UNIVERSITY OF STIRLING

IMPACT OF MITOCHONDRIAL GENETIC VARIATION AND IMMUNITY COSTS ON LIFE-HISTORY TRAITS IN DROSOPHILA MELANOGASTER

SUMAYIA BASHIR-TANOLI

BIOLOGICAL AND ENVIRONMENTAL SCIENCES

SCHOOL OF NATURAL SCIENCES

UNIVERSITY OF STIRLING

A thesis submitted in partial fulfillment of the degree of Doctor of Philosophy

November 2014

Abstract

Immune activation is generally acknowledged to be costly. These costs are frequently assumed to result from trade-offs arising due to the reallocation of resources from other life-history traits to be invested in immunity. Here, I investigated the energetic basis of the costs associated with immune activation in *Drosophila melanogaster*. I found that immune activation significantly reduced fly fecundity (45%) and also caused a decline in metabolic rate (6%) but had no effect on body weight. To understand the factors behind reduced fecundity and metabolic rate I measured feeding and found that food intake was reduced by almost 31% in immune-challenged *D. melanogaster*. These findings suggest that fecundity costs of immune activation result not from the commonly accepted resource reallocation hypothesis but probably because resource acquisition is impaired during immune responses.

The individuals of any animal population generally vary greatly in their ability to resist infectious disease. This variation arises due to both environmental heterogeneity and genetic diversity. Genetic variation in disease susceptibility has generally been considered to lie in the nuclear genome. Here, for the first time, I explored the influence of mitochondrial genetic (mtDNA) variation on disease susceptibility. I crossed 22 mitochondrial haplotypes onto a single nuclear genome and also studied epistasis interactions between mitochondrial and nuclear genomes (mitonuclear epistasis) by crossing five haplotypes onto five different genetic backgrounds. I found that fly susceptibility to *Serratia marcescens* was influenced significantly by mtDNA allelic variation. Furthermore, the effect of mitonuclear epistasis on fly susceptibility to *S. marcescens* was twice as great as

the individual effects of either mitochondrial or nuclear genome. However, susceptibility to *Beauveria bassiana* was not affected by mtDNA allelic variation. These findings suggest the mitochondrial genome may play an important role in host-parasite coevolution.

The Mother's Curse hypothesis suggests that sex-specific selection due to maternal mitochondrial inheritance means that mitochondria are poorly adapted to function in males, resulting in impaired male fitness. Mother's Curse effects have previously only been studied for two phenotypic traits (sperm-infertility and ageing) and their generality for broader life-history has not been explored. I investigated the impact of mtDNA allelic variation on 10 phenotypic traits and tested whether the patterns of phenotypic variation in males and females conformed to the expectations of the Mother's Curse hypothesis. I found that seven of the 10 traits were significantly influenced by mtDNA allelic variation. However, there was no evidence that the effects of this variation differed between males and females. I therefore concluded that Mother's Curse is unlikely to be a general phenomenon, nor to provide a general explanation for sexual dimorphism in life-history traits.

Overall, this thesis explored the impacts of immunity costs, mitochondrial genetic variation, mitonuclear epistasis and sex-specific mitochondrial selection on *D. melanogaster* life-history.

Declaration of authorship

I, Sumayia Bashir-Tanoli, declare that I have composed this thesis and conducted all the relevant research work and experimentation. I confirm, that it embodies the results of my own research. However, I acknowledge, where appropriate, the nature and amount of work carried out in collaboration with others.

Signed ....

Date ....

Acknowledgements

I use this opportunity to express my appreciation to everyone who supported me during my PhD; I am beholden for their inspiring direction, constructive criticism and friendly advice throughout my PhD work. I am sincerely grateful to them for sharing their truthful and illuminating views on various issues related to this study.

Initially I would like to acknowledge Almighty Allah (swt) for giving me the strength and courage to complete my PhD research and to produce this thesis, this PhD would not be possible without His kind support. After that I am grateful to my principal supervisor Dr Matthew Tinsley for letting me work under his thoughtful guidance. Thank you Matt for your enormous support throughout my learning: without the perfect balance of your trust in me and positive criticism to do better I would not have been able to complete this colossal process. I am also obliged to Dr Luc Bussière for his support during my study, especially his suggestions for statistical analysis of my data. I also give thanks to Prof. Phil Wookey and Dr Jens-Arne Subke for letting me use their lab facilities for measurements of metabolic rate.

I would like to express my sincere appreciation to all my colleagues for their friendly suggestions and support during the course of my studies. Particularly, Dr Tom Houslay, Dr Danielle Mackenzie, Karlien Gootzen, Dr Stuart Auld, Dr Andrew Dobson, and Rebecca Fellows for their kind support. I am especially indebted to my lovely friend Dr Lislie Solis-Montero for her encouragement and support during tough and rough times of my PhD and listening to my ‘inner thoughts’ over a cup

of coffee and it always worked as a magical remedy. Along with all these people I could not forget to acknowledge the essential contribution of the Higher Education Commission (HEC) Pakistan, and the University of Stirling for providing financial support to my PhD.

I cannot finish without acknowledging my parents, especially my beloved late father Muhammad Bashir, who was not only the source of inspiration for me but was actually the man behind this PhD, alas now he is not with me to share the moment. Finally, I am very grateful to my husband Usman Ahmed Khan, not only for washing fly food bottles and counting fly mortality with me during my late night experiments but also for his enormous love and support throughout this study.

Table of Contents

Chapter 1: General Introduction 1

1.1. General features of invertebrate immunity 2
1.2. Costs of immunity 11
1.3. Natural variation in disease susceptibility 18
1.4. The biology of mitochondria
1.5. Characteristics of mtDNA 25
1.6. Mitochondrial-nuclear epistasis 34
1.7. Mother's Curse 35

Thesis overview 38

Chapter 2: Immune response costs are associated with changes in resource acquisition and not resource reallocation 40

2.1. Abstract 40
2.2. Introduction 42
2.3. Materials and methods 47
2.4. Results 53
2.5. Discussion 60

Chapter 3: Disease susceptibility is influenced by mitochondrial DNA genetic variation and mitochondrial-nuclear epistasis 67

3.1. Abstract 67
3.2. Introduction 69
3.3. Materials and methods 73
3.4. Results 77
3.5. Discussion 85

Chapter 4: Sex-specific phenotypic effects of mitochondrial genomic variation: a test of the Mother’s Curse Hypothesis 93

4.1. Abstract 93
4.2. Introduction 95
4.3. Materials and methods 100
4.4. Results 109
4.5. Discussion 125

5. Chapter 5: Discussion 134

5.1. Costs of immune activation: resource reallocation or resource acquisition? 134
5.2. The influence of mtDNA allelic variation on disease susceptibility 138
5.3. The significance of the phenotypic non-neutrality of mtDNA polymorphisms for mitochondrial evolution 142
5.4. No evidence for Mother’s Curse affecting sexual dimorphism in *D. melanogaster* 145
5.5. Conclusion 148

6. References 149

Chapter 1: General Introduction

In this section I review the literature to discuss the mechanisms and importance of the innate immune system in invertebrates. I describe the costs that are associated with activating the immune response during infection, with particular focus on the mechanisms underpinning these costs. The factors influencing variability amongst individuals in natural populations to resist pathogenic attack will also be discussed, with particular emphasis on host-parasite genetic variation, environmental heterogeneity and their interactions. Energetic demands may play a role shaping the performance of the immune system and other biochemical/physiological processes. I move on to discuss the mitochondrion, its unique genome and its influence on phenotypic variability in various life-history traits. Finally I introduce the theory of why male fitness may be compromised due to maternal inheritance of mitochondrial DNA (mtDNA).

1. The immune system and immune responses

The immune system is essential to defend an organism against potentially pathogenic attack from a wide range of microbes and other parasitic organisms. Immune responses can broadly be divided into innate immune responses and acquired immune responses (Janeway *et al*. 2001). Innate immunity ranges from protection by barrier epithelia, through to complex responses such as phagocytosis for clearing invading microbes. The vertebrate acquired or adaptive immune response displays highly specific pathogen recognition by specialised immune cells and the molecules they secrete. Both responses involve homoral

and cell-mediated immune system components. Invertebrates solely rely on the innate arm of the immune system and lack adaptive immunity; whereas vertebrates depend on both innate and acquired immune response for their defence against pathogenic attack (Janeway *et al*. 2001).

There is strong homology between vertebrate and invertebrate innate immune systems (Hoebe *et al*. 2004). Most of these homologies are found in the signalling pathways/receptors such as the Toll molecule (Hoffmann 2003). A wide variety of invertebrate model organisms, such as earthworms, oysters, horseshoe crabs, beetles and the fruit fly *D. melanogaster* have been employed in immunological research, although of these, research on the insect immune system is most advanced (Yamaura *et al*. 2008, Cavaliere *et al*. 2010, Valanne *et al*. 2011). Strong immunological homology across organisms' means that research with generalised significance for human and animal health can be performed on many organisms in addition to the most widely used animal model, the mouse.

1.1. General features of invertebrate immunity

The innate immune system is a general and ancient form of host defence, which provides the first line of inducible protection against pathogenic attack by bacteria, fungi and viruses (Hoebe *et al*. 2004). For vertebrates, the innate immune response not only protects directly against pathogens but also preceded and guides the adaptive immune response (Beckage 2011). Invertebrate innate defensive mechanisms involve physical barriers (epithelia), local immune molecule synthesis, systemic humoral responses and specialised immune cells

(haemocytes) (Medzhitov and Janeway 1997). Epithelia provide the front line of defence against pathogens: the lining of the tracheae, malpighian tubules and reproductive tract all produce antimicrobial peptides (AMP) that suppress and manage microbial growth; however, the highest concentration of AMP is produced by the fat body and secreted into the haemolymph (Beckage 2011).

1.1.1. Cellular and humoral immune responses

Microbes and other macroparasites that are not excluded by front line immune defences and successfully enter the haemocoel are then dealt with by both the cellular and humoral responses. The cellular response is based on coagulation, agglutination, encapsulation and phagocytosis of parasites. Whereas, humoral immune responses consist of AMP production, synthesis of lectins and activation of the pro-phenoloxidase (proPO) system. However, it is difficult to perfectly discriminate humoral and cellular immune system components because humoral factors also influence cellular defence and vice versa. Thus, insects are capable of mounting a multi-faceted immune response directed at the type of parasitic organism that needs to be attacked (Lemaitre and Hoffmann 2007, Beckage 2011).

1.1.2. Coagulation

Invertebrates possess an open circulatory system; therefore, coagulation is not only important to prevent blood loss but also to contain microbes that have entered the host body through wounds (Theopold *et al*. 2004, Dushay 2009). In general, coagulation is triggered by factors released from injured tissues and/or foreign material, which initiate biochemical changes in plasma proteins. In some animals, due to their low levels of plasma proteins, the blood cells form a thick cellular clot by adhering to each other, for example coelomocyte clots in sea urchins and *Themiste petricola* (a marine worm) (Hillier and Vacquier 2003, Lombardo and Blanco 2012). However, many invertebrates display true coagulation that is triggered by haemocyte-derived factors, for example freshwater crayfish and horseshoe crabs (Kawabata and Muta 2010). Lipophorin is a common coagulation factor in insects: apolipophorins I and II have been identified as putative coagulation factors in *Drosophila* larva (Scherfer *et al*. 2004). The immune function of coagulation is well demonstrated in many invertebrates including horseshoe crabs (Isakova and Armstrong 2003) and *Drosophila* (Bidla *et al*. 2005) this highlights the importance of coagulation as a basic and well defined immune process across the animal kingdom.

1.1.3. Agglutination

Agglutination is an immune mechanism that triggers pathogen aggregation by binding pathogen-associated molecules with host immune molecules, thereby preventing microbial spread within the body. In invertebrates lectins and cytokines are involved in the agglutination of microbes. Lectins are glycoproteins that are divided into many types based on their structure and function. Lectins involved in immune defense can be soluble or surface bound and are found in circulating haemocytes and in the haemolymph (Malagoli *et al*. 2010). The C-type lectins and 'Pentraxin' lectins are common in invertebrates and play a crucial role in innate immunity (Wang *et al*. 2011). Another lectin, galectin, occurs in *D. melanogaster* and *Anopheles gambiae* (Pace and Baum 2002). Several cytokine-like molecules are found in invertebrates such as molluscs, annelids, tunicates, echinoderms and insects (Malagoli *et al*. 2010). A variety of structural and functional studies have demonstrated that these invertebrate cytokines are homologues of mammalian cytokines, for example interleukin-2 (IL-2), interleukin-8 (IL-8) and growth factors (Franchini *et al*. 1996, Ottaviani *et al*. 2004). These cytokine-like molecules are involved in many immunological functions, such as triggering agglutination, phagocytosis, cytotoxicity, chemotaxis, wound repair and cell death (Ottaviani *et al*. 2004).

1.1.4. Phagocytosis and encapsulation

Most invertebrates have a population of immune cells that assist in clearing infection (Williams 2007). Cell morphology and classification varies considerably between different invertebrate groups. In *D. melanogaster* there are four cell types that defend against foreign invaders. 1) Plasmatocytes, which phagocytose microbes, have functional similarity to macrophages and make up 95% of circulating haemocytes. 2) Crystal cells, which constitute nearly 5% of circulating haemocytes and are involved in melanization. 3) Lamellocytes, which are rarely found in healthy larvae, differentiate in response to parasitism and are responsible for encapsulation. 4) Prohaemocytes, which are present in all larvae and are the precursors of the mature haemocyte classes (Williams 2007, Wood and Jacinto 2007). Encapsulation is a common invertebrate defence mechanism against multicellular parasites that are too large to be phagocytized. The lamellocytes encapsulate multicellular parasites and release cytotoxic products, such as free radicals and degradative enzymes, to destroy the foreign body (Batista *et al*. 2009, Siddiqui and Al-Khalifa 2014). During invertebrate phagocytosis the recognition of non-self invaders is achieved by either lectins, soluble factors present in plasma (which play a role as opsonins) and/or other membrane bound receptors to trigger engulfment. Over four decades ago the role of serum proteins as opsonins was documented by Mckay and Jenkin (1970); using an in-vitro phagocytosis study they showed that crayfish haemocytes phagocytosed erythrocytes more rapidly when they were incubated with crayfish serum, compared to those that were not exposed to serum. In *Drosophila* the transmembrane protein eater was shown to be involved in bacterial phagocytosis: studies in a haemocyte-derived cell line

demonstrated that silencing transcription of the gene *eater* caused significantly reduced bacterial binding and engulfment (Kocks *et al.* 2005).

1.1.5. Humoral immunity

The humoral immune response is characterized by the systemic production of a variety of antimicrobial peptides. These antimicrobial peptides are produced by the fat body (an organ equivalent to mammalian liver) and secreted into the haemolymph; in infected flies the combined concentration of antimicrobial peptides can reach 300µM (Boman 2003). Seven antimicrobial peptide gene families have been found in *D. melanogaster*: diptericin, drosocin, defensin, attacin, cecropin, drosomycin and metchnikowin**.** The promoter sequence for all these antimicrobial peptide genes contains $_{K}$B sites that are the binding site for transcription factors of the NF-$_{K}$B/Rel family: factors that also play an important role in mammalian inflammatory responses (Hoffmann *et al.* 1999, Hoffmann 2003). Mostly these antimicrobial peptides consist of 15-45 amino acid residues and contain a positive net charge. The expression of antimicrobial peptide genes depends on the nature of microbial attack; antifungal peptides are dominant during fungal attack, whereas antibacterial peptides are strongly induced by bacterial pathogens. Drosocin and defensin exhibit anti-bacterial activity and provide defence against gram-negative and gram-positive bacteria respectively (Diamond *et al.* 2009)**.** Drosomycin is exclusively associated with antifungal defence, however, cecropin and metchnikowin are effective against both fungal and bacterial pathogens (Diamond *et al.* 2009)**.** Attacin and diptericin are close homologues of related antibacterial peptides in other insects (Diamond *et al.* 2009). *D. melanogaster* mutants that

are unable to produce antimicrobial peptides are more susceptible to microbial infection: it was demonstrated that a mutation in the 18-wheeler receptor (a *D. melanogaster* protein related to IL-1R) makes larvae more vulnerable to infection (Williams 1997). Thus, antimicrobial peptides are crucial in invertebrate innate immune defence.

1.1.6. The phenoloxidase cascade

In addition to antimicrobial peptide production, humoral defence in invertebrates depends strongly on the activation of the ProPO cascade. The *Drosophila* genome codes for three forms of Phenoloxidase (PO); out of these, two (PO1 and PO2) are produced by crystal cells as a zymogen or prophenoloxidase (ProPO1 and ProPO2), which are then converted into active PO by the action of ProPO-activating enzyme. Activated PO catalyses the oxidation of phenolic compounds to quinones: these have joint roles in pathogen killing and, following polymerisation into melanin, in wound healing. Recently, it was demonstrated in *Drosophila* larvae that all PO activity in the haemolymph is down to ProPO1 and ProPO2: ProPO1 is responsible in early delivery of PO and ProPO2 is stored in crystal cells to be provided for later-phase responses. Both of these ProPO forms play a crucial role in survival following bacterial and fungal infections (Binggeli *et al.* 2014). Mutations in the β-1, 3-glucan recognition protein receptor, which initiates melanisation via the ProPO cascade after binding β-1, 3-glucan on the surface of fungal cells, significantly reduced survival following fungal and bacterial infection in *Locusts* (Zheng and Xia 2012).

1.1.7. Immune recognition in *D. melanogaster*

The recognition step is the hallmark of immune system; immune cells and soluble receptors differentiate between self and non-self, recognising foreign intruders and efficiently inducing immune responses against them. In invertebrates recognition of foreign intruders is carried out by microbial recognition receptors (MRR) such as peptidoglycan recognition proteins (PGRPs) and gram-negative binding proteins (GNBPs) (Kim 2000, Werner 2000).

1.1.8. Toll and IMD signalling pathways

The involvement of the Toll signalling pathway in *Drosophila* immunity was identified in 1996, when it was demonstrated that induction of drosomycin requires a wild-type Toll transmembrane receptor (Lemaitre *et al*. 1996). The Toll pathway is predominantly triggered by PGRP receptors following infection by gram-positive bacterial or fungal infections. The Toll receptor shares structural and some functional homology with the mammalian Toll-like receptors (Hoebe *et al*. 2004). The Toll receptor is a transmembrane protein, the extracellular domain consist of lucine-rich repeats and the intra-cytoplasmic region demonstrates high sequence homology with part of the interleukin 1 receptor (IL-1R) and is known as a Toll/IL-IR (TIR) domain (Valanne *et al*. 2011). Spätzle (a cystine-knot cytokine-growth factor) functions to activate the Toll pathway (Mizuguchi *et al*. 1998).

The IMD pathway is primarily stimulated by GNBPs, mainly in response to gram-

negative bacterial infection and is a relish-dependent pathway (Hetru and Hoffmann 2009). The gene product of IMD is a 25-kD protein comprising of a death domain which has close homology with mammalian tumor necrosis factor alpha (TNF-α) receptor interacting protein (Georgel *et al.* 2001). The transcription of some antimicrobial peptides is driven by both Toll and IMD signalling pathways, such as drosomycin.

Once a bacterium or fungus enters the fly's body the innate immune response is activated by either PGRPs or GNBPs, recognising the repetitive microbial surface determinants which are conserved in microbes but not found in the host genome, such as peptidoglycans, β glucans and manan (Hetru and Hoffmann 2009). Upon recognition of infection the host receptors activate the appropriate signalling cascade for the transcription of effectors molecules to target that particular invader.

In all organisms an efficient immune system is crucial to defend against infections. However, mounting an effective immune response is costly. Our understanding of the immune system, its mechanism and functionality is considerably more advanced compared to our knowledge of immune system costs and the mechanisms underlying these costs. Nevertheless, adequate understanding of the mechanistic basis of immune costs is important because the optimum level of defence depends on the balance between benefits and costs.

1.2. Costs of immunity

The optimal degree to which an organism should invest in parasite defence is shaped by the risk of being attacked, the virulence of infections, the efficiency of defence responses, and the magnitude of the costs of these responses. Immune system costs can be divided into two types. First, those related to constitutive immunity: costs associated with forming, maintaining and indeed evolving the immune system. These costs arise through negative genetic correlations between the immune response and other life-history traits (Rolff and Siva-Jothy 2003), and need to be paid even in the absence of infection. Selection for increased encapsulation ability against parasitoids has been shown to result in such constitutive costs, reducing larval competitive ability (Kraaijeveld and Godfray 1997) and lowering survival rates (Fellowes *et al*. 1998). The second type of cost is related to inducing the immune response upon infection (Ahmed *et al*. 2002, Derting and Compton 2003, Schwartz and Koella 2004, Graham *et al*. 2011). For example, in *D. melanogaster* reduced longevity was associated with increased resistance against *Pseudomonas aeruginosa* infection (Yixin *et al*. 2009). The costs related to induced immunity are only paid following pathogen attack, thus selection to minimise their magnitude may be weaker than for constitutive costs. However, these costs have proven difficult to quantify experimentally for various reasons, such as the nature of the pathogen, timing of immune responses (Fellowes and Travis 2000, Schwartz and Koella 2004), and difficulty in experimental manipulation of defence level in the field or the laboratory (Graham *et al*. 2011).

There are two potential mechanisms that might explain the costs of activating the immune response: firstly, the energetic (or resource) costs of immune molecule and cell synthesis; secondly, the non-energetic costs associated with pleiotropic effects of immune system activity. Life-history theories commonly propose that immune activation costs are mainly energetic or nutritional, and involve the transfer of energy or reallocation of resources between the immune system and other fitness-related traits (Freitak *et al*. 2003, Schwartz and Koella 2004, Boggs 2009). In addition, specific non-energetic costs may also apply to the activated immune system: molecules produced to attack parasites may cause collateral damage to host tissues, and many immune responses lead to acute or chronic inflammation (Sitkovsky and Ohta 2005, Licastro *et al*. 2005, Sadd and Siva-Jothy 2006). Innate immune effectors such as haemolymph cells and phenoloxidase (PO) defend against microbial attack but cause self-harm as well (Sadd and Siva-Jothy 2006). The non-energetic costs of activated immune response have been studied in invertebrates (Markovic *et al*. 2009, Eleftherianos and Castillo 2012) as well as in vertebrates (Svensson *et al*. 1998). Reactive oxygen species and other free radicals produced by activated immune cells (phagocytes) can cause severe damage to biomolecules such as lipids, proteins and nucleic acids (Splettstoesser and Schuff-Werner 2002, Halliwell 2005).

1.2.1. Resource-based mechanisms underlying the costs of immune activation

If resources are invested in one physiological function such as immunity, they will be unavailable for other processes. This life-history hypothesis, termed resource reallocation, is principally based on the transfer of energy and resources between life-history traits depending on the demands placed on an organism. For example, during immune activation energy or resources may be drawn away from other non-essential traits to fuel the immune response and help fight against a parasitic infection (Freitak *et al*. 2003, Boggs 2009). A decrease in survival of bumblebees was observed upon immune activation triggered by lipopolysaccharide (LPS) and micro-latex beads, which was exacerbated by nutritional limitation (Moret and Schmid-Hempel 2000). Resource reallocation underpinning immune activation costs may also result from competition between physiological processes for limiting bio-molecules, as well as from energetic resource constraints. For example, immune function and flying are both energy demanding life-history traits that compete for the protein apolipophorin III (apoLpIII) in crickets (Adamo *et al*. 2008). Similarly, the shared role of PO in immune defence and creating a sexually selected cuticular melanisation pattern in damselflies results in a potential trade-off between immunity and reproductive success (Siva-Jothy 2000).

An alternative paradigm to explain resource-mediated life-history costs is that investment in one trait may impair the ability of the organism to acquire resources to fuel other physiological processes. The resource-reallocation vs resource acquisition framework has been used to provide general explanations for phenotypic diversity between individuals, as well as the presence and

absence of trade-offs between life-history traits (van Noordwijk and de Jong 1986, Reznick *et al.* 2000). Impaired resource acquisition, for example reduced food intake, in immune-challenged individuals could be a primary factor that limits function of other life-history traits during immune responses.

Infection-induced anorexia is a phylogenetically conserved general response following pathogen attack, where food intake is reduced during the time an individual is mounting an immune response (Scrimshaw 1991). These decreases in food intake even occur following mild immune challenges related to vaccination (Gandra and Scrimsha 1961). This leads to energy depletion, during which catabolic responses in the host start to break down large molecules like lipids, proteins and nucleotides to release energy to fuel energetic demands. There are several hypotheses to explain the adaptive significance of the infection-induced anorexia response. One states that reduced feeding enhances host survival by increasing host tolerance to parasite infection (that is, the ability of infected hosts to limit the fitness costs of infection) (Exton 1997, Ayres and Schneider 2009, Medzhitov *et al.* 2012). A second hypothesis suggests that infected hosts become more selective in their feeding habits to diminish the risk of further oral infections (Kyriazakis *et al.* 1998).

There are two main approaches to measure these energetic costs of immunity. First, the indirect approach: investigating the trade-offs between immunity and other physiological systems such as reproduction, growth and life span. Second, the direct approach: by making metabolic measurements that take energetic parameters as a potential currency for the cost of immunity (e.g. metabolic rate).

The details of these approaches are discussed below.

1.2.2. The indirect approach to measure the energetic cost of immunity

Triggering the immune response alters other life traits, such as reproduction, growth, food intake and ageing (Kraaijeveld *et al*. 2001, Ahmed *et al*. 2002, Schwartz and Koella 2004, Zerofsky *et al*. 2005). Furthermore, experimentally manipulating the investment of animals in non-immune parameters has been shown to reduce immune function (Siva-Jothy *et al*. 1998, Fedorka *et al*. 2004). Alternations in fecundity can be seen as the fundamental cost of immune upregulation. Organisms utilise a major proportion of their energy budget in growth, maintenance and reproduction (Kearney 2012). Immune upregulation either against infectious agents (Gwynn *et al*. 2005) or immune activators (Ahmed *et al*. 2002) can significantly reduce host fecundity and growth. In female *Drosophila*, immune challenge with *Escherichia coli* and *Micrococcus luteus* triggered reduced fecundity through innate immune activation. Females receiving the same treatments but with essential immune pathways inactivated (IMD and relish deficient) did not display fecundity declines, confirming that fecundity costs originated from immune activation (Zerofsky *et al*. 2005).

Ahmed *et al.* (2002) reported reduced yolk protein content in the ovaries and lower egg output following injection of LPS in *Anopheles gambiae*. Mouatcho *et al*. (2011) found that the fungal infections *B. bassiana* and *Metarhizium anisopliae* caused reproductive costs in *Anopheles funestus.* Female *D. melanogaster*

artificially selected for ability to survive *P. aeruginosa* infection over 10 generations showed stronger Toll and IMD immune responses at the cost of shortened life span and reduced larval viability (Yixin *et al*. 2009). In addition to this, there is evidence that when animals undertake physiologically challenging activities this can cause a decline in the efficiency of the immune system. For example, increased reproductive activity (copulation and oviposition) caused reduced rates of encapsulation in damselflies (Siva-Jothy *et al*. 1998); in male crickets decreased PO-activity and impaired resistance to bacterial infections were correlated with reproductive activities (Adamo *et al*. 2001).

The trade-off between immunity and other life-history traits has been observed in higher animals as well as in invertebrates. In house sparrows (*Passer domesticus*), LPS injection resulted in a significant reduction in feeding rate, body mass and reproductive success (Bonneaud *et al*. 2003). House sparrows also suffer compromised feather regrowth following immune challenge (Martin 2005), which may mean that passerines with elevated immune system activity show reduced development of sexual ornaments. Studies in pied flycatchers (*Ficedula hypoleuca)* (Ilmonen *et al*. 2000) and blue tits (*Parus caeruleu)* (Råberg *et al*. 2000) have also shown trade-offs between immune activation and reproductive traits. In mice, adult immune challenge resulted in reduced mass of the testes and small intestine (Derting and Compton 2003); similarly, reduced somatic and reproductive tissue growth was reported in peripubertal Siberian hamsters (Prendergast *et al*. 2004) and Western bluebirds (*Sialia sialis*) (Fair and Myers 2002).

1.2.3. Direct approaches to measure the energetic costs of immunity

If immune responses are energetically costly to maintain or mount then it should be possible to make direct measures of altered metabolism. However this phenomenon is rarely studied in either vertebrates or invertebrates; thus far only a few studies have taken this approach. The implantation of nylon filaments into pupae of white cabbage butterfly to induce an encapsulation response increased their standard metabolic rate by 8% compared to controls (Freitak *et al*. 2003). Antibody production against a non-pathogenic immune stimulus (sheep red blood cells) increased basal metabolic rate (BMR) by 8.5% in immune stimulated collared doves compared to controls 7 days after injection (Eraud *et al*. 2005). In contrast, immune activation by diphtheria-tetanus vaccine injection in blue tits had no significant metabolic cost over 42 days following immune challenge (Svensson *et al*. 1998). Another study, on mice, failed to find a metabolic cost of immune response induction following challenge with LPS and keyhole limpet hemocyanin (KHL); this research also shown that hypoxia enhanced the immune response in mice, but that mice with elevated immune responses had lower metabolic rate compared to controls (Baze *et al*. 2011). Furthermore, the prediction that there would be a significant dose-dependent correlation between immune upregulation and metabolic rate was not supported in great tits injected with different doses of phytohemagglutinin (Nilsson *et al*. 2007).

In summary, studies using the direct approach to investigate the energetic cost of mounting an immune response have produced contradictory results. It is therefore

unclear whether energy limitation during immune responses is a universal cause of the life-history costs of immune activation (Mendes *et al.* 2006, Scantlebury *et al.* 2007).

1.3. Natural variation in disease susceptibility

Variation in host defence systems for resisting pathogens is a ubiquitous feature of natural populations (Lazzaro *et al.* 2004, Auld *et al.* 2010). This phenotypic variation originates in genetic differences between host and parasite, differences in the environmental conditions that individuals experience, and the combination of these factors.

1.3.1. Genetic variation for disease susceptibility

Genetic variation for disease resistance is well documented in many species (Hill 1998, Kover and Schaal 2002, Tinsley *et al.* 2006). Lazzaro and colleagues (2004) found that genetic polymorphism between 101 *D. melanogaster* chromosome 2 genotypes caused considerable variation in the ability to suppress systemic growth of *S. marcescens*. This study went on to associate this variation with nucleotide polymorphism in 16 immune system genes, demonstrating that variation in genes responsible for intracellular signaling and pathogen recognition influenced pathogen resistance. To generalise these results to other pathogens, Lazzaro *et al.* (2006) later demonstrated that sequence polymorphism in these genes was also associated with resistance variation against three additional bacterial

pathogens, *Enterococcus faecalis*, *Lactococcus lactis* and *Providencia burhodogranaria*. Variation in the nuclear genome also influences susceptibility to fungal pathogens: genetic variation for ability to survive *Beauveria bassiana* infection existed in flies from six different *D. melanogaster* populations (Tinsley *et al.* 2006). The extensive influence of host nuclear genetic variation on resistance to pathogen infection is also well established in vertebrates (Hill 1998) and plants (Rausher 2001).

1.3.2. Host-parasite coevolution and the maintenance of genetic variation for disease susceptibility

Immunity is an important life-history trait and is therefore thought to be under strong selection pressure. However, genetic variability for defending against infections is a common observation in natural populations. This suggests that, despite strong selection, there are mechanisms which maintain genetic variability for host susceptibility. First of these is environmental heterogeneity: in most laboratory-based immunological studies environmental variables are kept constant and genotypes are assumed to display fixed susceptibility phenotypes to infection. The environment plays a crucial role in host defence: for example, the availability of food resources to the host can affect host tolerance to infection or improve their resistance to pathogens, as well as affecting the development and function of the immune system (Coop 2001). Even more importantly, the interaction between genotype and environment (G x E) is a key player in maintaining genetic variability in susceptibility to disease. This means that in variable environmental conditions

(spatial and temporal), the optimum host genome for efficient defence differs; selection is therefore too inconsistent to erode the genetic variation for disease susceptibility present in a population (Lazarro and Little 2009). For instance, interactions between ambient temperature and host genotype determine host resistance to microbial infection in *D. melanogaster* (Lazzaro *et al*. 2008). *Daphnia magna* provides an excellent example of disease resistance variation determined by host-environment interactions: host genotypes dramatically changed their susceptibility when temperature increased from 15 to 25 °C (Mitchell and Read 2005).

Another important factor in the maintenance of genetic variation for disease susceptibility is the interaction between host and parasite genomes, which has been studied in many species (Schulenburg and Ewbank 2004, Lambrechts *et al*. 2005, Dubuffet *et al*. 2006). Specific interactions between the genomes of hosts and parasites have very strong impacts on the outcome of infections: a particular host genotype may only be susceptible to a small subset of all genotypes of a particular parasite species (Lazzaro and Little 2009). There are two principal paradigms of the evolutionary effects of these interactions, one of these reduces genetic variation, whereas the other maintains it. Arms race dynamics in host-parasite interactions suggest that selective sweeps cause the optimal defence allele to spread through populations, reaching high frequency and eroding genetic variability for disease susceptibility (Obbard *et al*. 2011). Alternatively, according to red queen theory, host-parasite interactions are a mechanism that maintains genetic variation through negative frequency-dependent selection (Lazzaro and Little 2009). A study in *D. magna*, revealed not only significant genetic variation

for resistance to its bacterial parasite (*Pasteuria ramosa*), but also considerable variation in the ability of the parasite to infect its host and a significant host genotype – parasite genotype interaction (Carius *et al*. 2001).

Finally, the maintenance of genetic variation in natural populations is made more complex by three-way interactions between the host genome, the parasite genome and environmental conditions: G x G x E, (Tack *et al*. 2012). A study of the fungus *Cryphonectria parasitica* and its parasitic virus at different temperatures demonstrated strong effects of G x G x E interactions in determining virulence (Bryner and Rigling 2011). This highlights the potentially important role of G x G x E interactions for maintaining genetic variation for disease resistance.

Environmental inconsistency creates variation in the phenotypes produced by host and parasite genotypes, reducing the long term efficiency of selection in driving directional adaptive change. However, antagonistic coevolutionary interactions are probably the reason that immune genes undergo more rapid adaptive evolution than non-immune genes, as has been reported in *Daphnia pulex*, in which adaptive evolution in 30 immune genes was compared with 24 non-immune system genes (McTaggart *et al*. 2012). Host-parasite genetic interactions are therefore important, not only because they can maintain genetic variation in host resistance or parasite virulence, but also because they provide strong directional selective forces that shape organismal life-history.

So far in this introduction I have described the mechanisms of immune defence and how immune system activity can invoke costs on other physiological functions

of an organism. I have reviewed the evidence that costs of immunity arise due to resource-mediated trade-offs with other fitness traits. At the most fundamental level, the energy budget of an organism is determined by the mitochondria within each cell, which generate adenosine triphosphate (ATP) to power all cellular processes. For this reason, I focused part of my PhD research on how variation in mitochondrial function influences immune defence phenotypes.

1.4. The biology of mitochondria

1.4.1. Mitochondrial structure biology

Mitochondria are descended from prokaryotic ancestors following an ancient endosymbiotic event and are one of the key organelles found in the cells of eukaryotic organisms. The numbers of mitochondria per cell vary from just one to 10,000 mitochondria depending on the structure and function of the cell; however, more typically 200 mitochondria are present. Mitochondria are usually elongated cylinders 1-2 μm in length and 0.5-1 μm in diameter. However, they are plastic in nature and have the ability to reshape, fuse and then separate. Mitochondria consist of an inner and an outer membrane, cristae (folds of the inner membrane), an inter-membrane space and the mitochondrial matrix. The mitochondrial ribosomes are of 70s nature rather than 80s, indicative of their prokaryotic ancestry. Unlike all other animal cell organelles mitochondria possess their own deoxyribonucleic acid (DNA); they also have the ability to synthesize ribonucleic acids (RNA) and proteins.

1.4.2. Functional biology of mitochondria

Mitochondria are involved in a variety of functions depending on the cell type they are present in. In animal cells mitochondria are not only involved in the vital function of energy production to generate ATP by oxidative phosphorylation (Kinosita *et al*. 2004) but also work as an important reservoir for calcium ions and thus help to maintain the correct concentration of calcium inside the cell (Menon *et al*. 1985). In liver cells mitochondria possess an enzyme that is crucial to detoxify ammonia. Mitochondrial play a central role in apoptosis (programmed cell death of unwanted and excess cells) (Jeong and Seol 2008). Mitochondria also contribute in building, breaking and recycling of different products required for proper cell function. For example, mitochondria are a site for biosynthesis of certain haem molecules (Oh-Hama 1997) as well as steroid sex hormones such as estrogen and the testosterone precursor pregnenolone (Papadopoulos and Miller 2012, Miller 2013). Mitochondria are also critical in neurotransmitter (Stavrovskaya and Kristal 2005) and cholesterol (Duchen 2004) metabolism.

1.4.3. Molecular biology of mitochondrial DNA

The size of the mitochondrial DNA (mtDNA) genome is far smaller than the size of the nuclear genome in any organism. For example, the human nuclear genome consists of almost 3.9 billion base pairs (bp), whereas there are only 16,500 bp of mtDNA; in *Drosophila*, the nuclear DNA consists of 122 million bp, whereas the mitochondrial genome is only 19,500 bp. In the vast majority of animal species the

mitochondrial genome is between 15 and 20 kb. However, in some exceptional cases much larger mtDNA genomes are found; this increased size is not due to variation in gene content but to duplications in mtDNA genes (Fuller and Zouros 1993). Each mitochondrion contains 2-10 copies of the mtDNA genome.

In contrast to nuclear DNA, mtDNA is a circular, covalently closed and double stranded; however, in many unicellular (green algae) and a few multicellular organisms (such as cnidarian species in the Hydrozoa, Scyphozoa and Cubozoa), the mtDNA is linearly organised (Bridge *et al*. 1992). Mostly the animal mitochondrial genome consists of 37 genes: two code for the 12S and 16S rRNAs of the mitochondrial ribosome, 13 code mRNAs of 13 polypeptides involved in oxidative phosphorylation, and 22 genes are for tRNAs that are crucial for translation of mtDNA-encoded proteins (Boore 1999). These 37 mtDNA genes are homologous in animals, plants, fungi and protists (Boore and Brown 1994, Levings III and Vasil 1995, Paquin *et al*. 1997). The two strands of mtDNA are distinguished by their nucleotide content, the heavy strand is rich in guanine and light strand is rich in cytosine. The mtDNA also contains a large non-coding region called the D-loop, which in some animals is believed to contain replication and transcription controlling elements (Shadel and Clayton 1997). The exact function of D-loop is not yet well studied, however, recent work proposes a role in the organisation of mtDNA nucleotides (He *et al*. 2007).

1.5. Characteristics of mtDNA

Mitochondrial DNA is often described as being uniquely different from the nuclear genome in a number of ways: 1) the mtDNA mutation rate, 2) mtDNA is uniparentally inherited, 3) in general it lacks recombination, and 4) mtDNA polymorphism has been considered to be largely neutral, not strongly affecting fitness. However, a good body of evidence has emerging to contradict most of these conventional concepts of mtDNA biology (Ballard and Whitlock 2004).

1.5.1. Higher mtDNA mutation rate

Mitochondrial DNA has a higher mutation rate than nuclear DNA. The rate of mutation in mtDNA varies across species, but on average it is 10 times faster than nuclear DNA (Galtier *et al*. 2009). This high rate of mutation in mtDNA is due to the damage caused by free radicals, lack of repair mechanisms and proofreading capabilities. Together these factors make mtDNA sensitive to base substitution that leads to a high mutation rate. However, within mtDNA all regions are not equally variable; some regions are hyper-variable, such as the control region that has double the mutation rate of the rest of the mtDNA (Galtier *et al*. 2009). Because of the absence of recombination, the haploid nature of mtDNA and its asexuality, natural selection is inefficient at purging mitochondrial mutations (Neiman and Taylor 2009). Therefore, deleterious mtDNA mutations are eradicated slowly from the population and cause a greater fitness loss. The high mutation rate of mtDNA (combined with inefficient selection) makes it more variable than the nuclear genome. A number of specific mutations in mtDNA are

the cause of non-communicable hereditary diseases in humans (Bender *et al.* 2006).

1.5.2. Maternal mitochondrial inheritance

The concept that mitochondria are transmitted through the maternal lineage in animals was originally founded on parallel evidence of chloroplast inheritance in plants (Weier and Stocking 1952), and mitochondrial inheritance in plants and fungi (Barr *et al.* 2005). Support for the cytoplasmic, maternal mode of mitochondrial inheritance initially came from the work of yeast researchers in the 1950s, when they observed petite colonies of baker's yeast, *Saccharomyces cerevisiae*, caused by defects in the respiratory chain (reviewed by Williamson 2002). In the 1960s mtDNA was formally identified and localised (Mounolou and Lacroute 2005), and the first direct evidence for maternal inheritance of mtDNA in animals was presented in 1972 (Dawid and Blackler 1972). The hypothesis of strict mitochondrial maternal transmission in animals received additional support from many early studies that failed to identify paternal mtDNA transfer across a range of animal species (Hayashi *et al.* 1978, Reilly and Thomas 1980, Gyllensten *et al.* 1985).

Despite the broad principle of maternal mitochondrial transmission, occasional paternal leakage has been observed in various vertebrate and invertebrate species (Gyllensten *et al.* 1991, Kvist *et al.* 2003, Nunes *et al.* 2013). The rate of paternal leakage has not been well studied across the animal kingdom. However, there is a range of animal species where the paternal transmission of

mtDNA has been reported: *Drosophila* (Kondo *et al.*1992), birds including great tits (Kvist *et al.* 2003), mice (Gyllensten *et al.* 1991) and humans (Bandelt *et al.* 2005). These studies suggest an approximate estimate of 10^{-3} to 10^{-4} incidents of paternal leakage per fertilisation; in contrast a high paternal leakage (about 14%) was found in *D. melanogaster* (Nunes *et al.* 2013). Besides low rate paternal leakage, paternal inheritance of mtDNA occurs in some species. For example in bivalve mussels, such as *Mytiloida* (marine) and *Unionoida* (freshwater), female progeny inherit mitochondria from their mothers but both parents transmit it to male offspring. This phenomenon is known as doubly uniparental mtDNA inheritance, meaning that female offspring are homoplasmic and male offspring are heteroplasmic (Zouros *et al.* 1994).

1.5.3. Mechanisms that underpin maternal inheritance

Maternal mitochondrial transmission might be due to several different reasons (Sato and Sato 2013). Firstly, the numbers of mitochondria are greater in eggs (from 10^5 to 10^6) than in a sperm (from 10^1 to 10^2). Secondly, sperm mitochondria may fail to enter the egg during fertilisation. Thirdly, mitochondria from sperm that do enter the egg may be denatured due to the presence of ubiquitin, a universal proteolytic marker. Overall, uniparental mitochondrial inheritance has probably evolved as a mechanism to reduce cytonuclear genetic conflict.

In most mammalian species, during fertilisation some sperm mitochondria enter the oocyte and are then present in the zygote (Ankel-Simons and Cummins

1996). However, because sperm mitochondria are relatively uncondensed, it is likely they are easily damaged by reactive oxygen species during the fertilisation process (Aitken *et al*. 2012). A recent study in *Drosophila* demonstrated the destruction of paternally-derived mitochondria after fertilisation by endocytic and autophagic pathways (Politi *et al*. 2014). This supports the viewpoint that sperm mitochondria and their mtDNA are eliminated at the early embryo stage to protect embryos and offspring from the effects of damaged paternal mtDNA (Marchesi and Feng 2007). Nevertheless, other studies provided evidence for the pre-elimination of mtDNA from sperm mitochondria during spermatogenesis. For example, a study of *Drosophila* showed that sperm mtDNA was eliminated in the early stages of spermatogenesis by the action of endonuclease G and cell remodelling processes (DeLuca and O'Farrell 2012). This was not only found in insects, but a recent study in mice confirmed sperm mitochondria were not eliminated through early embryonic degradation (Luo and Sun 2013); instead, sperm mitochondria could persist in several cells until the morula stage. Luo and Sun (2013) used allele-specific nested PCR and restriction digest analysis, and demonstrated that most of the active motile sperm cells eliminated their mtDNA during spermatogenesis. They also found that if mtDNA occasionally remained and entered the zygote, it could be transmitted to offspring.

1.5.4. Mitochondria as selfish genetic elements

Selfish genetic elements are genes within, or genomes associated with, an organism that evolves to favour their own fitness, with detrimental consequences for the fitness of the organism as a whole. The non-nuclear inheritance of

mitochondria has led to the evolution of mitochondrial genotypes, which favour their own replication or transmission at the expense of their host's fitness. The best known example of mitochondrial selfish elements in plants is the group of genes responsible for cytoplasmic male sterility (CMS): the phenomenon of total or partial male sterility that is determine by either the mitochondrial genome or an interaction between mitochondrial and nuclear genomes to abolish the development of pollen in plants; subsequently enhancing seed development (Frank 1989). However, the nuclear genome has evolved suppression genes for CMS called restoration of fertility genes (*Rf*), which suppress the CMS phenotype in plants. This gene is only expressed when sterile cytoplasm is present (Schnable and Wise 1998). Another well-known example of a mitochondrial selfish genetic element is petite yeast mutants (*S. cerevisiae*): these are characterised by their inability to undertake oxidative respiration, resulting in no growth in the presence of a non-fermentable carbon source and compromised small colonies in the presence of fermentable carbon. As the mtDNA of petite mutants has many more origins of replication than wildtype mtDNA, these mitochondria outcompete wildtype mitochondria in heteroplasmic cells and subsequently have an inheritance advantage (Williamson 2002).

1.5.5. Lack of recombination in mtDNA

The other broadly established feature of animal mtDNA is the lack of recombination, which decreases the efficacy of natural selection and can lead to the accumulation of deleterious mutations (Felsenstein and Yokoyama 1976). However, some evidence disputes this link between recombination frequency

and the efficacy of selection (Bullaughey *et al*. 2008). Initially, it was believed that mtDNA lacked recombination, primarily due to the failure to find clear evidence of mitochondrial heteroplasmy in natural populations (Avise 2000). Due to the lack of normal recombination, the mitochondrial genome is supposed to have a single genealogical history that is shared by all genes. Therefore, evolutionary forces acting on one site in the mtDNA will affect the entire genome equally, potentially driving a hitchhiking effect when selection acts on any one locus (Galtier *et al*. 2009). However, the absence of mtDNA recombination is now in debate. Heteroplasmy of mtDNA is important to facilitate recombination (Tsaousis *et al*. 2005). A recent study demonstrated a high frequency (~14%) of heteroplasmy in natural populations of *D. melanogaster* (Nunes *et al*. 2013). Heteroplasmy is the rule in bivalve mussels, where recombination is common (Ladoukakis and Zouros 2001). Overall, evidence for mtDNA recombination is growing in vertebrates (Kraytsberg *et al*. 2004) invertebrates (Ujvari *et al*. 2007) and plants (Barr *et al*. 2005, Hecht *et al*. 2011). While mtDNA recombination has been found across taxa, the extent to which it is widespread across species is still unclear.

1.5.6. MtDNA – a neutral or non-neutral marker?

Mitochondrial DNA has been widely embraced for phylogeographic studies. This was partly because of an implicit assumption that polymorphism in this genome, with such a conserved cellular function, would be selectively neutral. It was expected that haplotype frequencies should be determined principally by genetic drift and patterns of gene flow. The assumption of neutrality was based partially on

the observation that the mean rate of divergence over the whole mtDNA molecule is on average 5 to 10 times greater than nuclear DNA in various species, yet its function was assumed to be strongly conserved (Ferris *et al*. 1983). For sequences evolving under neutral processes, a positive correlation is expected between the amount of genetic variation with in population and population size (Gillespie 1999, Meiklejohn *et al*. 2007). However, Bazin *et al*. (2006) demonstrated extremely constant mtDNA polymorphism levels across a wide range of population sizes and taxa, casting doubt on the neutrality of mtDNA polymorphism.

The increasing number of studies demonstrating a significant influence of mtDNA sequence variation on many phenotypic traits across the animal kingdom has challenged the neutral nature of mtDNA polymorphism. MacRae and Anderson (1988) studied haplotype frequencies in 12 experimental populations of *Drosophila pseudoobscura*, which were polymorphic for their mtDNA haplotypes, to test mtDNA non-neutrality. In an initial population formed by two strains with different mtDNA haplotypes, they found the frequency of a one haplotype increased by 46% after 3 generations and subsequently reached equilibrium at 82% after 32 generations. The effects of mtDNA polymorphism on male infertility and reproduction are well established across animal and plant kingdoms (Kao *et al*. 1995, Rand *et al*. 2001, Venkatesh *et al*. 2009, Smith *et al*. 2010). The role of mtDNA variation in determining oxidative stress levels and apoptotic activity is also well established (Lee and Wei 2007), providing potentially important influences on animal ageing and carcinogenesis (Birch-Machin 2006, Clancy 2008).

A range of other studies have characterised the impact of mtDNA polymorphism on life-history traits and organism fitness. A study involving 11 mitochondrial haplotypes of *Drosophila subobscura* demonstrated a significant effect of mtDNA variation on survival time, fertility and larval-to-adult viability (Christie *et al*. 2011). A study in *Drosophila simulans* using three mitochondrial haplotypes, revealed that starvation resistance, recovery from chill coma, egg size, egg production and metabolism were significantly influenced by mtDNA sequence variation (Ballard *et al*. 2007). Additionally, mtDNA polymorphism has economic importance too: in livestock, variation in mtDNA has shown to be correlated with milk production (Schutz *et al*. 1994) carcass traits (Mannen *et al*. 1998) and reproduction efficiency (Reicher *et al*. 2012). Recently, a meta-analysis carried out by (Dobler *et al*. 2014) gathered data from 61 published studies of animals, fungi and plants and reported that in general the effects of cytoplasmic genome polymorphism (mtDNA in the case of animals and combined mtDNA and chloroplast DNA for plants) are of moderate size. Dobler *et al*. (2014) also found that the effects of variation in cytoplasmic genomes are stronger on morphological traits compared to traits

associated with life-history or metabolism. It is therefore clear that mtDNA polymorphism frequently has measurable phenotypic effects; however, the extent to which mtDNA variation is generally strongly shaped by selection is still an ongoing debate (Mitrofanov *et al*. 2002, Dowling *et al*. 2008).

One arena in which it is well established that mtDNA polymorphism is non-neutral, is the role of rare mtDNA alleles and recurrent mutation in heredity human conditions associated with mitochondria. Mitochondrial disease is associated with

a broad range of non-communicable disorders. The first hereditary disease known to be caused by mtDNA mutation was reported over 20 years ago (Wallace *et al.* 1988). Since then, hundreds of mtDNA mutations (substitutions, deletions and insertions) have been studied and are associated to various diseases in humans and animals, such as myopathies, neurodegenerative diseases, Leber's hereditary optic neuropathy, Kearns Sayre syndrome and ischemic reperfusion (Taylor and Turnbull 2005).

1.6. Mitochondrial-nuclear epistasis

Proper function of mitochondria depends on the physical interaction between proteins coded by both the nuclear and mitochondrial genomes. Therefore, studying the genetic interactions between the mitochondrial and nuclear genomes (mitonuclear epistasis) is crucial for understanding how mtDNA variation influences the phenotypic variability of traits in natural populations. Although high mtDNA mutation rates and relaxed purifying selection contribute to allelic variation

in mtDNA genomes, selection for compensatory alleles in the nuclear genome may buffer the phenotypic effects of mtDNA variation and result in dynamic coevolution between these genomes.

Mitonuclear interactions influence metabolic rate because both genomes play vital roles in the formation of enzyme complexes that are responsible for ATP generation. In an insect model (*Callosobruchus maculatus*) whole organism metabolic rate was influenced by mitonuclear interactions in an

environment specific manner (Arnqvist *et al.* 2010). Recently, a study has shown, in *Drosophila* hybrids, that an epistatic interaction between an incompatible nuclear-encoded mitochondrial tyrosyl-tRNA (tRNA synthetase) of *D. melanogaster* and a mitochondrially encoded tRNA (tyr) of *D. simulans* led to reduced oxidative phosphorylation, resulting a compromised development, bristle formation and reproduction in flies. However, these fitness effects were strongly temperature dependent (Hoekstra *et al.* 2013). Dowling *et al.* (2007b) found that the main effects of either the mitochondrial or nuclear genome did not influence female fitness of *Drosophila*. However, mitonuclear epistasis had a strongly significant effect on fitness, which was also mediated by environmental variation between block replicates (Dowling *et al.* 2007b). An increasing body of evidence suggests that mitonuclear epistasis caused by naturally segregating genetic variants not only has a strong influence on mitochondrial function, but also affects a range cellular process and life-history traits (Zeyl *et al.* 2005, Dowling *et al.* 2007a, Montooth *et al.* 2010, Kenney *et al.* 2014).

1.7. Mother's Curse

According to the Mother's Curse hypothesis compromised male fitness may be associated with maternal inheritance of mitochondria. Because of the maternal mode of mtDNA inheritance, natural selection can only act on mitochondrially-associated mutations through females and deleterious mtDNA mutations that have male-specific effects cannot be purged by selection (Frank and Hurst 1996). This sex-specific selective sieve may result in phenotypic and fitness differences

between males and females and may therefore be responsible for sexual dimorphism. Nuclear DNA is transmitted through both parents, enabling selection to act on nuclear DNA in both sexes and to shape the evolution of sexually dimorphic and sex-specific traits. However, natural selection is unable to purge mtDNA mutations that are harmful when expressed in males but have beneficial, neutral or only mildly deleterious effects in females (Frank and Hurst 1996, Zeh and Zeh 2005, Burt and Trivers 2006). In contrast, selection on mtDNA may be relatively efficient at influencing the evolution of traits controlled by mtDNA that are either female-specific, or for which mtDNA mutations affect the phenotype in both sexes equally (Nielsen and Weinreich 1999, Stewart *et al*. 2008, Pereira *et al*. 2011). Whilst selection acts on mitochondria in females, males are an evolutionary dead end (Zeh and Zeh 2005, Nakada *et al*. 2006). The effect of this sex-specific selection on the mtDNA genome is known as Mother's Curse (Gemmell *et al*. 2004).

To investigate the existence of Mother's Curse, researchers have investigated whether the history of female-specific selection on mtDNA results in mtDNA causing fitness loss in males, but not in females. Apart from mitochondrial diseases good evidence of this effect only exists for two traits: male fertility and male longevity. Different studies in invertebrates (Dowling *et al*. 2007c) and vertebrates (Ruiz-Pesini *et al*. 2000) have demonstrated the link between specific mtDNA variants and impaired male reproductive fitness (Smith *et al*. 2010). Convincing support for these male-specific effects of mtDNA comes through a study where five mitochondrial variants of *D. melanogaster* were expressed on a

single nuclear background (Innocenti *et al.* 2011). Innocenti *et al.* (2011) examined genome-wide variation in expression levels of nuclear genes when the nuclear genome was expressed alongside five different mitochondrial haplotypes. They found clear asymmetry in the pattern of this variation between the sexes. The expression of 9.3% of nuclear genes varied between the haplotypes in males, whereas only 0.06% varied in females; most of the differential expression in males was driven by genes associated with male reproductive tissues (Innocenti *et al.* 2011). Generally, this study raises the possibility that mtDNA variation may have stronger effects on male fitness than female fitness.

Although it is not universal, in many species females exhibit longer life span than males. Some research suggests that this dimorphism may be the result of male-specific deleterious effects of mtDNA (Clancy 2008, Wolff and Gemmell 2013). In a recent study of *D. melanogaster*, 13 different mitochondrial haplotypes were crossed onto the same nuclear genome; in these flies there was greater variation in life span between the haplotypes in males than there was in females (Camus *et al.* 2012). Furthermore, this research suggested that these male-specific effects of mtDNA on male longevity were associated with the degree of sequence divergence in the mitochondrial genome. These authors concluded that mtDNA might also underpin the fact that females outlive males in humans (Camus *et al.* 2012).

Thesis overview

This PhD used *Drosophila melanogaster* as a model organism to study questions related to immune defence and the evolutionary biology of mitochondria. It is well established that immune activation is costly, I began by investigating the energetic cost of, and the mechanisms underpinning, immune activation. For this, I first measured trade-offs between immunity and fecundity in flies challenged with live and dead fungi and bacteria. To dissect the energetic basis of this trade-off I studied three aspects of energy flow: food consumption (energy intake rate), metabolic rate (the rate at which energy is utilised) and body weight (storage of energy) in immune challenged flies. This information helped me understand the primary mechanism underlying the costs of immune activation.

This developed my interest in the relationship between metabolism and parasite defence. I therefore moved on to study whether naturally segregating genetic variation in the mitochondrial genome influenced the ability of flies to survive infectious disease. Interactions between mitochondrial and nuclear genomes (mitonuclear epistasis) are commonly important in nature due to their effects on mitochondrial function. I used 22 mitochondrial haplotypes expressed on single nuclear genome, and 25 mitonuclear epistasis lines generated by backcrossing 5 mitochondrial variants onto 5 different nuclear genomes, to study the impact of mtDNA allelic variation and mitonuclear epistasis on susceptibility to fungal and bacterial pathogens in *D. melanogaster*.

Mitochondria are the powerhouse of the cell and all biological process from the

simplest to the most complicated require energy. The final section of this work involved considering the impacts of mitochondrial genome polymorphism more broadly. I studied 10 sexually different phenotypic traits in 22 mitochondrial haplotypes. By quantifying the extent to which mtDNA polymorphism differentially influenced phenotypic variation in males and females, I tested whether the mitochondrial Mother’s Curse hypothesis provides a general explanation for sexual dimorphism.

Chapter 2: Immune response costs are associated with changes in resource acquisition and not resource reallocation

This chapter has been published as:

Bashir-Tanoli S, Tinsley MC. 2014. Immune response costs are associated with changes in resource acquisition and not resource reallocation. *Functional Ecology* 28 (4): 1011-1019.

2.1. Abstract

Evolutionary ecologists frequently argue that parasite defence is costly because resources must be reallocated from other life-history traits to fuel the immune response. However, this hypothesis is rarely explicitly tested. An alternative possibility is that immune responses impair an organism's ability to acquire the resources it needs to support metabolism. Here we disentangle these opposing hypotheses for why the activation costs of parasite resistance arise. We studied fecundity costs associated with immune stimulation in *Drosophila melanogaster*. Then, by measuring correlated changes in metabolic rate, food consumption and body weight, we assessed whether responses were consistent with immunity costs originating from altered resource allocation or from impaired resource acquisition. Microbial injection resulted in a 45% fecundity decrease; it also triggered a mean decline in metabolic rate of 6% and a mean reduction in food intake of 31%, body

weight was unaffected. Metabolic rate down-regulation was greater in males than in females, whereas declines in food ingestion were of similar magnitude in both sexes. These physiological shifts did not depend on whether microbial challenges were alive or dead, thus they resulted from immune system activation not pathogenesis.

These costs of immune activation are significant for individuals that successfully resist infection and might also occur in other situations when immune responses are upregulated without infection. Whilst we found significant activation costs of resistance, our data provide no compelling evidence for the popularly argued hypothesis that immune deployment is costly because of reallocation of energetic resources to the immune system. Instead, reduction in resource acquisition due to 'infection-induced anorexia' may be the principal driver of metabolic changes and fecundity costs resulting from immune response activation.

2.2. Introduction

Immune responses are generally considered to be costly: a fact that is central to many fundamental concepts in evolutionary ecology, such as sexual selection (Hamilton and Zuk 1982), the maintenance of genetic variation for parasite resistance (Flor 1956) and host-parasite coevolution (Haldane 1949). These costs of immunity can be divided into two broad categories: First, the costs of forming and maintaining constitutive immune mechanisms, such as barrier defences and immune cell populations (Fellowes *et al.* 1998, Kraaijeveld *et al.* 2001). Second, the costs of activating inducible immune responses upon infection, such as immune molecule synthesis and fever development (Schulenburg *et al.* 2009, Martin *et al.* 2011). Here we focus on the activation costs of immunity.

Life-history concepts suggest that immune activation costs are principally energetic or nutritional, involving reallocation of resources to parasite defence at the expense of other fitness-related traits (Moret and Schmid-Hempel 2000, Schulenburg *et al.* 2009). However, evolutionary trade-offs may be governed both by variation in resource allocation between different traits and also by variation in resource acquisition ability (van Noordwijk and de Jogn 1986). The relative magnitudes of variation in resource allocation and resource acquisition can profoundly shape population responses to selection and the nature of associations between life-history traits (Reznick *et al.* 2000). Here we use this evolutionary framework to investigate the causes of immune activation costs, testing the relative importance of resource budget reallocation and alterations in resource acquisition ability in driving the costs of immune system deployment. This

distinction is important because resource reallocation can adaptively withdraw resources from particular traits to minimise overall fitness loss, whereas the consequences of impaired resource acquisition are potentially more widespread.

Costs of immunity are primarily realised as a decline in the quality or quantity of an individual's offspring. In *Drosophila melanogaster*, immune-challenged females suffer reduced fecundity; study of flies with genetically manipulated immune responses demonstrates that this cost arises specifically from immune system activation (Zerofsky *et al*. 2005). Similarly, in *Anopheles gambiae* immune stimulation with lipopolysaccharide (LPS) significantly reduces fecundity (Ahmed *et al*. 2002). As well as fecundity effects, immune challenge by LPS injection reduced survival of bumblebee workers under starvation conditions (Moret and Schmid-Hempel 2000). However, it is not clear why activating the immune system to attack parasites should invoke these fitness costs.

Total resource expenditure can be assessed by measuring metabolic rate. Some studies suggest that energetic resources are indeed reallocated, perhaps from stored reserves, to support immune system activity. Antibody production following immune challenge in collared doves increased basal metabolic rate by 8.5% 7 days after injection (Eraud *et al*. 2005). Similarly, in invertebrates, cabbage white butterfly pupae increased metabolic thermogenesis by 8% in response to the immunogenic stimulus of a nylon filament implant (Freitak *et al*. 2003). Nevertheless, in the collared dove study the authors concluded this metabolic cost was small and of similar magnitude to other normal homeostatic processes (Eraud *et al*. 2005). Furthermore, mice did not experience elevated metabolic rate when

injected with immune elicitors, either in standard conditions, or under hypoxia designed to cause metabolic stress (Baze *et al.* 2011). Thus, immunity-induced increases in metabolism are not universal; whether increased resource expenditure in the immune system is the major factor which causes declines in other fitness traits remains to be determined.

Mounting an immune response may also alter resource acquisition, changing the ability of organisms to support fecundity. Some studies have suggested that organisms increase food consumption when infected by pathogens to fuel the immune response (Moret and Schmid-Hempel 2000). However, the opposite, reduced food intake, is a common behaviour in animals upon immune challenge; a phenomenon termed infection-induced anorexia (Exton 1997). It is counterintuitive that animals as diverse as humans, mice and flies should adaptively decrease their food intake when infected. Nevertheless, the suggestion that this is a maladaptive symptom of illness has been challenged by work in *Drosophila*, which indicated that survival of flies following infection by some (but not all) pathogens is enhanced by this anorexic response (Ayres and Schneider 2009).

Although immune costs are frequently argued to be resource-mediated (DiAngelo *et al.* 2009), non-energetic costs can have significant fitness effects. Immune defence molecules produced to attack parasites can also cause collateral damage to host tissues, including inflammatory responses. In invertebrates, the cellular encapsulation response can attack host tissues causing pseudo-tumours (Govind 1996, Minakhina and Steward 2006), the synthesis of melanin for immunity can cause dispersed tissue damage (Sadd and Siva-Jothy 2006) and immune

responses against enteric microbes frequently cause extensive damage to the gut lining (Buchon *et al*. 2009).

Here we investigate costs of immune upregulation in *D. melanogaster*. Studying this model ectothermic invertebrate enabled us to investigate metabolic changes specifically associated with immune system deployment whilst avoiding the potentially confounding thermal impact of fever, which is commonly associated with pathogen infection in endotherms. The *D. melanogaster* immune system mounts a complex attack on invading microbes comprising coordinated cellular and humoral responses. Two key signalling cascades principally drive this attack: the Toll and the immune deficiency (IMD) pathways. The Toll pathway is activated preferentially by fungi and Gram positive bacteria, whilst the IMD pathway is stimulated primarily by Gram negative bacteria (Lemaitre and Hoffmann 2007). Each pathway triggers transcription of an appropriate subset of the fly's antimicrobial genes to defend against the type of microbe encountered (Hoffmann 2003). The enzyme phenoloxidase (PO) catalyses melanin production, which possesses cytotoxic properties as well as assist in wound healing and clotting (Eleftherianos and Revenis 2011). In adult *Drosophila*, cellular immune responses involve phagocytosis and parasite encapsulation by plasmatocyte cells circulating within the haemolymph (Williams 2007).

In this study we use a fungus (*Beauveria bassiana*) and a bacterium (*Escherichia coli*) to trigger either Toll-dependent or IMD-dependent immune responses. First we assess the magnitude of fecundity costs associated with these immune defences. Then we study how the resource budget of flies alters during immune

system deployment by quantifying correlated changes in metabolic rate, food intake and body mass. We use these measures to dissect the importance of altered resource allocation and resource acquisition in mediating fecundity declines. We predicted that if immune activation costs are principally due to resource reallocation, then either there would be no change in overall metabolic rate (if resources are withdrawn from non-essential traits and perfectly reallocated to immunity), or alternatively metabolic rate might go up if resources are reallocated from stored reserves to be spent on immune function. However, if compromised energy acquisition underpins immune costs, immune activation should be accompanied by reduced feeding rate and potentially a decline in other metabolic-related traits.

2.3. Materials and methods

2.3.1. Fly stocks and rearing

The wildtype genotype Samarkand (from Bloomington Stock Centre) was used throughout. Flies were bred in bottles; all rearing and experimentation was on Lewis food medium (Lewis 1960) at 25 °C, 70% RH on a 12 h L/D cycle. For all the experiments flies were allowed to mate following eclosion, then 3 day old flies were sorted into vials without additional live yeast, in single-sex groups of 10 using light CO_2 anaesthesia the day before immune challenge. Each vial of flies was only used in one of the following experiments.

2.3.2. Immune challenges

The impacts of microbial injection on fly fecundity, metabolic rate, food ingestion and body weight were studied. Microbes were prepared as both live and dead suspensions to permit separation of the physiological effects of immune activation from those of microbial replication. *B. bassiana* spores were grown on potato dextrose agar (PDA) from an existing strain using standard procedures (Tinsley *et al.* 2006); live and dead conidiospores were suspended in oil (87.5% Shellsol T, 12.5% Ondina EL). *E. coli* were cultured overnight in Luria Broth (LB) at 37 °C with continuous shaking, both live and dead *E. coli* were suspended in sterile LB. Heat-killed suspensions of *B. bassiana* and *E. coli* were prepared by boiling at 100 °C for 15 min. The absence of growth was confirmed for heat-killed suspensions by

plating 100 µl on PDA and LB agar respectively; the viability of live suspensions was similarly verified. Live fungal spores (2.0 x 10^6 spores ml^{-1}), heat-killed fungal spores (2.0 x 10^7 spores ml^{-1}) and 2.0 x 10^6 cells ml^{-1} of live and heat-killed bacteria were used to trigger immune responses in flies by injection into the thorax using a fine tungsten wire needle. The terminal 0.3 mm of the needle was bent slightly to provide a marker ensuring consistent penetration. Flies received CO_2 anaesthesia for injections, but then not during any subsequent assays. Four day old flies received one of six treatments: gas control (GC, flies anaesthetised with CO_2 but not injected), injection control (IC, flies injected with a needle dipped in blank oil), dead fungal (DF, dead *B. bassiana* injection), live fungal (LF, live *B. bassiana* injection), dead bacterial (DB, dead *E.* coli injection) and live bacterial injection (LB, live *E. coli* injection). Microbial suspensions were vortexed frequently to prevent microbes settling and needles were sterilised with ethanol and flaming.

2.3.3. Effect of immune challenge on fecundity

Immediately following the four immune challenge and two control treatments, groups of 10 female flies were allowed to oviposit in vials containing standard fly food containing blue food colouring (0.1% v/v) to aid egg counting. Flies were tipped into fresh vials after two 24 h periods, providing fecundity estimates for three consecutive days after immune treatment. Flies that escaped or died during vial transfers were recorded and fecundity measures adjusted accordingly. After oviposition vials were frozen and eggs counted later under a stereomicroscope. In total 300 flies were studied: five independent groups of 10 flies for each of treatment.

2.3.4. Measuring metabolic rate

This study was conducted using 410 independent 10-fly groups in seven blocks; each block contained multiple replicates of five or six of the different immune treatments. The effect of immune activation on fly metabolic rate was assessed by respirometry, measuring CO_2 production with an infrared gas analyser (IRGA: EGM-4, PP Systems). Day one measurements were made on 5-day old male and female flies 16-18 h after immune treatment; further measurements were made at 24 h intervals. Flies were housed in a plastic chamber connected in a circuit to an IRGA with tubing (total system volume 40.5 cm^3). Air circulated within this sealed system and CO_2 accumulation was measured. In each assay the metabolic rate of a group of 10 flies was measured at 25 °C over 5 min, recording CO_2 every 1.6 seconds; data from the first 2 min whilst flies settled were discarded. Measurements on each 10-fly group were repeated on three or four consecutive days. CO_2 efflux per minute was calculated by linear regression, then converted to nmole CO_2 min^{-1} fly^{-1} using knowledge of the apparatus volume.

2.3.5. Effect of immune challenge on food intake

Food consumption assays followed protocols of previous authors by measuring pigment intake from food (Libert *et al.* 2007, Ayres and Schneider 2009). Immediately after administering one of the six treatments, 70 groups of 10 flies were transferred to food medium comprising 0.5% v/v bromophenol blue (Sigma), 5% w/v sugar, 5% w/v yeast, 2% w/v agar, and water. After 24 h the head of each fly was removed using a scalpel (to exclude red eye pigments), then bodies were homogenised on ice in five-fly groups in 500 µl ice-cold TE buffer. Homogenate samples were centrifuged at 13362 x g at 4 °C for 10 min; the supernatant was then similarly re-centrifuged. The amount of blue pigment in 100 µl supernatant was measured in 96-well plates using a Versa Max microplate reader (Molecular Devices) to record absorbance at 520 nm. The flies in each original vial were split between two five-fly replicates; these replicates were measured in different 96-well plates. To convert absorbance values into food mass eaten per fly a calibration relationship was determined by measuring the absorbance of serial dilutions of a known food mass (n = 6 samples). The linear regression equation for this mass-absorbance plot (y=0.0008x-0.0048) had an R^2 value of 0.9998.

2.3.6. Effect of immune challenge on fly body mass

Flies were divided into 120 single-sex 10-fly groups when 3 days old and weighed whilst anaesthetised on a PI 225D balance (Denver Instruments) reading to 0.01 mg. The next day each group received one of three injection treatments: injection

control, dead fungal spores or dead bacteria. On the three subsequent days each group was reweighed; flies that died or escaped were recorded and each weight was converted to a per-fly mass. Flies were maintained on Lewis medium throughout.

2.3.7. Statistical analysis

All analyses were conducted in R version 2.15.1 (R Development Core Team 2013); linear mixed effects models were executed using lmer from the lme4 package (Bates, Meachler and Bolker 2013). Our principal aim was to assess the impact of the six immune challenge treatments on fly life-history traits. Data from the six treatments were progressively pooled by a systematic process to produce minimally complex models that adequately explained trait variation. We concluded that the treatment differences were important if the more complex model had improved explanatory power (see below). The impact of breaching the cuticle was tested by pooling data from the gas control and injection control treatments. We tested whether trait variation was due to pathogenesis or immune activity by pooling data from live and dead microbial treatments, and we tested if microbial identity influenced immune costs by pooling bacterial and fungal treatments. Finally, tests for a general effect of immune stimulation compared control groups to data pooled from across all microbial injected flies.

For analyses involving repeated measures on vials of flies over successive days the term ‘vial’ was included as a random effect, whilst temporal changes were

assessed using the fixed effect of ‘day’ and its two-way interaction with treatment. With the exception of fecundity studies, models also included fly ‘gender’ and a ‘gender by treatment’ interaction. When analysing metabolic rate data, models contained an additional random effect of ‘block’, accounting for variation between the seven blocks over which the investigation was conducted. We also tested the impact of time of day and the air CO_2 concentration when each metabolic rate measurement was made. Finally, for investigations of variation in food ingestion after immune challenge, ‘vial’ was used as a random effect to associate the two five-fly batches from each vial. The number of flies in assay vials for fecundity, metabolic rate and body weight experiments varied slightly due to escapes; in each case we tested whether fly number influenced the trait measured.

All models employed Gaussian errors. Models were serially simplified by eliminating terms for which inclusion did not enhance model explanatory power by 2 AIC units. Likelihood-ratio tests comparing models with and without the term of interest were used to calculate *P*-values. Results are presented as means ± standard errors.

2.4. Results

2.4.1 Fecundity costs of immune activation in *D. melanogaster*

Fecundity was recorded from 30 groups of 10 flies, observed daily for three days after receiving immune treatments. Immune stimulation by microbes was associated with a major reduction in fecundity (Figure. 1; control vs immune challenged flies, χ^2_1 = 72.42, P = 2.2 x 10^{-16}). Mean fecundity of flies receiving a microbial injection of any type was 2.16 eggs fly^{-1} day^{-1} (± 0.109), approximately half that of flies receiving control treatments, which laid 4.16 eggs fly^{-1} day^{-1} (± 0.150). The egg output of control injected IC flies (4.22 eggs fly^{-1} day^{-1} ± 0.153) was not different from the control anaesthetised GC flies (4.12 eggs fly^{-1} day^{-1} ± 0.146), demonstrating the injection process itself had no significant effect on fecundity (χ^2_1 = 0.41, P = 0.521). The flies receiving microbial immune challenges all responded similarly, with no individually significant differences between treatments (χ^2_3 = 3.44, P = 0.329). Indeed there was no significant fecundity difference between flies injected with live and dead microbes (χ^2_1 = 3.08, P = 0.079), nor between flies injected with bacteria and fungi (χ^2_1 = 0.26, P = 0.613). Fecundity did not change notably across the days of the experiment (day, χ^2_1 = 1.71, P = 0.190) and the fecundity reduction associated with immune stimulation remained significant three days after microbial injection (χ^2_1 = 17.71, P = 2.2 x 10^{-5}). The exact number of flies in each vial varied slightly (mean = 9.56, SE = 0.133), however this variation did not influence the per-fly fecundity (χ^2_1 = 0.27, P = 0.60).

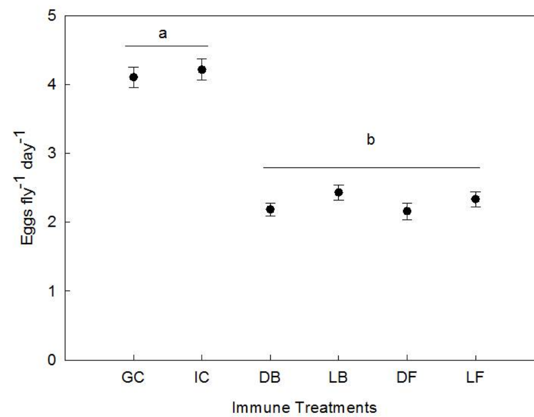

Figure. 1: Microbial injections decreased the fecundity of *D. melanogaster*. Eggs were counted from 30 groups of 10 flies for three consecutive days after immune treatments. Treatments were gas control (GC), injection control (IC), dead bacteria (*E. coli*: DB), live bacteria (LB), dead fungus (*B. bassiana*: DF) and live fungus (LF). Points represent daily means for each treatment and error bars show mean standard errors. Different letters (a/b) denote significantly different groups of treatments.

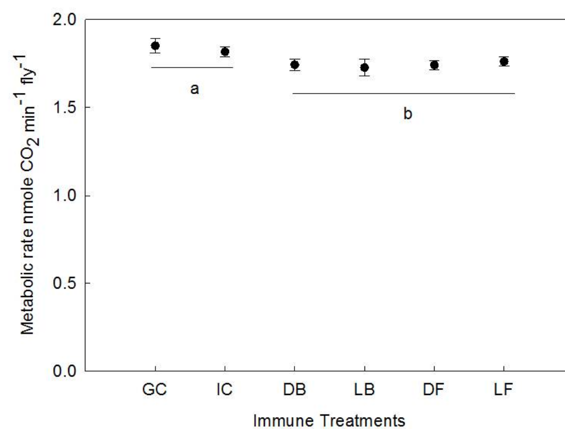

Figure. 2: Metabolic rate of *D. melanogaster* decreased after immune stimulation by microbial injection. Letters (a/b) indicate that the control treatments (GC and IC) differed significantly from the flies receiving bacterial (DB, LB) and fungal (DF, LF) immune challenges. Data points show means ± standard errors from 410 independent replicate groups of 10 flies assayed daily for between two and four days after treatment.

2.4.2. Immune activation decreased the metabolic rate of *D. melanogaster*

To investigate the effects of immune upregulation on metabolic rate 4100 flies in single-sex groups of 10 were subjected to metabolic rate measurements after immune challenge or control treatment. The metabolic rate of immune activated flies was 6% lower than control flies (Figure. 2): a highly significant decline (χ^2_1 = 25.42, $P < 4.0 \times 10^{-7}$). The four microbial treatments reduced metabolic rate by similar amounts (χ^2_3 = 1.16, P = 0.763). There was no difference either between live and dead microbial injections (χ^2_2 = 0.67, P = 0.717), or between fungal and bacterial injections (χ^2_2 = 0.80, P = 0.671). As with fecundity experiments, metabolic rate declines were associated with microbe exposure, not the injection process: metabolic rate of control injected IC flies (1.83 nmol min^{-1} fly^{-1} ± 0.045) was almost the same as anesthetised GC flies (1.85 nmol min^{-1} fly^{-1} ± 0.041) and the difference was not significant (χ^2_1 = 1.60, P = 0.206). The metabolic rate reduction associated with immune activation persisted during our experiment: following initial reduction there was no consistent metabolic rate change across the three days post-treatment (χ^2_1 = 0.63, P = 0.427).

The CO_2 levels in the laboratory fluctuated naturally during the study; higher CO_2 concentrations at the start of an assay were associated with slightly lower metabolic rates (χ^2_1 = 8.40, P = 0.003): an increase of 1 ppm CO_2 was associated with a metabolic rate decrease of 0.004 nmol min^{-1} fly^{-1} (95% CI 0.003-0.007). Time of day at which measurements were taken did not affect fly metabolic rate (χ^2_1 = 0.01, P = 0.918). A very small number of flies escaped from vials during transfers, therefore the mean flies per vial was 9.99; (SE = 0.002), this variation

had no effect on the per-fly metabolic rate (χ^2_1 = 0.51, P = 0.477). The metabolic rate of male flies was significantly less than females (Figure. 3; χ^2_1 = 25.42, P = 2.2 x 10^{-16}).

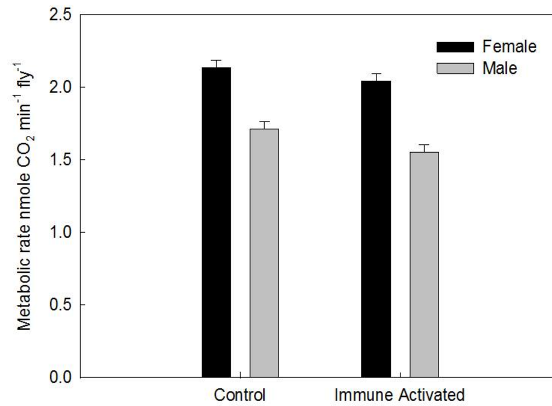

Figure. 3: Immune stimulation caused a greater metabolic rate decline in male flies than in females. Bars show mean metabolic rate of immune activated (DB, LB, DF, LF) and control (GC, IC) flies with their standard errors.

Furthermore, a significant gender by immune activation interaction demonstrated that immunity-induced metabolic declines were 50% greater in males than females (Figure. 3; χ^2_1 = 8.55, P = 0.003). For females, control metabolic rate was 2.14 nmol min^{-1} fly^{-1} (± 0.050), which declined by an average of 0.10 nmol min^{-1} fly^{-1} following immune activation; whereas in males control metabolic rate was 1.71 nmol min^{-1} fly^{-1} (± 0.050) and immune treatments caused a 0.16 nmol min^{-1} fly^{-1} decline.

2.4.3. Immune activation reduced the food intake of *D. melanogaster*

I measured food intake by assessing pigment uptake into the gut from coloured food. There were 70 independent feeding assays, each on a single-sex group of 10 flies; each group was then split in half for 140 pigment assays on five-fly samples. Microbe injected flies ate 72.4 µg fly^{-1} day^{-1} (± 2.65), 30.9% less than control flies, which ate 104.7 µg fly^{-1} day^{-1} (± 3.75) (Figure. 4; $\chi^2{}_1 = 60.89$, $P = 6.0 \times 10^{-15}$).

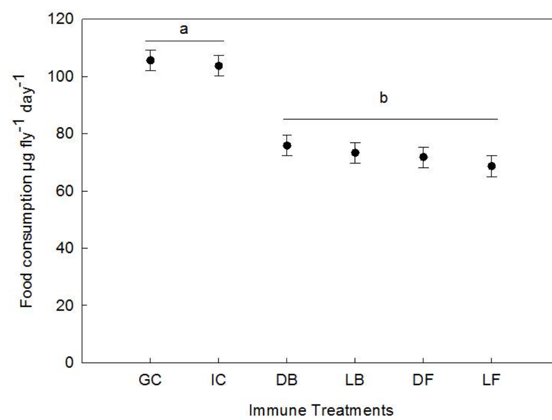

Figure. 4: Microbial injection reduced food ingestion in *D. melanogaster*. Data points represent means (± standard errors) from 140 measurements of food consumption on five-fly pools. The letters (a/b) show that all immune challenged flies (DB, LB, DF, LF) responded similarly, but were significantly different from control treatments (GC, IC).

There were no significant differences in feeding rate between bacterial and fungal treatments ($\chi^2{}_1 = 1.41$, $P = 0.235$), live and dead microbial injections ($\chi^2{}_1 = 0.58$, $P = 0.445$), nor between the IC and GC control groups ($\chi^2{}_1 = 0.11$, $P = 0.74$). Whilst male flies ate significantly less than females ($\chi^2{}_1 = 34.98$, $P = 3.3 \times 10^{-9}$),

the extent of the feeding decline was of similar magnitude in both sexes (Figure. 5; χ^2_1 = 0.02, P = 0.888): males and females suffered 32.9 and 31.5 µg fly^{-1} day^{-1} reductions respectively.

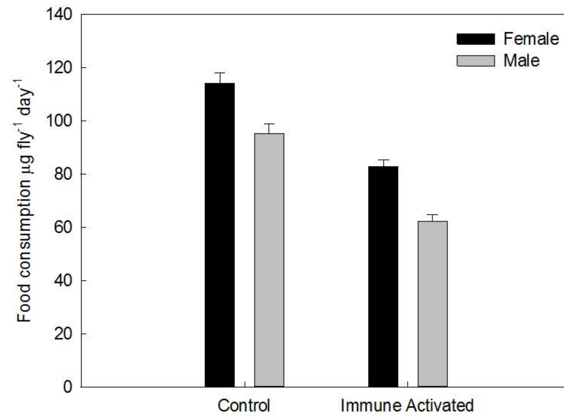

Figure. 5: The extent of feeding reduction caused by immune challenge was the same for males and females. Bars represent the mean food ingestion for immune activated (DB, LB, DF, LF) and control treatments (GC, IC) with their standard errors.

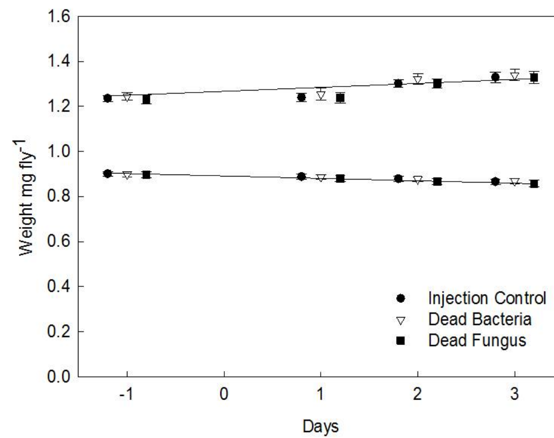

Figure. 6: Immune activation had no detectable effect on the rate at which flyweight changed. Flies were weighed the day before immune treatment (Day -1) and for three days afterwards (Day 1, 2 & 3). Female flies gained weight, whilst male flies lost weight during this period. However, immune challenge with dead bacteria (DB) or dead fungi

(DF) did not influence this temporal pattern compared to controls. 120 independent 10-fly groups were repeatedly weighed; points show means ± 2 x the mean standard error.

2.4.4. Immune activation did not affect body mass in *D melanogaster*

Experiments testing the impact of immune activation on fly body weight assessed mass for 10-fly groups of males (n = 64) and females (n = 62). In this case we only compared injection control, dead bacteria and dead fungal spore treatments. Flies were weighed the day before immune challenge and for three days afterwards. Female flies gained 7.6% weight during the experiment, whereas male flies lost 3.9% weight (Figure. 6; sex by day interaction (χ^2_1 = 327.97, $P < 2.2 \times 10^{-16}$). However, considering just the post-injection data, the immune treatments had no effect on absolute weight, nor on the temporal pattern of weight change for either sex (treatment effect, males χ^2_2 = 1.57, P = 0.456, females: χ^2_2 = 0.50, P = 0.778; day by treatment interaction, males: χ^2_2 = 0.74, P = 0.691, females: χ^2_2 = 0.32, P = 0.854). There was slight variation in the exact number of flies in each vial (mean = 9.95, SE = 0.014) but this did not affect the per-fly body weight (χ^2_1 = 0.29, P = 0.589).

2.5. Discussion

In this study we investigated the validity of the hypothesis that the costs of defending against parasites arise because resources normally invested in other physiological processes must be diverted to fuel the demands of the immune response. We demonstrated clear immune system costs in female flies, which suffered a sustained 45% reduction in fecundity across the three days following immune challenge. However, our findings challenge the common notion that this fecundity decline results from reallocation of resources to immunity.

We studied the three corners of the energy budget triangle: the rate at which energy is used (metabolic rate), the rate at which energy is acquired (feeding) and the dynamics of resource accumulation (body weight). We predicted that if mounting an immune response requires mobilisation of additional stored resources then fly metabolic rate would increase during immune system activity. Instead, metabolic rate fell by an average of 6% and remained low up to four days after immune challenge. If resource expenditure were perfectly reallocated from fecundity to immunity then this need not require an overall increase in metabolic rate. However, at the same time, resource acquisition fell dramatically: flies entered an anorexic state after immune challenge, with feeding rate falling by an average of 31%. Against this backdrop of depressed physiological activity we detected no effect of immune stimulation on body weight, providing no evidence that metabolism during immune activation depletes stored reserves. Nevertheless, flies are 70% water (Burr and Hunter 1969) and may gain water and loose fat

during lethal pathogenic infections (Arnold *et al.* 2013). We cannot rule out that similar alterations could have occurred due to immune activation by dead microbes in our experiments, potentially confusing detailed interpretation of total body weight trends.

The most parsimonious explanation of our findings is that reduced food ingestion in response to immune challenge restricts resource availability, resulting in depressed metabolic rate and limited fecundity. Therefore, fecundity costs associated with immune stimulation are probably not because the immune response requires increased energy expenditure, but because anorexia induced by the immune system reduces acquisition of resources that are normally required for egg production. This interpretation is supported by comparison of physiological changes in males and females. The reduction in metabolic rate was significantly greater in males than females, whereas feeding reductions were similar in both sexes. We hypothesise that females mobilised energetic resources by resorbing eggs from the ovarioles, as has been shown in both *Drosophila* and mosquitoes suffering infections (Ahmed and Hurd 2006, Thomson *et al.* 2012). Egg resorption may provide females with additional energetic reserves, not available to males, which support metabolism when food acquisition is restricted during immune responses. We note that this is a form of resource reallocation, but emphasise our conclusion that immune activation costs originate from reduced food intake; if egg resorption occurs in this manner; it only partially ameliorates some of these costs.

Our experiments only measured food intake for 1-day post-immune challenge, whereas other traits were measured for three days. This was because the assay

involved sacrificing flies to measure food ingestion. This limits our understanding of how feeding behaviour is affected by immune challenge beyond 24 hours. However, immune response-dependent trends in fecundity, metabolic rate, and food intake established rapidly during the first day post-challenge, and at least for fecundity and metabolic rate did not reverse by day three.

If infection-induced anorexia is a key driver of the fecundity costs associated with immune upregulation, this questions why the anorexic response exists. This phenomenon is phylogenetically conserved, which perhaps points to a fundamental function and a variety of adaptive benefits have been proposed (Exton 1997). Experiments in insects suggest anorexia can enhance survival during pathogen attack (Ayres and Schneider 2009) and may function to mediate conflicts between processing food and immune activity (Adamo *et al*. 2010).

One mechanistic factor shaping these immune-induced metabolic shifts is that some immune system molecular pathways have pleiotropic roles in other physiological processes. For example, in crickets, the lipid transport molecule apolipophorin III is involved in immune function, as well as in fuelling energetic demands of locomotion. This generates a trade-off between lipid transport and immune defence causing immunosuppression following exercise (Adamo *et al*. 2008). Also, the Toll pathway's immune activation role may conflict with nutrient storage and growth as Toll activity can depress insulin signalling (DiAngelo *et al*. 2009). Therefore, a variety of proximate mechanisms may be responsible for metabolic rate suppression in *D. melanogaster* following immune activation.

There were no differences in the responses of flies to live or dead microbes. Therefore, surprisingly, the fecundity, feeding and metabolic rate reductions apparently all resulted solely from activity of the immune response (or other downstream systems) and not from infection pathology. Whilst *B. bassiana* is highly pathogenic to flies (Tinsley *et al* 2006), *E. coli* inoculation does not normally cause mortality (Lemaitre and Hoffmann 2007); immune responses caused by other pathogens or increased infection doses may cause different effects. Our studies revealed no impact of immune stimulation on body weight; however, here we only tested the effect of dead microbes, it remains possible that responses to live microbial infection might be different. Recent studies have used *D. melanogaster* as a model to understand the physiological changes which take place in the lead up to death by lethal bacterial and viral infections (Chambers *et al*. 2012, Arnold *et al*. 2013). Chambers *et al.* (2012) reported that flies dying of *Listeria monocytogenes* suffered depleted energy stores and underwent major changes in the transcription and activity of key metabolic pathways. Arnold *et al*. (2013) concluded that pathology caused by *Drosophila* C virus resulted in metabolic rate reduction. However, both these studies compared flies infected with live microbes to unmanipulated flies. Our data challenge these conclusions, as we have found that immune system activity alone can drive similar metabolic shifts of considerable magnitude. Furthermore, our data show very similar costs and metabolic responses to fungal and bacterial inoculation. Thus, it seems likely that these major physiological changes are not specifically trigged by either the Toll or IMD immune signalling pathways, but represent a generic response to immune activation.

The fitness reduction associated with immune system activation is potentially substantial. Our data show that, not only does fecundity fall by 45% following immune challenge, but also this fecundity depression persists for three days. Indeed Zerofsky *et al.* (2005) showed fecundity was reduced for up to six days after immune activation. Thus, depressed fecundity persists for a substantial fraction of a fly's life after acute immune upregulation. Whilst some aspects of the fly immune response can be long-lived, IMD pathway transcriptional upregulation following Gram negative bacterial challenge generally only persists for ~24 hours (Lemaitre and Hoffmann 2007). Thus, the persistent nature of these fecundity costs might possibly provide additional evidence against the hypothesis that resource reallocation to immune molecule synthesis drives fecundity reduction. We note our measures of fecundity are low for *D. melanogaster*, probably because our food vials were not supplemented with live yeast. Fitness is determined by the quality as well as the quantity of offspring; further studies might address the trans-generational impacts of immune activation on general fitness traits.

For an organism that is infected by a potentially lethal microbe, these immune activation costs may be worth paying; the inducible nature of these defences protects the organism from these fitness consequences except when they are necessary. When epidemics sweep through population resistant individuals may survive, whilst susceptible individuals die. Our data suggest that the survivors may still suffer considerable fitness reduction as a consequence of resisting infection by deploying immune responses. Selection should shape the magnitude of immune defence costs; high costs of resisting pathogen infection may select for the alternative strategy of tolerance to the presence of microbes (Little *et al.* 2010).

Sizeable immunity costs may have profound consequences when the immune system is activated in anticipation of infection. Some organisms adaptively upregulate immune defence when environmental cues enable prediction of elevated pathogen risk: for example density dependent prophylaxis in desert locusts (Wilson *et al*. 2002). Immune responses are also activated in the absence of pathogen infection during courtship and in response to mating (McGraw *et al*. 2004, Immonen and Ritchie 2012). Thus, immune system upregulation may be a major cost of copulation that could generate selective forces governing the evolution of polyandry and female willingness to mate.

The sizeable nature of this immune response-induced fecundity cost has an important applied dimension. Entomopathogenic fungi, such as *B. bassiana*, which we used here, are currently being trialled for control of the mosquito vectors of human pathogens, such as the malaria parasite *Plasmodium*. Unlike the problems associated with the rapid evolution of resistance to chemical insecticides in vector populations, these biopesticides have been proposed to be 'evolution proof' (Read *et al*. 2009). This is because fungal biopesticides kill mosquitoes slowly. Thus although mosquitoes die before they can transmit human infections, they still have substantial opportunities to lay eggs post-exposure, reducing the fitness loss caused by pesticide control compared to conventional chemical insecticides. However, our data demonstrate that immune system activation by fungi results in a substantial fitness reduction. Similar findings have been reported for *Anopheles* mosquitoes (Mouatcho *et a*l. 2011). We therefore urge caution that even if fungal biopesticides result in slow vector mortality, substantial fecundity loss following exposure could still generate strong selection pressure for the evolution of novel

mechanisms to reduce mortality from biopesticides. Nevertheless, we acknowledge that our experiments administered microbes by injection and that immune responses following infection by natural routes could differ.

We hope that this study stimulates further critical evaluation of the role resource-reallocation plays in generating the costs of life-history trait investment. It is appealing to assume that fitness costs result from switches in resource allocation decisions. However, for the activation costs of resisting parasite infection, immune system deployment causes major impairment of resource acquisition, of sufficient magnitude to explain fecundity costs.

Chapter 3: Disease susceptibility is influenced by mitochondrial DNA genetic variation and mitochondrial-nuclear epistasis

This chapter is in revision following review at PLoS Genetics as:

Sumayia Bashir-Tanoli, Luc F. Bussière and Matthew C. Tinsley. (2014) Disease Susceptibility Is Influenced By Mitochondrial DNA Genetic Variation And Mitochondrial-Nuclear Epistasis.

3.1. Abstract

Genetic differences cause substantial variation between individuals in ability to survive infectious disease. Until now, research has focussed on the nuclear genome as a source of this variation. We investigated whether polymorphism in the mitochondrial genome also influences infection susceptibility. We took 22 *Drosophila melanogaster* mitochondrial haplotypes originating from the same wild population, introgressed all haplotypes onto the same nuclear background, then compared their mortality following pathogen infection. There was striking variation between haplotypes in susceptibility to the bacterial pathogen *Serratia marcescens*, but not the fungus *Beauveria bassiana*. By taking the phylogeny of the haplotypes, derived from the whole mitochondrial genome sequence, We demonstrated that this variation in susceptibility to *S. marcescens* was associated with DNA sequence variation. we also crossed a set of haplotypes to a set of nuclear genotypes to investigate the impact of epistasis interactions between

nuclear and mitochondrial genomes. Susceptibility of mitotypes to *S. marcescens* was strongly dependent on the nuclear genotype with which they were expressed. The magnitudes of the nuclear and mitochondrial effects on infection susceptibility were similar, whereas epistasis explained twice the variance of each genome individually. Our finding that mtDNA polymorphism can strongly affect pathogen susceptibility informs medical and ecological science and suggests mitochondrial genes could be a hot-spot for host-parasite coevolution. Missing heritability in previous parasite resistance genome wide association studies may be explained by these mitochondrial effects.

3.2. Introduction

The relationships between parasites and their hosts generate some of the most intense evolutionary forces (Haldane 1949). Furthermore, segregating genetic variation in natural populations frequently causes considerable variability between individuals in their susceptibility to infectious diseases (Hill 1998, Lazzaro *et al.* 2004, Tinsley *et al.* 2006). However, to date, researchers have focused on the nuclear genome as the cause of genetic variation in pathogen resistance (Wilfert *et al.* 2007, Bangham *et al.* 2008, Abel *et al.* 2014). Here, to our knowledge for the first time, we test whether variation in the mitochondrial genome (mtDNA) causes differences between individuals in disease susceptibility.

Despite early opinions that most sequence variation in mitochondrial genomes was phenotypically neutral, it is now well established that this genetic variation influences a range of traits, including metabolic rate, lifespan and reproductive success (Chan 2006, Ballard *et al.* 2007, Clancy 2008, Arnqvist *et al.* 2010, Christie *et al.* 2011). Rare mtDNA variants also cause debilitating hereditary human diseases (Wallace *et al.* 1988, Taylor and Turnbull 2005) and influence the onset of a number of non-communicable conditions (Hudson *et al.* 2014). The extent to which this variation in mtDNA is maintained by selection, or whether it is governed principally by non-adaptive processes is debated (Meiklejohn *et al.* 2007, Dowling *et al.* 2008). Purifying selection undoubtedly plays a major role in mtDNA evolution because of the essential function of mitochondrial oxidative phosphorylation. However, the efficacy of selection on mtDNA is relatively weak,

due to its low effective population size and the absence of frequent recombination (Ballard and Whitlock 2004). High mtDNA mutation rates also influence the position of the mutation-selection balance. Nevertheless, some studies indicate that positive selection has a significant impact in driving evolution of mtDNA (Ballard and Whitlock 2004, Bazin *et al.* 2006, Meiklejohn *et al.* 2007, Oliveira *et al.* 2008). Furthermore, because phenotypes are influenced by mtDNA genotype-by-environment interactions, some authors have speculated that mitochondrial genomes may contribute to adaptation in novel or changing environments (Dowling *et al.* 2007b). Whilst the efficacy of selection on mtDNA may be generally low, the strong selection pressure generated by parasites may nevertheless be able to efficiently drive adaptive change in mitochondrial genomes.

Mitochondrial function requires precise interactions between proteins and other gene products coded by the nuclear genome and those coded within mtDNA. Evolution in mtDNA (both due to adaptive forces and fixation of mildly deleterious alleles because of inefficient purifying selection) may select for compensatory evolutionary change in the nuclear genome (Dowling *et al.* 2007b). Different trajectories of mitochondrial evolution in different populations can trigger population-specific evolution in the nuclear genome, leading to inter-population and inter-species reproductive incompatibilities (Burton and Barreto 2012). Within populations, the segregating variation in nuclear and mitochondrial genomes can interact to cause strong epistasis for a variety of traits (Zeyl *et al.* 2005, Ballard *et al.* 2007, Dowling *et al.* 2007a, Dowling *et al.* 2007b, Montooth *et al.* 2010, Hoekstra *et al.* 2013).

Many theories exist for why there is considerable genetic variation for disease resistance in natural populations, despite the acknowledged strength of selection from parasites that might purge allelic variation (Lazzaro and Little 2009). One candidate explanation is that a significant amount of the standing genetic variation might be non-additive (Falconer *et al.* 1996), reducing the strength of phenotypic resemblance between offspring and parents. For example, some researchers have found that parasite resistance is strongly influenced by epistasis between nuclear loci (Wilfert and Schmid-Hempel 2008); however, others suggest that the role of epistasis in determining disease resistance phenotypes may have been overstated (Hall and Ebert 2013). A strong effect of mitochondrial-nuclear (mitonuclear) epistasis might contribute to low heritability of pathogen resistance phenotypes and facilitate the maintenance of genetic variation.

Here we investigate the influence of mtDNA variation, and also the effects of mitonuclear epistasis, on disease susceptibility in the model organism *Drosophila melanogaster*. We studied a set of mitochondrial and nuclear genotypes that had all been isolated from the same population. We introgressed 22 different mtDNA haplotypes onto the same homozygous nuclear genetic background. To verify that phenotypic differences between our mtDNA haplotype lines were not due to residual nuclear genetic variation, we also used an identical process to generate seven 'introgression control' lines which all carried the same mtDNA haplotype. At the same time we crossed five mtDNA haplotypes onto each of five nuclear genotypes to investigate epistasis. We then quantified the extent to which mtDNA-encoded allelic variation and mitonuclear epistasis influenced *D. melanogaster* susceptibility to two pathogens, *Serratia marcescens* and *Beauveria bassiana.*

3.3. Materials and Methods

3.3.1 Fly genotypes and rearing conditions

Fly lines used in this study originated from the *Drosophila* Genetic Reference Panel (DGRP); these were collected in Raleigh, North Carolina, and made homozygous for their nuclear genomes by 20 generations of sib-sib mating (Mackay *et al*. 2012). More than 10 generations prior to starting this study these lines were treated for two generations with tetracycline to remove symbiotic bacteria (eg *Wolbachia*) and subjected to embryo dechorionation to clear natural viral infections (Magwire *et al*. 2012). We constructed a set of 22 'mitochondrial lines', each carrying a mitochondrial haplotype from the DGRP set, replacing their original nuclear genomes by backcrossing for 20 generations to a single nuclear genome from the DGRP, which we termed 'background'. To assess the efficiency of our backcrossing process in replacing the original nuclear genomes we created seven 'introgression control' lines carrying identical mtDNA. We crossed males from seven of our DGRP genotypes to females of the background genotype, then backcrossed the resulting female offspring for a further 19 generations to homogenise their nuclear genomes with the background. To investigate the effects of mitochondrial-nuclear epistasis on disease susceptibility we constructed 25 mitonuclear epistasis lines. We used five DGRP lines to provide five different mitochondrial haplotypes and backcrossed these onto five different DGRP nuclear genomes for 20 generations.

All fly crossing, rearing and experimentation was performed on Lewis medium (Lewis 1960) in a controlled environment room at 25 °C, 70% relative humidity and

a 12 hour light/dark cycle. For crosses, a single virgin female was placed with two males and allowed to oviposit in a vial for 3 days. For disease susceptibility assays, flies were bred in bottles with added live yeast: three females with two males oviposited for only three days to ensure low larval density. When offspring eclosed into adults, three-day old flies were sorted into single-sex 10-fly groups in vials of Lewis medium without additional yeast, then subjected to infection assays the following day. For *B. bassiana* survival experiments flies were tipped into new vials every 2 days.

3.3.2. Culturing *S. marcescens* and *B. bassiana*

Strain DB11 of *S. marcescens* (Flyg *et al*. 1980) was grown at 37 °C overnight in Luria Broth (LB) with 10 mg ml^{-1} streptomycin. Bacteria were then washed three times by centrifuging and re-suspending in fresh LB, then prepared at 8.0 x 10^8 cells ml^{-1} in LB. The *B. bassiana* strain originated from an earlier experiment (Tinsley *et al*. 2006). Conidiospores were grown on potato dextrose agar supplemented with chloramphenicol using standard procedures, then suspended in oil (87.5% Shellsol T, 12.5% Ondina EL) at 2.0 x 10^8 spores ml^{-1}.

3.3.3. Infecting flies

Microbes were injected into the thorax of four-day old flies using a fine tungsten needle (25 µm diameter) dipped into the microbial suspensions. To ensure consistent penetration the terminal 0.3 mm of the needle was bent slightly as a marker. Flies received brief CO_2 anaesthesia for injections. Needles were sterilised with ethanol and flaming. Control flies were injected with sterile LB or oil,

in bacterial and fungal studies respectively.

The bacterial and fungal susceptibility experiments were performed separately, as were studies on the mitochondrial lines (22 mtDNA lines and 7 introgression controls) and epistasis lines (25 lines). In each case, experiments were performed in four replicate blocks, with 10-fly groups of every line and each sex represented in each block. For each pathogen, per line, the infected treatment had eight replicates, whereas there were four replicates for controls: each replicate vial had an average of 9.8 flies. In total we used 12,736 flies.

3.3.4. Statistical analysis

All data analysis was carried out in R version 3.0.2 (R Development Core Team 2013). We investigated mortality variation for each experiment at the time-point P.I. when mean mortality of infected flies first exceeded 50%. To study disease susceptibility variation across the mitotype line-set we ran mixed effects models with binomial error distributions using the MCMCglmm package (Hadfield 2010); proportional mortality was expressed as a two-vector response. We tested whether the between-line variance across the 22 mitotype lines was greater than the variance across the 7 introgression control lines by specifying a line by line-set (mitotype or introgression control) interaction, generating a P-value by calculating the proportion of our posterior samples for which the mitotype line variance exceeded that of the introgression controls. A further random effect of block was included in the model, as were fixed effects of sex and line-set. For parameter

estimates, we took the mode of the posterior distribution alongside its 95% credible interval. Following Hadfield (Hadfield 2010) we used weakly informative priors and performed prior sensitivity analysis. Models were run for 300,000 iterations, including a burn-in of 50,000 iterations; the Markov chain was sampled every 250 iterations. Richardson *et al*. (Richardson *et al*. 2012) assembled the whole mtDNA genome sequences for the DGRP genotypes and used these to determine the phylogeny of the mtDNA haplotypes. For 20 of the 22 mitochondrial lines that we created, for which unambiguous matches were present, we took the tree topology from Richardson *et al*. (Richardson *et al*. 2012) and assessed the extent to which this was associated with the pattern of variation in pathogen susceptibility across the mitochondrial lines. Using MCMCglmm with parameter expanded priors; we partitioned the mitotype variance into a component associated with the phylogeny and a residual line-specific component. Then, we calculated the phylogenetic heritability for pathogen susceptibility: the proportion of the between-line variance explained by phylogenetic effects (Longdon *et al*. 2011).

I used general linear models with binomial errors to study interactions between mitochondrial and nuclear genomes in the epistasis-line experiments. We treated all factors as fixed effects and assessed their contribution to pathogen susceptibility variation. We included main effects of block, sex, nuclear genotype and mitochondrial genotype. Our models tested for genotypic epistasis by including a mitotype x nuclear genotype interaction, and further interactions with sex to test for sex specific effects.

3.4. Results

3.4.1. Impacts of mtDNA variation on pathogen susceptibility

By 18 hours post infection (P.I.) with the bacterium *S. marcescens*, over half the flies had died (mortality: 0.551 ± 0.013 SE); during this time no fly died in the uninfected control group. Bacterial infection caused slightly higher male mortality (0.574 ± 0.017 SE) than female (0.528 ± 0.018 SE; P=0.040). Mean mortality following *S. marcescens* injection varied considerably between the 22 mitotype lines, ranging from 0.388 (± 0.071 SE) to 0.662 (± 0.049 SE); in contrast, mortality differences between the introgression control lines were small: range 0.588 (± 0.022 SE) to 0.623 (± 0.035 SE) (Figure 1a). The between-line variance associated with the mitotype line-set (0.146; 95%CI: 0.051–0.331) was considerably and significantly greater than the variance across the introgression control lines (0.00067; 95%CI: 0.00013–0.031, P = 0.006). For the mitotype line-set, variation between haplotype lines accounted for 46.8% (95%CI: 7.1–86.3) of the total residual variance after fitting the fixed effects. We took the phylogeny of the mitochondrial haplotypes derived from the entire mitochondrial genome (Richardson *et al.* 2012) and tested whether the between-line survival variation was associated with the topology of the mtDNA phylogeny. The bacterial infection susceptibility tended to be most similar for haplotypes that shared recent ancestry: the phylogeny explained approximately two thirds of the between-mitotype variation in *S. marcescens* susceptibility (phylogenetic heritability was 0.645; 95%CI: 0.023–0.998).

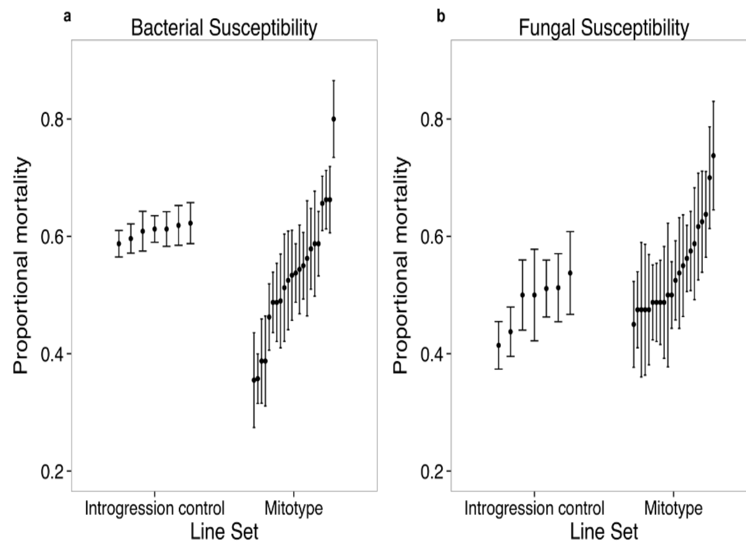

Figure. 1: The influence of mitochondrial genetic variation on *D. melanogaster* susceptibility to (a) the bacterium *S. marcescens* and (b) the fungus *B. bassiana*. Plots show mortality following pathogen infection of 22 mitotype lines and 7 introgression control lines. Mitotype lines each carried a different mitochondrial haplotype, whereas introgression control lines carried identical haplotypes; all were crossed onto the same nuclear background. For susceptibility to the bacterium *S. marcescens*, the between-line variance across the mitotype lines significantly exceeded the variance for the introgression control lines; this was not the case for the fungus *B. bassiana* (see text). Data points correspond to line means ± se.

Injection of the fungus *B. bassiana* caused mortality of over 50% of flies by day 9 P.I. (0.518 ± 0.022 SE), whereas by the same time the control treatment showed only slight mortality (0.030 ± 0.015 SE). For the fungal infected flies, there was no difference in mean mortality between males (0.517 ± 0.020 SE) and females (0.542 ± 0.021 SE; P = 0.240). Whilst the variation between mitotype lines in survivorship following *B. bassiana* infection was of similar magnitude to the *S. marcescens* experiment, the degree to which this variation in the mitotype line-set exceeded the introgression control lines was more modest in comparison (Figure 1b). Mean mortality between mitotypes in the infected treatment ranged from

0.450 (± 0.073 SE) to 0.738 (± 0.092 SE), and for the introgression control lines from 0.414 (± 0.040 SE) to 0.538 (± 0.062 SE) (Figure 1b). The variance between mitotype lines (0.0010; 95%CI: 0.0001–0.058) was twice that in the introgression control lines (0.00054; 95%CI: 0.00015–0.055), however there was substantial overlap in the credible intervals of these estimates and the variances were not significantly different (P = 0.424). We did not consider the pattern of mortality variation between mitotype lines in the control infection treatment because so few flies died (33 of 831) that the accuracy of the between-line variance estimate was extremely low. To further emphasise the lack of variation between mitotype lines for *B. bassiana* infection survivorship, the random effect of mitotype was associated with only 4.6% (95%CI: 0.0036–20.2) of the residual variance after accounting for the fixed effects.

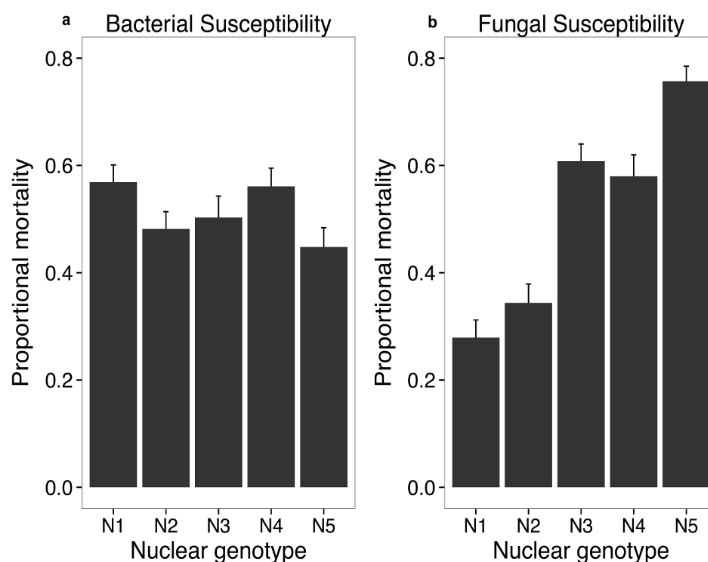

Figure. 2: Variation between five nuclear genotypes in mortality following (a) bacterial (*S. marcescens*) and (b) fungal (*B. bassiana*) injection. Susceptibility of each nuclear genome was estimated five times, in combination with five different mitochondrial haplotypes: bars show nuclear genotype means (± se). The variation is significant for both pathogens (Tables 1 and 2).

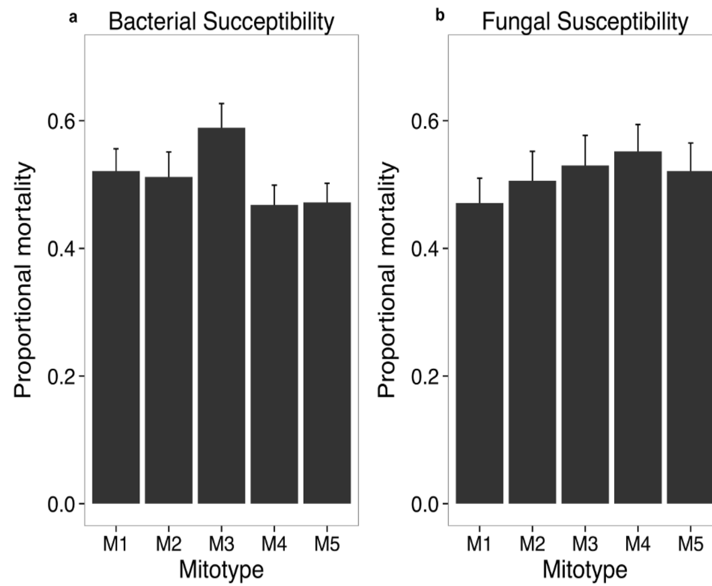

Figure. 3: Variation between five mitochondrial haplotypes in mortality following (a) bacterial (*S. marcescens*) and (b) fungal (*B. bassiana*) injection. Susceptibility of each mitochondrial haplotype was estimated five times, when backcrossed onto five different nuclear genotypes: bars show mitotype means (± se). The mitotype variation is significant for bacterial, but not fungal susceptibility (Tables 1 and 2).

3.4.2. Mitonuclear epistasis strongly influenced pathogen susceptibility variation

I investigated the influence of mitonuclear epistasis on survival following *S. marcescens* and *B. bassiana* infection by assaying infection susceptibility in 25 lines, comprising five mitochondrial haplotypes crossed onto five nuclear backgrounds. We partitioned the infection susceptibility variation into variance caused by differences in the nuclear genome (Figure 2), variance caused by mitochondrial genetic differences (Figure 3), and the epistasis effect caused by specific interactions between the two genomes (Figure 4). Mortality following *S. marcescens* injection reached 0.512 (± 0.016 SE, n = 1994) by 18 hours P.I., whilst over this time no control flies died (n=1000). Mean mortality in the bacteria-

injected flies varied significantly between the five nuclear genomes (from 0.448 (± 0.036 SE) to 0.569 (± 0.032 SE); Figure 2a, Table1); a comparable level of mortality variation existed across the five mtDNA haplotypes (from 0.472 (± 0.300 SE) to 0.589 (± 0.038 SE); Figure 3a, Table 1).

Table. 1: A variance components analysis for susceptibility to *S. marcescens*

Variable	d.f	Deviance	% Total deviance	*P*
Block	3	89.86	20.22	$2.2x10^{-16}$
Sex	1	6.94	1.56	0.0085
Nuclear genotype	4	17.53	3.94	0.0015
Mitotype	4	16.09	3.62	0.0029
Sex x nuclear genotype	4	22.85	5.14	0.0001
Sex x mitotype	4	2.26	0.51	0.6884
Nuclear genotype x mitotype	16	45.08	10.14	0.0001
Sex x nuclear genotype x mitotype	16	27.74	6.24	0.0339
Residual	147	216.16	48.63	-

A variance components analysis testing the contribution of different genetic effects to variation in susceptibility to the bacterium *S. marcescens.* We infected 25 different *D. melanogaster* lines, comprising five mitochondrial haplotypes crossed to five nuclear genetic backgrounds. d.f stands for degrees of freedom.

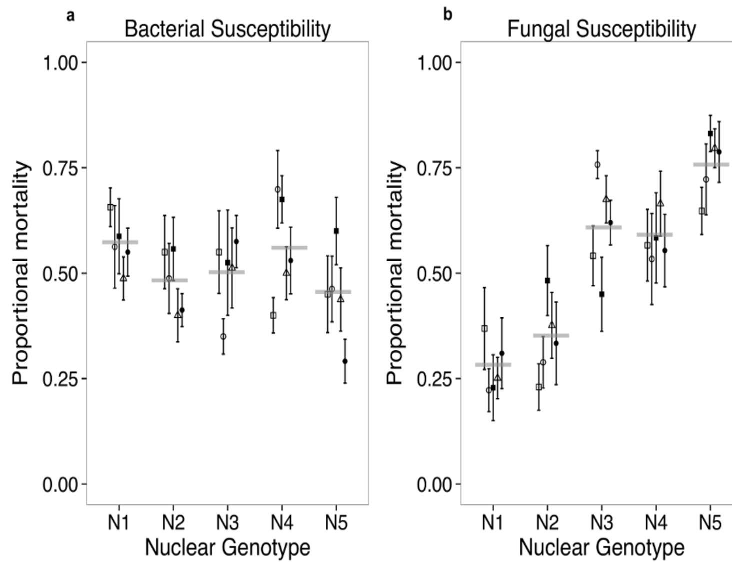

Figure. 4: The role of mitonuclear epistasis in driving pathogen susceptibility variation to (a) the bacterium *S. marcescens* and (b) the fungus *B. bassiana*. Five mitochondrial haplotypes were each backcrossed to five nuclear genotypes. The five mitotypes are each shown by a different symbol (open/closed square, circle or triangle) grouped according to the nuclear genotype they were crossed to, which is indicated by the x-axis (N1-N5). Points show mean mortality ± se. Grey horizontal lines denote the overall mean mortality for each nuclear genotype. For both pathogens, the pattern of mortality variation between mitotype lines was significantly influenced by which nuclear genotype they were crossed to (see text and Tables 1 and 2).

Furthermore, susceptibility of particular mitotypes to *S. marcescens* depended sharply on the particular nuclear genotype with which they were expressed (Figure 4a): the statistical interaction between mitochondrial and nuclear genomes was highly significant (Table 1). Our analysis revealed that the magnitudes of the effects of nuclear and mitochondrial genetic variation on infection survival were similar, whilst variation in bacterial susceptibility associated with mitonuclear epistasis was more than twice as great as the individual effects of either genome (Table 1). In this experiment female mortality following bacterial injection (0.541 ±

0.022 SE) was significantly greater than male (0.484 ± 0.022 SE, Table 1). The impacts of nuclear genetic variation and the mitonuclear epistasis effect were sex-specific (interactions between sex and the genotype terms were significant), but susceptibility differences between mitotypes were consistent across the sexes (Table 1).

Table. 2: A variance components analysis for susceptibility to *B. bassiana*

Variable	d.f	Deviance	% Total deviance	*P*
Block	3	11.97	1.78	0.0075
Sex	1	23.35	3.47	1.35×10^{-6}
Neuclear genotype	4	261.67	38.85	2.20×10^{-16}
Mitotype	4	8.00	1.19	0.0915
Sex x nuclear genotype	4	14.78	2.20	0.0052
Sex x mitotype	4	7.78	1.15	0.1001
Nuclear genotype x mitotype	16	41.01	6.09	0.0006
Sex x nuclear genotype x mitotype	16	16.54	2.46	0.4156

This table shows a variance components analysis testing the contribution of different genetic effects to variation in susceptibility to the fungus *B. bassiana.* Twenty-five different *D. melanogaster* lines were infected, comprising five mitochondrial haplotypes crossed to five nuclear genetic backgrounds. d.f stands for degrees of freedom.

In the fungal susceptibility experiment, mortality of *B. bassiana* injected flies was 0.52 (± 0.019 SE, n=1939) seven days P.I., considerably greater than in the control treatment (0.02 ±0.007 SE, n=951). Significant susceptibility variation

existed between the five nuclear genetic backgrounds (range from 0.279 (± 0.033 SE) to 0.757 (± 0.028 SE); Figure 2b, Table 2), but the variation between mtDNA haplotypes was not individually significant (Figure 3b, Table 2). However, the mortality rank order of the mitochondrial genotypes varied when they were crossed onto different nuclear backgrounds: there was a significant interaction term between the nuclear and mitochondrial genotypes (Figure 4b, Table 2). We attempted to verify that the mortality variation between lines following pathogen injection was due to differences in infection susceptibly, rather than intrinsic survival variation unrelated to infection. That only 32 control flies died across this entire experiment argues against pronounced intrinsic viability variation between lines. To investigate further, we also tested for a nuclear genotype x mitotype x infection treatment interaction, which did not quite reach significance ($\chi^2_{(16)}$ = 24.75; P = 0.074); however, the low control mortality makes this test conservative. In this experiment, males (0.572 ± 0.027 SE) were significantly more likely to die than females (0.460 ± 0.028 SE) following fungal spore injection (Table 2). The magnitude of this sex difference varied between nuclear genotypes but there was no sex specific component to the impact of mtDNA or mitonuclear epistasis on fungal susceptibility variation (Table 2).

3.5. Discussion

Here, to our knowledge for the first time, we report that naturally occurring genetic variation in the mitochondrial genome influences survival following pathogenic infection. In a set of experimental *D. melanogaster* lines challenged with the bacterial pathogen *S. marcescens* we found significant survival differences between mtDNA haplotypes. This occurred both in an experiment comparing 22 haplotypes on a single nuclear genetic background and when five of these haplotypes were introgressed onto each of five nuclear genomes. However, in parallel experiments we found no strong evidence that mitochondrial genetic variation influenced susceptibility to the entomopathogenic fungus *B. bassiana.* In addition, for *S. marcescens*, the disease susceptibility differences between mtDNA haplotypes were significantly influenced by the nuclear genetic background on which they are expressed, demonstrating a strong effect of mitonuclear epistasis on infection susceptibility.

Many studies have characterised genetic variation in host resistance to infectious disease, yet these have never considered whether the genetic variation is encoded in the nuclear or the mitochondrial genome (Hill 1998, Lazzaro *et al*. 2004, Tinsley *et al*. 2006). Association studies have made major progress in identifying the nuclear loci at which variation driving individual differences in pathogen susceptibility exists; however, these still reveal substantial missing heritability. For example, the genome wide association study of Magwire *et al*. (2012) demonstrated that viral resistance in *D. melanogaster* was influenced by a

small number of major-effect loci, but these explained less than 50% of the genetic variation. It has been suggested that the missing heritability results from the sum effect of minor loci (Hill 2012). Our data provide an alternative explanation: this variation may be explained by the previously neglected mitochondrial genome.

3.5.1. The evidence that mtDNA variation influences pathogen susceptibility

We used 20 generations of backcrossing to introgress mitotypes onto isogenic nuclear genetic backgrounds in order to compare their disease susceptibility. This method benefits from the ability to replace the entire original nuclear genome; however, it has been criticised because selection for nuclear alleles that are co-adapted with particular mtDNA haplotypes might potentially prevent full genome replacement (Clancy 2008). To assess if this was the case, we used the same backcrossing process and the same nuclear background to generate a set of control lines that shared identical mitochondrial genomes. This enabled us to experimentally verify that the marked variation in *S. marcescens* susceptibility across our mtDNA haplotypes substantially exceeded that in the control lines and was not due to incomplete nuclear genome replacement. In contrast, although variation in survival following *B. bassiana* infection across our mtDNA haplotype lines was greater than across the control lines, this difference was not significant, suggesting that any effect of mtDNA haplotype on susceptibility to this pathogen was small in comparison.

To provide further evidence that sequence polymorphism in the mitochondrial genome caused these disease susceptibility differences, we tested whether the

pattern of pathogen susceptibility variation between the mtDNA haplotypes was associated with their phylogenetic relatedness. For *S. marcescens*, the topology of the mitochondrial phylogeny (based on the entire mtDNA genome sequence) explained approximately two thirds of the between-haplotype susceptibility differences, indicating a mitochondrial genetic basis for variation in susceptibility to infection. We do not have the power to identify the individual mtDNA nucleotide differences influencing pathogen defence. However, sequence variation across the phylogeny was low: 45 sites were variable, the maximum pairwise divergence between any two haplotypes was 21 bases, and the mean pairwise divergence was 5.90 (±0.40 SE) bases. Therefore, it is likely that the differences in disease susceptibility are caused by relatively few mtDNA loci of relatively large effect.

3.5.2. The magnitude of mtDNA effects on pathogen susceptibility

I studied five mitochondrial haplotypes, each crossed onto five different nuclear backgrounds, revealing that the impact of mtDNA variation on *S. marcescens* susceptibility was of a similar magnitude to the impact of variation in the nuclear genome. The equal contribution of mitochondrial and nuclear genomes seems remarkable given that the nuclear genome has three orders of magnitude more genes than the mitochondrial genome. However, mitochondrial function is probably particularly important for host responses to pathogens. Hosts undertake major infection-induced shifts in metabolism (Bashir-Tanoli and Tinsley 2014); also, mitochondrially-derived reactive oxygen species and other molecules are frequently involved in regulating immune defence (West *et al.* 2011). Mitochondrial

genetic variation may be made even more important because many microbial pathogens have evolved virulence mechanisms that directly target mitochondrial function, principally due to the mitochondrion's role in integrating pro- and anti-apoptotic signals. Bacteria that produce toxins that specifically interact with mitochondrial pathways to induce host cell apoptosis include *Clostridium difficile*, *Helicobacter pylori*, *Neisseria gonorrhoeae* and *Staphylococcus aureus* (Jiang *et al*. 2012). Viral pathogens, including Hepatitis C Virus, Human Immunodeficiency Virus and Influenza A Virus also directly alter mitochondrial function during infection (Anand and Tikoo 2013). It is notable that the fungus *B. bassiana*, for which we did not find evidence of mtDNA effects on susceptibility, is not known to interact with mitochondria. In contrast, the bacterium *S. marcescens* exerts virulence by synthesising prodigiosin, which induces apoptosis in host cells by interfering with mitochondrial membrane permeability (Llagostera *et al*. 2003). These specific interactions between pathogen toxins and host mitochondria, combined with our data, raise the possibility that the mitochondrial genome may be an overlooked central player at the forefront of many host-pathogen coevolutionary relationships.

3.5.3. Implications of mtDNA-encoded variation for host-pathogen evolution

The mitochondrial genomes we studied all came from a single wild population and differed considerably in their pathogen susceptibility. This demonstrates the potential for natural selection to act directly on mtDNA haplotype frequencies during disease epidemics and for disease resistance to evolve through mtDNA evolution. Mitochondrial DNA is inherited as a single linkage group and influences

a number of phenotypes (Ballard *et al.* 2007, Clancy 2008, Christie *et al.* 2011); therefore, such pathogen-driven mtDNA evolution could drive non-adaptive change in a variety of unrelated traits. However, the significance of the mtDNA-encoded infection variation we discovered for the evolution of pathogen resistance is more complex. On the one hand, the selection pressures generated by pathogens during disease epidemics can be amongst the strongest evolutionary forces (Haldane 1949). On the other, the efficacy of selection on the mitochondrial genome is generally considered weak due to haploidy, uniparental inheritance and infrequent recombination (Ballard and Whitlock 2004).

We investigated the potential for mitonuclear epistasis to influence pathogen susceptibility variation by studying a set of nuclear genomes with which our mtDNA chromosomes shared recent coevolutionary history. We found that *S. marcescens* defence was influenced by strong mitonuclear epistasis interactions, which explained more susceptibility variation than the nuclear and mitochondrial main-effects put together. Given this, pathogen-driven evolution in mtDNA could trigger dynamic evolutionary responses at nuclear loci, and *vice versa*. For epistasis to play a major part determining disease resistance in natural populations the alleles concerned must be at appreciable frequencies; however, for most nuclear loci, alleles are at extreme frequencies, suggesting that the general role of epistasis in influencing phenotypic variation may be small (Hill *et al.* 2008, Hall and Ebert 2013). Our panel of genotypes provides little information about likely allelic frequencies in the field. However, it is possible that mitonuclear epistasis may be a special case because many species have mitochondrial haplotypes at intermediate frequencies (Torroni *et al.* 1996, Garcia-Martinez *et al.* 1998). We

note that our nuclear genotypes were fully inbred, preventing assessment of how dominance influences this phenotypic variation and potentially influencing our quantification of epistasis effects. Our analysis suggested that susceptibility to the fungus *B. bassiana* may also be influenced by interactions between particular nuclear and mitochondrial genotypes. However, our comparison between fungal susceptibility variation across the introgression control lines with that across the mitochondrial lines did not verify that the line variation in *B. bassiana* survival originated in the mitochondrial genome, therefore we hold back from drawing firm conclusions about how mitonuclear epistasis influences infection with this pathogen.

One of the most-debated topics in host-parasite coevolution is how considerable genetic variation for disease resistance persists in natural populations despite strong selection from pathogens. Explanations range from genetic specificity and negative frequency dependent selection, through to the impact of genotype by environment interactions in spatially or temporally variable habitats (Lazzaro and Little 2009). Our data provide an alternative, powerful explanation for why genetic variation for pathogen resistance is not always eroded by parasite-mediated selection. A role for mitochondrial genetic variation, combined with strong epistasis between mitochondrial and nuclear genomes, may result in inefficient selection and relatively low heritability for disease resistance traits.

Both *S. marcescens* and *B. bassiana* are naturally pathogenic to *D. melanogaster* (Flyg *et al*. 1980, Tinsley *et al*. 2006); however, neither is a coevolved pathogen, thus it will be interesting to extend these studies to host-parasite associations with

long coevolutionary histories. The role for mitochondrial genetic variation in host-parasite coevolution will depend on the genetic specificity of interactions between mitochondrial genotypes and individual pathogen strains. Further studies are needed to investigate whether strain-specific mitotype-pathogen interactions occur.

3.5.4. Conclusion of discussion

Our data provide proof of principle that mtDNA variation can have a major role shaping the outcome of host encounters with infectious disease. This adds to increasing evidence that mtDNA variation strongly affects a range of phenotypes with strong relevance to host ecology and life-history evolution, as well as of medical and economic importance (Schutz *et al*. 1994, Cummins *et al*. 2002, Birch-Machin 2006, Ballard *et al*. 2007, Clancy 2008). We argue that host-pathogen interactions represent a particularly special case of mtDNA non-neutrality, partly because of the strength of pathogen-mediated selection and partly because of the large magnitude of the effect we observed for *S. marcescens.* Recent work indicates that naturally segregating mtDNA haplotypes in human populations influence variation in immune-relevant phenotypes (Kenney *et al*. 2014). Given the range of medically significant pathogens for which infection pathology results from interactions with host mitochondria, we predict that mtDNA variation will be found to be a widespread and important determinant of disease susceptibility.

Chapter 4: Sex-specific phenotypic effects of mitochondrial genomic variation: a test of the Mother's Curse Hypothesis

In preparation for submission to Evolution

4.1. Abstract

Maternal mitochondrial inheritance means that selection operates on mitochondrial DNA (mtDNA) through its effects on females. Thus, selection is unable to purge mutations with male-specific deleterious effects. The expected male fitness loss due to this selection asymmetry is known as Mother's Curse and may explain aspects of sexual dimorphism. We tested a central prediction of the Mother's Curse Hypothesis: that mtDNA polymorphism should cause greater phenotypic variation in males than in females. We took 22 mitochondrial haplotypes from the same natural *Drosophila melanogaster* population, all backcrossed onto the same nuclear genome. We tested for sex-specific patterns in the effect of mtDNA allelic variation on 10 different phenotypic traits. Our results offer strong confirmation of the non-neutrality of mtDNA polymorphism: seven of the 10 traits were significantly influenced by mitochondrial genetic variation. However, mtDNA-driven phenotypic variation was not greater in males than in females for any of these traits. Furthermore, for most traits, we found relatively high intersexual mtDNA genetic correlations, arguing against large sex-specific phenotypic effects of mtDNA allelic variation. These data challenge the generality of the Mother's Curse hypothesis. We suggest two principal reasons why Mother's Curse is absent. Either mtDNA

mutations may, for mechanistic reasons, only rarely have male-specific deleterious effects, or the nuclear genome may frequently evolve modifiers that buffer these effects, meaning they are not evident within populations. Our work indicates that Mother’s Curse is unlikely to be a general explanation of sexual dimorphism.

4.2. Introduction

Mitochondria are generally inherited only through females, meaning that selection on the mitochondrial genome is sex-biased. This leads to the prediction that mitochondrial genomes should be better optimised to function in females than in males (Frank and Hurst 1996). The observation of sexually asymmetric selection on the mitochondrial genome has given rise to the 'Mother's Curse Hypothesis' (Gemmell *et al*. 2004). This suggests that relaxed or absent selection for mitochondrial function in males may result in reduced male fitness (Zeh and Zeh 2005), causing maladaptive sexual dimorphism with potentially profound consequences for population persistence (Gemmell *et al*. 2004). Genetic variation in mitochondrial DNA (mtDNA) was once thought to be largely neutral, however, overwhelming evidence now demonstrates that sequence polymorphism in mitochondrial genomes influences a wide diversity of traits (Dobler *et al*. 2014). Therefore, the potential exists for Mother's Curse to have major pervasive impacts on male fitness.

The Mother's Curse Hypothesis relies on the occurrence of mitochondrial genome mutations that have sex-specific effects. Mutations that have deleterious effects in males but leave female fitness relatively unaffected may not be purged by selection and reach high population frequencies (Frank and Hurst 1996). There is evidence of traits that appear to suffer from this phenomenon. Some hereditary mitochondrial diseases of humans show male-biased incidences (Kirches 2011), although this sex-bias is not universal (Sheridan *et al*. 2000). In addition,

mitochondrial genotypes can have antagonistic effects on fitness in males and females (Rand *et al.* 2001). Furthermore, genetic variation in the *Drosophila melanogaster* mitochondrial genome influences the lifespan of male flies more strongly than it does females (Clancy 2008, Camus *et al.* 2012). Variation in *Drosophila* mitochondrial DNA (mtDNA) also causes transcriptional variation in considerably more nuclear genes in males than it does in females (Innocenti *et al.* 2011). A different class of traits potentially shaped by Mother's Curse are male-limited phenotypes such as sperm function, for which direct selection on mitochondrial function is generally not possible. Indeed the cause of some human sperm fertility defects lies in the mitochondrial genome (Ruiz-Pesini *et al.* 2000; May-Panloup *et al.* 2003; John *et al.* 2005). However, whilst some studies in other organisms have found strong effects of mtDNA variation on sperm traits (Dowling *et al.* 2007c, Smith *et al.* 2010), mtDNA control of male fertility is not found universally (Dowling *et al.* 2007a, Friberg and Dowling 2008).

Whilst the predicted evolutionary consequences of uniparental mitochondrial inheritance are clear (Frank and Hurst 1996), the extent to which these are generally realised will be dependent on several factors. First, limited direct selection on mtDNA in males may operate if some paternal transmission occurs: whilst maternal mitochondrial inheritance undoubtedly predominates, significant levels of paternal transmission have been recorded (Nunes *et al.* 2013). Second, selection for mitochondrial function in males might, in some circumstances, occur indirectly through selection on female siblings if kin selection or inbreeding contribute strongly to female fitness (Wade and Brandvain 2009). Third, whilst male-specific deleterious mitochondrial alleles may persist in populations, their

effects may be rapidly ameliorated by selection for nuclear genes that modify their effects; thus these loci may not influence male phenotype when mitochondria are expressed in their natural coevolved nuclear genetic background (Beekman *et al.* 2014). Fourth, from a mechanistic perspective, Mother's Curse requires that mutations in mtDNA should commonly have sex-specific effects; given the principal fundamental function of mitochondria in oxidative phosphorylation, this need not be the case and has yet to be universally demonstrated.

The Mother's Curse Hypothesis suggests that uniparental mitochondrial inheritance may lead to poor average male fitness. However, sexually dimorphic phenotypes also evolve due to divergent selection on males and females (Chen and Maklakov 2014), meaning that it is difficult to determine the role of sub-optimal mitochondrial performance in shaping average male phenotype. For this reason, the impact of Mother's Curse, and the existence of un-purged mitochondrial alleles with male-specific deleterious effects, can instead be assessed by testing whether genetic variation in mtDNA is associated with greater phenotypic variation in males than in females (Innocenti *et al.* 2011, Camus *et al.* 2012).

Here we present the results of a multi-trait test of the Mother's Curse hypothesis, investigating whether naturally-segregating genetic variation in the mitochondrial genome causes more phenotypic variation in males than females. We deliberately excluded sex-limited traits and instead focused on traits expressed in both sexes. We studied four traits concerning ability to overcome environmental stress (desiccation resistance, starvation resistance, recovery from carbon dioxide (CO_2) anaesthesia and chill coma recovery); two traits relating to activity (metabolic rate

and the innate response to escape a stimulus – negative geotaxis performance); two morphological traits (body size and wing size); and two pathogen susceptibility assays (survival following injection with the fungus *Beauveria bassiana* and the bacterium *Serratia marcescens*).

We studied a set of 22 *D. melanogaster* mitochondrial haplotypes that were isolated from the same wild population and were all introgressed onto the same nuclear genetic background. Unlike in some similar studies (Innocenti *et al*. 2011, Camus *et al*. 2012), this nuclear genome was isolated from the same population as the haplotypes, sharing recent coevolutionary history. We also used an introgression process to homogenise the nuclear genetic background in seven 'introgression control' (IC) lines that all carried identical mitochondrial haplotypes. These IC lines enabled us to assess the level of phenotypic variation that might arise in these lines that was unrelated to mtDNA effects. For each trait we tested whether phenotypic variance between the 22 haplotypes exceeded that between the IC lines, to verify that trait variation was influenced by mtDNA polymorphism.

We first tested which of the 10 traits were influenced by mitochondrial genetic variation; then, for seven traits, which were affected by mtDNA genotype we compared the mtDNA affect in males and females. To strengthen our argument that phenotypic variation originated in the mitochondrial genome we took the phylogeny derived from the whole mtDNA sequences of our haplotypes and tested whether the pattern of phenotypic variation was associated with the phylogeny topology. Mother's Curse predicts that mtDNA alleles with male-specific deleterious effects mean that haplotypes have inconsistent effects on males and

females; therefore we tested the hypothesis that inter-sexual mtDNA genetic covariances for each trait would be low. Finally, we reasoned that if the magnitude of the Mother's Curse effect varies between traits, then the traits affected most strongly (with greatest excess mitochondrial haplotype variation in males), would be those traits with lowest inter-sexual mtDNA genetic covariances: we tested for this association. By conducting this study we also present an unbiased screen for the proportion of traits influenced by mtDNA sequence polymorphism.

4.3. Materials and methods

4.3.1. Fly crossing and stock rearing

The Drosophila genomic reference panel (DGRP) fly genotypes used in this study were originally collected in Raleigh (North Carolina) and made homozygous for their nuclear genome by 20 generations of sib-sib mating (Mackay *et al.* 2012). These had subsequently been treated to remove symbiotic bacteria such as Wolbachia (two generations with tetracycline) and to eliminate viral infections (embryo dechorionation with sodium hypochlorite); treatments were conducted at least 10 generations prior to this research (Magwire *et al.* 2012).

We made 22 mtDNA lines, each carrying a mitochondrial haplotype selected from the DGRP set by replacing the original nuclear genomes through 20 generations of backcrossing to a single nuclear genotype from the DGRP. To provide a measure for the efficiency of our backcrossing process to eliminate residual nuclear genetic variation we created seven 'introgression control' (IC) lines by taking DGRP lines, crossing their males to females of the back-cross genotype used above, then backcrossing to this genotype for a further 19 generations. Further details of this crossing scheme were described previously in chapter three.

All fly rearing and experiments were done in a controlled environment room at 25°C, 70% relative humidity and 12 h light/dark cycle using Lewis food medium (Lewis 1960) unless otherwise stated. For all experiments flies were bred in bottles: using only three females with two males, and allowing oviposition for only three days ensured low larval density. Following offspring eclosion, 3-day old flies

were sorted into single-sex groups of 10 flies after short CO_2 anaesthesia and placed in food vials without additional dry yeast. Unless otherwise stated, flies were 4 days old (~18 h after sorting) when the following experiments began.

4.3.2. Desiccation resistance

Single sex 10-fly groups were tipped from food vials into empty vials; dead flies were first recorded after 6 h, then every 2 h until complete mortality at 71 h. In total 120 flies were studied per genotype, split between six replicate blocks. Mortality times for each fly were used as the response variable in statistical analyses.

4.3.3. Starvation resistance

Sterile cotton wool balls saturated with sterile distilled H_20 were placed into empty vials and single sex 10-fly groups tipped into them. Subsequent mortality was recorded every 5 h between 7 h and 92 h post set-up, until all flies were dead. The sample size and response variable were the same as the desiccation resistance experiment.

4.3.4. Recovery from CO_2 anaesthesia

Flies were subjected to CO_2 anaesthesia for 10 min. To achieve this six fly vials (without food) were connected in parallel using tubing, single sex 10-fly groups were tipped into these vials, then humidified CO_2 was passed through. To assess

recovery, single anaesthetised flies were transferred into individual wells of a 24-well microtitre plate with lid; the time until flies recovered (when they first stood up) was recorded. Flies were observed every minute for 60 min, until all recovered. 100 flies per genotype were used across five replicate blocks. Data were recorded as recovery time in minutes.

4.3.5. Chill coma recovery

Recovery from chill coma was investigated following methods of Linderman *et al.* (2012). Briefly, single sex 10-fly groups were sorted into food vials when 3 days old, then stored for approximately 40 h until 5 days old. Flies were then transferred to empty fly vials, which were placed in melting ice for 4 h. Afterwards, single chilled flies were transferred into individual wells in chilled 24-well microtitre plates with lids; plates were then transferred to 25°C and the time for each fly's recovery was recorded. Sample sizes, recovery criteria and recording time interval were identical to the CO_2 anaesthesia recovery experiments.

4.3.6. Negative geotaxis

We assessed the speed of response to stimulus using negative geotaxis assays following Gargano *et al.* (2005). A total of 90 flies per line were subjected to climbing speed assays in three replicate blocks. Flies were placed into vials in single-sex 10-fly groups, then 18 h later (without further CO_2 anaesthesia) were tipped into 12 cm long clear plastic tubes, made by joining two standard fly vials; five such tubes were arranged in an array to allow simultaneous assessment of 50

flies. The array was placed upright in front of a light box. After a 1 min acclimation period the tube array was tapped sharply on the bench three times (in ~4 sec), then the distance flies climbed was recorded by taking a photograph 4 sec after the last tap (camera: Fujifilm Finepix S4500, 14 mega pixel); this procedure was repeated a further two times for each set of flies with a 1 min recovery interval between assays. ImageJ (Schneider *et al*. 2012) was used to manually plot the position of each fly on photos to determine the distance flies had climbed.

4.3.7. Metabolic rate

A total of 180 flies per genotype in single sex 10-fly groups were subjected to metabolic rate measurements in nine replicate blocks. Metabolic rate was assessed using published methods (Bashir-Tanoli and Tinsley 2014). Briefly, an infra-red gas analyser (IRGA, EMG4 – PP systems) was used to detect the rate of CO_2 production by single-sex 10-fly groups at 25°C in a sealed chamber of known volume. After a 2 min acclimation phase, the IRGA assessed CO_2 concentration every 1.6 sec for 3 min; CO_2 efflux rate was calculated by liner regression and converted to nmole CO_2 per h per fly for use in further analyses.

4.3.8. Body weight

Flies were weighed 3 days after eclosion. Twelve 10-fly groups were weighed per line, six of each sex: there were six experimental blocks, with both sexes and all lines represented in each. Flies were sorted into 10-fly single-sex groups when 2

days old, then on day three each group was anaesthetised by brief CO_2 exposure and weighed using a digital balance (PI 225D, Denver Instruments) recording to an accuracy of 0.001 mg.

4.3.9. Wing size measurement

Flies were frozen at -80°C 3 days after eclosion. Later, pairs of wings were cut from the thorax using fine scissors and stuck to white paper. Left and right wings were photographed at 40x magnification using a stereomicroscope (Leica MZ12.5); images were captured with a camera (Olympus) controlled by the computer software Eye TV Recorder 3.5.2 (Elgato). Both wings from ten flies of each sex per line were measured: 1160 wings in total. Wing area was determined by tracing the edge of the wing using the ImageJ polygon function (Schneider *et al*. 2012) and later converted to mm^2.

4.3.10. Disease susceptibility

We assessed variation in susceptibility to both a fungal pathogen (*B. bassiana*, strain from Tinsley *et al*. (2006)) and a bacterial pathogen (*S. marcescens*, strain DB11 from Flyg *et al*. (1980)). These methods, and some of the data have previously been described in chapter three. Briefly, each fly was anesthetised with CO_2 and injected with either live *B. bassiana* conidiospores suspended in oil or live *S. marcescens* in Luria Broth; controls received blank oil or sterile broth injections respectively. For each pathogen, eight and four vials of flies (mean 9.8 flies per vial) per line were used in the infected and control treatments respectively. Data

presented are proportional survival, 9 days post-injection for *B. bassiana* and 18 hours post-injection for *S. marcescens*. In total we used 6,852 flies.

4.3.11. Statistical analysis

All data analysis was carried out in R version 3.0.2 (R Development Core Team 2013) using the MCMCglmm package (Hadfield, 2010). Mitochondrially encoded genetic variation for the 10 phenotypic traits was investigated using general linear mixed effects models. Weakly informative inverse wishart priors (following Hadfield (Hadfield, 2010)) were selected after verifying efficient Markov chain sampling. Unless otherwise stated, models were run for 300,000 iterations; this included a burn-in period of 50,000 iterations, after which the Markov chain was sampled every 250-iterations. To formally test if variances differed between paired treatment groups, we generated P values by calculating the proportion of the 1000 posterior samples for which one variance exceeded the other. Parameter estimates were calculated as means from the posterior distribution alongside their 95% credible intervals (CI).

Testing for phenotypic differences between mtDNA haplotypes. For each trait we assessed whether the phenotypic variance across the 22 mtDNA lines (which had different haplotypes) differed from the variance across the seven IC lines (which all carried the same haplotype): 'line set' (mtDNA or IC) was a fixed effect in all analyses, along with the effect of fly sex. For the wing size analysis we also had an additional fixed effect of 'side' (right or left); for pathogen susceptibility

analyses the effect of treatment (infected or uninfected) was also included. All models contained a random effect of 'line'. For experiments where individually-assayed flies were housed in the same vial during experiments (desiccation and starvation resistance; recovery from chill coma and CO_2 anaesthesia; and negative geotaxis) 'vial' was a random effect. Models also included a random effect of 'block', except for wing size analyses, where 'fly' was used as an individual–level random effect. To compare variances across the mtDNA and IC line sets we specified an interaction between 'line' and 'line set', constraining the covariance between line sets to zero; residual error variances were heterogeneous for the two line sets. We then repeated these analyses using only the mtDNA line-set to test for sex-specific effects of mtDNA variation. We specified an interaction between 'sex' and the random effect of line, using an unstructured covariance matrix to assess the association between patterns of variation in males and females.

Testing for inter-trait genetic covariances. To investigate if all the traits responded independently to the mtDNA-encoded allelic variation, the data from only the mtDNA lines for the traits that varied between haplotypes were pooled together, using an additional variable 'trait' to distinguish the different assays in the data frame. Models included the same terms as above, but also an additional random effect interaction between 'trait' and 'line', using an unstructured covariance matrix to calculate individual covariances for each trait pair.

Testing the association between mtDNA genome polymorphism and trait variation. Richardson *et al.* (2012) determined the phylogeny for all the

mitochondrial haplotypes in the DGRP flies based on their whole mtDNA genome sequences. We pruned the Richardson *et al.* (2012) tree to yield the tree topology for 20 out of the 22 mtDNA lines for which we could find definitive matches. We used MCMCglmm with parameter-expanded priors to subdivide the between-haplotype variance into a variance term associated with the phylogeny and a residual line-specific component. For seven traits that were affected by mtDNA variation we calculated the phylogenetic heritability: the proportion of the between-line variance explained by the phylogenetic effect (Longdon *et al.* 2011). For these analyses only data from the mtDNA lines were used. Fixed and random effects were the same as described above; however, these analyses also included an additional random effect for the phylogeny (using the 'animal' specification). To determine whether the phylogeny topology influenced phenotypic variation in males and females differently, we took the data for the two sexes separately and calculated the phylogenetic heritability for each. The phylogenetic effect models were run for 1,000,000 iterations with a burn-in period of 50,000 iterations; the chain was then sampled every 400-iterations.

4.4. Results

4.4.1. Variation in the mitochondrial genome influences phenotypic variation for many phenotypic traits

We first evaluated the evidence that mitochondrial haplotypes differ in their phenotypic effects for each of the 10 traits we studied. We assessed whether the phenotypic variance between the 22 different mtDNA lines exceeded that of the seven IC lines carrying the same mitochondrial haplotype. Six of the 10 traits demonstrated significantly higher phenotypic variance amongst the mtDNA lines compared to the IC lines (Figure. 1 and Table 1). These traits were: desiccation resistance, recovery from CO_2 anaesthesia, chill coma recovery, body weight, wing size and bacterial infection susceptibility (Figure. 1 and Table 1). For a seventh trait, metabolic rate, whilst the mtDNA line variance considerably exceeded that of the IC lines, this comparison did not quite reach significance (P = 0.053). However, for three traits there was no evidence of an effect of mtDNA variation: the mtDNA line variance clearly did not exceed that for the IC lines for starvation resistance, negative geotaxis performance and fungal pathogen susceptibility (Figure. 1 and Table 1). Variance comparisons for metabolic rate (P= 0.053) and body weight (P=0.043) were both close to the significance threshold; therefore, to account for Monte Carlo error in our estimates, the models were run 10 times and the P values reported here represents the average significance that was calculated from 10 model runs.

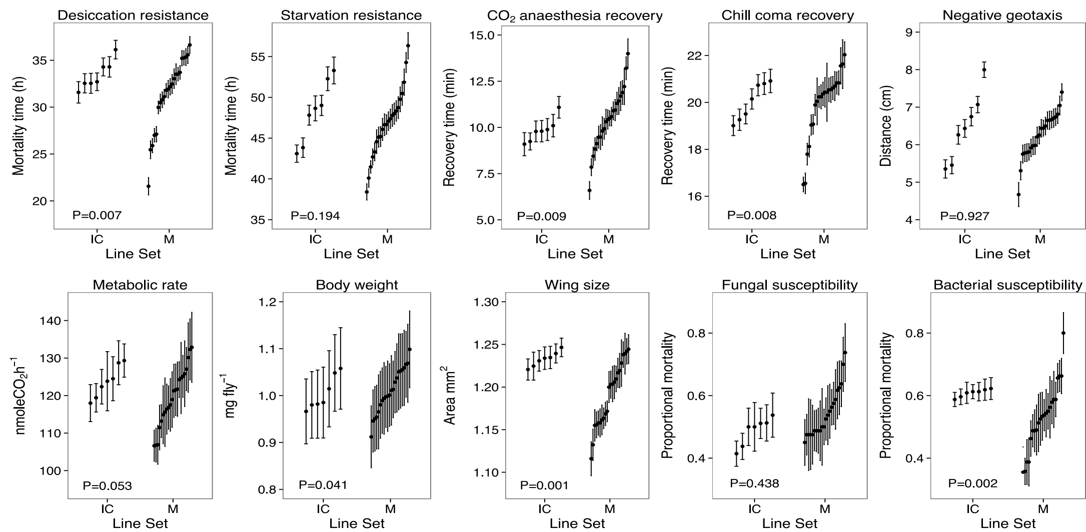

Figure.1: The impact of mtDNA variation on 10 different traits in *D. melanogaster*. Each panel shows the line means and standard errors for 22 mtDNA lines (M, which carried different mitochondrial haplotypes introgressed onto the same nuclear genome) and seven (IC) introgression control lines (which all carried an identical mitochondrial and nuclear genome). The significance of the difference in the between-line variance for the mtDNA and IC lines is given by the P-values, derived from linear mixed effects models (see text).

Table. 1: Phenotypic variance of mtDNA and IC lines for 10 different traits

Phenotypic trait	Variance with 95% CI		*P*
	Introgression controls	mtDNA lines	
Desiccation resistance	1.262 [2.10x10^{-4} – 4.685]	13.77 [5.906 – 24.38]	0.007
Starvation resistance	10.12 [4.01x10^{-3} – 27.58]	16.32 [6.907 – 29.39]	0.194
CO_2 anaesthesia recovery	0.186 [4.10x10^{-4}– 0.783]	2.556 [0.805 – 4.664]	0.009
Chill coma recovery	0.076 [1.26x10^{-5}– 0.396]	1.778 [0.266 – 3.625]	0.008
Negative geotaxis	0.961 [0.036 – 2.461]	0.199 [7.02x10^{-3} – 0.445]	0.927
Metabolic rate	2.649 [1.01x10^{-4} – 13.58]	34.29 [3.02x10^{-4} – 76.42]	0.053
Body weight	0.510 [1.01x10^{-3} – 1.802]	2.40 [0.890 – 4.3]	0.041
Wing size	0.054 [1.10x10^{-4} – 0.193]	1.360 [0.530 – 2.383]	0.001
Fungal susceptibility	0.004 [1.38x10^{-6} – 0.019]	0.007 [1.36x10^{-6}– 0.038]	0.438
Bacterial susceptibility	0.004 [1.55x10^{-6} – 0.015]	0.181 [0.045 – 0.348]	0.002

Comparison of the phenotypic variance for 10 traits across the 22-mtDNA lines (which carried different haplotypes) with the seven (IC) introgression control lines (which carried the same haplotype). 95% credible intervals of variances and P values for each comparison are given.

Table. 2: Assessing the impact of mtDNA phylogenetic ancestry on phenotypic variation for 10 traits

Phenotypic trait	Variance with 95% CI		Phylogenetic heritability
	Phylogeny	mtDNA lines	
Desiccation resistance	18.17 [6.28×10^{-5} – 54.33]	8.091 [0.020 – 17.61]	0.57 [4.76×10^{-6} – 0.960]
CO_2 anaesthesia recovery	2.652 [2.99×10^{-7} – 9.091]	1.033 [1.63×10^{-5} – 28.49]	0.57 [0.011 – 1]
Chill coma recovery	2.350 [2.93×10^{-6} – 9.052]	2.891 [0.433 – 6.211]	0.32 [0.011 –1]
Metabolic rate	90.46 [9.87×10^{-8} – 0.932]	15.27 [2.93×10^{-6} – 54.39]	0.76 [0.081 – 1]
Body weight	0.010 [4.78×10^{-5} – 255.35]	0.002 [4.69×10^{-10} – 4.01×10^{-3}]	0.56 [0.012 – 1]
Wing size	0.001 [3.69×10^{-10} – 5.01×10^{-3}]	0.001 [3.01×10^{-4} – 0.003]	0.33 [5.85×10^{-7} – 0.85]
Bacterial susceptibility	0.021 [3.96×10^{-9} – 0.051]	0.010 [2.09×10^{-9} – 0.012]	0.64 [0.021 – 1]

This table tests the extent to which the mtDNA phylogeny explains the pattern of variation across mitochondrial haplotypes. For each trait the between-line variance was separated into a component associated with the mtDNA phylogenetic tree (phylogeny) and a residual line-specific component (mtDNA lines). The phylogenetic heritability gives the proportion of between-line variance explained by the phylogeny. Errors are 95% credible intervals.

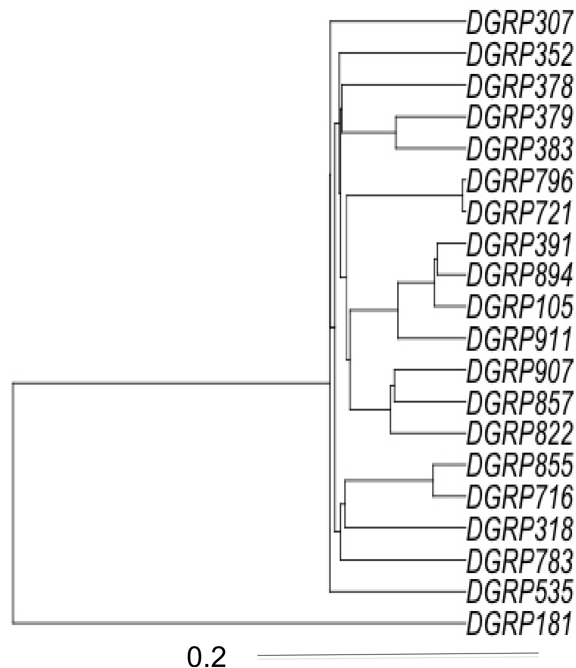

Figure. 2: The phylogenetic tree for the 20 haplotypes derived from the whole genome mtDNA sequences. The tree was pruned from a larger phylogeny created by Richardson *et al*. (2012).

4.4.2. The mitochondrial phylogeny explains phenotypic variance across mitochondrial haplotypes

To provide further evidence that sequence variation in the mitochondrial genome influenced this diverse array of phenotypes, we tested the degree to which the phylogeny of the whole mitochondrial genomes (Figure 2) explained the pattern of phenotypic variation across 20 of the mtDNA lines. By partitioning the between-line phenotypic variance into a component explained by the phylogeny and a residual component due to the line-specific factors, we calculated the phylogenetic heritability, giving the proportion of between-line variance associated with the

phylogeny (Table 2). Out of the seven traits for which the mtDNA line variance exceeded that of the IC lines (above), the highest phylogenetic heritabilities were found for metabolic rate and bacterial susceptibility (Table 2). Desiccation resistance, CO_2 anaesthesia recovery and body weight demonstrated intermediate phylogenetic heritabilities (Table 2). The lowest phylogenetic heritability was found for chill coma and wing size (Table2).

4.4.3. Mitochondrial haplotypes have independent effects on different phenotypic traits

Next we investigated the extent to which mitochondrially encoded genetic variation influenced the seven traits independently. By fitting bivariate-response mixed effect models for the 22-mtDNA line dataset we estimated the genetic covariances between the traits and also the inter-trait correlation coefficients (Table 3). There were no trait-pairs where the genetic covariance was significant: in all cases the 95% credible intervals overlapped zero. Nevertheless, some traits had relatively larger, but non-significant, inter-trait genetic correlation values. The strongest associations were a positive correlation between performance in the desiccation resistance and bacterial susceptibility assays (-0.465; 95%CI, -0.875 – 0.087), as well as a negative association between performance in the bacterial assay and metabolic rate (-0.447; 95%CI, -0.908 – 0.139).

Table. 3: An assessment of inter-trait genetic associations for seven traits across 22 mitochondrial haplotypes

Inter-trait correlation coefficient with 95% CI (above diagonal); Inter-trait covariance with 95% CI (below diagonal)

	DR	CO_2	CC	MR	BW	WS	BS
DR	–	0.264 [-0.244 – 0.759]	-0.057 [-0.598 – 0.510]	0.096 [0.594 – 0.709]	0.035 [-0.521 – 0.567]	-0.211 [-0.701 – 0.318]	-0.465 [-0.875 – 0.087]
CO_2	3.222 [-4.611 – 13.58]	–	0.303 [-0.246 – 0.807]	-0.365 [-0.897 – 0.255]	0.073 [-0.470 – 0.628]	0.091 [-0.432 – 0.661]	0.322 [0.276 – 0.8
CC	-0.781 [-9.031 – 5.670]	1.358 [-2.107 – 4.353]	–	-0.328 [-0.857 – 0.408]	-0.282 [-0.795 – 0.227]	-0.247 [-075 – 0.301]	0.010 [-0.570 – 0.626]
MR	0.088 [-0.651– 0.792]	-0.141 [-0.491 – 0.162]	-0.127 [-0.411 – 0.140]	–	0.287 [-0.291 – 0.531]	-0.036 [-0.666 – 0.531]	-0.447 [-0.908 – 0.139]
BW	0.026 [-0.252 – 0.374]	-0.056 [-0.191 – 0.073]	0.011 [-0.091 – 0.122]	0.004 [-0.013 – 0.021]	–	0.205 [-0.353 – 0.667]	-0.154 [-0.689 – 0.415]
WS	0.076 [-0.321 – 0.141]	-0.038 [-0.141 – 0.052]	0.010 [-0.080 – 0.082]	-3.02x10-4 [-0.010 – 0.011]	0.001 [-0.002 – 0.012]	–	0.294 [-0.229 – 0.772]
BS	-0.375 [-1.010 – 0.190]	0.004 [-0.270 – 0.240]	0.097 [-0.120 – 0.301]	-0.011 [-0.030 – 0.011]	-0.002 [-0.010 – 0.012]	0.003 [-0.004 – 0.011]	–

This table gives the inter-trait genetic covariances and the inter-trait genetic correlation coefficients with their 95% credible intervals. The phenotypic traits are: DR = desiccation resistance, CO_2 = CO_2 anaesthesia recovery, CC = chill coma recovery, MR = metabolic rate, BW = bodyweight, WS = wing size and BS = bacterial susceptibility.

Table. 4: Sexual dimorphism in the 10 phenotypes. Traits means are given along with 95% credible intervals and P values

Phenotypic trait	Trait mean with 95% CI		*P*
	Female	Male	
Desiccation resistance	39.194 [37.43 –40.93]	27.29 [24.94 – 29.97]	<0.001
Starvation resistance	57.84 [54.86 – 61.40]	38.46 [34.83 – 42.72]	<0.001
CO_2 anaesthesia recovery	10.90 [9.651 – 12.16]	9.870 [7.791 – 12.04]	<0.001
Chill coma recovery	21.93 [20.12 – 23.75]	18.86 [15.86 – 20.62]	<0.001
Negative geotaxis	5.691 [4.870 – 6.551]	7.162 [6.051 – 8.272]	<0.001
Metabolic rate	122.81 [74.83 – 162.36]	108.98 [57.54 –152.29]	<0.001
Body weight	1.250 [1.220 – 1.281]	0.780 [0.731 – 0.830]	<0.001
Wing size	1.311 [1.290 – 1.322]	1.151 [1.130 – 1.172]	<0.001
Fungal susceptibility	0.552 [0.361 – 0.732]	0.542 [0.270 – 0.792]	0.694
Bacterial susceptibility	0.591 [0.481 – 0.701]	0.631 [0.491 – 0791]	0.042

4.4.4. Most traits were strongly sexually dimorphic but intersexual mitochondrial genetic covariances were generally high

Nine of the ten traits studied were sexually dimorphic. In comparison to females, males had: significantly smaller bodies and wings; lower metabolic rate, desiccation and starvation resistance; quicker recovery from chill coma and CO_2 anaesthesia; faster climbing speed and higher susceptibility to bacterial infection (Table 4). There was no difference between males and females for fungal infection susceptibility (Table 4).

Table. 5: Between-sex trait associations. The intersexual covariances and intersexual correlation coefficients across the mtDNA haplotypes are given with their 95% CIs

Phenotypic traits	Inter-sexual genetic trait association with 95% CI	
	Covariance	Correlation coefficient
Desiccation resistance	10.59 [1.707 – 21.31]	0.604 [0.126 – 0.832]
CO_2 anaesthesia recovery	2.024 [-0.081– 4.492]	0.755 [-0.069 – 0.985]
Chill coma recovery	1.581 [0.260 – 3.421]	0.853 [-0.033 – 0.997]
Metabolic rate	26.86 [-19.99 – 71.89]	0.696 [-0.588 – 0.992]
Body weight	0.002 [0.001 – 0.004]	0.891 [0.421 – 0.993]
Wing size	0.001 [-1.02×10^{-4} – 0.002]	0.501 [-0.024 – 0.805]
Bacterial susceptibility	0.011 [-6.01×10^{-5} – 0.024]	0.487 [-0.787 – 0.892]

However, considering the seven traits that were influenced by mtDNA variation, all showed relatively strong intersexual mtDNA encoded genetic covariances, which were all positive (Table 5). The power to detect significant inter-sexual covariances is relatively weak in this data set; however, for two traits (body weight and desiccation resistance) the credible intervals on these covariances did not overlap zero. Regardless of significance, the intersexual mtDNA genetic correlations were particularly strong for body weight, chill coma recovery and CO_2 anaesthesia recovery; the lowest correlation (0.487) was for bacterial susceptibility (Table 5).

4.4.5. No evidence of excess male-specific mtDNA encoded genetic variation

A central prediction of the Mother's Curse hypothesis is that the persistence of mutations with male-specific effects should result in higher phenotypic variance between mitochondrial haplotypes when they are expressed in males than in females. However, among the seven traits influenced by mtDNA variation, none demonstrated considerably greater mitochondrially-derived phenotypic variation in males than in females across the 22 mtDNA lines. In most cases the mtDNA variance for males and females was similar and did not differ significantly (Figure. 3). For two traits, bacterial susceptibility and CO_2 recovery, males did exhibit greater mtDNA variance than in females, but only marginally so and this was not significant (Figure. 3, P = 0.656 and P = 0.610 respectively). Whereas, for three traits females displayed significantly greater mtDNA variance than males (Figure 3): metabolic rate (P = 0.012), body weight (P = 0.004) and wing size (P = 0.022).

For six of the seven phenotypes, females had a larger mean trait value than males on the measurement scale of our assays. The comparison of genetic variances in males and females could therefore be biased if the trait variances scale with the mean. For this reason we also calculated the genetic coefficient of variation for both sexes, which scales the index of variation by the mean (Houle 1992). However, in no case was the genetic coefficient of variation significantly different between the sexes and in general the point estimates were closely similar (Table 6).

Table. 6: Quantification of the extent of between-haplotype variation in males and females for each trait.

Phenotypic traits	Genetic coefficient of variation with 95% CI		*P*
	Female	Male	
Desiccation resistance	11.65 [8.142 –16.90]	14.31 [11.54–22.51]	0.930
CO_2 anaesthesia recover	12.52 [2.150 – 21.59]	17.10 [5.061–28.99	0.804
Chill coma recovery	7.39 [6.121 – 20.82]	5.06 [0.096–9.320]	0.199
Metabolic rate	6.45 [2.421 – 11.341]	2.60 [2.01×10^{-3}–7.041]	0.102
Body weight	4.45 [3.140 – 7.420]	3.58 [2.441–6.481]	0.300
Wing size	3.74 [3.01×10^{-3} – 26.87]	3.61 [1.832–4.271]	0.094
Bacterial susceptibility	4.22 [9.01×10^{-3} – 24.38]	0.655 [0.035–22.85]	0.501

In this table variation is given as the genetic coefficient of variation with 95% CIs and P values for the between-sex differences.

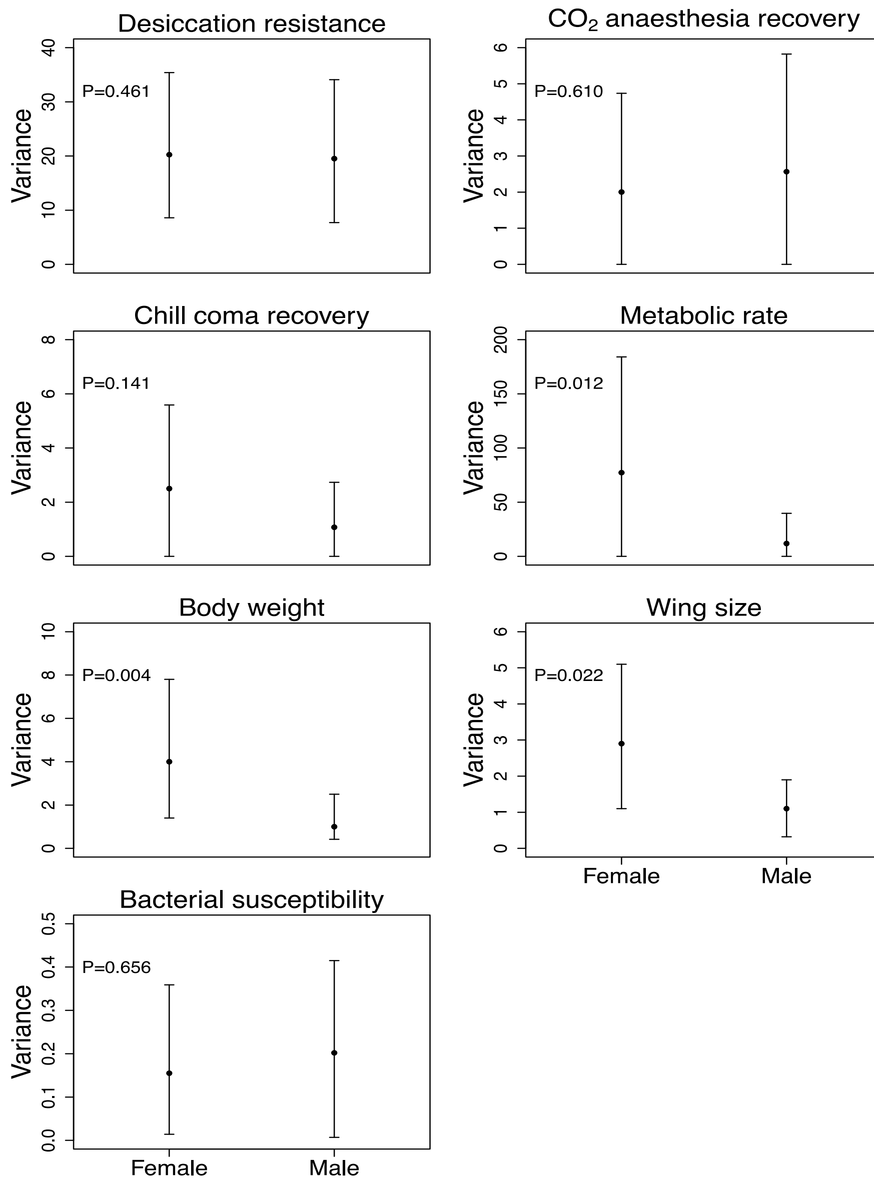

Figure. 3: Comparison of the effects of mtDNA variation on phenotypic variation in males and females for seven traits. Data points give trait variances across 22 different mitochondrial haplotypes with their 95% credible intervals. The P value indicates the significance of variance differences between the sexes of each trait.

4.4.6. The mitochondrial phylogeny does not explain more phenotypic variation in males

As an alternative and more direct way to assess the relative impact of mitochondrial sequence variation on phenotype in males and females, we compared the extent to which the phylogeny of the mitochondrial genomes explained phenotypic variation in the two sexes. For each sex we partitioned the variation between the mtDNA lines into a phylogeny component and a residual line-specific component, then compared the phylogenetic heritability estimates between males and females (Table 7). High phylogenetic heritability can result either because the phylogeny variance component is high or because the residual line variance component is low. To evaluate support for the Mother's Curse hypothesis we looked for traits where both the phylogenetic heritability and the phylogeny variance component were comparatively larger in males than females. For desiccation resistance, both the phylogenetic and line variances were higher in males and the phylogenetic heritability for males was twice that for females; this supports a male-specific effect of mtDNA variation for this trait, however 95% CIs on all these terms were very broad and overlapping (Table 7). The reverse was true for body weight: in this case, compared to males, females had higher phylogenetic and line variances, combined with higher phylogenetic heritability, again with broad CIs (Table 7). However, all other traits demonstrated broadly similar levels of mitochondrial phylogenetic heritability for the two sexes.

Table. 7: A comparison of the relative magnitude of mtDNA phylogentic effects and residual line-specific effects on phenotype for the two sexes

Phenotypic traits	Variance with 95% CI				Phylogenetic heritability	
	Phylogeny		mtDNA line		Female	Male
	Female	Male	Female	Male		
Desiccation resistance	14.27 [$3.73x10^{-6}$ – 51.21]	32.58 [$2.19x10^{-6}$ – 92.93]	14.75 [3.641 – 30.20]	9.00 [$5.69x10^{-5}$– 22.40]	0.37 [$3.74x10^{-7}$– 0.86]	0.65 [0.031 – 0.99]
CO_2 anaesthesia recovery	4.650 [$3.54x10^{-5}$ – 15.84]	2.81 [$3.67x10^{-8}$ – 10.01]	2.071 [$1.42x10^{-6}$ – 5.09]	0.797 [$1.15x10^{-5}$– 2.680]	0.531 [0.012 – 0.99]	0.64 [0.011 – 0.99]
Chill coma recovery	1.871 [$1.09x10^{-7}$ – 7.680]	3.59 [$8.97x10^{-7}$ – 13.39]	2.442 [$4.01x10^{-4}$ – 5.28]	3.171 [0.051 – 6.721]	0.33 [$1.16x10^{-7}$– 0.86]	0.39 [$2.18x10^{-7}$ – 0.93]
Metabolic rate	130.80 [0.0011 – 439.30]	63.92 [$3.78x10^{-6}$ – 482.01]	39.21 [$3.10x10^{-5}$– 122.90]	15.19 [$1.35x10^{-5}$– 54.78]	0.67 [0.031–1]	0.70 [0.022 – 0.99]
Body weight	0.006 [$2.20x10^{-11}$ – 0.022]	0.001 [$3.54x10^{-11}$ – 0.012]	0.003 [$8.41x10^{-9}$– 0.011]	0.001 [$3.12x10^{-8}$– 0.003]	0.54 [0.011 – 0.99]	0.38 [$3.25x10^{-8}$ – 0.97]
Wing size	0.003 [$1.64x10^{-11}$ – 0.011]	0.001 [$2.31x10^{-10}$ – 0.004]	0.003 [$3.09x10^{-7}$– 0.011]	0.001 [$3.12x10^{-8}$– 0.003]	0.34 [$4.261x10^{-9}$ – 0.89]	0.39 [$1.88x10^{-7}$ – 0.89]
Bacterial susceptibility	0.022 [$3.95x10^{-9}$ – 0.061]	0.022 [$3.95x10^{-9}$ – 0.061]	0.011 [$2.29x10^{-9}$– 0.022]	0.011 [$1.70x10^{-11}$–0.021]	0.63 [0.022 – 1]	0.64 [0.022 – 1]

This table demonstrates the sex-specificity in the effects of the mtDNA phylogeny. For each trait and each sex, the between-line variance was divided into at component associated with the phylogeny and a residual line-specific component. The phylogenetic heritability gives the proportion of between-line variance explained by the phylogeny. 95% credible intervals are given.

4.4.7. Evaluation of Mother’s Curse hypothesis

Mother’s Curse suggests that selection on the mitochondrial genome as it passes through females is inefficient at optimising mitochondrial function in males. However, the sex-specificity of allelic variation and thus the power of the Mother’s Curse mechanisms may vary between traits. Traits with high inter-sexual genetic covariance should be relatively unsusceptible to Mother’s Curse, whereas, a trait for which the phenotype produced in females is poorly correlated with that in males should be more susceptible. For the seven traits influenced by mtDNA polymorphism, we investigated whether an association existed between the strength of the inter-sexual genetic covariance and the relative magnitude of mitochondrially-encoded phenotypic variation in males and females (Figure 4). The pearson’s correlation coefficient analysis provided a very weekly positive association between them ($r = 0.252$, 95%CI: -0.45 – 0.76, $P = 0.586$). Whilst there is no statistical support for this association, the relationship that exists is in the reverse direction to that predicted by the Mother’s Curse hypothesis.

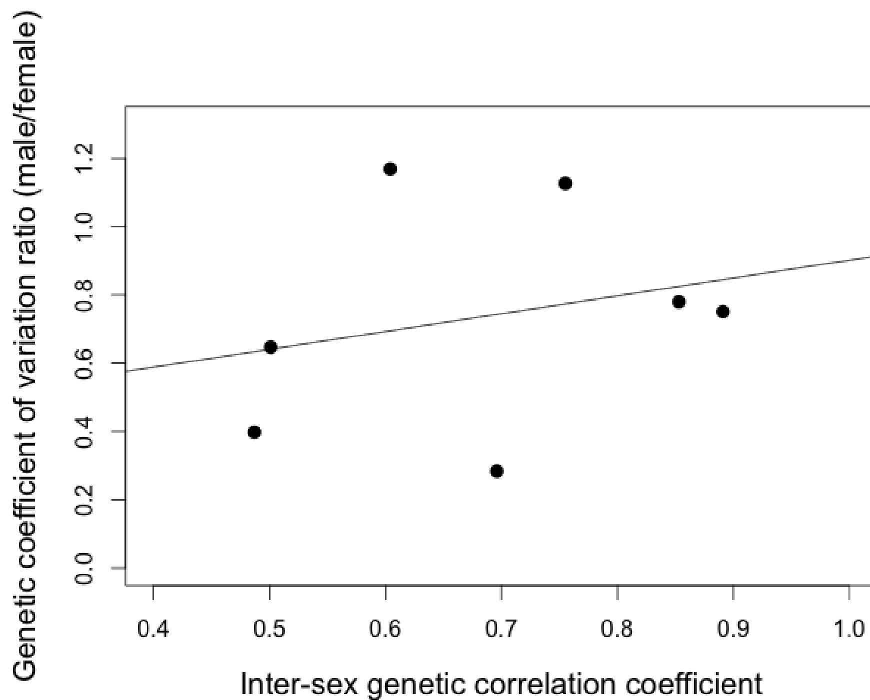

Figure. 4: Testing whether the correlation between the effects of mtDNA variation in males and females influences the susceptibility to traits to the Mother's Curse hypothesis. Sex-specific mitochondrial selection predicts that selection will be least efficient at shaping mitochondrial function in males for traits where the genetic intersexual covariance is lowest. Each data point shows a different trait: desiccation resistance, recovery from CO_2 anaesthesia, chill coma recovery, metabolic rate, body weight, wing size and bacterial infection susceptibility. The y-axis shows the magnitude of mtDNA-encoded variation in males relative to that in females: for data points above 1.0 on the y-axis, variation in males exceeds that in females. This graph shows no strong trend (P = 0.586, see text) and a gradient in the opposite direction to that predicted by the Mother's Curse hypothesis.

4.5. Discussion

The Mother's Curse hypothesis suggests that the fitness of males is compromised by sub-optimal mitochondrial function resulting from maternal mitochondrial inheritance and female-specific selection (Frank and Hurst 1996, Gemmell *et al.* 2004). We tested for the evidence of Mother's Curse by studying the extent to which mitochondrial genome polymorphism caused phenotypic variation in 10 different traits. Despite a comprehensive investigation, we found no evidence of Mother's Curse effects, casting doubt on the generality of this hypothesis.

A prerequisite for the Mother's Curse hypothesis is that the mtDNA allelic variation must be non-neutral. Phenotypic variation between mtDNA haplotypes has been found before (MacRae and Anderson 1988, Blouin 2000, Mishmar *et al.* 2003) and is now known to be widespread (Dobler *et al.* 2014). Nevertheless, we suspect that there may be a publication bias towards research that finds a given phenotype is influenced by mtDNA variation. Our study provided an unbiased screen for the impact of mtDNA polymorphism on 10 phenotypic traits. Of these, we found strong evidence that seven varied between mitochondrial haplotypes: desiccation resistance, CO_2 anaesthesia recovery, recovery from chill coma, metabolic rate, body weight, wing size and bacterial susceptibility. Other studies have also investigated the impacts of mtDNA variation on some of these traits. Previously mtDNA effects have been shown for metabolic rate (Pichaud *et al.* 2012), recovery from chill coma (Ballard *et al.* 2007), *S. marcescens* infection susceptibility (Chapter 3) and starvation resistance (Aw *et al.* 2011); notably, here we did not

find an impact of mtDNA variation for starvation resistance. However, to our knowledge, our findings of mtDNA variation effects for desiccation resistance, CO_2 recovery, body weight and wing size are entirely novel. In summary, for the traits we studied, a substantial majority is indeed influenced by mtDNA variation.

We present two lines of evidence that the mtDNA haplotype variants influence these seven traits. First, we compared the extent to which these traits varied across a panel of 22 haplotypes, all expressed on a uniform nuclear background. We created these fly lines by backcrossing haplotypes to an inbred nuclear genome for 20 generations. Whilst these lines differ in their mtDNA, it is possible that they might also vary due to residual nuclear genetic variation that was not eliminated during backcrossing, or alternatively due to non-genetic factors. We therefore used a set of seven ‘introgression control’ lines, produced using this same process, but carrying identical haplotypes, to assess the level of trait variation that might not be related to mtDNA differences. This provides a robust test of mtDNA effects that has not, to our knowledge, previously been used in similar studies (Dowling *et al.* 2007a, Dowling *et al.* 2007b, Dowling *et al.* 2007c, Friberg and Dowling 2008). By these criteria, the between-line variation that existed for three of our traits (starvation resistance, negative geotaxis response and fungal pathogen susceptibility) was not due to mtDNA variation. Furthermore, for metabolic rate the variation between mtDNA lines was only marginally significantly greater than in the controls. Our conclusion of a mtDNA polymorphism effect on metabolic rate is backed up by a previous studies in *Drosophila simulans* (Pichaud *et al.* 2012, Ballard *et al.* 2007) and also in humans (Tranah *et al.* 2011).

Our second line of evidence that mtDNA variation influenced the majority of the phenotypes we studied was that we tested for an association between the pattern of between-line trait variation and the pattern of relatedness between haplotypes (derived from the whole mtDNA genome phylogeny). With this method we calculated the phylogenetic heritability: the proportion of between-line variation explained by the phylogeny. By this measure, all of the seven traits had relatively high phylogenetic heritability. Of note, metabolic rate, which was only marginally significant in the mtDNA vs IC line test, had the highest phylogenetic heritability of any trait (0.76). It is worth noting that whilst high phylogenetic heritability is strong evidence that mtDNA variation influences phenotype, low phylogenetic heritability does not preclude this. The phylogeny topology is mostly strongly influenced by loci with relatively ancient allelic variation. Whereas, if recently derived haplotype-specific alleles in the phylogeny strongly influence trait variation, these will reduce the estimate of phylogenetic heritability.

Our finding that mtDNA-encoded variation influences many traits raises the question whether these effects are independent of one another. Does variation in a single mitochondrial process (such ATP generation efficiency) have pervasive impacts for multiple traits, or do different mechanisms underlie them? Chapter 3 argued that between-haplotype variation in susceptibility to infection with the bacterium *S. marcescens* might result from highly specific pathogen-mitochondrion interactions (Llagostera *et al*. 2003). We investigated whether the effects of mtDNA-encoded allelic variation were independent across the seven traits by testing for between-trait mitochondrial genetic covariance. We found no evidence that variation was significantly associated for any traits: none of the inter-trait

covariances was significantly different from zero. Nevertheless, with 22 haplotypes, our power to detect these covariances was not especially high. The haplotype phylogeny contained only 45 polymorphic sites, which must place an upper limit on the number of independent mechanisms that could be affected by this variation. Whilst this level of polymorphism does not exclude the possibility that variation in every trait is influenced by an independent mitochondrial mechanism, it does suggest that there may be some mitochondrial processes with diverse phenotypic effects. Nevertheless, even if the mitochondrial mechanisms driving variation in some traits are the same, our failure to find inter-trait genetic covariance does suggest that the phenotypic effects of haplotype differences on different traits are uncorrelated.

Our main test of the Mother's Curse hypothesis was to determine whether genetic variation in mtDNA caused more phenotypic variation in males than in females, as would be expected if the mitochondrial genome harbours deleterious alleles with male-specific effects (Frank and Hurst 1996). For the seven traits that were influenced by haplotype variation, none displayed significantly higher mtDNA-driven variation in males, compared to females. For three traits, body weight, wing size and metabolic rate, mtDNA genetic variation actually caused significantly larger phenotypic differences in females than in males. Female trait values tended to be larger than those in males; in case variances scaled with the mean, we repeated these analyses using the genetic coefficient of variation as the variability index. In these tests variability between haplotypes was never significantly different between the sexes, and for five of seven traits the trend was for greater variation in females. As a further test for the Mother's Curse phenomenon we

compared the mtDNA phylogenetic heritability of the two sexes for each trait; thereby testing if sequence variation in the haplotype phylogeny was associated with more phenotypic variation in males. This trend did exist for one trait, desiccation resistance, for which the mtDNA phylogenetic heritability in males was twice that in females; however, there was no statistical support that this difference was significant. For the remaining six traits, the phylogenetic heritability estimates for the two sexes were similar and not significantly different.

As a final test, we wondered whether some phenotypes might be more susceptible to Mother's Curse than others. If the phenotype that a haplotype produces in males is mechanistically well correlated to the phenotype in females, then female-limited mitochondrial selection should be efficient at optimising mitochondrial function for males. However, traits where the inter-sexual genetic correlation is low may be particularly susceptible to Mother's Curse. This rationale would predict that there should be a negative correlation across traits between the strength of the inter-sexual mtDNA genetic covariance and the relative amount of mtDNA genetic variation in males compared to females. We tested for this relationship across the traits we studied, but it was not present. In summary, across seven traits and using several analyses, we found no evidence that mtDNA harbours alleles with male-specific deleterious effects that result in greater phenotypic variation in males than in females. This clearly contradicts the predictions of the Mother's Curse hypothesis (Gemmell *et al*. 2004, Zeh and Zeh 2005).

Whilst this study does not support the Mother's Curse hypothesis, other research has found evidence consistent with its effects. Notably, *Drosophila* studies on

longevity and variation in nuclear gene expression have found compellingly greater mtDNA-encoded genetic variation in males than in females (Innocenti *et al*. 2011, Camus *et al*. 2012). Our study differed from these in two important respects. The haplotypes in our study all came from a single population and were expressed on a nuclear background from that population, with which they were coevolved. In contrast, studies finding strong Mother's Curse effects have used mitochondrial haplotypes that were collected from geographically distant populations and expressed alongside a foreign nuclear genome (Innocenti *et al*. 2011, Camus *et al*. 2012). It has been suggested that if mitochondrial genomes function poorly in males, this should select for nuclear modifiers that compensate for the effect of particular mtDNA loci (Beekman *et al*. 2014). The existence of strong epistasis between mitochondrial and nuclear genomes suggests that nuclear modifiers can buffer deleterious mtDNA effects (Zeyl *et al*. 2005, Bar-Yaacov *et al*. 2012, Kenney *et al*. 2014). We hypothesise that if that selection on the nuclear genome is efficient at counteracting male-specific deleterious mtDNA alleles , then it will only be apparent following inter-population crosses, when haplotypes are expressed on nuclear backgrounds with which they have not coevolved. Mitochondrial maladaptation might then contribute importantly to reproductive incompatibility between populations and species (Dowling *et al*. 2007c, Lee *et al*. 2008). However, whilst Mother's curse would have interesting evolutionary consequences, our data suggest it does not contribute substantially to male phenotypic differences in natural populations, nor provide a general explanation for sexual dimorphism.

An alternative explanation for why Mother's Curse effects were absent in our study

is that whilst selection on males may be week or absent, mtDNA mutations with male-specific effects arise only rarely, or remain at low frequency in natural populations. If this is true, Mother's Curse (Gemmell *et al*. 2004) may be a theoretical hypothesis that is not frequently realised in practice. We investigated the strength of mtDNA genetic covariance between traits in males and females. If mtDNA alleles with male-specific effects were common, the inter-sexual mtDNA covariance should be low. Nevertheless, these covariances were relatively high for most traits. This is consistent with there being few mtDNA loci with male-specific effects, but is also compatible with the hypothesis that nuclear modifiers buffer the effect of such loci.

Whilst this study found no evidence of a Mother's Curse bias in the effects of mtDNA genetic variation, it did reveal equivocal evidence for the opposite: that mtDNA variation might be the cause of more phenotypic variation in females. This could be explained if development in females is generally more susceptible to variation in the energy generating capabilities of mitochondria, as suggested before (Ballard *et al*. 2007, Dowling *et al*. 2007a). However, we suggest an alternative hypothesis that runs contrary to all the predictions of Mother's Curse. It is increasingly clear that mtDNA variation has pervasive impacts on phenotype (Dobler *et al*. 2014). Furthermore, mtDNA has played an important role in adaptation to novel environments (Ruiz-Pesini *et al*. 2004) and coevolution with the nuclear genome can maintain mtDNA variation through negative frequency dependent selection (Kazancıoğlu and Arnqvist 2014). If much genetic variation in mtDNA is indeed maintained by selection, this generates the opposite prediction to the Mother's Curse hypothesis. If mtDNA alleles with female-specific phenotypic

effects exist, then due to the asymmetry in the strength of selection on mitochondria between the sexes, mtDNA could cause greater phenotypic variation in females.

Certain sex-limited phenotypes such as sperm viability provide serious evolutionary conundrums because direct selection on mitochondrial function for these traits in males must normally be very weak or absent (Frank and Hurst 1996, Zeh and Zeh 2005). However, the extent to which Mother's Curse generally affects other life-history traits has been less clear. The Mother's Curse hypothesis attempts to explain general sexual dimorphism and the reason why males may often perform poorly relative to females for some sexually dimorphic traits such as ageing (Gemmell *et al*. 2004). Nevertheless, there are many theories that attempt to explain sexual dimorphism, not least generally sexually divergent life-history selection (Bateman 1948, Dowling 2014b, Chen and Maklakov 2014). We have found no evidence that the sex-specific selective sieve that operates on mitochondria results in a larger mitochondrial mutational load in males. We suggest that, either selection on mtDNA in females is relatively successful at optimising mitochondrial function for males, or that nuclear modifiers are efficient at ameliorating the effects of male-specific deleterious alleles. Whilst this study does not support the Mother Curse Hypothesis, it does demonstrate the widespread influence of mtDNA genetic variation on a diverse range of phenotypes.

5. Chapter 5: Discussion

5.1. Costs of immune activation: resource reallocation or resource acquisition?

Trade-offs between life-history traits such as immunity, reproduction and growth are common and have been studied across many species (Kraaijeveld *et al*. 2001, Armitage *et al*. 2003, Schwartz and Koella 2004, Zerofsky *et al*, 2005). One explanation of these trade-offs is competition between life-history traits for the allocation of limited resources (Stearns 1989, Boggs 2009). However, trade-offs between life-history traits can also be due to other reasons, for example physiological processes (the sharing of common biochemical or signalling pathways) or genetic factors (such as the effects of antagonistic pleiotropy) and may also be shaped by the environment (Flatt 2011, Van Straalen and Roelofs 2012). In chapter two I demonstrated that fecundity declined by 45% following immune activation, demonstrating a clear trade-off with immune defence. However, my data were not consistent with the theory that this trade-off resulted from the simple reallocation of resources from fecundity to immunity. Whilst metabolic rate declined slightly, activating the immune response caused a 31% reduction in food consumption. This suggests that impaired resource acquisition is the primarily driver of the trade-off between immunity and fecundity in *Drosophila melanogaster*.

At the most fundamental level, activating or investing in any life-history trait must necessarily consume resources, which must then be unavailable for other life-history functions. However, in the case of immunity, my data suggest that the effect of this resource consumption is small in comparison to the decline in resource acquisition that occurs when the immune system is activated. It is interesting to question the extent to which resource acquisition variation, instead of alterations in resource allocation decisions, explains other life-history trade-offs. Impaired resource acquisition does mediate trade-offs between a number of other life-history traits. For example, Tatar and Carey (1995) found that poor food consumption was behind the trade-off between reproduction and survival in the beetle *Callosobruchus maculatus*; in their study improving available food resources during peak reproduction reduced the severity of this trade-off. More generally, certain life-history activities may be costly because the behaviours involved reduce the opportunity of organisms to feed. For example, in mammals with highly polygynous mating systems where males hold harems, a strong driver of the costs of reproductive activity for males is that opportunities for resource acquisition are restricted whilst defending a harem (Bonenfant *et al.* 2004). Nevertheless, the mechanism of immune-induced fecundity costs that I have identified, where investment in one trait (parasite defence) triggers a physiological reduction in feeding rate, may perhaps still represent a relatively unique case. This leaves open the question as to whether costly life-history trade-offs in general are more frequently driven by resource reallocation, or changes in resource acquisition (van Noordwijk and de Jong 1986).

I found no evidence that the immune response consumes additional energetic resources, indeed metabolic rate dropped by ~6% following immune challenge. That flies reduced their food intake by 31% suggests that energy budgets become severely restricted during immune defence, which seems maladaptive. However, the infection-induced anorexia that I observed is a common behaviour found in animals and humans upon immune challenge (McDermott *et al*. 2006, Segerstrom 2007). Research has highlighted this behaviour, not as a maladaptive response but instead an evolved behavioural strategy of infected hosts to increase tolerance of pathogens and help overcome illness (Ayres and Schneider 2009). Quite a number of theories for why this response is adaptive have been suggested (Exton 1997). However one potential adaptive consequence of infection-induced anorexia relates to the interaction between diet and senescence. Dietary restriction has a conserved link with increased longevity and slowed senescence. Initially this was demonstrated in laboratory rodents (McCay *et al*. 1935) but other work shows this ageing phenomenon in a range of species from *Drosophila* (Metaxakis and Partridge 2013) to humans (Willcox *et al*. 2007) (but contradictory studies are also reported (Maxmen 2012)). Dietary restriction is, perhaps, a strategy to slow ageing during starvation conditions, thus increasing longevity and future reproductive opportunities (Kirkwood and Shanley 2005). In the context of infection-induced anorexia, reduced feeding during infection could potentially also function as a dietary restriction response, minimising senescence during a time when organisms have reduced reproductive potential. Protective mechanisms that slow senescence may be induced during infection, thereby preserving future fitness after the infection has been cleared.

I have provided evidence in chapter two that resource acquisition impairment may be a primarily driver of the trade-off between immunity and fecundity. However, this conclusion is indirect and correlative: the feeding rate reductions following immune activation appear to be of sufficient magnitude to explain fecundity declines, without needing to invoke resource reallocation as a mechanism. It would be possible to generate more experimental evidence that impaired resource acquisition drives immunity costs to strengthen this conclusion. For example, by demonstrating that experimentally reducing food intake by a similar magnitude to infection-induced anorexia causes a comparable fecundity reduction. Experiments could include three treatment groups: two unmanipulated fly groups and a third group of immune-challenged individuals. One of the unmanipulated fly groups and the immune-challenged group would be provided with normal food, whereas the second unmanipulated fly group would be fed a restricted diet (31% reduced calories by mixing the diet with an inert bulking ingredient such as agarose). Comparing fecundity between these three groups would help to determine if the decline in fecundity of immune challenged flies was entirely down to lower food consumption.

5.2. The influence of mtDNA allelic variation on disease susceptibility

Mitochondria play a crucial role in eukaryotic cells by generating ATP to fuel all physiological traits, ranging from basic growth to immunity and reproduction. Whether life-history trade-offs are due to the reallocation of limited resources, or altered resource acquisition, the ability of mitochondria to supply energy is central to eukaryote physiology. Much work has been done on the role of variation in mitochondrial function in cell processes (Feissner *et al*. 2009), ageing (Camus *et al*. 2012), sperm fertility (Luo *et al*. 2013) and non-communicable diseases (Dowling 2014a). Recently, mitochondrial functionhas been shown to influence a broad range of innate immune pathways: mitochondria not only regulate antiviral signalling but also facilitate antibacterial immunity by generating reactive oxygen species and contributing to innate immune activation (West *et al*. 2011).

Genetic variation in populations is a common cause of variation in all life-history traits, especially in the ability of an organism to mount successful immune responses (Lazzaro and Little 2009). In this thesis I undertook the first study of the role of mtDNA encoded allelic variation in influencing host susceptibility to infectious diseases. I found that susceptibility to *Serratia marcescens*, but not *Beauveria bassiana*, was significantly affected by mtDNA allelic variation. In addition, interactions between the mitochondrial and nuclear genomes (mitonuclear epistasis) had twice the effect on *S. marcescens* susceptibility than did variation in either the nuclear or mitochondrial genomes individually. This suggests that coevolutionary processes between the mitochondrial and nuclear

genomes may influence disease susceptibility.

The findings from the third chapter of this thesis demonstrate that specific interactions between pathogens and particular host mitochondrial genotypes can strongly influence host susceptibility to pathogenic attack. Previous studies of how genetic variation influences disease resistance in *Drosophila* have focussed only on the role of nuclear genome polymorphism and have ignored the potential role of mitochondrial DNA (Lazzaro *et al*. 2004, Lazzaro *et al*. 2006, Tinsley *et al*. 2006, Bangham *et al*. 2008). Nevertheless, in the Lazzaro *et al* (2006) study, three of the four bacterial pathogens for which nuclear genetic variation in susceptibility was demonstrated (*S. marcescens*, *Enterococcus faecalis* and *Lactococcus lactis*) have been reported to interact with mitochondrial proteins and even with mtDNA (Llagostera *et al*. 2003, Marreddy *et al*. 2011, Strickertsson *et al*. 2013). *S. marcescens* induces host cell apoptosis by interacting with mitochondrial proteins (Llagostera *et al*. 2003). Likewise, recently it was reported in an *E. faecalis* infected human gastric cell model, *E. faecalis* not only decreased mitochondrial respiration four-fold compared to uninfected cells but also caused significant instability in mtDNA (Strickertsson *et al*. 2013). *L. lactis* is known to interact with mitochondrial carrier proteins present in inner mitochondrial membrane that transport metabolites and cofactors across it (Marreddy *et al*. 2011). No study on the influence of nuclear genetic variation on pathogen susceptibility, prior to chapter 3 of this thesis, has controlled for differences between genotypes in the mitochondrial genome. Whilst studies have frequently found strong associations between nuclear genetic polymorphism and disease susceptibility, it seems reasonable to suggest that some of the between-genotype variation demonstrated

in studies such as those by Lazzaro *et al*. (2004, 2006), may not solely be caused by the nuclear genome and that additional variance may be explained by mtDNA differences. It is not only the pathogens used in experimental model systems that directly target mitochondrial proteins and mtDNA; many other human bacterial (Jiang *et al*. 2012) and viral (Corcoran *et al*. 2009) pathogens also modulate mitochondrial function as part of their virulence mechanism. For example, Herpes simplex virus degrades host mtDNA (Corcoran *et al*. 2009) and human immunodeficiency virus highjacks host mitochondrial proteins to grow and replicate inside the host cell (Kaminska *et al*. 2007). Thus, it seems safe to say that mitochondria are more than just the powerhouse of cells and are likely to have broad implications of medical importance in infection biology.

In chapter three I discussed why the observation that mitochondrial genetic variation influences pathogen susceptibility is likely to be of widespread evolutionary importance. Two of the possible implications I suggested were contradictory. On the one hand I suggested that because selection on mitochondrial DNA is generally considered to be inefficient (partly due to low effective population size (Ballard and Whitlock 2004)), a strong role of mtDNA in determining pathogen susceptibility might provide an explanation for why genetic variation for pathogen defence is not eroded by selection. On the other hand, I suggested that selection from pathogens might drive dynamic selective sweeps in mitochondrial genomes meaning that mtDNA plays an important role in the evolution of pathogen resistance, with correlated consequences for other traits. Whether selection from pathogens on mtDNA is inefficient or strong may depend on the pathogen in question and characteristics of the host population. It is

possible that both these implications are important in different host-parasite systems. However, mtDNA population genetics perhaps provides a clue to which is most generally true. If the frequency of mitochondrial haplotypes was often dynamically altered by selection, selective sweeps should reduce genetic variation and eliminate demographic signatures in patterns of mtDNA genetic diversity. However, the undeniable utility of mtDNA for phylogeographic studies (Ho *et al*. 2011) argues that if pathogen-mediated mtDNA selective sweeps do occur, they are not common. In contrast, relatively inefficient selection on mtDNA due to low effective population size, combined with non-additive effects of mitonuclear epistasis make it likely that mtDNA-encoded variation for pathogen defence may respond more weakly to selection than polymorphisms at nuclear loci. Thus, perhaps mtDNA variation should be added to the list of reasons why genetic variation for pathogen resistance is a pervasive feature of host populations despite short-term directional selection from many pathogens.

In some cases mtDNA evolution may occur via selection on selfish mitochondrial alleles, which spread through populations even at the cost of organism fitness, for example petite mutants in yeast (Williamson 2002). Possibly of more widespread importance in invertebrates, indirect selection may influence mitochondria through hitchhiking due to their coinheritance with cytoplasmic maternally inherited microbes such as *Wolbachia*. *Wolbachia* and other similar symbionts can spread deterministically through populations due to their ability to manipulate host reproduction, such as by sex ratio distortion (Zeh *et al*. 2005). When a *Wolbachia* strain initially invades an insect population it is associated with a single mtDNA haplotype, and as the bacterium spreads the mtDNA variant does too, depleting

mtDNA polymorphism during a selective sweep (Jiggins and Hurst 2011). Now that mtDNA is well established as being phenotypically non-neutral, especially with respect to important fitness traits such as pathogen susceptibility as shown here, the potential importance of endosymbiont-induced selective sweeps in shaping host evolution may need to be re-appraised.

5.3. The significance of the phenotypic non-neutrality of mtDNA polymorphisms for mitochondrial evolution

In this thesis I present robust evidence that mtDNA polymorphism has strong effects on a number of phenotypes. In chapter four, seven of the 10 traits were significantly influenced by mitochondrial allelic variation in *D. melanogaster*. Many of these traits seem strongly relevant for fitness, such as bacterial pathogen susceptibility, bodyweight, resistance to starvation and desiccation, and metabolic rate. These findings suggest that selection could act on mtDNA and it may evolve non-neutrally. It is worth noting that the magnitude of the mtDNA allelic variation influencing phenotypic variation, in the 22 *D. melanogaster* genomic reference panel (DGRP) lines, reported here was down to the sequence variation in only 45 nucleotides, which is much lower than the level of polymorphism segregating across fly populations. Additionally, this mtDNA sequence variation is also far smaller than the sequence variation in mitochondrial haplotypes of other species, such as humans (Van Oven and Kayser 2009). This suggests that the impact of mtDNA allelic variation on organism life-history is perhaps much greater than found here and may encourage future studies to investigate effects in more traits.

Recently, a review by Dobler *et al*. (2014) demonstrated the widespread nature of mtDNA effects on life-history traits, including longevity, reproduction and fitness. More evidence shows that different human mtDNA haplotypes differ functionally: for example haplotype H is responsible for the delayed onset of Alzheimer's disease (Shoffner *et al.* 1993), haplogroup J is correlated with improved longevity in European populations (Rose *et al*. 2001) and haplotype T is linked to impaired sperm functionality in European males (Ruiz-Pesini *et al*. 2000).

However, simple demonstration of phenotypic non-neutrality does not mean that these effects necessarily influence mtDNA evolution. The evolutionary forces acting on the mitochondrial genome are debated (as mentioned in section 5.2). mtDNA sequence analysis data provide us some insight into the nature of mitochondrial genomic evolution but these studies have conflicting results. For instance, sequence analysis of the human mtDNA hypervariable segment 1 from Ashkenazi Jews suggested that patterns of polymorphism are consistent with genetic drift (Behar *et al.* 2004). However, the Ashkenazi Jewish population is small and relatively genetically isolated, and perhaps one where genetic drift could be expected to predominate. In contrast, a study using 104 complete mtDNA sequences from various global regions (Asia, Europe, Africa, Siberia and North America) studied the ratio of non-synonymous to synonymous substitutions and found patterns consistent with non-neutral evolution in all regions except Africa. This suggests that selection perhaps plays a considerable role in shaping regional human mtDNA evolution (Mishmar *et al*. 2003). This may mean that demographic inferences from mtDNA are unreliable. Non-neutral mtDNA evolution is indicated by studies such as Bazin *et al*. (2006). These authors carried out an analysis

involving more than 1600 species testing the theoretical expectation of an association between population size and mtDNA genetic diversity. However, they found that despite variation in population size, average within-species mtDNA diversity is extremely similar across vertebrates and invertebrates; inconsistent with the view that mtDNA polymorphism is largely governed by drift. In summary, it seems likely that selection may be able to act effectively on mtDNA through some of the phenotypic effects I have shown in this thesis, providing potential explanations for molecular evolution studies suggesting non-neutral evolution.

In chapter four I showed that mtDNA haplotypes differ in their effects on metabolic activity; this has also been shown in *D. simulans* (Ballard *et al.* 2007). In chapter two I demonstrated that costs of immunity can be metabolic but are driven by limited resources. It would be interesting to investigate the role of mtDNA allelic variation on the magnitude of the costs of immune activation, by measuring how fecundity, food consumption and metabolic rate respond to immune stimulation across different mitochondrial haplotypes. This would provide information on the impact of the varying energy generating abilities of different haplotypes on immunity costs. It is likely that mtDNA variants might have a role in variation in tolerance to infection because tolerance, unlike resistance, concerns the ability of an individual to manage the pathology of infection instead of suppressing pathogen growth (Ayres and Schneider 2009). Tolerance may depend on the metabolic state of an organism and the extent it can cope with reduced resource acquisition associated with infection-induced anorexia. Thus, this thesis may encourage studies investigating the genetic basis of variation in tolerance to infection.

5.4. No evidence for Mother's Curse affecting sexual dimorphism in *D. melanogaster*

Maternal inheritance of mitochondria has been hypothesised to result in compromised male fitness due to sex-specific selection on mtDNA (Gemmell *et al*. 2004). However, I did not find evidence to support the existence of Mother's Curse in *D. melanogaster*. Among 10 traits, 9 of which were sexually dimorphic, seven were influenced by haplotype variation; however, none demonstrated significantly greater mtDNA-encoded genetic variability in males. I assessed the extent to which the mtDNA sequence phylogeny was associated with the pattern of phenotypic variation across haplotypes. By this measure, for only one trait (desiccation resistance) the phylogeny explained twice as much variation in males than females, nevertheless, in this case broad 95% credible intervals on the estimates provided no statistical support for a robust difference. That my work has found no support for the Mother's Curse hypothesis raises two possibilities: 1) Mother's Curse may not exist or 2) Mother's Curse may apply only to a narrow range of traits, such as male fertility (Smith *et al*. 2010) and ageing (Camus *et al*. 2012).

There can be several possible explanations for the discrepancy between my study of 10 traits, which did not detect the patterns of Mother's Curse, and other studies,

which claim to have found these effects. I have discussed these explanations at other points in this thesis. However, one compelling reason may be that previous

studies finding evidence of Mother's Curse have crossed haplotypes isolated from widely spaced geographic populations onto a nuclear background with which they do not share a history of coevolution (Camus *et al*. 2012, Innocenti *et al*. 2011). In contrast, my work crossed a set of haplotypes onto a nuclear genome from the same population. It has been suggested that compensatory evolution in the nuclear genome could rapidly buffer the effects of deleterious mtDNA mutations on either sex (Dobler *et al*. 2014). If this compensatory buffering is efficient, then Mother's Curse may only be evident following inter-population crosses and may not be relevant for explaining sexual dimorphism or sex-specific patterns of mtDNA genetic variation within natural populations. Whatever the cause, my data suggest that the Mother's Curse hypothesis neither strongly influences patterns of phenotypic variation within populations, nor to provide a general explanation for sexual dimorphism.

5.5. Conclusion

In conclusion, this thesis provides insight into the costs of mounting immune responses and the role of impaired resource acquisition in driving these costs. It has also demonstrated the considerable impact of mitochondrial genome allelic variation on susceptibility to infectious diseases, a phenomenon that has never previously been investigated. The results of this thesis strengthen the emerging argument that mtDNA polymorphism is phenotypically non-neutral. Furthermore it provides a powerful experimental test of the Mother's Curse hypothesis, suggesting that female-biased selection on mtDNA probably does not result in a general reduction in male fitness in natural populations. My data have allowed me to speculate on the evolutionary mechanisms shaping mitochondrial evolution and the contribution of mitonuclear epistasis as a source of non-additive mtDNA variation influencing important phenotypes. Moreover, this work provides a strong basis for future studies to explore the role of mitochondrial haplotype variation in immunity, disease susceptibility and other life-history traits.

6. References

Abel L, Alcaïs A, Schurr E. 2014. The dissection of complex susceptibility to infectious disease: Bacterial, viral and parasitic infections. *Current Opinion in Immunology* 30: 72-78.

Adamo SA, Bartlett A, Le J, Spencer N, Sullivan K. 2010. Illness-induced anorexia may reduce trade-offs between digestion and immune function. *Animal Behaviour* 79: 3-10.

Adamo SA, Jensen M, Younger M. 2001. Changes in lifetime immunocompetence in male and female *Gryllus texensis*: Trade-offs between immunity and reproduction. *Animal Behaviour* 62: 417-425.

Adamo SA, Roberts JL, Easy RH, Ross NW. 2008. Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets. *Journal of Experimental Biology* 211: 531-538.

Ahmed AM, Baggott SL, Maingon R, Hurd H. 2002. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. *Oikos* 97: 371-377.

Ahmed AM, Hurd H. 2006. Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis. *Microbes and Infection* 8: 308-315.

Aitken RJ, Jones KT, Robertson SA. 2012. Reactive oxygen species and sperm function: In sickness and in health. *Journal of Andrology* 33: 1096-1106.

Anand SK, Tikoo SK. 2013. Viruses as modulators of mitochondrial functions. Advances in Virology 2013.1-17. doi.org/10.1155/2013/738794.

Ankel-Simons F, Cummins JM. 1996. Misconceptions about mitochondria and mammalian

fertilization: Implications for theories on human evolution. *Proceedings of the National Academy of Sciences of the United States of America* 93: 13859-13863.

Armitage S, Thompson J, Rolff J, Siva-Jothy M. 2003. Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. *Journal of Evolutionary Biology* 16: 1038-1044.

Arnold PA, Johnson KN, White CR. 2013. Physiological and metabolic consequences of viral infection in *Drosophila melanogaster*. *Journal of Experimental Biology* 216: 3350-3357.

Arnqvist G, Dowling DK, Eady P, Gay L, Tregenza T, Tuda M, Hosken DJ. 2010. Genetic architecture of metabolic rate: Environment specific epistasis between mitochondrial and nuclear genes in an insect. *Evolution* 64: 3354-3363.

Auld SK, Scholefield JA, Little TJ. 2010. Genetic variation in the cellular response of *Daphnia magna* (crustacea: Cladocera) to its bacterial parasite. *Proceedings of the Royal Society B-Biological Sciences* 277: 3291-3297.

Avise JC. 2000. Phylogeography: The History and Formation of Species. *Harvard University Press.*

Aw W, Correa C, Clancy D, Ballard J. 2011. Mitochondrial DNA variants in *Drosophila melanogaster* are expressed at the level of the organismal phenotype. *Mitochondrion* 11: 756-763.

Ayres JS, Schneider DS. 2009. The role of anorexia in resistance and tolerance to infections in *Drosophila*. *PLoS Biology* 7: 1-10.

Ballard JWO, Whitlock MC. 2004. The incomplete natural history of mitochondria.

Molecular Ecology 13: 729-744.

Ballard JWO, Melvin RG, Katewa SD, Maas K. 2007. Mitochondrial DNA variation is associated with measurable differences in life-history traits and mitochondrial metabolism in *Drosophila simulans*. *Evolution* 61: 1735-1747.

Bandelt HJ, Kong QP, Parson W, Salas A. 2005. More evidence for non-maternal inheritance of mitochondrial DNA? *Journal of Medical Genetics* 42: 957-960.

Bangham J, Knott SA, KIM K, Young RS, Jiggins FM. 2008. Genetic variation affecting host–parasite interactions: Major-effect quantitative trait loci affect the transmission of sigma virus in *Drosophila melanogaster*. *Molecular Ecology* 17: 3800-3807.

Barr CM, Neiman M, Taylor DR. 2005. Inheritance and recombination of mitochondrial genomes in plants, fungi and animals. *New Phytologist* 168: 39-50.

Bar-Yaacov D, Blumberg A, Mishmar D. 2012. Mitochondrial-nuclear co-evolution and its effects on OXPHOS activity and regulation. *Biochimica Et Biophysica Acta (BBA)-Gene Regulatory Mechanisms* 1819: 1107-1111.

Bateman AJ. 1948. Intra-sexual selection in *Drosophila*. *Heredity* 2: 349-368.

Bates D, Meachler M, Bolker B. 2013. Lme4: Linear mixed-effects models using S4 classes. R package version 0999999–2. http://cran.r-project.org/web/packages/lme4/index.html.

Bashir-Tanoli S, Tinsley MC. 2014. Immune response costs are associated with changes in resource acquisition and not resource reallocation. *Functional Ecology* 28: 1011-1019.

Batista F, Boudry P, Santos DA, Renault T, Ruano F. 2009. Infestation of the cupped oysters *Crassostrea angulata*, *C. gigas* and their first-generation hybrids by the

copepod *Myicola ostreae*: differences in susceptibility and host response. *Parasitology* 136: 537-543.

Baze MM, Hunter K, Hayes JP. 2011. Chronic hypoxia stimulates an enhanced response to immune challenge without evidence of an energetic trade-off. *Journal of Experimental Biology* 214: 3255-3268.

Bazin E, Glémin S, Galtier N. 2006. Population size does not influence mitochondrial genetic diversity in animals. *Science* 312: 570-572.

Beckage NE. 2011. Insect Immunology. *Academic press, Oxford.*

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T. 2006. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nature Genetics* 38: 515-517.

Beekman M, Dowling DK, Aanen DK. 2014. The costs of being male: Are there sex-specific effects of uniparental mitochondrial inheritance? *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences* 369: 20130440.

Behar DM, Hammer MF, Garrigan D, Villems R, Bonne-Tamir B, Richards M, Gurwitz D, Rosengarten D, Kaplan M, Pergola DS. 2004. MtDNA evidence for a genetic bottleneck in the early history of the Ashkenazi Jewish population. *European Journal of Human Genetics* 12: 355-364.

Bidla G, Lindgren M, Theopold U, Dushay MS. 2005. Hemolymph coagulation and phenoloxidase in *Drosophila* larvae. *Developmental & Comparative Immunology* 29: 669-679.

Binggeli O, Neyen C, Poidevin M, Lemaitre B. 2014. Prophenoloxidase activation is

required for survival to microbial infections in *Drosophila*. *PLoS Pathogens* 10: e1004067.

Birch-Machin MA. 2006. The role of mitochondria in ageing and carcinogenesis. *Clinical and Experimental Dermatology* 31: 548-552.

Blouin MS. 2000. Brief communication. Neutrality tests on mtDNA: Unusual results from nematodes. *Journal of Heredity* 91: 156-158.

Boggs CL. 2009. Understanding insect life histories and senescence through a resource allocation lens. *Functional Ecology* 23: 27-37.

Boman HG. 2003. Antibacterial peptides: Basic facts and emerging concepts. *Journal of Internal Medicine* 254: 197-215.

Bonenfant C, Gaillard J, Klein F, Maillard D. 2004. Variation in harem size of Red deer (*Cervus elaphus* L.): The effects of adult sex ratio and age-structure. *Journal of Zoology* 264: 77-85.

Bonneaud C, Mazuc J, Gonzalez G, Haussy C, Chastel O, Faivre B, Sorci G. 2003. Assessing the cost of mounting an immune response. *American Naturalist* 161: 367-379.

Boore JL, Brown W. 1994. Mitochondrial genomes and the phylogeny of mollusks. *Nautilus* 108: 61-78.

Boore JL. 1999. Animal mitochondrial genomes. *Nucleic Acids Research* 27: 1767-1780.

Bridge D, Cunningham CW, Schierwater B, DeSalle R, Buss LW. 1992. Class-level relationships in the phylum cnidaria: Evidence from mitochondrial genome structure. *Proceedings of the National Academy of Sciences of the United States of America* 89: 8750-8753.

Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B. 2009. *Drosophila* intestinal response to bacterial infection: Activation of host defence and stem cell proliferation. *Cell Host & Microbe* 5: 200-211.

Bullaughey K, Przeworski M, Coop G. 2008. No effect of recombination on the efficacy of natural selection in primates. *Genome Research* 18: 544-554.

Burr MJ, Hunter AS. (1969) Effects of temperature on *Drosophila*- V. Weight and water, protein and RNA content. *Comparative Biochemistry and Physiology* 29: 647-652.

Burt A, Trivers R. 2006. Genes in conflict. *Cambridge*, *UK Belknap*.

Burton RS, Barreto FS. 2012. A disproportionate role for mtDNA in Dobzhansky–Muller incompatibilities? *Molecular Ecology* 21: 4942-4957.

Bryner SF, Rigling D. 2011. Temperature-dependent genotype-by-genotype interaction between a pathogenic fungus and its hyperparasitic virus. *The American Naturalist* 177: 65-74.

Camus MF, Clancy DJ, Dowling DK. 2012. Mitochondria, maternal inheritance, and male aging. *Current Biology* 22: 1717-1721.

Carius HJ, Little TJ, Ebert D. 2001. Genetic variation in a host-parasite association: Potential for coevolution and frequency-dependent selection. *Evolution* 55: 1136-1145.

Cavaliere V, Papademetrio DL, Alvarez EM, Blanco GA. 2010. Haemostatic and immune role of cellular clotting in the sipunculan *Themiste petricola*. *Cell and Tissue Research* 339: 597-611.

Chambers MC, Song KH, Schneider DS. 2012. *Listeria monocytogenes* infection causes

metabolic shifts in *Drosophila melanogaster*. *PLoS One* 7: e50679.

Chan DC. 2006. Mitochondria: Dynamic organelles in disease, aging, and development. *Cell* 125: 1241-1252.

Chen H, Maklakov AA. 2014. Condition dependence of male mortality drives the evolution of sex differences in longevity. *Current Biology* 24: 2423-2427.

Christie J, Picornell A, Moya A, Ramon M, Castro J. 2011. Mitochondrial DNA effects on fitness in *Drosophila subobscura*. *Heredity* 107: 239-245.

Clancy DJ. 2008. Variation in mitochondrial genotype has substantial lifespan effects which may be modulated by nuclear background. *Aging Cell* 7: 795-804.

Coop RL, Kyriazakis I. 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends in Parasitology* 17: 325-330.

Cummins J, Greeff J, Lymbery A. 2002. Mitochondrial DNA polymorphisms and fertility in beef cattle. *Theriogenology* 57: 1603-1610.

Corcoran JA, Saffran HA, Duguay BA, Smiley JR. 2009. *Herpes simplex* virus UL12.5 targets mitochondria through a mitochondrial localization sequence proximal to the N terminus. *Journal of Virology* 83: 2601-2610.

Dawid IB, Blackler AW. 1972. Maternal and cytoplasmic inheritance of mitochondrial DNA in *Xenopus*. *Developmental Biology* 29: 152-161.

DeLuca SZ, O'Farrell PH. 2012. Barriers to male transmission of mitochondrial DNA in sperm development. *Developmental Cell* 22: 660-668.

Derting TL, Compton S. 2003. Immune response, not immune maintenance, is

energetically costly in wild white-footed mice (*Peromyscus leucopus*). *Physiological and Biochemical Zoology* 76: 744-752.

Diamond G, Beckloff N, Weinberg A, Kisich KO. 2009. The roles of antimicrobial peptides in innate host defence. *Current Pharmaceutical Design* 15: 2377-2392.

DiAngelo JR, Bland ML, Bambina S, Cherry S, Birnbaum MJ. 2009. The immune response attenuates growth and nutrient storage in *Drosophila* by reducing insulin signalling. *Proceedings of the National Academy of Sciences of the United States of America* 106: 20853-20858.

Dobler R, Rogell B, Budar F, Dowling D. 2014. A meta-analysis of the strength and nature of cytoplasmic genetic effects. *Journal of Evolutionary Biology* 27: 2021-2034.

Dowling DK, Friberg U, Arnqvist G. 2007a. A comparison of nuclear and cytoplasmic genetic effects on sperm competitiveness and female remating in a seed beetle. *Journal of Evolutionary Biology* 20: 2113-2125.

Dowling DK, Friberg U, Hailer F, Arnqvist G. 2007b. Intergenomic epistasis for fitness: Within-population interactions between cytoplasmic and nuclear genes in *Drosophila melanogaster*. *Genetics* 175: 235-244.

Dowling DK, Nowostawski AL, Arnqvist G. 2007c. Effects of cytoplasmic genes on sperm viability and sperm morphology in a Seed beetle: Implications for sperm competition theory? *Journal of Evolutionary Biology* 20: 358-368.

Dowling DK, Friberg U, Lindell J. 2008. Evolutionary implications of non-neutral mitochondrial genetic variation. *Trends in Ecology & Evolution* 23: 546-554.

Dowling DK. 2014a. Evolutionary perspectives on the links between mitochondrial

genotype and disease phenotype. *Biochimica Et Biophysica Acta (BBA)-General Subjects* 1840: 1393-1403.

Dowling DK. 2014b. Aging: Manipulating sex differences. *Current Biology* 24: R996-R998.

Dubuffet A, Dupas S, Frey F, Drezen J, Poirie M, Carton Y. 2006. Genetic interactions between the parasitoid wasp *Leptopilina boulardi* and its *Drosophila* hosts. *Heredity* 98: 21-27.

Duchen MR. 2004. Mitochondria in health and disease: Perspectives on a new mitochondrial biology. *Molecular Aspects of Medicine* 25: 365-451.

Dushay MS. 2009. Insect haemolymph clotting. *Cellular and Molecular Life Sciences* 66: 2643-2650.

Eleftherianos I, Castillo JC. 2012. Molecular mechanisms of aging and immune system regulation in *Drosophila*. *International Journal of Molecular Sciences* 13: 9826-9844.

Eleftherianos I, Revenis C. 2011. Role and importance of phenoloxidase in insect hemostasis. *Journal of Innate Immunity* 3: 28-33.

Eraud C, Duriez O, Chastel O, Faivre B. 2005. The energetic cost of humoral immunity in the collared dove, *Streptopelia decaocto*: Is the magnitude sufficient to force energy-based trade-offs? *Functional Ecology* 19: 110-118.

Exton MS. 1997. Infection-induced anorexia: Active host defence strategy. *Appetite* 29: 369-383.

Fair JM, Myers OB. 2002. The ecological and physiological costs of lead shot and immunological challenge to developing western bluebirds. *Ecotoxicology* 11: 199-208.

Falconer DS, Mackay TF, Frankham R. 1996. Introduction to quantitative genetics (4th edn). *Essex*: *Longman*.

Fedorka KM, Zuk M, Mousseau TA. 2004. Immune suppression and the cost of reproduction in the ground cricket, *Allonemobius socius*. *Evolution* 58: 2478-2485.

Fellowes MD, Travis JM. 2000. Linking the coevolutionary and population dynamics of host–parasitoid interactions. *Population Ecology* 42: 195-203.

Fellowes M, Kraaijeveld A, Godfray H. 1998. Trade-off associated with selection for increased ability to resist parasitoid attack in *Drosophila melanogaster*. *Proceedings of the Royal Society B-Biological Sciences* 265: 1553-1558.

Feissner RF, Skalska J, Gaum WE, Sheu SS. 2009. Crosstalk signaling between mitochondrial Ca2+ and ROS. *Frontiers in Bioscience* 14: 1197-1218.

Felsenstein J, Yokoyama S. 1976. The evolutionary advantage of recombination. II. Individual selection for recombination. *Genetics* 83: 845-859.

Ferris SD, Sage RD, Huang CM, Nielsen JT, Ritte U, Wilson AC. 1983. Flow of mitochondrial DNA across a species boundary. *Proceedings of the National Academy of Sciences of the United States of America* 80: 2290-2294.

Flatt T. 2011. Survival costs of reproduction in *Drosophila*. *Experimental Gerontology* 46: 369-375.

Flor HH. 1956. The complementary genetic systems in flax and flax rust. *Advances in Genetics* 8: 29-54.

Flyg C, Kenne K, Boman HG. 1980. Insect pathogenic properties of *Serratia marcescens*: Phage-resistant mutants with a decreased resistance to *Cecropia* immunity and a

decreased virulence to *Drosophila*. *Journal of General Microbiology* 120: 173-181.

Franchini A, Kletsas D, Ottaviani E. 1996. Immunocytochemical evidence of PDGF-and TGF-β-like molecules in invertebrate and vertebrate immunocytes: An evolutionary approach. *The Histochemical Journal* 28: 599-605.

Frank SA, Hurst LD. 1996. Mitochondria and male disease. *Nature* 383: 224.

Frank SA. 1989. The evolutionary dynamics of cytoplasmic male sterility. *American Naturalist* 133: 345-376.

Freitak D, Ots I, Vanatoa A, Horak P. 2003. Immune response is energetically costly in white cabbage butterfly pupae. *Proceedings of the Royal Society B-Biological Sciences* 270: S220-S222.

Friberg U, Dowling D. 2008. No evidence of mitochondrial genetic variation for sperm competition within a population of *Drosophila melanogaster*. *Journal of Evolutionary Biology* 21: 1798-1807.

Fuller KM, Zouros E. 1993. Dispersed discrete length polymorphism of mitochondrial DNA in the scallop *Placopecten magellanicus* (Gmelin). *Current Genetics* 23: 365-369.

Galtier N, Nabholz B, Glémin S, Hurst G. 2009. Mitochondrial DNA as a marker of molecular diversity: A reappraisal. *Molecular Ecology* 18: 4541-4550.

Gandra YR, Scrimsha WNS. 1961. Infection and nutritional status II. effect of mild virus infection induced by 17-D yellow fever vaccine on nitrogen metabolism in children. *The American Journal of Clinical Nutrition* 9: 159-163.

Garcia-Martinez J, Castro JA, Ramon M, Latorre A, Moya A. 1998. Mitochondrial DNA haplotype frequencies in natural and experimental populations of *Drosophila subobscura*.

Genetics 149: 1377-1382.

Gargano JW, Martin I, Bhandari P, Grotewiel MS. 2005. Rapid iterative negative geotaxis (RING): A new method for assessing age-related locomotor decline in *Drosophila*. *Experimental Gerontology* 40: 386-395.

Gemmell NJ, Metcalf, V.J. & Allendorf, F.W. 2004. Mother's curse: The effect of mtDNA on individual fitness and population viability. *Trends in Ecology and Evolution* 19: 238-244.

Georgel P, Naitza S, Kappler C, Ferrandon D, Zachary D, Swimmer C, Kopczynski C, Duyk G, Reichhart JM, Hoffmann JA. 2001. *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defence and can promote apoptosis. *Developmental Cell* 1: 503-514.

Gillespie JH. 1999. The role of population size in molecular evolution. *Theoretical Population Biology* 55: 145-156.

Govind S. 1996. Rel signalling pathway and the melanotic tumour phenotype of *Drosophila*. *Biochemical Society Transactions* 24: 39-44.

Graham AL, Shuker DM, Pollitt LC, Auld SK, Wilson AJ, Little TJ. 2011. Fitness consequences of immune responses: Strengthening the empirical framework for ecoimmunology. *Functional Ecology* 25: 5-17.

Gwynn D, Callaghan A, Gorham J, Walters K, Fellowes M. 2005. Resistance is costly: Trade-offs between immunity, fecundity and survival in the pea aphid. *Proceedings of the Royal Society B-Biological Sciences* 272: 1803-1808.

Gyllensten U, Wharton D, Josefsson A, Wilson AC. 1991. Paternal inheritance of mitochondrial DNA in mice. *Nature* 352: 255-257.

Gyllensten U, Wharton D, Wilson AC. 1985. Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. *The Journal of Heredity* 76: 321-324.

Hadfield JD. 2010. MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. Journal of Statistical Software 33: 1.

Haldane JBS. 1949. Disease and evolution. *La Ricerca Scientifica* 19: S68-S76.

Hall MD, Ebert D. 2013. The genetics of infectious disease susceptibility: Has the evidence for epistasis been overestimated? *BMC Biology* 11: 79-83.

Halliwell B. 2005. Free Radicals and Other Reactive Species in Disease. *Wiley Online Library* DOI: 10.1038/npg.els.0003913.

Hamilton WD. Zuk M. 1982. Heritable true fitness and bright birds - a role for parasites. *Science* 218: 384-387.

Hayashi J, Yonekawa H, Gotoh O, Watanabe J, Tagashira Y. 1978. Strictly maternal inheritance of rat mitochondrial DNA. *Biochemical and Biophysical Research Communications* 83: 1032-1038.

He J, Mao CC, Reyes A, Sembongi H. 2007. The AAA protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. *Journal of Cell Biology* 176: 141-146.

Hecht J, Grewe F, Knoop V. 2011. Extreme RNA editing in coding islands and abundant microsatellites in repeat sequences of *Selaginella moellendorffii* mitochondria: The root of frequent plant mtDNA recombination in early tracheophytes. *Genome Biology and Evolution* 3: 344-358.

Hetru C, Hoffmann JA. 2009. NF-kappaB in the immune response of *Drosophila*.

Perspectives in Biology 1: a000232.

Hill AV. 1998. The immunogenetics of human infectious diseases. *Annual Review of Immunology* 16: 593-617.

Hill AV. 2012. Evolution, revolution and heresy in the genetics of infectious disease susceptibility. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences* 367: 840-849.

Hill WG, Goddard ME, Visscher PM. 2008. Data and theory point to mainly additive genetic variance for complex traits. *PLoS Genetics* 4: e1000008.

Hillier BJ, Vacquier VD. 2003. Amassin, an olfactomedin protein, mediates the massive intercellular adhesion of sea urchin coelomocytes. *The Journal of Cell Biology* 160: 597-604.

Ho SY, Lanfear R, Bromham L, Phillips MJ, Soubrier J, Rodrigo AG, Cooper A. 2011. Time-dependent rates of molecular evolution. *Molecular Ecology* 20: 3087-3101.

Hoebe K, Janssen E, Beutler B. 2004. The interface between innate and adaptive immunity. *Nature Immunology* 5: 971-974.

Hoekstra LA, Siddiq MA, Montooth KL. 2013. Pleiotropic effects of a mitochondrial-nuclear incompatibility depend upon the accelerating effect of temperature in *Drosophila*. *Genetics* 195: 1129-1139.

Hoffmann JA. 2003. The immune response of *Drosophila*. *Nature* 426: 33-38.

Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. 1999. Phylogenetic perspectives in innate immunity. *Science* 284: 1313-1318.

Houle D. 1992. Comparing evolvability and variability of quantitative traits. *Genetics* 130: 195-204.

Hudson G, Gomez-Duran A, Wilson IJ, Chinnery PF. 2014. Recent mitochondrial DNA mutations increase the risk of developing common late-onset human diseases. *PLoS Genetics* 10: e1004369.

Ilmonen P, Taarna T, Hasselquist D. 2000. Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. *Proceedings of the Royal Society B-Biological Sciences* 267: 665-670.

Immonen E, Ritchie MG. 2012. The genomic response to courtship song stimulation in female *Drosophila melanogaster*. *Proceedings of the Royal Society B-Biological Sciences* 279: 1359-1365.

Innocenti P, Morrow EH, Dowling DK. 2011. Experimental evidence supports a sex-specific selective sieve in mitochondrial genome evolution. *Science* 332: 845-848.

Isakova V, Armstrong PB. 2003. Imprisonment in a death-row cell: The fates of microbes entrapped in the limulus blood clot. *The Biological Bulletin* 205: 203-204.

Janeway CA, Travers P, Walport M, Shlomchik MJ. 2001. Immunobiology: The Immune System in Health and Disease. 5th edition. *New York Garland Science.*

Jeong SY, Seol DW. 2008. The role of mitochondria in apoptosis. *BMB Reports* 41: 11-22.

Jiang J, Tong J, Gabriel K. 2012. Hijacking mitochondria: Bacterial toxins that modulate mitochondrial function. *IUBMB Life* 64: 397-401.

Jiggins FM, Hurst GD. 2011. Microbiology: Rapid insect evolution by symbiont transfer. *Science* 332: 185-186.

John JC, Jokhi RP, Barratt CL. 2005. The impact of mitochondrial genetics on male infertility. *International Journal of Andrology* 28: 65-73.

Kao S, Chao HT, Wei YH. 1995. Mitochondrial deoxyribonucleic acid 4977-bp deletion is associated with diminished fertility and motility of human sperm. *Biology of Reproduction* 52: 729-736.

Kaminska M, Shalak V, Francin M, Mirande M. 2007. Viral hijacking of mitochondrial lysyl-tRNA synthetase. *Journal of Virology* 81: 68-73.

Kawabata S, Muta T. 2010. Sadaaki iwanaga: Discovery of the lipopolysaccharide-and β-1, 3-d-glucan-mediated cascade and unique proteins in invertebrate immunity. *Journal of Biochemistry* 147: 611-618.

Kazancloğlu E, Arnqvist G. 2014. The maintenance of mitochondrial genetic variation by negative frequency-dependent selection. *Ecology letters* 17: 22-27

Kearney M. 2012. Metabolic theory, life-history and the distribution of a terrestrial ectotherm. *Functional Ecology* 26: 167-179.

Kenney MC, Chwa M, Atilano SR, Falatoonzadeh P, Ramirez C, Malik D, Tarek M, de Carpio JC, Nesburn AB, Kuppermann BD, Udar N. 2014. Inherited mitochondrial DNA variants can affect complement, inflammation and apoptosis pathways: Insights into mitochondrial-nuclear interactions. *Human Molecular Genetics* 23: 3537-3551.

Kim YS, Ryu JH, Han SJ, Choi KH, Nam KB, Jang IH, Lemaitre B, Brey PT, Lee WJ. 2000. Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and β-1, 3-glucan that mediates the signalling for the induction of innate immune genes in *Drosophila melanogaster* cells. *Journal of Biological Chemistry* 275: 32721-32727.

Kinosita Jr K, Adachi K, Itoh H. 2004. Rotation of F1-ATPase: How an ATP-driven molecular machine may work. *Annual Review of Biophysical and Bimolecular structure* 33: 245-268.

Kirches E. 2011. LHON: Mitochondrial mutations and more. *Current Genomics* 12: 44-54.

Kirkwood TBL. 2005. Understanding the odd science of aging. *Cell* 120: 437-447.

Kirkwood TB, Shanley DP. 2005. Food restriction, evolution and ageing. *Mechanisms of Ageing and Development* 126: 1011-1016.

Kocks C, Cho JH, Nehme N, Ulvila J, Pearson AM, Meister M, Strom C, Conto SL, Hetru C, Stuart LM. 2005. *Eater*, a transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila*. *Cell* 123: 335-346.

Kondo R, Matsuura ET, Chigusa SI. 1992. Further observation of paternal transmission of *Drosophila* mitochondrial DNA by PCR selective amplification method. *Genetical Research* 59: 81-84.

Kover PX, Schaal BA. 2002. Genetic variation for disease resistance and tolerance among *Arabidopsis thaliana* accessions. *Proceedings of the National Academy of Sciences of the United States of America* 99: 11270-11274.

Kraaijeveld A, Godfray H. 1997. Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *Nature* 389: 278-280.

Kraaijeveld A, Limentani E, Godfray H. 2001. Basis of the trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *Proceedings of the Royal Society B-Biological Sciences* 268: 259-261.

Kraytsberg Y, Schwartz M, Brown TA, Ebralidse K, Kunz WS, Clayton DA, Vissing, J. &

Khrapko, K. 2004. Recombination of human mitochondrial DNA. *Science* 304: 981-981.

Kvist L, Martens J, Nazarenko AA, Orell M. 2003. Paternal leakage of mitochondrial DNA in the great tit (*Parus major*). *Molecular Biology and Evolution* 20: 243-247.

Kyriazakis I, Tolkamp B, Hutchings M. 1998. Towards a functional explanation for the occurrence of anorexia during parasitic infections. *Animal Behaviour* 56: 265-274.

Ladoukakis, E.D. & Zouros, E. 2001. Direct evidence for homologous recombination in mussel (*Mytilus galloprovincialis*) mitochondrial DNA. *Molecular Biology and Evolution* 18: 1168-1175.

Lambrechts L, Halbert J, Durand P, Gouania LC, Koella JC. 2005. Host genotype by parasite genotype interactions underlying the resistance of anopheline mosquitoes to *Plasmodium falciparum*. *Malaria Journal* 4: 3-10.

Lazzaro BP, Flores HA, Lorigan JG, Yourth CP. 2008. Genotype-by-environment interactions and adaptation to local temperature affect immunity and fecundity in *Drosophila melanogaster*. *PLoS Pathogens* 4: e1000025.

Lazzaro BP, Little TJ. 2009. Immunity in a variable world. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 364: 15-26.

Lazzaro BP, Sackton TB, Clark AG. 2006. Genetic variation in *Drosophila melanogaster* resistance to infection: A comparison across bacteria. *Genetics* 174: 1539-1554.

Lazzaro BP, Sceurman BK, Clark AG. 2004. Genetic basis of natural variation in *Drosophila melanogaster* antibacterial immunity. *Science* 303: 1873-1876.

Lee HC, Wei YH. 2007. Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging. *Experimental Biology and Medicine* 232: 592-606.

Lee HY, Chou JY, Cheong L, Chang NH, Yang, S.Y. & Leu, J.Y. 2008. Incompatibility of nuclear and mitochondrial genomes causes hybrid sterility between two yeast species. *Cell* 135: 1065-1073.

Lemaitre B, Hoffmann J. 2007. The host defence of *Drosophila melanogaster*. *Annual Review of Immunology* 25: 697-743.

Lemaitre B, Nicolas E, Michaut L, Reichhart J, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette spätzle/toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 86: 973-983.

Levings III CS, & Vasil IK. 1995. The Molecular Biology of Plant Mitochondria. *Kluwer Academic Publishers*.

Lewis EB. 1960. A new standard food medium. *Drosophila Information Service* 34: 117-118.

Libert S, Zwiener J, Chu X, Vanvoorhies W, Roman G, Pletcher SD. 2007. Regulation of *Drosophila* life span by olfaction and food derived odors. *Science* 315: 1133-1137.

Licastro F, Candore G, Lio D, Porcellini E, Colonna-Romano G, Franceschi C, Caruso C. 2005. Innate immunity and inflammation in ageing: A key for understanding age-related diseases. *Immunity and Ageing* 2: 1-14.

Linderman JA, Chambers MC, Gupta AS, Schneider DS. 2012. Infection-Related Declines in Chill Coma Recovery and Negative Geotaxis in *Drosophila melanogaster*. *PLoS One* 7:e41907.

Little TJ, Shuker DM, Colegrave N, Day T, Graham AL. 2010. The coevolution of virulence: Tolerance in perspective. *PLoS Pathogens* 6: e1001006.

Llagostera E, Soto-Cerrato V, Montaner B, Pérez-Tomás R. 2003. Prodigiosin

induces apoptosis by acting on mitochondria in human lung cancer cells. *Annals of the New York Academy of Sciences* 1010: 178-181.

Lombardo T, Blanco GA. 2012. Clot formation in the sipunculid worm *Themiste petricola*: A haemostatic and immune cellular response. *International Journal of Cell Biology* 2012: 1-7.

Longdon B, Hadfield JD, Webster CL, Obbard DJ, Jiggins FM. 2011. Host phylogeny determines viral persistence and replication in novel hosts. *PLoS Pathogens* 7: e1002260.

Luo SM, Sun QY. 2013. Autophagy is not involved in the degradation of sperm mitochondria after fertilization in mice. *Autophagy* 9: 2156-2157.

Luo SM, Schatten H, Sun QY. 2013. Sperm mitochondria in reproduction: Good or bad and where do they go? *Journal of Genetics and Genomics* 40: 549-556.

Mackay TF, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y, Magwire MM, Cridland JM. 2012. The *Drosophila melanogaster* genetic reference panel. *Nature* 482: 173-178.

MacRae AF, Anderson WW. 1988. Evidence for non-neutrality of mitochondrial DNA haplotypes in *Drosophila pseudoobscura*. *Genetics* 120: 485-294.

Magwire MM, Fabian DK, Schweyen H, Cao C, Longdon B, Bayer F, Jiggins FM. 2012. Genome-wide association studies reveal a simple genetic basis of resistance to naturally coevolving viruses in *Drosophila melanogaster*. *PLoS Genetics* 8: e1003057.

Malagoli D, Sacchi S, Ottaviani E. 2010. Lectins and cytokines in celomatic invertebrates: Two tales with the same end. *Invertebrate Survival Journal* 7: 1-10.

Mannen H, Kojima T, Oyama K, Mukai F, Ishida T, Tsuji S. 1998. Effect of mitochondrial

DNA variation on carcass traits of Japanese black cattle. *Journal of Animal Science* 76: 36-41.

Marchesi DE, Feng HL. 2007. Sperm DNA integrity from sperm to egg. *Journal of Andrology* 28: 481-489.

Markovic MP, Kylsten P, Dushay MS. 2009. *Drosophila* lamin mutations cause melanotic mass formation and lamellocyte differentiation. *Molecular Immunology* 46: 3245-3250.

Marreddy RK, Pinto JP, Wolters JC, Geertsma ER, Fusetti F, Permentier HP, Kuipers OP, Kok J, Poolman B. 2011. The response of *Lactococcus lactis* to membrane protein production. *PloS One* 6: e24060.

Martin II LB. 2005. Trade-offs between molt and immune activity in two populations of house sparrows (*Passer domesticus*). *Canadian Journal of Zoology* 83: 780-787.

Martin LB, Hawley DM, Ardia DR. 2011. An introduction to ecological immunology. *Functional Ecology* 25: 1-4.

Maxmen A. 2012. Calorie restriction falters in the long run. *Nature* 488: 569.

May-Panloup P, Chretien M, Savagner F, Vasseur C, Jean M, Malthiery Y, Reynier P. 2003. Increased sperm mitochondrial DNA content in male infertility. *Human Reproduction* 18: 550-556.

McCay C, Crowell MF, Maynard L. 1935. The effect of retarded growth upon the length of life span and upon the ultimate body size. *Journal of Nutrition* 10: 63-79.

McDermott JR, Leslie FC, D'Amato M, Thompson DG, Grencis RK, Mclaughlin JT. 2006. Immune control of food intake: Enteroendocrine cells are regulated by CD4(+) T

lymphocytes during small intestinal inflammation. *Gut* 55: 492-497.

McGraw LA, Gibson G, Clark AG, Wolfner MF. (2004) Genes regulated by mating, sperm, or seminal proteins in mated female *Drosophila melanogaster*. *Current Biology* 14: 1509-1514.

McKay D, Jenkin C. 1970. Immunity in the invertebrates: The role of serum factors in phagocytosis of erythrocytes by haemocytes of the freshwater crayfish (*Parachaeraps bicarinatus*). *The Australian Journal of Experimental Biology and Medical Sciences* 48: 139-150.

McTaggart SJ, Obbard DJ, Conlon C, Little TJ. 2012. Immune genes undergo more adaptive evolution than non-immune system genes in *Daphnia pulex*. *BMC Evolutionary Biology* 12: 63-74.

Medzhitov R, Janeway CA. 1997. Innate immunity: Minireview the virtues of a nonclonal system of recognition. *Cell Cambridge* 91: 295-298.

Medzhitov R, Schneider DS, Soares MP. 2012. Disease tolerance as a defence strategy. *Science* 335: 936-941.

Meiklejohn CD, Montooth KL, Rand DM. 2007. Positive and negative selection on the mitochondrial genome. *Trends in Genetics* 23: 259-263.

Mendes L, Piersma T, Hasselquist D. 2006. Two estimates of the metabolic costs of antibody production in migratory shorebirds: Low costs, internal reallocation, or both? *Journal of Ornithology* 147: 274-280.

Menon GK, Grayson S, Elias PM. 1985. Ionic calcium reservoirs in mammalian epidermis: Ultrastructural localization by ion-capture cytochemistry. J*ournal of Investigative*

Dermatology 84: 508-512.

Metaxakis A, Partridge L. 2013. Dietary restriction extends lifespan in wild-derived populations of *Drosophila melanogaster*. *PloS One* 8: e74681.

Miller WL. 2013. Steroid hormone synthesis in mitochondria. *Molecular and Cellular Endocrinology* 379: 62-73.

Minakhina S, Steward R. 2006. Melanotic mutants in *Drosophila*: Pathways and phenotypes. *Genetics* 174: 253-263.

Mishmar D, Ruiz-Pesini E, Golik P, Macaulay V, Clark AG, Hosseini S, Brandon M, Easley K, Chen E, Brown MD, Sukernik RI, Olckers A, Wallace DC. 2003. Natural selection shaped regional mtDNA variation in humans. *Proceedings of the National Academy of Sciences of the United States of America* 100: 171-176.

Mitchell SE, Read AF. 2005. Poor maternal environment enhances offspring disease resistance in an invertebrate. *Proceedings of the Royal Society B-Biological Sciences* 272: 2601-2607.

Mitrofanov V, Sorokina SY, Andrianov B. 2002. Variation of the mitochondral genome in the evolution of *Drosophila*. *Russian Journal of Genetics* 38: 895-907.

Mizuguchi K, Parker JS, Blundell TL, Gay NJ. 1998. Getting knotted: A model for the structure and activation of spätzle. *Trends in Biochemical Sciences Amsterdam* 23: 239-242.

Montooth KL, Meiklejohn CD, Abt DN, Rand DM. 2010. Mitochondrial–nuclear epistasis affects fitness within species but does not contribute to fixed incompatibilities between species of *Drosophila*. *Evolution* 64: 3364-3379.

Moret Y, Schmid-Hempel P. 2000. Survival for immunity: The price of immune system activation for bumblebee workers. *Science* 290: 1166-1168.

Mouatcho JC, Koekemoer LL, Coetzee M, Brooke BD. 2011. The effect of entomopathogenic fungus infection on female fecundity of the major malaria vector, *Anopheles funestus*. *African Entomology* 19: 725-729.

Mounolou J, Lacroute F. 2005. Mitochondrial DNA: An advance in eukaryotic cell biology in the 1960s. *Biology of the Cell* 97: 743-748.

Nakada K, Sato A, Yoshida K, Morita T, Tanaka H, Inoue S, Yonekawa, H, Hayashi, J. 2006. Mitochondria-related male infertility. *Proceedings of the National Academy of Sciences of the United States of America* 103: 15148-15153.

Neiman M, Taylor DR. 2009. The causes of mutation accumulation in mitochondrial genomes. *Proceedings of the Royal Society B-Biological Sciences* 276: 1201-1209.

Nilsson J, Granbom M, Råberg L. 2007. Does the strength of an immune response reflect its energetic cost? *Journal of Avian Biology* 38: 488-494.

Nielsen R, Weinreich DM. 1999. The age of nonsynonymous and synonymous mutations in animal mtDNA and implications for the mildly deleterious theory. *Genetics* 153: 497-506.

Nunes MDS, Dolezal M, Schlotterer C. 2013. Extensive paternal mtDNA leakage in natural populations of *Drosophila melanogaster*. *Molecular Ecology* 22: 2106-2117.

Obbard DJ, Jiggins FM, Bradshaw NJ, Little TJ. 2011. Recent and recurrent selective sweeps of the antiviral RNAi gene argonaute-2 in three species of *Drosophila*. *Molecular Biology and Evolution* 28: 1043-1056.

Oh-Hama T. 1997. Evolutionary consideration on 5-aminolevulinate synthase in nature. *Origins of Life & Evolution of the Biosphere* 27: 405-412.

Oliveira DC, Raychoudhury R, Lavrov DV, Werren JH. 2008. Rapidly evolving mitochondrial genome and directional selection in mitochondrial genes in the parasitic wasp *Nasonia* (Hymenoptera: Pteromalidae). *Molecular Biology & Evolution* 25: 2167-2180.

Ottaviani E, Malagoli D, Franchini A. 2004. Invertebrate humoral factors: Cytokines as mediators of cell survival. *Progress in Molecular & Subcellular Biology* 34: 1-25.

Pace KE, Baum LG. 2002. Insect galectins: Roles in immunity and development. *Glycoconjugate Journal* 19: 607-614.

Papadopoulos V, Miller WL. 2012. Role of mitochondria in steroidogenesis. *Best Practice & Research Clinical Endocrinology & Metabolism* 26: 771-790.

Paquin B, Laforest M, Forget L, Roewer I, Wang Z, Longcore J, Lang BF. 1997. The fungal mitochondrial genome project: Evolution of fungal mitochondrial genomes and their gene expression. *Current Genetics* 31: 380-395.

Pereira L, Soares P, Radivojac P, Li B, Samuels DC. 2011. Comparing phylogeny and the predicted pathogenicity of protein variations reveals equal purifying selection across the global human mtDNA diversity. *The American Journal of Human Genetics* 88: 433-439.

Pichaud N, Ballard JWO, Tanguay RM, Blier PU. 2012. Naturally occurring mitochondrial DNA haplotypes exhibit metabolic differences: Insight into functional properties of mitochondria. *Evolution* 66: 3189-3197.

Politi Y, Gal L, Kalifa Y, Ravid L, Elazar Z, Arama E. 2014. Paternal mitochondrial destruction after fertilization is mediated by a common endocytic and autophagic

pathway in *Drosophila*. *Developmental Cell* 29: 305-320.

Prendergast BJ, Hotchkiss AK, Bilbo SD, Nelson RJ. 2004. Peripubertal immune challenges attenuate reproductive development in male siberian hamsters (*Phodopus sungorus*). *Biology of Reproduction* 70: 813-820.

R Development Core Team. 2013. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. http://www.R-project.org/.

Råberg L, Nilsson J, Ilmonen P, Stjernman M, Hasselquist D. 2000. The cost of an immune response: Vaccination reduces parental effort. *Ecology Letters* 3: 382-386.

Rand DM, Clark AG, Kann LM. 2001. Sexually antagonistic cytonuclear fitness interactions in *Drosophila melanogaster*. *Genetics* 159: 173-187.

Rausher MD. 2001. Co-evolution and plant resistance to natural enemies. *Nature* 411: 857-864.

Read AF, Lynch PA, Thomas MB. 2009. How to make evolution-proof insecticides for malaria control. *American Journal of Tropical Medicine and Hygiene* 81: 165-165.

Reicher S, Seroussi E, Weller JI, Rosov A, Gootwine E. 2012. Ovine mitochondrial DNA sequence variation and its association with production and reproduction traits within an Afec-Assaf flock. *Journal of Animal Science* 90: 2084-2091.

Reilly J, Thomas Jr C. 1980. Length polymorphisms, restriction site variation, and maternal inheritance of mitochondrial DNA of *Drosophila melanogaster*. *Plasmid* 3: 109-115.

Reznick D, Nunney L, Tessier A. 2000. Big houses, big cars, superfleas and the costs of

reproduction. *Trends in Ecology and Evolution* 15: 421-425.

Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, Jiggins FM, Bergman CM. 2012. Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster*. *PLoS Genetics* 8: e1003129.

Rolff J, Siva-Jothy MT. 2003. Invertebrate ecological immunology. *Science* 301: 472-475.

Rose G, Passarino G, Carrieri G, Altomare K, Greco V, Bertolini S, Bonafe M, Franceschi C, De Benedictis G. 2001. Paradoxes in longevity: Sequence analysis of mtDNA haplogroup J in centenarians. *European Journal of Human Genetics* 9: 701-707.

Ruiz-Pesini E, Lapeña AC, Díez-Sánchez C, Pérez-Martos A, Montoya J, Alvarez E, Díaz M, Urriés A, Montoro L, López-Pérez, MJ, Enríquez JA. 2000. Human mtDNA haplogroups associated with high or reduced spermatozoa motility. *American Journal of Human Genetics* 67: 682-696.

Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC. 2004. Effects of purifying and adaptive selection on regional variation in human mtDNA. *Science* 303: 223-226.

Sadd BM, Siva-Jothy MT. 2006. Self-harm caused by an insect's innate immunity. *Proceedings of the Royal Society B-Biological Sciences* 273: 2571-2574.

Sato M, Sato K. 2013. Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. *Biochimica & Biophysica Acta Molecular Cell Research* 1833: 1979-1984.

Scantlebury M, Waterman JM, Hillegass M, Speakman JR, Bennett NC. 2007. Energetic costs of parasitism in the cape ground squirrel *Xerus inauris*. *Proceedings of the Royal*

Society B-Biological Sciences 274: 2169-2177.

Scherfer C, Karlsson C, Loseva O, Bidla G, Goto A, Havemann J, Dushay MS, Theopold U. 2004. Isolation and characterization of haemolymph clotting factors in *Drosophila melanogaster* by a pull out method. *Current Biology* 14: 625-629.

Schnable PS, Wise RP. 1998. The molecular basis of cytoplasmic male sterility and fertility restoration. *Trends in Plant Science* 3: 175-180.

Schneider CA, Rasband WS, Eliceiri KW, Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S. 2012. NIH image to imageJ: 25 years of image analysis. *Nature methods* 9: 671-675.

Schulenburg H, Ewbank JJ. 2004. Diversity and specificity in the interaction between *Caenorhabditis elegans* and the pathogen *Serratia marcescens*. *BMC Evolutionary Biology* 4: 49-55.

Schulenburg H, Kurtz J, Moret Y, Siva-Jothy MT. 2009. Introduction. Ecological immunology. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 364: 3-14.

Schutz M, Freeman A, Lindberg G, Koehler C, Beitz D. 1994. The effect of mitochondrial DNA on milk production and health of dairy cattle. *Livestock Production Science* 37: 283-295.

Schwartz A, Koella JC. 2004. The cost of immunity in the yellow fever mosquito, *Aedes aegypti* depends on immune activation. *Journal of Evolutionary Biology* 17: 834-840.

Scrimshaw NS. 1991. Rhoades lecture: Effect of infection on nutrient requirements. *Journal of Parenteral & Enteral Nutrition* 15: 589-600.

Segerstrom SC. 2007. Stress, energy, and immunity: An ecological view. *Current Directions in Psychological Science* 16: 326-330.

Shadel GS, Clayton DA. 1997. Mitochondrial DNA maintenance in vertebrates. *Annual Review of Biochemistry* 66: 409-435.

Sheridan LA, Poulin R, Ward DF, Zuk M. 2000. Sex differences in parasitic infections among arthropod hosts: Is there a male bias? *Oikos* 88: 327-334.

Shoffner JM, Brown MD, Torroni A, Lott MT, Cabell MF, Mirra SS, Beal MF, Yang C, Gearing M, Salvo R. 1993. Mitochondrial DNA variants observed in alzheimer disease and Parkinson disease patients. *Genomics* 17: 171-184.

Siddiqui M, Al-Khalifa M. 2014. Review of haemocyte count, response to chemicals, phagocytosis, encapsulation and metamorphosis in insects. *Italian Journal of Zoology* 81: 2-15.

Sitkovsky MV, Ohta A. 2005. The danger sensors that STOP the immune response: The A2 adenosine receptors? *Trends in Immunology* 26: 299-304.

Siva-Jothy MT, Tsubaki Y, Hooper RE. 1998. Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. *Physiological Entomology* 23: 74-277.

Siva-Jothy MT. 2000. A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *Proceedings of the Royal Society B-Biological Sciences* 267: 2523-2527.

Smith S, Turbill C, Suchentrunk F. 2010. Introducing Mother's Curse: Low male fertility associated with an imported mtDNA haplotype in a captive colony of brown hares.

Molecular Ecology 19: 36-43.

Splettstoesser WD, Schuff-Werner P. 2002. Oxidative stress in phagocytes–"the enemy within". *Microscopy Research and Technique* 57: 441-455.

Stavrovskaya IG, Kristal BS. 2005. The powerhouse takes control of the cell: Is the mitochondrial permeability transition a viable therapeutic target against neuronal dysfunction and death? *Free Radical Biology and Medicine* 38: 687-697.

Stearns SC. 1989. Trade-offs in life-history evolution. *Functional Ecology* 3: 259-268.

Stewart JB, Freyer C, Elson JL, Wredenberg A, Cansu Z, Trifunovic A, Larsson N. 2008. Strong purifying selection in transmission of mammalian mitochondrial DNA. *PLoS Biology* 6: e10.

Strickertsson JA, Desler C, Martin-Bertelsen T, Machado AMD, Wadstrøm T, Winther O, Rasmussen LJ, Friis-Hansen L. 2013. *Enterococcus faecalis* infection causes inflammation, intracellular oxphos-independent ROS production, and DNA damage in human gastric cancer cells. *PloS One* 8: e63147.

Svensson E, Råberg L, Koch C, Hasselquist D. 1998. Energetic stress, immunosuppression and the costs of an antibody response. *Functional Ecology* 12: 912-919.

Tack AJ, Thrall PH, Barrett LG, Burdon JJ, Laine A. 2012. Variation in infectivity and aggressiveness in space and time in wild host–pathogen systems: Causes and consequences. *Journal of Evolutionary Biology* 25: 1918-1936.

Tatar M, Carey JR. 1995. Nutrition mediates reproductive trade-offs with age-specific mortality in the beetle *Callosobruchus maculatus*. *Ecology* 76: 2066-2073.

Taylor RW, Turnbull DM. 2005. Mitochondrial DNA mutations in human disease. *Nature Reviews Genetics* 6: 389-402.

Theopold U, Schmidt O, Söderhäll K, Dushay MS. 2004. Coagulation in arthropods: Defence, wound closure and healing. *Trends in Immunology* 25: 289-294.

Thomson TC, Schneemann A, Johnson J. 2012. Oocyte destruction is activated during viral infection. *Genesis* 50: 453-465.

Tinsley MC, Blanford S, Jiggins FM. 2006. Genetic variation in *Drosophila melanogaster* pathogen susceptibility. *Parasitology* 132: 767-773.

Torroni A, Huoponen K, Francalacci P, Petrozzi M, Morelli L, Scozzari R, Obinu D, Savontaus ML, Wallace DC. 1996. Classification of European mtDNAs from an analysis of three European populations. *Genetics* 144: 1835-1850.

Tranah GJ, Manini TM, Lohman KK, Nalls MA, Kritchevsky S, Newman AB, Harris TB, Miljkovic I, Biffi A, Cummings SR. 2011. Mitochondrial DNA variation in human metabolic rate and energy expenditure. *Mitochondrion* 11: 855-861.

Tsaousis AD, Martin DP, Ladoukakis ED. 2005. Widespread recombination in published animal mtDNA sequences. *Molecular Biology and Evolution* 22: 925-933.

Ujvari B, Dowton M, Madsen T. 2007. Mitochondrial DNA recombination in a free-ranging Australian lizard. *Biology Letters* 3: 189-192.

Valanne S, Wang JH, Ramet M. 2011. The *Drosophila* toll signalling pathway. *Journal of Immunology* 186: 649-656.

Van Noordwijk, A.J, de Jong, G. 1986. Acquisition and allocation of resources - their influence on variation in life-history tactics. *American Naturalist* 128: 137-142.

Van Oven M, Kayser M. 2009. Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. *Human Mutation* 30: E386-E394.

Van Straalen NM, Roelofs D. 2012. An Introduction to Ecological Genomics. *Oxford University Press*.

Venkatesh S, Deecaraman M, Kumar R, Shamsi M, Dada R. 2009. Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. *Indian J Med Res* 129: 127-137.

Wade MJ, Brandvain Y. 2009. Reversing mother's curse: selection on male mitochondrial fitness effects. *Evolution* 63: 1084-1089.

Wallace DC, Zheng X, Lott MT, Shoffner JM, Hodge JA, Kelley RI, Epstein CM, Hopkins LC. 1988. Familial mitochondrial encephalomyopathy (MERRF): Genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. *Cell* 55: 601-610.

Wang L, Huang M, Zhang H, Song L. 2011. The immune role of C-type lectins in molluscs. *Invertebrate Survival Journal* 8: 241-246.

Weier T, Stocking C. 1952. The chloroplast: Structure, inheritance, and enzymology. II. *The Botanical Review* 18: 14-75.

Werner T, Liu G, Kang D, Ekengren S, Steiner H, Hultmark D. 2000. A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* 97: 13772-13777.

West AP, Shadel GS, Ghosh S. 2011. Mitochondria in innate immune responses. *Nature Reviews Immunology* 11: 389-402

Wilfert L, Gadau J, Schmid-Hempel P. 2007. The genetic architecture of immune defense and reproduction in male *Bombus terrestris* bumblebees. *Evolution* 61: 804-815.

Wilfert L, Schmid-Hempel P. 2008. The genetic architecture of susceptibility to parasites. *BMC Evolutionary Biology* 8: 187-2148-8187.

Williams MJ, Rodriguez A, Kimbrell DA, Eldon ED. 1997. The 18-wheeler mutation reveals complex antibacterial gene regulation in *Drosophila* host defense. *EMBO Journal* 16: 6120-6130.

Williamson D. 2002. The curious history of yeast mitochondrial DNA. *Nature Reviews Genetics* 3: 475-481.

Williams MJ. 2007. *Drosophila* hemopoiesis and cellular immunity. J*ournal of Immunology* 178: 4711-4716.

Willcox BJ, Willcox DC, Todoriki H, Fujiyoshi A, Yano K, He Q, Curb JD, Suzuki M. 2007. Caloric restriction, the traditional okinawan diet, and healthy aging. *Annals of the New York Academy of Sciences* 1114: 434-455.

Wilson K, Thomas M, Blanford S, Doggett M, Simpson S, Moore S. 2002. Coping with crowds: Density-dependent disease resistance in desert locusts. *Proceedings of the National Academy of Sciences of the United States of America* 99: 5471-5475.

Wolff JN, Gemmell NJ. 2013. Mitochondria, maternal inheritance, and asymmetric fitness: Why males die younger. *BioEssays* 35: 93-99.

Wood W, Jacinto A. 2007. *Drosophila melanogaster* embryonic haemocytes: Masters of

multitasking. *Nature Reviews Molecular Cell Biology* 8: 542-551.

Yamaura K, Takahashi KG, Suzuki T. 2008. Identification and tissue expression analysis of C-type lectin and galectin in the pacific oyster, *Crassostrea gigas*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 149: 168-175.

Yixin HY, Chenoweth SF, McGraw EA. 2009. Effective but costly, evolved mechanisms of defence against a virulent opportunistic pathogen in *Drosophila melanogaster*. *PLoS Pathogens* 5: e1000385.

Zeh JA, Zeh DW. 2005. Maternal inheritance, sexual conflict and the maladapted male. *Trends in Genetics* 21: 281-286.

Zeh D, Zeh J, Bonilla M. 2005. *Wolbachia*, sex ratio bias and apparent male killing in the harlequin beetle riding pseudoscorpion. *Heredity* 95: 41-49.

Zerofsky M, Harel E, Silverman N, Tatar M. 2005. Aging of the innate immune response in *Drosophila melanogaster*. *Aging Cell* 4: 103-108.

Zeyl C, Andreson B, Weninck E. 2005. Nuclear-mitochondrial epistasis for fitness in *Saccharomyces cerevisiae*. *Evolution* 59: 910-914.

Zheng X, Xia Y. 2012. Β-1, 3-glucan recognition protein (βGRP) is essential for resistance against fungal pathogen and opportunistic pathogenic gut bacteria in *Locusta migratoria manilensis*. *Developmental & Comparative Immunology* 36: 602-609.

Zouros E, Ball AO, Saavedra C, Freeman KR. 1994. An unusual type of mitochondrial DNA inheritance in the blue mussel *Mytilus*. *Proceedings of the National Academy of Sciences of the United States of America* 91: 7463-7467.