alternatively in this current study the bead injection may not have compromised phagocytosis.

A second complication is that in the immunologically wildtype flies, whilst pathogen induced mortality did elevate with age, this increase, indicative of immune senescence, was not significant. Also, in the case of the humoral immune comparison, the nature of the three-way age:infection:immune intervention interaction was not as predicted. This interaction arose due to the difference between the marginally increasing amount of infection-induced mortality with age in wildtype flies and a declining amount of infection-induced mortality in flies with a compromised Toll-pathway humoral response. Therefore, it is not possible to conclude that this indicates a role for humoral immunity in age-dependent changes in pathogen susceptibility. Notably, the reduced expression of a key Toll pathway gene (*Dif*) did have a dramatic impact on disease resistance. The proportional survival of one week old *Dif* knockdown flies 6 days post infection was 11% less than w1118 non-bead flies of the same age. This increased mortality in *Dif* knockdown flies could have been due to greater frailty in these immune compromised flies however there was no difference in the proportional survival of immunologically wildtype flies and the *Dif* knockdowns in the absence of infection.

In conclusion, this chapter has made progress towards defining some of the mechanisms that may underlie senescence in the cellular immune response in *D. melanogaster*. We now know that the decline in the total size of the circulating haemocyte population in female flies is not due to haemocytes being ‘used up’ following a bacterial infection. Additionally, the decline in plasmatocyte phagocytic function in ageing flies is a cell autonomous process that occurs independently of

potential alterations in the components within the haemolymph. However, these experiments were unable to establish whether changes in phagocytosis receptors and their associated molecular pathways have a role in haemocyte senescence, nor determine the involvement of humoral or cellular immunity in disease resistance senescence. Nevertheless, valuable progress towards the understanding of senescence in the cellular immune response in *D. melanogaster* has been made.

Chapter 4: Past immune insults determine future cellular immune responses in *Drosophila melanogaster*

4.1. Abstract

The immune response in ageing animals can decline in efficacy (immunosenescence) but can also improve in its effectiveness through the formation of immunological memories. Although invertebrates do not possess the same machinery behind immunological memory as vertebrates, a similar phenomenon occurs in many invertebrates and is termed 'immunological priming'. I investigated how multiple previous immune encounters influenced phagocytis activity in the *Drosophila melanogaster* cellular response. Flies received two priming injections three days apart, on each occasion receiving either a control, or dead *Escherichia coli* or dead *Beauveria bassiana* treatment; after a further three days the plasmatocyte phagocytic response was tested in a competitive assay using fluorescently labelled *B. bassiana* and *E. coli*. Phagocytosis was enhanced by up to 33% if one of the priming injections was homologous to the assay microbe, and by up to 50% if both primes were homologous. Phagocytic activity was reduced if flies received a heterologous immune challenge, indicating that the priming response was not caused by general immune upregulation. Subsequently, I investigated whether the priming response altered with age. The level of enhancement in plasmatocyte function declined as flies became older, indicating senescence in the ability of flies to develop a primed response. These results suggest that plasmatocytes play a key role in immunological priming in invertebrates; also they challenge the conventional view of immune senescence, since individual immune history was shown to shape later cellular immune responses.

4.2. Introduction

As animals age the efficacy of the immune response can change. Pathogen resistance may decline due to immune senescence (Nikolich-Žugich & Čičin-Šain, 2010; Weiskopf *et al.*, 2009). Alternatively, disease defence may also be enhanced as an animal's life progresses by the formation of immunological memories of prior microbial encounters. Whilst invertebrates were once thought to be unable to develop immunological memories, an increasing body of evidence reveals that this ability is not restricted to the acquired immune response of jawed vertebrates (Kurtz & Franz, 2003; McTaggart *et al.*, 2012b; Moret & Siva-Jothy, 2003; Roth *et al.*, 2009; Sadd & Schmid-Hempel, 2006). To distinguish between the phenomena in invertebrates and the mechanisms in vertebrates, the process by which invertebrates develop immune memories has been termed immunological priming.

In this chapter I investigate whether the *Drosophila melanogaster* cellular immune response can adapt following microbial challenges to mount a more efficient phagocytic response against secondary encounters with a specific pathogen. Such changes in phagocytic ability driven by prior microbial exposure could have a major impact on the age-related changes in phagocytosis shown previously (Chapter 2/(Mackenzie *et al.*, 2011)). Clearly immune priming could enhance phagocytic ability late in life relative to the performance of haemocytes in young naïve individuals. However, there is also the potential for a cost of immune priming. Organisms generally encounter a range of microbial challenges throughout their lifetime. If phagocytic ability becomes highly specialised towards certain microbes perhaps this would be at the cost of a reduced response towards novel pathogens? I therefore investigate the impact that multiple immune challenges have on the efficacy of phagocytosis both against novel and against previously-encountered microbes.

In the first chapter of this thesis I explored whether senescence occurred in the cellular immune response in *D. melanogaster*. The second chapter focussed on potential mechanisms behind an ageing cellular immune response and the impact of an ageing immune system on disease resistance. This chapter will concentrate on immune priming and the impact that ageing has on this.

Although *Drosophila* possesses the best-studied insect immune system and is a leading model organism in the study of innate immunity (Leips, 2009; Lemaitre & Hoffmann, 2007) it has not been used extensively in the study of invertebrate immune priming. Most studies investigating priming have been in other organisms, and date from as early as the 1920s. A review by Wagner (1961) concluded that although there was evidence that invertebrates displayed a phenomenon similar to acquired immunity in vertebrates, this seemed partial, transitory and lacked specificity. It is only in the last decade that studies demonstrating strong, long-lasting and specific priming have emerged.

Moret and Siva-Jothy (2003) led the recent resurgence in the study of invertebrate immune priming by showing strong though non-specific priming in mealworm beetles (*Tenebrio molitor*). When individuals were pre-injected with lipopolysaccharides (LPS) and subsequently challenged with spores of the fungus *Metarhizium anisopliae* they had enhanced survival (reduced death rate of 58%) compared to naïve beetles or beetles that had received a Ringer pre-injection. The effect lasted up to 7 days. Further, they showed that the phenoloxidase system was not responsible for improved survival, but that increased haemolymph antimicrobial activity following immune challenge may have underlain the primed immune response (Moret & Siva-Jothy, 2003).

In the same year, a high degree of specificity was demonstrated in the invertebrate priming response of the copepod *Macrocyclops albidus* against its tapeworm parasite *Schistocephalus solidus* (Kurtz & Franz, 2003). Homologous exposures resulted in significantly lower reinfection rates (Kurtz & Franz, 2003). In *Daphnia* priming was shown to occur across generations, termed 'transgenerational priming' (Little, *et al*., 2003). Replicate clones of *Daphnia magna* were exposed to one of two strains of the bacterial pathogen *Pasteuria ramosa*, infection by which causes reduced fecundity in its host. Offspring of infected hosts were collected a week later and exposed to either the same strain of the alternative strain of *P. ramosa*. If offspring received the same strain (homologous treatment) as their mother they had higher fitness than if they received an alternative strain. Individuals in the homologous treatment produced an earlier first clutch and cumulatively up to 21% more offspring compared to individuals in the heterologous treatment. A within-generation priming response in *D. magna* has also been demonstrated (McTaggart *et al*., 2012b). However, there was not the same level of specificity; both priming treatments lowered the subsequent rate of infection following secondary exposure to *P. ramosa* regardless of whether individuals received a homologous or heterologous treatment (McTaggart *et al*., 2012b).

A study on bumblebees conclusively demonstrated specific immunological priming that was also long-lasting; indeed nearly the full lifespan of a bee. *Bombus terrestris* workers were injected with either *Pseudomonas fluorescens*, *Paenibacillus alvei*, *Paenibacillus larvae* or saline (Sadd & Schmid-Hempel, 2006). Bees were then reinfected with one of these bacteria 8 or 22 days later so that combinations of homologous and heterologous infections could be investigated. Bees that received a homologous treatment regime had significantly greater survival compared to other treatments and they also had comparatively greater clearance of bacteria from their haemocoel. This suggested that the mechanism responsible for individual survival was

not due to increased immune tolerance but rather specific immune activity (Sadd & Schmid-Hempel, 2006).

For a given host organism, not all pathogens have been shown to stimulate immunological priming. Roth *et al*., (2009) found this in the red flour beetle (*Tribolium castaneum*). Individuals were either naïve or injected with one of a range of heat-killed bacteria or Ringer's solution. The bacteria included two strains of *Bacillus thuringiensis* (a natural pathogen of *T. castaneum*) and two non-pathogenic bacteria; *B. subtilis* and *E. coli*. Eight days later beetles were challenged with live bacteria following a reciprocal design to examine all infection combinations. Survival was then monitored for 17 days. Immunological priming (increased survival) occurred following previous exposure to either strain of *B. thuringiensis* for homologous but not heterologous combinations, thus demonstrating a remarkable level of specificity. This was also the case for homologous *B. subtilis* treatments (as opposed to heterologous combinations of *Bacillus* challenges) but there was no significant difference in survival following previous challenge with *E. coli* (Roth *et al.*, 2009). An obvious explanation for this lack of observed priming for *E. coli* is that it is not a natural pathogen of *T. castaneum*, therefore has no history of coevolution with this host. Roth *et al*., (2009) also report that the level of immune priming for *B. thuringiensis* was stronger than for *B. subtilis*.

Some authors have expressed scepticism in the results of immunological priming studies in invertebrates and have cautioned against drawing close parallels between immune priming and the acquired immune system in vertebrates (Hauton & Smith, 2007). One principle criticism is that invertebrate immunological priming studies have been mainly phenomenological in design and have not, as yet, uncovered the mechanisms behind improved resistance following immune challenge (Hauton & Smith, 2007). However, usually it is the discovery of a phenomenon that leads to the pursuit of possible mechanisms (Little *et al.*, 2005). Clearly the mechanisms behind

priming in invertebrates must vary from those mechanisms underpinning the acquired immune response in vertebrates. Acquired immunity in vertebrates is driven by the presence of B and T cells which, through somatic recombination, generate highly specific immunoglobulin pattern recognition receptors. This cellular and genetic machinery employed by vertebrates to acquire immune memory and mount specific immune responses is not thought to be present in invertebrates (Litman *et al.*, 2010). However, some candidate mechanisms have emerged which may explain how invertebrates achieve immune priming.

One candidate mechanism that has received much attention is the Down syndrome cellular adhesion molecule (Dscam) (Brites *et al.*, 2008; Dong *et al.*, 2006; Dong *et al*., 2012; Kurtz & Armitage, 2006). *Dscam* is a member of the immunoglobulin superfamily and through splicing of alternative exons it is able to generate over 38,000 proteins making it an attractive candidate for explaining receptor diversity (Schmucker *et al.*, 2000). Indeed it has been demonstrated that knock-down of the Dscam receptor in *Drosophila* haemocytes through RNA interference substantially reduced their phagocytic activity (Watson *et al.*, 2005). Work in mosquitoes (*Anopheles gambiae*) corroborated this: Silencing of *Dscam* interfered with *A. gambiae* resistance to infection and the expression of splice-variants varied with pathogen type (Dong *et al*., 2006). However, more recent work suggests that this specificity between splice-variants and pathogens may not be as extensive as originally thought (Smith *et al*., 2011). *Dscam* splice-form was compared in mosquitoes that had been fed blood lacking the malaria parasite (*Plasmodium falciparum*), or containing a single or multiple genotypes of this parasite. Diversity of splice-form increased following exposure to *P. falciparum* but there was not a significant difference in diversity between mosquitoes exposed to single compared to multiple genotypes (Smith *et al.*, 2011). However, it has now been demonstrated that instead of being expressed as a single highly specific

molecule in response to immune challenge, Dscam is produced as a ‘cloud of effectors’ comprised of a different combination of splice-variants depending on the infection type (Dong *et al*., 2012). The repertoire of splice forms varied for up to eight different immune stimuli (including two malaria parasite species: *P. faciparum* and *P. berghei*), and it was argued that Dscam continues to remain the most likely component in the mechanisms driving innate immune memory and specificity. Whether Dscam is the ultimate mechanism behind receptor-mediated immune specificity has yet to be proven but its link with haemocyte immune function corresponds well with recent studies suggesting that the cellular immune response in invertebrates is a key mechanism behind invertebrate immune priming (Pham *et al.*, 2007; Powell *et al.*, 2011; Rodrigues *et al*., 2010; Roth & Kurtz, 2009).

There have been only two priming studies published on *Drosophila.* Pham *et al*., (2007) showed that priming with a sub-lethal dose of *Streptococcus pneumoniae* or *Beauveria bassiana* protects those flies from an otherwise lethal dose subsequently. The protective effect of previous exposure was shown to last up to two weeks for *S. pneumoniae*. However, as found in red flour beetles (Roth *et al*., 2009), this effect was not universal as prior challenge with other bacteria: *Salmonella typhimurium*, *Listeria monocytogenes* or *Mycobacterium marinum* did not produce a subsequent protective effect (Pham *et al.*, 2007). Key in this study was some indirect evidence that haemocytes played a role in immune priming. Flies in which phagocytosis by plasmatocytes was blocked by injecting polystyrene beads became extremely sensitive to *S. pneumonia* regardless of whether they were naïve or had been previously challenged with the microbe. Thus, flies with inactive plasmatocytes were unable to be immunologically primed. Additionally, this link between plasmatocyte activity and immune priming was shown to be specific as flies that had been primed

with *S. pneumonia* did not demonstrate increased phagocytosis activity following challenge with *E. coli* (Pham *et al*., 2007).

More recently, priming in *D. melanogaster* was demonstrated by studying the increase in survival to a lethal strain of *Pseudomonas aeruginosa* if flies had previously been exposed to a heat-killed version or a sub-lethal strain of *P. aeruginosa* (Christofi & Apidianakis, 2013). Additionally, if the phagocytic receptor Eater (known to be important in the recognition, binding and engulfment of bacteria through phagocytosis (Kocks *et al.,* 2005)) was inactivated then flies had a significantly compromised priming response: with either little or no survival benefit of previous exposure during secondary infection. However, a possible concern about this paper is that the wildtype lines potentially varied from the background genotypes of mutant fly lines and the survival benefit of pre-exposure was only a matter of hours, therefore possibly explained by factors other than priming.

Work in other organisms has been very valuable in describing the relationship between haemocyte phagocytosis and immunological priming. Mori and Stewart (2006) investigated whether increased phagocytic activity in lobsters (*Homarus americanus)* was specific or general following a particular immune stimulus. They compared injection with LPS to injection with *Aerococcus viridans* var. *homari* (a pathogenic bacterium of particular interest in aquaculture due to the extensive economic losses infection can cause). LPS challenge induced a non-specific elevation in phagocytic activity, whereas individuals exposed to *A. viridans* var. *homari* over 30 days previously showed specificity in subsequent levels of phagocytosis (Mori & Stewart, 2006). A later study using woodlice (*Porcellio scuber*) demonstrated an even greater level of specificity (Roth & Kurtz, 2009). Woodlice were either given a prior injection of *E. coli* or one of two different strains of *B. thuringiensis*, then two weeks later their phagocytic activity towards a combination of all three bacteria was measured. The

resulting phagocytic activity was highly specific with homologous combinations inducing a greater cellular immune response than a heterologous challenge (Roth & Kurtz, 2009).

Similarly, three recent studies have shown specificity and memory in the phagocytic response of shrimp (*Litopenaeus vannamei*) (Fu *et al.*, 2011; Pope *et al.*, 2011; Powell *et al.*, 2011). In one study, juvenile shrimp were injected with a mixture of inactivated vibrio bacteria present in a commercially available vaccine (Powell *et al*., 2011). Two weeks later the shrimp haemocytes were extracted and challenged with a 1:1 combination of vaccine vibrios and the novel immune challenge of *B. subtilis*. Again specificity in the phagocytic response was observed; vaccine challenged individuals contained a higher number of vibrio inclusions per 100 cells compared to *B. subtilis* inclusions (Powell *et al.*, 2011). Related experiments were conducted in the same year except that the 'vaccine' used consisted of an inactivated strain of *Vibrio harveyi* (Pope *et al*., 2011). Once again specificity in the number of internalised bacteria as well as the percentage of phagocytically active haemocytes was seen (Pope *et al.*, 2011). The third study used the novel method of 'vaccinating' shrimp against the White Spot Syndrome Virus (WSSV) by introducing spores of *B. subtilis* containing a recombinant form of the viral protein VP28 through feeding (Fu *et al*., 2011). Again, those that were 'vaccinated' displayed significantly greater phagocytic activity against WSSV compared to controls (Fu *et al.*, 2011). Specificity was shown by incubating the same cells with an unrelated virus where no increase in phagocytic activity was observed (Fu *et al.*, 2011).

Regarding the potential role of haemocytes in immune priming, work by Rodrigues *et al*., (2010) on the cellular immune response in mosquitoes (*A. gambiae*) to *Plasmodium* infection is particularly interesting. Unlike *Drosophila,* adult mosquito haemocytes are able to differentiate following infection (King & Hillyer, 2013).

Rodrigues *et al*., (2010) demonstrated that following initial infection re-exposure to *P. falciparum* up to two weeks later led to a much reduced intensity of infection compared to the first immune challenge. This was due to a combination of the differentiation of pro-haemocytes into granulocytes (phagocytic haemocytes) and a greater anti-bacterial response due to ookinetes breaching the gut epithelium and releasing gut microbiota. If the gut bacterial flora was removed through the use of antibiotics or if granulocyte function was disrupted using Sephadex beads then the priming response was prevented (Rodrigues *et al*., 2010). Of greatest interest was the fact that the priming response was able to be transferred to naïve individuals through the transfer of haemocytes from challenged mosquitoes, demonstrating how key these were to the primed response (Rodrigues *et al*., 2010).

This thesis investigates immune senescence in *Drosophila*, focussing on the cellular immune response. It has been shown that phagocytic ability declines with age in *Drosophila*; the number of phagocytically active haemocytes decreased during a fly's lifetime (Mackenzie *et al*., 2011). This could be straightforwardly due to immune senescence: age-related deterioration in cell function. Alternatively, if phagocytic priming of the cellular immune response occurs following microbial encounters, then declining phagocytic ability with age could be explained by an adaptive shift in the immune system to defend specifically against pathogens previously experienced. This would potentially reduce the ability of plasmatocytes to phagocytose novel immune challenges.

Priming studies generally challenge hosts with a single microbe and study how this changes pathogen resistance or immune function. However, organisms are likely to encounter many immune challenges throughout their lifetime adding complexity to the development of immune memory. In this chapter I investigated the impact of multiple microbial encounters on the priming response. Flies received two priming injections,

either with identical or different microbes, and then I assessed how these multiple exposures influenced the development of the primed response.

When considering ageing, one key aspect of immune senescence in vertebrates is senescence in the ability of individuals to develop an acquired immune response (reviewed in Lang *et al*., 2011). A combination of factors decreases immune specificity and immune memory as vertebrates age. An age-related reduction in thymus size (thymic involution) reduces naïve T cell production, and naïve T cells in elderly humans do not differentiate into effector cells as efficiently as cells from younger individuals (Weiskopf *et al.*, 2009). The B cell population also declines as vertebrates age; their ability to interact with T cells decreases, reducing their expansion and differentiation following antigen recognition, as well as impairing antibody production. Together this undermines the ability of the acquired immune system to respond to infection and vaccination in elderly people (Lang *et al*., 2011). As immune memory is affected by ageing in vertebrates, I questioned whether immunological priming in invertebrates may also be influenced by ageing.

The first aim of this chapter was to investigate whether the cellular immune response in *D. melanogaster* could be specifically enhanced or 'primed' following a microbial encounter. Second, I tested if the phagocytic response of plasmatocytes was influenced by multiple homologous and heterologous immune challenges. Finally, I attempted to determine how fly age affected the enhancement of plasmatocyte phagocytic function through priming, by comparing the development of a primed phagocytosis response in flies aged from one to four weeks of age.

4.3. Materials and Methods

4.3.1. Fly culturing

The wildtype genotype Samarkand was used throughout (obtained from Bloomington Stock Centre). Flies were reared on standard Lewis medium (Lewis, 1960) at 25°C, 70% RH, with a 12hr L/D photoperiod. Cultures were set up following a standard procedure (Mackenzie *et al.*, 2011). After eclosion flies were transferred to 11 litre demography cages (~400 mixed-sex flies per cage). Flies removed that died were counted, and constant density was maintained by topping up cages from separate, similarly aged, populations maintained for that purpose

For the first experiment, 2 independent demography cages were represented. Flies were aged within the demography cage until they were two weeks old and then were all CO_2 anesthetised and the females sorted into three-fly batches within which flies originated from the same cage. Vials were randomly allocated to treatments while maintaining an equal representation of each demography cage per treatment.

In the second experiment, a total of 8 independent demography cages were used, two cages per age. All flies in each cage were CO_2 anesthetised and the females sorted into five-fly batches within which flies originated from the same cage and therefore were the same age. These vials were randomly allocated to treatments while maintaining an equal representation of each age class and demography cage per treatment.

4.3.2. Preparation of microbes

Escherichia coli (strain BB4) was grown and prepared following methods used previously (Mackenzie *et al.*, 2011). Following culture in LB broth, bacteria were washed and resuspended in sodium carbonate buffer (0.1M $NaHCO_3$, pH 9). After the

final wash the bacteria were resuspended in 1ml of PBS (pH7) and diluted to 2 x 10^7 cells ml^{-1}. *Beauveria bassiana* spores originating from a strain used in the past (Tinsley *et al.*, 2006) were prepared as described in earlier work (Mackenzie *et al.*, 2011). The final concentration was the same as for *E. coli* (2 x 10^7 cells ml^{-1}). Both microbe suspensions were heat-killed by immersion in boiling water for 10 minutes. The resulting suspensions were confirmed to be non-viable by plating out onto suitable media and ensuring no growth occurred before being used to inoculate flies.

For use in assessing the phagocytic ability of *Drosophila* haemocytes, *E. coli* (strain BB4) was grown as before then labelled with Fluorescein isothiocyanate (FITC) (Sigma) following a standard method (Mackenzie *et al.*, 2011). *B. bassiana* spores were also prepared as before, and then fluorescently labelled using lissamine™ rhodamine B sulfonyl chloride (Invitrogen). 100µl of reactive dye solution (10mg/ml lissamine™ rhodamine B sulfonyl chloride dissolved in Dimethylformamide) was added to 900µl of spore suspension and incubated for one hour at 4°C in the dark with continual shaking. The final suspension was then washed and resuspended twenty times to remove excess dye, and finally resuspended in 1ml of PBS (pH 7) before being prepared to the same concentration as for *E. coli* (2 x 10^7 cells ml^{-1}). The two fluorescent microbe suspensions were then combined at a 1:1 ratio to produce the final suspension used in the phagocytosis assay. A competitive assay was performed to investigate haemocyte ability to differentiate and potentially preferentially phagocytose a particular microbe in a multi-microbe environment.

4.3.3. The effect of multiple different immune challenges on phagocytic ability

Only two-week old female Samarkand flies were used in this experiment. Flies were separated into three-fly batches and injected with 0.05µl of PBS or one of the

unlabelled microbes using a Drummond Scientific oocyte microinjection pipette. Flies were given a second injection of one of these three treatments three days later. The second injection followed a reciprocally designed protocol so that an equal number of flies were challenged with each dose combination (Table 1). Flies were maintained as 3 flies per vial on Lewis medium (Lewis, 1960) at 25°C, 70% RH, with a 12hr L/D photoperiod at all times (except during actual injection). Fly haemocyte phagocytic activity was assessed 3 days after the second injection.

Table 1: Treatment combinations to stimulate the cellular immune response in *Drosophila melanogaster*. Adult female *D. melanogaster* were treated with a control injection of phosphate buffered saline (C), or primed by injecting either heat-killed *Beauveria bassiana* (B), or *Escherichia coli* (E). This was done once at the beginning of the experiment, and again three days later.

DAY 0	DAY 3	Abbreviation
B. bassiana	*B. bassiana*	**BB**
B. bassiana	PBS	**BC**
PBS	*B. bassiana*	**CB**
E. coli	*E. coli*	**EE**
E. coli	PBS	**EC**
PBS	*E. coli*	**CE**
PBS	PBS	**CC**
B. bassiana	*E. coli*	**BE**
E. coli	*B. bassiana*	**EB**

4.3.4. The influence of fly age on immune priming

One to four-week old female Samarkand flies were separated into five-fly batches then each fly was injected in the lateral thorax using an ethanol and heat-sterilised tungsten needle dipped in either sterile PBS or the prepared unlabelled suspensions of either *E. coli* or *B. bassiana*. Flies were maintained in environmental conditions described above for 24hrs before their haemocyte phagocytic activity was assessed.

4.3.5. Assessing fly haemocyte phagocytic activity

A Drummond Scientific oocyte microinjection pipette was used to inject one to four-week old Samarkand flies with 0.05µl of the mixed suspension of fluorescently labelled *B. bassiana* spores and *E. coli* bacteria described above. Flies in their three-fly (or five-fly) batches were injected into the lateral thorax on the opposite side to their previous injection to minimise cumulative damage. In the case of the multiple immune stimulation experiment this was not possible as flies had already been injected on both sides; therefore the injection was done on whichever side appeared least damaged. Flies were then incubated at 25^{o}C for 30 min. The combined haemolymph of each group of flies was bled using a standardised pulled glass capillary needle. Haemolymph volumes were measured and recorded using standard methods (Mackenzie *et al*., 2011), before being processed following methods used previously (*in vivo* assay (Chapter 3: section **3.3.**)). Cells were fixed and the fluorescence of any un-engulfed particles was quenched before plates were viewed on an Axiovert 135 epifluorescence microscope. All cells were inspected in each well and the number and type of fluorescent included particles per cell recorded. 90 biological replicates (wells containing the combined haemolymph of three flies) were used in the multiple immune challenge experiment. 120 biological replicates (wells containing the combined haemolymph of five flies) were used for the experiment testing the influence of fly age on immune priming.

4.3.6. Statistical Analysis

Data were analysed using the Lme4 package (Bates *et al*., 2013) for R statistical software, version 3.0.0 (R Core Team, 2013). The first experiment was on two-week old female flies receiving a double immune stimulation before the phagocytic ability of their haemocytes was assessed. For these analyses I constructed linear mixed-effects models with two random effects: the cage in which flies were housed, and the day on which experiments were carried out. The fixed effects included the prime combination the flies received, the volume of haemolymph extracted per three-fly batch, and the time of day for the assay. Models with Gaussian errors were used to ascertain how these factors influenced the total number of microbes that haemocytes phagocytosed. Several different analyses were conducted to address different questions about haemocyte function; the same model structure was used for the analyses of total haemocyte number, the mean number of particles engulfed per haemocyte, and the mean number of inclusions per active haemocyte (analysing only those cells that had successfully phagocytosed a microbe). To calculate the proportion of active haemocytes, data were analysed with each cell as a replicate. Binary errors were used with the response variable of active or inactive cells; sample was also included in the model as a random effect. This model generated the probability of cell activity and hence the proportion of active cells within the haemolymph.

After uncovering significant heterogeneity in phagocytosis rates amongst the priming treatments, I investigated which combinations of the multiple priming treatments differed significantly from which others. Instead of considering all nine treatments and performing standard post-hoc comparisons, I selectively explored the more biologically relevant subset of treatment combinations. This involved a systematic treatment-pooling process to produce minimally complex models which explained the variation in the dataset. Using likelihood ratio tests, models in which the priming treatments to be

tested were specified separately were compared to models in which these were pooled. For example, to compare the total number of inclusions in flies that had received two *B. bassiana* injections (BB) verses controls (CC), the data for these two treatments were merged (BB + CC = BBCC). The fixed effect of prime combination in this model now had one fewer parameters (BBCC instead of BB and CC). This altered model was then compared to the original model (where prime combination contained no merged treatments) using a likelihood ratio test thus demonstrating whether haemocytes from BB primed flies phagocytosed differentially to those from CC treated flies. Alternatively, many treatments could be merged (EB+BE+CB+BC) and compared to a model where fewer treatments were combined (EB+BE and CB+BC) to test whether two groups of treatments were significantly different from one another. Considering the most conservative method of post-hoc analysis, a Bonferroni-adjusted significance level of 0.0056 was also calculated and any p-values which may represent a type-I error have been highlighted in the results.

In the second experiment five-fly batches from four different age classes (1, 2, 3 and 4 weeks of age) were injected with a single immune stimulus. Data from this experiment were analysed as before, except that to study senescence the fixed effect of age and the two-way interaction between age and prime, and between age and the volume of extracted haemolymph were added to the models. Comparisons were conducted between treatments in the same way as described above.

All models were sequentially simplified by selecting terms for which the parameter estimate was closest to zero and eliminating them if their presence did not improve the explanatory power of the model by 2 AIC units. Significance of terms was determined using likelihood ratio tests. Means are given ± their standard errors throughout.

4.4. Results

4.4.1. Prior pathogen exposure increases phagocytosis to a homologous challenge

To determine whether phagocytic priming occurred in *Drosophila,* female Samarkand flies were pre-injected with one of three primes: sterile phosphate buffered saline (C), heat-killed *Escherichia coli* (E), or heat-killed *Beauveria bassiana* (B) (Prime 1). Three days later flies were again injected with one of these primes (Prime 2). The phagocytic ability of haemocytes was assessed after a further three days using a competitive assay injecting a 1:1 mix of fluorescein-labelled *E. coli* and rhodamine-labelled *B. bassiana*. Phagocytic ability was calculated as the total number of bacterial or fungal inclusions that the haemocytes from each sample contained (each sample consisted of the haemolymph extracted from three flies).

Considering only the double-dose treatments (BB, CC and EE) (Fig 1), the mean number of inclusions per sample varied strongly depending on which pre-injection flies had received (phagocytosis of *B. bassiana*: $\chi^2_{(2)} = 59.22$, $P < 0.0001$; phagocytosis of *E. coli*: $\chi^2_{(2)} = 53.71$, $P < 0.0001$). Previous exposure strongly enhanced phagocytosis against homologous microbes (Fig 1). In these analyses I first report data on the ability of flies to phagocytose *B. bassiana*. Haemocytes from flies which previously received *B. bassiana* primes phagocytosed the most *B. bassiana* spores, 44% more inclusions than haemocytes from flies that received control injections (*B. bassiana*: BB vs CC, $\chi^2_{(1)} = 49.67$, $P < 0.0001$). However, this could be considered to represent general immune upregulation as heterologous priming did not reduce phagocytic efficacy (*B. bassiana* phagocytosis: CC vs EE, $\chi^2_{(2)} = 4.71$, $P = 0.03$) (Bonferroni-adjusted significance level = 0.0056). These trends were mirrored for the ability of flies to clear *E. coli*: haemocytes from homologously primed flies phagocytosed the most microbes,

controls phagocytosed an intermediate number of inclusions (*E. coli*: EE vs CC, $\chi^2_{(2)}$ = 32.13, $P < 0.0001$). However, in this case heterologous priming produced significantly diminished phagocytic activity towards *E. coli* (*E. coli*: EE vs CC, $\chi^2_{(2)}$ = 32.13, $P <$ 0.0001; CC vs BB, $\chi^2_{(2)}$ = 10.70, $P = 0.0047$).

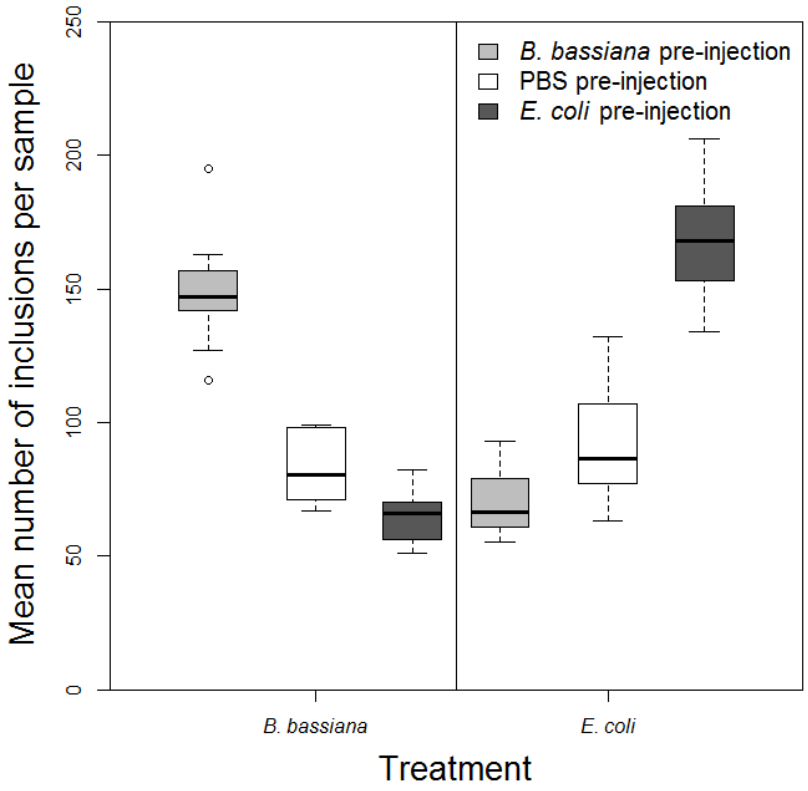

Figure 1: Variation in phagocytosis ability following immune priming. Adult female *Drosophila melanogaster* were pre-injected with a double-dose of either PBS (white), or heat-killed *Beauveria bassiana* (light grey) or *Escherichia coli* (dark grey). The treatment axis indicates the microbe being phagocytosed. Boxes illustrate inter-quartile ranges, the dark line highlights the median, bars define maximum and minimum values and empty circles represent outliers.

I also assessed the influence of the time of day the assay was conducted to investigate any evidence of circadian rhythms. The phagocytic activity of haemocytes

did not change throughout the day (*B. bassiana* phagocytosis: $\chi^2_{(1)} = 5.07$; $P = 0.024$ (Bonferroni-adjusted significance level = 0.0056); *E. coli* phagocytosis: $\chi^2_{(1)} = 0.21$; $P = 0.647$). Additionally, larger numbers of microbes were phagocytosed in assays where greater haemolymph volumes had been extracted for *E. coli* phagocytosis ($\chi^2_{(1)} = 10.30$; $P = 0.001$) but not *B. bassiana* phagocytosis ($\chi^2_{(1)} = 3.90$; $P = 0.048$)

4.4.1a. Alternative measures of phagocytic ability

Although the total number of microbes engulfed per sample increased following prior exposure to a homologous challenge, providing clear evidence of immunological priming, the processes behind priming remains unclear. To examine the details I interrogated the data further by addressing four questions: Does the proportion of actively phagocytosing haemocytes vary between priming combinations? Does the mean activity per haemocyte vary, or do the numbers of engulfed particles only differ within those haemocytes that are active? Lastly, does the total population size of haemocytes differ between treatments?

To answer the first question: following a homologous prime the proportion of phagocytosing haemocytes increased relative to controls (*B. bassiana* phagocytosis: BB vs CC $\chi^2_{(1)} = 9.81$; $P = 0.002$; *E. coli* phagocytosis: EE vs CC $\chi^2_{(1)} = 20.13$; $P < 0.0001$). There was a 30% increase in the proportion of haemocytes phagocytosing *B. bassiana* following a double-dose of the fungus compared to a double-dose of PBS (0.27 ± 0.020 compared to 0.19 ± 0.015), similarly a 47% increase in the proportion of haemocytes phagocytosing *E. coli* following a homologous priming regime (EE) compared to controls (CC) (0.34 ± 0.024 compared to 0.18 ± 0.018). A heterologous priming procedure resulted in a potentially non-significant 21% drop in the proportion of haemocytes phagocytosing *B. bassiana* if they had been exposed to a double-dose of *E. coli* relative to PBS (0.15 ± 0.011 compared to 0.19 ± 0.015) (CC vs EE $\chi^2_{(1)} =$

3.83; $P = 0.05$) (Bonferroni-adjusted significance level = 0.0056). Likewise, a 28% reduction in the proportion of haemocytes phagocytosing *E. coli* following exposure to a double-dose of *B. bassiana* compared to PBS (0.13 ± 0.015 compared to 0.18 ± 0.018) (CC vs BB $\chi^2_{(1)} = 4.51$; $P = 0.034$). Models were also fitted with the time of day the assay was conducted and the volume of haemolymph extracted per sample (three-fly batch), but these were not significant ($P > 0.05$).

The mean number of microbial inclusions per haemocyte also varied with prime (*B. bassiana*: $\chi^2_{(2)} = 27.00$, $P < 0.0001$; *E. coli*: $\chi^2_{(2)} = 33.05$, $P < 0.0001$). As before, haemocytes in homologous treatment categories phagocytosed the most inclusions per cell (*B. bassiana*: 0.42 ± 0.05, *E. coli*: 0.56 ± 0.05), followed by controls (*B. bassiana*: 0.28 ± 0.05, *E. coli*: 0.32 ± 0.05), though heterologous treatments resulted in a non-significant reduction in phagocytic activity (*B. bassiana*: 0.21 ± 0.05, *E. coli*: 0.22 ± 0.05) (*B. bassiana*: BB vs CC $\chi^2_{(1)} = 13.72$; $P < 0.001$, CC vs EE $\chi^2_{(1)} = 3.77$; $P = 0.052$; *E. coli* phagocytosis: EE vs CC $\chi^2_{(1)} = 22.98$; $P < 0.0001$, CC vs BB $\chi^2_{(1)} = 4.41$; $P = 0.036$) (Bonferroni-adjusted significance level = 0.0056). Again, neither the time of day the assay was conducted nor the volume of extracted haemolymph significantly influenced phagocytosis ($P > 0.05$).

When I investigated only those haemocytes that had successfully phagocytosed a microbe, there was no influence of prime on the efficacy of phagocytosis for either microbe (*B. bassiana*: $\chi^2_{(2)} = 4.71$; $P = 0.10$; *E. coli*: $\chi^2_{(2)} = 1.40$; $P = 0.496$). Active haemocytes engulfed roughly the same number of spores regardless of prime (mean inclusions were, BB: 1.55 ± 0.035, CC: 1.52 ± 0.035, EE: 1.46 ± 0.035), and their affinity for bacteria was similarly comparable (mean inclusions were, EE: 1.68 ± 0.044, CC: 1.73 ± 0.044, BB: 1.69 ± 0.044). Models included the time of day and the volume of extracted haemolymph but these were only marginally significant.

To answer the last question of whether the total size of the population of haemocytes varied between treatments I compared the total number of haemocytes per sample in each group of primed flies, irrespective of whether cells had engulfed a microbe or not. The total number of cells per sample did not vary between primes ($\chi^2_{(2)}$ = 2.09; P = 0.35) (BB: 373.9 ± 58.4, CC: 316.5 ± 58.4, EE: 316.2 ± 58.4). The time of day the assay was conducted had no effect on cell number ($\chi^2_{(1)}$ = 0.21; P = 0.65). These tests did not find a relationship between the volume of haemolymph extracted per sample and the number of haemocytes recorded ($\chi^2_{(1)}$ = 0.92; P = 0.34).

4.4.2. Repeat immune stimulation can enhance the level of phagocytic enhancement

So far I have only considered flies that received repeat immune challenges (BB, CC and EE); however the full experimental design consisted of 9 treatment combinations to investigate the impact of multiple different immune encounters (Fig 2). Double priming with a homologous microbe produced the greatest phagocytic enhancement (as described in **4.3.3.**). For *B. bassiana* phagocytosis, a single injection with a homologous prime led to an intermediate improvement in phagocytosis towards that microbe compared to two homologous injections. This was still 28% more inclusions than haemocytes from flies which received the control treatment (BC+CB vs CC: $\chi^2_{(1)}$ = 19.05; P < 0.0001), but 22% fewer inclusions than haemocytes from flies that had received a double prime of the homologous (BC+CB vs BB: $\chi^2_{(1)}$ = 18.31; P < 0.0001). This was also the case for *E. coli* phagocytosis following a single *E. coli* prime, haemocytes phagocytosed 33% more inclusions than controls (EC+CE vs CC: $\chi^2_{(1)}$ = 14.78; P < 0.001), but 21% less than haemocytes from flies that had received two homologous injections (EC+CE vs EE: $\chi^2_{(1)}$ = 9.66; P < 0.001).

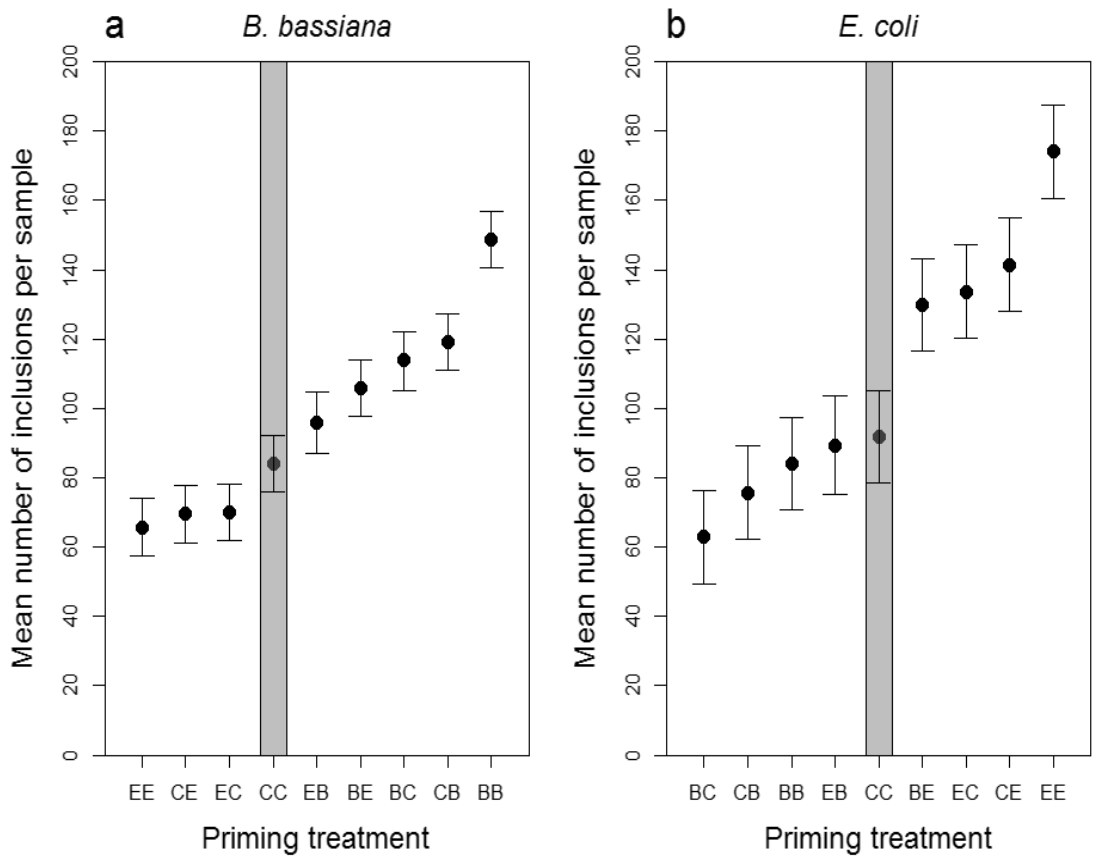

Figure 2: Variation in phagocytosis efficacy between priming treatments. Adult female *Drosophila melanogaster* were treated with a control injection: PBS (C) or a priming treatment: heat-killed *Beauveria bassiana* (B), or *Escherichia coli* (E), once at the beginning of the experiment and again three days later. Flies phagocytic ability was measured three days after the second inoculation via a competitive assay involving an injection of a 1:1 mix of fluorescently labelled *B. bassiana* and *E. coli*. Bars represent standard errors. (**2a**) *B. bassiana* phagocytosis. (**2b**) *E. coli* phagocytosis.

4.4.3. Heterologous priming reduces phagocytosis

Priming enhances a fly's phagocytic performance in a secondary encounter with the same microbe compared to naïve individuals. But this is potentially at the cost of a lower immune response towards a heterologous immune challenge. The next set of

analyses attempted to explore how multiple different immune encounters shapes the cellular immune response towards novel microbial challenges.

Though priming with a homologous treatment improved haemocytes' affinity for that microbial target it impeded haemocyte ability to phagocytose other novel microbes. Any exposure to a heterologous microbe without mitigation by exposure to the homologous treatment reduced phagocytic efficacy (*B. bassiana*: EC+CE+EE vs EB+BE: $\chi^2_{(1)} = 29.16$; $P < 0.0001$, *E. coli*: BC+CB+BB vs EB+BE: $\chi^2_{(1)} = 15.24$; $P < 0.0001$). The number of phagocytosed fungal spores per sample dropped by 33% in *E. coli* primed flies compared to flies also treated with *B. bassiana*. Likewise, phagocytosis of bacteria decreased by 32% in fungal treated flies relative to *E. coli* primed flies.

4.4.4. Senescence in the ability of haemocytes to develop a primed response

Having demonstrated that phagocytic priming does occur in *Drosophila* and that multiple microbial exposures influence immune system performance, I next investigated whether the age of the fly impacted the level to which phagocytosis was enhanced by microbial exposure. For this experiment a simpler treatment regime was followed: Female Samarkand flies from four age classes (1, 2, 3 and 4 weeks old) were injected with a single control dose (PBS) or a single injection of a heat-killed microbe (*B. bassiana* or *E. coli*). The competitive assay (1:1 mix of fluorescently labelled *B. bassiana* and *E. coli*) investigating flies' phagocytic ability and specificity was carried out 24hrs later. Each sample contained the pooled haemolymph of five flies.

As before there was a significant difference in the mean number of inclusions per sample depending on which prime flies received (*B. bassiana* phagocytosis: $\chi^2_{(2)} =$

46.4; $P < 0.0001$; *E. coli* phagocytosis: $\chi^2_{(2)} = 39.8$; $P < 0.0001$). Though the homologous prime improved phagocytosis compared to other treatments, phagocytosis declined with age regardless of priming (*B. bassiana* phagocytosis: $\chi^2_{(1)} = 14.9$; $P < 0.001$; *E. coli* phagocytosis: $\chi^2_{(1)}$ 16.1; $P < 0.0001$) (Fig 3). First considering *B. bassiana* phagocytosis; haemocytes from one week old flies primed with *B. bassiana* phagocytosed 32% more spores than controls, but in four week old flies there was no significant difference between treatments in the number of inclusions per sample. Equally, though senescence in phagocytosis occurred in the unprimed controls ($\chi^2_{(1)} = 10.1$; $P = 0.002$), there was a steeper decline in phagocytosis ability with age in flies primed with the homologous microbe (shown by an age by prime interaction: *B. bassiana* phagocytosis: age x prime interaction: $\chi^2_{(2)} = 14.1$; $P = 0.0021$; *E. coli* phagocytosis: age x prime interaction: $\chi^2_{(2)} = 8.02$; $P = 0.018$). This shows that, as flies age, priming is becoming less efficient at stimulating a response and generating a 'primed' phenotype.

Though in the previous experiment there was a negative effect of being primed with a heterologous treatment this was not the case here. Flies treated with *E. coli* phagocytosed the same number of *B. bassiana* spores as did the controls (C vs E: $\chi^2_{(1)} = 0.04$; $P = 0.84$).

Comparable to the results for *B. bassiana* phagocytosis, considering haemocyte ability to phagocytose *E. coli*, flies primed with *E. coli* phagocytosed the greatest number of bacteria at all ages ($\chi^2_{(2)} = 39.8$; $P < 0.0001$). Haemocytes from one week old flies phagocytosed 32% more bacteria than controls, and though haemocytes in four week old flies phagocytosed considerably fewer bacteria they still engulfed significantly more than controls ($\chi^2_{(2)} = 10.3$; $P = 0.006$). Though there was a significant difference in the phagocytic ability of haemocytes between the *E. coli* primed flies and the controls, this difference became less pronounced as flies aged. This again suggests that the priming

effect diminishes with fly age. Finally, just as *E. coli* primed flies phagocytosed a similar number of spores as controls, so *B. bassiana* treated flies engulfed an equivalent number of bacteria to controls (C vs B: $\chi^2_{(1)} = 0.003$; $P = 0.96$).

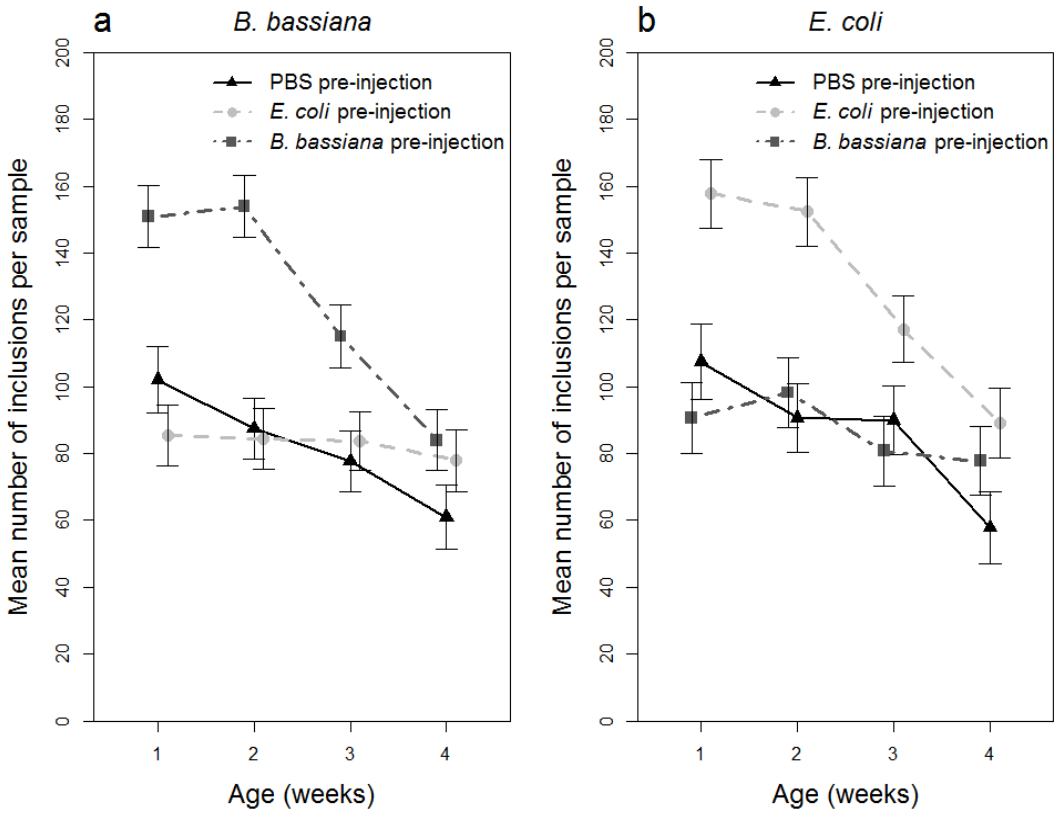

Figure 3: Decline in the effect of phagocytic priming with age. Adult female *Drosophila melanogaster* from four age classes (1, 2, 3 or 4 weeks old) were treated with either a control injection (PBS) or a priming treatment of heat-killed *Beauveria bassiana* or *Escherichia coli*. (**a**), *B. bassiana* phagocytosis, (**b**), *E. coli* phagocytosis. Bars show mean standard errors.

The time of day the assay was conducted strongly impacted the level of phagocytosis measured with more microbes being phagocytosed later in the day (*B. bassiana* phagocytosis: $\chi^2_{(1)} = 21.3$; $P < 0.0001$, *E. coli* phagocytosis: $\chi^2_{(1)} = 8.66$; $P = 0.003$). A greater level of phagocytosis was recorded when a larger volume of haemolymph was extracted per sample (*B. bassiana* phagocytosis: $\chi^2_{(1)} = 6.13$; $P = 0.013$, *E. coli* phagocytosis: $\chi^2_{(1)} = 8.88$; $P = 0.003$).

4.4.4a. Alternative measures of phagocytic ability

Unlike other experiments, the total number of haemocytes per sample substantially increased following a priming treatment compared to controls ($\chi^2_{(2)} = 17.2$; $P < 0.001$) (Fig 4). There was a 19% rise in the number of cells in one week old flies treated with a microbe compared to controls (B vs C: $\chi^2_{(1)} = 14.5$; $P = 0.0001$, E vs C: $\chi^2_{(1)} = 11.6$; $P < 0.001$), but there was no difference in the level of enhancement between microbe types (B vs E: $\chi^2_{(1)} = 0.24$; $P = 0.62$). Although the total number of haemocytes declined as flies aged ($\chi^2_{(1)} = 7.61$; $P = 0.006$), the magnitude of the cell number elevation associated with microbe exposure remained constant (age x prime: $\chi^2_{(2)} = 0.17$; $P = 0.92$). The time of day the assay was conducted had no effect on cell number ($\chi^2_{(1)} = 2.64$; $P = 0.10$). The volume of haemolymph extracted per sample did not impact the number of haemocytes recorded either ($\chi^2_{(1)} = 0.35$; $P = 0.55$).

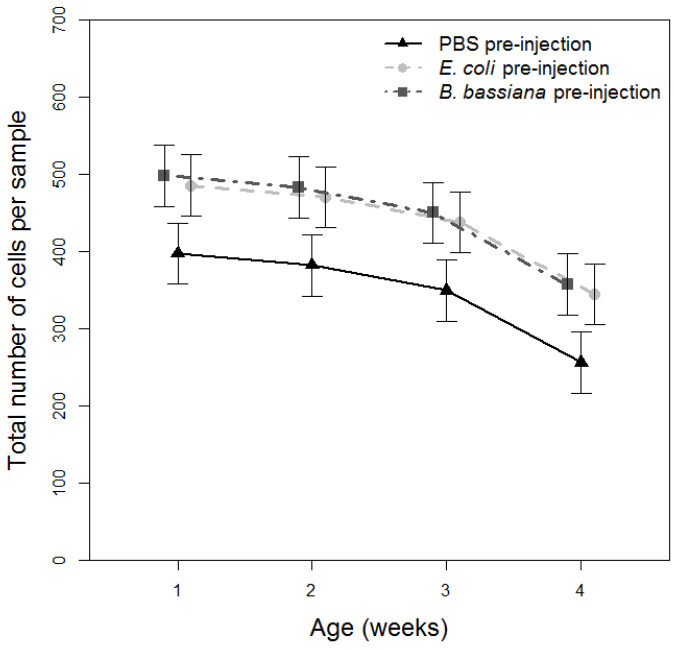

Figure 4: Variation in the total circulating haemocyte population with age and prime. Adult female *Drosophila melanogaster* from four age classes (1, 2, 3 or 4 weeks old) were treated with either a control injection (PBS) or a priming treatment of heat-killed *Beauveria bassiana* or *Escherichia coli*. Bars show mean standard errors.

The previous analyses showed that in young flies there was an improvement in total phagocytic capacity of the haemocyte population during a secondary immune challenge if flies had previously received that particular microbial stimulus. Next, I tested how the age of the fly impacted the effect of priming on the proportion of circulating haemocytes which phagocytosed a microbe. Again the greatest proportion of haemocytes which engulfed a microbe were from flies that received a homologous prime compared to controls (*B. bassiana* phagocytosis: B vs C $\chi^2_{(1)} = 12.03$; $P = 0.002$; *E. coli* phagocytosis: E vs C $\chi^2_{(1)} = 12.46$; $P < 0.001$). In one week old flies there was a 23% increase in the proportion of haemocytes phagocytosing *B. bassiana* following a fungal prime compared to a control treatment of PBS (0.22 ± 0.01 compared to 0.17 ± 0.02), similarly a 29% rise in the proportion of haemocytes phagocytosing *E. coli* following a homologous treatment (E) compared to controls (0.21 ± 0.01 compared to 0.15 ± 0.01). However, by four weeks of age, this enhancement in the proportion of haemocytes phagocytosing *B. bassiana* decreased by 23% (0.17 ± 0.02) ($\chi^2_{(1)} = 5.36$; $P = 0.02$), and dropped by 19% for *E. coli* phagocytosis (0.17 ± 0.01) ($\chi^2_{(1)} = 4.70$; $P = 0.03$), compared to their one week old counterparts.

In this analysis a lower proportion of haemocytes phagocytosed both microbes if flies had received the heterologous prime compared to the control injection (*B. bassiana* phagocytosis: C vs E $\chi^2_{(1)} = 9.97$; $P = 0.007$; *E. coli* phagocytosis: C vs B $\chi^2_{(1)} = 9.92$; $P = 0.002$). Haemocytes from one week old flies exposed to *E. coli* phagocytosed 29% fewer spores than controls (0.12 ± 0.01 compared to 0.17 ± 0.02), though there was no evidence of a reduction in the phagocytosis of spores at four weeks old (0.16 ± 0.01 compared to 0.16 ± 0.02). Likewise, haemocyte activity towards bacteria decreased by 20% in one week old flies primed with *B. bassiana* compared to those that received the PBS injection (0.12 ± 0.01 compared to 0.15 ± 0.02). Again the proportion of phagocytosing haemocytes equalised between treatments at four weeks old (0.14 ±

0.02 (B) compared to 0.15 ± 0.01 (C)). Models were also fitted with the time of day the assay was conducted which was marginally significant but the volume of extracted haemolymph per five-fly batch was not a significant factor.

The mean number of microbial inclusions per haemocyte also varied with prime (*B. bassiana* phagocytosis: $\chi^2_{(2)} = 25.8$; $P < 0.0001$; *E. coli* phagocytosis: $\chi^2_{(2)} = 25.5$; $P < 0.0001$). However, by this metric the enhancement of phagocytosis through priming was unaffected by the age of the fly (*B. bassiana* phagocytosis: $\chi^2_{(1)} = 0.69$; $P = 0.40$; *E. coli* phagocytosis: $\chi^2_{(1)} = 1.88$; $P = 0.17$). Cells in flies that received the homologous treatment phagocytosed the greatest number of microbes (one week olds: *B. bassiana*: 0.32 ± 0.03, *E. coli*: 0.36 ± 0.03), followed by controls (one week olds: *B. bassiana*: 0.24 ± 0.03, *E. coli*: 0.26 ± 0.03); again heterologous treatments phagocytosed the least inclusions per cell (one week olds: *B. bassiana*: 0.18 ± 0.03, *E. coli*: 0.20 ± 0.03) (*B. bassiana*: B vs C $\chi^2_{(1)} = 9.36$; $P = 0.009$, C vs E $\chi^2_{(1)} = 8.01$; $P = 0.02$; *E. coli* phagocytosis: E vs C $\chi^2_{(1)} = 9.12$; $P = 0.01$, C vs B $\chi^2_{(1)} = 7.97$; $P = 0.02$). Models were fitted with the time of day and the volume of extracted haemolymph but these were only marginally significant.

Analysis of the number of inclusions only in the successfully phagocytosing haemocytes showed that haemocyte affinity for *B. bassiana* was not influenced by prime ($\chi^2_{(2)} = 0.16$; $P = 0.92$), or by the age of the fly ($\chi^2_{(1)} = 0.66$; $P = 0.42$). Haemocytes engulfed a comparable number of spores regardless of treatment (one week old flies: B: 1.453 ± 0.08, C: 1.446 ± 0.08, E: 1.446 ± 0.08). Similarly, prior microbial exposure had no effect on haemocyte affinity for *E. coli* compared to controls (one week old flies: E: 1.554 ± 0.09, C: 1.608 ± 0.09, B: 1.651 ± 0.09) (E vs C: $\chi^2_{(1)} = 2.09$; $P = 0.15$, C vs B: $\chi^2_{(1)} = 1.33$; $P = 0.25$), and did not vary with the age of the fly ($\chi^2_{(1)} = 0.01$; $P = 0.92$). Once again models were fitted with the time of day and the volume of extracted haemolymph but these were only marginally significant.

4.5. Discussion

4.5.1. Key findings

This study has demonstrated that the cellular immune response in *Drosophila melanogaster* provides a strong, specific and relatively long-lasting immunological priming response. Flies' phagocytic ability was significantly improved during secondary immune challenge if they had previously received a homologous prime. This was sufficiently specific to differentiate between *Beauveria bassiana* fungal spores and the bacterium *Escherichia coli*. Additionally, the priming enhancement was fairly long-lasting as some treatments received a single homologous priming injection six days before the immune assay. Finally, I showed that the priming response declined as flies aged; aged flies were less able to display an enhanced immune response to a secondary homologous immune challenge.

Immunological priming occurred following pre-exposure to both microbes in the experiment: *B. bassiana* and *E. coli*. Haemocytes phagocytosed 50% more inclusions per cell compared to controls if flies had received two homologous injections. A single immune stimulus of a homologous prime produced an intermediate effect; a third more particles were phagocytosed if flies had encountered that microbe before.

4.5.2. Priming response may be driven by a cohort of specialised cells

When investigating the potential processes behind immunological priming I interrogated the data to answer three specific questions. The first question asked if the proportion of actively phagocytosing haemocytes varied between priming combinations. The second question looked at whether there was any variation in the mean activity of haemocytes. The third question asked whether the number of engulfed particles within only successfully phagocytosing haemocytes varied between

primes. The proportion of circulating haemocytes containing phagocytosed microbes was greatest when haemocytes were extracted from flies that had received the homologous priming injection. The mean number of inclusions per haemocyte was also enhanced towards the microbe from a homologous prime with up to a third more of that microbe being engulfed compared to controls. However, when I investigated only the haemocytes that had successfully phagocytosed a microbe there was no evidence of heightened activity towards microbes that flies had previously encountered for these active haemocytes.

These findings suggest that the priming effect is not driven by an improvement in the ability of individual cells to phagocytose greater numbers of a microbe during a secondary homologous encounter. However, the proportion of haemocytes that phagocytosed a particle did increase following a homologous prime. This hints at possible options for how the priming effect occurs. Perhaps a greater number of haemocytes become phagocytically active so that cells that had not phagocytosed before do so following secondary exposures to the same microbe. Alternatively, a new population of specialised haemocytes might enter circulation in response to priming.

The answer to my fourth question of whether the total size of haemocyte population varied between primes could suggest which hypothesis is more likely. In the first experiment there was no effect of treatment on the total number of haemocytes per sample. However, in the second experiment haemolymph samples from flies which had been injected with either *B. bassiana* or *E. coli* had 19% more circulating haemocytes than controls. The variation between these experiments was possibly due to the differences in experimental technique. In the first experiment flies were assayed three days after their second prime, whereas in the second experiment the phagocytic ability of flies' haemocytes was tested 24hrs after the immune stimulus. The reason for

this in the second experiment was due to age-related mortality that would occur in the oldest age class of flies had the assay been conducted later.

The results here (**4.4.4a**) are at odds with the findings of an experiment in Chapter 3 specifically designed to determine whether infection altered the size of the circulating haemocyte population (**3.4.1.**). Potential reasons for this are discussed in the final chapter in this thesis (Chapter 6).

The benefit in this current study is that it has highlighted a possible explanation for the role of haemocytes in immune priming. Potentially, following an immune challenge a cohort of haemocytes could enter circulation that are specifically enhanced towards phagocytosing the microbe that has invaded the haemocoel. As cell proliferation has not been observed in *Drosophila* and adult flies do not possess a hematopoietic organ (Lanot *et al*., 2001), novel cell division is not likely to be the origin of these specialised haemocytes. The assays used in this chapter only observed circulating haemocytes, although *Drosophila* also possess a sessile population of tissue-bound haemocytes (Williams, 2007). Potentially, specialised tissue-bound cells are released into circulation in response to a microbial encounter. If after a period of time non-specialised circulating cells dropped out of circulation, perhaps adhering to tissues, there would be a shift in the proportion of specialist compared to naïve circulating haemocytes which could explain the length of the priming response and support this study's findings of a temporary increase in the size of the non-adherent haemocyte population. Twenty-four hours post priming there was an increase in the total circulating haemocyte population in flies that had been injected with *B. bassiana* or *E. coli* but by 72hrs post priming haemocyte numbers were unaffected by microbial exposure (there was no difference in haemocyte numbers between treatments).

4.5.3. Potential mechanisms behind enhanced plasmatocyte specificity

To alter the specificity of phagocytosing haemocytes there is a range of elements that could be modified. Phagocytic haemocytes (plasmatocytes) detect, engulf and degrade pathogens, apoptotic and dead cells as well as secrete AMPs and produce signalling molecules to activate other parts of the immune response (Agaisse *et al*., 2003). Targets for phagocytosis are mainly recognised by receptors within the plasmatocytes, including the scavenger receptors SR-CI, Peste and Croquemort, as well as the EGF-like repeat containing receptors Eater, Nimrod C1 and Draper (reviewed by Ulvila *et al*., 2011). One possible mechanism behind immune priming could be that variation exists in the expression of recognition receptors in tissue-bound haemocytes. If sessile cells become phagocytically active they might enter the circulating population which would lead to an alteration in the profile of the circulating haemocyte population to predominately include cells that had phagocytosed a particle. This would appear as an increase in specificity towards a microbe as only those cells that expressed particular receptors engulf a microbe and remained in circulation. This hypothesis relies on heterogeneity in the haemocyte population for phagocytic specificity; there is no current evidence that such heterogeneity exists (Lemaitre & Hoffmann, 2007).

Additional factors influencing plasmatocyte activity are opsonins (molecules which enhance phagocytosis) such as the complement-like opsonin family of TEPs (thioester-containing proteins). Finally there are Down syndrome cell adhesion molecule (Dscam) isoforms (reviewed by Ulvila *et al*., 2011). Dscam isoforms operate as phagocytic receptors and partially as opsonins (Watson *et al*., 2005). Recent work demonstrated that the repertoire of Dscam splice-variants varied for up to eight different immune stimuli indicating a potential source of specificity and immune memory in the innate immune response of invertebrates (Dong *et al*., 2012).

Plasmatocytes are known to stimulate other elements of the immune response through the production of signalling molecules (Agaisse *et al*., 2003). Perhaps they also cause increased specific activity in other plasmatocytes through the production of a particular range of Dscam isoforms which increase the phagocytosis of microbes via opsonisation and enhanced recognition during a secondary homologous encounter.

Although variation in haemocyte activity following priming demonstrates specificity it is important to note that these experiments have only investigated specificity at the level of distinguishing fungal spores and bacteria. Fungi and gram-negative bacteria stimulate the *Drosophila* immune system in different ways (thoroughly reviewed by Lemaitre & Hoffmann, 2007). The diaminopimelic acid-containing peptidoglycan from the inner membrane of gram-negative bacteria causes the activation of the immune deficiency (IMD) pathway to induce the production of an array of antimicrobial peptides (AMPs) to combat the infection (Choe *et al*., 2002; Gottar *et al*., 2002; Ramet *et al*., 2002; Wu *et al*., 2001). Fungal infection stimulates the Toll pathway by the detection of glucan (Gottar *et al*., 2006) and fungal proteases (Ligoxygakis *et al*., 2002). Activation of the Toll pathway induces the production of another specific cohort of AMPs (Hoffmann, 2003). However, whilst this dichotomy of the immune response to fungi and gram-negative bacteria is well characterised for humoral immunity, there is currently no evidence that these divergent signalling pathways play similar roles in stimulating phagocytosis by the cellular immune response (Lemaitre & Hoffmann, 2007). Therefore the level of specificity I have uncovered probably does not result from upregulation of either the Toll or IMD pathway.

The humoral immune response does not act in isolation. As previously described there are a wide range of recognition molecules involved in pathogen detection by plasmatocytes which respond either to microbial signals or signals from other parts of the *Drosophila* immune system to give a coordinated humoral and cellular immune

response (Nehme *et al*., 2011; review by Reumer *et al*., 2010). Plasmatocytes express the Toll ligand Späztle, the cleaved form of which activates the Toll immune pathway (Irving *et al*., 2005; Shia *et al*., 2009). However, though experiments have shown that plasmatocytes in *Drosophila* larvae can stimulate the production of AMPs following an oral infection of the gram-negative bacteria *Erwinia carotovora* (Charroux & Royet, 2009), in adult flies AMP activation following injections with a range of bacteria did not depend on plasmatocyte activity (Defaye *et al*., 2009). Alone, this evidence suggests that in adult flies the humoral and cellular immune responses occur separately in their specific activity against bacteria but in a co-ordinated manner against fungi. However, this area of *Drosophila* immunity clearly requires further investigation.

Other studies have demonstrated a greater degree of specificity in insect immune priming. Sadd and Schmid-Hempel (2006) showed that *Bombus terrestris* workers' immune systems could differentiate between two species of bacteria: *Paenibacillus alvei* and *P. larvae*. Bees which received a homologous infection had increased survival and bacterial clearance from their haemoceol compared to bees with heterologous infections even with closely related bacterial challenges (Sadd and Schmid-Hempel, 2006). Roth *et al*., (2009) found that the immune system of the red flour beetle (*Tribolium castaneum*) could be specifically primed by different strains of a bacterium (*Bacillus thuringiensis*), with increased survival following homologous but not heterologous treatment combinations. Whether this level of specificity exists for *Drosophila* immune priming has not yet been investigated.

4.5.4. *The cost of immunological priming*

In this study, haemocyte activity was not universally increased following priming. Heterologous priming treatments dramatically hindered the phagocytosis of novel microbes compared to the phagocytic ability of flies injected with PBS. Haemocytes

from flies exposed to a heterologous prime phagocytosed significantly fewer microbes during the phagocytosis assay compared to control treated flies (*B. bassiana* phagocytosis: -22% following an *E. coli* prime; *E. coli* phagocytosis: -8% following a fungal prime). If flies were primed with both microbes (EB and BE treatments) haemocytes were less able to phagocytose spores or bacteria compared to controls or flies that had only been exposed to a single microbial type. These results lead to the suggestion that multiple microbial exposures determine how priming develops. Once a fly mounts a response towards an infection this shapes their cellular immune response to become more aggressive towards a secondary homologous exposure, but at the cost of reduced activity towards a novel microbial encounter.

One obvious benefit to the development of a primed cellular immune response is to defend against future encounters with the same pathogen. However, an alternative benefit is suggested by the work of Haine *et al*., (2008) who reported that following the initial immune response to clear an infection small numbers of resistant bacteria tended to persist within individuals. Thus priming may be partly tuned to cope with long-term or repeat infections.

4.5.5. *The role of plasmatocytes and the effect of fly age on the priming response*

Plasmatocytes have recently received greater scrutiny in their role in the invertebrate innate immune response and are now considered to play a key role in immune priming (Pham *et al*., 2007; Powell *et al*., 2011; Rodrigues *et al*., 2010; Roth & Kurtz, 2009). Rodrigues *et al*., (2010) crucially demonstrated that the transference of resistance to *Plasmodium* infection could be achieved solely through transplanting haemocytes from primed mosquitoes into naïve individuals. They additionally showed that the transference of cell-free haemolymph also had a protective effect. The authors

suggested that perhaps some form of immune signal was released into the circulating haemolymph of immune challenged mosquitoes causing the observed differentiation of haemocytes and subsequent resistance to malarial infection. My work strengthens this growing body of research by supporting their findings that plasmatocytes are key to the observed improvement in the invertebrate immune response against secondary homologous infections.

A recent study on *D. melanogaster* found that the survival benefit of pre-exposure to a sub-lethal strain of *Pseudomonas aeruginosa* lasted roughly 5 days and could be 'boosted' to last 7 days if flies received two separate primes instead of one (Christofi & Apidianakis, 2013). This suggested that this prime had a short-term, transitory benefit. Comparatively, Pham, *et al*., (2007) report that the protective effect of *Streptococcus pneumonia* priming lasted for the lifetime of the fly (demonstrated for at least 2 weeks post-priming) and *B. bassiana* priming improved survival for at least a week. This chapter partly corroborates that of Pham, *et al*., (2007) as the increase in haemocyte activity towards *B. bassiana* following a fungal pre-injection lasted for up to six days (BC treatment). This was also the case for *E. coli* (EC treatment). However I did not examine the full potential timescale for the priming effect. Nonetheless, it can be suggested that the protective effect of pre-exposure to sub-lethal doses of *D. melanogaster* pathogens may be in part driven by the increase in phagocytosis of that pathogen by their haemocytes.

For the first time in *D. melanogaster* I have shown that as flies age there is a reduced effect of immune priming. In the same way that elderly humans have reduced immune-responsiveness to vaccination (Chen *et al*., 2009; Lang *et al*., 2011; Lang *et al*., 2012), haemocytes in older *Drosophila* are less able to form immunological memories to novel pathogens. As with previous experiments (Chapter 2; Mackenzie, *et al.,* (2011)), senescence (age-dependent decline) was demonstrated in haemocyte number and

phagocytic ability. From one week old to four weeks old, control flies had a 40% decline in the phagocytosis of *B. bassiana* and a 47% drop in *E. coli* phagocytosis. In addition, their haemocyte population diminished by 36% which culminated in a severely compromised cellular immune response by four weeks of age. Young flies primed with fungal spores or bacteria demonstrated a dramatic improvement in haemocyte phagocytic ability towards the microbe they had been exposed to but by four weeks of age there was a much reduced (or non-existent) enhancement of phagocytosis. The rate of age-related decline in haemocyte function in flies which received a homologous microbial prime was double that of the controls, so there must be more driving this effect than just cellular loss-of-function.

It could be that haemocytes in older flies are functionally impaired in the same way that vertebrate macrophages show a decline in phagocytic function (Sebastián *et al*., 2009). Unpublished work by Lucas Horn (referred to in a review of current research on the *Drosophila* immune system by Eleftherianos and Schneider (2011)) reported that plasmatocytes in five-week old flies were unable to digest and degrade microbes and through becoming 'choked' with inclusions they became immunologically irrelevant. In my experiment flies were aged in non-sterile, mixed-sex cages which would have likely shaped their immune history in addition to the prime treatments I used in the experiment. If haemocytes in four-week old flies were already becoming 'choked' with past immune insults then perhaps this meant that when flies were challenged with *B. bassiana* or *E. coli* they were unable to phagocytose these efficiently and so were unable to produce a 'primed phenotype'. Alternatively, the rapid decline in the ability of haemocytes to be primed may be because they have already become specialised to focus on other microbes. Perhaps, through experiencing the general bacterial flora present in the environment, the oldest flies' haemocyte population had already developed specificities and were therefore less able to be primed towards a novel pathogen.

4.5.6. A potential link between immunological priming and the senescent decline in plasmatocyte phagocytic function

Initially I suggested that perhaps the senescence previously observed may be partially influenced by the cumulative exposure of flies to multiple microbes priming their immune systems until no ‘naïve’ cells remain to combat novel infections. This would be perceived as a ‘loss-of-function’ with age and therefore interpreted as senescence. From this current work, it does appear that this may be a contributory factor to late-life phagocytosis impairment and that our conventional interpretation of senescence of the cellular immune response may have to be adapted to include individual immune history shaping later cellular immune responses.

Chapter 5: Testing for a genetic link between ageing and investment in immunity

5.1. Abstract

Effective immune responses are necessary to defend against pathogens but immune defence is also considered to be costly. These costs may either be due to resource limitation, potentially imposing trade-offs with other physiological systems, or via collateral damage to host tissues through immune system activation. However, immune activity may also protect against infection-induced damage and late-life pathogen induced mortality. Therefore, the dynamic nature of immune defence traits may have important evolutionary and ecological consequences for ageing. I studied whether naturally segregating genetic variation for ageing traits was present in *Drosophila melanogaster* populations, then investigated if selection on these traits is likely to be constrained by genetic links between immune and ageing life history traits. I examined a panel of outcrossed genotypes of *D. melanogaster* derived from a natural population. Their median lifespan was measured, as well as five physiological traits across a range of ages: fecundity, susceptibility to *Beauveria bassiana* infection, ability to phagocytose *B. bassiana* spores, total phenoloxidase activity, and total protein content. Although substantial genetic variation was present in each of these traits, no significant correlations between the genetic variation in life history traits and the genetic variation in immune traits were identified. The absence of genetic correlations between the immunity and ageing traits investigated here challenges the conventional view that selection, and therefore the evolution of ageing, is constrained by trade-offs between these physiological systems.

5.2. Introduction

The field of biogerontology is focussed on two aspects of ageing: demographic senescence and functional senescence. Much research is aimed at mitigating the effects of ageing to increase human lifespan and 'healthspan' (reviewed by Mercken *et al*., 2012). A key feature of increased healthspan is the attempt to reduce the impact of ageing on the immune system. However, some elements of an active immune system are documented phenotypes of ageing: heightened innate immune responses ('inflamm-ageing', Franceschi *et al*., 2000) and the production of reactive oxygen species (ROS) ('free radical theory of ageing', Sohal & Orr, 2012), being prime examples. Therefore, understanding the links between the immune system and senescence is an important part of understanding the mechanisms of ageing.

The previous chapters of this thesis have been focussed on age-related changes in the cellular immune response of adult *Drosophila melanogaster*. The mechanistic systems that may underlie these senescent declines have been explored, with an attempt to put these into the context of disease resistance. This chapter investigates the genetic links between immune system activity and phenotypic measures of ageing. Two key questions will be addressed. Firstly, is there genetic variation in life history, immunity and ageing traits on which natural selection can act? Secondly, do genetic trade-offs exist between investment in immune parameters and investment in life history traits related to ageing?

The force of natural selection declines as a population ages (Medawar, 1952). A key factor in the evolution of ageing is that selection to optimise fitness early in life can have detrimental consequences for late-life traits (Williams, 1957). This is the central premise of two main theories on ageing; 'antagonistic pleiotropy' (AP) and the 'disposable soma theory' (DST). General life history theory proposes that the activity of

one physiological process often compromises the outcome of another. One example of this is that early-life investment in reproduction can lead to a shorter lifespan (Rose & Charlesworth, 1980). AP proposes that this trade-off results because the same alleles that are favoured by selection to elevate early-life fecundity have negative consequences for lifespan (Williams, 1957). Whereas DST takes a life history perspective and suggests that to achieve high early fecundity, energy and resources must be diverted away from the maintenance and repair functions that are necessary for long lifespan (Kirkwood, 1977). Specific cases in which this trade-off between lifespan and reproductive investment have been demonstrated include laboratory model organisms such as *Drosophila* (Rose & Charlesworth, 1980), *Daphnia* (Dudycha & Tessier, 1999) and *Caenorhabditis elegans* (Kenyon, 2010), as well as in wild animals including great tits (Bouwhuis *et al*., 2012), red squirrels (Descamps *et al*., 2006) and Asian elephants (Robinson *et al*., 2012).

Organisms require an immune system principally because of their constant conflict with parasites and pathogens. We might expect interactions between the immune system and ageing, either because immunity imposes costs that negatively impact late-life survival, or alternatively because efficient immune performance may be protective against infection-induced damage and late-life pathogen-induced mortality (DeVeale *et al*., 2004). The costs of immunity may arise for two broad reasons: either due to mechanistic constraints, such as pleiotropy and collateral damage, or because of conflicts over resource investment.

Immune activity can cause damage to the host as well as the pathogen, a type of collateral damage associated with pathogen attack. An example is the prophenoloxidase cascade in invertebrates: although this is a potent immune defence system (Leclerc *et al*., 2006) it is also a prominent source of ROS, which are strongly linked to ageing (Dowling & Simmons, 2009). Immune upregulation can also restrict

resource intake, thereby interfering with the energetic budget for other metabolic processes (Bashir-Tanoli & Tinsley, 2014). When resources are limited, organisms adopt different resource allocation strategies that can appear as variation in investment in physiological processes (Gadgil & Bossert, 1970). It has been proposed that differences in resource allocation strategies are the dominant explanation for variation in immune responses and rates of ageing. Some theorists even consider genetic influences on metabolism rates to be the principal determinants of ageing and lifespan (Blagosklonny, 2010). Genetic influences on metabolism rates, especially in regards to the target of rapamycin (TOR) signalling pathway, have been demonstrated to dramatically influence lifespan and phenotypes of ageing (review by Stipp, 2012).

There is good evidence that the immune system is also protective against some aspects of ageing. A failure of the immune system to defend against infection is a hallmark of ageing: *Streptococcus pneumoniae* infection was the cause of 80% more mortality in men aged 85-89 years old, compared to 65-69 year old men in the United Kingdom from 2009-2011 (Millett *et al*., 2013). Thus, individuals in which immune system senescence occurs more slowly may live longer. Furthermore, a proportion of the molecular and cellular damage that characterises ageing may result from pathogen infection early in life; efficient immune defences may offer protection from this. A recent study assayed the bacterial clearance ability of one and four week old flies in 20 inbred lines of *D. melanogaster* and examined gene expression profiles from 12 of these lines (Felix *et al*., 2012). Control and infected flies of one and four weeks of age were compared to identify genes that contributed to natural variation in the senescence of the immune response. There was significant variation in bacterial clearance ability; however the only significant correlation between variation in gene expression and bacterial clearance ability was in four week old flies (Felix *et al*., 2012). Interestingly, metabolism genes including those involved in the insulin signalling/ target

of rapamycin (TOR) pathway were identified which had significant associations with bacterial clearance in older individuals (Felix *et al*., 2012).

Upregulation of the immune response can have negative consequences for lifespan and reproduction. In mealworm beetles (*Tenebrio molitor*) activation of the melanisation response negatively correlates with lifespan (Krams *et al*., 2013). In cotton bollworm moths (*Helicoverpa armigera*) a larval immune challenge caused reduced reproductive investment in adult males but not adult females (McNamara *et al*., 2012). Yellow dung flies (*Scathophaga stercoraria*) selected for high phenoloxidase (PO) activity had shorter lifespans when starved compared to flies selected for decreased PO activity (Schwarzenbach & Ward, 2006).

Mechanistic studies in *Drosophila* have shed useful light on the processes that might link immune activity and ageing. Flies overexpressing the peptidoglygan recognition protein PGRP-LE have reduced lifespans (DeVeale *et al*., 2004; Libert *et al*., 2006). Additionally, microarray studies have demonstrated that immunity gene expression is up-regulated in older flies (Landis *et al*., 2004; Pletcher *et al*., 2002; Seroude *et al*., 2002). Just as heightened immune activity can decrease lifespan, lower immune activity in the absence of infection can slow ageing rates: the rate at which mortality accelerates with age slows in flies with *Dif* or *Rel* expression downregulated compared to immunologically wildtype controls (Lintott & Tinsley, unpublished).

It is clear that immune system activity can have strong negative consequences for senescence and lifespan. However, compelling evidence that the selective processes governing the evolution of immunity and ageing are intertwined requires demonstration that evolutionary change in immune traits could drive evolutionary change in ageing. Selection experiments provide one means to demonstrate this. Selection for longevity in *Drosophila* was associated with correlated declines in antimicrobial peptide gene

expression (Remolina *et al*., 2012; Sarup *et al*., 2011), suggesting an evolutionary trade-off between immune activity and lifespan.

An alternative approach is to investigate phenotypic correlations across genotypes from wild populations. Cotter *et al*., (2004) compared investment in multiple immune and life history traits in the Egyptian cotton leafworm (*Spodoptera littoralis*). The heritability of a broad range of traits was explored including: larval and pupal development rates, pupal weight, adult longevity, larval cuticular melanisation, haemolymph PO activity, antimicrobial activity and haemocyte density (Cotter *et al*., 2004). There was a negative genetic correlation between antibacterial activity and haemocyte number, and haemocyte number positively correlated with PO activity and cuticular melanisation (Cotter *et al*., 2004). The authors concluded that there was a trade-off between investing in immune traits that specifically defended against parasite infection (antimicrobial activity) and traits that lower larval conspicuousness to predators (mediated by larval colour through PO activity and cuticular melanisation) (Cotter *et al*., 2004). However, they found no correlations between the life history traits investigated and the immune traits measured (Cotter *et al*., 2004).

In this chapter I undertook a study to investigate whether there was an association between ageing and immunity traits across genotypes within *Drosophila* populations. Thirty-six inbred *D. melanogaster* lines derived from a natural population were crossed to produce 18 outcrossed genotypes. Using outcrossed wildtype flies, rather than inbred or mutant flies, means that the results are indicative of the effects of naturally segregating genetic variation on which natural selection acts in the wild. Additionally, inbred lines may suffer from inbreeding depression caused by rare alleles which would only occur homozygously exceptionally rarely in natural populations (Mackay, 2010). The purpose of this study was not to investigate the effects of rare deleterious alleles that could have large fitness consequences, but to explore the genotypes on which

selection is likely to act in natural populations. A logistical constraint against using flies caught from the wild was that in order to assess genetic variance individual flies could only be measured once and therefore numerous genetically identical flies were required to conduct this study.

Flies were aged into four age classes: 1, 2, 3 and 4 weeks old (age posteclosion). *Ad libitum* nutrient conditions were maintained during rearing and throughout the experiment to minimise variation produced by differences in resource acquisition. Each line's median lifespan was measured. Also, every line and age class had five physiological traits assessed: fecundity, susceptibility to *Beauveria bassiana* infection, ability to phagocytose *B. bassiana* spores, total phenoloxidase activity, and total protein content. The presence of genetic variation in each trait was tested for, as well as age-related changes in these traits. The extent of genetic co-variance was explored between each physiological trait and median lifespan, as well as between these traits and rates of senescence in fecundity.

5.3. Materials and methods

5.3.1. Fly culturing

Thirty-six fly lines from the *Drosophila* Genetic Reference Panel (DGRP) (Mackay *et al*., 2012) were used in this experiment. The DGRP lines are derived from flies collected from a natural population in Raleigh, North Carolina, and then fully inbred by 20 generations of sib-sib mating. Up to 20 generations prior to their use in this experiment these fly lines had been treated with the antibiotic tetracycline for two generations to eliminate *Wolbachia* and other symbionts as variation in their presence could confound any variation in traits observed in this study (Jiggins & Hurst, 2011). For the same reason, these flies also went through a dechorionation treatment (see Brun & Plus, 1998) at a similar time to eliminate any potential viruses present (Longdon *et al*., 2011).

For this experiment, pairs of fly genotypes were picked randomly and crossed to produced 18 outcrossed lines; therefore the phenotypes studied were the result of naturally segregating genetic variation and not the result of inbreeding. The experiment used a set of 4 temporal blocks with crosses conducted in groups of five or six at a time, whilst one cross was repeated each time to monitor block variation.

Flies were reared on Lewis medium (Lewis, 1960) at 25 °C, 70% RH, with a 12hr L/D photoperiod. Cultures were set up following a standard procedure (Mackenzie *et al.*, 2011). During crosses virgins from one line were crossed with males from another and larval density controlled following Clancy and Kennington's method (2001): 13µl of collected eggs suspended in saline were pipetted into bottles of food (~24 bottles per genotype). After eclosion flies were transferred to 11 litre demography cages (~400 mixed-sex flies per cage): 12 cages per genotype. A total of 168 independent demography cages were represented in the experiment (8 cages per genotype); one

cage per age per genotype was maintained to replace any flies that died or escaped so that a constant density was preserved within the experimental cages.

5.3.2. Determination of the median lifespan of fly lines

In addition to the flies placed in demography cages, 50 females and 50 males were taken from each cross to assess longevity. Within two days of eclosion, flies were placed in a bottle with fresh food and allowed to mate for four days (100 mixed sex flies in a bottle) before being separated into single-sex vials, 10 flies per vial. There was no fly mortality prior to this point. Corpses were recorded every 3-5 days until all 100 flies had died. Flies were transferred onto fresh food once a week.

5.3.3. Preparation of *Beauveria bassiana* spores

Beauveria bassiana spores originated from a strain used previously (Tinsley *et al.* 2006). Fungal material was grown following Mackenzie *et al*., (2011) methods and diluted to 2.8×10^7 spores ml^{-1}. This was vortexed and agitated briefly using a probe sonicator prior to use in fungal pathogen susceptibility assays.

For assessing phagocytosis, spores were prepared following a standard protocol (Mackenzie *et al*., 2011). Following labelling with Fluorescein isothiocyanate (FITC) (Sigma), spores were washed and resuspended in 1ml of phosphate buffered saline (PBS) and diluted to 5×10^6 spores ml^{-1} before use in the assay.

5.3.4. Assessing genetic variation in the rate of functional senescence for life history traits

i. Pathogen susceptibility

Flies were removed from demography cages at appropriate ages and one hundred males and one hundred females from each cross and age (one, two, three and four

weeks of age) were separated into single-sex batches of 10-flies per vial. Using the methods described previously (*in vivo* assay in Chapter 3; **3.3.3.**), flies were maintained on standard food medium lacking the anti-fungal agent 'Nipagen' for the duration of the experiment. Flies were injected using a tungsten needle following a standard protocol (**3.3.5.**), and received either a dose of the prepared live *B. bassiana* spores or blank oil (87.5% Shellsol T, 12.5% Ondina EL). Flies were maintained in their vials and checked every second day for mortality, corpses were recorded and the living flies transferred onto fresh food in a new vial.

ii. Phagocytosis assay

Forty males and forty females from each cross and age (one, two, three and four weeks of age) were separated into single-sex batches of 5-flies per vial. Flies were injected with 0.05µl of the prepared FITC labelled *B. bassiana* following a standard protocol (Mackenzie *et al*., 2011). Haemolymph was extracted and processed following the methods of Mackenzie *et al*., (2011). Samples on a 96-well plate were viewed with an Axiovert 135 epifluorescence microscope; 100 cells were inspected in each well and the number of fluorescent included particles per cell recorded.

iii. Fecundity assay

One hundred female flies were taken from the mixed-sex demography cages at each of the four ages (one, two, three and four weeks of age) for each genotype. These were separated into batches of 5-flies per vial, and fecundity was assessed for these 5-fly batches. Flies were kept on Lewis food (Lewis 1960) seeded with yeast for 24hrs before being transferred to vials containing apple juice agar seeded with yeast. The five-fly groups remained in these vials for 24hrs to lay eggs. Vials were then stored at -20^{o}C to prevent larval development. Subsequently, vials were defrosted overnight at 4^{o}C, then viewed under a microscope and their egg number recorded.

iv. Phenoloxidase (PO) assay

Eighty female flies from each cross and age were separated into 10-fly groups in 1.5ml microcentrifuge tubes. These were immediately stored at -80^{o}C until needed. The PO activity assay was adapted from that used for assessing PO activity in honey bees (Brown *et al*., 2003). Flies were defrosted on ice and a tissue grinder used to homogenise each 10-fly batch in 100µl of PBS, the homogenate was then centrifuged at 15.7*g* (4^{o}C for 10min). The tissue grinder was cleaned in ethanol and thoroughly dried before each homogenisation. The supernatant was used to measure total PO activity and protein content (see methods **3.4.v**). Reaction mixtures for determining PO activity contained 40µl of supernatant, 43µl distilled water, 7µl PBS, 10µl L-DOPA solution (4 mg ml^{-1} distilled water) and 5µl of bovine α-chymotrypsin (Sigma, C4129; 2.1mg ml^{-1} distilled water). This was first incubated for 5min at room temperature, then plates were maintained at 25^{o}C for 40 min in a microplate reader (Versamax, Molecular Devices, USA), for the duration of the reaction. Absorbance readings were taken every 10 s at 475nm and analysed using SOFT$_{MAX}$PRO 4.0 software (Molecular Devices, USA). Enzyme activity was measured as the slope (V_{max} value) of the reaction curve during the linear phase of the reaction. Each homogenate sample was assayed twice.

v. Protein content

To calculate fly protein content, 20µl of supernatant from the female fly homogenate in PBS (**3.4.iv.**) was added to a further 80µl PBS. Protein was measured by reaction with Coomassie Brilliant Blue and correlated to a standard curve using bovine serum albumin (BSA) (Bradford, 1976). Briefly; 50µl of the diluted supernatant was mixed with 50µl of 0.06% Coomassie Brilliant Blue and the absorbance measured at 620nm and 465nm. The reading from 620nm was divided by the reading at 465nm and the result

compared to the standard curve generated using known concentrations of BSA, giving the protein content of the sample in question.

5.3.5. Statistical Analysis

Data were analysed using Bayesian Markov chain Monte Carlo (MCMC) generalised linear mixed models in the MCMCglmm package (Hadfield, 2010) for R statistical software, version 3.0.0 (R Core Team, 2013). To calculate the genotypic variance for each trait (except pathogen susceptibility), models with a Guassian distribution were used and non-informative priors, derived following the method of Wilson *et al*., (2010) were set for each analysis. A binomial error distribution was used (method in Chapter 3 (**3.3.6.**)) for the data from the pathogen susceptibility assay. All models had a burn-in period of 10,000, a total of 110,000 iterations and a thinning interval of 100. Low autocorrelation and good model convergence was confirmed through a visual examination of the model output. Prior sensitivity analysis was conducted following the methods of Wilson *et al*., (2010) to verify the final models were robust to variation in the values of priors.

Multiple blocks were present in the experiment which contained different batches of genotypes. I verified that no differences existed in the traits between these blocks by including one common genotype in each set. For this genotype, there was no consistent difference between blocks for any trait. Therefore the term ‘block’ was excluded from further analyses.

All models contained fixed effects for fly ‘sex’ (when both sexes were present in the experiment) and fly ‘age’, which was specified as a covariate (except for the model for median lifespan). For the analysis of median lifespan the data points were the median lifespan calculated for each vial. For the analysis of haemocyte phagocytic ability the ‘time’ of day the assay was performed was also included as a fixed effect. An

interaction between ‘genotype’ and ‘age’ was included as a random effect in every model (except for analyses of median lifespan). Other random effects included were the ‘genotype’, as well as the ‘cage’ or ‘vial’ where flies were housed prior to assay. For the protein content and phenoloxidase activity analyses additional random effects were the 96-well ‘plate’ used for each sample and ‘sample’ itself to account for repeat measures of the same sample. The model for genetic variation in disease resistance also contained ‘treatment’ (oil or fungal injection), as a fixed effect and an interaction between ‘treatment’ and ‘age’. The random effects for this model differed from other models in that they contained only a three-way interaction between ‘treatment’, ‘genotype’ and ‘age’; a two-way interaction between ‘treatment’ and ‘age’; and ‘cage’ on its own.

I tested whether the random effect genotypic variance components in these models could have arisen by chance. Models were run 100 times with genotype repeatedly randomly reassigned to each data point. A loop function was designed which used the transform function in R to randomly allocate the genotypes to each data point, then repeat the relevant model with this new dataset; 100 such ‘random’ models were run per trait. The variance components from these ‘random’ models were compared with those generated by the real model: the number of times out of 100 the ‘random’ variance components were equal to or exceeded the ‘real’ model was taken to generate a p-value.

Multivariate models were used to explore genotypic covariances between traits. I investigated the covariance between median lifespan and all other traits as well as between fecundity and the other physiological traits. The ‘age’ term was centred prior to analyses so that the intercept for age was at the middle of the age range studied. The response variable error structures were not changed. Variance-covariance matrices were unstructured (using the ‘us’ specification) and residual variances were

heterogeneous (using the 'idh' specification) between traits. When both genders were present in the dataset for both response variables, 'sex' was included as a fixed effect in the model. If only one gender was measured for one response variable then 'sex' was not included in the model and the data for the missing sex were removed from the second dataset.

As the infection 'treatment' term was only present for measures of fungal pathogen defence, this could not be included in multivariate models. Therefore for multivariate models assessing pathogen defence only the data from the fungal infected flies were included.

Parameter estimates reported are modes from the posterior distribution with 95% lower and upper credible intervals (CI). Means ± their standard errors in the text are generated from the raw data. Figures are simplified to represent only age classes 1 and 4, and data means in figures are generated using the lsmeans function in R. These means are based on linear mixed effects models using the Lme4 package (Bates *et al*., 2013), with 'age', 'genotype' and an interaction between these factors as fixed effects, and 'cage' or 'vial' as random effects.

The coefficient of genetic variation (CV_G) was also calculated: 100 x ($\sqrt{\sigma^2_G}$) /mean (Houle, 1992), where σ^2_G is the genetic variance due to genotype. The coefficient of genetic variation allows for comparisons in the σ^2_G of different traits standardised for differences in the mean and measurement units of each trait. Potential correlations between the σ^2_G of different traits were also explored.

5.4. Results

5.4.1. Genetic variation in multiple traits

I first investigated the extent of genetic variation for each of the life history traits measured.

5.4.1a. Genotypic variance in longevity

The first life history trait assessed was median lifespan; measured for samples of males and females from each genotype. There was considerable variation between the genotypes for this trait: the longest median lifespan was 83.0 ± 1.71 days and the shortest was 45.2 ± 2.72 days. The genetic variation in median lifespan had a large parameter estimate (σ^2_G = 64.44, Lower CI = 34.52, Upper CI = 158.88; $P < 0.01$) (Fig 1; Table 1), and explained 40.26% of the total random effect variance.

Across all genotypes, males lived longer than females (median lifespan: males = 66.1 ± 1.68; females = 63.3 ± 2.07; $P = 0.032$). Of the 18 genotypes investigated in this current study, 5 did not display sex-specific differences in median lifespan, male median lifespan was greater than female in 7 lines, and in the remaining 6 lines female median lifespan was greater than male.

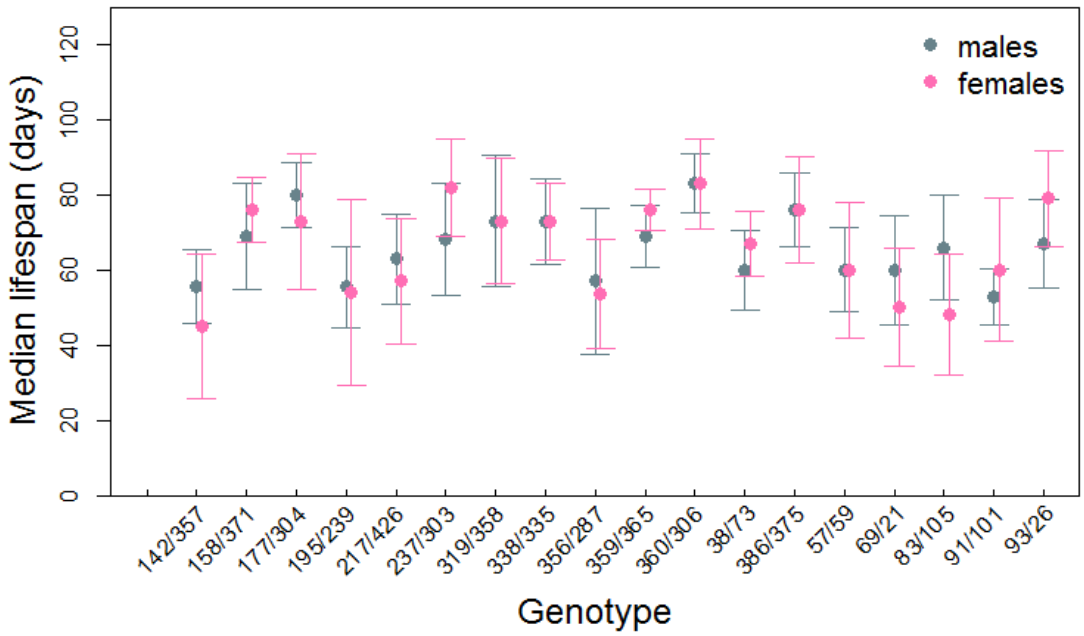

Figure 1: Genetic variation in the median lifespan of 18 outcrossed *Drosophila melanogaster* lines (per point n = 50: median calculated from raw data per gender per genotype). Bars represent the standard deviation per gender per genotype.

5.4.1b. Genotypic variance in fecundity

The mean fecundity of female flies varied dramatically: one week old flies from the most fecund genotype laid 79.8 ± 6.47 eggs in 24hrs, whereas females of the same age from the least fecund genotype laid less than half as many eggs (32.1 ± 4.65) in the same time period. There was strong genetic variation in fecundity between genotypes (σ^2_G: 81.83, Lower CI = 25.26, Upper CI = 178.50; $P < 0.01$), which explained 19.76% of the total random effect variance (Table 1).

Across all genotypes, fecundity dropped by nearly half as flies aged through the experiment (one week old mean: 54.5 ± 2.79 eggs per 24hrs, four week old mean: 27.7 ± 2.16 eggs per 24hrs; $P < 0.001$) (Fig 2). There was considerable genetic variance in the rate that fecundity changed with age (σ^2_G = 101.45, Lower CI = 54.92, Upper CI = 164.52; $P < 0.01$). This explained 24.50% of the total random effect variance (Table 1).

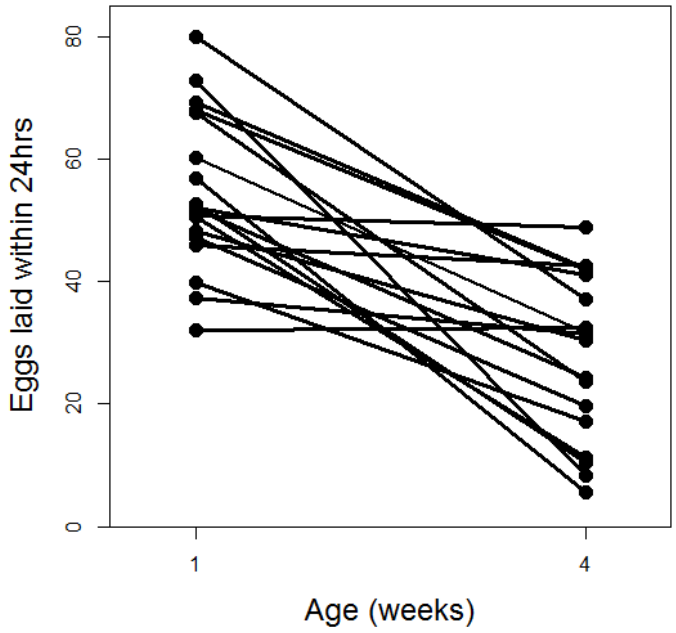

Figure 2: Variation in the rate of age-dependent decline in female fecundity across 18 outcrossed *Drosophila melanogaster* lines. Only data for one and four week old flies are plotted. For each data point n = 20.

Considering only the fecundity in one week olds and four week olds, there was a positive genotypic covariance between the fecundity of young and old flies (genotypic covariance = 16.95, Lower CI = 6.76, Upper CI = 33.76).

5.4.1c. Genotypic variance in pathogen defence

There was a lot of variation between genotypes in their susceptibility to *Beauveria bassiana* infection. By 9 days post treatment, the proportional mortality of one week old infected flies ranged from no mortality in one genotype to 0.63 ± 0.01 in another. Across the whole dataset, by 9 days post-treatment, infected flies suffered almost twice the mortality that uninfected flies experienced (Proportional mortality: infected = 0.28 ± 0.02; uninfected = 0.16 ± 0.01; $P = 0.04$). Proportional mortality increased as flies aged (four week old flies 9 days post treatment: infected = 0.30 ± 0.03; uninfected = 0.23 ± 0.04; compared to one week old flies: infected = 0.23 ± 0.03; uninfected =

0.02 ± 0.01; *P* = 0.02). However, infection did not cause more mortality in older flies, therefore senescence in pathogen defence was not detected (there was no age x infection interaction: *P* = 0.53). There was a significant difference in disease susceptibility between the sexes: 10% more females than males died following infection with *B. bassiana* (*P* = 0.014).

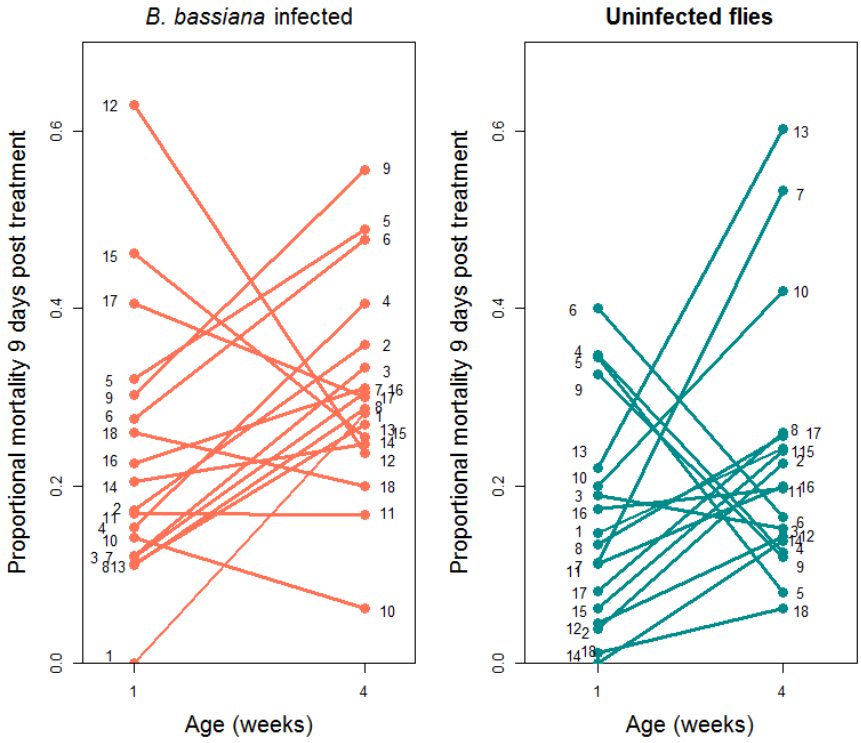

Figure 3: Variation in the rate of age-dependent change in proportional mortality following pathogen challenge. Eighteen outcrossed *Drosophila melanogaster* lines from one and four weeks of age received a control injection or the fungal pathogen *Beauveria bassiana*. Graphs show proportional mortality 9 days post treatment. Lines labelled with genotype numbers, n = ~100 flies per genotype, per treatment, per age.

Though immune senescence was not observed, there was still evidence of genetic variation in susceptibility to infection (σ^2_G: 0.66, Lower CI = 0.27, Upper CI = 1.24; *P* < 0.01), and the pattern of age-dependent change in infection susceptibility differed between genotypes (Fig 3). The genotypic variance represents over 11% of the total

random effect variance and the interaction between age, genotype and treatment (infected or uninfected) comprised an additional 9% (σ^2_G: 0.54, Lower CI = 0.26, Upper CI = 1.00; $P < 0.01$) (Table 1).

5.4.1d. Genotypic variance in total phagocytosis ability

As well as assessing the wider aspect of disease resistance by assaying susceptibility to fungal infection, I also investigated individual immune traits. Fly plasmatocytes' phagocytosis ability was measured to determine potential variation in investment in cellular immunity between the fly genotypes.

The total number of spores engulfed per sample of 100 haemocytes was the measure used to describe overall phagocytosis ability. Mean plasmatocyte phagocytic activity differed by up to 26% between genotypes in a single age-class (range in the genotypic means of one week old flies: 57.7 ± 2.7 - 42.5 ± 1.8 spores per 100 haemocytes). As flies aged, there was a 25% decline in haemocytes' phagocytic ability across all genotypes (total spores included per sample: one week olds = 49.5 ± 0.48; four week olds = 36.9 ± 0.32; $P < 0.001$). There was no significant difference in phagocytic ability between the sexes ($P = 0.88$) and the time of the assay did not influence the observed number of internalised fungal spores ($P = 0.19$). There was variation between the genotypes in their observed total phagocytic ability (σ^2_G: 2.53, Lower CI = 1.16, Upper CI = 7.99; $P < 0.01$), although limited evidence of variability in their rates of senescence (σ^2_G: 2.09, Lower CI = 0.74, Upper CI = 4.81; $P = 0.11$) (Fig 4). Although these are not large parameter estimates, together they represent 10% of the total random effect variance. The CV_G, which helps compare variances between traits, is relatively small for total phagocytosis ability: only 2.3 (Table 1).

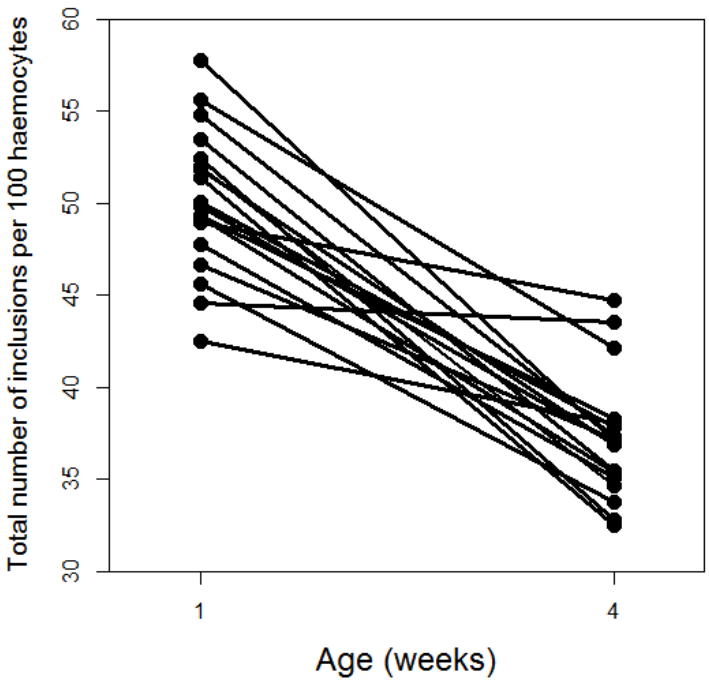

Figure 4: Genetic variation in the total phagocytic ability of one to four week old flies from 18 outcrossed lines of *Drosophila melanogaster* (each point: n = ~16).

5.4.1e. Genotypic variance in total phenoloxidase activity

Another immune trait measured was the total phenoloxidase (PO) activity within flies in the 18 outcrossed genotypes of *D. melanogaster*. Only female flies were assayed in this study. Genotypes varied in their mean total PO activity in one week old flies from 4.63 ± 1.74 (V_{max}) up to 9.59 ± 1.66 (V_{max}). Across the genotypes the mean total PO activity did not decline with age (mean total PO activity in one week olds = 6.71± 1.64 (V_{max}); and four week olds = 6.63 ± 1.63 (V_{max}); P = 0.74). There were differences in the mean total PO activity between genotypes (σ^2_G: 1.62, Lower CI = 0.74, Upper CI = 4.41; P = 0.01), but the pattern of age-related change in total PO activity did not vary across the genotypes (σ^2_G: 1.26, Lower CI = 0.75, Upper CI = 2.66; P = 0.52) (Fig 5; Table 1).

The genotypic variance measured explained slightly over 6% of the total random effect variance. Whilst 6% might suggest the influences of genotype on PO activity was small, the CV_G for this trait is 19.43 which is the greatest CV_G in all the traits measured.

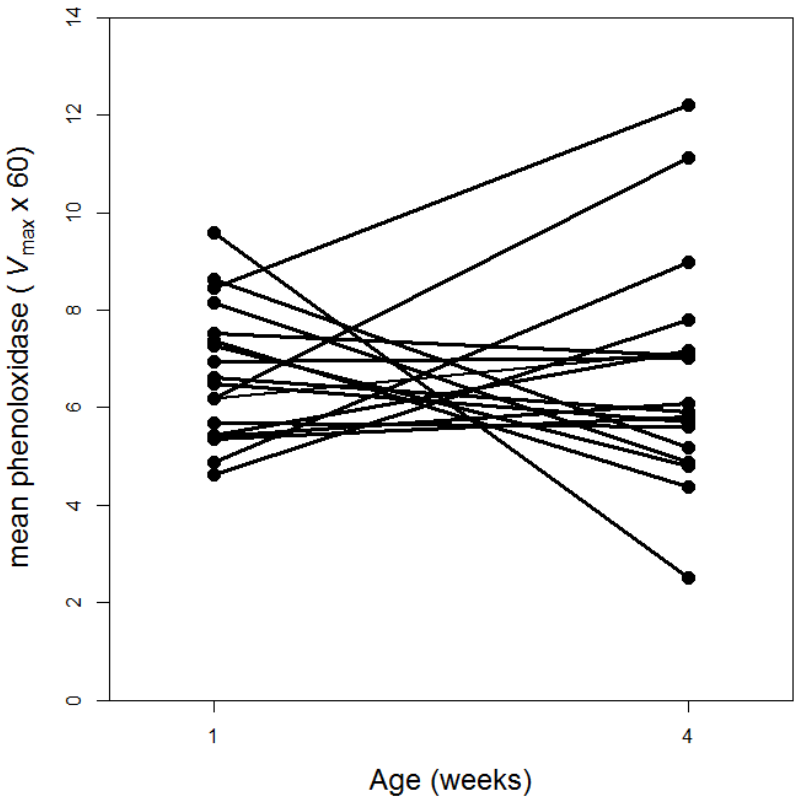

Figure 5: Genetic variation in the total phenoloxidase activity of one and four week old flies from 18 outcrossed lines of *Drosophila melanogaster* (each point: n = 8).

5.4.1f. Genotypic variance in protein content

The final trait recorded was protein content in females from the four age classes (one, two, three and four weeks old) of the 18 outcrossed fly lines. Protein content in one week old flies varied between genotypes by 60% (genotypic means varied between 26.5 ± 1.7 and 10.6 ± 0.9 µg/ml). The mean protein content did not vary with age when pooling across all genotypes (mean protein content of one week olds = 15.03 ± 1.72

µg/ml; four week olds = 16.12 ± 1.69 µg/ml; *P* = 0.25). The genotypic variation in protein content was significant (σ^2_G: 3.70, Lower CI = 1.13, Upper CI = 11.03; *P* < 0.01), and there was evidence of variation in age-dependent protein content changes between genotypes (σ^2_G: 3.34, Lower CI = 1.66, Upper CI = 6.52; *P* < 0.01) (Fig 6; Table 1). These combined genotypic variance terms explained nearly 23% of the total random effect variance, and the CV_G for the mean variance in this trait was 12.14 (Table 1).

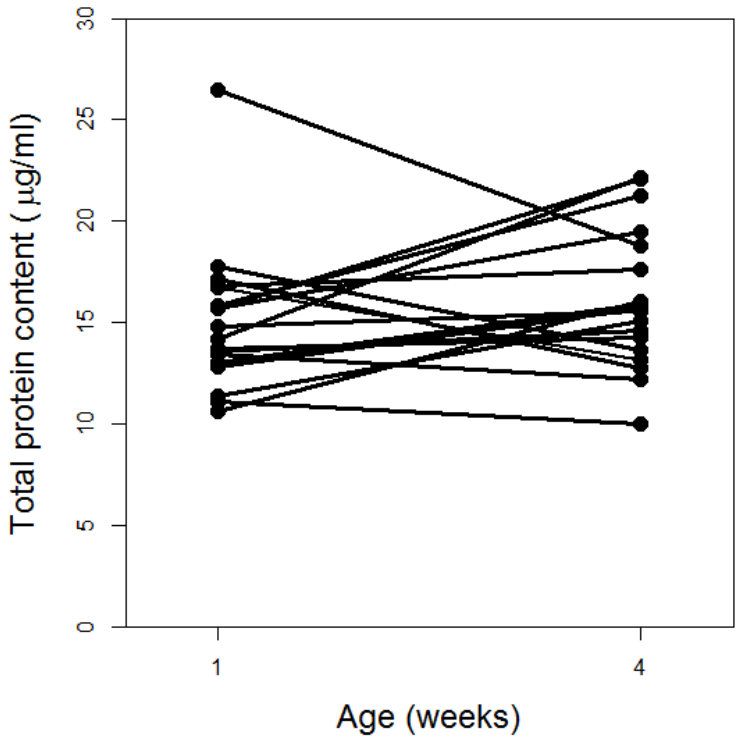

Figure 6: Genetic variation in the total protein content of one to four week old flies from 18 outcrossed lines of *Drosophila melanogaster* (each point: n = 8).

Table 1: Summary of the genetic variation present in six life history traits and the effects of age within 18 outcrossed lines of *Drosophila melanogaster*.

		Lifespan	Fecundity	Pathogen defence	Total Phagocytosis ability	Total Phenoloxidase activity	Total Protein
Fixed Effect of Age	estimate	-	-9.79	0.28	-4.30	0.04	0.32
	95% CI (lower, upper)	-	(-12.05, -6.97)	(0.07, 0.45)	(-5.32, -3.60)	(-0.37, 0.52)	(-0.21, 0.88)
	P value	-	< 0.001	0.018*	< 0.001	0.74	0.25
Genotypic Variance	variance component	64.44	81.83	0.66	2.53	1.62	3.70
	95% CI (lower, upper)	(34.52, 158.88)	(25.26, 178.50)	(0.27, 1.24)	(1.16, 7.99)	(0.74, 4.41)	(1.13, 11.03)
	P value**	< 0.01	< 0.01	< 0.01	< 0.01	0.01	< 0.01
	CV	12.68	15.06	***	2.29	19.43	12.14
Genotypic Variance in the Rate of Ageing	variance component	-	101.45	0.54	2.09	1.26	3.34
	95% CI (lower, upper)	-	(54.92, 164.52)	(0.26, 1.00)	(0.74, 4.81)	(0.75, 2.66)	(1.66, 6.52)
	P value**	-	< 0.01	< 0.01	0.11	0.52	< 0.01

* Survival declined as flies aged regardless of infection treatment (oil or *Beauveria bassiana*), there was therefore a significant fixed effect of age. However, there was not a steeper age-dependent decline in survival in infected flies compared to uninfected flies (no infection x age interaction) which indicated that senescence in pathogen defence did not occur (see text for further detail).

** 100 models were run with genotype randomly assigned to each data point. 'P value' represents how many of the 100 models had a variance component ≥ to that produced by correctly assigning genotype to each data point, i.e. the percentage time the variance component quoted could have arisen by chance.

*** No CV_G was calculated for the genotypic variance component for pathogen defence as the trait mean represents the difference in the mean proportional mortality of uninfected and infected flies.

5.4.2. Associations between life history traits across wildtype genotypes

I investigated the genotypic covariance between variability in immune traits and variation in ageing traits, measured either as lifespan, or as rate of decline in physiological and life history parameters. A degree of genotypic covariance was found for every trait. However, for not one association was the covariance term significantly different from zero when judged by the overlap of the 95% credible intervals with zero (Tables 2a-2c). For the majority of trait pairs, the magnitude of the covariance term between them was low, giving no indication of genetic associations. Whilst this data set provides no statistical support for genotypic covariances between any of the traits studied, for some trait pairs the overlap of the parameter estimate credible intervals and zero was relatively low.

5.4.2a. Investment in immunity may not influence median lifespan

The first set of analyses explored the potential genetic links between median lifespan and the other life history traits assessed. In this experiment, there did not appear to be a strong correlation between a genotype's female median lifespan and their early-life investment in fecundity (genotypic covariance: 15.28, Lower CI = -68.30, Upper CI = 146.43) (Table 2a), or between median lifespan and mean lifetime reproductive investment (genotypic covariance: -28.26, Lower CI = -95.52, Upper CI = 54.28) (Table 2b). The covariance estimate for the correlation between the age-related decline in female fecundity and a genotype's female median lifespan had credible intervals with a slightly smaller overlap with zero, which may suggest a potentially positive association between these traits (genotypic covariance: 70.80, Lower CI = -33.21, Upper CI = 133.42) (Table 2c).

Table 2a: Genotypic covariance between median lifespan and investment in traits in one week old flies.

Trait 1	Trait 2	Genotypic Covariance	95% CI (lower, upper)
Median lifespan	Fecundity	15.28	(-68.30, 146.43)
"	Infection related mortality*	0.13	(-5.30, 6.57)
"	Total phagocytosis ability	7.85	(-12.24, 37.18)
"	Total phenoloxidase content	4.00	(-12.07, 23.20)
"	Total protein	-12.24	(-41.51, 12.20)

The median lifespans of 18 outcrossed genotypes of *Drosophila melanogaster* were measured, and the genetic variation in this trait (Trait 1) was compared to the genetic variation present for five other physiological traits (Trait 2) measured in one week old flies from each genotype. This table describes the measure of genotypic covariance present between each pair of traits as well as the 95% credible intervals surrounding this value.

* NOTE * For models testing for evidence of genotypic covariance between median lifespan and investment in pathogen resistance only the mortality of infected flies was investigated.

Table 2b: Genotypic covariance between median lifespan and lifetime investment in traits (across one to four weeks of age).

Trait 1	Trait 2	Genotypic Covariance	95% CI (lower, upper)
Median lifespan	Fecundity	-28.26	(-95.52, 54.28)
"	Infection related mortality*	0.33	(-2.97, 5.61)
"	Total phagocytosis ability	2.12	(-14.95, 13.87)
"	Total phenoloxidase content	-1.19	(-11.36, 10.20)
"	Total protein	-6.37	(-31.68, 17.16)

Five physiological traits were measured in 18 outcrossed genotypes of *Drosophila melanogaster* from four different age classes (one, two, three and four weeks old). The genetic variation in the median lifespans of these genotypes (Trait 1) was then compared to the genetic variation in the total value of each physiological trait (Trait 2) representing each genotypes' lifetime investment per trait. This table describes the measure of genotypic covariance present between median lifespan and lifetime investment per trait as well as the 95% credible intervals surrounding this value.

Table 2c: Genotypic covariance between median lifespan and the rate of age-related change in various traits (across one to four weeks of age).

Trait 1	Trait 2	Genotypic Covariance	95% CI (lower, upper)
Median lifespan	Fecundity	70.80	(-33.21, 133.42)
"	Infection related mortality*	-0.82	(-2.78, 2.72)
"	Total phagocytosis ability	2.19	(-7.60, 14.82)
"	Total phenoloxidase content	0.70	(-5.12, 10.21)
"	Total protein	-12.14	(-30.34, 12.54)

The genetic variation in the median lifespans of 18 outcrossed genotypes of *Drosophila melanogaster* (Trait 1) was compared to the genetic variation present in the rate of age-related change in investment for five physiological traits (Trait 2) across four different age classes of flies from each genotype (one, two, three and four weeks old). This table describes the measure of genotypic covariance present between median lifespan and the rate of age-related change per trait as well as the 95% credible intervals surrounding this value.

There was clearly no correlation between genotypes' pathogen susceptibility and their median lifespan or between either of the immune parameters measured and median lifespan (Tables 2a-2c). Furthermore, there was no correlation between median lifespan and mean lifetime protein content (genotypic covariance: -6.37, Lower CI = -31.68, Upper CI = 17.16) (Table 2b). However, there are weak, non-significant trends for the association between lifespan and the two other protein content measures. These may suggest a negative correlation between median lifespan and early-lifetime protein content (genotypic covariance: -12.24, Lower CI = -41.51, Upper CI = 12.20) (Table 2a), and between median lifespan and the rate of age-related change in protein content across one to four weeks of age (genotypic covariance: -12.14, Lower CI = -30.34, Upper CI = 12.54) (Table 2c).

5.4.2b. No trade-offs between fecundity and immune traits were identified

The second set of analyses tested associations between measures of female fecundity and the other life history traits measured in female flies. There were no correlations between a genotypes' susceptibility to *Beauveria bassiana* infection and early-life or lifetime reproductive investment, or age-related changes in either of these traits (Tables 3a-4b). Likewise, there were no associations between phagocytosis ability and fecundity (Tables 3a-4b). Genotypes with high early-life female fecundity tended to have higher protein content (genotypic covariance: 17.79, Lower CI = -5.35, Upper CI = 50.36) (Table 3a), but there was no relationship observed between early-life reproductive investment and mean lifetime protein content (genotypic covariance: 3.98, Lower CI = -22.80, Upper CI = 7.87) (Table 3b).

Table 3a: Genotypic covariance between fecundity and immune parameters in one week old flies.

Trait 1	Trait 2	Genotypic Covariance	95% CI (lower, upper)
Fecundity	Infection related mortality*	0.70	(-11.30, 9.95)
"	Total phagocytosis ability	0.37	(-30.04, 33.13)
"	Total phenoloxidase content	0.55	(-17.18, 17.21)
"	Total protein	17.79	(-5.35, 50.36)

Table 3b: Genotypic covariance between fecundity in one week olds and their lifetime investment in various immune traits (across one to four weeks of age).

Trait 1	Trait 2	Genotypic Covariance	95% CI (lower, upper)
Fecundity	Infection related mortality*	1.87	(-5.04, 9.60)
"	Total phagocytosis ability	2.03	(-17.68, 29.3)
"	Total phenoloxidase content	-4.98	(-22.80, 7.87)
"	Total protein	3.98	(-16.13, 40.98)

* For models testing for evidence of genotypic covariance between fecundity and investment in disease resistance only the mortality of infected flies was investigated.

No correlation was demonstrated between phenoloxidase (PO) activity and fecundity in one week old flies (genotypic covariance: 0.55, Lower CI = -17.18, Upper CI = 17.21) (Table 3a), but the span of the credible intervals associated with the genotypic covariance term for early-life reproductive investment and lifetime PO activity may possibly suggest a negative association exists between these traits (genotypic covariance: -4.98, Lower CI = -22.80, Upper CI = 7.87) (Table 3b).

There were no correlations between the rate of age-related change in fecundity and any of the other life history traits investigated (Table 4b). However, there may be a potential negative relationship between lifetime female fecundity and PO activity (genotypic covariance: -3.75, Lower CI = -17.85, Upper CI = 5.19) (Table 4a). Genotypes with higher lifetime protein levels also appeared to have higher lifetime reproductive investment (genotypic covariance: 6.65, Lower CI = -8.80, Upper CI = 2.83) (Table 4a). Although the associated credible intervals span zero for these genotypic covariance terms, the overlap is slightly smaller than for the other comparisons between lifetime investment in fecundity and other traits, and between age-related change in fecundity and other traits (Tables 4a and 4b).

Table 4a: Genotypic covariance between mean lifetime fecundity and mean lifetime investment in various immune traits (across one to four weeks of age).

Trait 1	Trait 2	Genotypic Covariance	95% CI (lower, upper)
Fecundity	Infection related mortality*	0.44	(-6.02, 5.63)
"	Total phagocytosis ability	1.61	(-15.63, 20.31)
"	Total phenoloxidase content	-3.75	(-17.85, 5.19)
"	Total protein	6.65	(-8.80, 35.19)

* For models testing for evidence of genotypic covariance between fecundity and investment in disease resistance only the mortality of infected flies was investigated

Table 4b: Genotypic covariance between the rate of age-related change in fecundity and various immune traits (across one to four weeks of age).

Trait 1	Trait 2	Genotypic Covariance	95% CI (lower, upper)
Fecundity	Infection related mortality*	-0.57	(-2.92, 4.31)
"	Total phagocytosis ability	-1.12	(-11.10, 12.25)
"	Total phenoloxidase content	-0.04	(-7.31, 6.04)
"	Total protein	2.53	(-8.69, 13.40)

* For models testing for evidence of genotypic covariance between fecundity and investment in disease resistance only the mortality of infected flies was investigated.

5.5. Discussion

This study conducted a large scale experiment investigating genetic variation in immune and life history traits using nearly 41,000 *Drosophila melanogaster*. It has demonstrated that a significant degree of genetic variation is present in each trait, as well as in how these traits change throughout the lifetime of flies. The answer to the question of whether there is genetic variation in these traits on which natural selection could act is definitely yes.

It is not possible to partition the estimates of genetic variance into additive, dominant or epistatic genetic variances. Although the advantages of using outcrossed compared to inbred fly lines were outlined in the introduction, the use of a sib-sib or half-sib design would have allowed for the calculation of the heritability of traits and the partitioning of genetic variances. However, even in the absence of these measures, my data demonstrated that fecundity and immune activity are influenced by naturally segregating genetic variation. Importantly, the general decline observed in these traits during ageing is not an evolutionarily-unavoidable phenomenon, because there was considerable genetic variation in the pattern of age-dependent change on which natural selection could potentially act on. Natural selection only strongly impacts genetic variation for early-life functions (Medawar, 1952). Nevertheless, the strongly inconsistent impact of age on life history traits in different genotypes shows that selection on early-life function could have considerable, and unpredictable, effects on late-life fitness.

The first life history trait measured was the median lifespan of each genotype. A significant amount of variation was detected, with a 46% difference between the longest and the shortest median lifespans. Overall there was a significant

difference between the sexes in their median lifespans. Of the 18 genotypes investigated in this current study, in the fly lines that displayed sex-specific differences in median lifespan, the slight majority of genotypes had males that lived longer than females. Generally, females live longer than males although this is not universal (Tower & Arbietman, 2009). Sex-specific longevity patterns can be influenced by genotype (as shown here) and by environmental factors. In the current study flies were stored in a high-mating environment for four days prior to the experiment and mating frequency is known to influence female lifespan in *Drosophila* (Gerrard *et al*., 2013).

The second life history trait measured in this study was the female fecundity of each genotype. Across the 18 lines mean fecundity nearly halved as flies aged from one to four weeks of age. Considering individual genotypes some lines experienced a 90% reduction in egg production between these ages, others demonstrated almost no change in fecundity between ages. Although there was a distinct genetic influence on genotype lifetime fecundity, there was also a strong genetic influence on the rate of senescence in fecundity observed for each line. Interestingly, there was a positive correlation between fecundity in one and four week old flies, suggesting that genotypes with high early-life reproductive investment did not experience more rapid senescence in fecundity compared to flies with lower early-life reproductive effort. Traditionally, there is expected to be a positive correlation between early-life investment and the subsequent rate of senescence in fecundity (Kirkwood & Austad, 2000). The results of this study do not support this prediction. However, due to there being no resource limitation in this experiment perhaps this masked a trade-off that would be evident under natural conditions.

Fecundity in *Drosophila* is known to be impacted by multiple factors, including (but not restricted to): mating frequency (Priest *et al*., 2008); timing of mating (Long *et al*., 2010); male factors, such as sex peptides (Gioti *et al*., 2012); female factors, such as body size (Turiegano *et al*., 2013) and age (Tan *et al*., 2013); environmental factors (Dev *et al*., 2013) and genetic influences (Remolina *et al*., 2013). Although environmental effects were kept constant, the flies in this experiment were aged in mixed-sex demography cages and their mating rates were not measured. Therefore, although genetic influences on lifetime fecundity and the rate of senescence in fecundity have been observed, diverse physiological mechanisms could have driven this variation.

Many factors influence *D. melanogaster* pathogen defence and susceptibility to infection. Infection with the entomopathogenic fungus *Beauveria bassiana* is a standard disease defence assay in *D. melanogaster* (Kraaijeveld *et al*., 2012; Le Bourg *et al*., 2011; Tinsley *et al*., 2006). A study on house flies (*Musca domestica*) reported that susceptibility to infection with *B. bassiana* did not vary between one day old and two week old flies (Kaufman *et al*., 2008). Similarly, the adult age of mosquitoes was not found to impact susceptibility to *B. bassiana* infection (Mnyone *et al*., 2011). There are no published studies on age-associated changes in *D. melanogaster* pathogen susceptibility using *B. bassiana*, however a study in this lab has been conducted that suggests that susceptibility to infection increases as flies age (Tinsley, unpublished). However, the results of the study I conducted do not corroborate those findings. Across the 18 genotypes, senescence in *D. melanogaster* disease resistance was not observed.

This average pattern of no disease defence senescence masks considerable between-genotype variation within the data. One of the genotypes studied

demonstrated a 46% increase in proportional mortality from one to four weeks of age. However, though 6 of the 18 genotypes had higher proportional mortality in older compared to younger flies, 9 genotypes experienced lower proportional mortality in four week olds compared to one week olds. The remaining 3 genotypes displayed negligible differences in proportional mortality across the age classes. Across the whole data set there was significant genetic variation in pathogen susceptibility and in the age-related changes in this trait. Although *B. bassiana* is not a coevolved pathogen of *D. melanogaster*, these results have highlighted differences in immune activity and age-related changes in immune response that are controlled by heritable variation on which selection could act.

The sex-specific differences in pathogen susceptibility are again not in keeping with theoretical predictions. In this study 10% more females died following infection with *B. bassiana* compared to males. The conventional theory, based on Bateman's principle of evolutionary divergence in male and female reproductive strategies, is that females are expected to have greater immune investment than males (Rolff, 2002). Theories differ as to why this might be. One proposal is that males invest in sexual ornaments and behaviours at the expense of immunity to promote mating opportunities rather than lengthen lifespan (Zuk, 1990). Selection on females may promote longevity through greater immune investment to increase their lifetime fecundity (Rolff, 2002). Alternatively, the availability of resources may be the predominant influence on sex-specific differences in immune investment (McKean & Nunney, 2005). The availability of dietary yeast has been demonstrated to dramatically alter egg production in females with a correlated reduction in lifespan and starvation resistance (Chippindale *et al*., 1993; Partridge & Farquhar, 1981). In this current study, flies were fed on a standard Lewis medium (Lewis, 1960) with no extra yeast provided during the pathogen susceptibility assay.

Although there are many elements of the immune response that could have been assayed to determine each genotype's immune investment the two that were measured in this study were plasmatocyte activity and phenoloxidase (PO) activity. Plasmatocyte phagocytic ability varied significantly between the genotypes, the genotypic means ranging from 57.7 ± 2.7 spores per 100 haemocytes down to 42.5 ± 1.8 in one week old flies. However, the phagocytic ability of plasmatocytes universally senesced and there was no significant genotypic variation in rate of senescence.

Contrary to plasmatocyte phagocytic ability, the measure of the humoral response investigated did not demonstrate an age-dependent change or senescence. Across the genotypes, the mean total PO activity did not decline with age and genetic variance in the pattern of age-related change in PO activity was not detected. Nine of the 18 lines had a decline in PO activity from one to four weeks of age, 9 lines had no change or had increasing PO activity as they aged. A limiting factor of this assay was that total PO activity was measured rather than infection-induced PO activity. The reason for this was a difficulty in detecting any PO in *D. melanogaster*, so all potential PO was activated by the enzyme chymotrypsin in order to attain a measure. This was also the reason male flies were not included in this assay, as no PO was detected using these methods. If the assay was sensitive enough to detect PO activity following infection as well as total PO activity, this would have given a result that more closely reflected fly line investment in this trait.

Despite these limitations, the genotypic variance in overall PO activity had the largest CV_G in all the traits measured. This suggests that there was more genetic control for the determination of PO activity than for any other trait. Whether this reflects selective processes maintaining genetic variation for PO investment, or

indicates relaxed or limited selection on this trait is not clear. In terms of ageing, PO activity can be a source for reactive oxygen species (ROS) production as it is generated in the formation of melanin (Nappi *et al*., 2009). Therefore, although investment in PO may be beneficial for fly immunity, it might be at the cost of accelerated ageing.

Finally, the protein content of each genotype and age class was assessed. Although there was no overall age-dependent change in protein content, genotypic variance in the lifetime protein content of each line and in the patterns of age-related change were detected. Protein content in one week old flies varied significantly across the genotypes (ranging from 26.5 ± 1.7 µg/ml down to 10.6 ± 0.9 µg/ml). Whilst 6 out of 18 lines lost protein throughout their lifetime, the majority gained protein as they aged, though 3 lines had a negligible change in protein content between one and four weeks of age.

Protein content can be a measure of the acquisition and allocation of resources (Kristensen *et al*., 2011). Studies have shown that animals can alter the ratio of protein and carbohydrate they ingest (Behmer *et al*., 2001), which can also influence the activity of their immune systems (Cotter *et al*., 2011). A recent study on *Daphnia* found variation in life history traits among clones supplied with abundant resources (Olijnk *et al*., 2013). The authors suggested that the variation might be derived from differences in what they termed ‘differential resource utilisation’ (Olijnk *et al*., 2013). The results for *D. melanogaster* demonstrate genetic variation in lifetime protein content and for patterns of age-related change in this trait. It is possible that protein content varied due to genetic influences on acquisition rates and utilisation strategies.

A lack of correlations between immune and life history traits is not uncommon (Burger & Promislow, 2006; Cotter *et al*., 2004; Wit *et al*., 2013a). The absence of a correlation between lifespan and fecundity in this study suggests that there is not a strong genetic link between the activities of these two traits. The results from other studies are in conflict with this conclusion. *Drosophila* caught from natural populations have demonstrated correlations between lifespan and fecundity (Klepsatel *et al*., 2013). Additionally, selection experiments have often demonstrated a link between longevity and fecundity (Djawdan *et al*., 1996; Partridge & Fowler, 1992; Rose & Charlesworth, 1980). Laboratory selection can magnify phenotypic traits and highlight trade-offs that might otherwise be difficult to detect but sometimes studies can be contradictory (reviewed by Harshman & Hoffmann, 2000).

Introducing a stressor can highlight physiological trade-offs that were not otherwise apparent. Recently a *D. melanogaster* line selected for longevity that had not displayed negative correlations with other life history traits in the laboratory revealed lower competitive ability compared to non-selected controls in field conditions, thus highlighting a trade-off that had not been identified under laboratory conditions (Wit *et al*., 2013c). Placing selected lines in field conditions represented the introduction of a stress that was not present in the laboratory. In this current study, under optimum conditions, no trade-offs were detected between the life history traits measured; perhaps under stressful conditions trade-offs would be identified.

Conventional theory suggests that high early-life fecundity leads to a shorter lifespan. Some *D. melanogaster* longevity selection lines have lower early-life fecundity compared to lines selected to be short-lived (Luckinbill *et al*., 1984; 1985; 1987), but some longevity lines do not display this trade-off (Khazaeli *et*

al., 2010; Khazaeli & Curtsinger, 2013). This current study did not demonstrate a negative correlation between early-life reproductive investment and median lifespan. However, a non-significant trend existed suggesting that flies with rapid senescence in fecundity tended to have longer lifespans, potentially suggesting that as these genotypes aged resources were directed away from reproduction towards the maintenance and repair systems that increase longevity.

There was no evidence of a genetic link between investment in immunity, as reflected by genotypic differences in infection induced mortality, and lifespan. Perhaps this was because this was not a good measure of overall immune investment. The increased expression of immunity genes correlates with lifespan in a number of studies on *Drosophila* (Landis *et al*., 2004; Pletcher *et al*., 2002; Remolina *et al*., 2012; Seroude *et al*., 2002). Nevertheless, the lack of a correlation may indicate that responses to selection pressures influencing immune investment are not constrained by genetic correlations between loci influencing immunity and longevity.

Abundant resources and unrestricted resource acquisition may result in positive correlations between physiological processes that are predicted to be costly, such as reproduction and the maintenance and repair systems that extend lifespan (Reznick *et al*., 2000). However, from the perspective of life history evolution, greater fecundity is always beneficial for an organism especially if there is not a negative impact on lifespan. Immune activity is both beneficial for lifespan in regards to protection from infection and detrimental in regards to collateral damage to the organism; both friend and enemy (DeVeale *et al.,* 2004). Therefore, even with *ad libitum* resources, there is unlikely to be a positive correlation between pathogen defence and longevity. The lack of a negative correlation between these two traits is interesting in terms of life-history evolution

as it suggests that the genetic variation present within these traits is not due to genetic constraints between them (Reznick, 1985). Of the immune parameters measured, the only potentially negative, but non-significant, trend was between fecundity and PO activity. In flies in sub-optimal conditions, a trade-off between reproduction and immune investment might possibly be demonstrated.

I assessed relationships between the protein content of flies from each genotype and fly lifespan. Whilst these relationships were not significant, their directions do support the findings of other work. In this current study, genotypes with higher protein levels have a trend towards having shorter lifespans. High protein diets are associated with a shorter lifespan in *D. melanogaster*, compared to a high carbohydrate diet (Lushchak *et al*., 2012). Genotypes might trade long life for a shorter lifespan and higher protein levels as this may assist mounting stronger immune responses following infection (Cotter *et al*., 2011). Equally the potential trend of a genetic correlation between fecundity and protein content was in the predicted direction. Flies with high fecundity had a trend towards higher protein levels. Flies fed a high protein diet have been shown to have greater fecundity compared to flies fed on a low protein diet (Lushchak *et al*., 2012). Perhaps the possible relationship indicated by the current study reflects the high protein content of eggs stored in ovarioles.

In summary, for all the life history traits measured genetic variation was detected in their lifetime investment of each trait and in three out of five traits there was also genetic variation in the patterns of age-related change in investment or activity. This demonstrates considerable variation in the *D. melanogaster* genome that could be moulded by natural selection. As these fly lines were derived from a natural population, the genetic variation observed in this study can be expected to represent the potential for evolutionary change in the wild.

Although genetic correlations between traits were not identified, it perhaps suggests that genetic constraints between physiological processes are not as common as has been proposed. Evolutionary trade-offs between systems may not be as inevitable as some theories suggest.

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Chapter 6: Final Discussion

There is still a lot unknown about the processes and mechanisms of ageing in vertebrate systems. The complexity of the vertebrate immune system adds to the difficulty of investigating links between immune activity and ageing. Though some elements of the immune response decline in activity as individuals age (such as T cell and B cell number and activity (Aberle *et al*., 2013)), other parts increase in activity in older individuals (overly active inflammatory responses in elderly humans (Pereira *et al*., 2013)).

The use of model organisms is a standard practice in elucidating the complex processes that occur in other animals. *Drosophila* is one of the most well studied insect models to date; many immune traits are conserved between *Drosophila* and vertebrates (Eleftherianos & Castillo, 2012; Ramet, 2012). Understanding of the physiological processes in *Drosophila* cannot be straightforwardly extrapolated into vertebrate systems (Flajnik & Pasquier, 2004; Hauton & Smith, 2007). However, short lifespan, amenability to genetic manipulation, ease of rearing large numbers and the plethora of genetic, cellular and proteomic techniques available, all promote *Drosophila*'s use as a model for addressing questions related to ageing and immunity that are difficult to examine in vertebrate systems (Leips, 2009; Schneider, 2000).

This thesis has focussed on studying how the cellular immune response of *D. melanogaster* changes whilst flies age. In specific regard to the study of phagocytosis in *Drosophila,* 70% of the proteins in *Drosophila* phagosomes have mammalian orthologues (Stuart *et al*., 2005). The identification of the scavenger receptor Croquemort in *Drosophila* was one of the first proteins involved in phagocytosis in this insect that was found to be homologous to a receptor with a

similar function in mammals (Franc *et al*., 1996). Further study using *Drosophila* models and often *Drosophila* S2 cells (cultured embryonic cells physiologically closest to plasmatocytes) has been crucial for investigating the molecules and processes involved in phagocytosis in mammals (reviewed by Stuart & Ezekowitz, 2008).

6.1. Senescence in the cellular immune response in *Drosophila melanogaster*

Plasmatocyte haemocytes in adult *Drosophila* have closest similarity to the monocyte lineage that gives rise to macrophages in vertebrates (Williams, 2007). Vertebrate macrophages are less efficient at phagocytosing larger particles (Champion *et al*., 2008) and show an age-related decline in number and phagocytic function (Sebastián *et al*., 2009). In this thesis, I demonstrated that the effect of ageing on plasmatocyte number and function in *Drosophila melanogaster* follows the same trend as observed in vertebrate macrophages. Firstly, the ability of plasmatocytes to phagocytose particles declined as individuals aged from one to four weeks old. Secondly, in female flies there was a reduction in plasmatocyte numbers during ageing.

The number of *Beauveria bassiana* spores or *Escherichia coli* bacteria phagocytosed per haemocyte was a third less in four week old flies compared to one week olds. This was due to an age-dependent decline in the number of phagocytically active cells; the proportion of phagocytosing haemocytes dropped by 30% from one to four week old flies but the number of microbial inclusions per active cell remained constant. This suggests a level of heterogeneity in the haemocyte population as, instead of all the cells demonstrating an age-dependent reduction in phagocytosis ability, some cells remained as active in

aged flies as in younger individuals and other cells appeared to completely lose their phagocytosis ability. As well as assessing the influence of age on plasmatocyte ability to phagocytose microbes, flies were also challenged with latex beads of a range of sizes (0.5µm to 4.5µm in diameter). Though less than half the number of cells phagocytosed 4.5µm beads compared to the smaller sizes (0.5-2.0µm), the rate of senescence in plasmatocyte function was not influenced by the size of the particle or whether it was a latex bead or a microbe. This suggested that the age-related decline in plasmatocyte phagocytic function was due to changes in general aspects of the cell machinery.

6.2. Alterations in the size of the circulating haemocyte population

Plasmatocyte number declined in ageing adult females in experiments throughout this thesis (Chapter 2; **2.4.4**; Chapter 3; **3.4.1**; and Chapter 4; **4.4.4a**). An attempt to determine whether an immune challenge 'used up' plasmatocytes showed no evidence of cell number being influenced by a treatment of live *E. coli* (Chapter 3; **3.4.1.**). However, the results of a later experiment in Chapter 4 suggested that plasmatocyte number *was* influenced by immune challenge, albeit in the unexpected direction of temporarily increasing the size of the circulating cell population (Chapter 4; **4.4.4a**).

A few aspects varied between these two experiments which may account for the conflict in the results. Firstly, before their cell number was assessed flies were treated with live *E. coli* in one study and dead *E. coli* in the other. The immune effects of a live infection often differ from that of a dead immune challenge (Schmidt *et al*., 2008). Perhaps there was not enough live *E. coli* phagocytosed 24hrs post-challenge, possibility because of immune evasion by the bacteria, to

induce the alteration in cell number observed when dead *E. coli* had been used to stimulate the cellular immune response.

Secondly, different techniques were used to count the plasmatocytes in each experiment. Potentially the differences in plasmatocyte number could be due to the technique used to count them. Counting cells using a haemocytometer is standard practice and was also the technique used to originally identify the phenomena of declining plasmatocyte numbers with age (Chapter 2). The assay determining plasmatocyte phagocytic function in **4.4.4a** included the counting of cells during the assessment of phagocytosis efficiency, but did not use a haemocytometer. An experiment which compared the techniques would determine whether the difference in the results was because of the methods used.

However, even though a microbial challenge increased the number of circulating plasmatocytes in the experiment in Chapter 4, there was still an age-dependent decline in the size of this cell population that followed the same trend as control (saline pre-injected) flies (**4.4.4a**). Therefore, plasmatocytes are being removed from the circulating population by an unknown means.

6.3. Mechanisms that underlie senescence in the cellular immune response

Parts of plasmatocyte machinery that may experience age-associated alterations in function are the receptors that recognise and bind to targets for phagocytosis. *Drosophila* does not possess a hematopoietic organ and haemocyte differentiation has not been observed (Lanot *et al*., 2001), so perhaps part of the detrimental effect of ageing is a decline in plasmatocyte ability to identify

phagocytic targets. However, an attempt to investigate the influence of phagocytic receptors and their associated molecular pathways on the age-related decline in plasmatocyte phagocytic function was inconclusive (**3.4.3.**).

Though plasmatocyte activity is influenced by soluble components in the haemolymph, such as opsonins, the age-related decline in their phagocytic ability was shown to be a cell autonomous process (**3.4.2.**). Again the results suggest that a proportion of the haemocyte population loses its ability to engulf microbes, while other cells retain the same efficacy regardless of the age of the fly. So, why are some plasmatocytes unable to phagocytose anything while others demonstrate no age-dependent decline in function?

Functionally youthful haemocytes in aged flies could arise from the sessile haemocyte population. The assays of the efficacy of phagocytosis in this thesis did not measure cell function in the sessile population of haemocytes but only the function of cells circulating in the haemolymph. Sessile haemocytes are also phagocytically active (Elrod-Erickson *et al*., 2000), but the effect of ageing on their function has not been measured in *Drosophila*. The migration of haemocytes between these two cell populations would explain the observed increase in the size of the circulating haemocyte population following infection (**4.4.4a**) and the age-dependent reduction in the number of non-adherent plasmatocytes.

Additionally, plasmatocyte specificity increased in a secondary encounter of a homologous immune insult (immunological priming) (Chapter 4). One explanation for the preferential engulfment of microbes that flies have previously been exposed to is an alteration in plasmatocyte receptor expression. Alternatively, if sessile haemocytes vary in their intrinsic specificity, once they

have phagocytosed microbes they might enter circulation, while circulating plasmatocytes without an inclusion bind to tissues. This would generate the observed increase in proportional haemocyte activity following a homologous priming treatment, whilst allowing the mean number of inclusions in active cells to remain constant (**4.4.1a.**). The temporary increase in the size of the circulating haemocyte population 24hrs after immune challenge supports this hypothesis (**4.4.4a**).

If the sessile haemocyte population is indeed a reservoir of 'healthy' plasmatocytes, it presumably does not possess a limitless supply. Though functionally youthful plasmatocytes are present in aged flies, a larger proportion of the circulating haemocyte population is phagocytically inactive at later ages compared to in younger individuals. So there is still an age-dependent alteration in cellular machinery that has detrimental consequences for overall cell phagocytosis ability.

Studies of plasmatocyte phagocytic activity in five-week old flies observed that these cells accumulated inclusions but appeared unable to degrade or digest them (Lucas Horn, unpublished). The suggestion was that due to age-related decline in the degradation mechanisms within plasmatocytes, these cells became 'choked' with engulfed particles and so became immunologically irrelevant (Eleftherianos & Schneider, 2011). For all the experiments investigating ageing in this thesis, flies were aged in non-sterile, mixed-sex cages that probably permitted exposure to commensal bacteria. Potentially, the older individuals assayed in my work had already accrued a number of immune insults that could have compromised their plasmatocyte phagocytic machinery. This would have diminished the number of 'naïve' youthful haemocytes and so

produced the observed age-dependent change in the proportion of active plasmatocytes.

Another potential mechanism for the observed age-related decline in plasmatocyte phagocytic ability is the cost of the priming response demonstrated in Chapter 4. Although plasmatocytes became more phagocytically active towards microbes that flies had previously encountered, this appeared to be at the cost of phagocytic activity towards novel immune insults. There was a negative effect of pre-exposure to a heterologous immune challenge. When flies had previously been primed with *E. coli* their plasmatocytes phagocytosed up to 22% fewer fungal spores compared to controls. The opposite was also true; flies pre-exposed to *B. bassiana* phagocytosed fewer *E. coli* compared to controls.

The priming response also declined as flies aged. Cells from one week old flies that received a *B. bassiana* priming injection phagocytosed 32% more spores than controls, but there was no enhancement of plasmatocyte phagocytic activity in four week old flies. Additionally, the rate of age-related decline in haemocyte function in flies that had received a homologous injection was double that of the controls, indicating that the age-dependent reduction in the priming response was more than just generally compromised phagocytosis ability.

This gives rise to the possibility that the cost of developing immune memory against previous immune challenges may be the principal driver in the senescence of plasmatocyte phagocytic function in older flies. If the reservoir of sessile haemocytes is being used up for specialisation towards particular immune insults as well as cells being ‘choked’ and becoming immunologically irrelevant, these two processes would combine to generate the decline in plasmatocyte phagocytosis ability observed throughout this thesis.

6.4. The role of the cellular immune response in pathogen defence

This thesis has demonstrated that plasmatocyte number and functional ability decline as flies age. However, what does plasmatocyte phagocytic activity mean for pathogen defence? Furthermore, what does an age-dependent decline in this trait represent?

Plasmatocyte phagocytosis ability influences survival following bacterial infection. Bead injected flies were extremely sensitive to *Streptococcus pneumoniae* infection, with over 50% mortality ~6 days earlier than for non-bead flies (Pham *et al*., 2007). This suggests that survival may be crucially determined by the ability of plasmatocytes to clear *S. pneumonia* from the haemocoel. Additionally, although wildtype flies had increased survival to a lethal *S. pneumoniae* challenge if they had received a priming treatment of the dead bacterium beforehand, if these flies were injected with beads before the priming treatment they died at the same rate as naïve individuals (Pham *et al*., 2007). This demonstrated the importance of plasmatocyte phagocytic activity in the process of immunological priming. Pham *et al*., (2007) also demonstrated a protective benefit of prior injection with dead *B. bassiana* in increased survival to a subsequent lethal challenge with this fungus. Although they did not develop this further to explore the role of plasmatocytes in this priming effect, work in this thesis has shown that plasmatocytes do preferentially phagocytose more *B. bassiana* spores following a priming treatment with this fungus (Chapter 4). The work of Pham *et al*., (2007) and my work in Chapter 4, strongly suggest that survival to *B. bassiana* infection is specifically influenced by plasmatocyte phagocytic activity and not just the activity of other functions that plasmatocytes perform.

In another study, bead injected flies suffered significantly greater susceptiblity to infection with *Micrococcus luteus*, *Enterococcus faecalis* and *Staphylococcus aureus* (Nehme *et al*., 2011). In this case the authors also measured bacterial titres in the haemolymph and showed that microbial clearance was compromised by bead injection; there was no evidence of the immune response being stimulated by the presence of latex beads (Nehme *et al*., 2011).

In this thesis an experiment in Chapter 3 (**3.4.4.**) attempted to determine the role of plasmatocyte phagocytic activity in disease resistance senescence. Although the control genotype used was not fully wildtype (w1118) it was expected to have a wildtype immune response. This genotype was used as the control because it was the background genotype of the RNAi knockdown used to concurrently investigate the role of the humoral immune response in disease resistance senescence (for the full methods refer to **3.3.1.** and **3.3.5.**). However, there were two major set-backs in this experiment. Firstly, there was no evidence of disease resistance senescence in w1118 flies. This was demonstrated by the lack of an interaction between age and infection (injection with oil or *B. bassiana*). Secondly, the bead injection did not increase pathogen susceptibility. There was no interaction between treatment (beads or no beads) and infection.

The experiment is open to criticism in a few aspects. Firstly, no *in vivo* assays were conducted to confirm that bead injection inhibited the phagocytosis of fungal spores. In the same way, unlike the previous study where bacterial titres were monitored (Nehme *et al*., 2011), this experiment did not determine whether the burden of fungal material in the haemolymph was influenced by bead injection. The presence of polystyrene beads in the haemolymph may have up-regulated the humoral immune response, and enhanced Toll pathway activation, compensating for reduced plasmatocyte phagocytosis activity (Nehme *et al*.,

2011). Measuring differences in antimicrobial peptide expression between bead and non-bead flies would have clarified whether this mechanism was the reason for bead injection not increasing fly susceptibility to *B. bassiana* infection. Finally, the genotype w1118 is not wildtype but the reason for its use has already been explained. Potentially, although it was expected that this genotype would be immunologically wildtype, there is the possibility that this was not the case. However, I repeated this experiment on the wildtype genotype Samarkand. Crucially, this is the genotype used to identify that senescence in plasmatocyte phagocytic ability occurred (Chapter 2) so there was a greater expectation that senescence in disease resistance would also be observed in this genotype. However, this was not the case (Appendix 1).

The prior concerns about the methods of these two experiments remain and could be addressed in the future. The conclusion from the experiment in Chapter 3 and Appendix 1 is that the cellular immune response does not have a strong role in determining survival following *B. bassiana* infection.

Genetic variation in susceptibility to *B. bassiana* infection and in plasmatocyte phagocytic ability was identified in the 18 outcrossed genotypes of *D. melanogaster* that were assessed in Chapter 5. Although my bead-injection studies had failed to demonstrate a direct link between the cellular immune response and pathogen susceptibility, this provided an opportunity to identify an association between variation in phagocytosis and variation in susceptibility. Potentially, variation in pathogen defence might be caused by allelic variation in genes influencing plasmatocyte activity. I investigated this possibility using the same statistical analyses that were used to explore correlations between the other immune and life history traits in Chapter 5 (see Appendix 2). However, there was no evidence for strong genetic correlations for any of the comparisons.

The weak, non-significant trends that existed were largely in the predicted direction of a negative correlation between pathogen susceptibility and plasmatocyte phagocytsis ability. However, this retains the caveat that the width of the associated credible intervals preclude any robust conclusions being drawn (Appendix 2).

Perhaps it was naïve to expect there to be a clear link between a single element of the immune response and overall pathogen susceptibility. The immune response of *Drosophila* is a complex amalgamation of multiple systems and processes that contain many redundancies (Lemaitre & Hoffmann, 2007). The humoral immune response can compensate for a compromised cellular immune response through the upregulation of AMP gene expression (Nehme *et al*., 2011). In the context of ageing, microarray studies have shown that AMP gene expression increases as flies age (Landis *et al*., 2004; Pletcher *et al*., 2002; Remolina *et al*., 2012; Seroude *et al*., 2002). Additionally, multiple studies have not found a decline in bacterial clearance ability in ageing *Drosophila* (Felix *et al*., 2012; Lesser *et al*., 2006; Ramsden *et al*., 2008), although survival following infection has been demonstrated to decline with age (Ramsden *et al*., 2008).

Perhaps the phagocytic properties of plasmatocytes are secondary in importance to their role in immune signalling and stimulation of the humoral response in determining pathogen resistance. In addition to phagocytosis, plasmatocytes also have other immune roles including the production of small quantities of AMPs (Irving *et al*., 2005) and opsonins which promote phagocytosis (Lagueux *et al*., 2000). These cells can also produce signalling molecules that interact with other parts of the immune system, such as the Toll ligand Spätzle (Irving *et al*., 2005; Shia *et al*., 2009) and Upd3 which triggers JAK/STAT pathway activity (Agaisse *et al*., 2003). Clearly phagocytosis has a role in immunological priming

(Pham *et al*., 2007; Chapter 4 of this thesis), but in this thesis I have not demonstrated a strong link between plasmatocyte phagocytosis ability and disease resistance, at least for defence against *B. bassiana* infection.

6.5. Genetic variation in immune effectiveness is independent of genetic variation in ageing

Chapters 2, 3 and 4 all identified senescence in the cellular immune response of *D. melanogaster*. However, they did not investigate whether this was a phenomenon all fly genotypes suffer, or whether there is genetic variation in natural populations with some genotypes being less affected by senescence. The force of natural selection declines with age which might mean genetic variation for these late-life traits is not strongly shaped by selection (Medawar, 1952). However, many genes have pleiotropic effects and selection on early-life traits can result in unpredictable effects on late-life traits. Therefore if there is genetic variation present for immune traits then it might be influenced by selection, which could have interesting consequences for late-life and ageing.

Genetic variation was detected in the mean level of investment or activity of each immune parameter and life history trait measured in Chapter 5 (see Table 1; **5.4.**). Genetic variation in the rate of senescence of these traits was also detected for three out of five traits. Interestingly, there was no evidence of correlations between genetic variation in median lifespan and the other traits measured, nor between female fecundity and investment in immune traits. Therefore, although there is evidence of considerable genetic variation in *Drosophila* populations that could be moulded by natural selection, there was no evidence of predicted genetic constraints between physiological processes. Potentially, the genetic variation in immune effectiveness in natural populations may not negatively trade-off with ageing. In this work there was no evidence of a

positive or negative correlation between investment in immune parameters and the measures of ageing investigated.

Additionally, of potential greatest importance to this thesis, there was no evidence of genetic variation in the rate of senesence for plasmatocyte phagocytosis ability in these 18 genotypes. All the genotypes experienced a decline in phagocytic function (Figure 4; **5.4.1d**). The two potential explanations for this finding are that the impacts of ageing on this process are mechanistically fixed across genotypes, or natural selection acts strongly to eliminate variation in this trait.

However, there was an apparently immunologically wildtype genotype in this thesis that did not demonstrate an age-dependent reduction in plasmatocyte phagocytic function. In the experiment in Chapter 3 plasmatocytes from the background control genotype w1118 maintained their phagocytic function regardless of the age of the fly (Chapter 3; **3.4.3.**). Potentially the inbred status of this fly line has unexpected consequences on rates of ageing.

The probable reality is that the results for the investigation into the role of plasmatocyte receptors and their associated pathways in the age-related decline in phagocytic function in wildtype flies were an anomaly. However, possibly a genetic influence that determines senescence in plasmatocyte phagocytic function could be identified in w1118 control cross flies (Act5C-Gal4/+). An experiment that compared plasmatocyte function in one and four week old flies of this genotype against similarly aged Samarkand flies, or the genotypes used in Chapter 5 would clarify whether this was a reasonable possibility.

6.6. Conclusions

The continued use of *Drosophila* for elucidating systems and processes involved in ageing and immunity in vertebrates has been supported by the work in this thesis. Plasmatocytes decline in number and function as flies age, in a similar manner to vertebrate macrophages. The lack of haemocyte proliferation in *Drosophila* suggests that as flies age plasmatocytes may experience mechanistic 'wear and tear' as well as alterations in specificity following immune encounters that potentially impairs their ability to recognise and internalise novel microbes. Though phagocytic cell proliferation occurs in vertebrates and not in *Drosophila*, macrophages still experience an age-dependent reduction in number and function that may suggest a conserved senescent process between the two taxa.

Although a clear link between plasmatocyte phagocytic function and pathogen defence was not demonstrated in this thesis, plasmatocyte activity does significantly respond to immunological priming and may be the key mechanism behind this phenomenon in invertebrates. Due to the additional immune roles of plasmatocytes, perhaps it is not their clearing of pathogens from the haemocoel that is most immunologically important, but rather their stimulation of other aspects of the immune response.

Finally, although evidence of naturally segregating genetic variation was identified for every immunity and ageing trait investigated, evidence for genetic constraints between physiological processes was not found. Potentially this could indicate that though there may be significant genetic variation present within natural fly populations on which selection could act, evolutionary trade-offs between systems may not be as tightly linked as some theories suggest.

Appendix 1

Bead injection improved survival to *Beauveria bassiana* infection in a wildtype genotype

In Chapter 3 of this thesis I attempted to investigate the contribution of age-dependent changes in the cellar immune response to senescence in pathogen defence. I sued the technique of injecting beads into the haemocoel to inhibit phagocytosis by plasmatocytes. I found unexpected results: neither bead injection, nor ageing had clear influences on pathogen susceptibility. Potentially, the lack of an age-dependent increase in non-bead w1118 flies' pathogen susceptibility (Chapter 3; **3.4.4.**) could be an ususual characteristic of this genotype. Therefore, the experiment was repeated using Samarkand flies, as senescence in the phagocytic response had been previously observed in this genotype (Chapter 2; **2.4.1.**). The methods for this experiment were the same as described in Chapter 3. Briefly, flies were reared for two generations at low density following Clancy and Kennington's (2001) method. Adults were then placed in 11 litre demography cages (~400 mixed-sex flies per cage) and aged for between one and four weeks. Density was maintained as previously described (**3.3.1.**). A total of 8 independent cages were used in this experiment.

Flies were separated into single sex vials of 10-flies per vial and flies from each age class (one, two, three and four weeks old) were injected with 0.05µl of 0.2µm beads carboxylate-modified blue fluorescent polystyrene microspheres (Invitrogen) (following Elrod-Erickson *et al*., 2000) (5×10^9 beads ml^{-1}) or 0.05µl of PBS 24hrs before the infection assay. All flies were maintained on standard Lewis food (Lewis, 1960) without the anti-fungal agent 'Nipagen' for 24hrs prior to the pathogen susceptibility assay. This assay was performed as described in

Chapter 3 (**3.3.5.**) and the data were analysed in the same way as the bead injected flies in the previous experiment (**3.3.6.**).

The trends observed in the experiment in Chapter 3 were repeated in this experiment. *B. bassiana* infection severely lowered survivorship ($\chi^2_{(1)} = 6.04$; $P = 0.01$). Pooling across the dataset, the proportional survival of one week old oil injected flies (controls) was 63% higher than that for fungal infected flies of the same age (uninfected proportional survival: 0.76 ± 0.09; infected: 0.36 ± 0.07) (Fig 1).

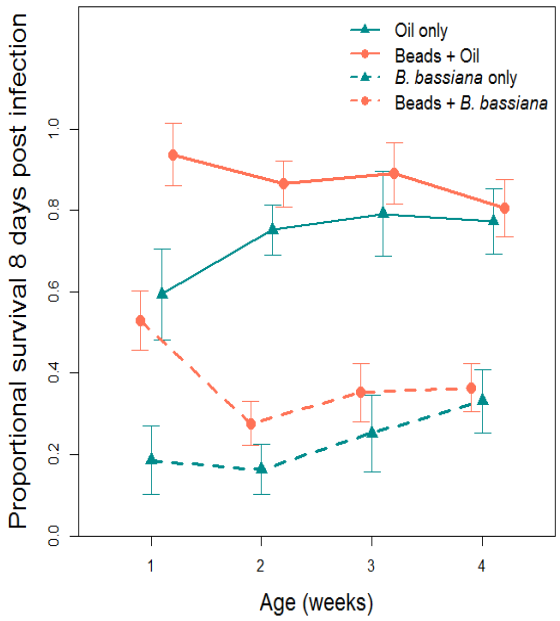

Figure 1: Age-related variation in disease resistance of flies following immune manipulation. Adult Samarkand *Drosophila melanogaster* from four age classes (one to four weeks old). Flies were either injected with 0.2µm polystyrene beads (orange lines) or did not receive a pre-injection (blue lines). 24hrs later, all flies either received a control injection (solid lines) or injection with the fungal pathogen *Beauveria bassiana* (dashed lines). The bead injection aimed to impair the phagocytic cellular response. Bars represent standard errors.

Considering both treatments (beads and no beads), bead injection did not increase pathogen susceptibility. There was a significant treatment x infection interaction ($\chi^2_{(1)} = 4.19$; $P = 0.04$), but this was due to there being higher survival in bead injected flies compared to non-bead flies (proportional survival in one week old infected bead flies was 0.53 ± 0.07, but for infected non-bead flies this was 0.18 ± 0.08). Additionally, the pattern of age-related change in disease resistance between bead and non-bead flies was not significantly different (age x treatment x infection interaction: $\chi^2_{(1)} = 1.16$; $P = 0.28$) (Fig 1). In non-bead flies senescence in disease resistance was not observed. Although there was a significant age x infection interaction ($\chi^2_{(1)} = 6.04$; $P = 0.01$), the direction of this effect was not an age-dependent decline in survival (disease senescence) but an improvement in post-infection survival in older flies. The proportional survival of four week old infected wildtype flies was 45% more than for one week old infected flies (0.33 ± 0.08 compared to 0.19 ± 0.08) (Fig 1).

Therefore the conclusion of this experiment was that once again senescence in disease resistance was not observed. Additionally, prior injection with beads improved survivorship following infection. Potentially, prior bead injection stimulated the immune response instead of impairing it. If the phagocytosis ability of haemocytes was actually enhanced following bead injection this may have caused the observed result. Alternatively, phagocytosis ability may have remained unchanged or impaired (as was expected) but the humoral immune response may have been up-regulated by a bead injection as opposed to a dose of PBS. The increased production of antimicrobial peptides has been demonstrated to improve survival following a bacterial infection when the cellular immune response was impaired in *D. melanogaster* (Nehme *et al*., 2011), so this may be what has happened in this current experiment.

Although bead-induced improvement in survival following a fungal infection was not observed in the experiment in Chapter 3, bead injection did not increase pathogen susceptibility either. Therefore, between the two experiments it seems clear that the method used in this thesis to compromise plasmatocyte phagocytic ability has not resulted in increased pathogen susceptibility or altered the pattern of age-related change in disease resistance in *D. melanogaster*.

Appendix 2

Study of the association between pathogen susceptibility and plasmatocyte phagocytosis ability in 18 outcrossed genotypes of *Drosophila melanogaster*

This thesis has studied a variety of aspects of cellular immune performance in *D. melanogaster*. However, how the quantitative variation in the efficacy of the cellular immune response influences disease susceptibility has not been investigated. The study in Chapter 5 provided an opportunity to explore this link. I assessed variation in the efficacy of plasmatocyte phagocytosis across a panel of wildtype genotypes and studied whether this was associated with variance in susceptibility to the fungal pathogen *Beauveria bassiana*. Thirty-six fly lines from the *Drosophila* Genetic Reference Panel (Mackay *et al*., 2012) were picked randomly and crossed to produce 18 outcrossed lines. Pathogen infection, phagocytosis assessment and multivariate modelling techniques were as described in Chapter 5 (**5.3.5**).

There were no significant genotypic covariance terms between any of the comparisons in pathogen defence and plasmatocyte phagocytosis ability (Table 1). The largest covariance term that did exist reflected a non-significant negative correlation between pathogen defence and plasmatocyte phagocytosis ability in one week old flies (genotypic covariance: -1.60, Lower CI = -5.48, Upper CI = 1.29). This suggested that genotypes in which plasmatocytes phagocytosed a low number of fungal spores suffered higher fungal-induced mortality.

In fact, the trends for all the comparisons are in the expected direction, suggesting the possibility that lower plasmatocyte activity correlates with increased mortality following *B. bassiana* infection.

Table 1: Genotypic covariances between pathogen defence and plasmatocyte phagocytosis ability. Eighteen outcrossed genotypes of *Drosophila melanogaster* from four age classes (one, two, three and four weeks of age) were assessed for susceptibility to *Beauveria bassiana* infection and the mean number of *B. bassiana* spores phagocytosed per 100 plasmatocytes.

	Genotypic covariance	**95% CI (lower, upper)**	**Total %**
Wk1F vs Wk1P	-1.60	(-5.48, 1.29)	4.69
Wk1F vs lifetime P	-0.67	(-3.41, 1.06)	1.71
Lifetime F vs Wk1 P	-0.79	(-2.93, 1.30)	2.28
Lifetime F vs Lifetime P	-0.15	(-0.90, 0.93)	0.54
RSF vs RSP	0.17	(-0.80, 1.02)	0.64

F = fungal-induced mortality

P = plasmatocyte phagocytosis ability

Wk1 = trait values in one week old flies

Lifetime = trait values for all ages

RS = rate of age-related change in each trait

7. References

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