Investigating the pathways of pathogen defence senescence in *Drosophila melanogaster*

Marco Kubiak

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Abstract

Animals commonly become more susceptible to infection as they age. This thesis focused on determining the extent to which deterioration of individual immune mechanisms in *Drosophila melanogaster* contribute to overall pathogen defence senescence.

I investigated how barrier defences and systemic pathogen defences senesce in flies infected with the fungal pathogen *Beauveria bassiana*, testing if these senescent processes are sexually dimorphic. For flies infected by dusting fungal spores onto the cuticle, both sexes became substantially more susceptible to infection while they aged. However, when fungal spores were injected through the cuticle directly into the body, only females demonstrated an age-dependent decline in pathogen defence. This suggests that pathogen defence senescence in females is largely due to impaired systemic defences, whereas for males barrier defence deterioration is mostly responsible. Sex-specific selection on males and females may have led to senescence of pathogen defence occurring by different routes in the two sexes.

Exposure to some pathogens can be reduced through hygiene behaviours; I subsequently investigated if ageing-impairment of hygienic grooming behaviour underlies *D. melanogaster* pathogen defence senescence. Using a dye-dusting assay, I found no evidence that cuticle cleaning ability declined with age. However, although flies of all ages cleaned rapidly, considerably more dye

particles initially attached to the cuticle of older flies, an ageing effect that was stronger in females than males. This may indicate that as individuals age, they must invest more resources in grooming behaviour to maintain body hygiene.

I next tested whether age-associated changes in the cuticular hydrocarbon layer explains the increased adherence of material to the cuticle of older flies. Solvent extraction techniques were used to remove cuticular hydrocarbons and subsequently exchange them between young and old flies. Old flies washed with solvent extract from young flies took on the dye powder adherence characteristics of young flies, and *vice versa*; an effect that was more pronounced in females than in males. These data strongly suggest that the age-dependent increase in particle adherence to the *D. melanogaster* cuticle is driven by cuticular hydrocarbon changes.

Most previous insect pathogen defence senescence studies focussed on changes in the cellular immune response and expression of humoral immune genes. My research demonstrates that senescence of additional non-immunological processes is also important.

Declaration of authorship

I, Marco Kubiak, 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

The ageing human population in many developed countries presents new challenges for us to understand the processes of senescence, in order to treat or manage the many debilitating conditions that are associated with old age. With an average life expectancy of over 70 years (World Health Organization 2016), longitudinal *in vivo* age-related studies in humans are challenging because the process of ageing is extremely slow. This frequently makes it necessary to study model systems like *Caenorhabditis elegans*, *Drosophila melanogaster* or mice.

The use of model species in ageing research presents many challenges (Partridge and Gems 2007). Using model organisms has advantages because they are typically available in large quantities for experimentation and are relatively cheap or easy to rear, thus making it practical to use them in large-scale genetic screening experiments (Hamilton et al. 2005). On the other hand, model organisms have clear disadvantages. The findings of studies on model organisms may sometimes have limited applicability for human ageing: whilst some mechanisms are highly conserved between humans and the primary model systems (Gershon and Gershon 2000), others differ to a great extent (Longo and Fabrizio 2002). Whilst humans exhibit a slow ageing process, many models are chosen due to their short lifespan, and are furthermore often raised under laboratory conditions that select for high fecundity in early life, which further

shortens lifespan and potentially renders some model organisms unrepresentative of wild populations (Spencer and Promislow 2002).

Even if there are certain disadvantages to studying model systems, these are frequently outweighed by their benefits. *D. melanogaster* is one of the most-studied model systems for ageing research. *D. melanogaster* shares hundreds of gene-counterparts with humans (Fortini et al. 2000) and immune pathways in particular have been shown to be highly conserved between *D. melanogaster* and vertebrates (reviewed in: Kimbrel and Beutler (2001), Hoffmann and Reichhart (2002), Eleftherianos and Castillo (2012), Rämet (2012)) . Moreover, studies in *D. melanogaster* have become one of the principal models to study the biological interactions between the ageing process and immune system function (Grotewiel et al. 2005, Zerofsky et al. 2005).

1.1 Why study insect immunity?

Despite serving as a valuable model in biomedical research, the study of insect immunity is important in its own right. Economic concerns gave this research field early attention: Bassi (1836) discovered that white muscardine disease in the commercially important silk worm *Bombyx mori* was caused by *B. bassiana*, whereas Metchnikow in 1880 initiated the first trails of the fungus *Metarhizium anisopliae* to control grain beetles (Lord 2005). Damage of crops by insects is still

a major problem in agriculture with millions of pounds worth of damage globally every year (Savary et al. 2012), for this reason understanding the interactions between insects, including *Drosophila*, and their natural enemies is crucial (e.g. Alnajjar et al. 2017). Moreover, insects play ecologically important beneficial roles as pollinators: global declines in honeybee abundance are to some extent because of compromised immunity to their parasites (Gregory et al. 2005). Finally, a wide range of vector-borne diseases are transmitted by insects, for example mosquitos and tsetse flies, representing a major problem in global health (Gubler 1998). Understanding the ability of these parasites to survive the vector's immune system is crucial to address disease prevention.

1.2 Immunosenescence

The term immunosenescence describes the age-related deterioration of a host's immune system defence mechanisms against pathogens. Generally, the immune system can be divided into two major parts: the innate immune system and the adaptive immune system. The innate immune system comprises the epithelia as a physical barrier, phagocytic cells (haemocytes in invertebrates and leukocytes in vertebrates), dendritic cells and natural killer (NK) cells in vertebrates, as well as circulating plasma proteins (Abbas et al. 2015). In contrast to invertebrates, which have only an innate immune system, the immune system of vertebrates additionally comprises adaptive immunity, which is epitomised by 'immune

memory' (Murphy and Weaver 2016). Adaptive immunity has two components: humoral immunity provided by B cells that secrete antibodies after activation, and cellular immunity by T cells (including helper and cytotoxic cells) (Gemsa et al. 1991, Litman et al. 2010, Spiering 2015). The outstanding role of adaptive immunity is to provide immune memory of antigens derived from pathogens that have previously been encountered. This memory means that a highly specific and faster immune response can be mounted upon secondary activation by the same antigen (Delves and Roitt 2000, Zanetti 2001). Due to a decline in the abundance and activity of B and T cells (Riley et al. 2005, Prelog 2006, Weng 2006), older humans tend to have impaired immune function making them more susceptible to diseases and providing poorer responses to vaccination (Gardner 1980, Yamaya et al. 2001, Hof 2010). This age-related decline in vaccination response has been shown for a number of immune challenges, including Hepatitis B vaccination, where antibody formation was significantly reduced (Rosenberg et al. 2013) and has also been demonstrated by lower responsiveness to Influenza vaccination (Deng et al. 2004, Goodwin et al. 2006).

Invertebrates lack an adaptive immune system but otherwise share similarity in how many immunological processes operate, making them highly-valuable model study systems. Within invertebrates, *D. melanogaster* is clearly one of the most studied immunological models and shares highly conserved molecular pathways with humans, for example hematopoietic factors (Fossett

and Schulz 2001, Evans et al. 2003) and the NF-κB orthologues involved in *D. melanogaster* anti-microbial peptide (AMP) expression (Georgel et al. 1993, Kappler et al. 1993). Age-related changes in immunity have been show in various invertebrates, including: in *Aedes aegypti* where ageing results in a decline in haemocyte numbers (Hillyer et al. 2005) but also (depending on diet) in lower susceptibility to *B. malayi* nematode infection (Ariani et al. 2015); in an age-related decline in survivorship of wildtype *C. elegans* after infection by various bacterial pathogens (Laws et al. 2004); and in *D. melanogaster* where an age-related decline in the ability to clear a bacterial infection was associated with changes in insulin signalling/TOR pathway gene expression (Felix et al. 2012).

1.3 Immunity in *Drosophila melanogaster*

Within their natural environment insects continuously face immune challenges from pathogens (Kaya and Vega 2012). Pathogen defence by the epithelial barriers is an important first line of defence. The epithelial barriers not only comprise the cuticle, which cannot easily be penetrated (Tsakas and Marmaras 2010), but also the gut, reproductive tract and tracheal system, which are potential entry points for microbes (Lemaitre and Hoffmann 2007, Lemaitre and Miguel-Aliaga 2013). Beside these routes for horizontal transmission of infections, pathogen infection can also occur via vertical transmission, as in the case of the α-proteobacterium *Wolbachia*, that is usually vertically transmitted

from the mother to her offspring (Werren 1997, Hong et al. 2002) and for vertical transmission of the Sigma virus in *D. melanogaster* (Longdon et al. 2011).

1.4 Humoral immune defence in *Drosophila melanogaster*

The insect humoral immune response leads to rapid production of AMPs in response to a microbial challenge. AMP production in *D. melanogaster* originates from different sources, including the epithelia and the plasmatocyte blood cells (Lavine and Strand 2002, Govind 2008), however, the major production site is the fat body (Hoffmann 1995), which is functionally equivalent to the mammalian liver (Tsakas and Marmaras 2010). After the first discovery of the antibacterial immune response in *D. melanogaster* (Boman et al. 1972), Cecropin was the first isolated AMP, from the moth *Hyalophora cecropia*. Since then, AMPs have been discovered not only in insects but also in plants and vertebrates. In *D. melanogaster* AMPs comprise three groups with a) antifungal, b) antibacterial, or c) general antimicrobial properties (Lemaitre et al. 1997). AMP genes contain an upstream promoter sequence that shows high sequence similarity to the mammalian NF-κB transcription factor binding sites and these genes rapidly transcribe AMP mRNA after an immune challenge (Engstrom et al. 1993, Kappler et al. 1993). In *D. melanogaster* three NF-κB transcription factors have been identified, Dorsal, Dif and Relish (Ip et al. 1993, Reichhart et al. 1993, Hultmark

2003), which drive expression of seven different classes of AMP genes, reviewed by Imler et al. (2005).

1.5 Recognition of microbes

Different classes of pathogens activate different immune pathways in *D. melanogaster*, which ultimately lead to the expression of the appropriate AMPs to fight an invading pathogen (Lemaitre et al. 1997). Detection of a pathogen requires particular receptors in the host, called pattern recognition receptors (PRRs) which bind matching microbial molecules. The main regulatory pathways in *D. melanogaster* for the expression of AMPs are Toll and Imd. In the Toll pathway, receptors are principally peptidoglycan recognition proteins (PGRPs), whereas in the Imd pathway Gram-negative binding proteins (GNBPs) are primary responsible (Lee et al. 1996, Yoshida et al. 1996, Medzhitov and Janeway 1997, Janeway and Medzhitov 2002, Medzhitov 2007). Structurally, peptidoglycan (PGN) contains a carbohydrate backbone of alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid, with the N-acetylmuramic acid residues cross-linked to peptides (Mengin-Lecreulx and Lemaitre 2005). In terms of immune activation the major difference between the two bacterial groups is that in Gram-negative bacteria, the lysine (Lys) residue at the third position in the peptide chain is substituted with a meso-diaminopimelic acid (DAP) residue (or an amidated-DAP in Gram-positive bacilli), whereas the

majority of Gram-positive bacteria have a Lys residue in this position. *D. melanogaster* PGRPs can discriminate between DAP-type PGNs or Lys-type PGNs (Kaneko et al. 2006).

In *D. melanogaster*, the detection of fungal pathogens does not solely rely on PRRs: a dual detection system exists. First, the PRR GNBP3 detects β-(1,3)-glucans which are found in the fungal cell wall and activates the Toll pathway. It also has been shown that overexpression of GNBP3 in the absence of an infection activates this pathway. The second mechanism is independent of GNBP3. Fungi can penetrate the cuticle of a host through enzymatic degradation and it has been shown for the entomopathogenic fungus *Beauveria bassiana*, that one of its proteases directly activates the Toll pathway in this case (Ligoxygakis et al. 2002, Gottar et al. 2006).

1.6 The Toll pathway

In *D. melanogaster*, the Toll pathway, named for the cell surface receptor Toll, activates the immune system through an NF-κB transcription factor action (Lemaitre et al. 1996). Toll was first identified by Rosetto et al. as an activator of the immune response in *D. melanogaster* (Rosetto et al. 1995). It predominantly triggers immune responses against Gram-positive bacteria or fungi (Lemaitre et al. 1997). Homologous Toll-like receptors (TLRs) are found in mammals, but in contrast to the *D. melanogaster* Toll receptor they directly interact with and detect

microbial molecules during an infection and don't rely on other proteins (e.g. PGRPs) to detect an infection first and signal the information to Toll, as in *D. melanogaster* (Medzhitov et al. 1997). Moreover, *D. melanogaster* Toll not only plays an important role in immunity but also in developmental processes (Halfon et al. 1995, Belvin and Anderson 1996) whereas no evidence of a developmental role has been found in mammals (Kimbrell and Beutler 2001).

1.7 The Imd pathway

In contrast to the Toll pathway, the Imd pathway is initiated by peptidoglycan recognition proteins (including PGRP-LC) that mediate DAP-type peptidoglycan recognition from Gram-negative bacteria (Choe et al. 2002, Ramet et al. 2002). Following the activation of Imd, the NF-κB transcription factor Relish is activated and the active N-terminal part of this molecule translocates to the nucleus to trigger the transcription of genes coding for AMPs, such as *Diptericin* and *Cecropin* (Silverman et al. 2000, Stoven et al. 2000, Stoven et al. 2003).

1.8 The JAK/STAT pathway

The third main immune signalling pathway in *D. melanogaster* is the JAK/STAT pathway, which has been shown to play an active role in wound healing after epithelial damage (Agaisse and Perrimon 2004). Briefly, the pathway comprises

four major components: the *dome* gene that encodes a transmembrane protein that activates the pathway, the secreted protein Udp that activates the tyrosine kinase Hopscotch (HOP) and STAT that then activates target genes after translocation into the nucleus (Agaisse et al. 2003, Agaisse and Perrimon 2004).

1.9 Cellular immune responses in *Drosophila melanogaster*

In contrast to mammals, *D. melanogaster* has an open circulatory system (haemocoel), that is filled with haemolymph. Blood cells, called haemocytes, are either attached to different tissues or circulate freely (Markus et al. 2009). There are three classes of mature haemocyte: plasmatocytes, lamellocytes and crystal cells (Rizki 1978). Plasmatocytes are phagocytes that can be found in the *D. melanogaster* embryo, larva and adult, where their main functions are to engulf foreign pathogen cells or apoptotic host cells (Rizki and Rizki 1980, Lavine and Strand 2002). As well as pathogen engulfment, they also synthesise AMPs and cytokines (Pastor-Pareja et al. 2008). Crystal cells carry out a humoral immune response that ultimately leads to the synthesis of melanin around a foreign invader, or at the site of an injury or infection. To achieve this crystal cells synthesise and release prophenoloxidases (PPO) into the haemolymph where they are activated by serine proteases (Bidla et al. 2007). The resulting phenoloxidase (PO) molecules then participate in the formation of melanic compounds from tyrosine precursors (González-Santoyo and Córdoba-Aguilar

2012). Binggeli et al. (2014) have recently shown that two out of three prophenoloxidase genes in *D. melanogaster*, PPO1 and PPO2 play an important role in survival of flies following infection with Gram-positive bacteria and fungi (Binggeli et al. 2014). So far, crystal cell have not to be shown to exist in adult *D. melanogaster* (Honti et al. 2014), but debate exists in the literature (Kurucz et al. 2007, Ghosh et al. 2015). The third cell type, lamellocytes, are only produced in larvae and are induced following parasite attack; these cells can encapsulate particles that are too large for engulfment by phagocytes (Lanot et al. 2001).

1.10 Immune Responses in the barrier epithelia

For insects such as *D. melanogaster*, which is continuously in contact with potentially harmful microbes, protection though epithelial defences to prevent systemic infection is of great importance. Part of this barrier defence involves the localised expression of AMPs in epithelia (Tzou et al. 2000). Localised AMP expression is under control of the Imd pathway: flies in which Imd function has been interrupted in the gut show higher susceptibility to bacterial infection (Onfelt et al. 2001, Liehl et al. 2006, Nehme et al. 2007). In addition to AMPs, reactive oxygen species (ROS) are rapidly produced in the *D. melanogaster* gut in response to immune challenge. ROS production depends exclusively on expression of the *dDuox* gene; flies in which *dDuox* is silenced by RNAi showed significant higher mortality rates after infection (Ha et al. 2005). However, a tight

regulation of ROS expression is necessary to avoid oxidative damage (Ley et al. 2008, Kim and Lee 2013). The role of the *dDuox* gene in gut inflammation has recently been reviewed extensively in the *D. melanogaster* system (Kim and Lee 2013).

1.11 Immunosenescence in *Drosophila melanogaster*

Not many studies have addressed immunosenescence in *Drosophila*. An early study found increased numbers of *E. coli* in older flies after inoculation, which they concluded was associated with a loss of xanthine dehydrogenase activity in older flies (Kim et al. 2001). Zerofsky et al. (2005) found increased transcription of the AMP diptericin in older *D. melanogaster* after septic infection with live bacteria; however, in contrast, when flies received killed bacteria older females induced less *diptericin* expression. This may indicate age-dependent senescence in the ability to transcribe *diptericin*. Results of another study showed that the bacterial burden with aerobic and anaerobic bacteria inside and outside the fly increases with age but that this has no effect on the lifespan; it was concluded that old flies may mount a strong immune response with no costs to their lifespan (Ren et al. 2007). Ramsden et al. showed that survival of bacterial injection decreased with increasing age of the flies but that bacterial clearance ability did not change (Ramsden et al. 2008), leading the authors to conclude that infection tolerance declines with age rather than resistance. More recent studies have

focused on the cellular and genetic processes underlying *Drosophila* pathogen defence senescence. The number of circulating plasmatocytes decreased in old female flies but not in males, moreover the ability of blood cells to phagocytose *E. coli* and *B. bassiana* decreased significantly in older individuals (Mackenzie et al. 2011). These results show that immune senescence occurs at the cellular level, however the difference in age-dependent changes in haemocyte numbers between the sexes is still unexplained. Another study investigated the impact of ageing on phagocytosis by heart-associated haemocytes in *D. melanogaster*, unlike Mackenzie et al. (Mackenzie et al. 2011) this work found no change in initial activity of haemocytes with age, but suggested that these cells became clogged with phagocytic inclusions during the immune response, impairing further microbial clearance (Horn et al. 2014).

1.12 Non-immunological pathogen defence

The term hygiene describes a range of processes that are important to maintain health and prevent diseases, especially through cleaning. The process of grooming can be observed in vertebrates and invertebrates and is an important behavioural process in host-pathogen interactions; the behaviours involved have been described by Sachs (Sachs 1988). Grooming has been characterized in many different species, including insects (Hlavac 1975), birds (Cotgreave and Clayton 1994) and crustaceans (Martin and Felgenhauer 1986). Regardless of the species,

self-grooming or allo-grooming serves a variety of functions, but a principal purpose is to clean pathogens or other particles from the cuticle (Bret and Ross 1986, Currie and Stuart 2001, Invernizzi et al. 2015). In *D. melanogaster*, there are only a few reports on grooming behaviour. These demonstrate that grooming is induced by contact with chemicals like LPS from *E. coli* as well as quinine and NaCl in high concentrations (Yanagawa et al. 2014). The grooming process comprises various movements that are carried out in a highly organized sequence, for example the fly balances on four legs while cleaning the ventral side of the thorax with the first two legs (Szebenyi 1969). It has also been demonstrated that flies increase grooming when maintained in groups (Connolly 1968). Inducing mutations in flies has led to the characterisation of six mutants with impaired grooming ability. These mutations most likely influence the central nervous system (CNS) or the muscular system (Phillis et al. 1993).

1.13 Cuticular hydrocarbons in insects, their role and how chemical composition changes in the aging organism

Hydrocarbons (HCs) serve many important functions in insects. Long-chain hydrocarbons contribute to waterproofing the cuticle and are crucial for survival in most insects to protect the body from desiccation (Kühnelt 1928, Ramsay 1935). On the other hand, insect cuticular hydrocarbons also play an important role in insect communication, where they serve as pheromones, as well as being

involved in gender and nestmate recognition. Chemical composition has been studied extensively using gas chromatography (GC) as an analytic tool: Baker et al. (Baker et al. 1963) analysed the cuticle hydrocarbon profile of the American cockroach (*Periplaneta americana*). Their analysis revealed three major classes of insect HCs: *n*-Pentacosane (an alkane), 3-Methylpentacosane (a methyl-branched alkane) and (*Z*,*Z*)-6,9-Heptacosadiene (an unsaturated hydrocarbon), which represent 97% of the insect hydrocarbons. Improved analytical techniques have led to analysis of cuticular hydrocarbons from hundreds of insects and this area still shows considerable research activity (Cvačka et al. 2006), potentially revealing previously unknown compounds that play a role in host defence.

I will provide an overview of various important aspects of insect hydrocarbon chemistry, focusing mostly on specific examples from *D. melanogaster*. There are three major classes of cuticular hydrocarbons. The n-alkanes (which represent the simplest organic molecules consisting of only carbon and hydrogen, with only single bonds between the carbon atoms), with a chain length between C21 to C31, that are not only present in almost every insect but also have great variation in the quantity and distribution of carbon chain lengths across species (Jackson 1972, Nelson et al. 1988). Unsaturated hydrocarbons (with double bonds between some carbon atoms), also referred as olefins, can be found in most insects. These compounds have important functionality in chemical communication where they act as pheromones, first shown with (Z)-9-Tricosene in the house fly (Carlson et al. 1971), whereas in

D. melanogaster (*Z*,*Z*)-7,11-heptacosadiene plays an important role as a pheromone (Jallon and David 1987). The third class are methyl-branched alkanes which are not as abundant in *Drosophila* as the other two hydrocarbon classes.

The distribution of hydrocarbons on the cuticle and the mechanisms of how transport to the cuticle and molecular arrangement take place have not been determined in great detail due to limitations in current techniques. However, work on hydrocarbons from *Megacyllene robiniae* (Ginzel et al. 2003) provides evidence that pheromonic compounds, which are essential in communication, are located close to the surface. In *Drosophila*, extensive research has been carried out on chemotaxonomy and sex pheromones, demonstrating that hydrocarbons can serve important roles in species discrimination, with examples including *D. simulans* (Jallon 1984), *D. sechellia* (Cobb et al. 1990) and *D. paulistorum* (Kim et al. 2004).

Hydrocarbons are vital for nestmate recognition in social insects, helping identification of non-nestmates or other invading species; for example nestmates showed more aggressive behaviour after cuticular hydrocarbons from two different nests of worker termites were exchanged (Takahashi and Gassa 1995). Hydrocarbons also play a vital role in reproduction in the ant *Camponotus floridanus*: as queen fertility declines, this is perceived by worker ants through changes in surface hydrocarbon profile, triggering changes in worker behaviour (Endler et al. 2006). In *Drosophila*, hydrocarbons play an important role in courtship and mating (Marcillac and Ferveur 2004).

Studies have demonstrated that hydrocarbon profiles in *Drosophila* change while the fly is ageing. This shift results from the increased synthesis of hydrocarbons with longer chain length (Everaerts et al. 2010, Kuo et al. 2012). One study reported the opposite result (Jackson and Bartelt 1986) but these authors only tested changes in flies up to 8 days of age; whereas studies showing increased hydrocarbon chain lengths tested truly old flies, up to an age of 71 days. Unfortunately, there are no data available to identify whether these senescent alterations in the composition of cuticular hydrocarbons in the ageing insect impact pathogen defence. A speculation would be that the described age-associated shift in hydrocarbon composition may, for some pathogens, provide and environment that enhances infection ability and therefore contributes to host pathogen defence senescence (Boucias and Pendland 1991, Doss et al. 1995).

1.14 Natural *Drosophila* fungal pathogens

Studies about fungal infections in natural *Drosophila* populations are rare. However, a recent study investigated total microbial diversity in wild caught *D. melanogaster*: the authors collected flies from tropical fruits in Puerto Rico and isolated 314 different microorganisms, including 171 associated fungi (Ramírez-Camejo et al. 2017). However, this study did not test if these fungi were pathogenic, or if alternatively they were commensal and only use the fly as a transmission vector (as described in Keebaugh and Schlenke (2014)).

Additionally, *Entomophthora muscae*, a behaviour-manipulating fungal fly pathogen, has recently been found in *Drosophila* in the wild (Elya et al. 2017). Nevertheless, the scarcity of knowledge of fungal fly pathogens in nature doesn't undermine the experimental power of *D. melanogaster* in infection research e.g. (Apidianakis et al. 2004, Lionakis et al. 2005, Lamaris et al. 2007, Alnajjar et al. 2017): a considerable number of humoral immune defence molecules in *Drosophila* target fungi indirectly (Lemaitre and Hoffmann 2007), indicating that fungal pathogens are an important aspect of this insect's natural host-parasite interactions.

1.15 The entomopathogenic fungus *Beauveria bassiana*

My research work used the entomopathogenic fungus *B. bassiana* to assess infection susceptibility in *D. melanogaster* flies. This fungus was first investigated by Bassi in 1835 as the cause of a disease in the silkworm *Bombyx mori*, reviewed by (Porter 1973); whereas the first detailed review about taxonomy and morphology was published later (Macleod 1954). *B. bassiana* naturally occurs in various soil types across the globe, it has been isolated from a range of locations, including: orchards in the United States (Shapiro-Ilan et al. 2003), soil samples from Moroccan forests (Imoulan et al. 2011), farmland in Poland (Tkaczuk et al. 2014), as well as in forest, pasture and cropland in New Zealand (Barker and Barker 1998). Alongside its wide geographic range, the fungus infects a wide

diversity of insect host species (with more than 700 reported (Li 1988)) and is a potent pathogen against many of them. Pathogenicity is not universal among entomopathogenic fungi and their isolates. This has been shown in cereal aphids where different fungal isolates differ greatly in their virulence (Feng et al. 1990) and in *D. melanogaster*, where different strains of *B. bassiana* and *Metarhizium anisopliae* induce different levels of mortality (Toledo et al. 2006). An evaluation of the effects of entomopathogenic fungi against the onion maggot *Delia antiqua* also showed strong differences in pathogenicity across a set of fungal isolates (Davidson and Chandler 2005). Indeed the pathogenicity and virulence of various entomopathogenic fungi has been reviewed in (Vestergaard et al. 2003). However, the application of entomopathogenic fungi as bio-control agents in the field must be carefully considered because the high virulence and broad host range of fungi such as *B. bassiana* can present dangers for non-target species (Howarth 1991).

The main route by which entomopathogenic fungi enter the host is via penetration of the host cuticle (Charnley 1984). To start penetration, spores must first adhere to the host cuticle (Boucias and Pendland 1991, Holder and Keyhani 2005, Dong et al. 2009). Next, spores germinate and use various chitinase, protease and lipase enzymes to digest the cuticle and subsequently allow fungal material to enter the host haemolymph (Stleger et al. 1986). Following invasion of the host, entomopathogenic fungi secret secondary toxins to induce pathology before killing the host (Vey et al. 2001).

1.16 The contribution of this work to the research field

Organisms get more susceptible to infection as they age (e.g. Muller et al. 2013). Changes in the immune system, such as changes in the ability to phagocytose foreign invaders (Mackenzie et al. 2011) and changes in gut immune responses (Jasper 2015) may play a strong role in driving this senescence of infection resistance. However, beside senescent changes in classic immune pathways, deterioration of more general determinants of infection susceptibility might also be responsible, for example: changes in ability to acquire energy resources for infection defence; behavioural changes that alter exposure to infections; changes in hygiene; or changes in barrier defence integrity. Thus far, no study has investigated the consequences of, or processes underlying, the senescence of barrier defences against pathogens in *D. melanogaster*, nor has age-dependent deterioration of hygiene been studied: I will address these topics in this thesis.

Thesis Aims

1. **Explore to what extent ageing-associated changes in barrier and systemic immune defences contribute to senescence of pathogen defence in *Drosophila melanogaster*.**

   Recent studies about immune senescence in *Drosophila melanogaster* focused primarily on systemic changes in the expression of immune genes (Zerofsky et al. 2005) or on changes in the ability of immune cells to phagocytose microbes (Mackenzie et al. 2011). However, the barrier epithelia also represent an important aspect of pathogen protection. The first chapter of this thesis investigates quantitative age-dependent changes in *D. melanogaster* pathogen defence against *B. bassiana*, when infections occur either via the epithelia or via systemic injection. I aimed to analyse whether the impact of senescence on pathogen defence via these two infection routes varies with age, and if these processes differ between the two sexes.

2. **Explore if a decline in hygienic behaviour occurs with age and if changes in cuticle properties might contribute to pathogen defence senescence.**

Pathogen defence senescence in *D. melanogaster* occurred in part by changes in barrier defences (see Chapter 2). To explore this observation further, I tested if this results from an ageing-decline in the ability of flies to clean foreign particles off the body. Grooming is an important process in insect disease defence (Zhukovskaya et al. 2013) and can be induced by foreign particles in *D. melanogaster* (Zhukovskaya et al. 2013). Secondly, I tested for age-dependent changes in the cuticle composition of flies, specifically by extracting and reapplying cuticular hydrocarbons from flies of different ages.

Chapter 2 : Sex-specific routes to immune system senescence in *Drosophila melanogaster.*

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2.1 Abstract

Animal immune systems change dramatically during the ageing process, often accompanied by major increases in pathogen susceptibility. However, the extent to which deteriorations in canonical systemic immune processes are causally responsible for senescent elevations in infection mortality is unclear. We studied *Drosophila melanogaster* and compared the relative contributions of impaired systemic immune defences and deteriorating barrier defences to increased pathogen susceptibility in aged flies. To test changes in systemic immune response efficacy we injected one and four-week old flies with the entomopathogenic fungus *Beauveria bassiana* and studied subsequent mortality; whereas to include the role of barrier defences we infected flies by dusting the cuticle with fungal spores. We show that the processes underlying pathogen defence senescence differ between males and females. Both sexes became more

susceptible to infection as they aged: for males, this was principally due to deterioration in barrier defences, whereas for females systemic immune defence senescence was mainly responsible. We discuss the potential roles of sex-specific selection on the immune system and behavioural variation between males and females in driving these different senescent trends.

2.2 Introduction

Advanced age is often accompanied by increased infection burden (Gardner 1980). Whilst many immunological processes show patterns of senescence (Castle 2000), the extent to which changes in any individual immune parameter drive age-dependent elevations in infection susceptibility is often unclear. Much research focusses on senescence of conventional immune response pathways (Chaplin 2010). However, the contribution of changes in other factors, such as barrier defence efficacy, hygiene behaviours and parasite exposure patterns, to age-dependent elevations in infection rates are often overlooked. In this study, we used the model insect *Drosophila melanogaster* to investigate the relative contributions of age-dependent declines in systemic immune responses and ageing deterioration in barrier defences to overall senescence of pathogen defence.

Strong sex-specific selection for reproductive strategy and other aspects of life history, including parasite resistance, means that males and females often differ greatly in immunity, lifespan and ageing. In humans, males have shorter lifespan and are generally found to suffer earlier immunosenescent degeneration than females (Caruso et al. 2013). Divergent life-histories in males and females might lead one to predict that different aspects of pathogen defence would show sex-specific changes during the ageing process (Zajitschek et al. 2009). However, explicit comparisons of immunosenescent processes in males and females are rare outside humans (Rolff 2001, Schmid et al. 2008). We addressed this by investigating whether the relative rates of barrier defence senescence and systemic immune senescence differ between the two sexes in *D. melanogaster*.

In comparison to vertebrates, the patterns of age-dependent change in immune parameters and pathogen susceptibility in insects are less clearly established. Even for insects with strong applied relevance, the insect vectors of human disease, studies vary in whether they detect age-associated changes in infection susceptibility, as well as in the direction and the magnitude of these changes (Ariani et al. 2015). Senescent declines in immune function are reported in the worm *Caenorhabditis elegans*, the cricket *Gryllus assimillis* and the bumble bee *Bombus terrestris* (Kurz and Tan 2004, Moret and Schmid-Hempel 2009, Park et al. 2011). In the best-studied insect immunological model, *D. melanogaster*, the few studies on immunosenescence have produced diverse results (Zerofsky et al.

2005, Lesser et al. 2006, Ren et al. 2007, Ramsden et al. 2008, Mackenzie et al. 2011). Lesser et al. (2006) found an age-associated *increase* in the rate of bacterial clearance from the haemocoel in nearly half of wildtype genotypes, whereas other genotypes showed either no change, or evidence of senescence. Ramsden et al. (2008) found no decline in ability to clear bacterial infection with age, but suggested that infection-tolerance declines in older flies. The induction of humoral immune response genes following infection changes as flies age (Zerofsky et al. 2005), the ability of immune cells to phagocytose pathogens deteriorates (Ramsden et al. 2008, Mackenzie et al. 2011, Horn et al. 2014) and in females, but not males, immune blood cell numbers decline (Mackenzie et al. 2011).

Here we investigate how the ability of *D. melanogaster* flies to defend against infection by the entomopathogenic fungus *Beauveria bassiana* declines with age. This fungus will naturally infect *D. melanogaster*: when spores contact the fly cuticle they germinate, penetrating this barrier defence before growing systemically in the haemocoel (Clarkson and Charnley 1996). It is also possible to inject *B. bassiana* spores directly into the fly, circumventing cuticle defences to test the efficacy of systemic immune processes. We assessed age-dependent changes in fly mortality following these two infection routes, testing the relative contribution of declines in barrier defences and systemic immune responses to overall senescence of pathogen protection. Our work demonstrates that the

relative importance of these two senescent processes differs strongly between males and females.

2.3 Materials and methods

Fly culturing:

We reared flies at a constant density to the age of 1 and 4 weeks following the methods of (Mackenzie et al. 2011). We placed Samarkand genotype *D. melanogaster* adults (Bloomington Stock Centre) into a laying cage and collected eggs for 24 h on apple juice agar plates seeded with yeast. Following (Clancy and Kennington 2001), eggs were washed from the plate with PBS buffer then a 13 µl volume of packed eggs was added to the food medium in fly bottles with a pipette. When adult flies emerged, they were transferred to age in 11 litre fly cages at a 1:1 sex ratio with 200 flies per cage. Every two days cages were provided with a new petri dish of fly food and a vial of water. Cages were set up weekly in groups of three: two cages to supply known-age flies to experiments and a third to provide flies in order to maintain the other two cages at constant density by replacing flies that died or were used in experiments. All flies were fed Lewis *Drosophila* medium (Lewis 1960) and maintained at 25°C, 12 h L/D throughout.

Pathogen Infection:

B. bassiana spores originating from a previous experimental strain 193–825 (IMI 391510) (Tinsley et al. 2006) were grown by plating onto potato dextrose agar containing chloramphenicol antibiotic (5×10^{-5} g ml^{-1}). Plates were incubated at 25°C in the dark, then dried for five days at room temperature. After sporulation, spores were scraped off the plates, pooled and stored at 4°C in tubes containing silica gel to protect against moisture.

To infect flies via cuticle inoculation dry fungal spores were mixed 1% w/w with agar powder, then this mixture was scattered into a 9-cm petri dish. CO_2-anaestethized flies in single-sex batches of 10 were shaken in the petri dish until flies were uniformly covered, then put in vials with standard Lewis food medium that did not contain nipagin (to exclude possible interference of this anti-fungal agent with mortality patterns). An equal number of flies also received a control procedure, where flies received the same dusting treatment with agar only. To infect flies systemically, circumventing the cuticle defences, we injected the pathogen. For this assay fungal spores were suspended in oil (87.5%: Shellsol T, 12.5% Ondina EL) at 1.3 x 10^8 spores/ml (Tinsley et al. 2006); single-sex 10-fly groups were CO_2 anaesthetized and pricked in the thorax with a stainless needle (AgnTho's minutien pins No. 26002–20, 0.2 mm diameter) that had been dipped into the fungal spore suspension. Flies were then transferred to fresh nipagin-free food vials. Control flies received identical oil-only injections. Flies that died

within 2 hours were not considered to have died from the pathogen and excluded from analysis. For both the cuticle inoculation and injection experiments, vials were subsequently inspected at the same time daily and dead flies counted; flies were changed to new vials every 2 days.

Statistics:

To compare the age-dependent mortality trends between the two infection routes we studied the probability of fly survival until a cut off time closest to when 50% of infected flies had died in each assay: day 5 and day 7 for haemocoel injection and cuticle exposure treatments respectively. We first focussed on the data from flies in the infected treatments. We used the glmer function from Lme4 (Bates et al. 2015) in R (version 3.2.3) and built linear mixed-effect models using sex, age, infection route and all interactions up to 3-way as fixed factors and cohort (from each replicated experiment) as a random factor. Fly vial (each containing approximately 10 flies) was the unit of replication. Models had a binomial error structure and used a two-vector response containing the number of flies that were dead and alive in each vial. Models were sequentially simplified and assumptions of the statistical models verified by inspecting diagnostic plots. Likelihood-ratio tests were performed, comparing models with and without the term of interest, to calculate a χ^2 statistic and the significance of relevant terms in the models. To demonstrate that mortality variation resulted from pathogen

infection, we verified that the treatment group differences amongst the infected flies were not present in the control dataset, and further tested for the presence of higher level interactions with the term 'infection status' across the entire data set. Lastly, we took an independent analytical approach and used survival analysis to assess the temporal pattern of post-infection mortality using the Coxme package, which fits proportional hazards models to survival data and models random effects using a Gaussian error distribution. The fixed and random effect structure was fitted as described above. Relevant means and differences were calculated from raw data and are stated in the text with their standard errors.

2.4 Results

To quantify the relative contribution of declines in barrier defences and systemic immune responses to overall senescence of pathogen defence we monitored survival of 3,271 flies, either infected with the fungal pathogen *B. bassiana* or subjected to a control treatment (188 infected vials and 186 controls; mean flies per vial 9.36). Flies were 1 or 4 weeks old and were either infected through haemocoelic injection or by cuticle exposure. Averaging across both sexes and infection routes we found a significant decrease in survival probability of pathogen-exposed flies as they aged ($\chi_1^2 = 107.98$, $p < 0.001$, Tab. 1 (a)): 59.68% (±2.95%) of 1 week old flies survived until our assay cut-off time, whereas for 4-week-old flies this fell to 35.18% survival (±2.79%). However, this pattern

of senescent decline in pathogen defence differed strongly between males and females depending on infection route.

In female flies, percentage survival after pathogen inoculation decreased from 53.74% (±5.09%) to 23.58% (±5.21%) (χ_1^2 = 53.66, p < 0.001, Tab. 1(b)) between 1 and 4 weeks of age in the cuticle exposure treatment, and similarly by 32.08% (±5.93%) in the injection treatment (χ_1^2 = 31.78, p < 0.001, Tab. 1 (c)) (Figure 1). This pattern of age-dependent decline in pathogen defence did not differ between the two infection routes (χ_1^2 = 0.48, p = 0.49, Tab. 1 (d)). In contrast, for males there was only a marginal and non-significant decrease of 5.00% (±5.93%) in survival in the haemocoel injection treatment between 1 and 4 weeks of age (χ_1^2 = 0.41, p = 0.52, Tab. 1 (f)), whereas survival declined from 78.15% (±3.79%) to 50.72% (±4.99%) between 1 and 4 weeks of age in flies infected by cuticle exposure (χ_1^2 = 50.46, p < 0.001, Tab. 1 (e)). For males, the difference in the age-dependent change in pathogen susceptibility between these two infection routes was strongly significant (χ_1^2 = 17.12, p < 0.001, Tab. 1 (g)). This difference between males and females in the relative rate of pathogen defence senescence in the two infection routes was supported by a significant three-way interaction between sex, infection route and age (χ_1^2 = 7.33, p = 0.007, Tab. 1 (h)).

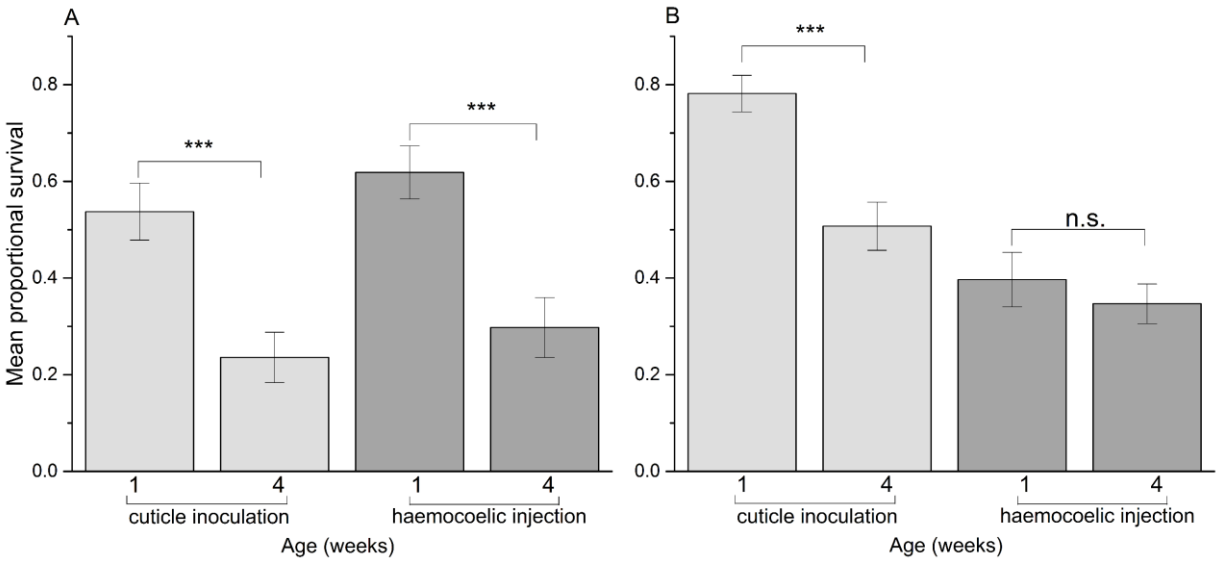

Figure 1: Mean proportional survival of 1 and 4-week-old flies after cuticle inoculation or haemocoelic injection with *Beauveria bassiana* spores. To make both infection treatments comparable, we selected data from the timepoint where overall mean proportional survival was closest to 50% for each treatment route: day 7 for the cuticle inoculation and day 5 for the haemocoelic injection. A) Female flies: both infection treatments showed the same decline in survivorship. B) male flies: cuticle inoculated flies showed a strong decrease in survival, while pathogen injected flies showed only a minimal change in survivorship while aging. Thus, pathogen-defence senescence in females can largely be explained by deterioration in the systemic defense, whereas male senescence is explained by changes in barrier defense systems. Uninfected control flies showed minimal mortality during this time frame (see text). Error bars show ± SE; significance of comparisons shown by *** = $P < 0.001$.

Table 1: Parameter estimate tables for analyses investigating how age, sex and infection route influence pathogen infection susceptibility. Model outputs are given for binomial mixed effects models using a 2-vector response variable that combined the number of dead and alive flies. Models were run on different subsets of the overall data set to investigate specific hypotheses as outlined in the text and in the table subheadings. P values were calculated using likelihood ratio tests (see text for details). These analyses only use the data from infected flies, as mortality rates in uninfected control flies were near-zero (see text).

Factor	Estimate	SE	$\chi 2$	df	P-value
(a) Age-dependent change in infection survival across both sexes and infection routes					
Intercept (1 week)	0.305	0.244			
age (4 weeks)	-1.101	0.109	107.98	1	<0.001
(b) Age-dependent change in infection survival in female flies receiving cuticle inoculation					
Intercept (1 week)	-0.070	0.673			
age (4 weeks)	-1.528	0.217	53.68	1	<0.001

Factor	Estimate	SE	$\chi 2$	df	P-value
(c) Age-dependent change in infection survival in female flies receiving haemocoelic injection					
Intercept (1 week)	0.492	0.197			
age (4 weeks)	-1.256	0.229	31.78	1	<0.001
(d) Comparing the age-dependent change in infection survival in female flies between the two infection routes					
Intercept (1 week, cuticle inoculation route)	-0.048	0.445			
age (4 weeks)	-1.508	0.215			
route (injected)	0.528	0.573			
age (4 weeks) x route (injected)	0.22	0.316	0.48	1	0.49
(e) Age-dependent change in infection survival in male flies receiving cuticle inoculation					
Intercept (1 week)	1.389	0.580			
age (4 weeks)	-1.509	0.224	50.46	1	<0.001
(f) Age-dependent change in infection survival in male flies receiving haemocoelic injection					
Intercept (1 week)	0.501	0.254			
age (4 weeks)	-0.152	0.237	0.41	1	0.52
(g) Comparing the age-dependent change in infection survival in male flies between the two infection routes					
Intercept (1 week, cuticle inoculation)	1.376	0.435			
age (4 weeks)	-1.491	0.224			
route (injected)	-1.879	0.561			
age (4 weeks) x route (injected)	1.338	0.326	17.12	1	<0.001
(h) Comparing the sex-specific age-dependent change in infection survival in flies between the two infection routes.					
Intercept (1 week, female, cuticle inoculation)	-0.011	0.423			
sex (male)	1.399	0.221			
age (4 weeks)	-1.477	0.213			
route (injected)	0.501	0.548			
sex (male) x age (4 weeks)	-0.068	0.304			
sex (male) x route (injected)	-2.395	0.314			
age (4 weeks) x route (injected)	0.182	0.314			
sex (male) x age (4 weeks) x route (injected)	1.216	0.450	7.33	1	0.007

Rather than studying the outcome of infection as the proportion of flies that survived until a single time point, we also used survival analysis (Figure 2) to test whether the entire pattern of post-infection mortality supported these trends. This alternative approach verified that averaging across all infected treatments older flies died at a faster rate post-infection than younger flies (χ_1^2 = 508.56, p < 0.001), that for females there was no difference in senescence of pathogen defence between the two infection routes (χ_1^2 = 8.12, p < 0.15), whilst for males, age dependent deterioration in pathogen defence occurred more strongly in cuticle-exposed flies than in injected flies (χ_1^2 = 11.34, p = 0.04).

In order to demonstrate that this senescence in post-infection survival was specifically due to declines in pathogen defence, we investigated these same age-dependent trends in the control treatments, where flies were not exposed to the *B. bassiana* pathogen. Survival of these controls was very high. Averaging across both sexes and infection routes, mean survival for 1 week old flies was 98.55% (±0.44%), compared to 97.88% (±0.42%) for 4-week-old flies, a difference that was not significant (χ_1^2 = 1.76, p = 0.184). Further analyses with either linear-mixed-effect models or Coxme survival models found no significant survival differences between the sexes, age groups or infection routes within the control flies.

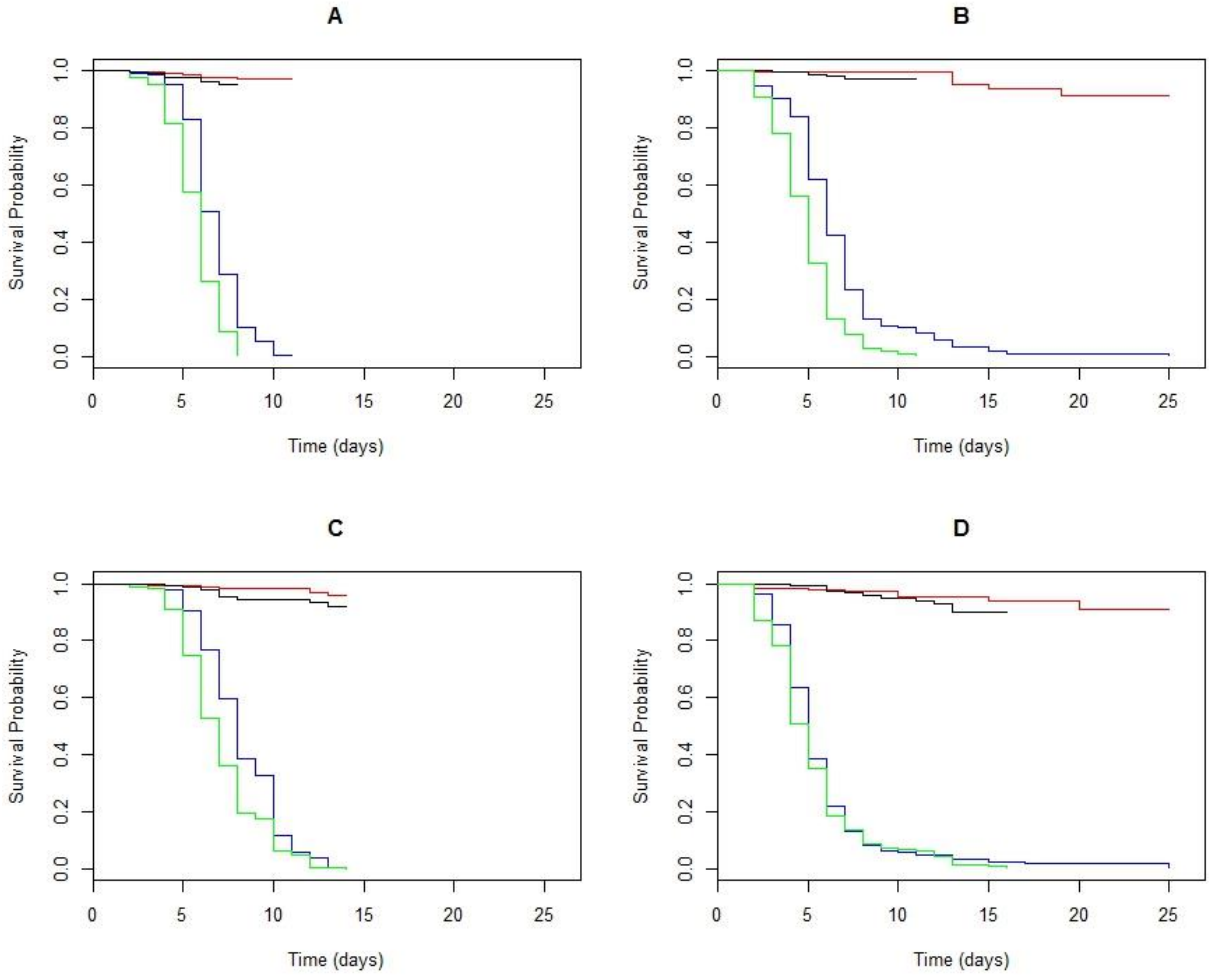

Figure 2: Survival analysis for female and male *D. melanogaster* after cuticle inoculation or haemocoelic injection with *B. bassiana* spores. A) Female survival after cuticle inoculation B) Female survival after haemocoelic injection C) Male survival after cuticle inoculation D) Male survival after haemocoelic injection. Coloured lines show**:** blue (age 1 week) and green (age 4 weeks) survival of flies that received a pathogen infection treatment, whereas red (age 1 week) and black (age 4 weeks) show survival of uninfected control flies. The comparisons between 1 and 4 week flies infected with spores were significant for A, B & C, but not for D. Control observations ceased at the time point when no infected flies for the corresponding treatment category remained alive, therefore the time-course of observations for assays A-D varies.

2.5 Discussion

In this study, we demonstrate that pathogen defence senescence occurs by different process in males and females. We conclude that whilst flies of both sexes become more susceptible to fungal infection as they age, for males this occurs principally due to deterioration in barrier defences, whereas for females it is most likely that systemic immune senescence is mainly responsible.

Our experiments assessed overall senescence of pathogen defence by comparing the infection susceptibility of 1 and 4-week-old flies dusted with entomopathogenic fungal spores. In parallel we isolated the contribution of changes in systemic immune responses to this senescent process by directly injecting fungal spores into the haemocoel, circumventing cuticle barrier defences. For male flies, pathogen-induced mortality did not increase during ageing when we injected spores into the haemocoel, but there was a strong age-associated increase in infection susceptibility when we dusted spores onto the cuticle. We conclude that, for males, whilst the efficacy of systemic defences changes little, ageing leads to deterioration of the cuticle barrier defences, so that a larger pathogen dose overwhelms the immune response in older flies. In contrast, female flies showed exactly the same ageing-decline in survivorship in both these assays. Given that systemic and barrier defences combine to influence mortality in the dusting assay, but only systemic defences are relevant to the injection assay, we conclude that changes in the systemic immune response are

sufficient to explain senescence of infection defence, with no additional contribution from changes in barrier defences.

Previous research on invertebrate immunosenescence has focussed on studying changes in systemic immune responses, demonstrating ageing-associated alterations in antimicrobial peptide expression, microbial phagocytosis by haemocytes and haemocyte abundance (Zerofsky et al. 2005, Moret and Schmid-Hempel 2009, Mackenzie et al. 2011, Park et al. 2011). We know of no studies investigating how senescence influences barrier defence efficacy in invertebrates. However, the external epithelia, such as the trachea, reproductive tract, and gut do all mount vigorous local immune responses (Lemaitre and Hoffmann 2007), which might alter with age, and it seems possible that the integrity of cuticle barriers could become compromised. Our other investigations indicate that sex-specific differences in senescence of hygiene behaviours such as cuticle grooming are unlikely to drive these effects (Chapter 3). Whilst invertebrate research on how immune-senescence varies between males and females is rare, Mackenzie et al. (2011) found that the density of haemocytes circulating in fly haemolymph showed an ageing decline in female flies, but not in males. If the density of haemocytes in the blood is a major determinant of successful pathogen defence against *B. bassiana*, it is possible that this sex-specific senescent change in cellular immunity could explain our current observations that systemic pathogen defence senesces in females but not males.

The ultimate reason for this difference in the pattern of pathogen defence senescence between males and females may lie either in divergent behaviour or differential life history selection between the two sexes. Our data show that for young flies, males are more susceptible than females when they are injected with *B. bassiana* spores, but less susceptible than females when spores are dusted onto the cuticle. This could indicate that young males invest relatively more than females in barrier defences, but less in systemic immunity. If pathogen barrier defences result mostly from cuticle integrity, males may be selected to have more resilient cuticle structure to defend against aggressive encounters with other males (Dow and von Schilcher 1975). Our conclusion that males suffer pathogen defence senescence because of barrier defence deterioration could be explained by repeated wounding during a lifetime of aggressive male-male encounters. Lower investment in costly systemic pathogen defence by males is consistent with much of the sizeable body of literature on sex biased parasitism (Rolff 2001, Rolff 2002). If females do invest more than males in systemic immune defences when they are young, it may be that females experience a larger senescent decline in these immune responses due to a higher starting point at young ages. Whilst our infection protocol was identical for males and females, the sexes do differ in size and in their surface area, therefore the relative impact of a standardised spore injection and cuticle exposure may have differed.

The extent to which our finding of sex-specific routes to immune senescence can be generalised to other pathogens and to other animal species will need to be assessed. Nevertheless, there are some parallels in medical science where cellular senescence and impaired repair mechanisms in respiratory epithelia may predispose to infection in vertebrate models of ageing (Zhou et al. 2011, Yin et al. 2014). Furthermore, impact of ageing on human traits such as senescence of infection resistance and vaccine responsiveness differ strongly in males and females (Klein et al. 2015). Understanding sex-specific routes to immune senescence in insects may be of strong applied relevance to the control of vector borne human diseases, where it is predominantly older female mosquitos that are responsible for infection transmission (Read et al. 2009). Our results demonstrate the overarching principle that patterns of immune senescence can vary strongly between males and females: the causes and consequences of this deserve further investigation.

Chapter 3 : Testing whether ageing-associated changes in cuticle hygiene underlie senescence of pathogen defence in *Drosophila melanogaster*.

This chapter is in preparation for submission as:

Kubiak, M., Tinsley, M. C. (2017) Testing whether ageing-associated changes in cuticle hygiene underlie senescence of pathogen defence in *Drosophila melanogaster*.

3.1 Abstract

Susceptibility to pathogen infection increases with advancing age in many animals. We studied *Drosophila melanogaster* and investigated whether age-dependent changes in fly hygiene contribute to this pathogen defence senescence. As a proxy for studying pathogen exposure, we covered flies with bromophenol blue dye and then measured their ability to clean this from the cuticle. Significantly more dye initially adhered to the cuticle of older flies before cleaning activity commenced. However, after two hours cleaning, dye loads on flies of all age groups did not differ, providing no evidence for senescence of cleaning ability. We next tested whether the high initial dye loads on old flies could be explained by changes in cuticular hydrocarbons. We used solvent to

extract cuticle hydrocarbons from one and four week old flies, then reapplied these to dead flies of both ages, before assessing bromophenol blue adherence to the cuticle. One week old flies receiving a hydrocarbon wash from four-week-old flies took on the dye adherence characteristics of old flies, and *vice versa*; however, this effect was stronger for females than for males. In the context of pathogen defence senescence, our data suggest that whilst pathogen propagules, such as infectious fungal spores, may be more likely to attach to the cuticle of older flies, aged flies retain the ability to rapidly clean their cuticle. Nevertheless, we speculate that as flies age they may have to invest more in maintaining cuticle hygiene.

3.2 Introduction

Many pathogens and parasites are adapted to penetrate or colonise the cuticular, epidermal or epithelial barriers that separate animals from their external environment. Animals have a range of physiological and behavioural processes that help maintain body hygiene, thereby reducing the burden of infection associated with their barrier defences (Zhukovskaya et al. 2013). In insects, much of the external chitinous cuticle provides a tough mechanical barrier that limits the ability of many infectious agents to gain entry the body, thus it is common for parasites and pathogens to exploit specialised internal epithelial sites to initiate infection, such as the gut, the reproductive tract and the trachea

(Kirkpatrick et al. 1994, Cox and Gilmore 2007). However, some pathogens have evolved specialised mechanisms to directly penetrate the external barrier defences before establishing systemic infection; in insects, these include entomopathogenic fungi, such as *Beauveria sp*. and *Metarhizium sp*. (Stleger et al. 1986, Stleger et al. 1988, Pedrini et al. 2007, Ortiz-Urquiza and Keyhani 2013). A major defence against these infections is cuticle grooming, which can efficiently reduce or eliminate the burden of fungal spores that might subsequently germinate and lead to systemic infection. Grooming behaviour occurs in a wide range of insect species and serves a variety of functions in addition to pathogen defence. However, the role of grooming behaviours in preventing infection has been best characterised in ants and termites, where auto- and allo-grooming provide important mechanisms to prevent infection of individuals by fungal pathogens and to limit the spread of disease within a social colony (Yanagawa and Shimizu 2007, Westhus et al. 2014, Theis et al. 2015). These defences against infection are not confined to insects and are relevant for a wide variety of infections of humans and livestock that are commonly associated with unsanitary conditions when hygiene may be compromised, including ectoparasites Hengge et al. (2006), skin fungal infections (Adams 2002), some arthropod vectored infections (Bitam 2012), as well as infections of the gut (Reidl and Klose 2002).

It is common for animals to become more susceptible to infection as they age (Muller et al. 2013). This is partly because aspects of immune system function become impaired in older individuals, a process termed immunosenescence (Doums et al. 2002, Eleftherianos and Castillo 2012, Roberts and Hughes 2014). However, other aspects of disease defence may also deteriorate during the ageing process. Behavioural defences against parasites, including hygiene behaviours, play a general and vital role in limiting the frequency of infection (de Roode and Lefèvre 2012). In this investigation, we study the model insect *Drosophila melanogaster* and investigate whether elderly individuals suffer from compromised hygiene, which might contribute to their elevated pathogen susceptibility. The only study to directly investigate how grooming behaviour in *D. melanogaster* is influenced by age showed that grooming responsiveness increased up to 10 days of age, but did not study further time points at which senescence occurs (Yanagawa et al. 2014). Nevertheless, Ren *et* al. (2007) found that: the number of bacteria associated with the *D. melanogaster* cuticle increased steadily as flies age; that these bacteria were concentrated in areas least accessible to grooming; and that these bacteria may have been associated with fibrous microbial biofilms. Whether this age-associated increase in cuticle microbial burden was driven simply by the increased time available for elderly flies to accumulate microbes, or whether failures in hygiene behaviour or physiology were responsible was not investigated. Whilst, Ren *et* al. (2007), found no impact of these commensal microbes on fly lifespan, when flies encounter pathogens

specialised to infect through the cuticle, such as entomopathogenic fungal spores, age-dependent changes in fly hygiene might drive higher mortality in elderly flies.

Grooming behaviour in *Drosophila melanogaster* is highly stereotyped and is induced when contact chemoreceptors are triggered by a variety of stimuli, including by Lipopolysaccharide (LPS) from *E. coli* and by *E. coli* bacteria (Yanagawa et al. 2014). However, grooming can only protect the individual to a certain extent from potential microbial invaders as this mechanical process will never perfectly clean the exoskeleton due to its complex three-dimensional structure.

The sclerotized insect cuticle covered in cuticular hydrocarbons (CHCs) cannot be penetrated by most pathogens. However, for specialists, such as entomopathogenic fungi, the CHC composition of the outer cuticle can influence the adhesion, germination and penetration of fungal spores. The principal function of the insect CHC layer is in preventing desiccation (Chown et al. 2006, Benoit 2010, Chown et al. 2011). CHCs also play an important role in insect communication, including nestmate recognition, formation of social groups, courtship and mating: this has been extensively reviewed for social insects and *Drosophila* (Ferveur and Cobb 2010, Greene 2010, Liebig 2010, van Zweden and d'Ettorre 2010).

The previous chapter showed that both sexes became more susceptible to fungal infection while they age. However, there was a strong sex-specific effect: our data suggested deterioration of the barrier defences in males but not females. This could be driven by several processes: impaired cuticle immune responses, wounding of males in mating contests, changes in the nutritional environment of the cuticle that favours fungal growth, or for reasons of impaired hygiene. Here we focussed on the latter possibly and hypothesised that a decline in hygiene, perhaps because of impaired grooming behaviour, might be a potential driver of increased infection susceptibility. The experiment was designed as a model, mimicking the natural exposure of the cuticle to fungal spores. Furthermore, the findings of these experiments led us to design an experiment to determine the role of changes in cuticular hydrocarbons in driving senescent alterations in cuticle properties.

Rather than studying how pathogen propagules interact with the cuticle, we used the dye bromophenol blue as an analogue, dusting flies with this powder and then assessing how they cleaned it off. Whilst we found no evidence of age-related deterioration of grooming, more dye adhered to the cuticle of flies as their age increased. We went on to assess whether changes in the CHC profile during ageing might be responsible for this, by solvent-extracting CHCs from flies and then reapplying them to flies of different ages. Our results suggest that changes in the hydrocarbon composition of the fly's cuticle during senescence

may increase the adhesion of pathogen propagules and necessitate greater investment in cuticle grooming as flies age.

3.3 Material and Methods

Fly stocks:

All flies were *D. melanogaster* Samarkand wildtype genotype, reared on standard Lewis medium (Lewis 1960) and maintained at 25°C on a 12 h L/D cycle. To make age-matched cohorts we followed the methods of Mackenzie et al. (Mackenzie et al. 2011). Parental flies were placed in laying cages and allowed to lay eggs for 24h on an apple juice agar plate seeded with yeast. To set up fly bottles with a controlled low density of larvae we used the methods from Clancy and Kennington (Clancy and Kennington 2001): eggs were washed from the agar plate with PBS buffer and 13 μl of packed eggs was added to a fly bottle with standard medium using a pipette. Resulting adult flies were transferred into 11 litre fly cages in groups of 200 at a 1:1 sex ratio. Flies were maintained on petri dishes of fly food and a vial of water, which were both changed every two days. Three cages were setup weekly: two cages with age-controlled flies for the experiments, the third to house additional flies used to replace flies in the other cages that died or were used in the experiment in order to keep cage densities constant.

Cleaning assay:

Flies were removed from ageing cages and prepared in single-sex five-fly groups of known age (1 to 4 weeks) in vials 24 hrs before experiments using CO_2 anaesthesia; after this they received no further anaesthesia to avoid potential influences on behaviour. To assess fly cleaning ability flies were dusted with bromophenol blue powder (Sigma): approximately 2 mg of dye (previously stored in a drying cabinet at 50 °C), was weighed into empty 30 ml fly vials, five-fly groups were tipped into these vials and shaken for 20 sec. Flies were then transferred into a sieve tube and shaken briefly to remove excess unattached dye. Thereafter, fly groups were transferred into fresh vials with standard food and kept at 25 °C for either 30, 60 or 120 minutes to clean themselves. At each time point flies were tipped to empty vials, which were then sealed with parafilm and flies killed at -80 °C. Additional flies were also transferred to -80 °C immediately after the sieve stage to assess dye load at a zero-hour time-point.

Dye load measures:

We assessed dye load by washing bromophenol blue powder from flies in a standard fluid volume and then measured its concentration using a spectrophotometer. Frozen flies from the cleaning assay were transferred individually into 96-well DeepWell™ Polypropylene Microplates (Fisher Scientific), 120 µl NaOH (10 mM) was added to each well and the plate vortexed

for 45 seconds to wash off the dye from the flies. 100 µl of the dissolved dye solution was taken from every well, transferred to a new well on a 96-well microtiter plate (Fisher Scientific) and absorption measured at 590 nm using a microplate reader (Molecular Devices SpectraMax 340PC). Samples with absorption readings greater 1.2 were diluted individually and measured again. The dye load on each fly was calculated using a bromophenol blue standard curve.

Cuticular hydrocarbon assay:

After identifying changes in dye powder adherence as flies aged, we tested the potential role of senescence-associated alterations cuticular hydrocarbons in driving this change. We used an established solvent wash technique (Antony and Jallon 1982, Ferveur 1991, Savarit et al. 1999) to remove cuticular hydrocarbons from dead flies of known age and transfer them to the bodies of other flies that had previously been washed of their original hydrocarbons. 1 and 4-week-old male and female flies were taken from the ageing cages described above. Flies were killed at -80 °C, thawed at room temperature, dried on filter paper, and groups of five flies (of single sex and age) were placed into 3.5ml glass vials. 50 µl of hexane (HPLC grade, Fisher Scientific) was added; vials were left standing for 5 min, mixed on a Vortex mixer for 2 min, then left standing for another 5 min before the hexane wash was removed and transferred with a glass pipette into a

clean glass vial. The extraction process was repeated for a second time with fresh solvent on the same group of flies, then the combined hexane washes were evaporated to dryness in the vial with a gentle CO_2 stream. The washed flies, with cuticular hydrocarbons removed, were dried on filter paper and stored in Eppendorf tubes. If not immediately used, flies and hydrocarbon extracts were stored at -80°C.

We then reapplied hydrocarbons to 1 and 4-week-old hydrocarbon-stripped flies, either using hydrocarbons from flies from the alternative age group, or applying hydrocarbons from flies of the same age to act as a control. Flies always received hydrocarbons washed from donor flies of the same sex. Previously-washed flies were put into clean glass vials in single-sex batches of five. Dried samples of hydrocarbons were reconstituted in 100 μl hexane and added to vials, which were then left standing for 5 min, whilst the hexane was slowly evaporated as described before. Flies were then removed from the vials and dried on filter paper. To assess the adherence of particles to these dead flies with experimentally manipulated cuticle hydrocarbons, the bodies were subjected to the same bromophenol blue dye dusting treatment as described before; dye loads for all flies were assessed immediately after dye application and sieving.

Statistics:

The data for the dye load grooming assays showed a right-skewed distribution, so we used the glmer.nb function from Lme4 to fit models with a negative binomial distribution (Bates et al. 2015) in R (version 3.2.3) . We built mixed-effect models using, where appropriate, the fixed effects sex, age and time as well as relevant interactions; all models contained random effects for 'vial' and 'cage', accounting for the non-independence of flies that were dusted in the same vial and which aged in the same demography cage, respectively. To analyse the data from the CHC re-application experiment, we used the lmer function from Lme4 (Bates et al. 2015) to fit models with the same effects-structures as above. Models were sequentially simplified and assumptions of the statistical models verified by inspecting diagnostic plots. Likelihood-ratio tests were performed, comparing models with and without the term of interest, to calculate a χ^2 statistic and the significance of relevant terms in the models. Figures 1 and 2 were generated from raw means of dye load with their standard errors for each treatment category.

3.4 Results

Grooming behaviour:

We investigated the grooming behaviour of 1839 female and male flies aged 1, 2, 3, and 4 weeks; we allowed these flies different time periods to groom bromophenol blue dye from their cuticles (0, 30, 60 and 120 min). The amount of dye that initially stuck to a fly's cuticle (at time = 0 min) was greater in flies of the older age classes (χ_3^2 = 67.74, p < 0.001, Tab. 2 (a)): immediately after dusting, 4 week old flies carried approximately three times the dye load of 1 week old flies (Figure 1). Males had significantly lower initial dye loads than females (χ_1^2 = 7.24, p < 0.01, Tab.2 (a)), but this difference did not vary between ages (sex by age interaction: χ_3^2 = 2.69, p = 0.44, Tab. 2 (a)).

Our data demonstrated a rapid cleaning phase within the first 30 minutes post-dusting during which flies cleaned the majority of dye from their cuticles, after this the dye load reached a steadily declining plateau (Figure 1). Despite the high initial dye load on older flies, later in the experiment there were no significant differences in dye load between the age classes after either 30, 60 or 120 minutes of grooming (30 min: χ_1^2 = 0.01, p = 0.92; 60 min: χ_1^2 = 1.27, p = 0.26; 120 min: χ_1^2 = 0.20, p = 0.66, Tab. 2 (b-d)). The slight decline in the dye load on flies during this 30 to 120 minute period was significant (χ_1^2 = 7.65, p < 0.01, Tab. 2 (e)), but the rate of decline did not vary between the age groups (time by age interaction: χ_1^2 = 2.06, p = 0.15).

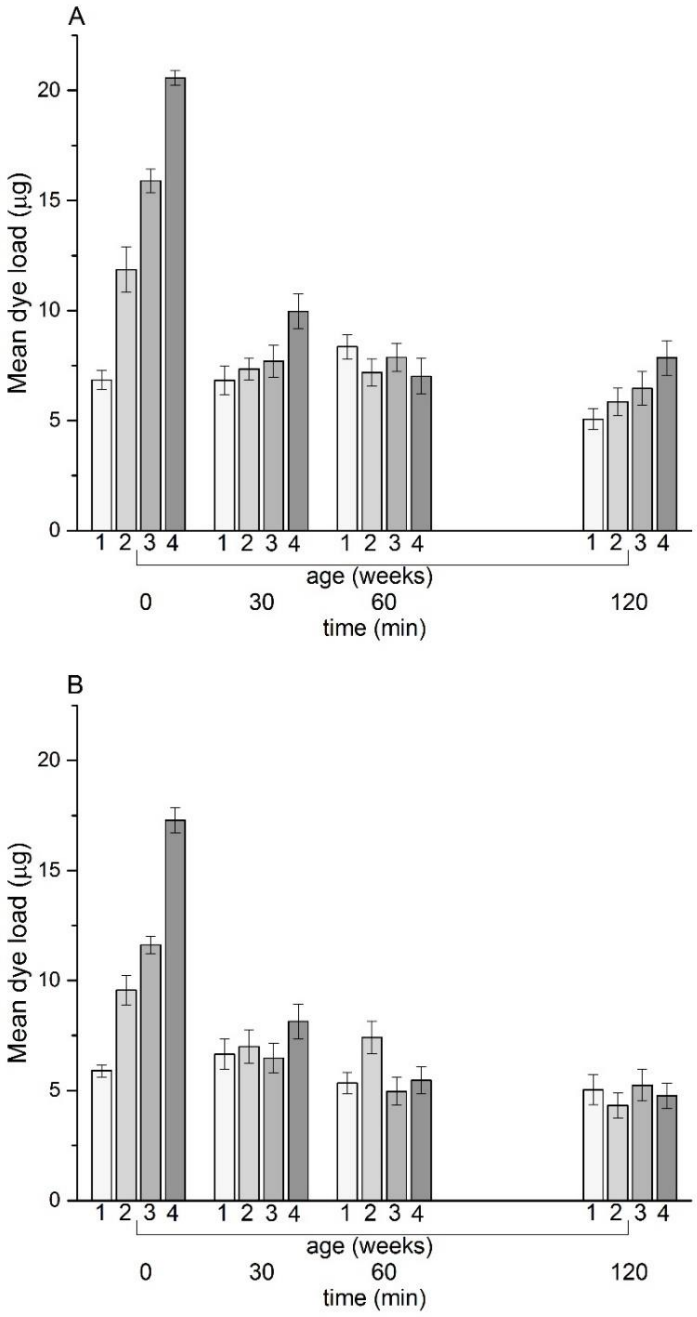

Figure 1: Testing for an impact of ageing on cuticle grooming ability. Flies were dusted with bromophenol blue dye powder. Mean dye load on flies aged 1, 2, 3, and 4 weeks after they were allowed to clean for 0 min, 30 min, 60 min or 120 min. A) Females: older flies carried significantly higher amounts of dye on the cuticle at t = 0 but did not show a significant difference between ages at any other measured time point. B) Males: older flies carried significantly higher amount of dye on the cuticle at t = 0 but the overall amount is significant lower than for females. At every other time point for males the dye load differences between ages were not significant. See text for statistical analysis.

Table 2: Parameter estimate tables for analyses of the impact of ageing on cuticle grooming ability. Model outputs are given for negative binomial mixed effects models using the quantity of adhered dye as the response variable. Models were run on different subsets of the overall data set to investigate specific hypotheses as outlined in the text and in the table subheadings. P values were calculated using likelihood ratio tests (see text for details).

Factor	Estimates	SE	$\chi 2$	df	P-value
(a) Assessing sex- and age-dependent changes in cuticle adhered dye at t = 0					
Intercept (1 week, female)	4.177	0.107			
age (weeks)	0.381	0.039	64.74	3	<0.001
sex (male)	-0.193	0.070	7.24	1	<0.01
age (weeks) x sex (male)	-0.185	0.197	2.69	3	0.44
(b) Assessing the age-dependency of dye load at t = 30 min					
Intercept (1 week, female)	4.183	0.200			
age (weeks)	-0.007	0.067	0.01	1	0.92
sex (male)	-0.235	0.108	4.55	1	0.03
(c) Assessing the age-dependency of dye load at t = 60 min					
Intercept (1 week, female)	4.205	0.211			
age (weeks)	-0.076	0.67	1.27	1	0.26
sex (male)	-0.355	0.111	10.61	1	<0.01
(d) Assessing the age-dependency of dye load at t = 120 min					
Intercept (female)	3.910	0.275			
age (weeks)	-0.040	0.090	0.20	1	0.66
sex (male)	-0.428	0.148	7.93	1	<0.01
(e) Assessing the age-dependency of dye load between t = 30 and t = 120 min					
Intercept (female)	3.900	0.134			
scale (time)	-0.129	0.046	7.65	1	<0.01
sex (male)	-0.397	0.092	17.65		<0.001
scale (time) x age (weeks)	0.059	0.046	2.06	1	0.15

Cuticular hydrocarbons:

To test whether the cause of the initially-elevated levels of adhered dye in older flies that we observed in our grooming assays was related to cuticular hydrocarbons we hexane-extracted CHCs from known-age flies and then reapplied them to flies that had been stripped of CHCs. The dye load on 1 and 4-week-old female control flies (that had been stripped of CHCs and then received a CHC wash from flies of the same age) differed significantly (χ_1^2 = 6.61, p = 0.010, Tab. 3 (a)). These 4-week-old female control flies had cuticle dye loads that were approximately double that of the 1 week old control flies (Figure 2), a result which mirrors the data from our grooming assays on flies that had un-manipulated CHC profiles. In the case of males in this CHC-control category, whilst four week controls did carry 22.7% more dye than 1 week old controls, this difference was not quite significant (χ_1^2 = 3.09, p = 0.079, Tab. 3 (b)).

Next, we assessed the effect of coating 1 week old flies with CHC extracts from 4-week-old flies, and compared these to our 1 week control flies, which had received a hydrocarbon wash from 1 week flies. Week-old females coated with 4 week CHCs had around double the dye load of the 1 week controls (χ_1^2 = 11.60, p < 0.001, Tab. 3 (c)), whilst in the case of males dye loads increased by 48.8% (χ_1^2 = 3.99, p = 0.046, Tab. 3 (d)) (Figure 1). We saw the reverse trend when the CHCs were removed from 4-week-old flies and the bodies were washed with CHCs from week-old flies. Washing old females with CHCs from young flies caused a

reduction in dye load of 48.38% compared to the 4-week old control group (χ_1^2 = 4.97, p = 0.026, Tab. 3 (e)), whereas for males this decrease was only 5.99% and not significant (χ_1^2 = 1.22, p = 0.27, Tab. 3 (f)).

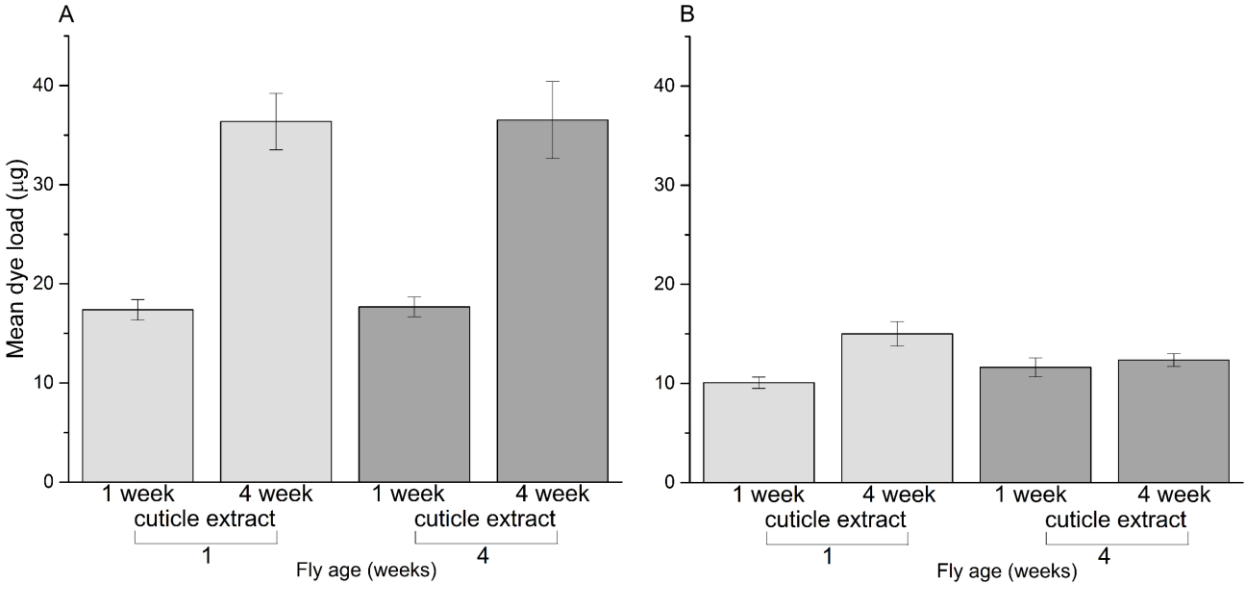

Figure 2: The impact of experimentally manipulating the cuticular hydrocarbons of old and young flies on the propensity of bromophenol blue powder to attach to the fly cuticle. A) Females: 1 week old flies had strongly increased dye loads when washed with CHCs from 4 weeks old flies, whilst the dye load on 4 week old flies declined when they received CHCs from 1 week flies. B) Males: 1 week old flies had moderately increased dye load when receiving CHCs from 4 week old flies, however, the decline in dye load when 4 week old flies received CHCs from 1 week old flies was small and not significant. Error bars show standard errors of the mean. See text for statistics.

Table 3: Parameter estimate tables for analyses of differences in dye load on the cuticle after exchange of hydrocarbons between age groups. Model outputs are given for linear mixed effects models using the quantity of adhered dye as the response variable. Models were run on different subsets of the overall data set to investigate specific hypotheses as outlined in the text and in the table subheadings. Control flies had their cuticular hydrocarbons extracted and then re-applied. P values were calculated using likelihood ratio tests (see text for details).

Factor	Estimates	SE	$\chi 2$	df	P-value
(a) Comparing the difference in dye load between 1- and 4-week old female control flies					
Intercept (1 week)	17.478	5.138			
age (4 weeks)	19.281	7.136	6.61	1	<0.010
(b) Comparing the difference in dye load between 1- and 4-week old male control flies					
Intercept (1 week)	10.079	0.938			
age (4 weeks)	2.282	1.327	3.09	1	0.079
(c) Comparing the difference in dye load between 1-week old control flies vs. 1-week old flies that received CHCs from 4-week old flies (females)					
Intercept (1 week with 4 week CHCs)	36.684	3.460			
age (1 week)	-19.225	4.839	11.60	1	<0.001
(d) Comparing the difference in dye load between 1-week old control flies vs. 1-week old flies that received CHCs from 4-week old flies (males)					
Intercept (1 week with 4 week CHCs)	15.067	1.783			
age (1 week)	-4.998	2.510	3.99	1	0.046
(e) Comparing the difference in dye load between 4-week old control flies vs. 4-week old flies that received CHCs from 1-week old flies (females)					
Intercept (4 week with 1 week CHCs)	20.904	4.728			
age (4 weeks)	14.459	5.982	4.97	1	0.026
(f) Comparing the difference in dye load between 4-week old control flies vs. 4-week old flies that received CHCs from 1-week old flies (males)					
Intercept (4 week with 1 week CHCs)	15.066	1.845			
age (4 weeks)	-2.705	2.298	1.22	1	0.27

3.5 Discussion

We investigated the potential contribution of age-dependent changes in fly hygiene to the increase in pathogen susceptibility that accompanies ageing. We were motivated to discover whether older flies might have impaired ability to remove potentially infectious microorganisms from their cuticle, thereby contributing to increased disease burdens in older individuals. We demonstrated that as flies aged particles stick to the cuticle in greater numbers, but that even the oldest flies we tested had an efficient grooming response which rapidly cleaned the cuticle; our data suggest that this increased adhesiveness of the cuticle is due to age-dependent changes in the CHC profile.

Our grooming assay tested the ability to flies to remove bromophenol blue dye: flies of all ages cleaned their cuticles rapidly. There was no evidence for senescence of grooming behaviour; in fact, older flies removed more dye from their cuticles than younger flies in the 30 minutes after dusting. However, this was simply due to that fact that more dye initially adhered to the cuticles of older flies, meaning they had more dye to remove. For this reason, our dye grooming assay did not provide a perfectly fair test of the ability of flies of different ages to clean. It is not possible to conclude with complete certainty that grooming behaviour does not change with age, because we were unable to generate comparably high starting dye loads on young flies as we did on old flies. Furthermore, we did not assess dye loads at any time point between zero and 30

minutes, which might have revealed subtle differences between age groups in the speed at which dye was removed. Nevertheless, flies from 1 to 4 weeks of age all cleaned off the majority of dye in less than 30 minutes, demonstrating an efficient hygiene response and indicating that there is no major failure of grooming ability in elderly flies.

We acknowledge that we did not quantify cuticle grooming behaviours directly and that processes other than active grooming could have contributed to the decline in dye load on flies after dusting. For example, dye may have fallen off flies as a consequence of locomotion, or because flies made contact with other surfaces. However, we did observe flies cleaning vigorously using their limbs immediately after dusting and it seems most likely that the majority of the decline in dye load after dusting was the result of grooming.

How our dye dust removal assay parallels the hygiene behaviours required to remove pathogen propagules from the cuticle before they penetrate to establish a systemic infection is not clear. When bacteria or bacterial molecules contact the cuticle flies do respond by grooming (Yanagawa et al. 2014). A study on the thrips *Frankliniella occidentalis* (Wu et al. 2014) demonstrated that after first contact with the insect, *B. bassiana* fungal spores take approximately 2 h to adhere to the cuticle, then 36 h to germinate and finally penetrate the cuticle. If the timing of this process is similar in *Drosophila*, then for all age classes we studied, the rapid grooming response we observed in the first 30 minutes of our assay

would probably remove the majority of spores before they represent an infectious threat.

The abundance of commensal bacteria on the *D. melanogaster* cuticle increases with age (Ren et al. 2007); therefore clearly the grooming response is not efficient at removing all microbes in ageing flies. Potentially, intensive grooming may not be able to clean microbes from all parts of the body (Hutchins and Barash 1976). Our data support this, as flies of all ages had a residual dye load after 2 hours of cleaning that they seem to have limited ability to remove. Ren et al.'s data (Ren et al. 2007) could be explained if flies of all ages clean themselves equally well, except for certain regions of the cuticle which elderly flies have poor ability to reach; these regions may be too small for our dye-load grooming assay to detect, but they might still be relevant for microbial infection. Alternatively, Ren et al (Ren et al. 2007) speculated that the microbes they observed accumulating on older flies may have been associated with biofilms; such biofilms could make the fly grooming response inefficient at removing these commensal microbes.

We demonstrated that the change in the properties of the cuticle that drives more dye to attach to older flies is associated with changes in the solvent-soluble fraction of CHCs. We used a standard method (Jallon 1984) to extract CHCs from flies aged 1 and 4 weeks of age and reapplied these extracts in a pairwise manner to flies of both age classes. Interestingly, when we applied the

hexane washes from 1 week old flies to the cuticle of CHC-stripped 4 week flies, dye adhered at the level seen on 1 week flies. In parallel, there was an increase in cuticle-adhered dye when 1 week old flies received a hydrocarbon wash from 4 week flies. This strongly implicates age-dependent changes in the CHC profile in causing an alteration in how particles interact with the cuticle. Previous work has shown that the molecular composition of cuticular hydrocarbons in *D. melanogaster* changes as flies age: molecules with shorter carbon chains become less abundant, whilst the abundance of molecules with longer carbon chains increases (Everaerts et al. 2010, Kuo et al. 2012). We speculate that these changes in CHC profile could drive increased adhesiveness of the cuticle as flies age and could explain the elevated dye levels we observed in old flies. Nevertheless, we cannot exclude the possibility that other material, in addition to CHCs, was transferred between flies in our solvent-wash experiments, which might have contributed to changes in dye adherence. Bacteria and associated biofilms may grow on ageing flies (Ren et al. 2007): we do not know whether these were removed during the solvent wash process, nor how they could have been deposited on recipient flies, but it is possible they might have contributed to the increased adherence of dye on young flies that received a CHC wash from older flies.

In the context of senescence in the ability of flies to defend against entomopathogenic fungi, the changes in the hydrocarbon profile of the cuticle

may have consequences beyond an increase in adhesive properties. When *B. bassiana* spores attach to the cuticle they metabolise hydrocarbons as a carbon source for initial growth; this fungus has stronger ability to utilise long-chain hydrocarbons (Stleger et al. 1988, Napolitano and Juarez 1997, Crespo et al. 2000). Therefore the shift in chain length of *D. melanogaster* CHCs as flies senesce (Kuo et al. 2012) might make the cuticle a more favourable environment for fungal infection, contributing to an age-dependent deterioration in pathogen defence.

In our experiments, as flies aged from 1 through to 4 weeks the amount of dye that initially adhered to the cuticle after dusting increased dramatically; in contrast, little dye attached to the cuticles of young flies. If, in nature, flies are constantly exposed to particles that could stick to their cuticle, then older flies may have to invest substantially more in grooming activities than younger flies in order to maintain the same level of cuticle hygiene. This could represent a considerable energetic cost that increases as flies age. Furthermore, grooming behaviour probably has an associated opportunity cost because flies may be unable to feed effectively whilst also cleaning their cuticle. Our study therefore suggests the possibility that ageing is associated with escalating costs to preserve cuticle hygiene.

Chapter 4 : Final discussion

Studies investigating increased insect susceptibility to infection during ageing have previously focussed mainly on mechanistic changes in humoral and cellular immunity. Whilst much recent research on *Drosophila* immunity provides in depth understanding of the details of immune response function, the processes of immunosenescence are still poorly understood. In addition, senescence of other non-immunological pathogen defences against infection may have a stronger role in determining how infection susceptibility alters with age than has previously been assumed.

In this thesis, I investigated age-dependent deterioration in barrier and systemic defences in female and male *Drosophila melanogaster* and examined factors that may be responsible for this degeneration. This study produced the following conclusions: a) senescence in pathogen defence in female flies is mainly due to systemic immune response changes, whereas in males barrier defences deteriorate; b) the ability to clean artificially applied particles from the cuticle does not decline with age and thus impaired grooming is unlikely to be a driver for pathogen defence senescence; c) I demonstrated that artificially applied dye dust adheres better to older flies and that changes in cuticular hydrocarbons are probably responsible.

Overall, the combination of these experiments shows that disease defence senescence involves changes in more processes than simply the humoral

signalling cascades and the cellular responses to microbial challenge. The additional contribution of barrier defence deterioration in older flies may mean that the systemic immune responses are more likely to become overwhelmed following pathogen exposure, leading to poorer defences against infection in elderly insects.

4.1 Pathogen defence senescence in *Drosophila melanogaster* after cuticle inoculation or haemocoelic injection of *B. bassiana* spores.

In Chapter 2, I investigated pathogen defence senescence for two different infection routes. Flies were infected with *B. bassiana* fungal spores, either via cuticle inoculation or haemocoelic injection. Pooling across the whole experiment, survival until our assay cut-off time was nearly 50% lower in four-week-old flies than one week olds. This finding is interesting because previous studies have created an ambiguous picture of whether there is a general decline in infection defence with age in *D. melanogaster*: Lesser et al. (2006) and Felix et al. (2012) found that bacterial clearance ability both declined and increased with age, depending on fly genotype; whilst Zerofsky et al. (2005) found a more vigorous AMP response in older flies. My work shows clearly that there is

significantly impaired pathogen defence in older flies, at least for the fungal pathogen I worked on, *B. bassiana*.

My sex specific analysis of pathogen defence senescence shows that cuticle-inoculated females suffered higher mortality than males at both ages. We also saw a strong decline in survival between one and four weeks of age in female flies whether the pathogen was injected into the haemocoel or if spores were dusted onto the cuticle. In contrast, males did not show an age-related decline in infection-survival after pathogen injection, but there was evidence of strong infection defence senescence after cuticle inoculation. These findings suggest that females have poorer overall barrier defences than males and that in females, pathogen defence senescence occurs via deterioration of systemic defences. In contrast, males exhibit clear barrier defence deterioration, but no change in systemic pathogen defence.

AMPs are expressed in the fly barrier epithelia (Tzou et al. 2000), therefore the decline in barrier defence efficacy I observed in male flies could be the result of an age-dependent decline in the local expression of AMPs in the epithelia underlying the cuticle. Such a decline might enable more extensive fungal invasion of the haemolymph in older flies. The systemic immune response in *Drosophila* adults relies to a large extent on AMP production in the fat body (Wicker et al. 1990, Dimarcq et al. 1994, Levashina et al. 1995) and on a range of cellular immune responses (Ferrandon et al. 2007). It would be tempting to

speculate that the reduced systemic disease defence I observed in females was driven by impaired AMP expression in older *D. melanogaster*, but this is contradicted by several studies which show that the opposite is true and that AMP expression is upregulated with age (Pletcher et al. 2002, Zerofsky et al. 2005). A more plausible explanation, both for this systemic pathogen defence senescence and for why I only observed it in females, is that there may be sex specific deterioration of the cellular immune response. The number of phagocytic blood cells declines with age in female *D. melanogaster*, but not males (Mackenzie et al. 2011) and age-dependent changes occur in the expression of relevant genes that regulate the phagocytic process (Horn et al. 2014).

The differences in the pattern of pathogen defence I observed between male and female flies may be influenced by sex specific selection on immune defence driven by the different ecologies of the two sexes (Sheldon and Verhulst 1996) reviewed in (Schmid-Hempel and Ebert 2003, Schulenburg et al. 2009). Female flies, at young and old ages, appeared to have poorer barrier defences to infection than males; this is in contrast to standard life history theories which suggest females are usually selected to invest more in maintenance traits than males (Stearns 1992). This discrepancy could be due to costly resource trade-offs between immunity and other fitness functions (Stearns 1992, Moret and Schmid-Hempel 2000) if investing in cuticle pathogen defences is, for some reason, more costly for females than for males. Alternatively, male ecology may specifically

select for greater investment in barrier defences than in females if the male cuticle is more likely to be damaged. Competitive fighting with other males for access to sexual partners (Saleem et al. 2014) could potentially cause cuticle damage, and this may select for a more robust cuticle in males, possibly with the inadvertent consequence of higher disease resistance.

Furthermore, some comments on the experimental design used in this work investigating sex-specific routes to immune senescence need to be made. Males and females were both infected with *B. bassiana* spores, either by cuticle dusting or by an injection using a wire needle (Neyen et al. 2014). The arguments I have made about sex differences in pathogen susceptibility might be undermined if the two sexes did not receive the same dose of pathogen. The infection administration protocol was identical for females and males. However, the sexes differ in their size, with females bigger than males (Partridge et al. 1994). Females have a greater absolute surface area on which to pick up fungal spores than males, so dose differences in the cuticle inoculation experiments might account for female higher mortality. On the other hand, the larger body volume of females and relatively smaller surface area corrected for body mass, may mean the mass-corrected pathogen burden is lower in females than in males. Even if these absolute or relative differences in dose between the sexes influenced the outcome of experiments, the sex differences revealed in this investigation

nevertheless represent the outcome of standardised pathogen encounters as might occur in the field.

The experimental design made the assumption that in the cuticle inoculation experiment, the likelihood of fly death was influenced by the combined efficacy of the barrier and systemic defences; however, in the haemocoelic injection treatment only the efficacy of systemic defences was relevant. To draw conclusions about the efficacy of barrier defences a subtractive approach was needed to account for the efficacy of systemic defence in preventing infection mortality. In the case of males, the result was clear cut: because there was no age-dependent change in infection survival after haemocoel injection, reduced survival in aged males receiving cuticle spore inoculations must be driven by barrier defence changes. However, for females, where I saw comparable levels of age-dependent survival declines in the cuticle infection and spore injection treatments, two interpretations are possible. The simplest explanation, which I have highlighted in Chapter 2, is that senescence of the systemic immune system is sufficient to explain the declines in survivorship following the injection and spore dusting treatments, therefore there is no evidence of senescence in barrier defences. However, an alternative explanation is that because survival declined with age following infection by both exposure routes, the efficacy of both the barrier and systemic pathogen defences senesce. This seems less likely, as if both defence systems suffer

senescence, I would have predicted greater age-related decline in infection survival in the cuticle inoculation treatment than following haemocoelic injection, which did not happen. Whichever explanation is the case, clearly the processes underlying pathogen defence senescence are different in males and females.

To test the validity of these conclusions further, other investigations could experimentally manipulate the immune response to provide more conclusive confirmation. To verify my speculation that senescence of the cellular immune response is responsible for survival differences between young and old female flies following fungal spore injection, the cellular phagocytic response could be inhibited by injection of polystyrene beads (Elrod-Erickson et al. 2000). This would leave humoral immunity, such as AMP production by the fat-body still intact. This manipulation should not alter the age-dependent trends observed for males that receive a haemocoelic spore injection (except that mean survival would probably be lower) because males did not display systemic immune senescence. However, for females, if senescence of cellular immunity explains pathogen defence senescence, the senescent trend seen following spore injection should disappear in flies in which phagocytosis is experimentally blocked.

4.2 Hygienic behaviour

Chapter 3 tested if senescence of pathogen resistance by the barrier defences can be explained by changes in grooming behaviour while flies are ageing. The experiment showed that coloured dye adheres more strongly to the cuticle of female and male *Drosophila* as they age (considerably more than a two-fold increase between 1 week and 4 weeks of age). Allowing flies to groom revealed that old flies clean the majority of dye from their cuticle within 30 minutes; after two hours there were no differences in the amount of adhered dye between the age groups. Generally, covering flies with a foreign material induces immediate grooming activity (Yanagawa et al. 2014). Whilst I wished to test whether flies suffer impaired grooming behaviour as they senesce, the results did not show any evidence that flies of either sex have an age-related decline in their ability to clean.

4.3 Cuticular lipid extraction and re-application

In my work dusting flies with dye powder, aged flies showed significantly elevated levels of dye stuck to their cuticle immediately after dusting. By using a solvent extraction technique (Chao et al. 2010) to switch cuticular hydrocarbons between young and old flies, I changed the dye-adhesion characteristics of the cuticle, causing young flies to resemble old flies and *vice versa*.

This result demonstrates that flies underwent changes in their cuticle while they aged and it seems most likely that a change of cuticular hydrocarbon profile is responsible. Cuticular hydrocarbons in *Drosophila* differ between the sexes and change dramatically while flies are ageing (Jackson and Bartelt 1986, Schaner et al. 1989, Everaerts et al. 2010, Kuo et al. 2012). Whilst it is possible that other materials, such as the biofilm and microbial burdens that are associated with the cuticle of aged flies (Ren et al. 2007), were also transferred alongside the cuticle hydrocarbons, it is less likely that these would be the cause of elevated dye adherence in old flies. It might be possible to verify this by electron microscopy and staining of potential biofilms, which could reveal where on the cuticle the increased dye load on aged flies adheres. An alternative method would be to analyse cuticular hydrocarbons on young and old flies by GC-MS and then create an artificial hydrocarbon mixture from the most abundant compounds found on the cuticle of each age group. This would enable a definitive experimental test of the role of cuticular hydrocarbons in driving the adherent cuticle phenotype in old flies.

I have shown that the hygienic grooming behaviour of flies did not senesce and that age-dependent cuticle changes made the fly exterior more adhesive to foreign particles. The validity of these model approaches using the dye bromophenol blue might be questioned, in terms of how well these assays mimic the interaction between pathogens, such as fungal spores, and the cuticle.

An artificial dye will never wholly reflect a real exposure of the cuticle to fungal spores, but given the fact that this dye has hydrophobic properties like *B. bassiana* spores (Zhang et al. 2011) this model approach can at least provide some valuable illumination.

4.4 Concluding perspectives

This study contributes fundamental knowledge to a research field that is still poorly understood. With increasing human life expectancy, the impact of age-related ill-health and elevated infectious disease incidence in the elderly have become major problems for our society. Sharing highly conserved gene families with humans, as well as analogous tissues and organs, *D. melanogaster* provides an excellent model to study the biological processes of ageing (Matthews et al. 2005). With a lifespan of only four to eight weeks, senescence and disease studies are simplified. Some ageing-related infection studies in *Drosophila* have addressed senescence of the intestinal barrier (Rera et al. 2012, Clark et al. 2015) but to my knowledge none has previously investigated the senescence of pathogen defence of the outer epithelia and cuticle. My work on *Drosophila* could shed new light on the processes and principles that may influence senescence of pathogen barrier defences in humans. Particularly, it seems probable that the principle that the mechanisms underlying pathogen defence senescence can be strongly sex-specific will be generalisable to a wide range of other organisms,

potentially including people. However, the fact that the structure of insect cuticle is so different to the epidermal barriers of humans may limit the utility of this study to drive new knowledge of the mechanisms of pathogen resistance senescence in humans.

Nevertheless, this work on *Drosophila* may provide useful model data to inform understanding of senescent processes in medically relevant insects, such as another group of Diptera, the mosquitos. Mosquitos vector a wide range of debilitating human pathogens including *Plasmodium* that causes malaria (Cox 2010). After an infectious blood meal, it takes between 8 and 35 days (depending on the environment and the parasite species) for *Plasmodium* to develop in the mosquito and reach the salivary glands to enable onward transmission (Becker et al. 2003, Heymann 2004). Therefore, mosquitos that survive longer into old age with an increased lifespan make more efficient vectors (Bruce-Chwatt 1980). Major efforts to develop new control strategies for malarial mosquitoes using entomopathogenic fungi are underway because fungi generally kill mosquito hosts slowly, often after host reproduction, minimising the strong selection for vector resistance evolution that has hampered control strategies using chemical insecticides (Read et al. 2009). The knowledge of the processes of insect immune senescence that my work provides may assist these efforts, enabling entomopathogenic biopesticides to be targeted to disproportionately kill older mosquitoes, which provide the greatest disease transmission threat.

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