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Evolutionary trade-offs with innate immune resistance; implications for ageing, oxidative stress resistance and motor function

by

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ABSTRACT

UNIVERSITY OF SOUTHAMPTON
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EVOLUTIONARY TRADE-OFFS WITH INNATE IMMUNE RESISTANCE; IMPLICATIONS FOR AGEING, OXIDATIVE STRESS RESISTANCE AND MOTOR FUNCTION

by Kirstin Joanna Williamson

Resistance to infection is essential to ensure survival and thus maximise offspring potential. However, resistance is not ubiquitous across the animal kingdom, or even within a population from the same species. It is thought that this is due, in part, to the costs involved in producing and maintaining a competent immune system and a corresponding decrease in other fitness-related characteristics. The focus of this research was to determine how immune resistance can impact upon mechanisms relating to ageing, resistance to oxidative stress and motor function. In order to do this a *Drosophila melanogaster* model system was implemented, selected for resistance to larval parasitism by the parasitoid wasp, *Asobara tabida*.

Firstly, it was necessary to gain a greater understanding of the immune mechanisms within the *Drosophila* model. This included how aspects of the immune system changed over time, in order to determine how these might act upon other processes at different stages of the ageing system. Resistance to larval parasitism corresponded to an increased number of circulating immune cells during the larval phase. This difference was no longer apparent in the adult *Drosophila*. Young resistant adult females revealed increased levels of overall cell metabolism, measured by the production of intracellular reactive oxygen species (ROS), a finding not seen in males or in the developing larvae. Lifespan was reduced in the resistant female, but not male, *Drosophila*. It was hypothesised that this reduction may in part be due to the augmented production of intracellular ROS in the young adult female, which at high concentrations can cause oxidative stress with known cytotoxic effects. However, differences in resistance did not translate to altered survival under acute oxidative stress, induced by the consumption of the toxin paraquat. Other factors may regulate these changes in longevity in the resistant females, such as genetic or resource-based trade-offs.

Functional assays were performed to assess motor function in the larvae and adult *Drosophila*. Resistant larvae showed less turning behaviour on a non-food background than their control counterparts, a trait generally linked to more proficient motor function. Differences in motor function continued into the adult females, where increased climbing velocities were found irrespective of age. This implies that changes in motor function may be determined during development, thus variations in resistance during this phase can cause life-long impacts on the individual, presumably by altering the development of other physiological systems.

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DECLARATION OF AUTHORSHIP

I, Kirstin Joanna Williamson declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

"Evolutionary trade-offs with innate immune resistance; implications for ageing, oxidative stress resistance and motor function"

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

7. None of this work has been published before submission.

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Date: 30.10.2013

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LIST OF ABBREVIATIONS

AIDS – acquired immunodeficiency syndrome

ANOVA – analysis of variance

BBB – blood brain barrier

CLC – complex life cycle

CPG – central pattern generator

CNS – central nervous system

DCF – dichlorofluorescein

DCFH – dichlorofluorecin

DCFH-DA – 2', 7' – dichlorofluorescin diacetate

DMSO – dimethyl sulfoxide

Dopa – dihydroxy-phenyl-alanine

ECM – extracellular matrix

EH – embryonically-derived hemocytes

Gcm/*gcm* – glial cells missing

GST – glutathione S-transferase

HSP – heat shock protein

IIS – insulin/IGF (insulin-like growth factor)-like signalling

JNK – c-jun n-terminal kinase

LGH – lymph gland-derived hemocytes

LOOH – lipid hydroperoxide

MAPK – mitogen-activated protein kinase

NOS – nitric oxide synthase

PBS – phosphate buffered saline

PBS-T – phosphate buffered saline-Tween

PH – proportional hazard

PHGPx – phospholipid hydroperoxide glutathione peroxidase

PO – phenoloxidase

ProPO – prophenoloxidase

ROS – reactive oxygen species

TNF – tumor necrosis factor

TOR – target of rapamycin

VNC – ventral nerve cord

1 General Introduction

1.1 Evolutionary trade-offs

Life on Earth encompasses an immeasurable amount of diversity displaying numerous examples of how organisms have adapted to survive and thrive in often extreme and hostile environments. Anglerfish have adopted an exceedingly sedentary lifestyle and exhibit low metabolic rates, which allows them to live 4000 metres beneath sea level where oxygen levels are very low (Cowles & Childress, 1995). Emperor penguins have a range of inbuilt thermoregulatory mechanisms, such as huddling behaviour and high levels of feather- and fat-based insulation, which enable them to withstand temperatures below -40°C (Mccafferty *et al.*, 2013). Underlying these adaptations lays one central theme that unites every living creature, and that is survival of the germ-line; an organism must survive and reproduce optimally, in order to maximise the transferral of their genes to the next generation (Hirshfield & Tinkle, 1975). Characteristics that help to fulfil these requirements will most likely allow the animal to thrive within its environment and thus these traits should be passed on to future generations. In contrast, characteristics that hinder an animal's chance of survival or reproduction will likely be lost along the way. Selection will therefore preserve the genes expressed in an organism that are optimally suited to the environment, coding for a 'fitter' individual. However, fitness is not universal and what may be suited to survival in one environment may not be optimal in another. Ideally an organism would be prepared for all potential threats within an environment and ensure successful mating, but in truth, an individual has a limited set of resources from which all physiological functions must be fuelled (Maynard Smith, 1974). Therefore, what underlies the fitness of all individuals is a careful balance of resource allocation. From this arises the trade-offs that are ubiquitous in all living creatures.

The principle of trade-offs is so fundamental to our outlook of the world that it precedes any modern understanding of biology or evolutionary theory. A trade-off can be viewed as a compromise between two optimal features, where a change in one optimal feature comes at a detriment to another optimal feature. Trade-offs can occur as a direct impact of one feature on another, but can also occur as an indirect result of competition for

shared resources; the allocation of resources to specific traits will mean a deficit in their availability for other traits. This theory extends through all natural and social sciences e.g. limitations of substrates and enzymes in chemistry or investment in healthcare versus public health education in politics. In the past one hundred and fifty four years trade-off theory has become integral in biology to explain why animals show reduced function and disease, ever since it formed the basis of our current understanding of natural selection and evolution as discussed by Darwin in 'the Origin of Species':

"As Goethe expressed it, 'In order to spend on one side, nature is forced to economise on the other side.' I think this holds true to a certain extent with our domestic productions: if nourishment flows to one part or organ in excess, it rarely flows, at least in excess, to another part; thus it is difficult to get a cow to give much milk and to fatten readily. The same varieties of the cabbage do not yield abundant and nutritious foliage and a copious supply of oil-bearing seeds. When the seeds in our fruits become atrophied, the fruit itself gains largely in size and quality." (Darwin, 1859, p147).

1.2 Research question

Although trade-offs will be observed throughout all aspects of an organism's phenotype, the areas most sensitive to selection will be those directly related to the organism's early survival and reproductive success. Allocation toward these areas would provide the animal with a competitive edge against its conspecifics and the pathogens that it might encounter, thus aiding the continuation of these genes into future generations. In this thesis I focus on trade-offs associated with a system on which selection pressures act heavily: the immune system.

Variation within the immune system can provide organisms with resistance against a wide range of pathogens (Schmid-Hempel & Ebert, 2003). However, immune resistance requires the upregulation of a series of costly immune mechanisms and, as such, is likely to come at a detriment to the individual (Lochmiller & Deerenberg, 2000, Sheldon & Verhulst, 1996). To date, experiments aimed at assessing the costs of immune resistance have focussed strongly on trade-offs in fitness-related traits, such as growth and fecundity (Fellowes *et al.*, 1998, Siva-Jothy *et al.*, 2005, Vijendravarma *et*

al., 2009). Far fewer experiments attempt to identify trade-offs in traits that are not classically linked to an organism's evolutionary fitness, consequently very little information exists in this area (Siva-Jothy *et al.*, 2005). This thesis was aimed at elucidating the impacts immune resistance has on characteristics that are less strongly associated to fitness, in particular looking for trade-offs in: ageing, oxidative stress resistance and motor function. Some of these traits may still play an indirect role in the animals' survival and/or reproductive success, for example proper motor function might be required for finding food or attracting a mate. In addition, it is important to understand how and why immune resistance affects these traits, due to the major impact these factors play on an individual's long-term health and function.

1.3 Introduction to immunity

It was in 1882 when Élie Metchnikoff, a Russian scientist, first stumbled across a lowly starfish larvae stranded on an Italian beach; an encounter that would go on to become the foundations of immunology. What Metchnikoff had noted was that when a foreign body (in this case a thorn) pierced an organism (a starfish larvae) it changed the behaviour of the cells within, causing them to locate to and surround the invading article (Figure 1.1) (Beck & Habicht, 1996). This simple observation led to a series of comparative animal studies that went on to revolutionise medicine and our understanding of the immune system (Kaufmann, 2008) .

Figure 1.1 Immune cells within the starfish larva surrounding a thorn
Metchnikoff later coined these cells "phagocytes" after the Greek words "phages" (to eat) and "cite" (cell), due to the cells unique ability to engulf or 'eat' invading pathogens (Tan & Dee, 2009). Image taken from Beck & Habicht (1996).

All organisms are at risk of infection from a range of pathogens, such as proteins, viruses, bacteria, fungi and parasites. In response to this, organisms have evolved a variety of immune defences (Akira *et al.*, 2006). The simplest form of immunity is the physical barrier defence, which incorporates the mechanisms that make it more difficult for pathogens to enter the body, such as the epidermis, mucous membranes and stomach acid (Siva-Jothy *et al.*, 2005). However, should these fail to prevent pathogen entry, then there are also a variety of immune defences in place within the organism ready to locate and kill the intruder (Sheldon & Verhulst, 1996).

1.3.1 Innate versus adaptive defence

The immune system consists of two different arms: innate and adaptive (Akira *et al.*, 2006). The reliance an organism has on these systems will depend partially on the risks imposed within its environment but also its evolutionary origins. Innate immunity can be seen as more reflective of a primitive immunological response, in that it can be

observed from the origins of the animal kingdom predating the advent of metazoic life (Beck & Habicht, 1996). Adaptive immunity arose with the Gnathostomata (jawed vertebrates) around 500 million years ago (Pancer & Cooper, 2006, Flajnik & Du Pasquier, 2004). Despite the more modern origins of the adaptive immune response it should not be interpreted as a 'better' or more effective immune response, in truth it is often the contrary. Unlike adaptive responses, innate immunity is often a response better suited to a more variable environment where the individual may be exposed to novel, fast-acting pathogens (Medzhitov & Janeway Jr, 1997, Davenport *et al.*, 2009). Innate responses of insect larvae have demonstrated the ability to withstand infection of pathogenic bacteria such as tuberculosis, tetanus, the plague and leprosy that would overwhelm the mammalian immune system (Metalnikov, 1924, Cameron, 1934, Salt, 1970). The benefits of an adaptive response are more apparent in longer-lived animals, which are more likely to experience repeated or more prolonged infections (Medzhitov & Janeway Jr, 1997). Allocation of resources to developing an adaptive response to infection may not immediately alter an organism's survival but could subsequently provide the animal with a long-term survival advantage.

1.3.2 Innate immunity

Innate immunity is an organism's first line of defence and is marked by a series of immediately available, non-specific mechanisms ubiquitously expressed across the animal kingdom (Akira *et al.*, 2006). This type of defence is coded on the germ-line and is passed through the generations from parent to offspring. Although there is variation between some of the systems in place, many of the fundamental mechanisms show a high level of conservation across the animal phyla (Akira *et al.*, 2006).

In all organisms the innate immune system comprises of two arms; cellular and humoral (chemical-based). Within the cell-mediated arm, there are subsets of immune cells (e.g. macrophages, dendritic cells, plasmatocytes) that act as the animal's surveillance system (Williams, 2007). These cells are able to distinguish self from non-self through a limited set of pattern-recognition receptors that can be found expressed on the immune cell membrane (e.g. Toll and toll-like receptors) or as soluble receptors in the blood (complement system) (Akira *et al.*, 2006). Upon recognition of pathogen-associated

molecular peptides, present on a range of pathogens, these cells activate a series of humoral and cell-mediated responses (Table 1.1). The responses activated include: recruitment of additional immune cells to the site of infection (through the release of chemical mediators e.g. cytokines), activation of phagocytic cells (e.g. monocytes, macrophages and neutrophils that release an assortment of antimicrobial peptides, enzymes and reactive oxygen species as well as actively engulfing infected cells and pathogens), and a series of inflammatory responses (Fialkow *et al.*, 2007, Hancock & Diamond, 2000).

Inflammatory responses can differ widely between animals. Vertebrate responses are characterised by swelling, heat and redness (Renshaw & Trede, 2012). This is caused by the rupture of granulocytes (eosinophils, basophils) and mast cells, which release cytotoxic reactive oxygen species, cytokines and histamine, the latter of which causes blood vessel dilation and the additional inflow of white blood cells into the area (e.g. neutrophils, lymphocytes and the macrophage precursor monocytes) (Alberts *et al.*, 2002). Once infiltrated, lymphocytes (natural killer cells and cytotoxic T cells) induce apoptosis in cells expressing low levels of the class I MHC proteins. MHC proteins are generally expressed on self cells unless the cell becomes infected or cancerous (Alberts *et al.*, 2002).

Humoral enzyme cascades found within vertebrates and invertebrate circulatory systems can also act to kill invading pathogens (Table 1.1) by enhancing the cellular responses and rupturing pathogen membranes (vertebrate and invertebrate complement cascade) and generating cytotoxic compounds (invertebrate phenoloxidase cascade) (Hoffmann, 1995, Alberts *et al.*, 2002). In addition, invertebrates display a range of additional cell-mediated responses, such as nodulation (against small pathogens) and encapsulation (against large pathogens), where immune cells surround and bind to invading pathogens, partitioning them off from the host (Lavine & Strand, 2002).

Table 1.1 Innate and adaptive immunity

Immune cells and cascades found within the invertebrate and vertebrate immune systems. Immune cells within invertebrates vary widely between organisms, listed below are some of the most commonly found invertebrate immune cells (Lavine & Strand, 2002). In vertebrates cytotoxic T cells and natural killer cells are involved in both innate and adaptive immune responses (shown under broken line). Any immune cell involved in phagocytosis has been marked with an asterisk (*).

<table>
<tr><td></td><td colspan="2">INNATE</td><td>ADAPTIVE</td></tr>
<tr><td></td><td>Invertebrate</td><td colspan="2">Vertebrate</td></tr>
<tr><td rowspan="2">Immune cells</td><td rowspan="2">Prohemocyte
Plasmatocytes*
Granulocytes*
Lamellocytes
Spherule cells
Oenocytoid cells:
Crystal cells</td><td>Monocytes*
Macrophages*
Dendritic cells*
Mast cells*
Granulocytes:
(Neutrophil*,
Basophil,
Eosinophil)</td><td>T helper cells
B cells</td></tr>
<tr><td colspan="2">Cytotoxic T cells
Natural killer cells</td></tr>
<tr><td>Cascades</td><td>Phenoloxidase
Complement - some memory function</td><td colspan="2">Complement</td></tr>
</table>

1.3.3 Adaptive immunity

The adaptive, or acquired, immune system is not a prominent part of this thesis and as such will not be covered in depth. However, it is important to understand that within the vertebrate immune system, the innate and adaptive arms are intrinsically linked and work together to provide protection against invading pathogens, thus the adaptive system plays a vital role in determining the outcome of infection in vertebrates (Werling & Jungi, 2003). Unlike innate immune responses, adaptive defences are slow to develop when presented with a novel pathogen (Davenport *et al.*, 2009). Instead, the key role of the adaptive arm is to enable the host immune system to 'remember' a prior experience with a pathogen, allowing much more rapid responses and more efficient eradication of the pathogen upon subsequent infection (Cooper & Alder, 2006, Kurtz, 2004).

When dendritic cells engulf and break down invading microbes, they migrate to peripheral lymphoid organs and present peptide remnants of the degraded pathogen (antigens) to resident T helper (T_H) cells (Alberts *et al.*, 2002). In response to this presentation, a number of activated T_H cells then migrate to the area of infection and help destroy the pathogens by activating macrophages and cytotoxic T cells (Alberts *et al.*, 2002). Some activated T_H cells stay within the lymphoid organ and prime B cells to proliferate and release antibodies against the initial antigen. Antibodies activate complement and bind to antigens directly in order to inactivate the pathogen (Parkin & Cohen, 2001).

1.4 Introduction to Ageing

An animal's lifespan is highly dependent on the risk imposed within its environment and the mechanisms that an organism has in place to help protect itself from extrinsic mortality (Kirkwood & Austad, 2000). Features that defend an organism from extrinsic hazards, such as protective shells and wings, have been shown to correlate with increased longevity (Kirkwood & Austad, 2000). Consequently, birds and bats tend to live longer than mammals of a comparable size (Partridge & Gems, 2002, Barzilai *et al.*, 2012). The time-frame in which an animal will survive can be extended by the reduction or removal of extrinsic hazards. When extrinsic mortality is eliminated an animal will exhibit senescence, characterised by reduced fertility, functional decline and an

increased risk of intrinsic mortality (Partridge & Gems, 2002, Kirkwood & Austad, 2000). The pattern of longevity between species generally remains the same from natural to protected environments, implying that senescence is intrinsically linked to survival in the wild (Partridge & Gems, 2002).

1.4.1 Theories of ageing

Since 1900 improved hygiene, nutrition and medicine have seen human lifespan increase by around 30 years in developed countries, but with this there has been an increase in the prevalence of senescence-related disease and functional decline (Christensen *et al.*, 2009). Ageing has therefore been the focus of intense research attention, but has proved to be a difficult phenomenon to elucidate. Many theories have been proposed, implicating a number of factors including, but not limited to: rate of living, wear-and-tear, mutation accumulation, antagonistic pleiotropy, oxidative stress and endocrine signalling (reviewed in Jin, 2010). Many of these theories centre around the evolutionary principle of trade-offs in some form, with differing emphasis on the role of genetics, resource allocation and other physiology that may act within an organism to cause senescence. Due to the expanse of the literature available the author has chosen three theories of ageing deemed to be relevant to changes in immune resistance on which to explain the findings found during this thesis, including the 'antagonistic pleiotropy', 'disposable soma' and 'oxidative stress' theories of ageing. Each of these theories is based upon the assumption that ageing is due to an imbalance between the accrual of damage throughout an organisms life and the mechanisms in place to protect against and repair this damage (Finch, 1994, Kirkwood & Austad, 2000).

1.4.2 Antagonistic pleiotropy theory of ageing

Although many factors are thought to influence the rate of ageing, such as diet and lifestyle, there is a large genetic component behind longevity with studies showing that siblings of humans living longer than 100 years (centenarians) are more likely to survive into their early nineties (Perls *et al.*, 1998). It is unlikely that an organism would evolve genes that specifically promote damage and ageing, as this would oppose the

rules set by natural selection. Natural selection generally eliminates genes that code for negative traits, as individuals expressing these genes will unlikely pass on their genes to future generations and as such these traits will be lost. One explanation as to why senescence exists is because it arises as a genetic trade-off from co-expression with another trait (Partridge & Gems, 2002).

Genetic changes arising from adaptations that organisms make to optimise their fitness can give rise to the expression of more than one trait; this is known as agonistic pleiotropy. In some cases, a gene coding for a beneficial effect can also lead to additional deleterious effects, which is termed antagonistic pleiotropy (Tower, 1996). In a similar fashion close physical linkage of two or more distinct genes could also result in opposing phenotypes (Stearns, 2010, Flint & Mackay, 2009). Based on this concept George Williams (1957) proposed the 'antagonistic pleiotropy theory of ageing', which implies that genes coding for fitness traits early in life, such as immunity, may also code for negative traits that become apparent in later life, such as those linked to protection against cellular damage (Williams, 1957). Genes that express negative traits in later life once many or all of the individual's potential offspring have been produced will not be subject to the same selection pressures (Drenos & Kirkwood, 2005). Therefore, if these genes are co-expressed with an early benefit then they will most likely remain in future generations.

Variation in immune resistance occurs due to differential expression of genes relating to the immune system (Wertheim *et al.*, 2011). Therefore, changes in expression of immune mechanisms are likely to have impacts within an animal, either via direct interactions between the immune system and other physiology or indirectly via competition for resources.

1.4.3 Disposable soma theory of ageing

In order to maintain long-term health and function, metabolic resources must be allocated toward somatic maintenance and repair, for example, through the expression of antioxidants and/or chaperone proteins (Kirkwood & Austad, 2000). However, in evolutionary terms, there is little to no selective advantage in buffering the effects of

ageing. Strategies that favour investment in somatic maintenance over early health or reproductive capacity will be at the detriment to the organism's fitness, as fewer individuals will reach sexual maturity and fewer offspring will be produced. Thus, when resources are limited, natural selection will favour investment towards components relating to fitness and away from somatic repair and maintenance. It is based upon this philosophy that the 'disposable soma theory of ageing' was proposed (Kirkwood & Holliday, 1979). Decreases in somatic protection and maintenance will be slow to take effect and may not become apparent until much later in life. If a negative phenotype arises past the point of selection, once most or all of the individual's potential offspring have been produced, then the genes coding for that trait will most likely remain in future generations.

In many environments investment into immune potential will directly affect the survival of the individual; a failure to successfully mount an immune response may result in a failure to successfully reach reproductive age. For this reason it is expected that the increasing resource expenditure on an effective immune system will divert resources that would otherwise be allocated to other traits. Consequently, if resources are required to maintain a competent immune system then they are likely to be reallocated away from the processes that buffer ageing.

1.4.4 Oxidative stress theory of ageing

All eukaryotic cells produce reactive oxygen species (ROS) as a byproduct of metabolism. Intracellular ROS production is therefore tightly regulated by a number of antioxidant enzymes, including superoxide dismutase, catalase and glutathione peroxidase, which convert ROS into the harmless end-products, such as water (Figure 1.2) (Sies, 1997). Oxidative stress occurs when there is an imbalance between the production of ROS (pro-oxidants) and the cells ability to counteract the build-up of these cytotoxic components (antioxidant defence) (Sies, 1991). If oxidative stress remains unregulated then this can lead to oxidative damage of the soma; the amalgamation of nucleic acid, protein and lipid damage, which can ultimately result in cell death. Sometimes mitochondrial function becomes upregulated and antioxidant

activity is insufficient to counteract the pathological effects of the associated ROS, as is the case in various life-threatening human diseases and ageing (Vafai & Mootha, 2012).

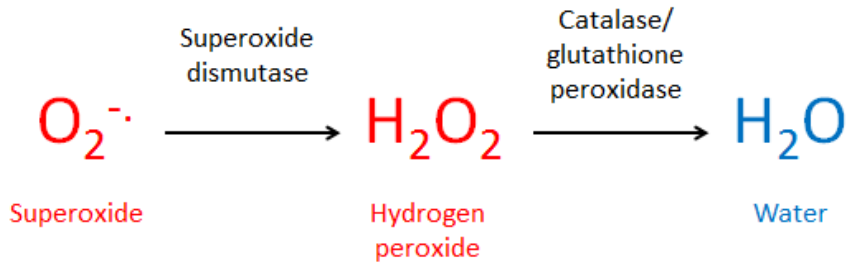


Figure 1.2 Intracellular ROS and their antioxidants

Conversion of the metabolic by-product superoxide into water; cytotoxic products (ROS) shown in red and harmless products in blue. The corresponding antioxidant enzymes that facilitate these reactions are written above the arrows in black.

Denham Harman (1955) suggested that the dysregulation between free radical production and antioxidant activity and the consequent accrual of oxidative damage to cellular macromolecules, such as DNA, proteins and lipids was one of the main causes of increased senescence ('the free radical theory of ageing' Harman, 1955, Gems & Doonan, 2009); a theory Harman later modified to incorporate other mitochondrial derived ROS (hydrogen peroxide (H_2O_2) and peroxynitrite ($OONO^-$)), which was termed the 'oxidative stress theory of ageing' (Harman, 1972). Although there has been some controversy about whether free radicals and/or mitochondrial derived ROS can directly lead to senescence, it is clear that they play a role in a decline in cell and tissue function and are thus likely to be contributing factors in the aging process. This subject has been well reviewed by Kirkwood and Kowald (2012) in 'The free-radical theory of ageing – older, wiser and still alive'.

1.4.5 Immunity and oxidative stress

Immunity and oxidative stress are physiologically linked, though the information surrounding this connection remains sparse (Lozano *et al.*, 2013). One main

contributing factor linking immunity to oxidative stress is the ability of immune cells to upregulate their ROS production as part of an immune response. When activated, vertebrate and invertebrate immune cells (neutrophils and macrophages) produce higher levels of ROS, as well as reactive nitrogen species, which they can release to kill pathogens; a process known as a 'respiratory burst' (Nappi & Vass, 1998, Nappi & Christensen, 2005, Moss & Allam, 2006, Rada & Leto, 2008). To counteract the damaging effects of immune-induced ROS an animal must therefore be able to upregulate the level of antioxidants within the cell. Animals with heightened immunity should therefore exhibit an increased ability to counteract increases in ROS.

1.4.6 Oxidative stress resistance

In addition to immune defence, chemical and environmental stress factors such as hypoxia, UV stress, pollutants and toxins can also increase the amount of ROS production (Blokhina *et al.,* 2003, Li *et al.,* 2003), shifting the balance in favour of the pro-oxidants and thus leading to oxidative stress within the cell. Therefore the ability to resist oxidative stress is important throughout the life of an organism to maintain health and function. Oxidative stress has been linked to the pathogenesis of diseases such as both types of diabetes mellitus and the following neurodegenerative disorders: Parkinson's disease, Alzheimer's disease and amylotropic lateral sclerosis (Andersen, 2004, Maritim *et al.,* 2003).

1.5 Motor function

Motor function is the effective use of the nervous system to stimulate muscle contraction in a coordinated manner to control the movement of the organism. Successful motor function can be essential for non-sessile animals, for the acquisition of nutrients, a mate and also to avoid threats within the environment, thus motor function can indirectly impact on fitness. In humans, possessing and retaining healthy motor function is also crucial for maintaining an individual's independence throughout life and into old age.

1.5.1 The role of the nervous system

The nervous system is comprised of two distinct cell types; the neuronal and glial cells. Neurons are the core component of the nervous system. They are specialised for the propagation of electrical and chemical signals. In both vertebrate and invertebrate systems neurons form complex circuits connecting large networks of interacting nerve cells, with a greater degree of complexity in the vertebrate systems (Katz *et al.*, 2013). Afferent (sensory) neurons transmit information about the internal and external environment toward the central nervous system, which in turn sends out signals along the efferent (effector) neurons to initiate an appropriate action (Ganong & Barrett, 2005). In motor circuitry, the effectors are the motor neurons. They convey information sent by the central nervous system resulting in the controlled contraction of muscle cells, which act to coordinate an appropriate motor response. Therefore, neurons are crucial regulators of motor function.

Glial cells regulate homeostasis of the neurons by providing insulation, supplementing them with growth factors and cytokines and by their role in establishing and maintaining the crucial barrier between the blood and the nervous system that protects the neural tissue; the blood brain barrier (BBB) (Jones *et al.*, 1995). Immune function within the nervous system is carried out by the glia, or specifically the microglia in vertebrates, which act by engulfing dying cells and pathogens.

1.5.2 Interactions between the immune and nervous systems

The immune and nervous systems are highly linked. In the first instance, immune cells (e.g. phagocytes and glia) from invertebrate and vertebrate organisms are crucial regulators of nervous system development, with involvement in brain and nervous system modelling (Olofsson & Page, 2005, Perry & Gordon, 1988).

Glial cells carry out the majority of immune functions within the CNS. However, upon infection there is also an influx of peripheral immune cells. Interactions between peripheral immune cells and the central nervous system (CNS) are tightly regulated by the blood-brain-barrier (BBB) (Engelhardt, 2008). This barrier provides the CNS with protection and ensures that nervous system function is not disturbed by systemic fluctuations. In mammals, microglia help coordinate and communicate with additional

immune cells that enter through the BBB barrier into the CNS upon infection. Although peripheral immune cell activity is vital to protect against infection and for repair, inflammatory mediators released by these cells can lead to profound damage to the neural tissue. Axonal injury can be caused by a number of neurodegeneration-inducing factors such as cytokines, enzymes, oxidative products and free radicals such as superoxide, all of which are released by activated glial cells and peripheral immune cells upon infection. Upregulation of nitric oxide synthase (NOS) in activated immune cells causes an overproduction of nitric oxide (NO). NO may act solely or in combination with superoxide ions to contribute to neurodegeneration (Brown & Borutaite, 2002, Liu *et al.*, 2002). Goureau and colleagues (1999) showed that addition of superoxide dismustase and peroxynitrite scavengers resulted in partial inhibition of NO-mediated neurotoxicity in murine retinal Müller glial cells suggesting the requirement of oxygen free radicals for NO toxicity. NO release in response to infection is a process conserved from invertebrates to vertebrates and has been shown to occur in *D. melanogaster* exhibiting the encapsulation response upon infection by the parasitoid *Leptopilina boulardi* and may therefore lead to increased vulnerability in the nervous system (Nappi *et al.*, 2000).

Defects in the immune system can lead to reduced motor function, for example, approximately 80% of acquired immunodeficiency syndrome (AIDS) patients also exhibit neuropathological symptoms with around half of all patients also suffering from AIDS-related dementia complex; exhibiting a series of neurological impairments including motor, cognitive and behavioural defects (Pugh *et al.*, 2001).

1.6 Measuring trade-offs with immune resistance

In recent years, many studies have attempted to establish the trade-offs that occur as a result of immunocompetance (Siva-Jothy *et al.*, 2005, Schmid-Hempel, 2005, Rolff & Siva-Jothy, 2003). From the literature it appears that there are four main methods frequently employed to identify such trade-offs (Siva-Jothy *et al.*, 2005, Norris & Evans, 2000). The approach taken by a study is likely to vary with regard to its intended goal; those looking at the trade-offs relevant to an organisms life history may attempt a more natural environment, whereas studies focussing on the mechanics of trade-offs may take

characteristics in a more isolated, and possibly less ecologically relevant environment. Inevitably each approach has its constraints but can also provide certain advantages. The four approaches used to elucidate trade-offs in a system are outlined below along with discussion of the constraints associated with such an approach.

1.6.1 Decrease resource availability for immune resistance

One approach has been to observe how resistance is affected when other life history traits are upregulated or when nutrient resources become limited. This indirect approach has been useful in demonstrating resource reallocation; how resources can become diverted away from one function, such as immunity, when other functions are upregulated. For example, it has been shown that lactating ewes, *Ovis canadensis*, reveal higher faecal lungworm larval counts than non-lactating ewes (Festa-Bianchet, 1989) and worker bumblebees, *Bombus terrestis*, inhibited from foraging exhibit a higher immune response to novel pathogens compared to those that are allowed to forage (König & Schmid-Hempel, 1995). If resources become limited a similar phenomenon is observed. A reduction of nutrients in the diets of young bobwhite quails, *Colinus* virginianus, can lead to reduced development of lymphoid organs (spleen and the avian bursa of Fabricius) and diminished cell-mediated immunity (Lochmiller *et al.*, 1993). Although these studies are useful to explain how resources can be pooled away from immune defence they offer little indication of the opposite scenario, when resources may be pooled away from other traits in favour of allocation toward immunity.

1.6.2 Artificial manipulation of immune resistance

Another method for assessing the costs of immunocompetance has been to artificially manipulate immunity either by genetic knockout or from chemically altering the immune system. Knockout studies have been useful for understanding resistance mechanisms, but less useful in establishing the costs involved in resistance, as one knockdown will only eliminate part of a very complex immune system. Dietary supplementation can be used, for example, the immunostimulant methionine is known to enhance avian T-cell responses (Soler *et al.*, 2003, Tschirren & Richner, 2006, Zuk & Stoehr, 2002). Conversely, immune downregulation can be accomplished by the

injection of immunosuppressive drugs, such as cyclophosphamide, which represses humoral but not cell-mediated immunity (Derting & Compton, 2003) However, with these studies it is not possible to tell what direct effects that these chemicals might be having on the animal. In addition, if naturally inherited trade-offs occur due to changes in the germ-line it may not be possible to induce the same trade-offs when stimulating the immune system in later life. Selection for immunity may instead be constrained by genetic correlations with other traits, rather than constraints on physiological mechanisms.

1.6.3 Correlations in natural experiments

Another approach to determining the cost of immune resistance has been to look at the correlations which exist in 'natural experiments' as opposed to those established in a laboratory environment. Monitoring studies sampling individuals from natural, wild populations with differing pathogenic exposures have been used to measure negative correlations between immunocompetence (e.g. immune cell number or activity) and other life-history traits. Møller *et al.* (2001) found that more social swallow species display greater immune investment in T- and B- cell responses and correspondingly longer development times. Longer development times were also shown in wild Indian meal moths (*Plodia interpuntella*) with higher levels of resistance against the granulosis virus (Vail & Tebbets, 1990). Unfortunately sampling from wild populations also produces a large number of uncontrolled variables. For example, it is not possible to determine whether a high lymphocyte count correlates to immunocompetance or to the level of infection encountered by that population at the time of sampling or indeed due to the influence of other external factors unrelated to immunity (Norris & Evans, 2000).

1.6.4 Selection for immune resistance directly

Finally, it is possible to artificially select for the immune trait of interest and to record the corresponding phenotypes. This method is useful for generating more ecologically relevant trade-offs associated with immune resistance. Norris and Evans (2000) argued that artificial selection was an important tool in determining trade-offs with immunocompetance and, as such, future work should utilise this method. However, few studies have taken this approach due to the complexity, ethical challenges, time and

financial costs involved (Verhulst *et al.*, 1999, Norris & Evans, 2000). To reduce the associated financial costs, time-frame required and avert ethical issues, most of the work performed in this area has utilised insect model systems (Cotter *et al.*, 2008, Kraaijeveld & Godfray, 1997, Fellowes *et al.*, 1998, Vijendravarma *et al.*, 2009). The relative simplicity of the invertebrate system can provide an additional advantage as results are less likely to be confounded by the maternal transfer of antibodies, hormones and nutrients associated with vertebrate systems (Hasselquist & Nilsson, 2009).

In this thesis, a selection-based (1.6.4) approach has been utilised to address our research question (see 1.2). Using this approach the experimenter can select for immunocompetance against one of its naturally occurring enemies and explore a range of potential trade-offs, whilst eliminating the confounding variables that would be encountered in a field environment.

1.7 Introducing the model system

The current understanding of trade-offs resulting from variation in resistance remains limited, mainly due to the difficulties in setting up an ecologically relevant model system (see 1.6) (Schmid-Hempel, 2003). Invertebrate models have been essential in filling this gap, allowing us to select these immune traits across multiple generations of pathogen exposure, in a relatively quick and cost-effective manner. Due to the lack of antibodies and lymphocytes invertebrates provide us with a simple system, which shares many traits with the vertebrate innate immune system (Vilmos & Kurucz, 1998).

The resistance model employed in this thesis was the fruit fly, *Drosophila melanogaster*, selected for resistance against its associated parasitic wasp, *Asobara tabida*. In order to understand *Drosophila* defence against parasitoid infection and how this might relate to trade-offs throughout the life of the model system it is first necessary to be familiar with the life cycle and immune system of *Drosophila*.

1.7.1 Drosophila life cycle

Drosophila exhibit complex life cycles (CLCs), which means that they display physiologically and morphologically discrete life stages (Wilbur, 1980). There are four phases of the *Drosophila* life cycle: egg, larva, pupa and adult (Figure 1.3). Within the larval phase *Drosophila* exhibit three instar periods, each separated by a moult, when the animal sheds its cuticle, mouth hooks and spiracles (breathing holes) (Flagg, 1988). The larval and adult phases are partitioned by the pupal phase when the fly undergoes metamorphosis; the breakdown of many larval features and the reformation into the adult structure. This transitional period gives the animal the distinct advantage of being able to amend its phenotype for the environmental and physiological requirements of the adult phase, in addition to allowing the animal to reallocate essential resources to optimise for each life stage.

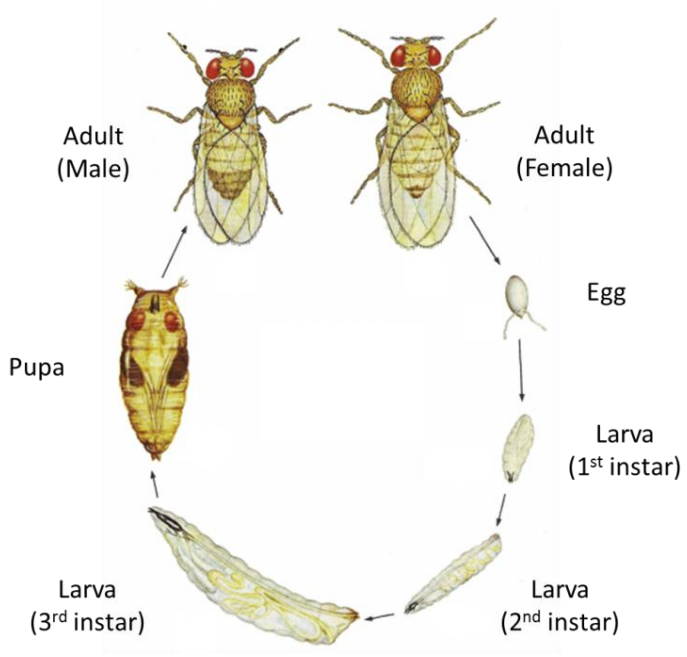


Figure 1.3 Drosophila life cycle

Showing the egg, three larval instars, pupal and adult phases (Adapted from Flagg, 1988).

1.7.2 Drosophila immunity

The *Drosophila* immune system is comprised of cellular and humoral (chemical-based) defences. The humoral response in *Drosophila* becomes activated upon microbial invasion through activation of NF-κB-like transcription factors and results in synthesis and release of lytic peptides from the liver-equivalent, the fat body, into the circulating hemolymph (Hoffmann & Reichhart, 2002, Hoffmann, 2003, Imler & Hoffmann, 2001, Libert *et al.*, 2006). There are at least 15 cationic peptides encoded for in the *Drosophila* genome capable of recognising and mounting an anti-microbial response (Hoffmann, 2003, Werner *et al.*, 2003), with two main pathways regulating their release (Hoffmann & Reichhart, 2002). Recognition of Gram-positive bacteria or fungal pathogens stimulates activation of the Toll pathway (Hoffmann & Reichhart, 2002). A Gram-negative bacterial infection will incite activation of the Imd pathway (Hoffmann & Reichhart, 2002).

The cellular defence includes the hemocyte-mediated responses such as surveillance, pathogen recognition, phagocytosis, encapsulation and wound healing (Lavine & Strand, 2002). The majority of these functions are undertaken by the most abundant immune cell found within *Drosophila*, the plasmatocyte, which makes up approximately 95% of all circulating immune cells (Meister, 2004). This cell type is most comparable to the mammalian monocyte/macrophage lineages, with the capacity to phagocytose invading pathogens and also to produce a series of antimicrobial peptides (Meister, 2004). In addition to plasmatocytes, there are two other immune cell types found in *Drosophila*, lamellocytes and crystal cells, neither of which have a defined mammalian homologue (Meister, 2004). Upon infection, additional hemocytes are differentiated and released from the *Drosophila* lymph gland; a specialised hematopoietic organ present in *Drosophila* larvae.

Lamellocytes are involved in encapsulating foreign articles that are too large to phagocytose and are only seen in circulation upon infection (Irving *et al.*, 2005). Crystal cells make up the other 5% of circulating immune cells and are one of the main producers of the enzyme prophenoloxidase (proPO) (Meister & Lagueux, 2003), which forms part of the humoral response. In order to become activated ProPO requires proteolysis by a serine protease. Once activated phenoloxidase (PO) will catalyse the hydroxylation of tyrosine to dihydroxy-phenyl-alanine (dopa), which is then further

oxidised by PO to quinones, which ultimately polymerise to form melanin (Cerenius & Söderhäll, 2004). The intermediates formed during this melanisation reaction are thought to be toxic to microorganisms, therefore the pathway must be strictly regulated to ensure limited self-toxicity (Cerenius & Söderhäll, 2004). In *Drosophila* regulation occurs by serine protease inhibitors (e.g. Serpin 27A), which limit the activity of the serine protease activating enzyme (De Gregorio *et al.*, 2002).

Melanin formation mainly occurs around encapsulated bodies and at wound sites where toxicity can be directed at invading pathogens (Cerenius & Söderhäll, 2004). It has also been suggested that melanin also has roles in reinstating the impermeable nature of the damaged epithelium and protecting against oxidative injury resulting from the killing of pathogens (Nappi & Christensen, 2005). As well as the melanisation reaction humoral defence also incorporates the production of antimicrobial peptides and reactive intermediates of nitrogen or oxygen by the immune cells (Lavine & Strand, 2002).

1.7.3 Immunity against parasitoids

Parasitic wasps, or parasitoids, are a natural enemy of *Drosophila* which, by definition as a parasitoid, result in the death of the host (Godfray, 1994) . *A. tabida* are one of the most common and well-studied parasitoids of *Drosophila* in Europe, known to infect *Drosophila* during the second instar larval phase of development (see 1.7.1) (Ellers & Van Alphen, 1997). Female *A. tabida* will insert an egg into the *Drosophila* larva during its 2nd instar (Figure 1.4a) where it will then develop, consuming internal organs of the *Drosophila* for sustenance and emerging as a fully matured adult. Due to the fatal consequences of this encounter for the *Drosophila*, there are strong selection pressures for populations exposed to this type of threat to evolve resistance mechanisms against parasitoid infection (Kraaijeveld & Godfray, 1999).

When the larval hemolymph patrolling cells (plasmatocytes) encounter a pathogen too large to phagocytose, as is the case with a parasitoid egg, an encapsulation response is initiated (Irving *et al.*, 2005). Encapsulation in *Drosophila* involves the collaborative efforts of all three *Drosophila* hemocyte types: plasmatocytes, crystal cells and lamellocytes (see 1.7.2). The plasmatocytes will detect the parasitoid egg within four to

six hours following infection (Wertheim *et al.*, 2005, Strand & Pech, 1995, Lemaitre & Hoffmann, 2007). Upon contact with 'non-self', detection molecules undergo conformation changes, inducing activation of Rac GTPases within the plasmatocyte. Once Rac GTPases are activated, they stimulate cytoskeleton reorganisation to obtain a flattened hemocyte-phenotype, triggering the structural modifications required for the hemocyte to adhere to the egg chorion and for the formation of septate junctions (Williams *et al.*, 2006, Burridge & Wennerberg, 2004). Altogether, these Rac GTPase mediated effects allow the plasmatocytes to wrap around the egg separating it from the hemocoel (Figure 1.4b), while also acting as a beacon bringing additional plasmatocytes into the area through Rac1- and Rac2-mediated filipodial migration (Williams *et al.*, 2006).

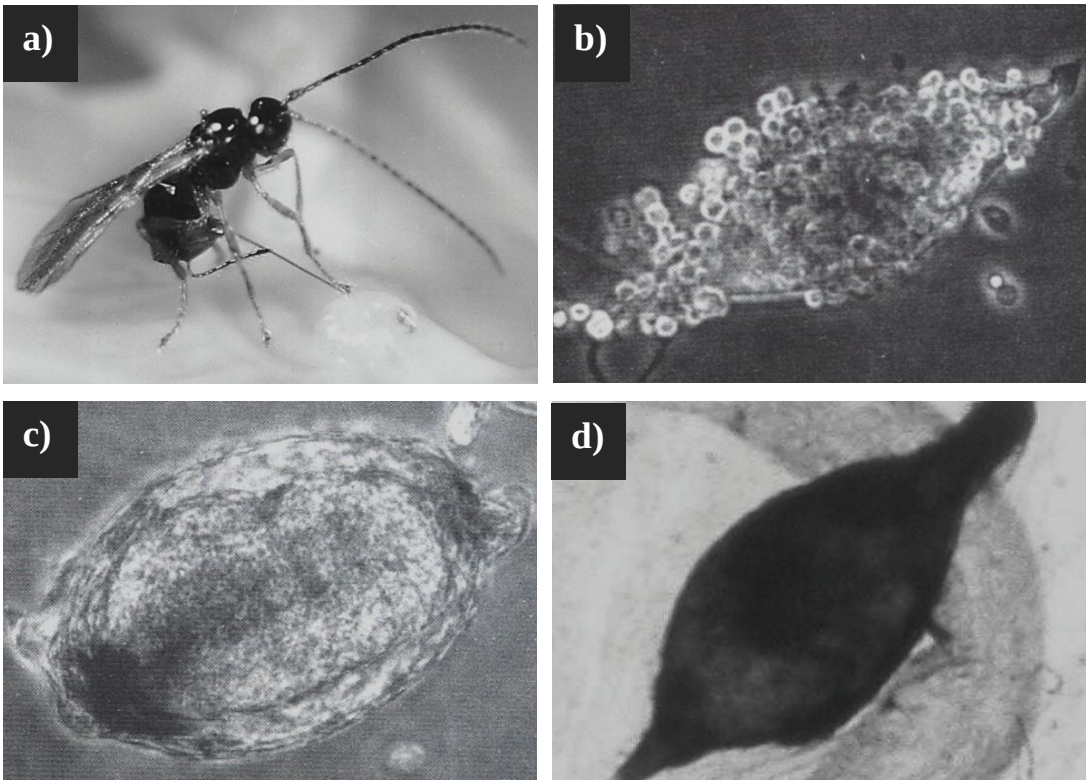


Figure 1.4 A successful encapsulation response

(a) the parasitoid inserts an egg into the larva, (b) upon detection by the plasmatocytes, lamellocytes are released and bind to the egg in an additive manner, (c) the egg becomes encased by the hemocytes, (d) the lamellocytes harden and melanise, the parasitoid larvae is killed within (Adapted from Wertheim et al., 2005).

Through unknown signalling molecules, the lymph gland is stimulated to induce the increased proliferation and differentiation of lamellocytes from pro-hemocytes in the primary lobe (medullary zone) and secondary lobes of the lymph gland (Lemaitre & Hoffmann, 2007, Meister, 2004, Williams *et al.*, 2006, Jung *et al.*, 2005). The newly synthesized lamellocytes are released from the lymph gland into the hemolymph where they can locate and bind to the already bound plasmatocytes in an integrin-dependent manner (Irving *et al.*, 2005). Binding is performed in an additive fashion resulting in the build-up of multiple layers, eventually entirely encapsulating the egg (Fellowes & Godfray, 2000). Crystal cells then locate to the area where they undergo Rho GTPase-mediated rupture, releasing proPOs into the surrounding hemolymph. This process is thought to be dependent on the c-Jun N-terminal kinase (JNK) pathway and the Drosophila TNFα homologue, Eiger (Bidla *et al.*, 2007). Upon rupture the proPOs enter a protease cascade leading to the production of melanin, which is deposited onto the lamellocytes resulting in a blackened capsule (Russo *et al.*, 2009) (Figure 1.1d).

Although the precise mechanisms by which the parasitoid is destroyed are unknown it is thought that a series of cytotoxic compounds (e.g. NO and ROS) and melanisation-intermediates could be produced to kill the invading organism (Nappi *et al.*, 1995) or that the parasitoid is suffocated within its melanised casing (Fellowes & Godfray, 2000).

1.8 Trade-offs associated with resistance to parasitism

There have been many attempts to determine the costs associated with immunocompetance in this model system. Two of the main findings include: *Drosophila* selected for resistance against larval parasitism show a lesser ability to compete against conspecifics, with lower survival when resources were limited (Fellowes *et al.*, 1998, Kraaijeveld & Godfray, 1997) and resistant males show increased mating success (Rolff & Kraaijeveld, 2003). There are also a number of traits that are not affected by immunocompetance in this *Drosophila* model (many of which directly relate to the animals fitness): size, development rate, egg viability, early fecundity, starvation resistance (Fellowes *et al.*, 1998).

Kolss *et al.* (2006) have already explored the effect of immunocompetance on one aspect of neural function; memory. No difference was found in the ability of *Drosophila* selected for parasitoid resistance to avoid an odour associated with a noxious mechanical stimulus, at 20 minutes (short-term memory) and 24 hours (long-term memory) following learning.

1.9 Thesis aims and hypotheses

To understand the potential costs involved in immune resistance it was first necessary to explore the *Drosophila* model system and any corresponding upregulation of immune mechanisms that occur as a result of artificial selection. Initial observations have already been conducted by Kraaijeveld *et al.* (2001), who found that *Drosophila* selected for resistance against parasitism show greater numbers of circulating hemocytes. The first step therefore was to reaffirm these findings, and then to expand on this work, measuring a series or cellular, humoral and immune defence characteristics in the resistant *Drosophila*. The next step was to elucidate whether these immune mechanisms remained intact into the adult, when there was no longer any risk from parasitoid attack, or whether these resources became reallocated upon metamorphosis. The thesis then went on to look for potential trade-offs exhibited throughout the life of the resistant *Drosophila*, with particular focus on ageing, oxidative stress resistance and motor function. In order to do this a series of survival, biochemical and motor function assays were implemented.

1.9.1 Overall aim of the research

The overall aim of this research was to determine whether immune resistance leads to trade-offs in mechanisms linked to ageing, resistance to oxidative stress and motor function.

1.9.2 Hypotheses for each experimental chapter

Chapter 2 explores humoral, cellular and immune defence characteristics within the control and resistant *Drosophila* expressed in early life (larva and young adult). Measurements were taken from the adult in order to determine whether immune mechanisms relating to larval parasitism persist or are lost during metamorphosis.

H1. Resistant *Drosophila* encapsulate a greater number of parasites than their control (more susceptible) counterparts

H2. Resistance corresponds to changes in immune-related machinery (humoral and/or cellular) and increased resistance to other immune challenges against the *Drosophila* larvae

H3. Differences in resistance/immune-related machinery are continued into adulthood

Chapter 3 investigates whether variation in resistance/immune-related machinery leads to differences in the overall cellular function, as assessed by detection of the metabolic by-product, reactive oxygen species (ROS). This was measured throughout life to assess the potential impact on the ageing system. Lifespan was also measured to give a direct read-out of the *Drosophila*'s ability to buffer senescence.

H1. Increased immune cell number in the resistant *Drosophila* will persist throughout life

H2. Increased immune cell number links to increased cellular metabolism/ intracellular ROS production

H3. Differences in intracellular ROS production will correspond to differences in immune cell number throughout life

H4. Resistance against larval parasitism will lead to a trade-off in longevity

Chapter 4 focusses on the ability of the control and resistant *Drosophila* to buffer toxin-induced rises in ROS and corresponding oxidative stress within the cell.

H1. Acute oxidative stress induced by paraquat consumption will further increase any differences between cell metabolism/ROS production in the resistant *Drosophila*

H2. Resistant *Drosophila* will show lesser survival when challenged with an acute dose of oxidative stress

Chapter 5 examines the motor function of the *Drosophila*, assessed by the ability of the larvae to crawl in an open-field and straight-track assay, and the adult flies' ability to climb following a startle-induced response.

H1. Resistant *Drosophila* larvae will show altered motor function

H2. Resistant adult *Drosophila* will show altered motor function

H3. Neurodegeneration will occur at a differential rate in resistant *Drosophila*

2 Exploring immune mechanisms in *Drosophila melanogaster* selected for parasitoid resistance

2.1 Introduction

Immunological competence is essential in all animals to ensure rapid protection against pathogens and parasites in an infectious environment. However, immune resistance is not ubiquitous and there is a great deal of variation; both between individuals and within the individual throughout its life (Shifrine *et al.*, 1982, Schmid-Hempel, 2003). It is thought that this is most likely due to the costs involved in producing and maintaining a competent immune system, which may take away vital resources from other functions leading to trade-offs with other life-history traits (Schmid-Hempel, 2003). In order to establish the potential costs of resistance, it is essential to understand the immune competence of the model system in use. This chapter explores the underlying immune mechanisms within *Drosophila melanogaster* selected for resistance to parasitism by the parasitoid wasp, *Asobara tabida*. Much of the work to date in this model has investigated the immediate effects of resistance on fitness-related traits, such as competitive ability, reproductive success and fecundity (Fellowes *et al.*, 1998, Kraaijeveld & Godfray, 1997). The ultimate goal of this research was therefore to use this model in the search for trade-offs throughout the organisms life, therefore it was necessary to elucidate immune mechanisms at later stages; late larval and adult phase. These phases are past the point when the animal is at risk from this type of parasitoid attack (during early larval phase).

The degree to which an animal can resist infection is dependent on the allocation of resources towards the humoral and cellular defence systems, which should ultimately relate to the degree of risk. The humoral and cellular *Drosophila* immune defences have been discussed in detail in Chapter 1 (see 1.7.2). Reeson *et al.* (1998) discovered that when African armyworm (*Spodoptera exempta*) larvae were reared at high densities they turned black by the third instar. It was found that this was due to an increased level of PO activity in the larval cuticle, which also corresponded to increased PO in the hemolymph and increased survival time against from viral infection by its nuclear

polyhedrosis virus. This study shows that the increased risk of infection, in this case posed by high population density, is sufficient to drive the increased resource allocation toward immunity. Eslin and Prévost (1998) demonstrated that 6 species from the *melanogaster* subgroup show variation in their responses to parasitism. They found that the ability to encapsulate an *Asobara tabida* egg was directly proportional to the hemocyte load within the *Drosophila* hemolymph. More recently, Kacsoh and Schlenke (2012) confirmed that increased hemocyte number provided immunity against a range of parasitoid wasps, with *D. suzukii* showing five times more circulating hemocytes than the less resistant *D. melanogaster*. All immune cell types were found to be upregulated proportionately, with a five-fold increase in plasmatocyte numbers and a three-fold increase in the prophenoloxidase containing crystal cells.

The model system utilised in this study has been artificially selected for increased resistance against the larval parasitoid *A. tabida* over five successive generations. Kraaijeveld and Godfray (1997) previously showed that individuals selected in such a manner (resistant *Drosophila*) were more likely to mount an encapsulation response compared to the unselected (more susceptible) controls. They later showed that this artificially selected increase in resistance was also correlated with an increase in circulating hemocyte numbers (Kraaijeveld *et al.*, 2001).

There are two separate lineages of hemocytes that arise during *Drosophila* development (Holz *et al.*, 2003). Embryonically-derived hemocytes (EHs) are initially produced during embryogenesis in the procephalic mesoderm (head region) (Lanot *et al.*, 2001). These hemocytes migrate and spread to occupy the entire germ prior to the final stage of embryonic development (Tepass *et al.*, 1994). Unless primed by an immune response, the overall number of EHs increases throughout larval phase from less than 200 to over 5000 due to mitotic division (Lanot, 2001). In an uninfected larva, EHs can be found freely circulating within the hemolymph, as well as sourced in sessile pools just under the larval cuticle (Williams, 2007). Another subset of hemocytes exists, but resides in an undifferentiated state, as pro-hemocytes, within the lymph gland; these cells are known as the lymph gland derived hemocytes (LGH's) (Holz 2003). Kraaijeveld *et al.* (2001) only measured differences in the number of hemocytes in circulation (EHs) and did not look at differences in overall hemocyte numbers (including LGHs and sessile hemocytes). In this study, hemocyte numbers were measured before and after a

wounding insult. Sterile wounding has been shown to induce an up-regulation of immune cell number comparable to that initiated by parasitoid infection (Márkus *et al.*, 2005). As such, sterile wounding was used to provide an indication of differences in hemocyte recruitment between the control and resistant flies.

EHs act as the critical surveillance force, ready to respond to an invader and induce production and release of LGHs from the lymph gland should a threat be detected. If this does not occur the LGHs will remain within the gland until late larval phase, at the brink of metamorphosis, at which point the lymph gland breaks down releasing LGHs into the hemocoel. This extra subset may be released at this particular phase to aid with the restructuring of the transforming *Drosophila* by the phagocytosis of larval fragments and tissue modelling (Whitten, 1964). After the lymph gland is broken down no further hematopoiesis can occur, thus no additional hemocytes can be created in the adult *Drosophila*. Some immune cells, plasmatocytes and crystal cells, have been shown to persist from larval into adult phase (Holz *et al.*, 2003, Kurucz *et al.*, 2007b). Lamellocytes are not present post-metamorphosis and therefore have not been addressed in this work. Although crystal cells persist into the adult fly (Kurucz *et al.*, 2007b), these cells are extremely difficult to quantify due to fact that they rupture very easily, including when the animal is injured for hemolymph collection. For these reasons, quantification was made solely for plasmatocytes; therefore it should be assumed that any mention of hemocytes from this point forward will refer to plasmatocytes, unless otherwise stated.

Metamorphosis allows a very distinct cut off period, when much of the fly's structure is broken down for restructure into the adult. At this stage most tissues and organs are broken down and rebuilt to ensure functioning is efficient in the new phase of life. Due to the costs involved in immune mechanisms one might assume that any additional immune cells in the resistant flies would therefore be lost during metamorphosis due to a redundancy in function in the adult. The 'adaptive decoupling theory' proposed by Moran (1994) suggests that independent development for larval and adult characteristics allows phase-optimisation and occurs due to different selective pressures at different life stages. As well as analysis of cellular changes, this study also went on to search for differences in the humoral-based immunity, via alterations in PO activity in the hemolymph. This factor has not yet been elucidated in this model system despite the

crucial role it plays in parasite toxicity and at the end point of encapsulation and its addition potential protective role of melanin against immune-induced oxidative injury (Nappi & Christensen, 2005).

Another key question posed in this chapter was whether the cellular or humoral mechanisms required for resistance to parasitism correlates with altered function in immune activation in later stages of the *Drosophila*'s life (late larval and post-metamorphosis). In nature the adult fly is at no risk from parasitoid attack, therefore testing the encapsulation ability in these older individuals is ecologically irrelevant and methodologically impossible. Instead it is more appropriate to test for other threats that the adult fly might encounter. To date, no differences have been found in the ability of the adults from a selected (resistant) background to mount a humoral response against fungal infection compared to individuals from a control (susceptible) background (Kraaijeveld *et al.*, 2012) . Neither were there altered effects in fecundity between the lines following microsporidian infection (known to induce declined fecundity), which relies on the ability of the humoral system to activate hemocytes for phagocytosis (Kraaijeveld *et al.*, 2012) . Another defence mechanism that requires the combined efforts of the humoral and cellular defences is wound healing.

Upon cuticle injury, damaged epithelial cells at the wound entrance release cell-aggregation factors to draw nearby hemocytes to the coagulating area (Stramer *et al.*, 2005). Surveillance hemocytes then release clotting proteins that accumulate into insoluble fibres, ultimately forming a 'soft clot' made up of insoluble fibres, which plugs the wound (Goto *et al.*, 2003). Protein cross-linking occurs as the fibres adhere together at the wound site forming a mesh-like structure. Through tightly regulated processes the phenoloxidase (PO) cascade ensues, killing pathogens and melanising clotted fragments to reinstate the non-permeable nature of the barrier (Rämet *et al.*, 2002). Many of the processes involved in wound healing are similar to those required for encapsulation of the parasitoid egg; in both scenarios the hemocytes are recruited to the critical point, becoming adherent and forming a mass culminating in their melanisation (Siva-Jothy *et al.*, 2005). It was therefore hypothesised that alterations in encapsulation ability might result in modifications within the wound healing response.

The first step taken in this chapter was to look for differences between cellular and humoral defence in the control and resistant *Drosophila* and to determine whether these features persist into the young adult when immunity against larval parasitism would be redundant. The next step was to determine whether resistance against parasitism in the larva related to defence against another closely linked immune defence, wound healing, which could then be used to look for ecologically relevant differences in immunity in the adult *Drosophila*.

2.2 Materials & Methods

2.2.1 Selection for resistance to parasitism and fly culturing

The flies used were kindly provided by K. M. Jalvingh of University of Groningen, the Netherlands. Eight populations were supplied (control (C) 1-4, selection (S) 1-4) all of which were derived from a single population of *D. melanogaster* collected around Leiden, the Netherlands by A. R. Kraaijeveld in 1995 (Figure 2.1). The original population was separated into four lines (1-4) and have continued to be cultured as such to date. In 2009 each of these four lines was sub-divided into two to provide four lines from which to select for immunity (S), each with an equivalent control line (C) (separation and 5 subsequent selections performed by K. M. Jalvingh). The four selection lines were subjected to parasitism by the *A. tabida* parasitoid wasp at every generation for 5 generations. Individuals within these lines that exhibited an encapsulation response were selected to produce the next generation. The corresponding controls were treated in the same fashion to its paired selection line at each point, except for the absence of *A. tabida* thus lacking the selection element.

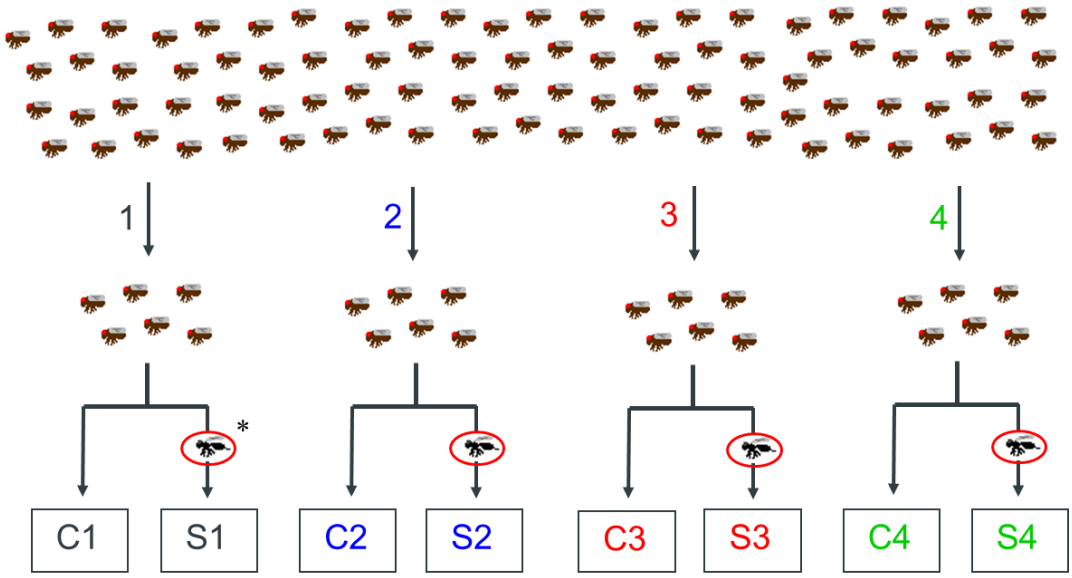


Figure 2.1 Establishing the Drosophila lines

*From the original wild population collected, four populations were formed. These four populations were then divided into pairs. A total of eight populations were formed; 4 selected for resistance to parasitism, paired with four control lines. *Within each pair one population was exposed to A. tabida parasitoids and selected for their potential to evade infection (S) the other kept free from parasitic attack (C).*

Upon receiving the 8 *Drosophila* populations (4 control and 4 selection lines; Figure 2.1), the lines were reared at medium density (between 100-200) in 250 ml bottles containing a yeast/sugar medium containing 0.5% propanoic acid. The 8 lines were cultured simultaneously in the same manner. Eggs were laid at 25 ^{o}C, larvae reared at 20 ^{o}C and flies held at 7.5 ^{o}C in temperature controlled incubators with 12-hour fixed light-dark cycles (at 25 ^{o}C and 20 ^{o}C, light: 8am – 8pm). The culture cycle was repeated every three weeks. In the first week approximately 600-800 flies from each line (C/S1-4) were divided up and transferred into three yeast-medium containing bottles, with additional fresh yeast to promote reproduction. *Drosophila* were allowed to lay for 24 hours at 25 ^{o}C, after which time the adults were removed from laying. Following a further 24 hours at 25 ^{o}C, larvae from the three bottles were pooled, prior to distribution into 6 new yeast-medium containing bottles (approximately 100-200 larvae per bottle). Developing *Drosophila* were then kept at 20 ^{o}C until eclosion. Eclosing adults of each

line (C/S1-4) were collected each day of the third week into a total of three new yeast-medium containing bottles per line. Once collected, culture flies were maintained at 7.5 ^{o}C until needed. Forty-eight hours prior to laying *Drosophila* were taken out of cool incubation and allowed to warm up at 20 ^{o}C.

For experimental procedures, further *Drosophila* could be sub-cultured from the culture stocks. Throughout this thesis the level of experimental replication was always 8, with each line (C/S1-4) acting as a biological replicate. For statistical analysis, comparisons were made from the differences between each control and selection line pair, e.g. C1 and S1.

Re-selection of the immune response against larval parasitism was carried out every 7-8 generations. The selection protocol was as follows: for each line (C/S1-4) approximately 600-800 adult *Drosophila* were divided up and placed into three yeast-medium containing bottles, with additional fresh yeast. *Drosophila* were allowed to lay for 24 hours at 25 ^{o}C, after this time adults were removed. The developing offspring were allowed to mature for a further 24 hours at 25 ^{o}C by which time many of the offspring should have reached L2 larval phase. At this point larvae from the three bottles were pooled, prior to distribution into 8-10 new yeast-medium containing bottles (approximately 100-200 larvae per bottle). For the selection lines (S1-4) 5 *Asobara tabida* females were added to each bottle, where they were left for 24 hours before removal. During late pupal phase the *Drosophila* were then examined for the presence of melanised capsules. Those that exhibited a lack of such capsules or the presence of parasitoid larvae were discarded and thus excluded from subsequent generations. For each selection line (S1-4) 300-400 capsule containing pupae were collected and positioned dorsal side up onto the side of three new yeast-media containing bottles. An equal number of pupae from the corresponding control line (C1-4) were randomly collected and positioned in the same manner. Upon eclosion *Drosophila* from each line (C/S1-4) were collected into three fresh yeast-medium containing bottles. Following re-selection flies were cultured 3 subsequent times prior to use in experimentation, to avoid maternal confounding effects.

A. tabida were originally collected near Sospel, Southern France in 1980's and since have been cultured on *Drosophila subobscura*, a species lacking the capacity to

encapsulate the wasp. Each week *A. tabida* were exposed to their *D. subobscura* host for a minimum period of 24 hours to ensure parasitism. *A. tabida* density and sex ratio were controlled (5 females plus 2 males per 100-200 *D. subobscura* larvae) to avoid superparasitism and ensure equal sex distribution of parasitoids upon emergence. Each week emerging parasitoids were collected into a 250 ml bottle with agar base and honey then stored at 7.5 ^{o}C for a maximum of 4 weeks.

D. subobscura were kept in two caged cultures (labelled A and B). On a weekday, two fresh medium bottles were labelled and placed in the cages for 24 hours to allow for laying. For weekend laying, four medium bottles were placed in the cages during the course of the afternoon and removed Monday morning. Weekend bottles provided the larvae necessary for *A. tabida* culturing. Weekday bottles were kept to maintain culture numbers. Flies emerging from 'A' bottles were transferred into cage B and flies from 'B' bottles transferred into cage A to ensure that the flies existed as one population between the cages. Technical assistance was provided for *D. subobscura* culturing (see acknowledgments 2.4.5).

2.2.2 Checking the immunity of the Drosophila populations

Prior to commencement of experimental work the lines were checked to verify the immune potential in each of the lines. Two hundred 2nd instar larvae of each line were subjected to exposure to parasitism from *A. tabida*. Six days following exposure the larvae were dissected under a dark-field microscope (x 10 magnification). Each larva was scored for the number of encapsulated parasitoid eggs (successful immune response) and the number of parasitoid larvae (unsuccessful immune response) present. Only singly parasitized larvae were used to calculate immunity (approximately 46 (± 4) larvae were analysed per line (C/S1-4)). Encapsulation rate was calculated as the proportion of singly parasitised larvae exhibiting a melanised capsule. This procedure was repeated 8 times for each biological replicate (C/S1-4).

2.2.3 Hemocyte counts

Hemocyte numbers were quantified with and without a prior wounding insult in order to elucidate whether overall hemocyte numbers within control and resistant flies differ and/or whether there are trade-offs between circulating and sessile immune cells. For a wounding insult, larvae and adult flies were stabbed once, as described in section *2.2.5*, 24 hours prior to hemolymph extraction.

For each larval hemolymph sample (wounded and unwounded), ten 3rd instar larvae were cut down the dorsal side with fine spring scissors (FST) and bled into a pool of 20 µl of *Drosophila* saline. Bleeding was performed on a glass plate cooled by ice to minimise larval movement. In total, 800 larvae were used (10 larvae bled per sample, with 10 samples analysed per biological replicate (C/S1-4)). *Drosophila* saline solution was prepared by adding the following reagents to 1 litre of distilled water:

Table 2.1 Drosophila saline

Ingredients to prepare a stock (X10) solution.

Reagent	X10 Stock solution
KCl	1.5g
NaCl	74.8g
MgCl2($6H_20$)	8.1g
CaCl2($2H_20$)	2.6g
Sucrose	123g
Hepes	11.9g

The pH of the saline solution was then adjusted to 7.4 using NaOH and aliquoted into 50 ml falcon tubes for long term storage at -20°C. Prior to use the aliquot was defrosted and diluted 1:10 (stock solution: distilled water).

For adult hemolymph collection, five-to-six day old *Drosophila* (wounded and unwounded) were lightly CO_2 anesthetised in batches of 6. *Drosophila* were decapitated and hemolymph was drained from the thorax of each batch using a 0.5 µl micro-capillary tube (Drummond Scientific, USA). Hemolymph quantity was calculated from the measurements taken of hemolymph inside the tube using an eyepiece graticule and the known diameter of the capillary tube. The hemolymph was expelled into 20 µl of *Drosophila* saline. In total, 960 adults were used (6 adults bled per sample, with 10 samples analysed for each sex. This process was repeated for each of the 8 biological replicates (C/S1-4)).

Cell density of each sample was counted on a Neubauer hemocytometer (Hawksley, UK) using a Kyowa Unilux-12 microscope (Tokyo, Japan). For this assay only the main surveillance cell in the insect, the plasmatocyte, was identified and counted. This type of cell is known to play a major role in larval and adult immunity, the other two immune cell types, lamellocytes and crystal cells, present in the larvae are not known to exist or are difficult to quantify in the adult fly and as such will unlikely affect the adult phenotype. The hemolymph solution was pipetted onto the edge of the coverslip, where it was drawn up by capillary action to cover the glass slide and counting chamber. Cells in each of the large corner squares, containing 16 smaller squares, were counted per sample.

2.2.4 Measurements of PO activity

For larval hemolymph collection, fifty 3rd instar larvae were bled into 40 µl pools of acidic phosphate buffer (pH 6.3) onto a glass plate cooled by ice. Bleeding was achieved by slicing the posterior cuticle with fine spring scissors (FST). Each sample was then pipetted into a 0.5 ml eppendorf and snap frozen on dry ice. In total, 1200 larvae were used (50 larvae bled per sample, with 3 repeats performed for each biological replicate (C/S1-4)).

For adult hemolymph collection, the hemolymph was drained from the thorax of the *Drosophila* using a 0.5 µl micro-capillary tube (Drummond Scientific, USA). The

hemolymph of 50 flies was added to 20 µl aliquots of phosphate buffer (pH 6.3) and snap frozen on dry ice in acidic phosphate buffer (pH 6.3). In total, 2400 adults were used (50 bled per sample; 3 samples for each sex were analysed for each biological replicate (C/S1-4)).

After thawing, the samples were vortexed prior to being spun down at 10,000 rpm for 20 seconds. The samples were then analysed for their protein concentration using a colorimetric protein assay kit (Bio-Rad). Following the method previously carried out by Ligoxygakis *et al*. (2002), 10 µg of protein was added to 40 µl of phosphate buffer. 120 µl of saturated L—3, 4-dihydroxyphenylalanine (L-dopa) was then added to each sample in a 96-well plate (Sterilin Ltd, UK). The samples were read immediately on a FLUOstar OPTIMA optical reader (BMG labtech) at 490 nm, and then read every 10 minutes for 2 hours to watch how the reaction developed over time.

Phenoloxidase activity was found to occur at the greatest rate in the first 30 minutes following addition of L-dopa. Therefore, rate was calculated using the change in absorbance at 490 nm between 0 to 30 minutes post-exposure to the L-dopa substrate.

2.2.5 Fly wounding

Larvae: Six to seven day old larvae were wounded (mid-later 3rd instar) using a 0.1 mm diameter steel needle (tip 0.01mm, FST). Prior to this, larvae were cooled on ice to minimise movement during the procedure. Larvae were stabbed once, between segments A6 and A7; care was taken to avoid the main internal breathing tubes. Unwounded larvae underwent the same procedure, without the wounding insult. Wounded and unwounded larvae were then transferred to vials with yeast/sugar medium at 20°C to develop for 48 hours. This allowed a check for larval viability, shown by the ability to enter pupation following injury. At this point, all pupated *Drosophila* were snap-frozen for imaging and analysis; any larvae yet to pupate were left in situ. Additional pupae were then collected at 24 hour intervals allowing all viable individuals to reach pupation. In total, 1600 larvae were used (100 for each treatment (wounded/unwounded) per biological replicate (C/S1-4)). None of the unwounded larvae exhibited spontaneous melanisation, at the cuticle or within the hemocoel

(unquantified observation). Out of the 800 larvae that received a wounding insult only 379 successfully reached pupation (approximately 47 (± 3) per biological replicate (C/S1-4), Figure 2.2). These larvae were later used to determine differences in the level of melanisation that occur as a result of a cutaneous injury.

During the wounding experiment it became clear that many of the wounded larvae exhibited further melanisation within the hemocoel in response to cutaneous injury (Figure 2.2b). The phenomenon noted has been previously described as melanotic masses; non-invasive black melanotic spots that occur within the *Drosophila*, often associated with mutations in genes relating to the immune system (Minakhina & Steward, 2006). Therefore, as an additional factor, it was recorded whether or not each larva showed these masses, and, if so, the size of each mass was measured.

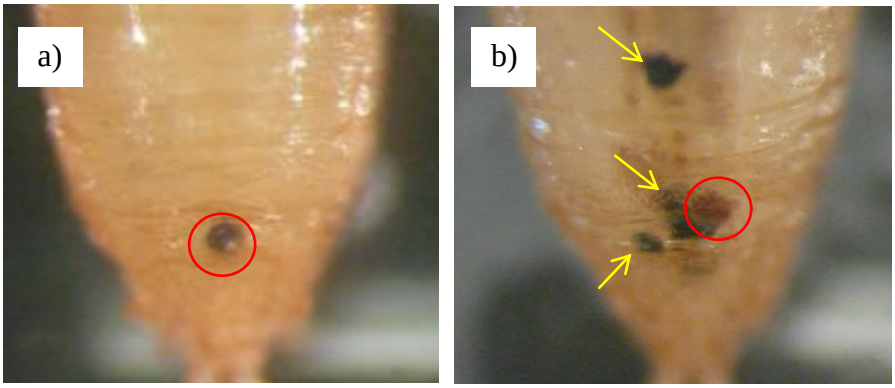


Figure 2.2 Larvae: melanised wound only (a) and melanised wound plus melanotic masses (b)

Pupated Drosophila with melanised wounds (circled in red) and melanised masses (indicated by yellow arrows).

Adults – single wounding: All adult wounding experiments were performed on five to six day-old mated male and female flies using a 0.2 mm diameter steel needle (tip 0.02mm, FST). In the first series of experiments, the abdomen was pierced once between the fourth and fifth abdominal segment (Figure 2.3). Wounding was alternated between the left and right side of the midline to ensure there was no side bias. Unwounded flies were treated in the same manner but lacking a cutaneous injury.

Wounded and unwounded flies were immediately placed into vials containing yeast/sugar medium at 20°C with a maximum of six flies per vial. In the adults we were also able to look an additional time variable, which we were unable to do with the larvae due to cuticle changes brought about by the onset of metamorphosis. After 0, 0.5, 1, 2, 3, 6, 12 or 24 hours, the flies were lightly anaesthetised with CO_2, placed in individual eppendorfs and snap-frozen in liquid nitrogen. The time point for the '0 hour' treatment was taken once all six flies had been wounded (hence, flies had actually been on the medium for up to 10 minutes). Preliminary experiments had shown that these time intervals were appropriate for following the entire wound melanisation process. Only living flies were snap-frozen; dead flies (of which there were very few) were discarded. A total of 2560 flies were used (10 flies for each treatment (wounded/unwounded) for each sex, at each time point (0, 0.5, 1, 2, 3, 6, 12 and 24 hours) for each biological replicate (C/S1-4)). No spontaneous melanisation was seen on the cuticle or in the hemocoel of the unwounded *Drosophila* (unquantified observation), as such, further melanisation analysis was only performed on wounded individuals. No melanised masses were noted in the hemocoel of the wounded or unwounded adult *Drosophila*.

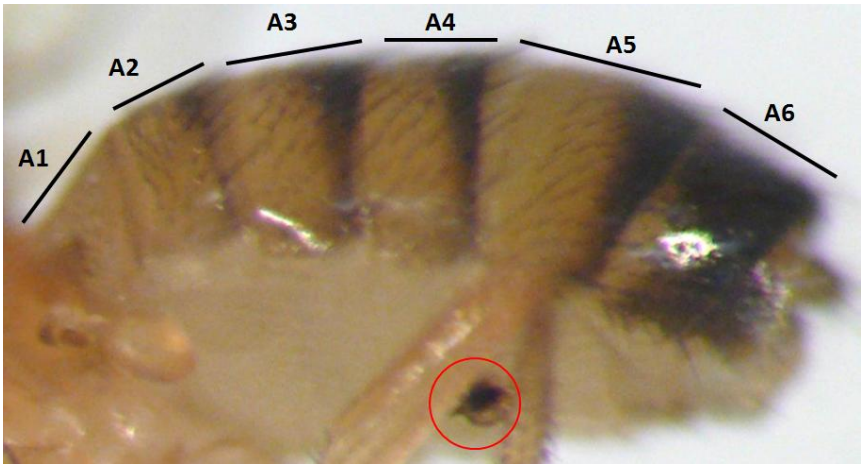


Figure 2.3 Melanised wound: adult (1 wound)

Adult Drosophila showing melanised wound (circled in red). Abdominal segments 1-6 marked out along the length of the fly (A1-6).

Adults - triple wounding: In the second series of experiments, female flies were stabbed three times, between the third and fourth, the fourth and fifth, and the fifth and sixth abdominal segments, as described above (Figure 2.4). Triple wounding was performed in order to deliver a more severe challenge. A significant interaction was shown between immunity and time on wound size following a single injury. It was hypothesised that an additional insult would augment any differences between the control and resistant *Drosophila*. Flies were kept at 20°C as described above and snap-frozen after 0.5, 2 or 6 hours. At each of these time points the proportion of flies showing one or more melanised wounds, and the size and intensity of these melanised wounds were measured. A total of 288 flies were imaged (12 flies for each treatment at each time point (0.5, 2 and 6 hours) for each biological replicate (C/S1-4)).

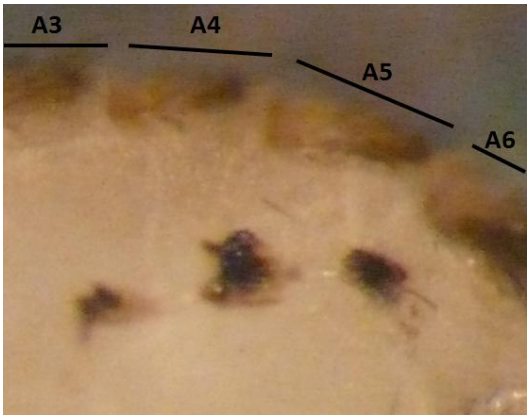


Figure 2.4 Melanised wound: adult (3 wounds)

Wounds of adult Drosophila stabbed three times between A3 and A4, A4 and A5, A5 and A6.

2.2.6 Image analysis

Following snap-freezing and subsequent defrosting, the legs of the adult flies were removed in order to aid visualisation. Each adult fly was then pinned to a Silguard plate using entomological pins (diameter 0.1mm, FST). Pupae required no additional handling prior to imaging. Images were captured with the aid of a Leica stereo microscope, using a Leica D-Lux 3 digital camera. Using ImageJ, each image was converted to a greyscale RGB stack, and the green field selected (as this was found to give the best wound-cuticle distinction (Figure 2.5). Each image was scaled using an image of a 10mm graticule (Pyser-SGI Ltd). The threshold was adjusted so that only areas reading a threshold above that of the background cuticle were registered. Only the wound site and additional melanised masses were quantified; registered background, for example, hairs and darkened cuticle - particularly in the adult flies - were excluded from analysis following visual inspection. The wound site was analysed for size and intensity. An area of cuticle adjacent to the wound site on each pupa/adult fly was also analysed for intensity to act as a control for cuticle variation between the flies. Intensity was then measured as a percentage of 'blackness' (white = 0, black = 100). Any individuals with additional regions detected above the registered background were assessed for the presence of melanotic masses. Melanotic masses were identified as regions of melanisation (increased 'blackness') that occurred away from the site of injury; these

areas were easily distinguishable due to their intensity or 'blackness' often equalling or exceeding that measured at the wound site (unquantified observation). The proportion of individuals exhibiting melanotic mass formation was calculated. In addition, individual melanotic masses were quantified for size determined by the area registered above the background cuticle. Melanotic masses were only visualised in the wounded larvae and not the adult fly.

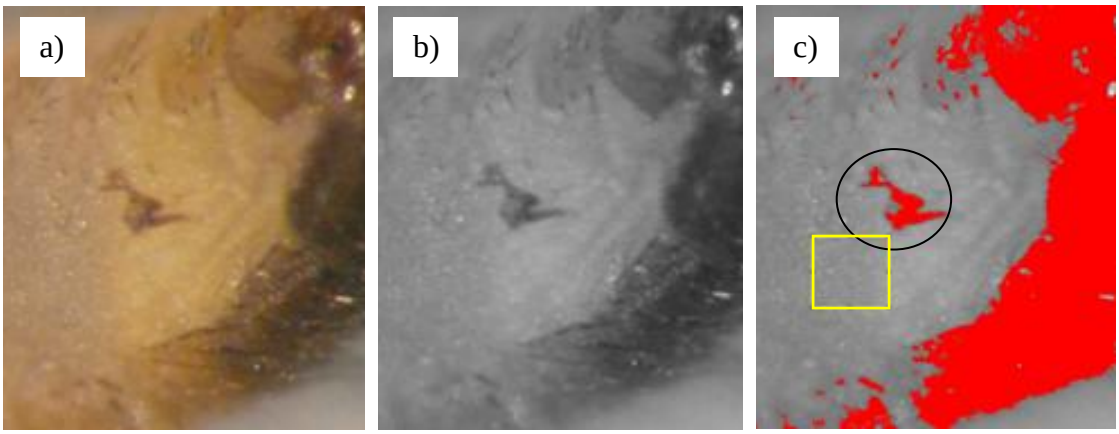


Figure 2.5 Image analysis

a) Original image of melanised wound on Drosophila abdomen, b) converted to greyscale in Image J, c)threshold adjusted to detect 'more black' areas, including wound (circled in black) and additional darker hairs/cuticle. An area of cuticle adjacent to the wound was also analysed for intensity as a control (outlined in yellow).

2.2.7 Statistical analyses

Encapsulation rates: Proportions were arcsine square-root transformed. A mixed effect linear model was fit in R with immunity set as a factor and line set as a random effect.

Hemocyte counts: An average was taken of the cell count across the 10 samples. A mixed effect linear model was then fit against the average cell count in R with immunity and treatment (wounded /unwounded) set as factors and line set as a random effect. This analysis was performed separately for larvae, adult males and adult females. Due to the difficulty in extracting hemolymph from the adult, all adult hemocyte counts were standardised against volume extracted.

PO assays: Rate was calculated as the change in fluorescence in the first 30 minutes. For both the initial PO activity and rate of PO conversion the following statistical analyses were applied: A mixed effect linear model was fit against the response in R with immunity set as a factor and line set as a random effect. Larval, adult female and adult male PO activity were analysed separately.

Wound healing: All proportion data was first arcsine square-root transformed and size data was log transformed to ensure that the data fit normality prior to analysis. For intensity data, the change in intensity between the background cuticle to the wound site was calculated (white = 0, black = 100). *Larvae (wounds and melanotic masses):* A mixed effect linear model was fit in R with immunity set as a fixed factor and line set as a random effect. An additional 'wounding treatment' factor was set to determine the effect of wounding on survival. *Adults (single wounded):* Each sex was analysed separately. For each sex, a mixed effect linear model was fit in R with immunity set as a fixed factor, time set as a covariate and line set as a random effect. *Adults (triple wounded):* Variables analysed included, proportion of flies exhibiting a) at least one wound, b) at least 2 wounds and c) 3 wounds, the average wound size per fly and the average wound intensity per fly. These variables were analysed separately for each sex as previously described for singly wounded adults.

2.3 Results

In order to establish whether *Drosophila* selected for increased resistance against larval parasitism was a suitable model system for life-history trade-offs it was first necessary to verify that the immune defences within the control and resistant *Drosophila* were different. In order to do this two of the main immune defence systems known to be implemented in the encapsulation response were evaluated; hemocyte numbers and PO activity. In addition to this, wound healing was also observed due to its close links with encapsulation. Wound healing allowed an easily observable phenotype with which to compare *Drosophila* immunity in the late larval and adult phase.

2.3.1 Checking the immunity of the Drosophila populations

The dissection experiments showed a greater ability of the larvae to encapsulate in response to parasitoid infection in all four selection lines relative to their paired control line (T = 8.72, df = 6, *P* = 0.0001) (Table 2.2).

Table 2.2 Encapsulation rate

Including the mean average encapsulation rate (%) for flies from a low resistance (C) and high resistance (S) background. Standard error calculated from the line means. Analysis: 46 (± 4) larvae were analysed per line (C/S1-4).

Control line	*Encapsulation rate (%)*	*Selection line*	*Encapsulation rate (%)*
C1	10.0	**S1**	66.7
C2	12.5	**S2**	62.7
C3	18.9	**S3**	47.1
C4	6.7	**S4**	55.0
Average (± SE)	**12.0 (± 2.6)**	**Average (± SE)**	**57.9 (± 4.3)**

2.3.2 Hemocyte counts in larvae and adults

Individuals wounded 24 hours prior to hemolymph extraction showed a 65.6% increase in hemocyte count compared to non-wounded individuals (Figure 2.6; T = 14.16, df = 6, *P* < 0.00001). Overall, more circulating immune cells were found in hemolymph extracted from individuals from a selected background, with 3 out of 4 selection lines revealing higher hemocyte counts than their control counterpart irrespective of treatment (see Appendix Table 1 for individual line means) (T = 3.19, df = 6, *P* = 0.019). Wounding treatment had an effect on the relationship between hemocyte number and immune background; shown by an interaction effect (T = -3.77, df = 6, *P* = 0.009). Unwounded individuals from a background of high parasitoid resistance displayed much greater numbers of hemocytes than those from a low resistance background (shown in 4 out of 4 line pairs), however, this relationship was reversed following a

wounding insult, where a greater number of immune cells were found in *Drosophila* from low resistance backgrounds (shown in 4 out of 4 line pairs).

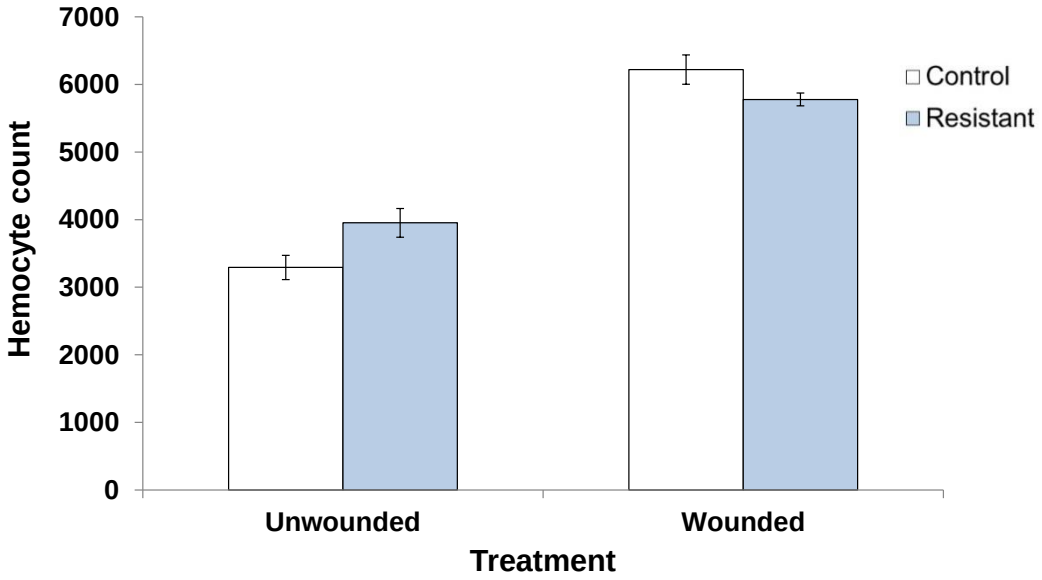


Figure 2.6 Larval hemocyte counts

Hemocyte counts from control (white) and resistant (blue) larvae, with or without prior wounding treatment (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 10 larvae were bled per sample, with 10 samples analysed per biological replicate (C/S1-4).

Unlike our larval findings, single wounding in adult flies 24 hours prior to hemolymph extraction did not lead to an increase in circulating hemocyte number (Figure 2.7a; females: T = 1.503, df = 6, $P = 0.184$, Figure 2.7b; males: T = -0.033, df = 6, $P = 0.975$). Overall, there was no effect of immunity in the adult male (T = -0.496, df = 6, $P = 0.638$) or female (T = 2.215, df = 6, $P = 0.069$), with increased numbers of hemocytes found in the selected flies compared to the control overall (shown in 3 out of 4 of the un-wounded line pairs and 4 out of 4 of the wounded line pairs). This result was not shown in the adult male, where no effect of immunity was found ($F_{1,14} = 0.24$, $P = 0.634$). There was no interaction between wounding treatment and immunity (males: T = 0.278, df = 6, $P = 0.790$; females: T = -1.14, df = 6, $P = 0.298$).

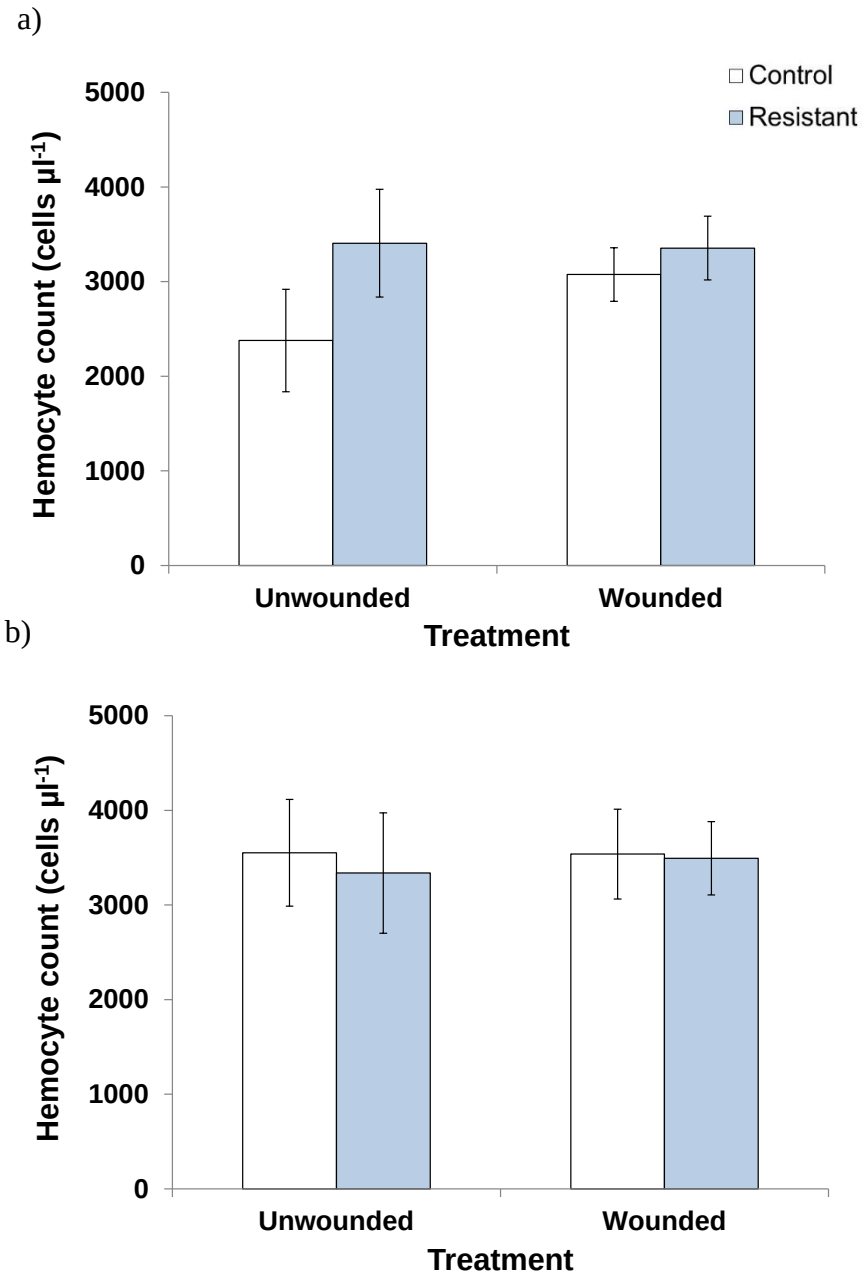


Figure 2.7 Adult hemocyte counts

Hemocyte counts from female (a) and male (b) adult Drosophila. Numbers from control (white) and resistant (blue) flies, with and without a prior wounding treatment (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 6 adults were bled per sample, with 10 samples analysed per sex for each biological replicate (C/S1-4).

2.3.3 PO activity in the larva and adult Drosophila

PO activity in the hemolymph was assessed from the absorbance reading after addition of L-dopa. We looked at two parameters: initial activity, which was taken from the first reading on the spectrophotometer immediately after L-dopa addition and rate, which was measured as the change in absorbance over the first 30 minutes. No difference was found in the PO activity of the larval hemolymph extracted from control and selection larvae; measured in terms of initial activity (Figure 2.8(i); T = 1.14, df = 6, *P* = 0.298) and rate (during the first 30 minutes following L-dopa addition) (Figure 2.8(ii); T = 1.245, df = 6, *P* = 0.259).

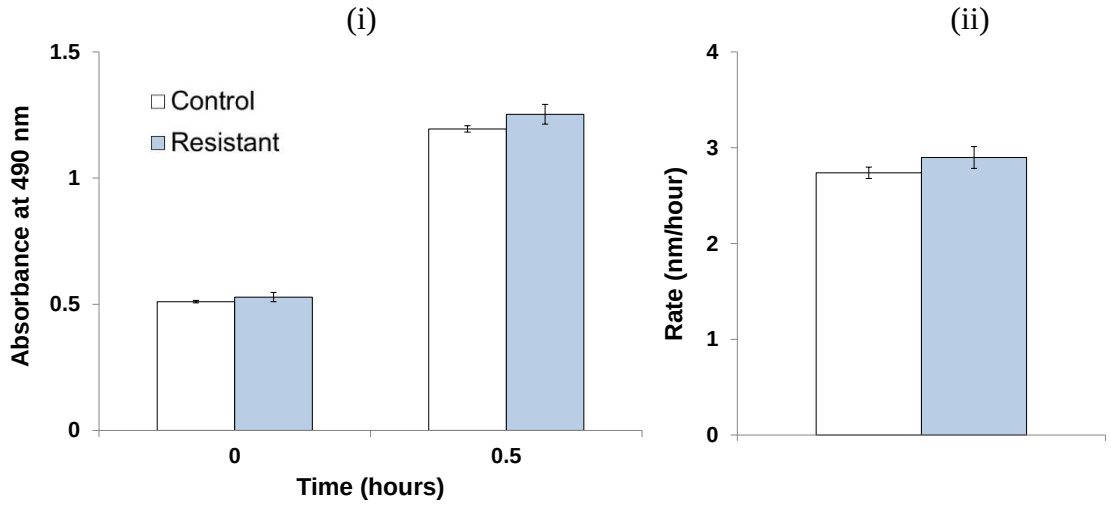


Figure 2.8 Larval PO activity

Measured by absorbance at 0 and 0.5 hours post exposure to the L-dopa substrate (i) and by the rate of change in absorbance per hour (ii) (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 50 larvae bled per sample, with 3 samples analysed for each biological replicate (C/S1-4).

There was no difference in the PO activity when measured as initial PO activity (females: Figure 2.9a (i); T = -2.15, df = 6, *P* = 0.075, males: Figure 2.9b (i); T = -1.304,

df = 6, *P* = 0.240) or when measured as the rate of PO conversion (females: Figure 2.9a (ii); T = -1.00, df = 6, *P* = 0.354, males: Figure 2.9b (ii); T = -1.416, df = 6, *P* = 0.207).

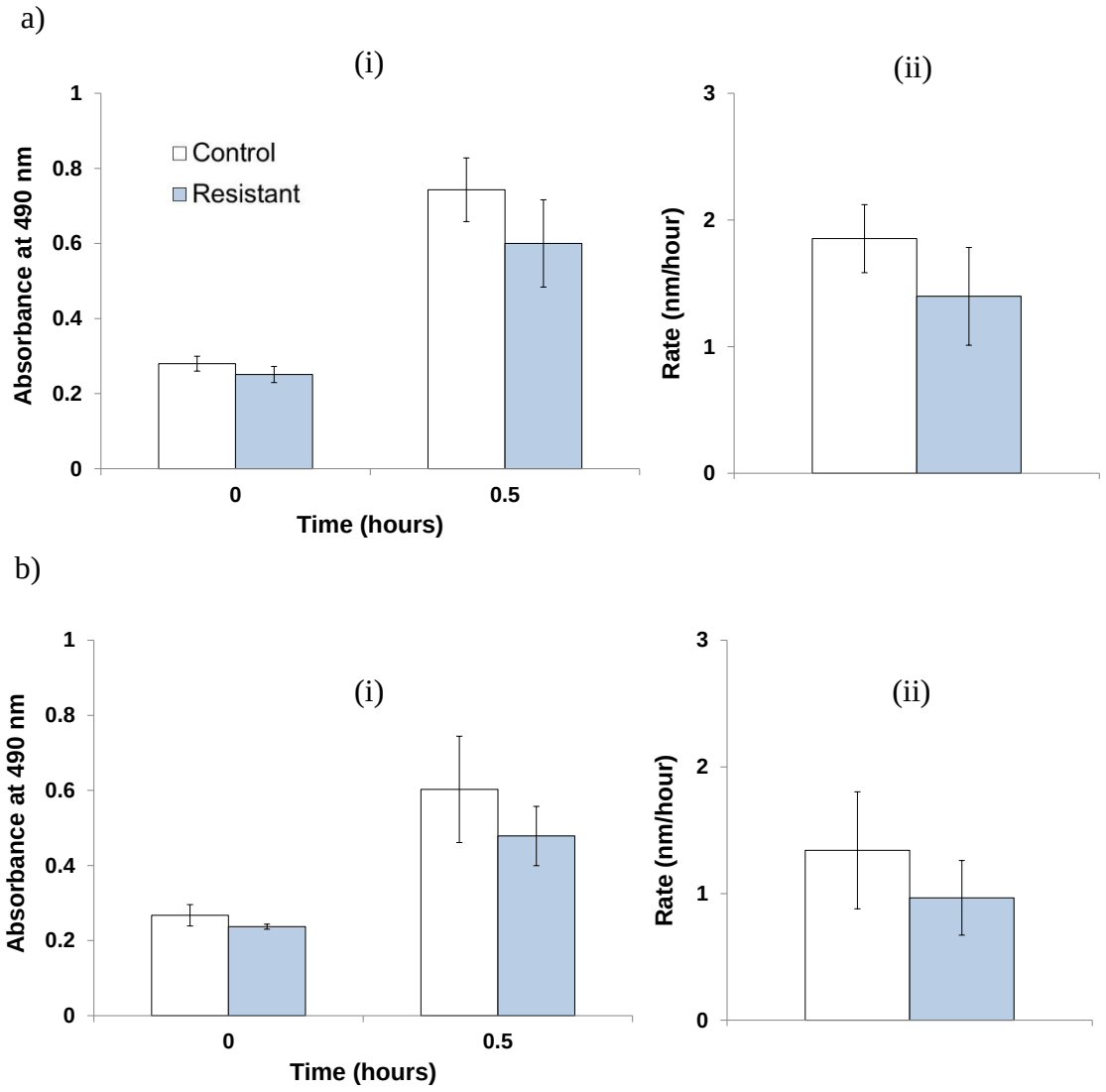


Figure 2.9 Adult PO activity

Adult a) female and b) male Drosophila: PO activity by absorbance at 0 and 0.5 hours post exposure to the L-dopa substrate (i) and rate of activity per hour (ii) (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 50 adults were bled per sample, with 3 repeats performed per sex for each biological replicate (C/S1-4).

2.3.4 Larval wound healing

To test whether larval wound healing is affected by the individual's ability to encapsulate upon parasitic infection, *Drosophila* larvae were wounded for both control and selection lines and four different parameters were measured: the proportion survival after stabbing, the proportion of larvae showing melanisation of the wound site, and the size and intensity of the melanised scab. Wounding larvae lead to around 50% mortality (Figure 2.10; T = -11.45, df = 6, $P < 0.0001$), but there was no difference in survival between control and selection larvae (T = 0.31, df = 6, $P = 0.767$) irrespective of stabbing treatment (T = 0.13, df = 6, $P = 0.901$).

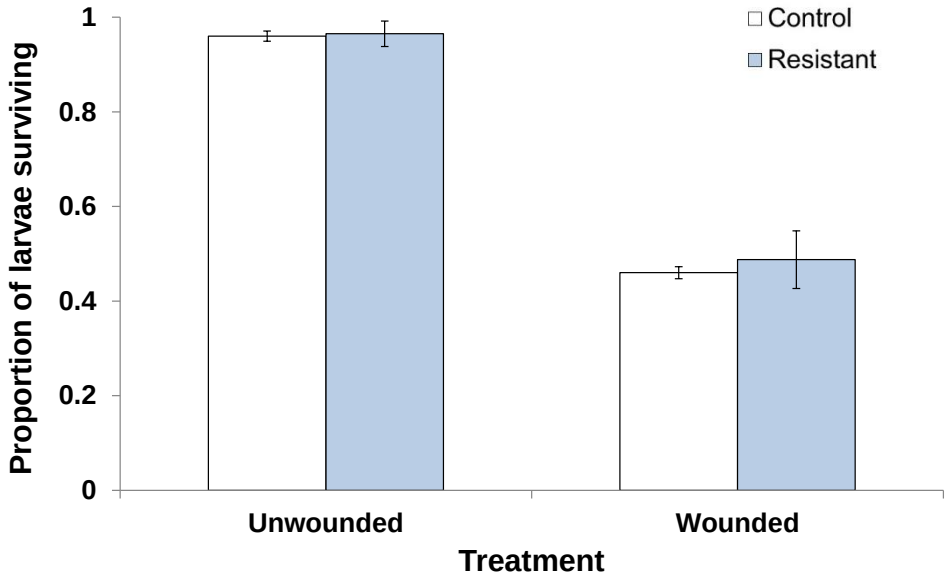


Figure 2.10 Larval wounding: Survival

Survival of wounded and unwounded larvae from a control (white) or immune resistant (blue) background (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 100 larvae of each treatment group (wounded/unwounded) were analysed for each biological replicate (C/S1-4)).

Of those larvae surviving a large number had initiated the full wound healing response, including melanisation of the wound clot (Figure 2.11). This response was seen to a greater extent in the selection larvae where a higher proportion of melanised scabs were

seen than with the control larvae (in 4 out of 4 of the line pairs, see Appendix Table 2 for individual line means) (T = 5.305, df = 6, *P* = 0.0018), accounting for percentages of 96.9% and 91.2% respectively.

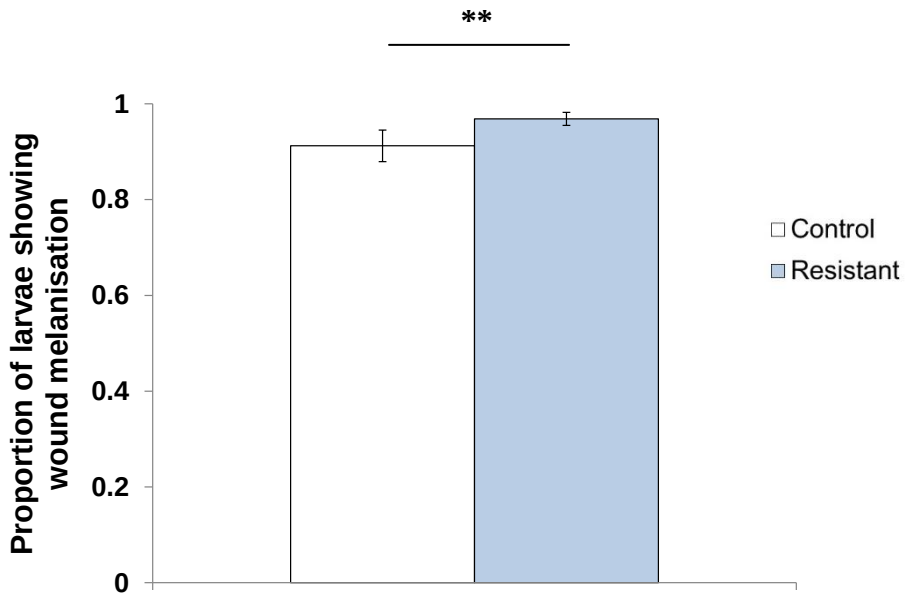


Figure 2.11 Larval wounding: Proportion showing wound melanisation

*Proportion of wounded larvae showing melanisation at the wound site in control (white) and resistant (blue) Drosophila (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 47 (± 3) pupae were analysed for each biological replicate (C/S1-4). ** P < 0.01*

There was no difference between the control and resistant larvae in the size (Figure 2.12a; T = -0.1, df = 6, *P* = 0.924) or intensity (Figure 2.12b; T = -0.775, df = 6, *P* = 0.468) of the melanised scab.

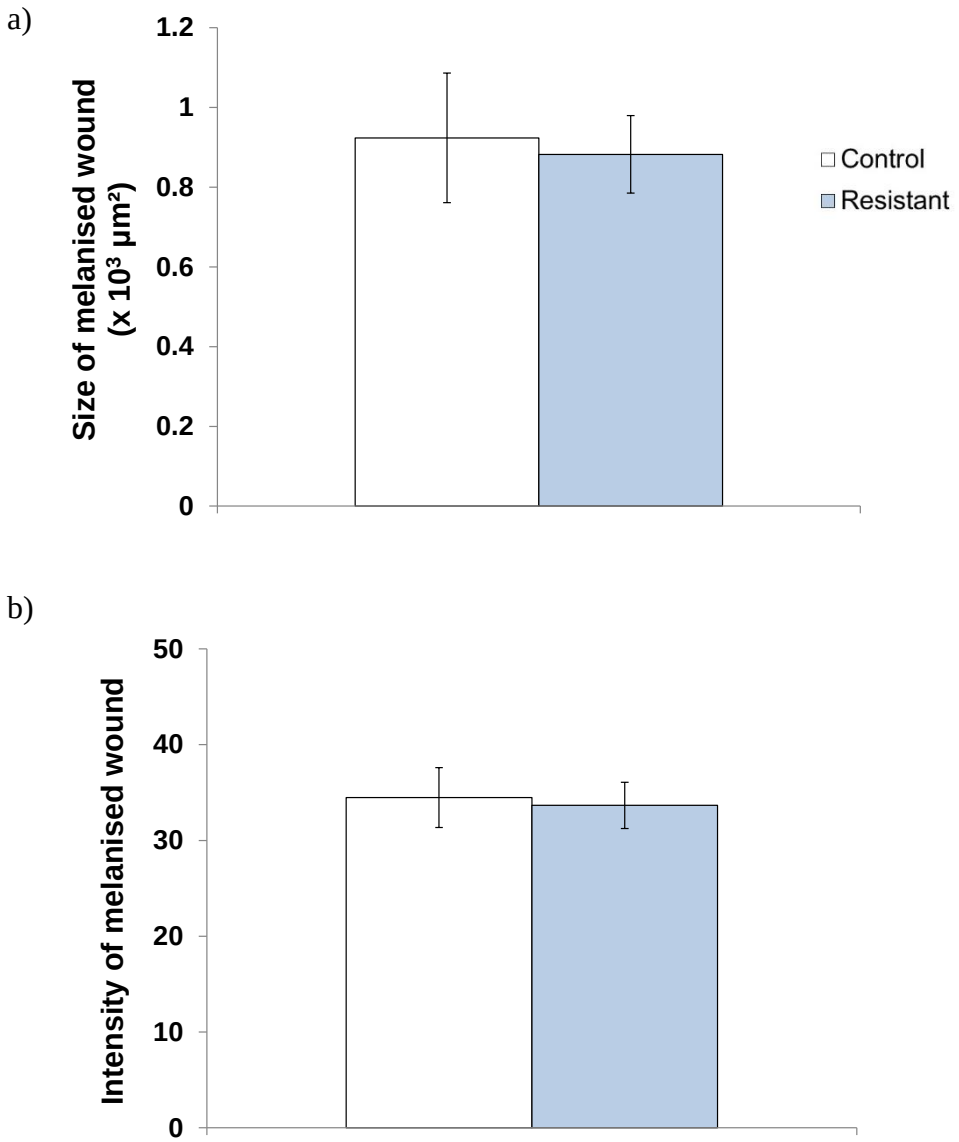


Figure 2.12 Larval wounding: Size and intensity

a) size and b) intensity of melanisation at the wound site in control (white) and resistant (blue) larvae (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 44 (± 1) wounds were analysed for each biological replicate (C/S1-4).

Selection larvae show a significantly higher proportion of melanotic masses (areas of melanisation that occur away from the wound site) per wounded individual (shown in 4

out of the 4 line pairs, see Appendix Table 3a for individual line means) (Figure 2.13a; T = 6.652, df = 6, *P* = 0.0006); 52.9% of wounded selected larvae exhibited masses compared to 31.9% of wounded control larvae. The average size of these masses was 69.2% larger in selection than in control larvae (Figure 2.13b; T = 11.159, df = 6, *P* < 0.0001); masses were consistently larger in the selection flies compared to the control (larger masses shown in 4 out of the 4 line pairs, see Appendix Table 3b for individual line means).

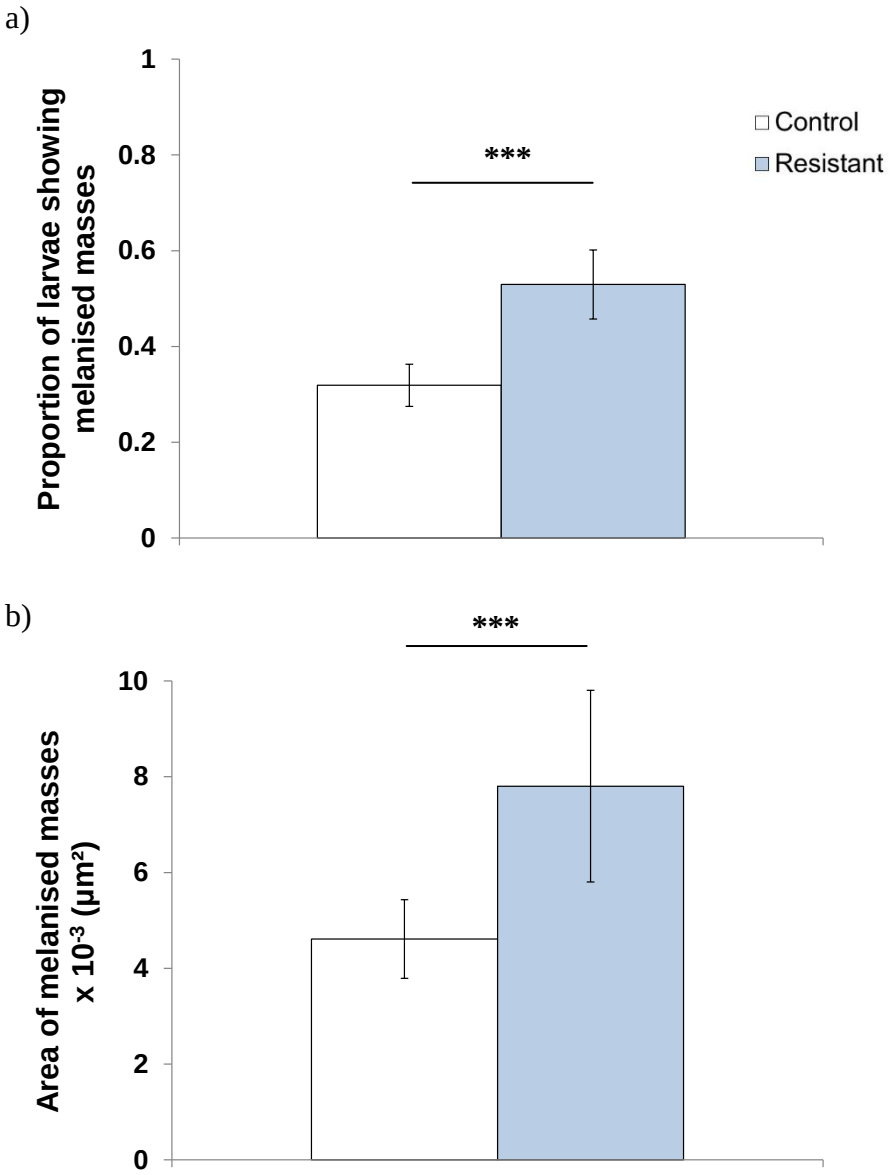


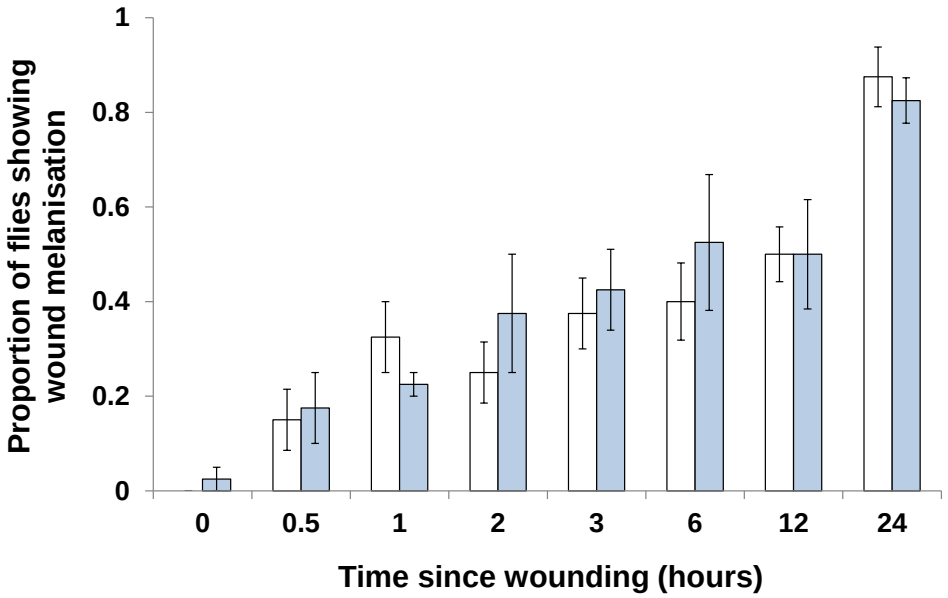
Figure 2.13 Larval wounding: melanotic masses

*a) Proportion of control (white) and resistant (blue) Drosophila larvae showing melanotic masses and b) the average area of melanotic masses per individual (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 47 (± 3) pupae were analysed for each biological replicate (C/S1-4), with 20 (± 3) pupae from each replicate (screened positive for melanised masses) then analysed for mass area. *** $P < 0.001$.*

2.3.5 Adult wound healing: single wounding

Male and female flies of both resistance backgrounds were wounded and three different parameters were measured over a 24 hour period: the proportion of flies showing melanisation of the stab wound, and the size and intensity of the melanised stab wound. Survival was also scored, and found that 99.5% of flies survived over the 24 hours after wounding (only 7 out of the 1280 flies died post-injury; 2 control and 5 selection). In both male and female flies, the proportion showing melanisation of the stab wound increased with time; overall 84.2% flies exhibited melanised scabs at 24 hours post-injury (females Figure 2.14a: T = 6.816, df = 6, P = 0.0005; males Figure 2.14b: T = 7.049, df = 6, P = 0.0004). In male flies, there was a significant effect of immunity level (T = -2.559, df = 6, P = 0.043): at each time interval, a higher proportion of control flies showed a melanised scab than selection flies (see Appendix Table 4 for line means). In female flies, there was no significant effect of immunity level on proportion of flies showing a melanisation at the wound site (T = 0.824, df = 6, P = 0.442). Interactions between time and immunity level were not significant in males or females (P = 0.986 and 0.472, respectively).

a)



b)

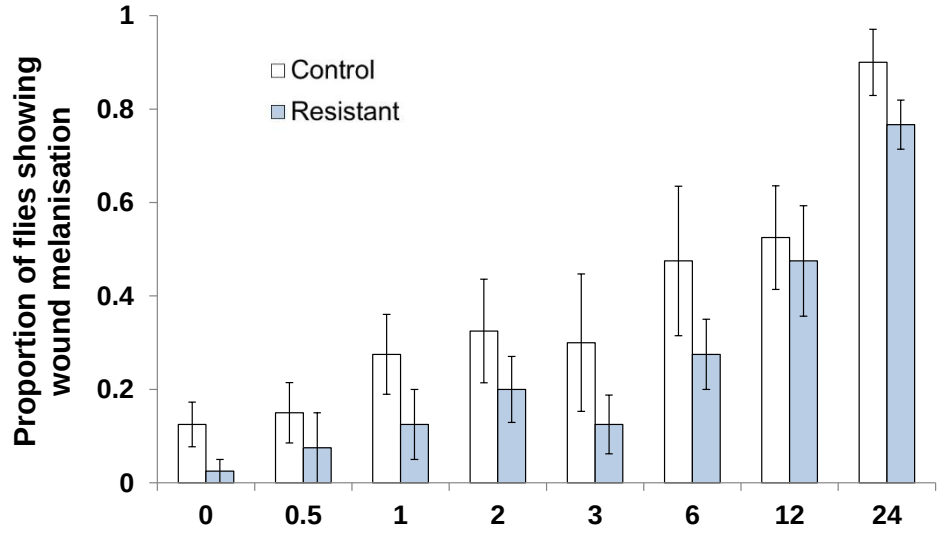


Figure 2.14 Adult wounding: Proportion showing wound melanisation

Proportion of singly-wounded adult female (a) and male (b) Drosophila from a control (white) and resistant (blue) immune background showing melanisation at the wound site (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 10 flies of each sex were analysed for each time point (0, 0.5, 1, 2, 3, 6, 12 and 24 hours) for each biological replicate (C/S1-4).

The size of the melanised scab increased over time in both sexes (females, Figure 2.15a: T = 3.915, df = 6, *P* = 0.0078; males, Figure 2.15b: T = 4.567, df = 6, *P* = 0.0038). However, there was no overall effect of immunity level in male (T = 0.563, df = 6, *P* = 0.594) or female flies (T = 0.544, df = 6, *P* = 0.606). The interaction between time and immunity level was not significant in male (T = 0.123, df = 6, *P* = 0.906) or female flies (T = -1.471, df = 6, *P* = 0.192).

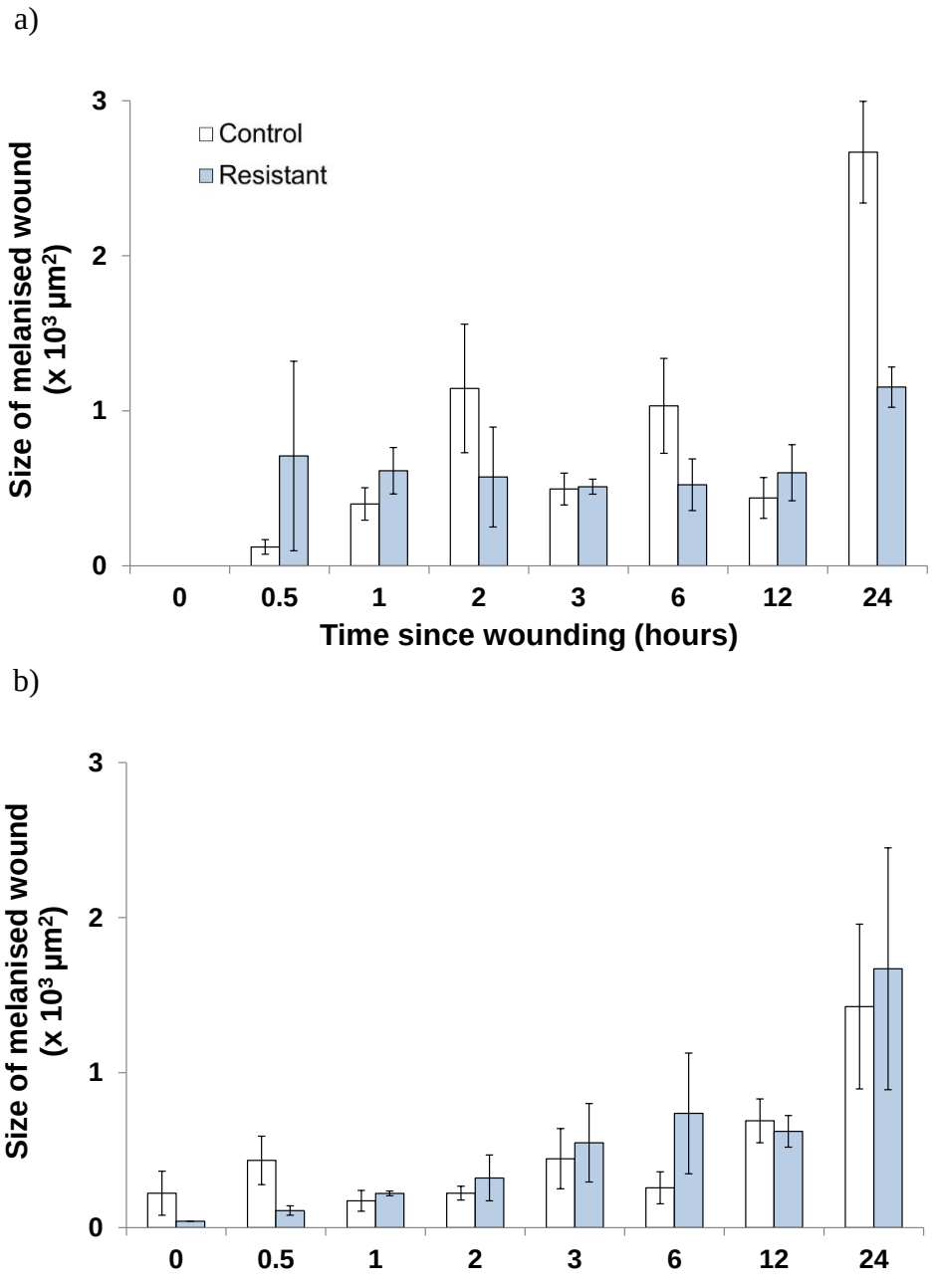
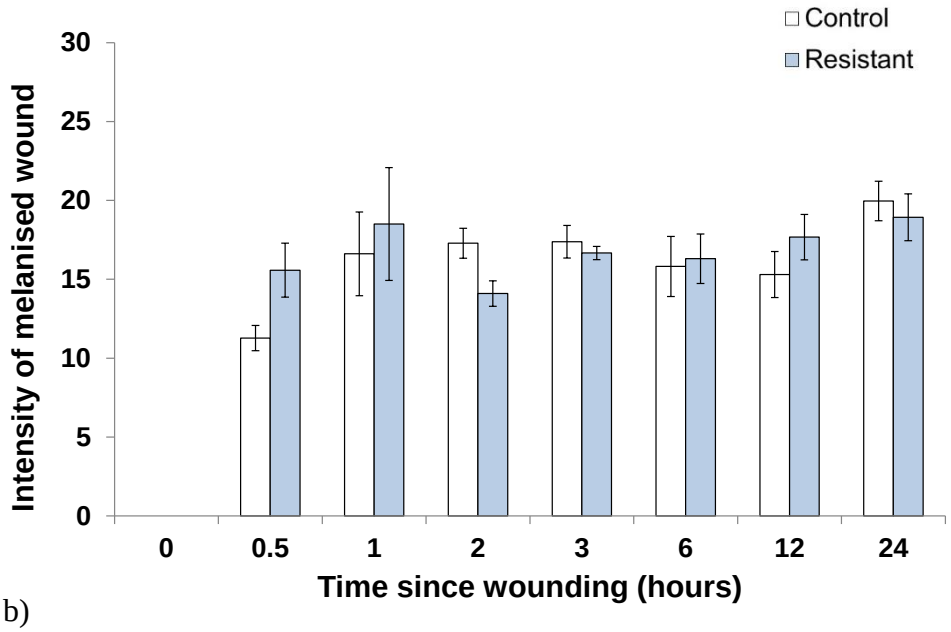


Figure 2.15 Adult wounding: Size

Size of melanised wound in singly-wounded adult female (a) and male (b) Drosophila from a control (white) and resistant (blue) immune background (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 29.6 (± 1.6) and 25.7 (± 3.3) wounds were analysed from females and males respectively for each biological replicate (C/S1-4).

Time since wounding had no effect on the melanisation intensity of the scab in male (Figure 2.16b: T = 0.212, df = 6, *P* = 0.839) or female (Figure 2.16a: T = 2.203, df = 6, *P* = 0.070) flies. Immunity level had no effect on melanisation intensity in either sex (males: (T = -0.571, df = 6, *P* = 0.589; females: (T = 0.571, df = 6, *P* = 0.589). Interactions between time and immunity level were not significant in either sex (p = 0.774 and 0.726 for males and females, respectively).

a)



b)

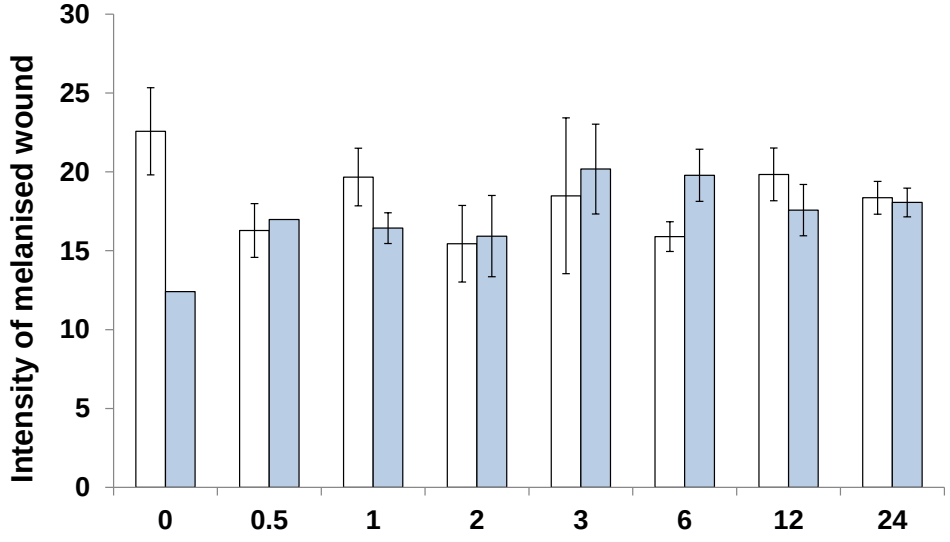


Figure 2.16 Adult wounding: Intensity

Intensity of wound melanisation from singly-wounded adult female (a) and male (b) Drosophila from a control (white) and resistant (blue) immune background (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 29.6 (± 1.6) and 25.7 (± 3.3) wounds were analysed from females and males respectively for each biological replicate (C/S1-4).

2.3.6 Adult wound healing: triple wounding

Survival over the 6 hour period was 98.3% (only 6 of the 350 flies died post-injury, 3 control and 3 selection). The proportion of all flies showing at least one melanised scab increased in size by 35.3% from 0.5 to 6 hours post-injury (Figure 2.17; T = 4.336, df = 6, *P* = 0.0049). The proportion of melanised flies showing more than one melanised scab also increased during this interval (T = 3.02, df = 6, *P* = 0.024), as did the proportion of multiply-melanised flies showing three melanised scabs (T = 3.655, df = 6, *P* = 0.0106); by 100.0% and 223.1% respectively. None of these three proportions was affected by immunity level (respectively: T = 0.802, df = 6, *P* = 0.453; T = -0.911, df = 6, *P* = 0.397; T = -0.522, df = 6, *P* = 0.620). There were also no significant time x immunity interactions (*P* = 0.243, 0.966, and 0.662, respectively).

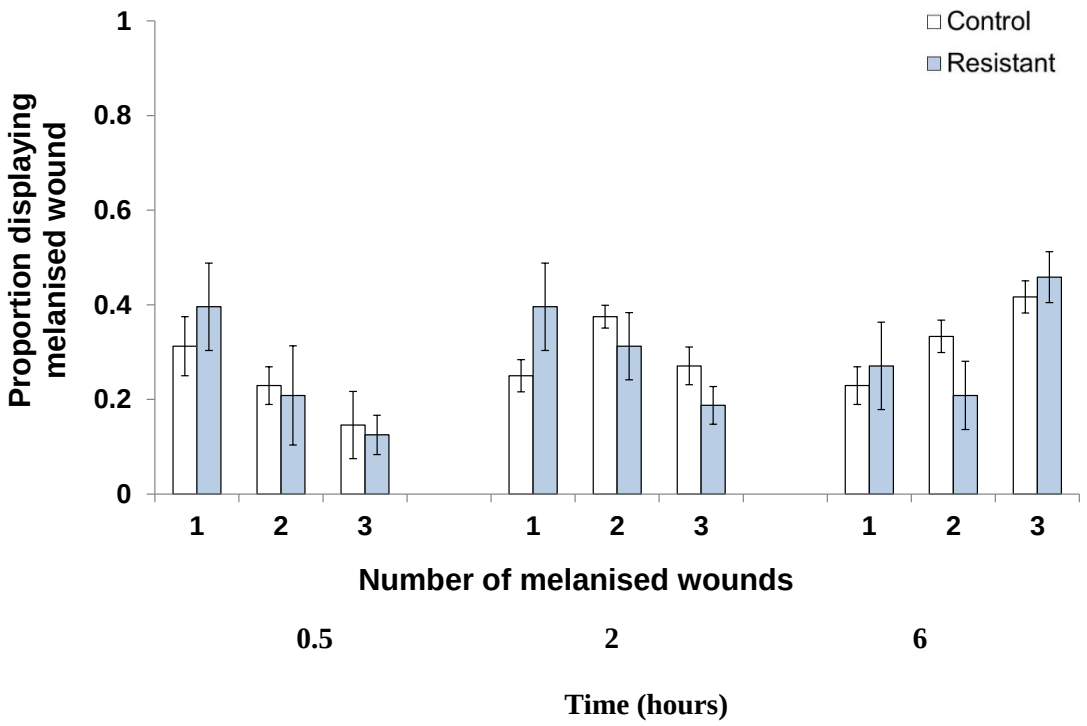


Figure 2.17 Adult wounding (x3): Proportion showing wound melanisation
Proportion of control (white) and resistant (blue) Drosophila displaying 1, 2 and 3 melanised wounds at three time points (0.5, 2 and 6 hours) following a triple stabbing injury (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 12 adult flies were analysed for each sex at each time point (0.5, 2 & 6 hours) for each biological replicate (C/S1-4).

As shown in the single wounding experiment, the average size of the melanised scab(s) per fly increased with time (Figure 2.18; T = 4.61, df = 6, *P* = 0.0037), with an increase of 166.6% in size between 0.5 and 6 hours. There was no effect of immunity on wound size (T = -0.84, df = 6, *P* = 0.433) or interaction between immunity and time (T = -0.39, df = 6, *P* = 0.710).

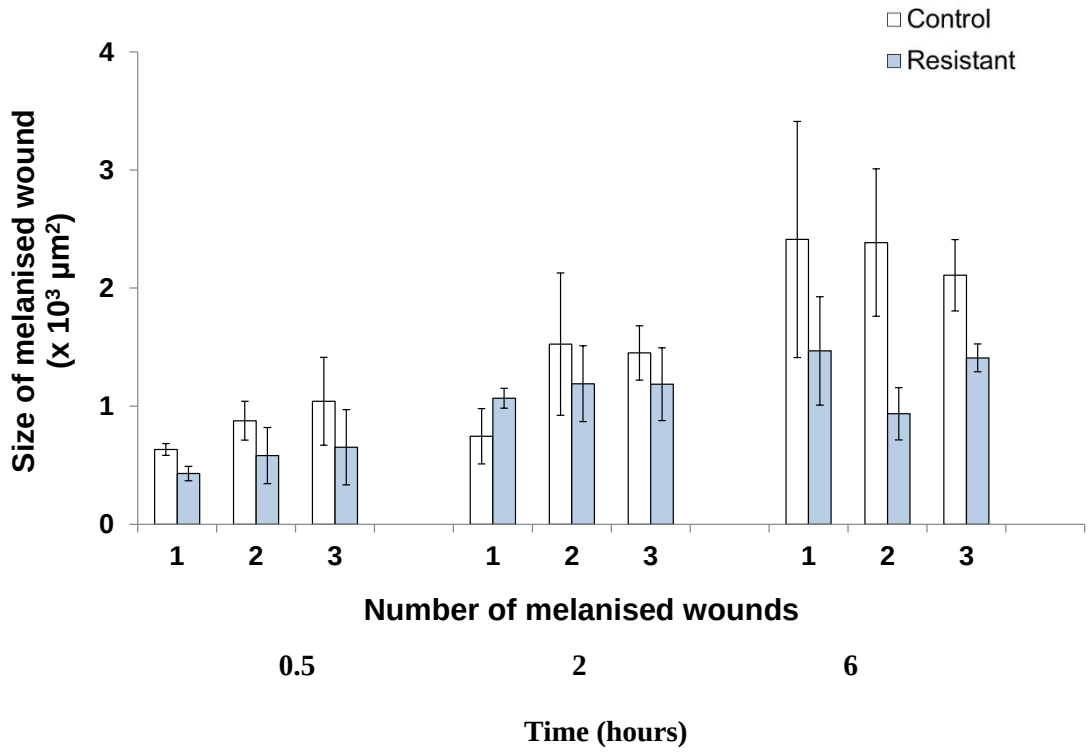


Figure 2.18 Adult wounding (x3): Size

Size of melanised wounds in adult female Drosophila showing 1, 2 and 3 wounds at three time points (0.5, 2 and 6 hours) after a triple stabbing injury, from a control (white) and resistant (blue) immune background (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 10.3 (± 0.2) wounds were analysed from wounded females for each biological replicate (C/S1-4).

The average melanisation intensity of the scab(s) per fly did not change over time (Figure 2.19; T = -0.602, df = 6, *P* = 0.569). Immunity level had no effect on the average melanisation intensity (T = -0.606, df = 6, *P* = 0.567). There was no interaction between the immunity level and time since wounding (T = 1.293, df = 6, *P* = 0.244).

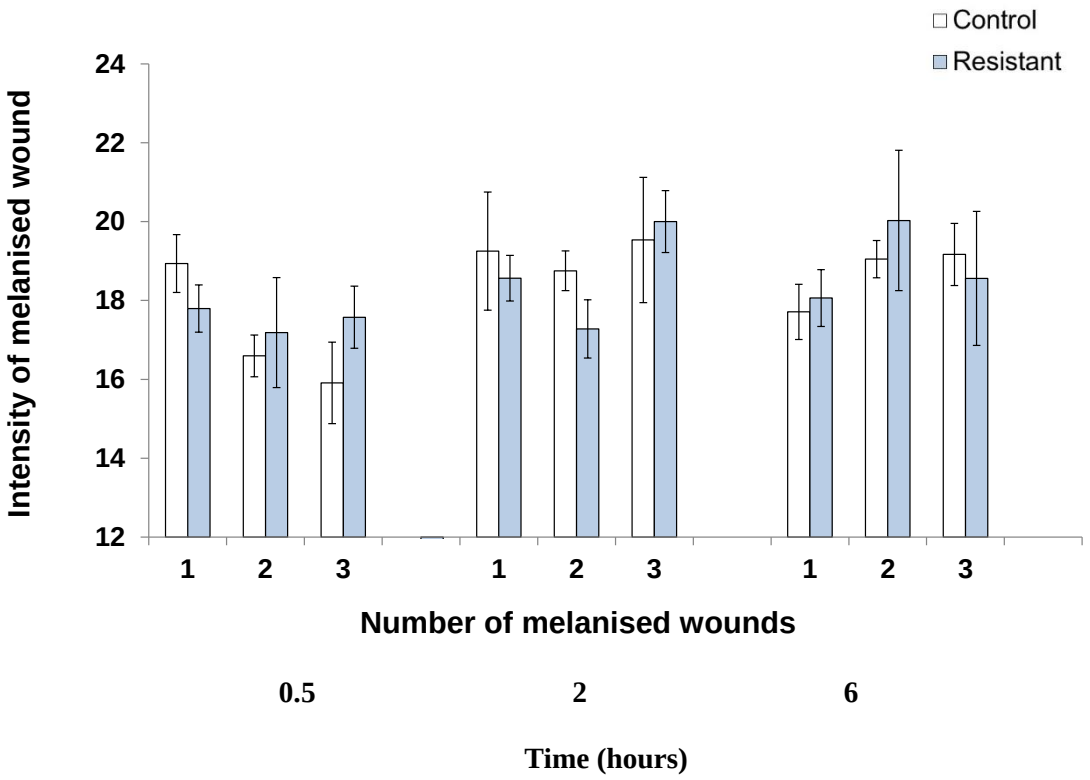


Figure 2.19 Adult wounding (x3): Intensity

Intensity of melanised wounds in adult female Drosophila showing 1, 2 and 3 wounds at three time points (0.5, 2 and 6 hours) after a triple stabbing injury - displayed from a control (white) and resistant (blue) immune background (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 10.3 (± 0.2) wounds were analysed from wounded females for each biological replicate (C/S1-4).

2.4 Discussion

The first aim of this chapter was to evaluate the immunocompetance of the model system; *D. melanogaster* selected for resistance against the larval parasitoid, *A. tabida*, and how this immunocompetance progresses into the adult phase. *Drosophila* larvae from lines exposed to parasitism showed a greater resistance against this kind of threat, with increased numbers mounting a successful encapsulation response upon infection. Further experimentation was then performed to determine whether corresponding changes in humoral and cellular defence systems were retained in these *Drosophila*. In

order to test this two parameters were measured, hemocyte counts and PO activity, in the late larval and adult phases of the control and resistant flies.

2.4.1 Hemocyte counts

Hemocyte numbers were measured with and without a prior cuticular insult. This allowed the direct comparison between circulating hemocyte numbers in the hemolymph of an unchallenged fly and the total number of hemocytes in circulation when a threat response has been triggered. A cuticular injury led to an increase in the numbers of hemocytes in the *Drosophila* circulation; a finding most likely attributed to the release of hemocytes from their sessile compartments. Generally, in a non-challenged state these cells would be found in a banded pattern along the length of the *Drosophila* larvae; a pattern which is fully established by the 3rd instar (Márkus *et al.*, 2009). Work by Markus *et al* (2009) using GFP-tagged hemocytes showed that by 12 hours after cutaneous injury the banded pattern is lost as hemocytes are released from these pools into the circulation, where they remain, for at least 3 days post wounding. The number of hemocytes released from sessile pools was found to be similar to the increase in general circulation, indicating that changes in circulating hemocyte numbers is dependent on the number stored in sessile pools, with some additional hemocytes released from the *Drosophila*'s hematopoietic organ, the lymph gland (Márkus *et al.*, 2009). Sessile and lymph-gland derived hemocytes are most likely released to aid with secondary infection within the hemolymph and to clear debris remaining from the initial defence.

Overall, hemocyte numbers were greater in the selected larvae than in their control counterparts. However, the relationship between hemocyte number and immune background was highly dependent on the wounding treatment. Upon wounding, fewer immune cells were released into circulation in the resistant *Drosophila* than in the control flies. This finding contrasts with that made by Eslin and Prévost (1998), who showed that more resistant *melanogaster* subspecies, with higher initial numbers of circulating hemocytes, showed greater increases in immune cell number upon infection. One potential explanation for this discrepancy is that the mechanisms up-regulated in our model system may be different to the variations found between the *melanogaster*

subspecies. More resistant *Drosophila* species may have evolved due to changes in expression in overall embryonic hemocyte production. Whereas, the short-term, intense selective pressures utilized to set up our model system may have induced changes by different mechanisms, for example by altering the allocation of hemocytes into their sessile and circulating lineages. Stofanko *et al*. (2008) conducted a misexpression screen in which they systematically misexpressed around 20% of larval hemocyte genes using a plasmatocyte-expressed GAL4 driver system. In conjunction with the screen they expressed a UAS-GFP reporter to tag the hemocytes for enhanced visualisation within the fly crosses. Among 3412 insertions screened 108 were found to disrupt hemocyte development. Of those 108 insertions, 37 led to altered overall hemocyte number, while 58 led to disrupted dorsal sessile compartmentalisation indicating that a greater number of genes are involved in recruitment at sessile pools than in the production of hemocytes. This also highlights a substantial number of potential candidate genes that may be responsible for the changes seen in our resistant flies.

When a parasitoid egg enters the circulation it will quickly become embedded into the host tissues, where it will become undetectable by the host surveillance system. Therefore, additional surveillance cells circulating within the larval hemolymph at the time of infection would increase the speed and likelihood of detection, whilst sessile hemocytes are likely to be less important in this initial recognition process and more important in the secondary phase of immune defence.

In addition to original hemocyte location, another possible suspect may be the *Drosophila* Jun amino terminal kinase homologue Basket (Bsk), which was previously found to be under-expressed in this *Drosophila* model system (Wertheim *et al.*, 2011). Bsk-dependent Rac1 signalling promotes recruitment of sessile hemocytes into the hemolymph (Zettervall *et al.*, 2004, Williams *et al.*, 2006). Decreased Rac1 activity in the resistant lines could therefore decrease hemocyte recruitment into general circulation thus leaving greater numbers of immune cells within sessile pools. Alternatively, more hemocytes may have become trapped within the wound clotting matrix in resistant individuals at the time of hemolymph extraction, which may indicate a greater role of the hemocytes at the wound site. In either case it would be necessary to quantify all hemocytes, including those trapped within the wound matrix and those remaining in sessile pools in both control and resistant flies. This could be done using a

more detailed fluorescent tagging approach, like that implemented by Brian Stramer's lab (Evans *et al.*, 2010).

In general, circulating hemocyte numbers in the adult *Drosophila* did not increase following a sterile wounding insult, perhaps indicating a lack of sessile hemocytes or a lack of recruitment of these cells in the adult. Mutations that affect the adherence or migratory properties of hemocytes can lead to disruptions in sessile pool formation (Stofanko *et al.*, 2008). At metamorphosis, most of the organs and tissues in the larva are broken down prior to the reconstruction of the adult phenotype. In this process important immune structures of the larva, such as the lymph gland (*Drosophila* hemopoitic organ), are lost thus negating further hemopoiesis, while hemocytes residing in sessile pools and the lymph gland are released into circulation to aid with restructure of the developing *Drosophila* (Abrams *et al.*, 1993). Males and females from a selected background no longer demonstrated disparity in hemocyte numbers compared to control flies, which suggests modification during metamorphosis; when crucial resources can be reallocated away from redundant immune functioning. A reorganisation of hemocyte numbers would agree with the 'adaptive decoupling hypothesis' proposed by Moran (1994).

2.4.2 PO activity

PO enzymes catalyse the conversion of the L-dopa substrate to quinones and then finally to melanin. Therefore, the rate of conversion of the substrate upon addition to a hemolymph sample, measured by changes in absorbency, should give an indication of the availability of PO in the hemolymph. As well as the PO already in circulation additional PO is also released from the crystal cells, which rupture during the hemolymph extraction process. Therefore, this assay measures both cellular and hemolymph derived PO. Melanin levels increased over time upon activation; however, this effect was independent of the *Drosophila* larvae's resistance background. This finding may indicate that initial PO levels and activity in the hemolymph does not correspond to the ability of the *Drosophila* larvae to encapsulate upon parasitoid infection, which may imply that hemocyte number is a greater regulator of these processes. Alternatively, it is possible that the flies differ in their ability to initiate

rupture of the crystal cells. In general, proPO would not be released unless prompted by strict regulatory mechanisms, such as JNK and tumor necrosis factor-alpha (TNFα), or Eiger protein, signalling (Bidla *et al.*, 2007), a factor that is not measured by the L-dopa assay. This relationship continued into the adult *Drosophila* where no effect of immune-background was found in PO activity in the adult males or females.

2.4.3 Wound healing

The other main objective of this study was to elucidate whether the ability of *Drosophila* larvae to encapsulate upon parasitic infection is linked to the capacity to mount a full wound healing response, quantified by the presence and extent of melanin deposition at the wound site. This attribute could then be used as a relevant indicator of immunocompetance in the control and resistant *Drosophila* in the late larval and adult phases.

Larvae: When wounded, *Drosophila* larvae from a population with increased resistance against parasitoid infection showed higher rates of wound melanisation. This finding infers that there is a link between the ability to mount an encapsulation response and a full wound healing response. This link may exist due to an overlap in the cellular defence system, which was found to be up-regulated in the resistant flies. During wound healing, hemocytes exude extracellular matrix (ECM) proteins that help plug the injured tissue and actively draw the edges of the punctured basal lamina back together narrowing the wound site (Olofsson & Page, 2005, Galko & Krasnow, 2004). Increased numbers of circulating hemocytes in the resistant flies means that more are immediately available, therefore allowing more rapid detection and increasing the likelihood and of a successful response. Once the wound site is sealed, melanin is laid down into the ECM mesh to reinstate impermeability and kill microbes at the wound entrance. Due to the fact that PO activity was found not to differ between the control and resistant flies, this might imply that this factor has less impact on the speed at which the wound site is melanised. The rate limiting factor of wound melanisation may instead be the time taken to initially plug the wound site. Alternatively, in situ PO activity may occur differentially between *Drosophila* from high and low resistance backgrounds due to variations in proPO quantity or activation (e.g. by serpins; a type of serine protease

inhibitors), factors not included in this study. Wertheim (2011) found increased expression of serpin-6 in the resistant *Drosophila* from our model system. This might suggest that additional serpins are in place to help regulate PO activity due to the increased chance of activation of the melanisation cascade through an immune induction.

In addition to increased wound melanisation, it was found that larvae with increased resistance to parasitism showed an increased susceptibility to form melanotic masses within the hemocoel upon wounding. Approximately 30 genetic alterations are known to result in melanotic mass formation in *Drosophila* larvae (Minakhina & Steward, 2006). Interestingly, one of the genes that has been implicated in the formation of melanotic masses in the hemolymph has also previously been shown to be upregulated in *Drosophila* selected for resistance to parasitism; that gene is su(var)205 (the function of this gene in immunity is currently unknown) (Wertheim *et al.*, 2011, Minakhina & Steward, 2006). Melanotic masses have previously been linked to changes in a number of signalling pathways including: Toll (Lemaitre *et al.*, 1995), Jak/STAT (Meister, 2004), Ras/MAPK (Zettervall *et al.*, 2004) and IMD/Relish (Takehana *et al.*, 2002). Melanotic masses formed in the hemocoel, including those linked to mutations in su(var)205, have also been linked to the involvement of hemocyte activity (Rizki & Rizki, 1983, Nappi *et al.*, 2005, Minakhina & Steward, 2006). Masses are thought to indicate an over-reactive immune response and can be harmful to the individual due to the excess production of toxic intermediates produced during the conversion of PO to melanin (Cerenius & Söderhäll, 2004). Generally, the melanisation cascade is strictly regulated by serpins, to ensure that toxic intermediates produced in the PO cascade do not spread from the site of injury to other regions within the host. Lack of serpin expression can lead to spontaneous melanisation and the formation of melanotic masses within the hemocoel; in some cases this can be lethal (Ligoxygakis *et al.*, 2002, De Gregorio *et al.*, 2002). In contrast, as discussed above, serpin-6 was found to be expressed more highly in the selected lines (Wertheim, 2011). One possible explanation is that serpin regulation targeted towards limiting the encapsulation response in the selected flies may have led to a trade-off in serpin regulation in other areas of the fly. In addition, melanotic mass formation could be linked to excessive hemocyte activity by one or all of the three hemocyte types (plasmatocytes, lamellocytes and/or crystal cells) (Watson *et al.*, 1991, Braun *et al.*, 1998). In order to determine the involvement of the

immune cells further investigation would be necessary, for example quantification of lamellocytes and crystal cells in the control and selected lines and closer inspection of the masses.

Irrespective of whether melanotic mass formation is due to hemocyte activity or PO regulation, increased mass prevalence in the selected flies signifies that resistance to disease may come at a cost of autoimmunity to the host. Excessive melanisation responses may explain why we still find a great deal of immune variation within *Drosophila* populations exposed to parasitoid attack, where encapsulation responses would be thought to be favourable (Kraaijeveld & Godfray, 1997). When parasitoid numbers are high the selective advantages of encapsulation should see ubiquitous resistance. However, in a population with repeated exposure to parasitism, resistance will typically only reach around 60% (Kraaijeveld & Godfray, 1997).

Increased wound melanisation responses did not correlate with survival, which was found to be the same in wounded control and resistant larvae. It is likely that sterile wounding will not directly relate to survival following an epidermal injury in a non-sterile setting. In reality, a series of bacterial, fungal and viral infections may be rife in the environment and thus pose a greater threat following cuticle damage. One might therefore assume that in an infectious environment a proficient wound healing system may indeed increase chances of survival. Alternatively, melanisation itself may not be solely responsible for survivorship following injury; instead it is a combination of factors, including the action of antimicrobial peptides that ensures survival (Braun *et al.*, 1998).

Adults: Adult *Drosophila* revealed produced much smaller scab sizes than those found in the larvae, even when injured with a needle of larger diameter (unquantified observation). This might be explained due to the additional hydrostatic pressure exerted onto the cuticle of the larvae, as well as the cuticle itself being less rigid than in the adult and more able to stretch at the wound site prior to the formation of the ECM clot by the hemocytes.

Upon injury, wound healing acted almost immediately; with initial melanisation observed in some flies within ten minutes following wounding. The proportion of

individuals exhibiting a response then increased over the next 24 hours, revealing a large disparity in rate of wound melanisation within a population. In contrast to the larval findings, it was found that the proportion of adult males from the selected lines displaying wound melanisation was reduced at each time point, indicating a subordinate healing process relative to the control lines. This relationship, however, was not found in females, where numbers exhibiting melanisation seemed to reflect those shown in the control males. These findings demonstrate how immune advantages can be lost during metamorphosis and in some cases that they can even prove detrimental to the adult in terms of a trade-off in the immune function. This trade-off may be due to the allocation of limited metabolic resources toward immune function in youth, leaving a deficit for immune function in later life, which may be more exaggerated in males who tend to allocate fewer resources toward defence mechanisms than females (Rolff, 2002, Zuk *et al.*, 2004). Alternatively, the additional mechanisms involved in generating a rapid response to larval parasitism and wound healing may themselves prove unfavourable in later life.

Possible culprits in the trade-offs seen between the larvae and adult males in their level of wound melanisation include the regulatory mechanisms that control the level of PO activity within the *Drosophila*, which may not be detected in the L-dopa assays, such as serpins or crystal cell regulation. Crystal cells in the resistant flies may be more fragile or more sensitive to regulatory signals, which might result in the increased speed or likelihood of rupture. An attribute such as this may aid in efficient melanisation of the encapsulated parasitoid or wound clot in the larval phase, however, the same attribute could see more crystal cells lost during metamorphosis, meaning less are available in the adult and subsequently lowering proPO stores. If crystal cells are assigned differentially between the sexes (e.g. less in male larvae than in female larvae) then this might explain why we do not see a difference in the melanisation of the adult females. In this study it was not possible to determine differences in the coordination of PO release between the lines as most, if not all, of the crystal cells would rupture during the hemolymph extraction process.

One potential flaw in determining whether there is cross-resistance between the encapsulation and wound healing responses is that when the *A. tabida* parasitoid inserts its egg into the larval host it pierces the cuticle with its ovipositor. Therefore, animals

more at risk of this type of parasitism are also more likely to experience a secondary threat from cuticular wounding. As a consequence, selection should also favour those animals that are able to successfully heal the wound, preventing excess exsanguination and secondary infection should the other host defence mechanisms succeed in encapsulating the parasitoid egg. The observed increase in ability to mount the full wound healing response in the resistant larvae might therefore not be due to direct links in the two immune processes; instead we may be seeing the effect of co-evolution of the two immune defences.

2.4.4 Summary

In summary, this research demonstrates that selection for resistance to larval parasitism also protects the larva against cuticle injury by increasing the likelihood of a melanisation response at the wound site, which may be linked to the increased number of hemocytes within these individuals at this phase or alternatively a further mechanism that has co-evolved due to the increased exposure to cuticle injury in a parasitoid-rich environment. However, as a consequence of resistance these individuals are more at risk of auto-immunity through the formation of melanotic masses, which can be noxious to the developing fly. In addition, this is a phase-limited response and the protection against wounding does not continue into adulthood, when the flies are no longer at risk of parasitoid infection, most likely due to a rearrangement of the wound healing machinery during metamorphosis. In contrast, resistant males displayed reduced melanisation at the wound sites, indicating a developmental phase trade-off in immune function. Adult females showed no difference between the control and selected individual's ability to mount a melanisation response at the wound site. Gender disparity may occur due to the females increased need to maintain immunity in order to prolong survival and thus maximise offspring potential; as suggested by the Bateman's principle (1948).

In conclusion, this model system would be suitable to assess trade-offs caused by immunocompetance during development. The disappearance of many of the immune traits, including increased resistance and hemocyte numbers, implies that this system

might be less well suited to discovering how maintained resistance can impact on processes within an individual.

2.4.5 Acknowledgements

Thanks go to Kirsten Jalvingh and Bregje Wertheim (University of Groningen, the Netherlands) for supplying the control and selection lines. Rachel Fitzearle, Roger Shannon and Elizabeth Robertson for assistance with culturing. Thanks also go to Hans Schuppe, in memory, for all his technical support and use of his imaging equipment.

3 Resistance to larval parasitism leads to altered cell metabolism and reduced longevity in female *Drosophila melanogaster*

3.1 Introduction

Within an organism metabolic resources must be allocated optimally toward functions that ensure the continued existence of the individual and its genes to the next generation. Optimal distribution should therefore favour strategies such as immunity and reproductive success that promote survival and maximise offspring potential. Enhanced immunocompetence provides immediate protection against the threat of infection and thus is a key factor in determining the fitness of an animal (Schmid-Hempel, 2003, Schulenburg *et al.*, 2009). However, in order for the immune system to exist at an increased state of readiness it requires the upregulation of defence mechanisms directed toward pathogen detection and eradication. As a result of an increased immune presence host physiology is altered, through genetic and mechanistic modifications. *Drosophila melanogaster* selected for resistance to parasitism possess heightened numbers of circulating immune cells in the larval phase (Kraaijeveld *et al.*, 2001; Chapter 2). Immune cells are metabolically expensive, requiring high levels of resources in order to function and producing large amounts of metabolic by-products, such as reactive oxygen species (ROS). This chapter examines how these immune cells persist in the ageing fly and explores differences between intracellular ROS production in control and resistant individuals. In addition, longevity has been measured in order to determine whether immune resistance can lead to trade-offs in lifespan.

Heightened immune resistance occurs due to differential expression of genes relating to the immune system. Wertheim *et al*. (2011) showed that *Drosophila* larvae selected for increased resistance against parasitism displayed altered expression levels in almost 900 genes. However, genetic changes coding for upregulated immune function may also code for other modifications within the organism (see 1.4.2). Schwarzenbach and Ward (2006) showed that increased genetic investment in humoral resistance, through enhanced PO activity, resulted in reduced longevity under starvation in yellow dung

flies (*Scathophaga stercoraria* (L.)). Williams (1957, 2003) proposed that genes coding for fitness aspects in early life may also correspond antagonistically to components required for somatic maintenance. Therefore, a decline in function and senescence may be a consequence of negative genetic covariance with traits relating to early fitness such as components of the immune system (Williams & Day, 2003, Siva-Jothy *et al.*, 2005).

The existence of the additional mechanisms required to provide immune resistance can directly or indirectly alter the physiology of the host. Immune cells are known to aid in initial detection and eradication of a number of pathogens. However, upregulation of immune cells can have other profound effects. Firstly, immune cells are metabolically expensive cells; macrophages have been shown to turn over Adenosine TriPhosphate (ATP) 10 times per minute (Newsholme & Newsholme, 1989). Cells with a high metabolism require a great deal of resources. In particular, immune cells utilise very high levels of glucose and glutamine (Lochmiller & Deerenberg, 2000, Newsholme & Newsholme, 1989). Trade-off theory predicts that an individual has a limited set of resources, from which all physiological functions must be fuelled (Maynard Smith, 1974). Heightened resistance mechanisms, for example increased numbers of nutrient-demanding immune cells, may therefore take away resources from other critical functioning and lead to trade-offs in other life history traits (see 1.4.3). A certain set of resources are required for somatic maintenance and repair. Increased investment in the immune system may take away resources from processes that buffer age-related damage, such as DNA repair and expression of antioxidants, leading to an accumulation of damaged soma and decreased longevity ('disposable theory of ageing' proposed by Kirkwood, 1990).

Immune cells utilise metabolism to generate antimicrobial oxygen radicals upon detection of invading pathogens. Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2) are created as by-products of all eukaryotic cellular metabolism, primarily from the energy generating respiratory chain reactions in the mitochondria (Harman, 1955, Kirkwood & Kowald, 2012). In response to infection immune cells can upregulate their metabolism to produce higher levels of these substances, which are toxic to the invading pathogen (see 1.4.5). However, the build-up of high concentrations of intracellular ROS can lead to oxidative stress within the cell

and consequently multiple noxious effects, such as protein, lipid and DNA damage, which can lead to an ageing phenotype (see 1.4.4).

Very little information exists as to whether immunocompetence, and the corresponding immune mechanisms required for resistance, can affect longevity. Reisen and Hahn (2007) used a 'natural experiment' (see 1.6.3) to show that two closely related bird species in the same family, the brown-headed cowbird (*Molothrus ater*) and the New World blackbirds, showed significantly different resistance to infection against West Nile virus. Hahn and Smith (2011) later went on to examine the relationship between resistance and lifespan in these birds using data from the North American bird banding database. They found that lifespan in the Red-winged blackbird and the Brewer's blackbird, both with low resistance against parasitism, showed increased longevity in comparison to the more resistant Brown-headed cowbird, although the processes involved are yet to be elucidated.

The aim of this chapter was to investigate the age-related changes in immune investment in *Drosophila melanogaster* populations selected for increased resistance to larval parasitism by the parasitoid wasp *Asobara tabida* (see 2.2.1). These *Drosophila* have already been shown to display increased circulating immune cells (hemocytes) compared to their control counterparts, during the time when the animal is most at risk; larval phase (Kraaijeveld *et al.*, 2001; Chapter 2). Firstly, we wanted to establish whether any differences in immune investment persist throughout life by ascertaining differences in hemocyte number between the selected *Drosophila* and their unselected, more susceptible counterpart, as the flies age. Secondly, it was necessary to investigate whether resistance, and the corresponding increase in immune cell number at larval phase, might come at a detriment to the individual. In order to do this, overall ROS production was measured using the intracellular probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is a redox sensitive probe thought to be an indicator of general cell metabolism due to its ability to detect metabolic byproducts created specifically within the cell, in particular H_2O_2 (LeBel *et al.*, 1992, Owusu-Ansah *et al.*, 2008). Non-fluorescent DCFH-DA can diffuse freely across cell membranes. Once in the cell the probe is hydrolysed by non-specific cellular esterases to the product dichlorofluorescin (DCFH), which due to its hydrophobic nature can no longer cross cell membranes and becomes trapped within the cell. Upon contact with H_2O_2 DCFH is oxidised to the

fluorescent dichlorofluorescein (DCF), which fluoresces directly proportionally to the amount of intracellular H_2O_2 produced (LeBel *et al.*, 1992). The majority of H_2O_2 is produced by the mitochondria as a by-product of cellular metabolism and thus generally provides an accurate read-out of the rate at which a cell is respiring (Harman, 1955, Kirkwood & Kowald, 2012). Finally, longevity was measured to determine whether resistance, or the underlying mechanisms involved, lead to trade-offs in senescence.

3.2 Materials and Methods

3.2.1 Drosophila stocks

All flies were cultured and kept as described previously in detail (Chapter 2). Briefly, for each experiment four control lines (C1-4) and four lines selected for parasitoid resistance (S1-4) were used, each line was cultured in exactly the same manner. *Drosophila* were kept on yeast-sugar medium containing 0.5% propanoic acid at 20°C with a 12:12 hour light:dark cycle. For all experiments performed, flies were allowed to lay for 24 hours at 25°C. Two days after egg laying larval density was moderated; per line a maximum of 100-200 larvae were transferred into each fresh medium bottle and allowed to develop. After eclosion flies were collected into fresh medium bottles. For the cell metabolism assay, 24 hours prior to the assay flies (larvae and adults) were transferred into new vials containing cellulose filters (Millipore) saturated with 5% sucrose solution.

3.2.2 Hemocyte counts

In order to establish whether ROS production was correlated to hemocyte number it was first necessary to quantify hemocyte numbers throughout life. Therefore, in these experiments larvae and adults aged 1, 4 and 8 weeks of age were assayed. Hemocyte counts were performed as described previously (Chapter 2) for larval and adult *Drosophila*, using some of the data obtained and presented in Chapter 2 for the larval and young adult (1 week old) counts. These two experiments were run simultaneously. In total, 800 3rd instar larvae were used (10 larvae bled per sample, with 10 samples quantified per biological replicate (C/S1-4) and 2880 adults were used to quantify

hemocyte numbers with age (6 adults bled per sample; repeated 10 times for each sex, at each age (1, 4 and 8 weeks) for each biological replicate (C/S1-4)).

3.2.3 Cell metabolism/ROS production with age

Flies were lightly anaesthetised (larvae: on ice, adults: with CO_2) prior to homogenisation. For each sample, twenty flies were homogenised using mini-pestles in eppendorfs containing 300 µl phosphate buffered saline (PBS) with Tween (PBS-T; PBS containing 0.1% Tween 20, Sigma Aldrich, UK). Samples were then centrifuged at 10,000 rpm for 1 minute to remove fly debris. A stock solution of the probe was made by dissolving DCFH-DA (Sigma Aldrich, UK) in dimethyl sulfoxide (DMSO, Sigma Aldrich, UK) to a final concentration of 20 mM and stored at -20°C until use. On each experimental day, a 300 µM working solution was prepared by defrosting the stock and diluting with distilled water (dH_2O); all probe solutions were stored in foil due to the photosensitive nature of DCFH. In this experiment black 96-well microplates (Griener Bio) were used to reduce cross-well fluorescence of the probe. To each well 20 µl of 300 µM DCFH-DA stock was added to 100 µl of the sample supernatant, to achieve a final concentration of 50 µM. Plates were placed in a FLUOStar OPTIMA spectrophotometer (BMG Labtech) at room temperature. The excitation filter was set at 485 nm and the emission filter was set at 612 nm. The fluorescence from each well was captured using top optics every 15 minutes for 90 minutes and then again at 255 minutes. Samples were shaken for 10 seconds prior to each reading to redistribute the sample within the wells. In total 480 larvae 3rd instar larvae were used (20 bled per sample, with 3 samples quantified per biological replicate (C/S1-4) and 2880 adults were used to test changes in overall cell metabolism with increasing age (20 adults bled per sample; repeated 3 times for each sex, at each age (1, 4 and 8 weeks) for each biological replicate (C/S1-4)).

In addition to the whole body samples a larval hemolymph sample was also analysed to determine whether cell metabolism in the hemolymph, where the hemocytes are located, showed any difference to the whole body analyses. For larval hemolymph collection, twenty 3rd instar larvae were cut along their dorsal side with fine spring scissors (FST) and bled into a pool of 120 µl of PBS-T and mixed to ensure even distribution. Bleeding was performed on a glass plate cooled by ice to minimise larval movement. To each

well 20 µl of 300 µM DCFH-DA stock was added to 100 µl of hemolymph solution and the plates were read spectrophotometrically as described above. A total of 480 larvae were used to test cell metabolism in larval hemolymph (20 larvae bled per sample, with 3 samples analysed per biological replicate (C/S1-4)). This protocol was not performed in the adult flies due to the amount of hemolymph required for the sampling process.

3.2.4 Longevity assays

Eclosing *Drosophila* were collected over a period of 24 hours and left for a further 48 hours to ensure copulation. *Drosophila* were then sexed and separated into vials containing standard yeast-sugar medium plus the anti-fungal agent propanoic acid. A maximum density of 10 flies per vial ensured minimal effects of population density, a factor which has been shown to alter lifespan (Graves & Mueller, 1993, Grandison *et al.*, 2009). *Drosophila* were kept in incubators regulating temperature at 20^{o}C and a 12 hour day-night cycle (light: 8am – 8pm). Vials were replaced every 2-3 days, death counts were made as frequently as possible (approximately 5-7 times per week). A total of 800 adult *Drosophila* were used to determine the impact of immunity on lifespan (50 adults of each sex for each biological replicate (C/S1-4)).

3.2.5 Statistical analyses

The statistical software Minitab was used to perform all analysis of variance (ANOVA). *Hemocyte counts:* A multivariate analysis (three-way ANOVA) was performed in the 1 and 4 week old adults to look for age- or sex- specific interactions (8 week old data had to be excluded due to lack of male data). Individual analyses were also carried out on each sex at each time point to ensure that sex-differences did not mask any immune-related effects and also to allow the inclusion of the 8 week old female data for analysis. A one-way ANOVA (Minitab) with line set as a factor was first performed against the average cell count across the 10 samples taken to eliminate differences between the lines. Subsequently, a one-way ANOVA (Minitab) was performed on the mean line residuals of the line means against immune-background. In adults, this analysis was performed for each sex and age. However, 8 week old male data was excluded from analysis due to insufficient males to sample from at this stage.

Cell metabolism/ROS production: Percentage increase in fluorescence was calculated using the formula [$(Ft_{255} – Ft_0)/Ft_0$ x 100], modified from the formula used by Wang and Joseph (1999). This method takes into account the change in fluorescence between time = 0 and time = 255 minutes, at which point any differences between well fluorescence were apparent. This method also provides a standardised fluorescence output from which to compare the samples, whilst also normalising the fluorescence intensities by initial values. To test the efficacy of this method regression analysis was performed on percentage fluorescence against fly number, using samples containing 0, 10, 15 and 20 control 1 week old flies. An R^2 value of 0.969 was achieved highlighting the validity of the assay and analysis combination. To measure the effect of resistance against ROS production, a multivariate analysis (three-way ANOVA) was performed in the 1 and 4 week old adults to look for age- or sex- specific interactions (8 week old data had to be excluded due to lack of male data). Again age and sex were found to have a significant effect on ROS production, therefore the effect of resistance was measured in each sex for each time point using separate one-way ANOVAs. For each analysis the percentage increase data was run in a one-way ANOVA fit against line as a factor. A second one-way ANOVA was then run on the line mean residuals against immunity to establish the effects of resistance.

Kaplan-Meier estimators were used to estimate the survival function from the collected data. From these estimates, log rank statistics were performed to establish any differences between survival distributions (SPSS). This type of statistical analysis takes into account the overall shape of the survival curve and allows inclusion of any subjects that survived past the duration of observation. It is, however, restricted in the fact that it only allows comparison of distributions with one factor (e.g. immunity, line or sex). To account for this, the Cox proportional hazard (PH) model was also used to determine the hazard at each time point. Hazard (or risk) and survival are two sides of the same coin and the degree of risk will directly inversely correlate to survival. Using the semi-parametric Cox PH model incorporating the parametric Wald test (SPSS), multivariate survival analyses could be performed on the data to incorporate all factors (immunity, line and sex).

In addition to the non-parametric analyses performed on the survival curves an additional parametric method could be utilised to look for differences in average and maximal lifespan (age of the oldest fly) a one-way ANOVA was performed against line to eliminate line-specific effects. Then a one-way ANOVA was performed on the line mean residuals against immune background.

3.3 Results

3.3.1 Hemocyte numbers

Selected L3 larvae possess 20% more hemocytes than the control larvae (shown in 4 out of 4 of the line pairs) (
Figure 3.1; $F_{1,6} = 52.69$, $P < 0.001$). The aim of this analysis was also to establish how this relationship changed over time; therefore hemocyte numbers were also sampled in adult *Drosophila* aged 1, 4 and 8 weeks (the latter performed in females due to a lack of males of this age). Overall, in 1 and 4 week old flies there was a decrease in hemocyte number with age ($F_{1,6} = 32.10$, $P = 0.0013$) and no effect of sex ($F_{1,6} = 3.18$, $P = 0.125$). There was no overall effect of immunity on hemocyte number ($F_{1,6} = 0.52$, $P = 0.498$). The were no two way interactions between age and sex, age and immunity or immunity and sex ($F_{1,6} = 0.24$, $P = 0.642$; $F_{1,6} = 0.79$, $P = 0.408$ and $F_{1,6} = 2.84$, $P = 0.143$ respectively) or three-way interactions between age, immunity and sex ($F_{1,6} = 0.72$, $P = 0.429$) on hemocyte number.

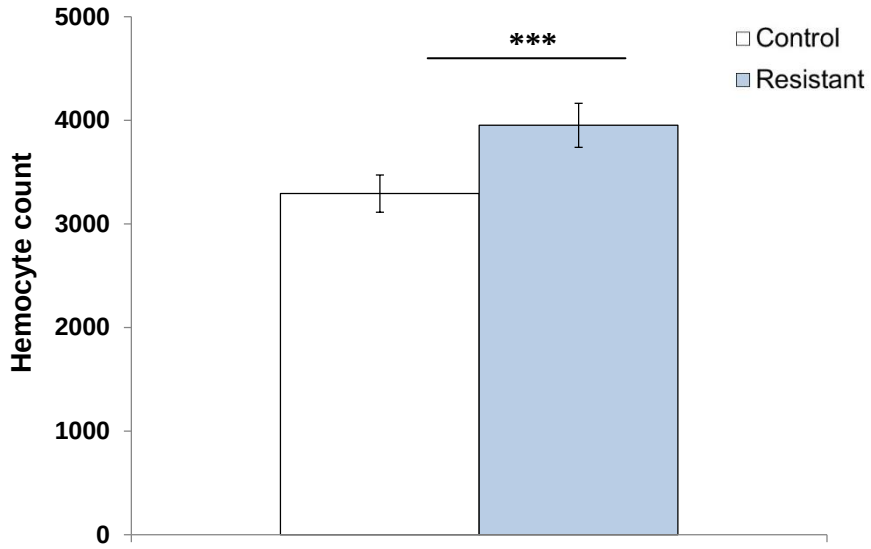


Figure 3.1 Larval hemocyte counts

*Hemocyte counts taken from larvae of control (white) and selection (blue) backgrounds (*** P < 0.001) (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 10 larvae were bled per sample, with 10 samples quantified for each biological replicate (C/S1-4).*

When analysed separately using multiple one-way ANOVAs the following results were found: In young females, there was no difference in hemocyte number between the control and selected lines at 1 week ($F_{1,6} = 4.01$, P = 0.092), 4 weeks ($F_{1,6} = 1.87$, P = 0.22) or 8 weeks ($F_{1,6} = 0.51$, P = 0.501) of age (Figure 3.2a). A comparison of the residual differences in the male flies also revealed no difference in the number of hemocytes between the control and selected lines at 1 week old ($F_{1,6} = 1.80$, P = 0.228) or 4 weeks old ($F_{1,6} = 5.06$, P = 0.065) (Figure 3.2b).

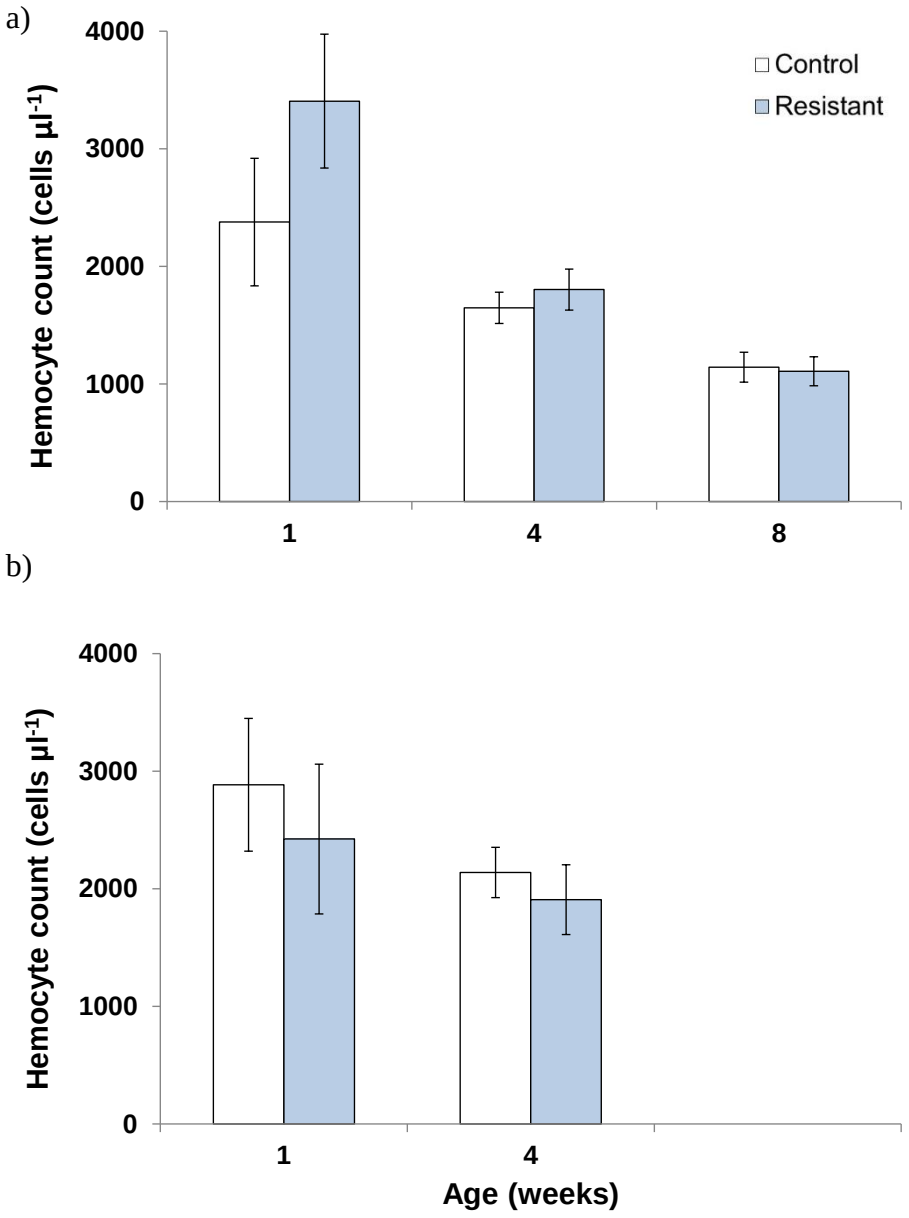


Figure 3.2 Adult hemocyte counts

Hemocyte count per µl of hemolymph in control (white) and resistant (purple) female (a) and male (b) adult Drosophila at 1, 4 and 8 weeks of age (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 6 adults were bled per sample, with 10 samples quantified for each sex, for each biological replicate (C/S1-4).

3.3.2 Measuring cell metabolism/H_2O_2 output

Cell metabolism was measured using the conversion rate of the probe DCFH to the fluorescent DCF. No difference was found in the cell metabolism between the control and selection larvae, from whole body or from hemolymph only analysis ((Figure 3.3a; $F_{1,6} = 0.33$, $P = 0.585$) and (Figure 3.3b; $F_{1,6} = 0.36$, $P = 0.569$) respectively).

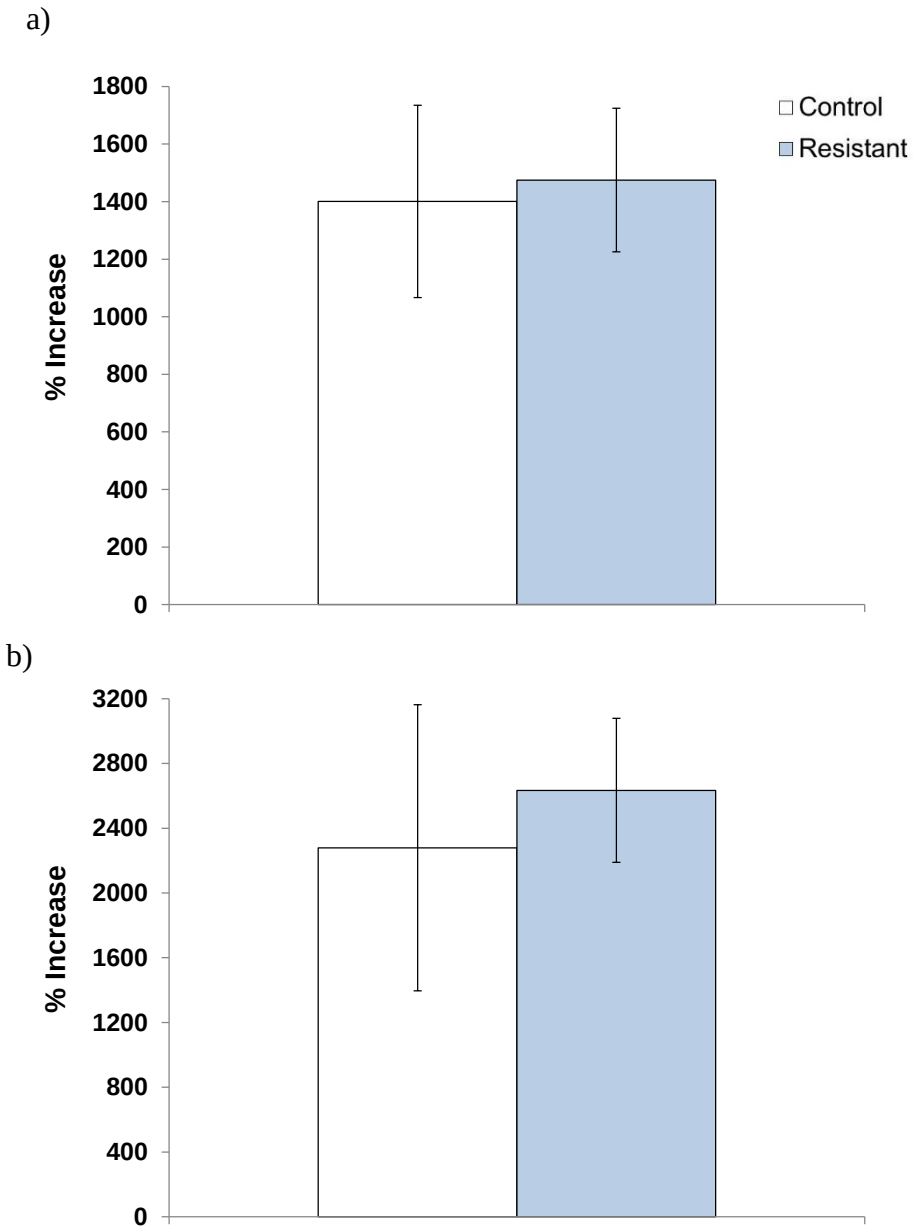


Figure 3.3 Larval cell metabolism/ROS output

Percentage increase in the level of DCF fluorescence in the control (white) and resistant (blue) larvae; (a) whole body and (b) hemolymph only (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: For both a) and b) 20 larvae were bled per sample, with 3 samples quantified for each biological replicate (C/S1-4).

Overall, ROS production in the adult flies increased between 1 and 4 weeks of age (Figure 3.4; $F_{1,6} = 13.95$, $P = 0.0097$). Males revealed higher levels of H_2O_2 overall than female *Drosophila* ($F_{1,6} = 59.44$, $P = 0.0002$). There was no overall effect of immunity ($F_{1,6} = 0.2$, $P = 0.670$). There was no two-way interactions between age and sex, age and immunity or immunity and sex ($F_{1,6} = 4.71$, $P = 0.073$, $F_{1,6} = 5.07$, $P = 0.0653$ and $F_{1,6} = 1.13$, $P = 0.329$ respectively). There was no three-way interaction between immunity, age and sex ($F_{1,6} < 0.01$, $P = 0.949$).

When separate analyses were performed in the adults for each sex at each age the following results were found: In 1 week old females, there was a higher percentage increase in fluorescence measuring cell metabolism in the selected than in the control flies (shown in 4 out of 4 line pairs, see Appendix Table 6a for individual line means) ($F_{1,6} = 18.64$, $P = 0.005$). This difference was not found in older females at 4 and 8 weeks old (($F_{1,6} = 0.31$, $P = 0.598$) and ($F_{1,6} < 0.01$, $P = 0.992$) respectively) (Figure 3.4a). Again in males, analysis could not be performed in male 8 week old flies due to too few remaining in some of the lines. There was no effect of immunity on the increase in DCF fluorescence in 1 week old ($F_{1,6} = 1.68$, $P = 0.242$) or 4 week old males flies ($F_{1,6} = 5.30$, $P = 0.061$) (Figure 3.4b).

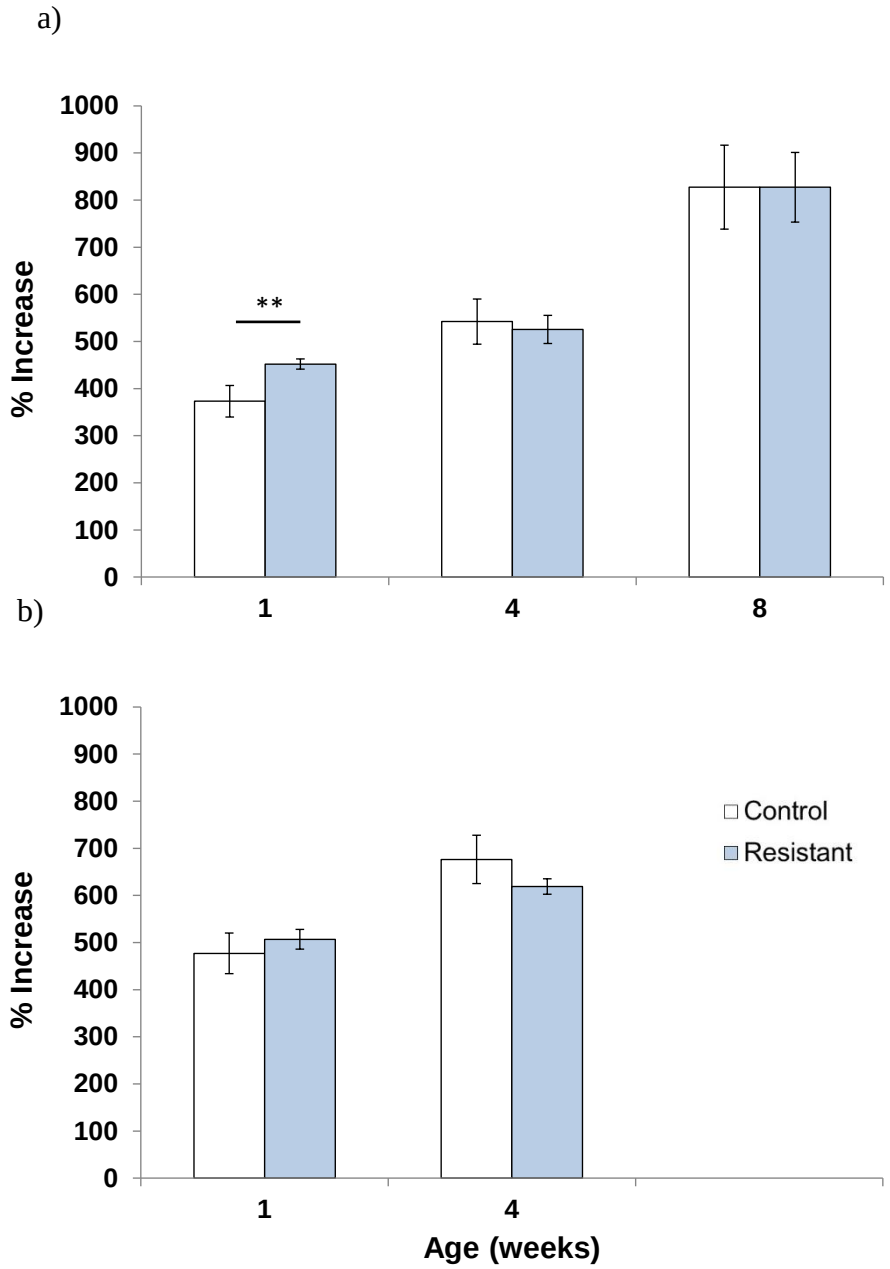


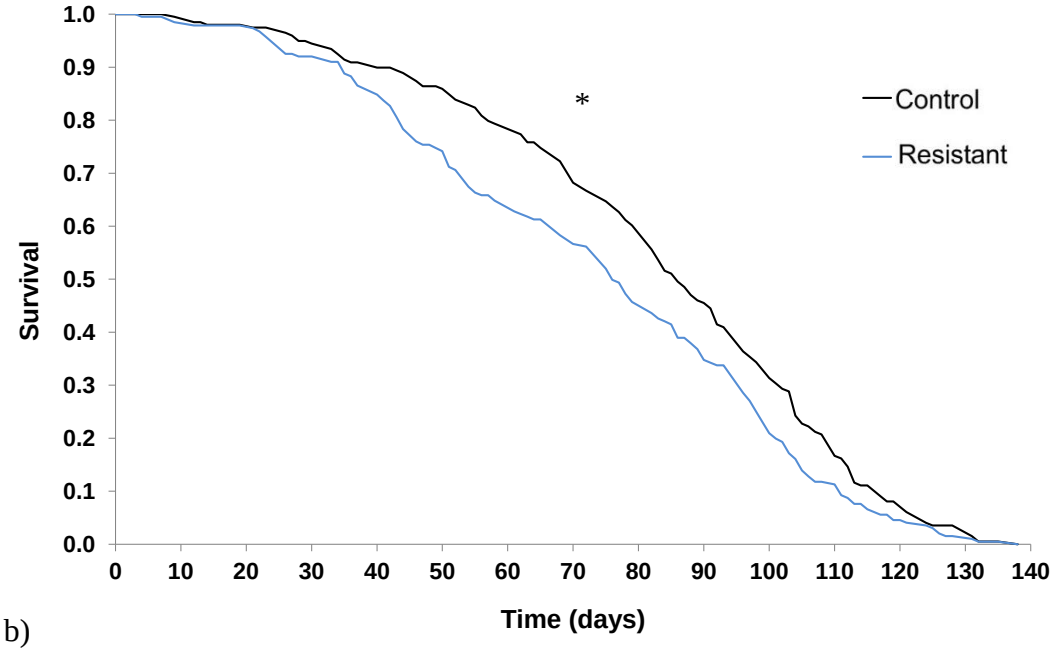
Figure 3.4 Adult cell metabolism/ROS output

*Percentage increase in DCF Fluorescence of female (a) and male (b) Drosophila in control (white) and selection (blue) lines at 1, 4 and 8 weeks of age (** $P < 0.01$) (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 20 adults were bled per sample, with 3 samples quantified for each sex, for each biological replicate (C/S1-4).*

3.3.3 Adult longevity

Resistance mechanisms are known to require resources and as such can lead to trade-offs in other functions. To test the effects of resistance against larval parasitism analysis of longevity was performed of males and females from each of the control and selection lines. Resistant females showed decreased longevity (Chi-Square = 5.066, df = 1, P = 0.024) compared to control females (Figure 3.5a) shown by an increased likelihood of death between approximately 20 and 55 days of age (Figure 3.5b). The average survival time (± standard error) of the selection lines was found to be 11.1% less than in the control flies at 72.6 days (± 6.6) and 81.7 (± 4.3) respectively (reduced longevity shown in 3 out of 4 of the line pairs, see Appendix Table 5 for individual line means) ($F_{1,6}$ = 12.41, P = 0.012). However, there was no difference in the maximal lifespan ($F_{1,6}$ = 1.73, P = 0.236), which were 129 (± 3.0) and 123 (± 6.3) for the control and resistant flies respectively.

a)



b)

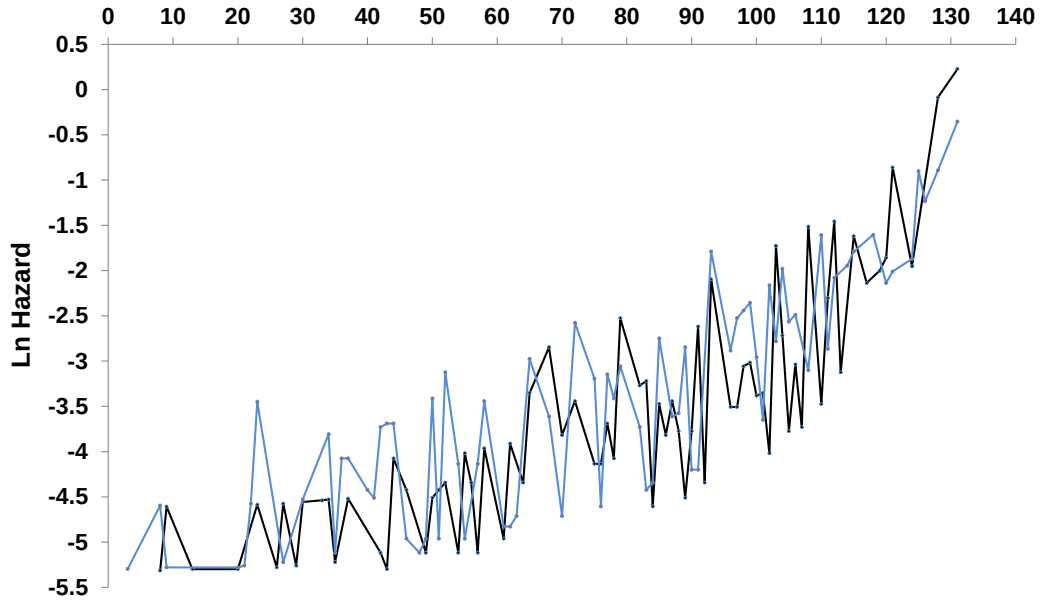


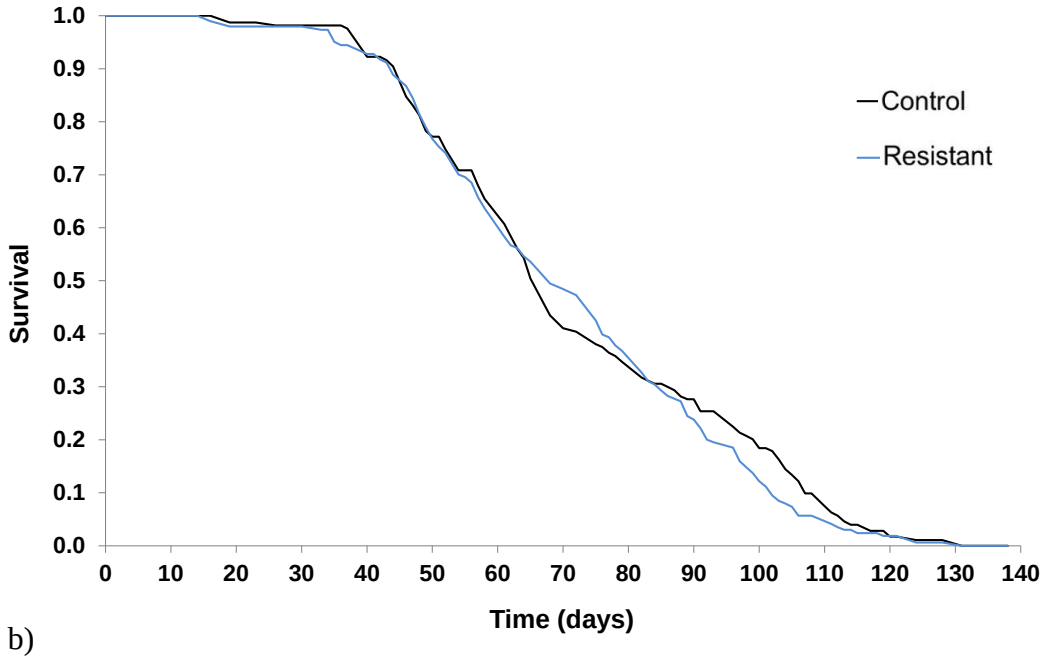
Figure 3.5 Female longevity

a) proportion surviving of Drosophila from a background of low immunity to parasitism (control) against a population selected for the increased potential to encapsulate upon parasitism (selection). b) Natural log of the hazard exerted on the population at each point. 50 adults were analysed for each of the 8 biological replicates. ($P < 0.05$).*

Males showed no difference in longevity between control and selection flies (Chi-Square = 1.086, df = 1, P = 0.297) (Figure 3.6). The average and maximal survival time of the flies also did not differ (($F_{1, 6}$ = 0.04, P = 0.855) and ($F_{1, 6}$ = 1.73, P = 0.236) respectively).

From the Cox PH analyses, there was no difference between the replicates for each parameter (Wald, P = 0.615), suggesting a repeatable response in survival distribution in the different populations. Overall, this revealed an effect of immune background (Wald, P = 0.02), although only distinguishable in the females (from log rank analysis) and a generic sex-related effect (Wald, P < 0.001), whereby males exhibited a 20% reduction in longevity compared to female (medians: 65 and 82 days respectively).

a)



b)

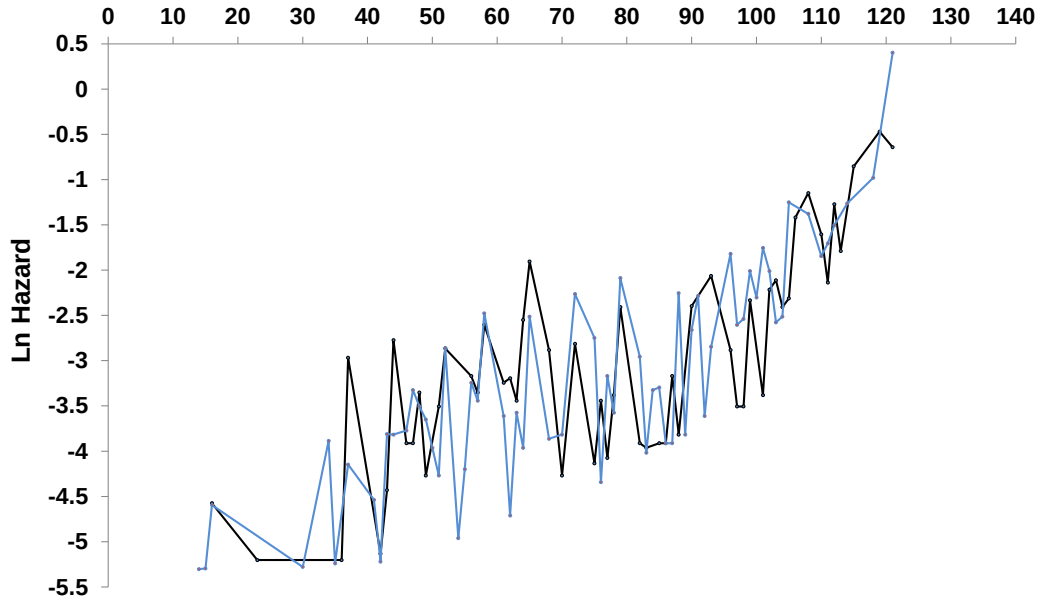


Figure 3.6 Male longevity

a) proportion surviving of Drosophila from a background of low immunity to parasitism (control) against a population selected for the increased potential to encapsulate upon parasitism (resistant). b) Natural log of the hazard exerted on the population at each time point. 50 adults were analysed for each of the 8 biological replicates.

3.4 Discussion

As previously shown by Kraaijeveld and colleagues (2001), *Drosophila* larvae selected for resistance against the larval parasitoid *Asobara tabida* contained higher numbers of circulating immune cells (plasmatocytes) than their control counterpart. This part of the study confirmed this difference in L3 stage larvae and went further to see how this difference changes with age; immune cell counts were performed with adult flies aged 1, 4 and 8 weeks old. Biochemical assays were also implemented to establish the overall cellular metabolism/intracellular ROS production in the control and resistant *Drosophila*. ROS detection assays were performed at two levels in the larvae; hemolymph only and whole body. This was done to assess whether resistance, or the upregulation of circulating immune cell number, may have physiological effects that can be detected at a localised (hemolymph only) and a systemic level (whole body). Due to experimental limitations, only whole body preparations were used to analyse cellular metabolism/intracellular ROS production in the adults. Finally, a longevity assay was performed to determine whether immune resistance led to a trade-off in lifespan due to the costs involved in the upregulation of immune-related processes.

3.4.1 Cell count and metabolism

The first aim of this chapter was to determine how hemocyte numbers change over time with respect to the insect's immune-background and to investigate whether increased resistance leads to changes in cell metabolism, measured by intracellular hydrogen peroxide production. As shown previously, resistant larvae possess increased immune cell numbers with respect to the control larvae (Kraaijeveld *et al.*, 2001, Chapter 2). This finding did not correlate to changes in cell metabolism/intracellular ROS production as detected by the oxidant-sensing probe DCFH-DA, which revealed no difference between the resistant and control flies at a localised (hemolymph) or systemic (whole body) level.

No immune-related difference was detected in adult hemocyte number at any age, indicating a decreased requirement for immunity in the selected lines in later life, past metamorphosis. The life- history theory predicts that immunity should reflect the current pathological risks and thus maintain a level of plasticity during the lifetime of an

individual (Ardia *et al.*, 2011). A loss of immune cell disparity between the control and resistant lines indicates that *Drosophila* immunity can show a degree of plasticity, through the loss of larval specific immune advantages.

One week old adult females revealed a significant increase in overall intracellular H_2O_2 production, as detected by the increased conversion of the probe DCFH to its fluorescent form DCF. H_2O_2 is a necessary metabolite and is vital for many cell functions, such as redox homeostasis, cellular transduction transcription factor activation, protein disulphide formation, cell proliferation and apoptosis (Dröge, 2002, Rojkind *et al.*, 2002, Wittmann *et al.*, 2012). However, at high concentrations H_2O_2 can become toxic, leading to protein, lipid and DNA damage, which can lead to disrupted cell function and even cell death by apoptosis or necrosis (Driessens *et al.*, 2009).

One possible explanation for an increase in H_2O_2 in the young selected females may be that the immune cells present in the female could exist at a more metabolically active state. Surveillance cells in selected individuals may be activated or 'primed' in order to achieve the successful encapsulation responses seen in the resistant lines, therefore each immune cell in these flies may have increased metabolism and thus a higher ROS output. In agreement to this, Nappi and Vass (1998) found that activated *Drosophila* immune cells fluoresce more intensely upon addition of DCFH-DA. Gender –specific differences may arise if selected females possess greater numbers of these 'active' cells than selected males. Azad at el. (2011) removed hemocytes from adult *Drosophila* by cell specific ablation and showed that much lower levels of hypoxia-induced ROSs were produced than in the control flies; these too were quantified by DCF fluorescence from whole fly extracts, indicating that differences in hemocytes alone are capable of producing detectable variation. Immune cells are known to produce high quantities of ROS as part of their defensive strategy (Nappi & Vass, 1998), as such one might propose that even a small difference in activity may be sufficient to cause the observed differences in the organism overall.

One key question raised by these results is: if selected adult females show enhanced H_2O_2 output due to an increased number of activated hemocytes, then why do we not see this phenomenon in the selected larvae? One possible explanation is that larvae were not separated by sex and therefore any gender-specific effects in the females may be

masked by the males. Another potential theory is that the variation in ROS output in the adult females may relate to differences in the activity of antioxidant enzymes between the control and resistant *Drosophila*. Wertheim *et al*. (2011) found that in our model system, selected larvae expressed higher levels of the glutathione metabolism and antioxidant enzymes; catalase, phospholipid hydroperoxide glutathione peroxidase (PHGPx) and a number of glutathione S-transferases (GSTs). Catalase is one of the key enzymes responsible for the breakdown of toxic hydrogen peroxide into its safe components water and oxygen and is thus essential in detoxification and protection from ROS-induced damage. Peroxidases too can eliminate H_2O_2 and its potential toxicity; this is done with a higher efficiency than catalase due to reportedly lower Km (Mishra & Imlay, 2012). PHGPx in particular, is important in the removal of lipid hydroperoxides (LOOHs) in cell membranes. LOOHs are produced though the action of ROS on polyunsaturated fatty acids and can lead to the degradation of the cell membrane (Blair, 2001). A lack of PHGPx leads to embryonic lethality, emphasizing the importance of its role in the cell (Yant *et al.*, 2003). Glutathione has been named as the central line of defence against oxidative damage by ROS in general (Marí *et al.*, 2009). It works by scavenging hydroxyl radicals and works through the actions of GST (Chen *et al.*, 2002).

During infection, immune cells become activated, increasing metabolic output as shown by increased ROS production (Nappi & Vass, 1998). Thus *Drosophila* larvae will be selected for their ability to buffer these potentially toxic ROS increases in addition to the resistance mechanisms necessary to eliminate the pathogen. This is shown by the upregulation of antioxidants in the resistant lines. Wertheim *et al*'s (2011) study looked solely at changes in larval gene expression and did not extend further to look at expression levels in adult flies. One hypothesis, therefore, is that resource allocation toward additional antioxidant enzyme production may only exist in the larvae, when differences in hemocyte number exist and at a time when immune cell activity may be required from an increased risk of parasitism. Expression of these antioxidant enzymes is expensive and production is generally limited to requirement (Felton & Summers, 1995, Korsloot *et al.*, 2010). Therefore, it is unlikely that this additional upregulation of antioxidants is continued into adulthood, by which time immune cell disparity no longer exists. In the females, we may actually be witnessing a trade-off in the allocation of

antioxidants between the larval and adult stages. Further work would be necessary to test this hypothesis (see section 6.7.3).

Overall, there was a decline in the number of hemocytes in both female and male *Drosophila* with increasing age, correlated with an increase in the level of overall intracellular ROS production. ROS release from mitochondria increases with age, which is accompanied by decreased antioxidant efficacy and the accumulation of oxidatively altered macromolecules (Sasaki *et al.*, 2010). Studies have also shown that as cells age, mitochondria are increasingly damaged by intracellular ROS and consequently generate higher levels of H_2O_2 (Sohal & Sohal, 1991). As the H_2O_2 output in all cells within the fly would be increasingly upregulated with age this would vastly overshadow any differences in fluorescence brought about by the age-associated decrease in hemocyte numbers. Immune mechanisms such as hemocyte number can decline with age (Mackenzie *et al.*, 2011), most likely due to a lack of selection toward immune maintenance at this stage in life. In addition, if hemocytes do generate increasingly greater levels of ROS, maintaining these cells may prove more detrimental to the individual than the risk posed by susceptibility to infection. Also, due to the high production of ROS within these cells as a result of lifelong respiratory burst, there may be a consequential increased risk of cell death thus decreasing the number of hemocytes found in circulation.

3.4.2 Lifespan

Another key aim in this chapter was to establish whether resistance to larval parasitism led to alterations in the longevity of the flies due to the increased costs involved in maintaining immunity. We found a marked decrease in survival of resistant females but no immune-related trade-off in male lifespan. It is important to note that the lifespan of an animal does not relate directly to the speed at which an animal is ageing. Instead, in the absence of risk from extrinsic risk (e.g. starvation, infection, predation, accidental death) the lifespan of an animal is determined by two parameters, as described by the Gompertz equation: the intrinsic frailty/vulnerability and the actuarial ageing rate (Kowald, 2002, Sas *et al.*, 2012). The former parameter relates to the level of protection that an animal carries against failure; the mechanisms in place that buffer damage. The

latter refers to the rate at which this protection declines (Kowald, 2002). Therefore, in our model system there are two possibilities, either the selected females start out with a lower rate of protection than their control counterparts, or the rate of deterioration of these mechanisms occurs at a faster rate. As intracellular ROS production was found to be higher in selected females than in the control females at an early age (1 week old, Figure 3.4a), this could infer that the reduction in lifespan is due to a difference in the initial level of protection between the flies. In agreement to this, the flies exhibited the greatest disparity in likelihood of death relatively early on (between 20 and 55 days of age, Figure 3.5b).

Additional immune resource investment in early juvenile life could therefore result in a reduction in initial investment in mechanisms that protect against damage in the adult female, for example, a reduced investment in the mechanisms that counteract excess production of ROS. Excessive production of ROS in hemocytes has already been shown to induce accelerated mortality in invertebrates (Azad *et al.*, 2011, Xian *et al.*, 2010). Research into trade-offs resulting from resource allocation at juvenile phase is relatively new and as such not much is known about the possible costs involved. McNamara *et al*. (2013) showed that investment in immune upregulation in the juvenile cotton bollworms (*Helicoverpa armigera*) lead to reduced allocation into adult male sperm production, an effect that alters depending on the time of challenge. This finding shows that costs of resource allocation in youth can be carried through development and into the adult phenotype.

In general, females are reported to possess greater immunological competence in comparison to males (Klein *et al.*, 1997, Zuk & McKean, 1996). Greater investment in immunity may cause a larger detriment to the animal thus leading to gender-specific effects of immunity on lifespan. Additional costs required in the adult female may also bring about this phenomenon, including metabolic expense of egg production, which may exaggerate the effect of an augmented immune response. In *Drosophila*, females also have an additional immune challenge caused during copulation, when the male will cause copulatory wounding and the transfer of seminal fluid to the female (Short *et al.*, 2012). Seminal fluid proteins and physical damage can cause a multitude of physiological responses including stimulation of the release of antimicrobial peptides, activating the Toll and Imd pathways (see 1.7.2), further increasing the immunological

pressures faced by the females. Short *et al.* (2012) showed that mated females show a decline in their ability to mount a humoral immune response following transferal of male seminal fluid proteins. Using virgin flies would have reduced this factor from the experiment. However, the use of virgin flies does not give an accurate representation of natural forces exerted on the organism. In reality, an unmated *Drosophila* would seldom be found and thus has little biological relevance.

One other variable to consider is the effect that the males may be having on female longevity. As females were mated with their immune counterparts (e.g. control female with control and selection female with selection male) it is not possible to rule out the possibility that longevity may be controlled wholly or in part by the males themselves. For example, selection males have shown differential mating rituals, it is therefore possible that the males may be more vigorous and thus injure the females to a greater extent, leading to increased mortality. Alternatively, the sperm fluid from selected males may be more dangerous to the females. In order to know for sure it would be necessary to repeat the experiment but keep the males that they are exposed to constant, such as, both control and selection females mate with control males prior to commencing the longevity experiment.

Overall, male survival was very high for the first 40 days before dropping off at a much greater rate, in contrast to female longevity where a steadier decline was noted from around 20 days of age. In males, reproduction occurs from an early age and continues very rapidly until death. It is beneficial for risk of death at this early phase to be low, to ensure sufficient copulations to pass on genes to the next generation. As sperm is cheap to produce a male can very quickly sire a high number of offspring. Offspring production for a female is a much more expensive, time-consuming process. It is therefore necessary for the female to survive for a greater time period in order to generate similar levels of offspring as the males, a phenomenon known as Bateman's principle (Bateman, 1948). This is reflected in the survival curves, where females on average survived longer than males. A similar effect is seen in humans, where lifespan is higher in women. It is thought that increased survival may be attributed to greater immune competence in females; a feature exhibited across the animal kingdom (Kraaijeveld *et al.*, 2008, Grossman, 1989).

3.4.3 Summary

In summary, Chapter 3 shows that female *Drosophila* selected for resistance to parasitism have an overall greater H_2O_2 output at 1 week of age. A possible explanation for this result is that increased metabolism in their hemocytes or other cells leads to increased ROS output in these individuals, which may not be correctly balanced with antioxidant enzyme presence in the adult fly. This might be more extreme in the females due to their increased need to maintain a competent immune system for maximal offspring production (Rolff, 2002). We also show decreased longevity in the females, which may in part link to our initial findings, with increased ROS output in the young female leading to greater cell damage and thus a faster decline in lifespan. The gender-specific effect of resistance on longevity may come down to increased resource allocation towards reproduction and immunity, leaving fewer resources to counteract age-related decline. Overall, the findings reveal that increased investment in immunity to parasitism has costs in the adult female, shown by early increased ROS output and reduced lifespan.

4 No effect of oxidative stress induced in *Drosophila melanogaster* selected for resistance to larval parasitism

4.1 Introduction

Immune competent individuals are better able to resist infection and therefore more likely to survive in a pathogen-rich environment. However, in order to resist infection an individual requires the upregulation of a number of immune-related mechanisms and pathways. Immune cells produce high levels of oxidants, which can lead to oxidative stress and eventually oxidative damage within the cell. *Drosophila melanogaster* selected for increased resistance to parasitism have been shown to display heightened numbers of immune cells in the larvae (Chapter 3). It was therefore hypothesised that immune-resistant *Drosophila* should possess altered levels of protective mechanisms against reactive oxygen species (ROS) and their associated damage due to the increased likelihood of immune-associated oxidative stress in these individuals. The aim of this chapter was to determine whether resistant *Drosophila* showed differing potentials to upregulate protective mechanisms in response to artificially increased ROS levels. In order to assess differences in these mechanisms, ROS production and survival were measured following consumption of the free-radical inducing agent paraquat.

Immune cells are highly metabolic, which means that they require a great deal of resources and have a high output of metabolic by-products, collectively termed as ROS. Approximately 2-5% of oxygen that is consumed through mitochondrial respiration produces ROS (Floyd & Hensley, 2002, Hensley *et al.*, 1998). In addition, as part of an immune response, phagocytes can increase their oxygen uptake by 10-20 times that of the resting levels (Costantini & Møller, 2009), generating significantly higher ROS outputs and oxidative stress within the cell (see 1.4.5). A range of antioxidant defence systems are in place to neutralise ROS or their actions (Sies, 1997). Metal-ion dependent enzymes such as superoxide dismutase, catalase and glutathione peroxidase as well as non-enzymatic antioxidants vitamins A, C and E, glutathione and β-carotene act to scavenge ROS (Finkel & Holbrook, 2000, Peng *et al.*, 2000). However, pathological, chemical and environmental stress factors such as hypoxia, UV stress, pollutants and toxins can increase the amount of ROS production within a cell

(Blokhina *et al.*, 2003), shifting the balance in favour of the oxidants and thus increasing the amount of damage caused to the proteins and lipids.

Mechanisms are in place to protect against stress-induced damage; mainly involving the actions of heat shock proteins (HSP) and mitogen-activated protein kinase (MAPK) signalling pathways. HSPs function to limit the aggregation of mis-folded proteins and to promote protein refolding, buffering the level of damage within the cell. Donaire and colleagues (2005) showed that in immortalised rat neuroblast cells upregulated HSP 27 and 70 proteins provided protection against toxin-induced cell death. Activation of MAPK pathways, such as p38 and c-Jun N-terminal kinase (JNK), regulates many aspects involved in the stress response including growth arrest, immunity and cell death (Chuang *et al.*, 2000, Stronach & Perrimon, 1999). In response to environmental stress, MAPK-signalling is thought to provide some early protection from oxidative stress, favouring survival of the cell. JNK signalling has been shown to act by antagonising the IIS pathway, which leads to the nuclear localisation of the transcription factor FoxO and the subsequent upregulation of stress defence genes, such as those relating to expression of small heat shock proteins (Wang *et al.*, 2005). Wang and colleagues (2003) found that flies with downregulated JNK signalling were more sensitive to toxin-induced oxidative stress; shown by decreased survival. However, this protective effect can be reversed with more heightened or prolonged stress-signalling leading to increased rates of apoptotic cell death (Wang *et al.*, 2003).

JNK and p38 signalling also play a vital role in immunity. These pathways respond to increases in stress-inducing inflammatory molecules and act to heighten the inflammatory response (Huang *et al.*, 2009, Stronach & Perrimon, 1999); inhibition of these pathways can be used to treat a range of inflammatory conditions (Kumar *et al.*, 2003, Manning & Davis, 2003). Wertheim *et al.* (2011) showed reduced levels of *Drosophila* JNK (Basket) and p38 in *Drosophila* selected for resistance against parasitism. This finding might suggest that heightened signalling induced by immune-related oxidative stress might be detrimental to the fly and therefore initial downregulation may act in a protective manner to avoid excessive apoptosis.

The aim of this chapter was to investigate whether *Drosophila melanogaster* selected for increased resistance to larval parasitism showed differing abilities to resist acute oxidative stress and the associated damage induced by the toxin paraquat. Upon consumption, paraquat becomes reduced to form paraquat radicals, which then react with oxygen to form the ROS superoxide (Bus & Gibson, 1984). Due to its propensity to produce heightened intracellular ROS, paraquat has been widely used in laboratory work to induce oxidative stress, including in many *Drosophila melanogaster* model systems (Arking *et al.*, 1991, Rzezniczak *et al.*, 2011). This study implements the oxidant-sensing probe dichlorofluorescein diacetate (DCFH-DA), as previously described in Chapter 3, to detect intracellular ROS levels in the flies. Azad and colleagues (2011) used DCFH-DA to detect ROS production in *Drosophila* larvae. They found that upregulation of HSP70 in *Drosophila* immune cells (hemocytes) was sufficient to reduce overall ROS levels following hypoxic shock, which suggests that hemocytes may by a key source of ROS production.

In the *Drosophila* model system, there are heightened numbers of immune cells in the larvae (Chapter 3). If hemocytes are major generators of stress-induced ROS, then we might expect to see a much greater ROS upregulation in the resistant flies upon stress challenge as measured by increased conversion of the DCFH probe into the fluorescent DCF. Chapter 3 showed greater levels of endogenous ROS in the young adult female; perhaps implying a trade-off in antioxidants or other protective mechanisms in these flies. If this were the case then we should see an even greater production of ROS in these individuals and reduced survival under stress. Azad *et al.* (2011) also found that increased HSP70 expression in *Drosophila* hemocytes improved the survival of flies under severe hypoxia as well as from oxidant stress brought about by paraquat consumption. Therefore any differences in survival found in our *Drosophila* model following paraquat treatment could indicate differential expression of protective mechanisms within the flies. Development times in larvae were also measured to determine whether the upregulated stress-signalling pathways show differing effects on toxin-induced growth arrest during development.

4.2 Materials & Methods

4.2.1 Drosophila stocks

All flies were cultured and kept as described previously in detail (Chapter 2). Briefly, for each experiment four control lines (C1-4) and four lines selected for parasitoid resistance (S1-4) were used, each line was cultured in exactly the same manner. *Drosophila* were kept on yeast-sugar medium containing 0.5% propanoic acid at 20°C with a 12:12 hour light:dark cycle. For all experiments performed, flies were allowed to lay for 24 hours at 25°C. For development to adulthood, two days after egg laying larval density was moderated; per line a maximum of 100-200 larvae were transferred into each fresh medium bottle and allowed to develop. After eclosion flies were collected into fresh medium bottles.

4.2.2 Cell metabolism/ROS production following acute paraquat treatment

Cell metabolism was measured using the DCFH-DA probe as previously described in Chapter 3. This experiment were performed in parallel to those ran in chapter 3, with the additional treatment of the stress-inducing toxin, paraquat, to assess the effects of acute oxidative stress in addition to endogenous levels expressed within the fly. Twenty-four hours prior to testing, the larvae and adults were transferred into new vials. In both age groups, half of the flies were placed into vials containing cellulose filters saturated with 5% sucrose solution, whilst the other half were placed into vials containing filters saturated with 5% sucrose with 20 mM paraquat (methyl viologen (1,1´-dimethyl-4,4′-bipyridinium dichloride)). In total, 960 3rd instar larvae were used (20 larvae bled per sample, with 3 samples analysed per treatment (with/without paraquat) for each biological replicate (C/S1-4)) and 1920 1 week old adults were used (20 adults bled per sample; repeated 3 times for each sex, for each treatment group (with/without paraquat) for each biological replicate (C/S1-4)) to determine the impact of immunity on intracellular ROS production.

4.2.3 Oxidative stress tests in larvae

The aim of this assay was to measure the survival of larvae fed on a control or paraquat-treated diet. In order to achieve this, a novel set-up was required. Due to the photo- and heat-sensitive nature of paraquat, the medium must be changed on a daily basis to ensure consistent toxin efficacy. Larvae require a yeast-based diet. This is generally given in a solid yeast, agar-containing medium. During consumption the larvae burrow themselves into the medium, making it very difficult to locate them up until the wandering phase (approximately 6 hours prior to pupation), where they emerge from the medium to seek a dry area more suitable for metamorphosis. For this reason it would not be feasible to extract the larvae on a daily basis in order to perform a medium change, particularly without causing disruption to the larvae. It was therefore decided to use a liquid medium which the larvae could feed on and that could also be washed and refreshed each day.

To ensure that larvae were not washed away a mesh sieve was needed, course enough to allow entry and exit of the yeast-medium but fine enough to contain the 2 day old larvae (2^{nd} instar). The fabric used in the sieve had a mesh opening of 70 µm (Sefar). This was initially tested to see how the medium could filter through. Agar was added to the medium in attempt to create a base for the larvae to rest upon, however, when adding agar at levels as low as 1.25 % the medium still clogged up the mesh once cooled. It was therefore decided that the medium should not include agar.

The first batch of tests ran without paraquat were unsuccessful as the larvae all died prior to pupation, in addition to a degree of bacterial/fungal growth in the sieves even though washed through once a day. Propanoic acid was added to future experimental set-ups to prevent this; however, the larvae were still not surviving. It was thought that the larvae may have been drowning within the liquid medium. In attempt to solve this problem the sieves were set at a tilt in the feeding tray to allow the larvae access out of the liquid (Figure 4.1). This design worked well in further larval trials.

Figure 4.1 Experimental set-up: larval sieves

Finished design of larval sieves. a) photograph of apparatus excluding lid. b) diagram with lid, showing tilt of sieve in its base during experiment to allow movement of larvae out of the liquid medium.

In the final protocol 16 sieves were used: 8 with bases containing 4 ml of liquid medium supplemented with 0.5% propanoic acid and 8 with bases containing 4 ml of liquid medium supplemented with propanoic acid and 5 mM paraquat. Two day old larvae were washed through a mesh and separated under a stereo microscope. Thirty 2nd instar larvae of each line (C & S1-4) were placed into separate labelled sieves. Sieves were set at an angle in the base to allow the larvae to enter and exit the liquid medium. Each day the sieves were lifted from their base and rinsed gently. At the same time the medium in the base was replaced with fresh liquid medium, with or without supplementation of 5 mM paraquat. The sieves were then returned to the base as described previously. Each day the numbers of pupae were counted. Pupae were transferred from the sieves into medium-containing vials in which they could emerge aiding adult collection. The date of emergence was noted and the flies were lightly CO_2 anaesthetised in order to ascertain gender under a light microscope to look for sex bias. In total, 480 larvae were used (30 larvae per treatment (with/without paraquat) for each biological replicate (C/S1-4)).

4.2.4 Oxidative stress tests in adult flies

Five day-old mated flies were separated by sex under light CO_2 anaesthesia. Flies were then transferred into empty bottles containing approximately 100 individuals. *Drosophila* were left for one hour to allow flies to awaken and encourage consumption of new diet upon transfer. *Drosophila* were then transferred into bottles with an agar base covered with a stack of 3 cellulose filters (Millipore); previously cut to fit, divided into six sections with a permanent marker and saturated in a 5% sucrose solution, with or without the supplementation of 10 mM paraquat (Figure 4.2).

Figure 4.2 Experimental set up: bottles for adults
Set-up within each bottle with paraquat saturated filter. Each filter was segmented to ease counting of dead flies.

Preliminary tests were performed to determine the appropriate paraquat concentration for this assay. For the adult flies we needed to ensure the survival spanned across a period of days rather than hours; survival less than this would reveal a more rapidly declining survival curve with less data points from which to assess patterns between lines. Preliminary experiments were performed with female flies from both control and selection lines aged 8-10 days old. The concentration found to be most suitable for the adult flies was 10 mM, which revealed a smooth decline in survival spanning across approximately 11 days, while stronger concentrations lead to a more rapid death in a

much shorter period, with over 90% dead after just 2 days with paraquat concentrations of 20 mM and above.

Survival counts were recorded at regular time intervals spaced evenly across each day (approximately 9 times per day for the first 10 days and then approximately 5 times per day following the death of all the paraquat treated flies). At each time point the number of dead flies was counted, aided by the markings on the filters, and recorded. Each day any surviving flies were transferred into new bottles with fresh filters saturated with sucrose or sucrose/paraquat solution, prepared as described above. In total 3200 adult *Drosophila* were used (100 flies of each sex, for each treatment (with/without paraquat) for each biological replicate (C/S1-4)).

4.2.5 Statistical analyses

Cell metabolism/ROS output: Analysis was performed as described previously in Chapter 3. Briefly, percentage increase in fluorescence was calculated using the formula $[(Ft_{255} – Ft_0)/Ft_0 \times 100]$, modified from the formula used by Wang and Joseph (1999). The percentage increase data was then run in a one-way ANOVA fit against line as a factor (Minitab). A second one-way ANOVA was then run on the line mean residuals against immunity.

Larval survival, development time and proportion of each sex to eclose: Proportions for larvae reaching pupation, pupae to eclose and each sex to eclose were arcsine square-root transformed to reach normality. Development time data was used without transformation. A one-way ANOVA was fit against each of these response values with line set as a factor. A two-way ANOVA was then performed on the line mean residuals fitted against the factors: immune-background, treatment and the interaction between immunity-background and treatment.

Adult survival: Cox proportional-hazards regression analysis was conducted on the survival data (SPSS). This allowed the comparison of the survival curves using a multivariate model. Firstly, a one-way ANOVA with line set as a factor was performed to eliminate differences between the lines (Minitab). A Cox model was fit to the line

mean residual data, followed by a forward stepwise model incorporating treatment, immune-background and the interaction between treatment and immune-background. The SPSS software tested all factors for entry into the model based on the significance of the score statistic. The order of entry was then according to the smallest significance value. Once in the model the variables were retested for the significance of the Wald statistic. Those with high probabilities were then removed until the model until only variables resulting in a value less than 0.1 (the POUT value).

4.3 Results

4.3.1 Cell metabolism/ROS production

Paraquat treatment caused an increase in the amount of cell metabolism ($F_{1,6}$ = 14.57, P = 0.0088). This effect was not dependent on immune-background ($F_{1,6}$ = 2.85, P = 0.142) and there was no interaction between immunity and treatment ($F_{1,6}$ = 0.73, P = 0.426) (Figure 4.3).

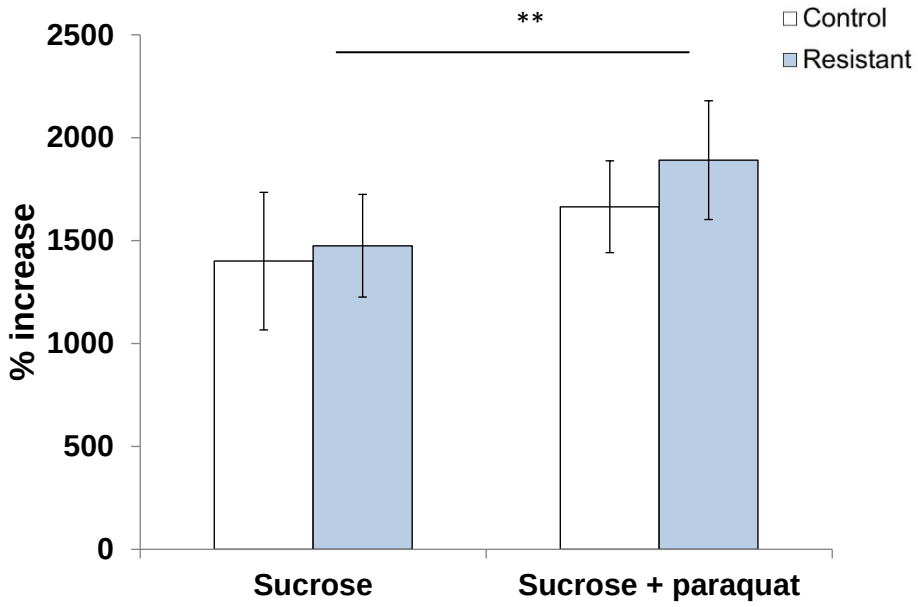


Figure 4.3 ROS output: larvae

Increase in fluorescence after four hours exposure with DCFH. Showing control (white) and resistant (blue) Drosophila larvae fed on sucrose and after treatment with the toxin paraquat (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 20 larvae were bled per sample, with 3 samples analysed per treatment (with/without paraquat) for each biological replicate (C/S1-4).

In the adult females, consumption of paraquat caused an increase in overall cell metabolism ($F_{1,6} = 28.44$, $P = 0.0018$). As shown in previously in chapter 3, overall ROS output was higher in resistant individuals than in control flies (shown in 4 out of 4 of the untreated line pairs and 3 out of 4 of the paraquat treated line pairs, see Appendix Table 6 for individual line means) ($F_{1,6} = 13.27$, $P = 0.0108$). There was no interaction between treatment and immune background ($F_{1,6} = 2.71$, $P = 0.151$) (Figure 4.4).

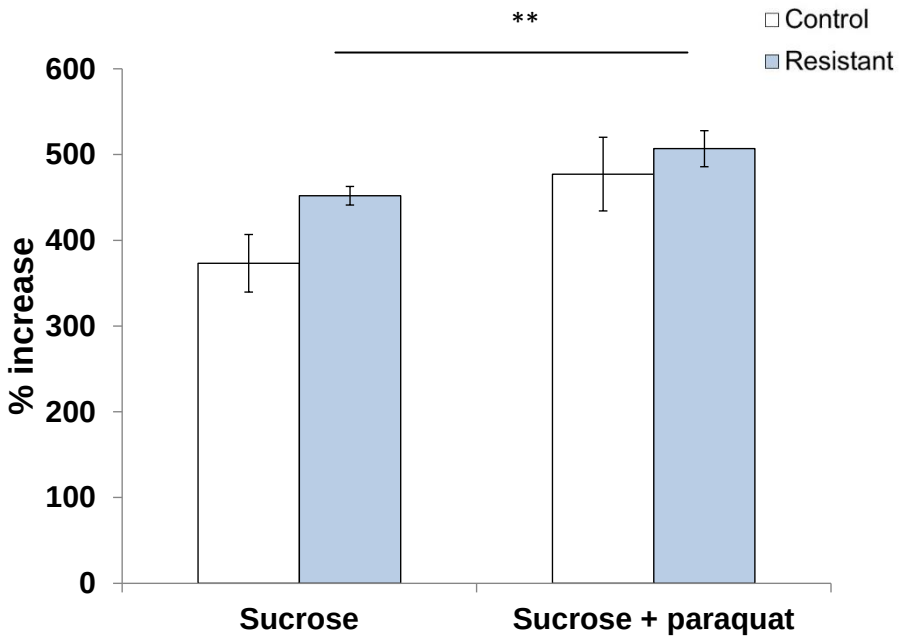


Figure 4.4 ROS output: adult females

Increase in fluorescence after four hours exposure with DCFH. Showing control (white) and resistant (blue) adult Drosophila fed on sucrose and after treatment with the toxin paraquat (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 20 adults were bled per sample, with 3 samples analysed per treatment (with/without paraquat) for each biological replicate (C/S1-4).

In the adult males, paraquat treatment produced a reduced cell metabolism ($F_{1,6}$ = 23.62, P = 0.0028). There was no effect of immune background ($F_{1,6}$ = 0.78, P = 0.411) and there was no interaction between treatment and immune background ($F_{1,6}$ = 0.45, P = 0.527) (Figure 4.5).

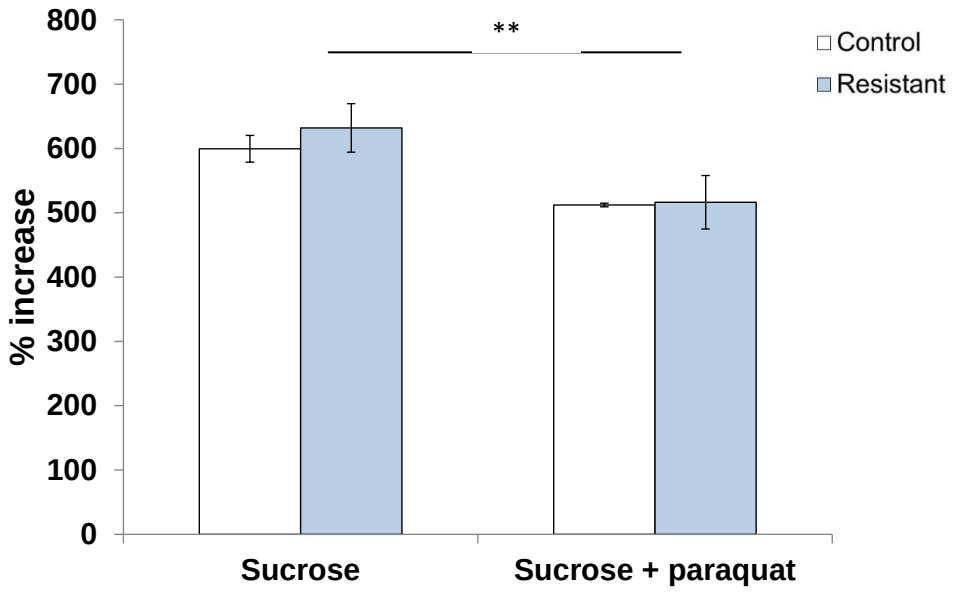


Figure 4.5 ROS output: adult males

Increase in fluorescence after four hours exposure with DCFH. Showing control (white) and resistant (blue) adult Drosophila fed on sucrose and after treatment with the toxin paraquat (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 20 adults were bled per sample, with 3 samples analysed per treatment (with/without paraquat) for each biological replicate (C/S1-4).

4.3.2 Oxidative stress tests in larvae

Paraquat treatment resulted in fewer *Drosophila* larvae reaching pupation stage ($F_{1,6}$ = 18.80, P = 0.0049), with a 16 % reduction in survival following paraquat treatment (Figure 4.6a). There was no effect in of immunity on survival to pupation ($F_{1,6}$ = 5.08, P = 0.0651) or interaction between immune-background and paraquat treatment ($F_{1,6}$ = 1.05, P = 0.345) (Figure 4.6a).

Treatment with paraquat lead to a reduced number of emergence in adult flies ($F_{1,6}$ = 23.21, P = 0.0029). However, this did not affect the proportion of *Drosophila* that

eclosed following successful pupation; showing little effect of treatment during this stage when paraquat was no longer consumed ($F_{1,6}$ = 2.67, *P* = 0.153) (Figure 4.6b).

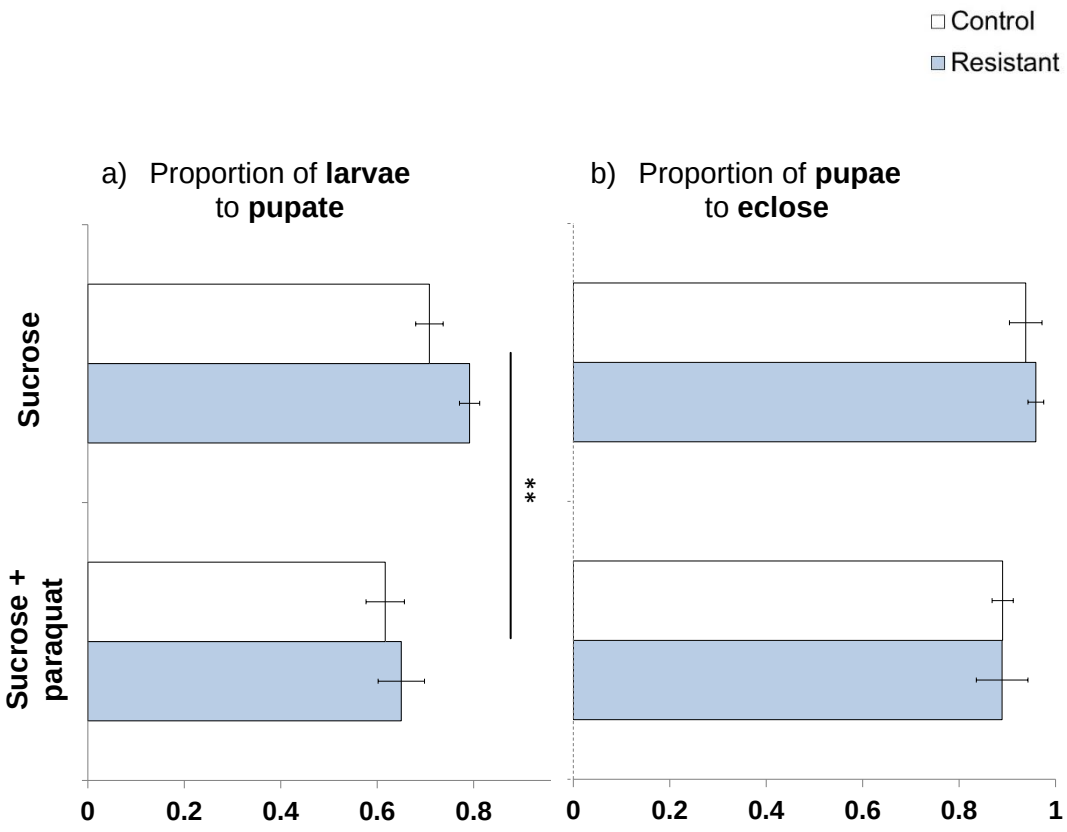


Figure 4.6 Larval survival

a) Proportion of larvae to reach pupation in control medium (no treatment) and medium supplemented with 5 mM paraquat. b) proportion of those larvae successfully reaching pupation to eclose. Comparisons made between individuals from a background with low immunity to parasitism (white) and individuals selected for increased resistance to parasitism (blue) (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: a) 30 larvae were analysed per treatment (with/without paraquat) for each biological replicate (C/S1-4) b) 20.8 (± 0.7) larvae were analysed per treatment for each biological replicate (C/S1-4).

There was an increase in the time taken to reach pupation and eclosion under oxidative stress (($F_{1,6}$ = 13.52, *P* = 0.0104) and ($F_{1,6}$ = 11.64, *P* = 0.0143) respectively); with a

delay in eclosion of almost one day (Figure 4.7). However, there was no effect of immunity (($F_{1,6}$ = 0.35, *P* = 0.576) and ($F_{1,6}$ = 0.09, *P* = 0.774)) or interaction between immune-background and treatment (($F_{1,6}$ = 0.55, *P* = 0.486) and ($F_{1,6}$ = 1.49, *P* = 0.268)) on development time to pupation or eclosion respectively (Figure 4.7).

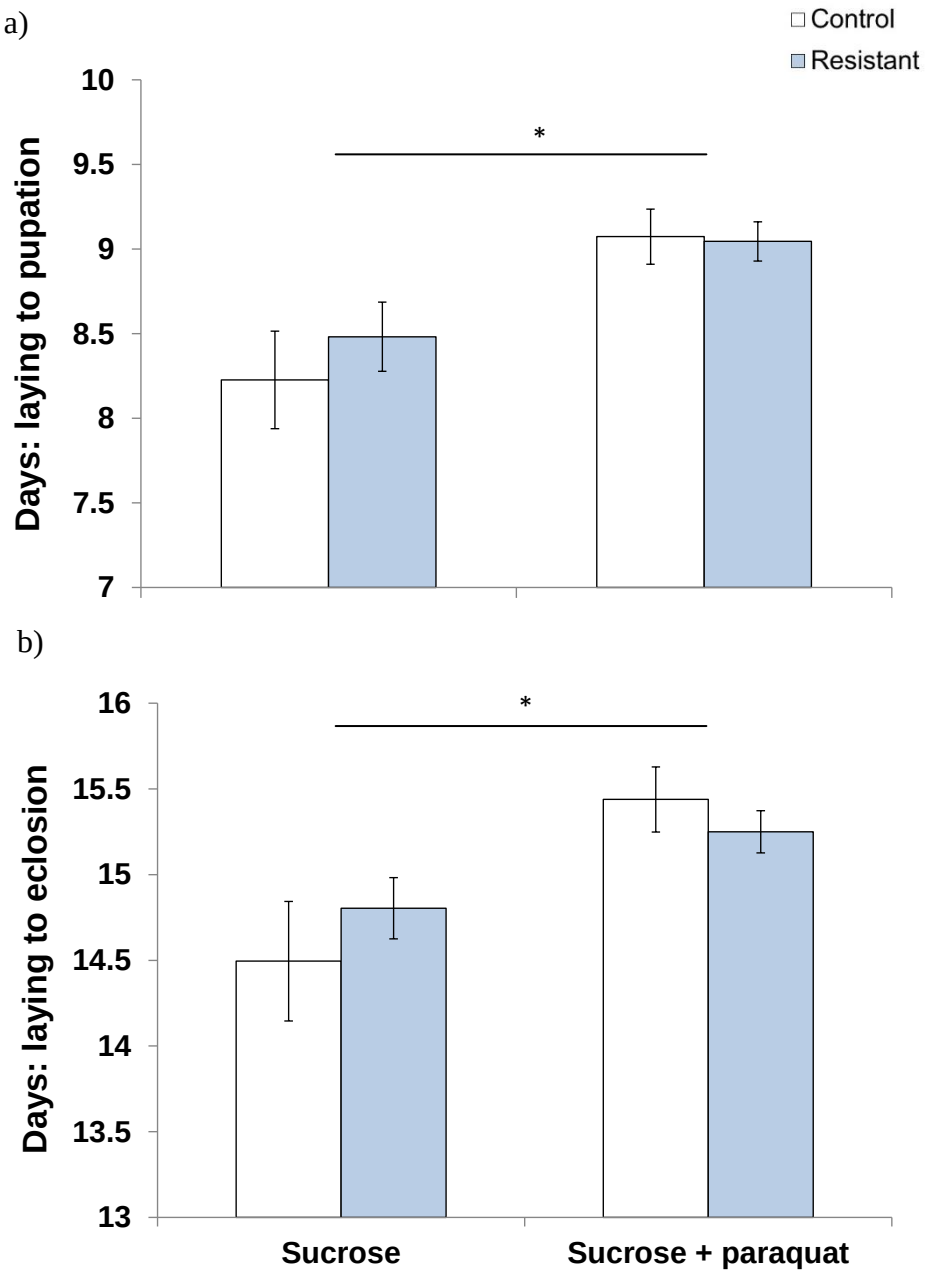


Figure 4.7 Larval development times

Average number of days from egg laying to (a) pupation and (b) eclosion in surviving control (white) and selection (blue) flies fed on a yeast-medium diet or a yeast-medium diet supplemented with paraquat (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: a) 30 larvae were analysed per treatment (with/without paraquat) for each biological replicate (C/S1-4).

The sex ratio of emerging flies was not dependent on the treatment given ($F_{1,6}$ = 1.94, *P* = 0.213) or immune background ($F_{1,6}$ = 1.00, *P* = 356); approximately two-fifths of flies eclosing were female irrespectively.

4.3.3 Oxidative stress tests in adult flies

Females died more quickly when fed on 10 mM paraquat than when fed on a sucrose (control) diet (Wald = 723.441, df = 1, P < 0.001) (Figure 4.8). However, the effect on lifespan was independent of immunity background (score = 2.627, df = 1, P = 0.105), with and without ingestion of paraquat (score = 2.683, df = 1, P = 0.101) (Figure 4.8).

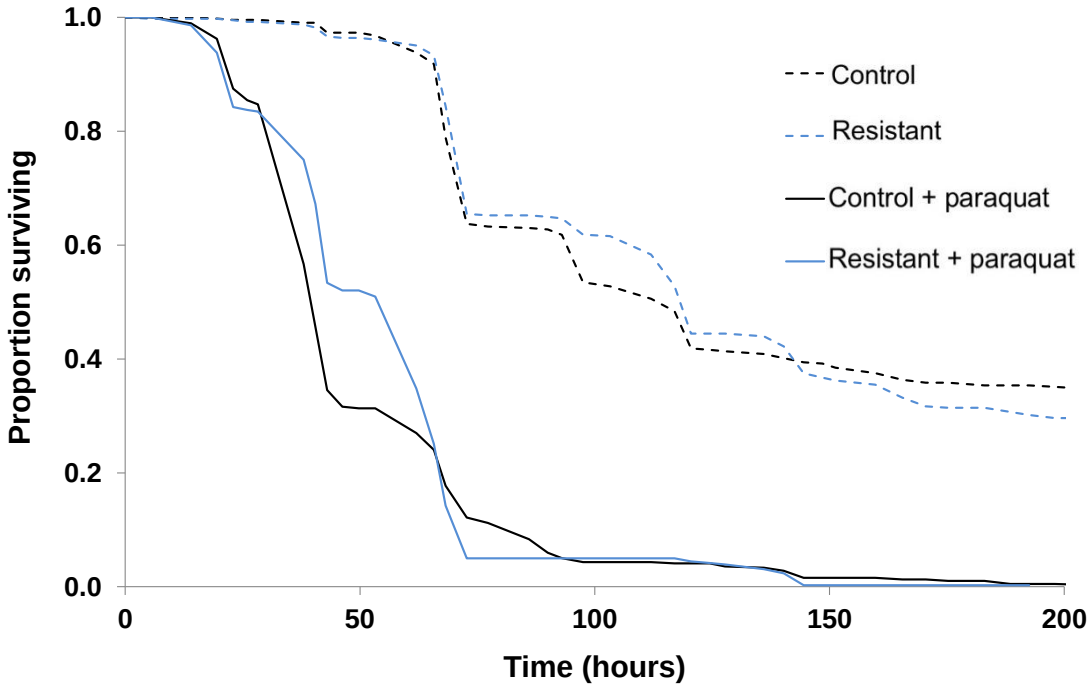


Figure 4.8 Adult female survival

Proportion of Drosophila females surviving over time when administered a control 5% sucrose diet or a 5% sucrose diet supplemented with 10 mM paraquat, known to induce oxidative stress. 100 flies were analysed for each of the 8 biological replicates (C/S1-4).

Male survival decreased when fed a paraquat-supplemented diet (Cox regression, Wald = 63.490, df = 1, $P < 0.001$) (Figure 4.9). Lifespan was found to be independent of immunity (score = 0.547, df = 1, P = 0.460), regardless of paraquat treatment (score = 0.033, df = 1, P = 0.857) (Figure 4.9).

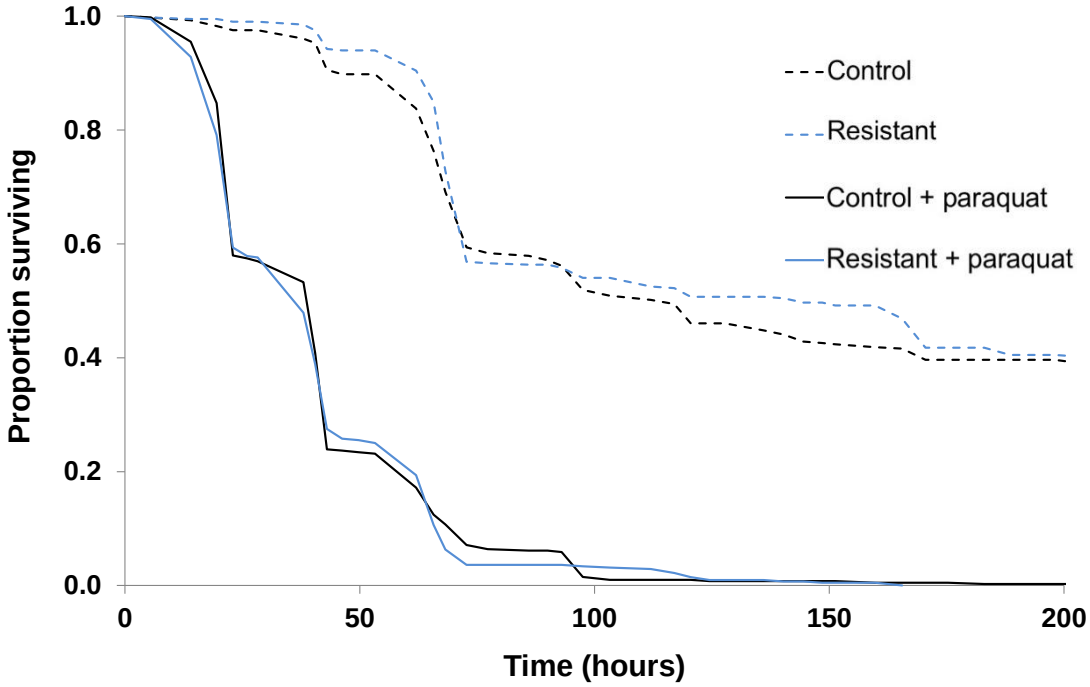


Figure 4.9 Adult male survival

Proportion of Drosophila males surviving over time when administered a control 5% sucrose diet or a 5% sucrose diet supplemented with 10 mM paraquat, known to induce oxidative stress. 100 flies were analysed for each of the 8 biological replicates (C/S1-4).

4.4 Discussion

The aim of this chapter was to determine whether immune-resistance, and the associated upregulation of immune mechanisms, alters an individual's ability to resist oxidative stress. The oxidant-sensing probe DCFH-DA was used to determine whether the control and immune resistant *Drosophila* contain differing levels of intracellular ROS following consumption of paraquat. In addition, development and survival assays were implemented to establish whether the flies differ in their ability to buffer growth arrest and the lethal damages induced by toxin consumption (respectively).

4.4.1 Cell metabolism/ROS output

Larvae: In the larvae, consumption of paraquat led to an increase in the total amount of intracellular ROS. Paraquat interferes with the intracellular electron transfer systems leading to the production of heightened levels of ROS, including superoxide and H_2O_2; the latter of which can be detected by the probe DCFH-DA. In this study we used the probe to determine whether *Drosophila* with increased resistance against parasitism showed varying abilities to counteract potentially damaging increases in ROS found within their cells. Resistant *Drosophila* larvae have greater numbers of circulating immune cells (Kraaijeveld *et al.*, 2001; Chapter 3); these cells can produce high levels of ROS due to their ability to increase their metabolic rate during infection (Newsholme & Newsholme, 1989). However, Wertheim (2011) previously showed that immune resistant *Drosophila* larvae express higher levels of anti-oxidants; catalase, phospholipid hydroperoxide glutathione peroxidise (PHGPx) and a number of glutathione S-transferases. Expression of protective mechanisms within the cell, such as antioxidants and heat shock proteins, can act to buffer this rise in ROS (Azad *et al.*, 2011, Sies, 1997). In this study, there was no difference in the overall amount of ROS found within the control and resistant *Drosophila* following paraquat consumption. This may infer that the increase in antioxidant expression is sufficient to counteract the excess production of ROS from the additional immune cells. The fact that there was no greater protection given by these antioxidants might suggest that they only provide adequate protection to buffer the effects of the immune cells and do not provide general protection in all cells; giving the resistant *Drosophila* and the control flies equal protection against stress.

Adults: There was no effect of immune-background on the level of ROS produced by the adult *Drosophila* in either sex, mirroring the result found in the larvae. In the adult the difference in immune cell number between the control and resistant *Drosophila* is no longer apparent (Chapter 3). In this study selected females showed an overall higher level of intracellular ROS. However, there was no effect of paraquat or interaction between the treatment of paraquat and the fly's ability to resist parasitism. This suggests that, although the females produce naturally higher endogenous ROS levels, further upregulation of intracellular ROS is not dependent on the fly's immune-background. This might imply that the processes in place to buffer increases in ROS generation, such as antioxidants and heat shock proteins, are upregulated equally in the cells of the control and resistant adults in response to oxidative stress.

In the males, we see a reduction in the level of overall intracellular ROS production with consumption of paraquat. This was an unusual observation as paraquat generally induces the production of increased intracellular free-radicals (Bus & Gibson, 1984). One potential explanation is that males are more susceptible to paraquat induced free-radical damage than the females and the larvae; either due to a lower expression of antioxidant activity or expression of the protective mechanisms that would generally buffer ROS damage, such as HSPs and other chaperone proteins (Söti & Csermely, 2002, Papp *et al.*, 2003).

Reactive free radicals can bind to electrons in DNA, lipids and proteins, including those found in the phospholipid bilayer (Pamplona, 2008). By binding to these electrons, the cell membrane integrity becomes disrupted and the membrane begins to breakdown, rupturing and killing the cell (Pamplona, 2008). Therefore, if the concentration of paraquat fed to the flies is great enough, and cells begin to rupture and die, there may be a reduction in the level of overall cell metabolism and consequent intracellular ROS production. Presumably if this were to occur, then this would mark the beginnings of functional decline and subsequent death of the fly as cells continue to break down. Palmeira *et al* (1995) demonstrated in isolated rat liver that the interplay between paraquat and mitochondrial bioenergetics is not always straightforward because, depending on the concentration given, the toxin can have both stimulatory and inhibitory effects. They found that very low toxin concentrations partially inhibited

ATPase activity, whilst higher concentrations increased this activity. Additionally, cytochrome-c reductase (complex III) and cytochrome-c oxidase (complex IV) are strongly inhibited by paraquat, which can lead to decreased ATP synthesis (Paleira et al., 1995). Karlsson *et al*. (2010) found that DCF-dependent fluorescence was highly dependent on the enzymatic activity of cytochrome-c. Therefore it appears that the concentration of paraquat can very much determine what metabolic activity is being examined. There seems to be a concentration window between which generates an increase in metabolism and fluorescence. If the concentration is too low then metabolism is limited by ATPase activity and if the levels are too high then cytochrome-c activity is disrupted. It appears that a concentration of 10 mM had differential effects between the adult males and females, implying that males might be more susceptible to the effects of paraquat. One way to avoid this problem might be to implement an alternative toxin with more consistent effects on ROS production, for example hydrogen peroxide.

4.4.2 Survival (& larval development time)

Larvae: Paraquat significantly reduced the proportion of larvae reaching pupation and subsequent eclosion. However, there was no effect of treatment on the number of larvae that eclosed following successful pupation. There are two potential explanations for this finding. The first is that if a larva can withstand acute oxidative stress up to point of pupation then there may be little effect of this treatment on their likelihood to emerge. The second is that, in general, 3rd instar larvae will leave the medium 6 hours prior to pupation, past this point no further food, or toxin, is consumed. Therefore, if the effects of paraquat transpire very quickly little to no further damage may occur 6 hours after the toxin is consumed once pupation has been reached. No immune-related differences were found in the numbers of larvae reaching pupation or eclosion, irrespective of treatment.

The time taken to reach pupation was increased in paraquat treated larvae; a finding most likely attributed to the influence of stress signalling pathways on growth regulatory systems within the developing fly. Development rate in *Drosophila* is primarily regulated by the insulin/IGF (insulin-like growth factor)-like signalling (IIS)

pathway (Walkiewicz & Stern, 2009), a pathway that is evolutionarily conserved between mammals and invertebrates. This pathway can be regulated by a number of pathways such as target of rapamycin (TOR) and the stress induced JNK pathway. The TOR pathway is an evolutionarily conserved pathway which acts by regulating cell growth and metabolism in response to a series of environmental cues and is essential for developmental growth (Kapahi *et al.*, 2010). As with JNK signalling, stress factors such as heat shock, DNA damage and ROS also cause decreased TOR activity and a resulting decrease in protein synthesis and growth (Reiling & Sabatini, 2006). If resistance leads to differences in stress signalling we might expect this to become apparent by their impacts on growth signalling and a consequent delay in development to pupation. However, no difference was found in development time between the control and resistant *Drosophila*, perhaps suggesting similar upregulation of these stress-induced growth inhibiting pathways. The time spent in pupal phase did not depend on treatment, which might suggest that IIS has less effect at this phase.

Adults: There was no difference in the ability of the control and resistant adult *Drosophila* to buffer the damaging effects induced by oxidative stress. Upon consumption, the toxin begins a redox cycle producing activated oxygen species such as H_2O_2, the superoxide anion or hydroxyl radical (Bus & Gibson, 1984). These products are highly reactive to cellular molecules and cause oxidation of essential reducing agents necessary for cellular functioning such as NADPH (Bus & Gibson, 1984). *Drosophila* possess internal clearance mechanisms to avoid the build-up of ROS. However, if the level of stress exerted on the individual outweighs the clearance rate then the amalgamation of ROS can be lethal. When consuming 10 mM paraquat, all *Drosophila* were dead in just under 10 days. Overall, males were more susceptible to lethality from acute oxidative stress. Within 24 hours of toxin consumption (10 mM paraquat) an average of 86.2% of females was still alive, while only 59.1% of males still remained.

4.4.3 Summary

In conclusion, this chapter shows that immune resistance to parasitism does not alter the individual's ability to resist acute oxidative stress induced by paraquat consumption.

Therefore, it is unlikely that protective mechanisms in place to counteract acute oxidative stress, such as the JNK stress-signalling pathway and chaperone protein expression, act differently within the resistant flies compared with the control flies. Any differences in expression are likely to directly correspond to buffering the potential damage from the upregulated immune mechanisms found in the resistant individuals, providing the *Drosophila* with equal protection against other external stresses.

5 Resistance to larval parasitism leads to altered motor function

5.1 Introduction

To survive and reproduce animals must optimally allocate resources towards immunity in order to match the risk from infection posed within their environment. However, the immune system has intricate links with other fundamental processes, such as those involved in the development of the nervous system and its function. The immune system and nervous system are intricately connected (see 1.5.2), thus changes in immune resistance and any corresponding up-regulation of immune-related mechanisms may disrupt the balance between these two systems. The nervous system is integral in the coordination of a variety of tasks, such as memory and motor function; therefore analysis of different behaviours should act as an accurate indicator of changes within the nervous system. This chapter investigates whether *Drosophila melanogaster* selected for increased resistance against parasitism have altered functional phenotypes throughout life. This was assessed by measuring differences in crawling behaviour in the late larval phase and altered climbing abilities in the adult fly.

The nervous system is comprised of two distinct cell types; the neuronal and glial cells (see 1.5.11.5.1). The nervous systems of organisms with a higher degree of neuronal complexity contain a greater proportion of glial cells to nerve cells. In the mammalian nervous system most cells are glial cells, whereas the *Drosophila* nervous system contains just 10% glial cells (Stork *et al.*, 2008). These cells are intimately connected with neurons and act as key regulators of neuronal development and function. Mammalian and *Drosophila* glia share many morphological and functional properties (Freeman & Doherty, 2006). In both systems exists a resident glia derived from hematopoietic lineages, which act as the primary immune cells of the nervous system by engulfing dying cells and pathogens. In mammals, this role is carried out by one of the glial subtypes; the microglia. Upon infection microglia become activated and locate to the diseased region of the nervous system, where they release immunomodulary factors, neurotrophins and cytoxins and begin to ingest cellular material (Neumann *et al.*, 2008).

Drosophila do not possess a distinct subset of glial cells, instead all glia are proficient in immune-like functions (Freeman *et al.*, 2003). These glia engulf apoptotic cells in the nervous system similarly to their mammalian counterparts (Sonnenfeld & Jacobs, 1995). In *Drosophila*, glial cell fate is determined by the genes *glial cells missing* (*gcm*) and *gcm2*; both of which promote glial cell proliferation (Jones *et al.*, 1995). Knock out mutants for both *gcm* and *gcm2* also produce decreased numbers of the main *Drosophila* immune cell, the plasmatocyte, which also fail to migrate to their correct location and to achieve full conversion to macrophage morphology (Alfonso & Jones, 2002).

In addition to their role in immunity, immune cells and glia are also crucial regulators of nervous system development. In mammals, macrophages moderate brain tissue modelling during development by the phagocytosis of dying cells (reviewed in Perry & Gordon, 1988). Macrophages also produce interleukins, which help to initiate angiogenesis and glial formation within the growing nervous system (Perry & Gordon, 1988, Giulian *et al.*, 1988). Insect immune cells (hemocytes) help shape the developing nervous system by phagocytosing neuronal cell corpses. *Drosophila* hemocytes are also involved in laying down extracellular matrix (ECM) during formation of basal membranes. They have been found to regulate production of the neural lamella, a thick layer of ECM that entirely surrounds the central and peripheral nervous systems. Reducing hemocyte motility through Pvr or Rac1 disruption results in severely depleted ECM components (Peroxidasin and Collagen IV), a total lack of lamella formation and a lack of CNS condensation (Olofsson & Page, 2005, Stork *et al.*, 2008). Condensation occurs during embryonic development, at which point ganglia fuse to become coordinated into the ventral nerve cord (VNC) (Olofsson & Page, 2005). Glia also play a major role in nervous system development by assisting in CNS condensation, aiding in nerve growth cone extension and providing essential trophic support to the developing neurons (Campbell *et al.*, 1994, Ebens *et al.*, 1993, Freeman & Doherty, 2006). They also secrete synaptogenesis stimulating factors and prune axons to modify dendrite morphology and shape synaptic connections (Freeman & Doherty, 2006). Disruption to glia prior to nerve development produces a number of nervous system defects and in the worst cases can lead to embryonic lethality (Nadon & Duncan, 1996, Dentinger *et al.*, 1985).

Due to the high level of interaction between the immune and nervous systems (see 1.5.2), changes in the expression of immune-related mechanisms are likely to have consequences in the development and continued function of the nervous system. There are very few studies existing that have looked at how increased immune resistance can impact on the nervous system. Kolss and colleagues (2006) used *Drosophila* specifically selected for immunity to parasitoids to look for trade-offs in associative learning ability. They found that immune resistance had no effect on the *Drosophila*'s ability to avoid mediums when previously conditioned to associate that medium with a noxious taste. Conversely, Alghamdi *et al*. (2009) found that bumble bee (*Bombus terrestris*) learning performance, as determined by correct flower colour choice following sucrose reward training, was more advanced in colonies with greater bacterial resistance, as measured by a zone of inhibition assay. The latter finding suggests that immune resistance can regulate the nervous system in a positive fashion.
In this chapter the aim was to determine the effect of resistance to parasitism on motor function by measuring different locomotor behaviours. Locomotion is a simple quantifiable readout of neuronal function and is therefore one of the key features used to measure the functional activity of the nervous system (Iliadi & Boulianne, 2010). As such, a range of simple locomotor assays have now been implemented and modified to assess nervous system function (Grotewiel *et al.*, 2005). In this study, locomotive behaviour was assayed during development in the larval phase and in the young to ageing adult.

Larval motor function: In the absence of external stimuli, for example visual or chemical cues, larval locomotor behaviour tends to follow a distinctive pattern: forward crawls, interspersed with pauses and turns (Berni *et al.*, 2012). Forward crawling is driven by peristaltic waves of muscular contractions that actively pick up each segment and drag the body forward. These muscular contractions are thought to be initiated by central pattern generator (CPG) circuits and can occur independently of input from the brain and sensory feedback (Gjorgjieva *et al.*, 2013). CPG circuits are groups of autonomous neurons that are distributed along the anterior-posterior axis of the nervous system. They produce rhythmic motor patterns and underlie many other rhythmic physiological processes found across the animal kingdom such as breathing, swimming, flying and walking (Marder & Bucher, 2001). Turning is an innate mechanism employed by the animal to help it navigate its way through its environment and to

maximise food discovery and intake. An organism will optimise its foraging behaviour (a series of turns and straight movements) to increase the time spent in a food patch and then, once that patch is depleted, to locate a new patch (Hill *et al.*, 2000). In general, when a larva is feeding, turning movements are increased to allow the individual to remain within the food patch (Troncoso *et al.*, 1987). However, away from food the behaviours are very different and more forward movements are employed, interspersed with random turns, as the larvae attempts to find another food source (Troncoso *et al.*, 1987). Reduced turning on a food background or increased turning on a non-food background can indicate problematic neuronal functioning, as shown in a number of neurodegenerative model systems (Steinert *et al.*, 2012, Jakubowski *et al.*, 2012, Sinadinos *et al.*, 2012, Quraishe *et al.*, 2013). In this study larval activity was assessed in an open-field plate assay using the tracking software Ethovision and in a straight racetrack assay. The former allows the precise tracking of the larvae as they navigate through their environment. Different parameters were then measured to determine changes in these innate larval behaviours; forward movement, pauses and turns. The latter allows the measurement of larval speed in one direction.

Due to the close links between the nervous and immune systems, it is likely that increased immunity would result in a change in the motor behaviour of the larvae, although it is not possible to estimate whether this will be in a positive or negative fashion. Both of the assays implemented in this study examine behaviour whilst the larvae are 'off-food', therefore we would expect more proficient motor function to include more forward movements and/or less turning movements.

Adult motor function: Stimulation of these motor nerves is directed in a coordinated fashion to achieve the synchronised leg movement required for walking and climbing (Büschges *et al.*, 2008). Similarly to larval locomotion, movement of the adult legs is generated by CPG circuits and requires little higher centre activity unless stimulated by changes in environmental cues (Duysens & Van de Crommert, 1998). In this assay adult locomotion was assessed by measuring the climbing velocity of *Drosophila* following initiation of the negative geotaxic response; movement away from the Earth's gravitational pull. In order to stimulate such a response a shock stimulus must by applied; this is generally performed via a mechanical 'tap' (Gargano *et al.*, 2005, Kamikouchi *et al.*, 2009). Climbing behaviour has commonly been used to assess motor

deficits in transgenic and ageing flies, when flies show decreased climbing activity (Watson *et al.*, 2008, Martinez *et al.*, 2007). In order to determine whether immune resistance has long term implications on the nervous system, this study measured the climbing activity over a series of weeks. Two assays were implemented, the first using a method commonly applied in the lab requiring the use of CO_2 anaesthesia to transfer the flies into their climbing chambers. The second assay was modified to eliminate the potential effects of CO_2 anaesthesia on the fly's climbing ability as well as to increase sampling numbers.

Due to the fact that most of the neuronal networking is established prior to metamorphosis, we would expect changes in the larvae to continue into the adult fly. In both of the adult assays, more proficient motor function should be apparent by increased climbing velocities.

5.2 Materials and Methods

5.2.1 Drosophila stocks

All flies were cultured and kept as described previously in detail (Chapter 2). Briefly, for each experiment four control lines (C1-4) and four lines selected for parasitoid resistance (S1-4) were used, each line was cultured in exactly the same manner. *Drosophila* were kept on yeast-sugar medium containing 0.5% propanoic acid at 20°C with a 12:12 hour light:dark cycle. For all experiments performed, flies were allowed to lay for 24 hours at 25°C. Two days after egg laying larval density was moderated; per line a maximum of 100-200 larvae were transferred into each fresh medium bottle and allowed to develop. After eclosion flies were collected into fresh medium bottles. For adult assays, eclosing *Drosophila* were collected over a period of 24 hours and left for a further 48 hours to ensure copulation. The flies were then sexed and separated into vials containing yeast-sugar medium plus the anti-fungal agent propanoic acid. Flies were supplied with fresh medium-vials every 2-3 days to remove offspring produced and minimise mortality caused by flies getting stuck on sticky food.

5.2.2 Larval motor function: Ethovision

Plate assay and recording: In order to monitor the movements of the larvae, the '4 plate' larval locomotion assay was used, as previously described by Sinadinos *et al*. (2012). Four square plastic plates containing 1% agarose with 0.1% *w/v* Alcian blue dye (Hopkin & Williams Ltd, UK) were prepared. The blue dye contrasts well with the pale cuticle of the larvae enabling the larvae to be distinctly visualised on the plate. To prepare the plates, the dye was dissolved in water and heated to increase dye solubility before mixing with the pre-dissolved and boiled agarose solution. Each plate was then filled to approximately 4 mm depth with the molten dye-containing agarose. The plates were allowed to cool and then stored at 4°C until use. Prior to experimentation the plates were allowed to warm up to room temperature for approximately 1 hour. On each experimental day, the four plates were lined up into a 2 by 2 square, as shown in Figure 5.1. A digital video camera (Ikegami) was positioned directly above the centre of the four plates using a Benbo Trekker camera tripod (Tracksys, UK). The camera was linked up to a digital video recorder (Panasonic Diga) and monochrome TV monitor (JVC), on which the recordings could be visualised live.

For this experiment, wandering third instar larvae were selected from the walls of the glass vials where they would have crawled to so as to pupate away from the food. If the larva displayed overly lethargic movements they were not selected since this was taken as an indication that those larvae were on the verge of pupation. For each assay, one larva was placed at the centre of each of the four plates and allowed to acclimatise for two minutes. Two resistant larvae and their corresponding controls were run in parallel on the four plates; larvae were randomly assigned between the plates. After this time the larvae were replaced into the centre of each plate and recorded for a period of 2 minutes. This was repeated a further two times, with a total of 6 minutes recording time. For data analyses, the motion of the larvae in the third trial was used; the other two trials ('practice trials') were saved and stored for reference. In total 128 wandering larvae were used (16 larvae per biological replicate (C/S1-4)) to determine larval crawling behaviour, all were approximately equal in size.

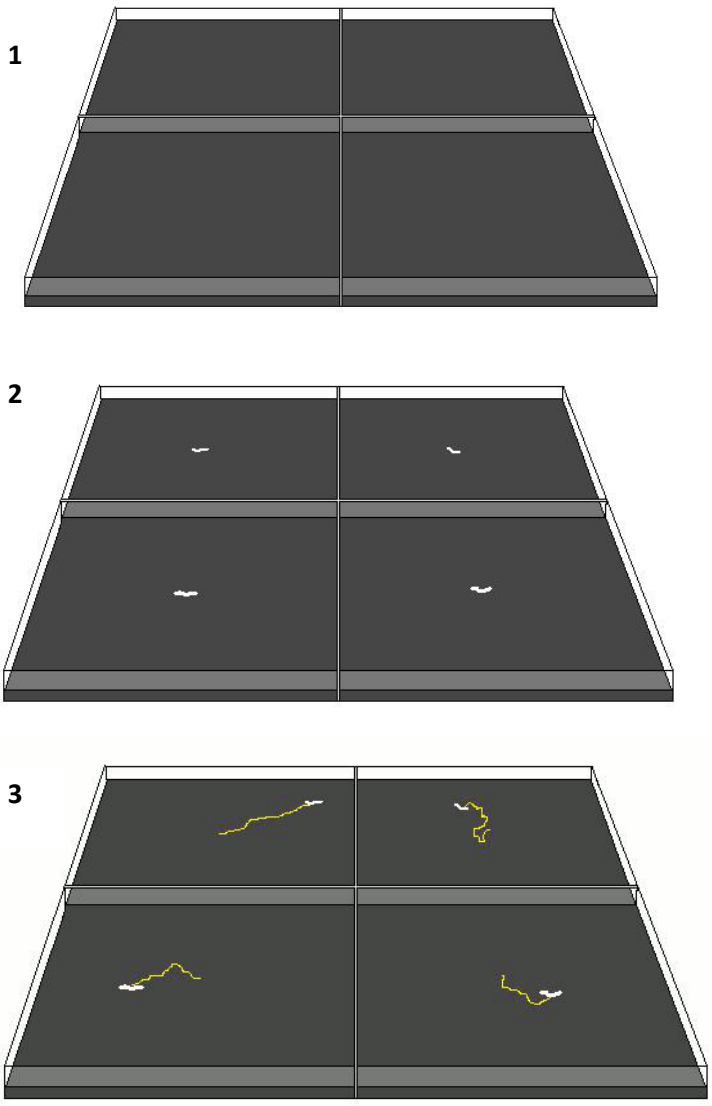


Figure 5.1 Experimental set-up: open field larval assay

1) Four plates with agarose plates (containing Alcian blue) were lined up directly under camera. 2) Larvae were placed onto the centre of each plate. 3) All movements made by the larvae were recorded. These were later analysed using the image tracking software Ethovision, to assess a number of parameters, such as velocity, angular velocity (degrees/s), and meander (degrees/cm).

Video analysis: Larvae in each arena were identified and tracked using the Ethovision image tracking software (version 3.1, Noldus Information Technology). Boundaries were set by defining the plates as 4 individual arenas marked out in the software. The detection levels were then adjusted to ensure that only objects reaching a certain threshold and size, as associated with the larvae, were tracked. Each video was run twice, once with the program tracking the subject once per second and the other time with the program tracking the subject 5 times a second. The reason for this is because

Sinadinos *et al*. (2012) previously showed that more frames per second were necessary to track minor movements of the larvae such as turning behaviours, whilst fewer frames per second gave a more accurate reading of velocity. Once the larvae had been tracked the desired read-outs could be selected and calculated by the software. Parameters measured were: velocity, angular velocity, meander, time spent moving. These parameters were selected as they allowed the measurement of the most common larval behaviours; forward motion, pausing and turning (Berni *et al.*, 2012). In order to establish contraction rate, each of videos was replayed and the number of contractions made in a 30 second time period were counted using a hand-held click counter.

5.2.3 Larval motor function: racetrack assays

Larvae at wandering phase were selected as described above. Each larva was given 5 minutes 'training' period during which time it was placed at one end of a moistened track with a small yeast ball (approximately 2-3 mm in diameter) located at the opposite end. Following training, the larva was moved into a fresh track, with a 30 mm interval marked out in between the larva and the yeast bait (Figure 5.2). The time taken for the larva to crawl across this 30 mm 'racetrack' was recorded. This process was repeated for each subject. In total, 200 larvae were tested (25 per biological replicate (C/S1-4)). This assay was performed by project student Liz Page.

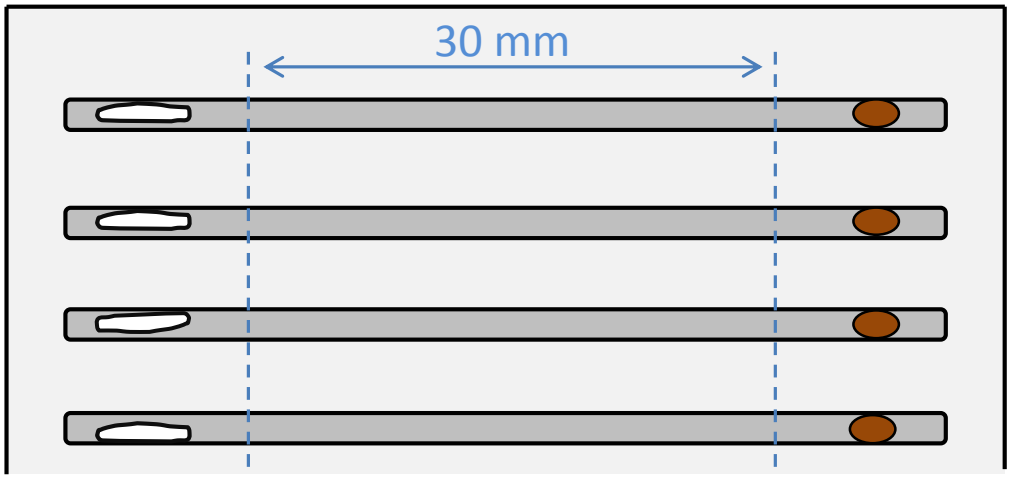


Figure 5.2 Experimental set-up: larval racetracks

Larvae (white) were placed at one end of each track and a ball of yeast (brown) was placed at the other. A 30 mm 'racetrack' was marked out along each track.

5.2.4 Adult motor function

Motor assays were performed by generating a startle-induced climbing response in the *Drosophila*; this was done by tapping the flies down and recording their climbing activity within a contained chamber. In the first instance, *Drosophila* climbing ability was measured using a method commonly implemented within the laboratory. For this assay twenty mated *Drosophila* of each sex for a single line pair were lightly anaesthetised with CO_2 for transfer into experimental climbing chambers (50 ml measuring cylinders, with additional lines drawn up to 70 ml point) (Figure 5.3). The flies were then allowed a 30 minute recuperation period before entering the experimental arena. The arena was set-up prior to commencement of the study and remained intact throughout to minimise experimental variation. In total, 320 adult flies were tested (20 adults of each sex were used for each biological replicate (C/S1-4)).

Arena set-up: In order to minimise *Drosophila* visual cues and aid experimenter visualisation, an all-white arena was used, with a standardised light source; a strip of light directly above the arena. A padded surface ensured that glass climbing cylinders were not damaged during the study. The video recording device (web-cam, Logitech)

was directed toward the arena and held in position using a clamp and stand. Videos were produced using the Logitech software package (web-cam standard), which could later be analysed to determine motor activity.

Three sharp downward taps were performed to initiate the negative geotaxic response and the resulting behaviour was recorded for approximately 15 seconds. This was done three times, with a break of 2 minutes between each stimulus. The four line pairs were assayed individually across 4 consecutive days, which was repeated on a weekly basis. A total of 640 flies commenced the experiment (2 repeats of 20 per line (C&S1-4), per sex). Liz Page provided technical support during this assay. Analysis was carried out at a later date by replaying the videos and manually pausing the sequence at chosen time points to calculate fly position. Only the third trial was analysed as the previous two were performed to allow flies to 'practice' climbing within the chamber.

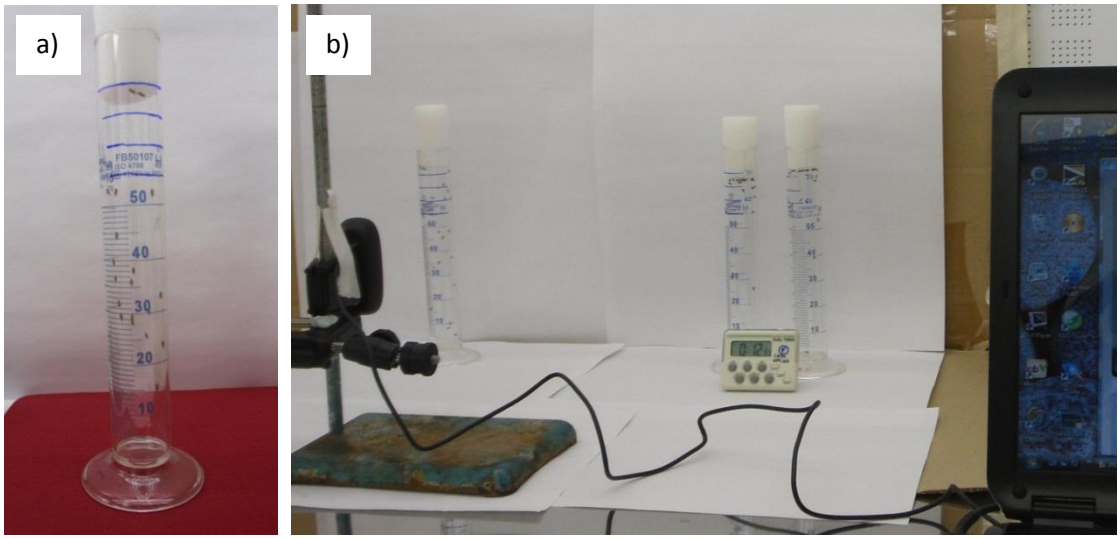


Figure 5.3 Experimental set-up: preliminary assay

a) climbing chamber containing Drosophila. b) arena set-up: camera held by clamp and directed at climbing chamber against a white background. The camera was connected to computer on which live recordings could be made.

It was recognised that the methodology used in this assay may have impaired the climbing ability. At later ages the flies took longer to 'come round' from anaesthesia so were given additional recovery time. Although times were kept constant within each age group it was clear that the use of light CO_2 anaesthesia to transfer the flies had a significant impact on the flies' ability to climb. Older *Drosophila* were often capable of climbing higher the day after the assay than they were during experimentation (unquantified observations). It was hypothesised that at the later ages this assay was measuring how well the flies recovered from anaesthesia rather than as a true effect of age. Carbon dioxide is known to affect the behaviour of invertebrates (Van Dijken *et al.*, 1977, Barron, 2000). However, even though CO_2 anaesthesia has been shown to impinge on the nervous system, laboratories still regularly use this technique to transfer flies before an assay (Coulom & Birman, 2004, Ali *et al.*, 2011). In many cases this is to minimise loss of potentially genetically modified, experimental flies into a controlled environment.

It was therefore decided that a second more reliable assay was required to accurately measure *Drosophila* motor function and how it changes over time. The second assay was inspired by the rapid iterative negative geotaxic (RING) assay. This assay was implemented by Gargano *et al*. (2005), who designed a piece of equipment with multiple chambers, which can be tapped down simultaneously and is thus capable of generating a much larger scale of data collection than our preliminary assay. The apparatus in this assay was designed and modified by the experimenter in line with those used by Gargano *et al.* (2005). Two multi-chamber tapping apparatuses were then produced. There were 6 glass chambers (diameter: 2.5 cm; height: 100 cm), with a plastic ventilated lid (Figure 5.4). Each chamber could slot into a padded well within the apparatus and was then held in place by a removable lid that was secured on each side with Velcro straps. To eliminate the potential effects of CO_2 on the *Drosophila* climbing activity, the chambers used in the experiment were also used to house the *Drosophila*, so that no gassing was necessary to move the flies on the day of the experiment. Approximately 10 mm of yeast-sugar medium lined the base of each chamber. To each chamber 20 flies were added and turned over to new chambers three times a week. Gargano *et al*. (2005) used similar sized chambers and showed that density was not a controlling factor on *Drosophila* climbing behaviour in numbers up to 25. The tapping assay was performed immediately prior to each turn-over giving the

flies 2-3 days to acclimatise to the chamber. Prior to each assay, each chamber was fitted with a 10 mm ruled sheet of paper, fixed to the back. The first line was aligned with the top of the medium to aid with measuring the height climbed from the base. A total of 1600 flies commenced this experiment (5 repeats of 20 per vial for each sex sex were analysed for each biological replicate (C/S1-4)).

Within the tapping apparatus 6 cylinders could be knocked down simultaneously. In each run 3 vials of resistant flies were tapped simultaneously with 3 vials of their corresponding control flies. Three sharp taps were performed. This procedure was then repeated and recorded 3 times as previously described for the first assay. Similarly, the all-white arena with controlled light source (shown in Figure 5.3) was used to minimise visual stimuli.

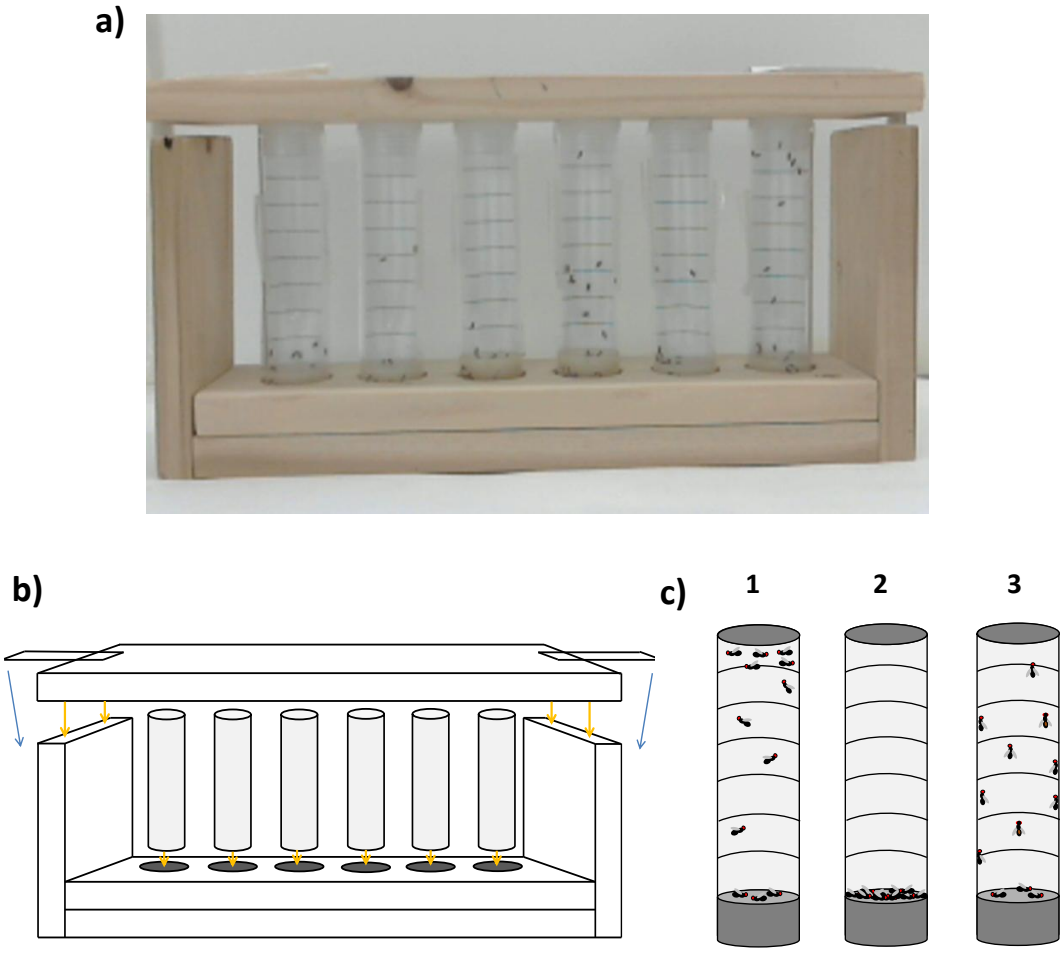


Figure 5.4 Experimental set-up: final adult climbing assay

Experimental set-up for adapted motor assay: a) Photograph taken of tapping apparatus during assay. b) Schematic diagram to show how the apparatus and cylinders fit together. Once in place Velcro is used to secure the lid (blue arrows). c) Tap down protocol: 1 = flies are flying around freely within chamber, 2 = three sharp taps send all flies to the bottom, 3 = following tap-down stimulus flies initiate negative geotaxic behaviour by climbing up the sides of the chamber.

5.2.5 Statistical analyses

The statistical software Minitab was used to perform all analysis of variance (ANOVA). *Larval motor function; Ethovision:* Data attained in Ethovision measuring 1 frame per second was used to calculate velocity. Data attained measuring 5 frames per second was used to calculate the turning behaviours (angular velocity and meander) and to assess the time spent in motion. Statistical analyses were performed using Minitab. For all measurements (velocity, angular velocity, meander, time spent moving and contraction rate) a one-way ANOVA with line set as factor was first performed to eliminate variations between the *Drosophila* lines. Subsequently, a one-way ANOVA was performed on the line mean residuals with immunity as factor.

Larval motor function; Racetrack assay: Statistical analysis was performed using Minitab. Average velocity was found by dividing the time taken to travel the length of the track by the 30 mm distance. A one-way ANOVA with line set as factor was fit against the average crawling velocity. This was followed by a one-way ANOVA on the line mean residuals against immune-background to determine immune-related effects.

Adult motor function: Velocity was calculated as height climbed per mm (averaged over the first 4 seconds (Gargano *et al.*, 2005)). A standard ANOVA design does not allow for repeated observations of the same subject. As such, data of this type is generally analysed using a repeated-measures ANOVA; as long as the data meets the assumptions of normality. Due to the lack of normality in the data at the latest ages, only log-transformed data for the climbing velocities of *Drosophila* aged up to 47 days for females and 67 days for males could be analysed. A one-way ANOVA with line set as factor was fit against the average climbing velocity against line. The mean line residuals were then run on two-way repeated-measures ANOVA in SPSS; the within-subject factor was age and between-subject factor was immunity. The analysis gives the estimates (epsilon) of sphericity, which refers to the equality of variances of the differences between the measurements. The Greenhouse-Geisser estimates were used as the epsilon values lay below 0.75 (Girden, 1992).

5.3 Results

5.3.1 Larval motor function; Ethovision

Motor ability was assessed in the larvae by observing their movement in an open field environment. Five different parameters were measured: velocity (mm/s), contraction rate (per minute), percentage of time in the arena spent moving, angular velocity (degrees turned per time elapsed) and meander (degrees turned per distance moved). Relative values for the turning behaviours of the selected larvae with respect to the control larvae were calculated to enable easier visualisation and comparison.

There was no difference in the average velocity of the larvae from high or low resistance background ($F_{1, 6} = 4.48$, $P = 0.079$) (Figure 5.5a). The turning of the larvae was addressed using two parameters: angular velocity and meander. The direction moved by the larvae per unit time (angular velocity) was not dependant on immunity background ($F_{1, 6} = 2.97$, $P = 0.135$) (Figure 5.5c). Selected larvae changed direction more relative to the distance travelled compared to those larvae from an unselected background (shown in 3 out of 4 line pairs, see Appendix Table 7 for individual line means) ($F_{1, 6} = 8.88$, $P = 0.025$) (Figure 5.5d). No effect of immunity on contraction rate ($F_{1,6} = 0.02$, $P = 0.883$) (Figure 5.5b).

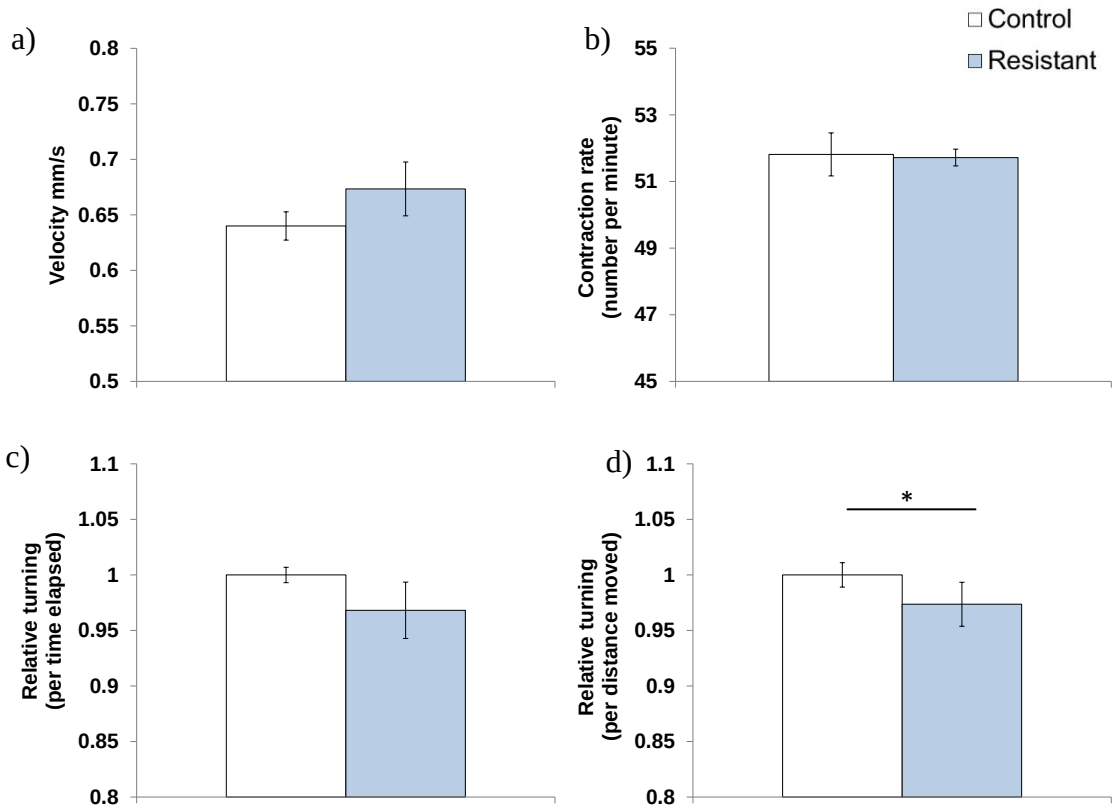


Figure 5.5 Larval movement: open field arena

Measuring different parameters of larval locomotion in an open field environment from populations with control (more susceptible) immunity to parasitism (white) and those from populations selected for the increased ability to encapsulate upon parasitic infection (blue). a) *Average velocity of larvae over 90 seconds. b) Average larval contractions per minute. c) Relative angular velocity (turns per time elapsed) = number of degrees deviation from a straight line made by the selected larvae per second relative to the control larvae. d) Relative meander (turns per distance moved) = number of degrees deviation from a straight line made by the selected larvae per cm moved relative to the control larvae (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 16 larvae were analysed for each of the 8 biological replicates (C/S1-4).*

The percentage of time in the arena spent travelling at particular velocities was calculated using the Ethovision software; the detection threshold was set to record 'movement' as any motion greater than 0.1mm/s. There was no immune-effect on the duration of inactivity ($F_{1,6}$ = 2.70, P = 0.151), which overall was found to be approximately 45.2% of the total time spent within the arena. Of the duration where the larvae were registered as 'moving', 52.3% and 24% of the time was spent travelling over 2mm/s and over 3mm/s respectively, this was not dependent on immune-background ($F_{1,6}$ = 0.51, P = 0.503 and $F_{1,6}$ = 0.02, P = 0.884). Approximately 22.6% of all the larvae tested were able to reach the highest speeds (greater than 5mm/s), irrespective of immune background ($F_{1,6}$ = 0.50, P = 0.504).

5.3.2 Larval motor function; Racetrack assay

Motor ability was assessed in the larvae by observing how long it took them to crawl a set distance when trained with a food reward, from which average velocity was calculated. Crawling velocity was found to be around 0.76 mm/s and was not affected by immune background in larvae ($F_{1,6}$ = 0.24, *P* = 0.644) (Figure 5.6).

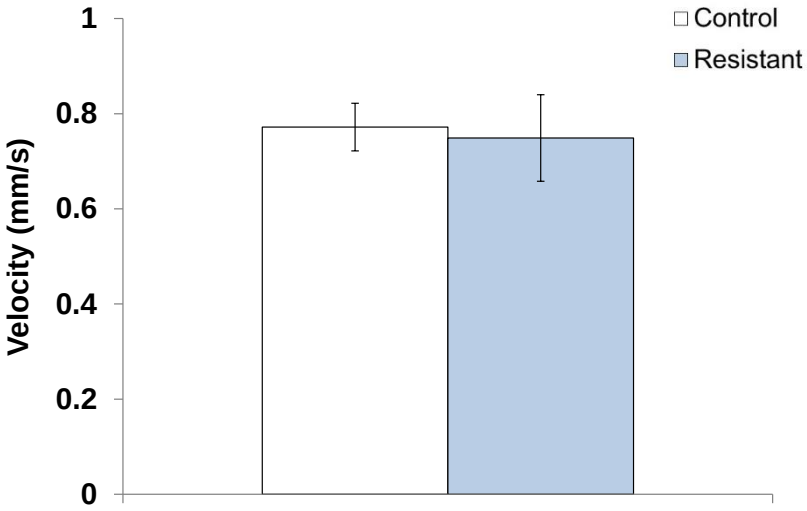


Figure 5.6 Larval movement: racetrack

Average velocity of larvae assessed across 30 mm racetrack of control (white) and resistant (blue) Drosophila (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 25 larvae were analysed for each of the 8 biological replicates (C/S1-4).

5.3.3 Adult motor function

Motor function declined with age in female *Drosophila* ($F_{1, 6} = 11.23$, $P = 0.015$) (Figure 5.7). Those females from a background selected for immunity to parasitism achieved consistently higher average climbing velocities than those from low resistance backgrounds (see Appendix Table 8 for line means) ($F_{1, 6} = 7.84$, $P = 0.031$). There was no interaction between immune-background and age ($F_{1, 6} = 0.456$, $P = 0.525$).

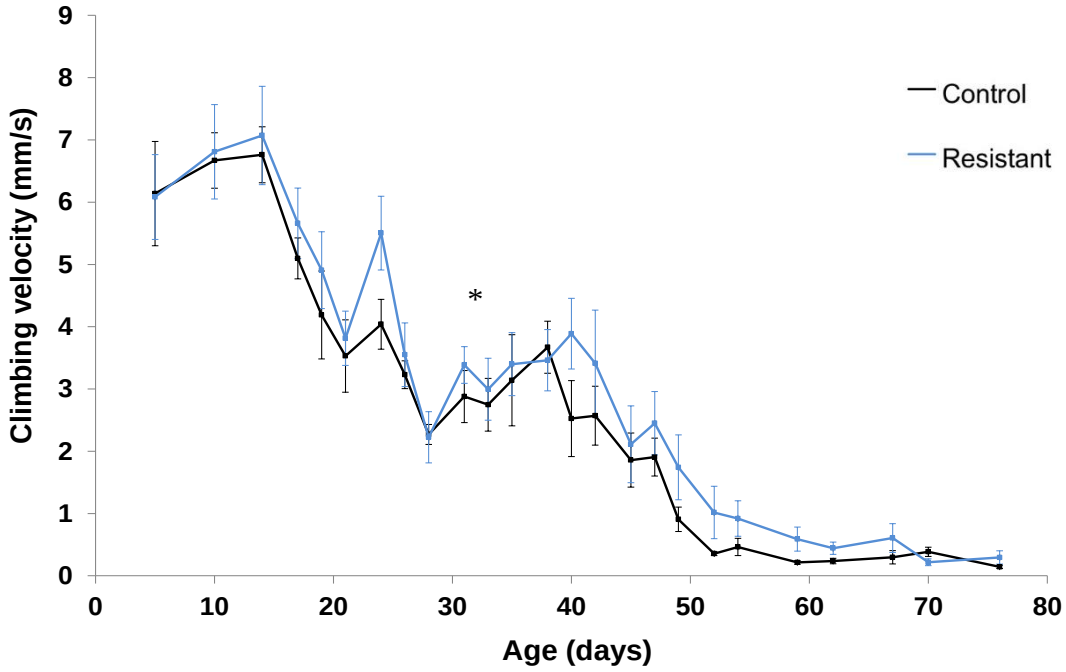


Figure 5.7 Adult female climbing ability

*Average climbing velocity in female Drosophila with increasing age (measured during first 4 seconds following a mechanical shock stimulus).Comparing control (black) and resistant (blue) flies (bars indicate ± standard error calculated from line means (biological replicates 1-4). 5 repeats of 20 flies per vial were analysed for each biological replicate (C/S1-4). * P < 0.05.*

Male climbing ability also declined with age ($F_{1, 6}$ = 21.02, *P* = 0.0038) (Figure 5.8). However, there was no effect of immunity background on average climbing velocity ($F_{1, 6}$ = 0.83, *P* = 0.783) and no interaction between age and immune background ($F_{1, 6}$ = 1.26, P = 0.305).

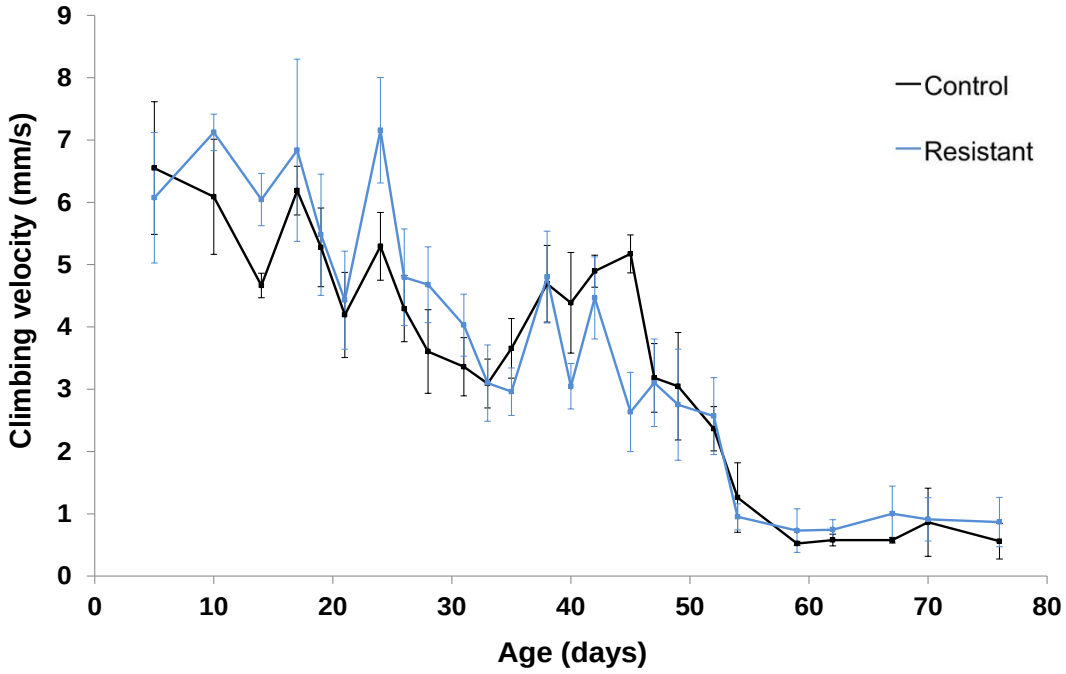


Figure 5.8 Adult male climbing ability

Average climbing velocity in male Drosophila with increasing age (measured during first 4 seconds following a mechanical shock stimulus). Comparing control (black) and resistant (blue) flies (bars indicate ± standard error calculated from line means (biological replicates 1-4). Analysis: 5 repeats of 20 flies per vial were analysed for each biological replicate (C/S1-4).

5.4 Discussion

In this chapter the aim was to observe the functional phenotypes arising from *Drosophila* with differing resistance to the larval parasitoid, *A. tabida*. More resistant *Drosophila* showed altered motor function in the late larval and adult phases, as measured by crawling and climbing ability respectively. The precise findings and possible mechanisms have been discussed below.

5.4.1 Larval motor function

Ethovision: Larval locomotion was first assessed in a homogenous, non-stimulatory, open-field setting to allow the larvae to move freely within their environment. Larval motion and locomotor behaviours were then quantified using the tracking software Ethovision. It was hypothesised that due to the close links between the nervous and immune systems we would expect to see a difference between the motor function in the control and selected larvae.

During this assay, larval movement was recorded approximately 45.2% of the overall tracking time irrespective of immune background. This shows that for the majority of time the larvae were in a paused state and not actively moving. Whilst in motion, the resistant and control larvae showed no difference in their average velocity, in the number of contractions per minute or in the percentage of time spent in motion. However, in agreement with our hypothesis, the amount of turning per distance covered (meander) was found to be decreased in the selected flies. Away from food larval movement generally favours more forward movements and less turning behaviour, which is optimal to help the larva find another food source, increasing the likelihood of survival in the wild (Troncoso *et al.,* 1987). Therefore, decreased turning on a non-nutrient substrate in the selected *Drosophila* could indicate more proficient locomotor activity.

Berni *et al*. (2012) showed that inhibiting synaptic activity in the brain did not decrease the amount of turns made by larvae when exploring a non-stimulatory environment. This observation suggests that turning behaviour in such environments is an autonomous process of the thoracic and abdominal nervous system and thus the variations seen in turning behaviour are likely due to adaptations to these systems. The differences in locomotor behaviour in the selected larvae, shown by a decrease in meander, may be due to interactions between the immune mechanisms required for resistance and aspects of the larval nervous system. These interactions may directly or indirectly bring about altered neuronal functioning. Larvae with increased resistance to parasitism contain higher numbers of circulating immune cells than the more susceptible control flies. As hemocytes are so highly linked with structural development of the nervous system in the embryo (as discussed above), a disparity in hemocyte

number could potentially lead to differential VNC organisation thus altering neuronal function. In order to confirm this it would be necessary to look more closely at the VNC structure, for example by implementing whole mount dissections.

In addition to the direct effect of hemocyte presence, impacts may also occur through pleiotropic gene expression. Pleiotropy is the term given when a gene codes for more than one phenotype (see 1.4.2). A number of genetic alterations are required to bring about the adaptations required for parasitic resistance thus yielding a large scope for pleiotropic effects (Wertheim *et al.*, 2011). Of particular interest are the genes *glial cells missing* (*gcm*) and *gcm2*, which are required for differentiation of the *Drosophila* surveillance hemocyte, the plasmatocyte, as well as the primary immune cell of the central nervous system, the glial cell (Alfonso & Jones, 2002). Knock out mutants produce fewer plasmatocytes and glial cells fail to differentiate (Alfonso & Jones, 2002, Jones *et al.*, 1995). Therefore decreased *gcm* expression would lead to an overall depreciation in immune cell function in the *Drosophila*. As glial and immune cell function are crucial for nervous system development and function (as discussed above), changes in *gcm* expression are likely to have impacts upon neural function. Freeman *et al*. (2003) characterised the expression and function of the gene *draper*, which is expressed in all Gcm positive glia and macrophages. They found that draper is a downstream target of Gcm and that it is required for corpse cell removal in the CNS. Due to the fact that the Draper protein exists in high levels on glial membranes that ensheath motor nerves, Freeman *et al*. (2003) then went on to assay the locomotor function of a series of draper mutants. They found that drpr$^{EP(3)522}$ and drpr$^{\Delta 19}$ mutations produced larvae with more uncoordinated behaviour and a decrease in crawling activity; quantified by the total number of gridlines (0.5cm grid) crossed in 5 minutes.

One potential hypothesis is that heightened immune potential and the increase in plasmatocyte numbers (Chapter 2) may result from changes in *gcm* and/or *gcm2* expression or their downstream targets, such as Draper. Increased expression in these genes would lead to upregulated numbers in the glia and/or Draper on the glial membranes that ensheath the motor neurons and thus could lead to increased cell corpse engulfment and a corresponding increase in nervous system function, shown by increased crawling ability. A decrease in turning found during this study may therefore suggest a more proficient nerve cell functioning associated with increased glial cell

presence. Further analysis would have to be made to determine whether resistance leads to a disparity in *gcm/gcm2/draper* expression and/or to differences in glial cell number and function within the *Drosophila* model system. Glial cells can be visualised and quantified by immunohistochemistry using the anti-Repo nuclear staining technique in conjunction with confocal microscopy or directly with electron microscopy.

An alternative hypothesis is that, instead of a trade-off, what we are actually seeing is an additional behavioural mechanism in the resistant larvae. Increased velocity and lessened turning behaviour could be favoured as an additional defence strategy against parasitoid attack. The parasitic wasp is attracted to the odour of host food as well as chemical cues (kairomones) released by the *Drosophila* larvae; this is due to the association of these scents with increased host aggregation (Vet & Dicke, 1992). Larvae that turn less frequently are more likely to move away from the foraging patch where they were originally laid, distancing themselves from other larvae and thus potentially reducing the likelihood of infection. This behavioural adaptation would have a selected advantage and therefore persist in future generations of resistant flies irrespective of the other immune mechanisms. Larvae at risk of parasitism have already revealed nocifensive behavioural phenotypes, including rolling upon intense stimulation of the class IV multidendritic nociceptive neurons by the parasitoid ovipositor (Hwang *et al.*, 2007). However, even though behavioural phenotypes may be selected for in nature, this type of selection could not have occurred in this study. During the selection process, 'resistant' individuals were selected on the basis that they were capable of mounting an encapsulation response. In order to display an encapsulation response the larvae must have been parasitised, thus the argument for differences in avoidance behaviours is redundant.

Racetrack assay: In the track assay the larvae are enticed toward a food reward. Berni *et al*. (2012) showed that upon detection of a food stimulus the brain can modulate autonomic larval locomotor behaviour. A yeast odor will therefore excite olfactory neurons in the brain, which will in turn elicit additional signalling to modify the CPG regulated exploratory movement, redirecting the larva toward the food stimulus. In conjunction to the food stimulus, the physical barriers of the racetracks should ensure that turning behaviour is minimised. It could therefore be said that this assay measures the maximal larval movement in one given direction. Although the assays are not

directly comparable, the average velocity was around 16% greater than that found in the Ethovision assay, reaffirming that when given a food reward and forced in one direction of travel the larvae can travel at higher rates, perhaps due to a decrease in time spent motionless or reduced velocity from turning. No difference was found in the average velocity of the control and resistant *Drosophila* larvae. This would indicate that the capacity to move forward is not hindered in either the control or the resistant larvae. However, the racetrack assay is not as accurate as Ethovision tracking, as shown by much greater standard error values, thus the assay itself may not be as capable of detecting minor differences between the lines. Overall, the findings from the larval assays may hint to differences in environmental navigation rather than in crawling capacity.

5.4.2 Adult motor function

The second part of this chapter set out to determine how resistance in early life can impact on motor function as the organism ages. It was hypothesised that due to the fact that most neural networking occurs prior to metamorphosis, that any differences apparent in the larvae should continue into the adult phase. The effects of resistance on adult motor function were assessed using the instinctual negative-geotaxic climbing movement of *Drosophila* following a startle response. This method is commonly used in labs to assess motor defects in neurodegenerative model systems and/or to analyse changes in motor function over time (Ali *et al.*, 2011).

Female *Drosophila* from a resistant background showed higher average climbing velocities than their more susceptible counterparts. This effect was not dependent on age and remained consistent throughout the lifetime of the flies. This finding is in agreement to our original hypothesis, implying that more proficient motor function in the larvae (shown by reduced turning behaviour) is continued through metamorphosis into the adult. Ninety percent of neuronal development occurs during the larval phase and relatively little alteration occurs after this point (Truman & Bate, 1988). Therefore, it is likely that we are witnessing alterations in nerve formation during the early phases of development. These findings would appear to show that resistance mechanisms can regulate nerve development and alter lifetime nerve functioning.

In males we found no effect of immune-background on climbing ability. The fact that this effect is not evident in the males might indicate minor rearrangement of motor function during metamorphosis a point where hemocyte disparity is lost in the male but not in the female (Chapter 2). Therefore the continued disparity in hemocyte number in the females may aid in the structure and restructure of the motor system at both stages of development. Alternatively, nerve development in the male may prioritise toward sexual behaviours which would increase the overall fitness, perhaps more so than general locomotor ability.

Resistant males from this model system have previously been shown to achieve higher mating success than their control counterparts (Rolff & Kraaijeveld, 2003). This may be in part due to differences in cuticular hydrocarbons; semiochemical signals which attract females for mating. However, in addition to pheromone attraction, males also exhibit a series of mating dances to entice the female; including orientation behaviour, foreleg tapping and wing vibrations (singing) (Ejima & Griffith, 2007). These complex courtship behaviours require input from the brain and other sensory organs, in addition to CPG activity to control the rhythmicity of these actions (von Philipsborn *et al.*, 2011). Increased sexual attractiveness as a mate may therefore result from increased aptitude to perform these rituals. If this is the case, this would involve adaptations to the resistant male's nervous systems. Resources allocated toward mating behaviour may lead to trade-offs in other neural functioning and may therefore leading to a greater decline in climbing ability with increasing age.

5.4.3 Summary

In summary, resistance to larval parasitism has resulted in altered motor function. This is first apparent in the larval phase where the resistant *Drosophila* exhibit an altered crawling phenotype and later apparent in the adult, where an increased climbing velocities were observed in the resistant females. These findings confirm that variations in an individual's ability to resist disease during development can have long-term impacts on the nervous system and highlights the importance of understanding how the immune system interacts with the nervous system in the absence of infection.

Recognition of how aspects of the non-activated immune system can affect the nervous system could help us gain further understanding of the links between these two critical processes. Further work would be needed to understand how these findings relate to potential variation in glial cell numbers and how they work within this model system.

5.4.4 Acknowledgements

Thanks to Stephen and Harry Aldridge, who helped with the construction of the two multi-chamber tapping apparatuses used in the second adult climbing assay.

6 General Discussion

All animals must optimally balance their metabolic resources in order to maximise their fitness (Kirkwood & Holliday, 1979, Hirshfield & Tinkle, 1975). As such, when pathogenic risk is high, selective pressures are in place to allocate resources toward immune resistance (Sheldon & Verhulst, 1996). However, resources within an organism are limited, therefore the allocation of resources toward immunity is likely to lead to trade-offs in other functions (Lochmiller & Deerenberg, 2000). In this thesis, research was conducted to establish how immune resistance can impact upon mechanisms relating to ageing, oxidative stress resistance and motor function. Changes in these traits may have a relatively low impact on the individual's fitness and therefore may not be protected by selection. However, these traits are important in determining an individual's long-term health, an area with particular relevance in today's world where there are an increasing number of aged individuals and a subsequent increased prevalence in age-related disease and function.

In order to assess trade-offs with immune resistance, this study implemented a *Drosophila* model that had undergone strong selective pressure from parasitoid infection. Utilising a selection-based model system allowed us to uncover naturally occurring, heritable phenotypic traits that arise as a result of resistance to infection (see 1.6.4). The first step of this thesis was to elucidate the immune-related differences between the resistant and susceptible *Drosophila* and how these differences varied with age, in order to assess their potential impact on the individual. Once the immune mechanisms had been elucidated, a series of assays were then implemented in the larvae and ageing adult to assess potential trade-offs in mechanism and/or function that manifest in the resistant model throughout life. Currently there is little information about the effects of naturally inherited resistance on an organism (Lochmiller & Deerenberg, 2000, Siva-Jothy *et al.*, 2005, Ferrari *et al.*, 2001, Vijendravarma *et al.*, 2009), providing a large scope for discovery in this area.

All results have been summarised in Table 6.1 showing significant differences between the control *Drosophila* and those selected for resistance against larval parasitism.

6.1 Improved understanding of the Drosophila model system

The first objective of this thesis was to gain a greater understanding of our *Drosophila melanogaster* model system (Chapter 2). It has been demonstrated that *Drosophila* selected for resistance against larval parasitoids have an increased number of circulating hemocytes (Kraaijeveld *et al.*, 2001; Chapter 3), which may aid in initial parasite detection and commencement of the encapsulation response during parasitoid infection (Chapter 2). As well as their role in immunity, immune cells are also important during metamorphosis to engulf larval fragments and shape the developing structure of the adult fly (Whitten, 1964). Hemocytes are produced solely during embryonic and larval phases of development, but many persist through metamorphosis and can be found circulating within the adult *Drosophila* (Holz, 2003). Chapter 2 shows that the immune cell advantage in the resistant *Drosophila* had disappeared by the young adult phase; no difference was shown between the control and resistant adults in either sex at 1, 4 or 8 weeks of age (Chapter 3). This may be achieved when the sessile and lymph gland derived hemocytes become incorporated into the *Drosophila* circulation, eliminating the difference between the control and selected lines. Alternatively there may be an active destruction of additional immune cells in the selected flies during metamorphosis. Surviving parasitoid infection will always be of benefit to the host, however, the protection against parasitoid attack is no longer required in the adult phase and therefore any expenditure of metabolic resources towards encapsulation immunity at this stage would be superfluous. The energetic cost involved in maintaining immunity may explain why additional immune cells are lost from the resistant *Drosophila* during metamorphosis.

Larvae from a selected background showed an increased ability to mount a melanisation response at the wound site. However, increased numbers of selected larvae also showed melanotic mass formation upon wounding when compared to the control larvae, demonstrating a trade-off within the immune system of these individuals. This is most likely due to a cross-over in immune mechanisms relating to encapsulation and melanotic mass formation.

Wound healing analysis confirmed that, although an immune cell advantage may confer benefits at the larval stage, there is no such advantage in the adult phase (Chapter 2). Conversely, resistant adult males showed less proficient wound healing than their controls (Chapter 2). Therefore, it seems reasonable to suggest that the additional resistance mechanisms in selected *Drosophila* are specifically adapted to larval defence and confer no immune advantage in the adult.

Increased intracellular ROS in the 1 week-old resistant females may exist directly or indirectly as a byproduct of an increased juvenile immune system. For example, the adult females from a selected background may have a lower capacity to buffer increases of ROS in the cell due to decreased resource allocation toward protective mechanisms, such as antioxidants. Alternatively, immune cells in the resistant females may be more metabolically active, perhaps due to an increased circulation activity. A trait such as this may aid in the detection of the parasitoid egg in the larvae and could perhaps continue into the adult, consequently resulting in higher outputs of intracellular ROS. To our current knowledge, resistant females show no increased resistance against pathogens in the adult (Kraaijeveld *et al.*, 2012), thus if activated immune cells exist they may remain as a vestigial immune trait. Vestigial traits occur because selection generally only acts on features that impact upon early survival and reproduction. A trait such as activated immune cells will be selected for if they provide an immediate benefit to the animal, for example to ensure survival against larval parasitism. These traits may become redundant, but will remain within the animal unless their presence causes a direct reduction to the individual's fitness. The fact that they are not removed completely indicates that they do not impact sufficiently to be detrimental to the fitness of the animal. In agreement to this Fellowes *et al.* (1998) showed that resistance to larval parasitism in *Drosophila* showed no effect on early fecundity or egg viability. Alternatively, any remaining difference in immune cell activity left over after metamorphosis may be due to constraints of selection linked to the processes that downregulate cell metabolism or to eliminate these overactive cells from the system, for example immune cell clearance may be limited by time and/or the availability of the mechanisms that break them down.

6.2 Assessing the costs of immune resistance

It is generally acknowledged that immune resistance and the related upregulation of immune-mechanisms are likely to come at a cost to the individual; otherwise, natural selection would have favoured proficient immunity against the whole spectrum of potential challenges (Siva-Jothy *et al.*, 2005). The aim of this thesis was to determine trade-offs associated with ageing, oxidative stress resistance and motor function.

6.2.1 Lifespan: An example of antagonistic pleiotropy

The reduction in longevity shown in the resistant females reveals a classic illustration of antagonistic pleiotropy; genetic expression of a gene coding for early resistance also codes for traits that negatively impact on those processes involved in buffering senescence. The most well-known example of antagonistic pleiotropy is that of sickle cell anaemia, whereby the expression of sickle red blood cells in heterozygous individuals results in increased resistance against the malarial parasite but decreases the oxygen carrying capacity of the blood (Carter & Nguyen, 2011). Similarly, the increased expression of larval immune cells found in Chapters 2 and 3 may confer an advantage in early immunity against parasitoid attack but a corresponding negative effect in later life, perhaps due to increased ROS production or through the deferral of resources away from protective mechanisms. Alternatively, immune cell increases may have no direct effect on lifespan but the two may occur in an indirect inversely correlative manner.

The results found in this study could support either the ‘disposable soma theory of ageing’ (see 1.4.3) and/or the ‘oxidative stress theory of ageing’ (see 1.4.4). Resources toward early defence may take away from those available to buffer senescence in later life and/or activated immune cells in the adult female may correspond to increased levels of intracellular ROS, which lead to increased senescence.

6.2.2 Endogenous ROS production

Resistant flies are known to possess increased numbers of immune cells (Chapter 2 & 3). To evaluate the cost of resistance and the associated increase in immune cell numbers,

Chapter 3 looked at differences in potentially harmful intracellular reactive oxygen species (ROS) production between the control and resistant flies. ROS are created as a by-product of metabolism in all eukaryotic cells and can be generated in high quantities by immune cells, particularly during an immune response (see 1.4.5) (Newsholme & Newsholme, 1989). A build-up of ROS can lead to protein, lipid and nucleic acid damage and ultimately cellular breakdown (Freeman & Crapo, 1982). Therefore, intracellular ROS must be regulated by a careful balance between ROS production and the actions of a series of antioxidants, which break down these metabolites into harmless products such as water. Hence, in an area where parasitism is rife, there is not only pressure for selection of resistance mechanisms, but also for the selection of mechanisms that buffer damage caused by the onset of an immune response. Consistent with this, control and resistant larvae showed no difference in their intracellular ROS concentrations (Chapter 3), indicating that antioxidant levels are synchronised with the increase in immune cells found in the larvae (Chapter 2). Wertheim (2010) correspondingly showed increased expression levels of antioxidants in larvae selected for parasitoid resistance. In contrast, 1 week-old resistant adult females that displayed no difference in hemocyte numbers exhibited an overall increase in intracellular ROS (Chapter 3). Increased ROS in these flies might suggest that there is a loss of the protective mechanisms put in place to counteract any immune-related ROS production (Chapter 2). A shift in the ROS/antioxidant balance allows harmful products to build up in the cells. If this occurs for prolonged periods, increased ROS levels can induce a state of oxidative stress, which leads to cellular damage associated with disease and ageing phenotypes (see 1.4.4). This finding could also be directly linked to the hemocytes if, for example, selected flies possess more actively metabolising immune cells.

6.2.3 Protection against increases in ROS

In order to further examine the mechanisms underlying the flies' ability to resist increases in ROS and any corresponding ROS-induced damage, Chapter 4 investigated how the flies respond to acute oxidative stress by consumption of the free-radical inducing toxin paraquat. In theory, any trade-offs between larval resistance and the defensive mechanisms in the flies, either by a reduction in antioxidants or the protective

proteins that buffer the resulting damage, should be shown by accentuated differences in exogenous ROS production and survival in the resistant flies.

Control and resistant *Drosophila* produced similar rises in ROS and lethality both in the larval and young adult phase, suggesting that there was no difference in the overall capacity to resist age-related damage. This seems to conflict with the hypothesis made in Chapter 3 that 1-week old females show increased endogenous ROS levels due to mismatched antioxidant protection. However, Kirkwood and Kowald (2012) recently suggested that external sources of ROS, such as those induced by toxin consumption, may have different effects to those produced by mitochondrial respiration. This is because mitochondrial DNA (mtDNA), which is arranged into complexes known as nucleoids, is attached directly to the inner mitochondrial membrane where the majority of intracellular ROS are produced. As antioxidants can only interact with ROS in the mitochondrial matrix, protection is not available for the nucleoids that receive the direct transfer of ROS from the membrane (Muller, 2000). Kirkwood and Kowald (2012) hypothesised that external oxidative stress may have a much smaller impact on DNA damage in comparison to that imposed by mitochondrial respiration. MtDNA deletions accumulate over the life of an animal and are thought to contribute to ageing, due to their ability to exaggerate respiratory chain function (Harman, 1972). One hypothesis, therefore, is that the resistant females have greater mitochondrial respiratory chain function causing a greater level of mtDNA damage and reduced longevity. When oxidative stress is externally induced by toxin consumption, antioxidants within the matrix regulate the production of ROS and the subsequent damage, therefore resulting in similar intracellular ROS measurements and survival numbers.

6.2.4 Motor function: An example of agonistic pleiotropy

Resistant *Drosophila* larvae have altered motor behaviour, a trait which is continued into the adult female (Chapter 5). This study demonstrates a positive correlation between these two traits, with selection for increased immune resistance leading to more proficient motor function. One potential explanation for this phenomenon may be linked to the role that immune cells play in the condensation of the ventral nerve cord (VNC) during *Drosophila* nervous system development. During VNC condensation, immune

cells secrete extracellular matrix to form a major component of the perineurium, the neural lamella (Homareda & Otsu, 2013), a process which is inhibited by blocking hemocyte migration (Olofsson & Page, 2005). Therefore, increased expression of immune cells in the developmental stages may lead to the generation of more competent nervous system function, perhaps due to changes in perineurium formation, which may alter the rate of nerve conduction (Pencek *et al.*, 1980). Alternatively, genetic expression required for the upregulation of the main surveillance immune cell, the plasmatocyte, also regulates the production of glial cells, which act to nurture and protect the nervous system (*gcm*/*gcm2*), as well as having a major role in nervous system development (Olofsson & Page, 2005).

Increased motor activity may itself lead to the rise in cellular metabolism shown by an overall increase in ROS output in the resistant *Drosophila* (Chapter 3). Therefore, the reduction in lifespan seen in the females may come as an indirect consequence of resistance (Chapter 3). The stabilisation of intracellular ROS by 4 weeks of age may be a result of mechanisms within the *Drosophila*, such as antioxidants or other protective chaperone proteins, which might be upregulated in response to the excess ROS in the young female. The costs involved in this upregulation could therefore be responsible for the reduced expenditure in somatic maintenance, and consequently accelerated ageing. Although plausible, this last theory seems unlikely as there would be little selection pressure acting to upregulate defence systems at this late stage, past the point when selection is likely to occur. The fact that defence systems would become upregulated also suggests that the level of ROS created in the system is harmful enough itself to reduce the overall fitness of the individual.

Table 6.1 Findings acquired during this thesis

Newly acquired data shown in red

TRADE-OFF	LARVAE	MALES	FEMALES
IMMUNE CELL COUNTS (Ch.2 & 3)	*(tested at L3 stage)* *Selected lines:* 20.0% more circulating immune cells (plasmatocytes) **Wounding** *Selected lines:* Overall 2.3% more immune cells (wounded and non-wounded combined) Interaction: Without wounding, 20.0% more circulating immune cells With wounding, 7.1% less circulating immune cells	**Wounding** No effect of immunity **Age-related** 1 week old No effect of immunity 4 weeks old No effect of immunity 8 weeks old n/a	**Wounding** No effect of immunity **Age-related** 1 week old No effect of immunity 4 weeks old No effect of immunity 8 weeks old No effect of immunity
PHENOLOXIDASE ACTIVITY (Ch.2)	**Initial activity** No effect of immunity **Rate** No effect of immunity	**Initial activity** No effect of immunity **Rate** No effect of immunity	**Initial activity** No effect of immunity **Rate** No effect of immunity
WOUND HEALING ABILITY (Ch.2)	*(tested at L3 stage)* **Wounded once** *Selected lines:* 1) 6.2% more displayed wound melanisation 2) 65.9% more exhibited melanotic mass formation 3) Melanotic masses were 69.2% larger	**Wounded once** *Selected lines:* On average, 12.6% less exhibited wound melanisation	**Wounded once** No effect of immunity **Wounded three times** No effect of immunity

LONGEVITY (Ch.3)	n/a	No effect of immunity	*Selected lines:* Average lifespan reduced by 11.1%
ENDOGENOUS INTRACELLULAR ROS LEVELS (Ch.3)	*(hemolymph and whole body tested)* No effect of immunity **With paraquat:** No effect of immunity	*(whole body tested)* **Age-related** 1 week old No effect of immunity 4 weeks old No effect of immunity 8 weeks old n/a	*(whole body tested)* **Age-related** 1 week old *Selected lines:* 21.1% more cell metabolism/ ROS output 4 weeks old No effect of immunity 8 weeks old No effect of immunity
ACUTE OXIDATIVE STRESS (Ch. 4)	**Intracellular ROS** No effect of immunity **Survival** No effect of immunity **Development time** No effect of immunity	**Intracellular ROS** No effect of immunity **Survival** No effect of immunity	**Intracellular ROS** *Selected lines:* 12.8% more cell metabolism/ROS output overall (with and without paraquat treatment) **Survival** No effect of immunity.
MOTOR FUNCTION (Ch.5)	**Ethovision:** *Selected lines:* Meander 2.6% less – take more direct routes **Racetrack assay:** No effect of immunity	**Climbing assay:** No effect of immunity	**Climbing assay:** *Selected lines:* Average climbing ability 15.6% higher overall irrespective of age

The key highlights of this thesis are: (i) increased resistance to larval parasitism leads to increased wound melanisation and increased autoimmunity in the larvae upon wounding (the latter shown by increased melanotic mass formation) (ii) resource allocation toward immunity was redistributed away from less essential processes at metamorphosis (iii) resistance in the larval phase led to a decrease in longevity and increased motor activity in the female, but not male, *Drosophila*. The important questions arising from this work

are therefore: What is the evolutionary rationale behind these phenomena? Why are many of these effects gender-specific? Do these observations have any medical relevance?

6.3 The importance of gender disparity

One consistent source of variation throughout the study was the sex of the flies involved. It is often taken for granted that gender serves as a confounding variable but it is important to address the question this raises: Why might there be such a great degree of gender disparity in the results? Hahn and Smith (2011) found that female Brown-headed cowbirds, a species which are known to be unusually resistant to infection with West Nile virus, had significantly shorter lifespans compared to their male counterparts. There was no gender disparity in the lifespans of closely related, but less resistant, species of Red-winged blackbird and Brewer's blackbird, perhaps implying that the cost involved in resistance was at a greater detriment to females, for example due to the increased expenditure in gamete production (Hahn & Smith, 2011). Alternatively, females may invest more heavily in immunity compared to the males, which could lead to a greater trade-off. Kraaijeveld *et al.* (2008) showed that female *Drosophila* larvae were better able to survive against parasitoid attack than males (65% versus 51% respectively). This may relate to an increased allocation of resources towards immunity, for example there may be an increased number of immune cells in the females relative to the males, or increased immune cell activation. Correspondingly, Beetz *et al*. (2008) showed that females of the Lepidoptera, *Manduca sexta*, appear to show a greater upregulation of plasmatocytes than males. In order to confirm or discount any involvement of sex related immune mechanisms in the resistant larvae it would be necessary to repeat the larval hemocyte counts analysing each sex independently.

6.4 Trade-offs: an evolutionary perspective

A trade-off was observed between the larval immune resistance and larval melanotic mass formation as well as between larval resistance and the lifespan of female *Drosophila*. The former trade-off may occur as a direct byproduct of selection for

parasitoid resistance. A more responsive immune system is a beneficial trait to ensure survival when the risk of parasitism is high. However, the increased quantity and/or more active immune mechanisms that are required to ensure this resistance may also lead to an exaggerated response to other stimuli, for example cutaneous injury. The trade-off between immune resistance and lifespan complies with our understanding of how the costs associated with certain functions relate to the organism's need to balance resources in order to optimise its fitness (Kirkwood & Austad, 2000, Kirkwood & Holliday, 1979, Hirshfield & Tinkle, 1975). Resources required for immunity, promoting survival of the *Drosophila*, appear to have expended resources required to buffer senescence.

As an additional level of complexity the *Drosophila* model system utilised in this study displays the reallocation of resources away from immunocompetance during metamorphosis. Although it is recognised that in a given environment a trait (e.g. immunity) will confer a selective advantage, for an organism to thrive in a more variable conditions, selection will occur not only on the traits themselves but rather the ability to allocate resources effectively throughout the organism's life. Since fitness is temporally linked, the traits that are advantageous to an organism are likely to change throughout its life. In this regard, all species are subject to these changing selection pressures, however, those with a more dramatic physiological change (e.g. *Drosophila* and other holometabolous insects) will experience this to a greater extent. The loss of resistance mechanisms in the selected flies indicates a level of phenotypic plasticity. Many animals with complex life cycles such as *Drosophila*, which exhibit more than one discrete phase, are thought to show phenotypic plasticity to allow phase-optimised resource allocation (Istock, 1967, Moran, 1994). This occurs because adaptive evolution acts independently at each life stage without negatively affecting the other stage(s). This is advantageous when selective forces vary throughout the animal's life (Moran, 1994). In addition to animals with complex life cycles, birds have also been shown to adjust their immune expenditure to optimise distribution of resources. Some small, non-tropical birds have evolved the ability to display seasonal immune variation, allowing them to adapt to the additional energetic demands, such as thermoregulation, imposed during extreme winters, by reducing the amount of resources allocated toward immunity (Nelson & Demas, 1996).

Hypothetically, one might assume that the loss of immune mechanisms in the resistant adult should make resources accessible to assist in the protection of the soma. However, female *Drosophila* still show reduced longevity, which could hint toward a quantitative limitation to the allocation of resources available throughout an organism's life. If the latter is true, the use of resources during development will therefore take away from the pool available to the adult *Drosophila*.

The appearance of an associated trait does not necessarily arise to the detriment of another. Two traits can arise simultaneously as a result of pleiotropic gene expression. Therefore, the selection pressure on one trait may be linked to the coexpression of another trait which may, or may not, confer an advantage to the individual. For example, increased proficiency was seen in both motor function and immune resistance, which may ultimately provide a selective advantage for the individual. Traits can also arise in a way that is not necessarily antagonistic or protagonistic to the fitness of the individual. A trait may have arisen concomitantly with another trait as has previously been described as an evolutionary spandrel by Gould and Lewontin (1979), after the resemblance with its architectural namesake. The phenomenon of concomitant trait expression was also noted by Darwin in 'The Origin of Species' (Darwin, 1859, p11-12). Darwin commented on various features such as hairless dogs having imperfect teeth and pigeons with short beaks having small feet, demonstrating that modification of some traits will result in changes in seemingly unrelated features. It is interesting that these examples of pleiotropic expression were noted before the wide-spread recognition of the genetic theory. A comprehension of genetic heritability has vastly expanded our knowledge of natural systems, and in the case of trade-offs has provided another level of understanding in that we can now trace phenotypes back to their genetic origin.

6.5 Medical relevance of understanding the costs of immunocompetance

As discussed previously, negative traits that manifest in later life will experience little selection pressure, which might explain why we see an increase in disease and degeneration in ageing humans. Therefore, even if a pathogenic risk is eradicated, immune traits may well persist in a population, as long as their effects do not impact on

early health or fecundity. In a study by Kraaijeveld *et al*. (2001), *Drosophila* that had been selected for resistance to larval parasitism around 40 generations beforehand, still showed heightened resistance and correspondingly increased immune mechanisms. It is highly feasible that the same thing is occurring within human populations. In developed countries, increased hygiene, preventative vaccinations and curative medicines may have eliminated many of the lethal risks from infectious diseases, including measles, whooping cough, tuberculosis, scarlet fever and gastroenteritis, which until around 50 years ago were the leading cause of death worldwide (Bickler & DeMaio, 2008). However, the immune mechanisms relating to these challenges, set in place after generations of selection pressures imposed on our ancestors, may still be in place. Therefore, our immune systems may now be mismatched to the level of threat imposed by the modern environment whilst ecologically redundant immune mechanisms may still persist.

A mismatched immune system can lead to increased prevalence of inflammatory, allergic and autoimmune disorders (Parker & Ollerton, 2013), as a lack of pathogenic risk leads to immune hypersensitivity and in some cases also leads to an attack on self-cells and/or tissues. Many allergies and autoimmune disorders, such as inflammatory bowel disease, have only appeared relatively recently and are virtually unseen in developing countries (Bickler & DeMaio, 2008). However, allergic and autoimmune disorders now account for around 54% and 8% of the US population respectively (Arbes Jr *et al.*, 2005, Fairweather *et al.*, 2008), 78% of those suffering from autoimmune disorders are female (Fairweather *et al.*, 2008). In this thesis, Chapter 2 shows how resistant larvae were more likely to exhibit autoimmune responses, shown by an increased formation of melanotic masses.

Deregulation of immune responses can have negative effects in the central nervous system. Parker & Ollerton (2013) hypothesised that a lack of environmental pathogens may be linked to a number of neuroinflammatory-related and/or neurodevelopment diseases, such as autism, schizophrenia, depression, bipolar disorder and migraine headaches. In addition, in a diseased or aged brain, activated microglia show more heightened responses (Perry *et al.*, 2007) and can lead to the aggravation of the pre-existing neurodegeneration (Perry *et al.*, 2007, Holmes *et al.*, 2009). Therefore, if excess immune mechanisms remain, they could lead to exaggerated responses within

ageing individuals, including nervous system function, and thus may be involved in the loss of memory and motor function. If we can determine what these redundant mechanisms might be, then this may provide potential targets for therapeutics.

The crossover between medicine and evolutionary studies is still very much in its infancy (Stearns, 2010), In a recent UK survey, only thirty seven percent of medical schools that responded included evolution in their medicine curriculum (Downie, 2004). However, in order to gain a full understanding of how disease and ageing might work, it is important to that future medical practitioners and researchers understand the limitations in place from natural selection and trade-offs.

6.6 Conclusions

In nature, traits will be selected due to a range of extrinsic pressures, including pathogenic risk. It is therefore important to understand how these factors can impact upon an individual in the short and long-term. This thesis has made important steps in uncovering some of the fundamental links between immune resistance and trade-offs in later life functioning. A trade-off between early resistance against parasitism and lifespan has now been established as well as a positive link between immune resistance and motor function.

The use of a selection-based model system to determine mechanisms underlying trade-offs involved in senescence, shows an alternative facet to the study of evolutionary gerontology (Partridge & Gems, 2007, Downie, 2004); an area in which the costs involved in immune resistance is still rather lacking. Whilst much information in the ageing field so far has come from functional genomic studies (Weinert & Timiras, 2003), there will always be limitations to what they can show due to the complexity of the ageing process, which is most likely to come as a result of a multitude of factors, thus it is important to explore more natural mechanisms underlying senescence (Partridge & Gems, 2007). Selection of a single trait can lead to a range of genetic changes (Wertheim *et al.*, 2011), and therefore shows a more relevant portrayal of how senescence may have evolved in natural systems. It is important to remember, however, that artificial selection based models can also carry limitations. For example, by

selecting individuals on the basis of their ability to mount an encapsulation response, there is a risk of a population bottleneck. Prior to selection only around 5% of the original *Drosophila* populations were able to encapsulate upon single parasitisation. Therefore in the first round of selection approximately 95% of the population would have been lost due to their inability to mount an immune response. It could hence be argued that there is a risk that the flies may end up with reduced genetic variability, as associated with inbred lines. Throughout this study, care has been taken to avoid inbreeding by maximising the number of flies selected and maintaining the flies in large populations. However, it is possible that selection for immunity may have also caused a loss of genes not actively being selected for.

In addition, selection methods such as these can generate a range of genetic and physiological alternations (Wertheim *et al.*, 2010), which make it almost impossible to look for the precise mechanisms underlying the changes that arise in these model systems. This type of model therefore cannot be used to determine exactly which, how or why mechanisms might lead to the trade-offs shown. For example, Drosophila selected for increased resistance to parasitism reveal increased numbers of hemocytes in the larval hemolymph, they also show a number of trade-offs such as reduced longevity and increased adult climbing function. It is not possible to deduce whether these trade-offs arise as a direct effect of the additional larval hemocyte numbers or whether both observations occur simultaneously due to linked or unlinked genetic or physiological changes within the organism. Nonetheless, these limitations should not take away from the fact that this model is a useful tool in determining correlative effects that arise when a population is under pressure to evolve resistance mechanisms.

The use of animal models in ageing research has been questioned (Ricklefs & Scheuerlein, 2001), due to the fact that animals in the wild will die from predation or disease and thus naturally tend not to exhibit ageing phenotypes. However, almost all animals exhibit senescence and intrinsic mortality when taken away from extrinsic risk (Partridge & Gems, 2002, Kirkwood & Austad, 2000), a fact that in itself highlights the ubiquitous nature of ageing and the validity of utilising model systems. As mentioned previously, just 50 years ago pathogenic risk was the leading cause of human mortality (Bickler & DeMaio, 2008). Therefore, ageing and age-related disease in humans are almost certainly trade-offs persisting from a time when humans came under the extrinsic

pressures of the environment at the time. It is of vital importance that as we continue to progress in the field of medicine, we take into account how evolutionary processes that occur and persist throughout all living creatures shape our modern day health.

6.7 Future work

This thesis has revealed a series of trade-offs that occur as a result of increased resistance to parasitism. The next step would be to take this model further to study some of the underlying mechanisms behind some of the phenomena uncovered in this work.

6.7.1 Hemocyte differences in the control and selected lines

One question that arose as a result of this investigation was why the selected lines, which possess greater numbers of circulating hemocytes, show reduced hemocyte recruitment after wounding. A number of possible scenarios that could lead to this finding were discussed in Chapter 2, including: a decreased number of hemocytes available in sessile pools, a reduced recruitment potential from downregulated Basket (Bsk)-dependent Rac1 signalling (Zettervall *et al.*, 2004, Williams *et al.*, 2006) and a greater recruitment of hemocytes at the wound entrance in the selected flies leading to less in circulation. In order to investigate this further, it would be necessary to more accurately determine the quantity and location of all the hemocytes residing in the control and selected larvae, prior to and following a wounding insult. Many previous studies have utilised plasmatocyte-expressed GAL4 driver systems with a UAS-GFP reporter to tag the hemocytes for in situ visualisation (Stofanko *et al.*, 2008, Honti *et al.*, 2010). However, this system is inappropriate for use with our selection based model system, due to the fact that outcrossing the lines severely reduces the immune capabilities of the selected lines (personal communication with Kraaijeveld, A.R). Another possible method is to use antibodies against the specific hemocytes, such as the plasmatocyte marker anti-NimC1, to quantify both circulating hemocytes in addition to those attached to the larval cuticle (quantified by fluorescence microscopy) (Kurucz *et al.*, 2007a, Márkus *et al.*, 2009). Attempting to quantify hemocytes at the wound site without the use of a live imaging in situ GFP tagging system (as previously used by Stamer and colleagues (2005)) would be a more complex task, due to the fact that

melanin begins to deposit at the wound site shortly after hemocytes have reached the wound entrance. Experimentation would be required to produce a suitable approach, for example, the use of PO cascade inhibitors, such as phenylthiourea (PTU), may prevent the formation of melanin around the wound site, which could then be used in conjunction with a hemocyte-antibody staining procedure to visualise and quantify these cells.

6.7.2 Increased melanotic mass formation upon wounding

Melanotic masses can form due to abnormalities in a number of genes and pathways (Minakhina & Steward, 2006). However, it would be difficult to elucidate the precise mechanisms involved in this instance due to the limitations in place within a selection based model (as discussed above in Section 6.6); as such it may not be possible to implicate a precise gene or pathway. It would, however, be interesting to investigate whether the formation of melanotic masses comes at a detriment to the individual, as hypothesised in Chapter 2. By wounding a number of control and selected *Drosophila* and allowing them to develop further, one could then look for phenotypic variations between individuals that were screened positive and negative for the presence of melanotic masses. For example, were there any short term trade-offs (e.g. growth, competitive ability, fecundity) or long term health implications (e.g. in lifespan or stress resistance) that may reduce these individuals' fitness and whether these trade-offs occur differentially between *Drosophila* taken form a control or selected background?

During the duration of this work it was noted that selected individuals occasionally exhibited melanotic masses in the absence of a cutaneous insult; a trait not notably observed in the control lines (personal observation). These masses occurred early on and were visible prior to the L3 larval phase. Once formed, the masses persisted through metamorphosis and into the adult phase. For this thesis, all larvae and adults containing melanotic masses were excluded from experimentation. However, if further work reveals that melanotic mass formation comes at a detriment to the animal's fitness, it would be necessary to quantify the proportion of control and selected *Drosophila* that exhibit a spontaneous melanotic mass phenotype, as this trait may in part be responsible for limiting the degree to which immunity against parasitism can be selected for.

6.7.3 Increased intracellular ROS output in young females

In Chapter 3 we found an increased intracellular ROS output in the 1 week old adult female *Drosophila*. Two theories were proposed as to why this might have occurred. The first theory proposed that immune cells in the selected female may exist at a more metabolically active state than in the less resistant control, perhaps due to increased circulation behaviour. This trait may not occur or occurs to a lesser extent in the resistant males and may not have been detected in the larvae due to a lack of gender-separation in the analysis. In the first instance the same methodology could be repeated in the larvae with separate analysis performed for each sex. However, more specific analysis would be required in order to firmly elucidate differences at a single cell level. This could either be done by measuring the DCF fluorescence levels of a solution with a known number of hemocytes or more specifically measuring the individual oxygen consumption or ROS production of a single cell using specialised non-invasive micro-probes, like those utilised by Peter J. S. Smith's lab on single mammalian cells (Osbourn *et al.*, 2005). However, both of these methods may prove difficult due to the small size of the cells involved; very low levels of metabolism occurring per individual cell may mean that detection thresholds cannot be reached.

The second theory to explain the increased ROS production in the adult females was that there may be differences in the allocation of antioxidants between the larval and adult phases. Wertheim and colleagues (2011) have already shown that larvae selected for resistance against parasitism express increased levels of antioxidant enzymes; catalase, phospholipid hydroperoxide glutathione peroxidase (PHGPx) and a number of glutathione s-transferases (GSTs). Antioxidant levels could be measured in the control and resistant adults (male and female) to establish immune- and sex-related differences. Glutathione levels could be detected in solution using 5,5'-Dithiobis (2-nitrobenzoic acid), also known as Ellman's reagent (Tietze, 1969). Alternatively, antioxidant expression could be quantified in the control and selected adults using polymerase chain reaction methodology.

Hydrogen peroxide (H_2O_2) is a necessary metabolite for many cell functions (Dröge, 2002, Rojkind *et al.*, 2002). It is only at high concentrations that H_2O_2 becomes toxic, leading to protein, lipid and DNA damage (Driessens *et al.*, 2009). The next step might therefore be to examine whether or not this transient increase in ROS in the young adult females is sufficient to induce damage in the selected individuals. In order to achieve this one could test the degree of protein oxidation in young adult females to determine whether the difference in ROS in the young female leads to corresponding protein damage that may accelerate the ageing processes. This could be done using the OxyBlot Protein Oxidation Detection Kit (Millipore) or by regularly weighing the *Drosophila* to assess immune-related differences in total body mass with age.

6.7.4 Reduced longevity in adult females

Processes that link to increased senescence are of particular interest in today's academic focus. In this thesis, we showed how resistant females display reduced longevity in comparison to their control counterparts. One variable that was not considered in this study was the impact of disparate immune-related male mating on the female longevity. Therefore, in order to remove this potential variable it would be necessary to repeat the lifespan experiment with females from a control and selected background mated males from a consistent immune background, for example all females mated with control males. This would ensure that differences between males e.g. in sexual behaviour and/or seminal fluid content are not acting as confounding variables.

Further work could be performed to elucidate some of the questions posed during this research, for example why do we see gender-specific trade-offs between immune resistance and lifespan? One hypothesis as to why resistant males show no trade-off in lifespan is that they require fewer resources than the females and thus resource allocation is not a limiting factor. This theory could be tested either by increasing the level of resources used in other fitness traits, e.g. by allowing the males to continue mating, or by limiting the nutrients available in the food supply. Partridge and Andrews (1985) showed that males kept with females had a shorter lifespan than those that had been allowed to mate briefly then stopped from mating.

The model utilised in this study was effective at determining trade-offs that occur due to immunocompetance during development. However, in order to establish the effects of maintained immunocompetance it might be necessary to set up a new model system against a pathogenic risk imposed during the adult phase. In conjunction to the results from this thesis, we could then begin to unravel how resistance can impact on the ageing organism, giving deeper understanding into the complexities of disease and ageing.

6.7.5 Altered motor function in larvae and adult females

In Chapter 5, *Drosophila* from a background with increased resistance against larval parasitism were shown to exhibit altered motor behaviour; decreased turning in the larvae and increased climbing velocities in the adult female. It was hypothesised that this may be due to altered interactions with the hemocytes during development or changes in genetic expression (e.g. in *gcm, gcm2* or *Draper*). Although it might not be possible to determine which of these processes is directly responsible, it would be interesting to investigate whether the changes shown in motor function are related to alterations in the nervous system. In order to test this theory, one could use a series of whole mount dissections to look for variations in the nervous systems of control and resistant *Drosophila*. Boerner and Duch (2010) prepared whole mount dissections in a number of 'average' *Drosophila* labelled with the nc82 antibody for detection of the Bruchpilot protein (Brp). These VNC preparations were then scanned using a laser scanning microscope in order to attain image stacks from which they could generate a standard 3D atlas for the *Drosophila* VNC. This atlas is now publically available and can be used to provide a reference against which changes in VNC structure can be compared (Boerner & Duch, 2010).

7 Appendix

This appendix includes a series of tables to show the individual line means (C/S1-4) for all the significant results found throughout this thesis. Averages and standard error values have also been given for control and selected lines.

Appendix Table 1: Line means for Figure 2.6 (wounded and unwounded) and Figure 3.1 (unwounded only)

Larval hemocyte counts: *Number of circulating hemocytes in larvae taken from low resistance (control lines) and high resistance (selection lines) backgrounds, with (b) or without (a) a prior wounding insult.*

a) Unwounded

Control line	*Hemocyte count*	*Selection line*	*Hemocyte count*
C1	3161	**S1**	3827
C2	3003	**S2**	3946
C3	3817	**S3**	4526
C4	3189	**S4**	3509
Average (± SE)	**3292.5 (± 179.6)**	**Average (± SE)**	**3952 (± 212.4)**

b) Wounded

Control line	*Hemocyte count*	*Selection line*	*Hemocyte count*
C1	6501	**S1**	6050
C2	5714	**S2**	5704
C3	6652	**S3**	5610
C4	6014	**S4**	5746
Average (± SE)	**6220.3 (± 216.8)**	**Average (± SE)**	**5777.5 (± 95.2)**

Appendix Table 2: Line means for Figure 2.11

Larval wounding: Proportion showing wound melanisation: *Proportion of wounded larvae showing melanisation at the wound site in control and resistant (selection) Drosophila.*

Control line	*Proportion displaying wound*	*Selection line*	*Proportion displaying wound*
C1	0.979	**S1**	1.000
C2	0.953	**S2**	0.975
C3	0.880	**S3**	0.966
C4	0.836	**S4**	0.934
Average (± SE)	**0.912 (± 0.033)**	**Average (± SE)**	**0.969 (± 0.014)**

Appendix Table 3: Line means for Figure 2.13

Larval wounding: melanotic masses: *a) Proportion of wounded control and selection Drosophila larvae showing melanotic masses and b) the average area of melanotic masses per individual.*

a)

Control line	*Proportion displaying mass*	*Selection line*	*Proportion displaying mass*
C1	0.354	**S1**	0.522
C2	0.418	**S2**	0.725
C3	0.213	**S3**	0.379
C4	0.291	**S4**	0.492
Average (± SE)	**0.319 (± 0.088)**	**Average (± SE)**	**0.529 (± 0.144)**

b)

Control line	*Area of mass* $(x10^3\ \mu m^2)$	*Selection line*	*Area of mass* $(x10^3\ \mu m^2)$
C1	5.66	**S1**	6.35
C2	6.15	**S2**	13.75
C3	4.12	**S3**	6.03
C4	2.52	**S4**	5.08
Average (± SE)	**4.61 (± 0.82)**	**Average (± SE)**	**7.80 (± 2.00)**

Appendix Table 4: Line means for Figure 2.14a

Adult wounding: Proportion showing wound melanisation (males only): *Average proportion of singly-wounded adult male Drosophila from a control and selection background showing melanisation at the wound site. Averages taken from each of the lines (C/S1-4) across all time points (0, 0.5, 1, 2, 3, 6, 12 & 24 hours).*

Control line	*Average proportion exhibiting melanised wound*	*Selection line*	*Average proportion exhibiting melanised wound*
C1	0.213	**S1**	0.263
C2	0.325	**S2**	0.288
C3	0.500	**S3**	0.250
C4	0.500	**S4**	0.233
Average (± SE)	**0.384 (± 0.071)**	**Average (± SE)**	**0.258 (± 0.011)**

Appendix Table 5: Line means for Adult longevity (Chapter 3)

Female longevity: *Average lifespan of adult female Drosophila from a control and selection background (excluding censored data).*

Control line	*Average lifespan (days)*	*Selection line*	*Average lifespan (days)*
C1	70.22	**S1**	57.39
C2	86.56	**S2**	87.31
C3	89.45	**S3**	78.67
C4	80.41	**S4**	66.94
Average (± SE)	**81.66 (± 4.25)**	**Average (± SE)**	**72.58 (± 6.56)**

Appendix Table 6: Line means for Figure 3.4a) (without treatment only) and Figure 4.4 (without treatment and paraquat treated)

Adult cell metabolism/ROS output: *Percentage increase in DCF fluorescence in 1 week old female Drosophila from control and selection backgrounds, with (b) and without (a) treatment with the toxin paraquat.*

a) Without treatment

Control line	*% increase*	*Selection line*	*% increase*
C1	382.5	**S1**	435.2
C2	279.5	**S2**	431.7
C3	438.6	**S3**	473.9
C4	392.4	**S4**	467.0
Average (± SE)	**373.2 (± 33.6)**	**Average (± SE)**	**452.0 (± 10.8)**

b) Paraquat treated

Control line	*% increase*	*Selection line*	*% increase*
C1	474.2	**S1**	516.3
C2	361.6	**S2**	446.3
C3	507.0	**S3**	543.4
C4	565.7	**S4**	521.7
Average (± SE)	**477.2 (± 42.9)**	**Average (± SE)**	**506.9 (± 21.0)**

Appendix Table 7: Line means for Figure 5.5d)

Larval movement: open field arena: *Relative meander (turns per distance moved) = number of degrees deviation from a straight line made by the selected larvae per cm moved relative to the control larvae.*

Control line	*Relative meander (turns per distance moved)*	*Selection line*	*Relative meander (turns per distance moved)*
C1	0.993	**S1**	0.996
C2	0.973	**S2**	0.915
C3	1.011	**S3**	0.982
C4	1.023	**S4**	1.000
Average (± SE)	**1.00 (± 0.01)**	**Average (± SE)**	**0.97 (± 0.02)**

Appendix Table 8: Line means for Figure 5.7

Adult female climbing ability: *Average climbing velocity in female Drosophila from a control and selection background (measured during first 4 seconds following a mechanical shock stimulus). Averages taken from each of the lines (C/S1-4) across all ages assayed (5 - 47 days old).*

Control line	*Average climbing velocity (mm/s)*	*Selection line*	*Average climbing velocity (mm/s)*
C1	2.70	**S1**	3.16
C2	2.74	**S2**	3.56
C3	2.62	**S3**	3.17
C4	2.57	**S4**	2.48
Average (± SE)	**2.66 (± 0.04)**	**Average (± SE)**	**3.09 (± 0.23)**

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