EXTRINSIC EFFECTS ON IMMUNITY IN *D. MELANOGASTER*

by

JODELL ELISE BOLI LINDER

(Under the Direction of Daniel Promislow)

ABSTRACT

The study of insect immunity is a rapidly expanding field in biology. Much work has been focused on the pathways and molecules that make up the insect immune system, and this has led to an extensive understanding of innate immunity. However, only recently have researchers begun to examine how the external environment affects the immune system of insects. In addition, other extrinsic factors, such as food limitation and resource allocation can have affects on the outcome of host-parasite interactions. In this dissertation, I examine several extrinsic influences on immunity in the fruit fly, *Drosophila melanogaster*. By combining the genetic tools we have available in this species with experiments that alter environmental variables, we are able to obtain a greater understanding of the innate immune system. Specifically, I examine how temperature, maternal effects, and very early age can influence immune system function and parasite resistance. The results presented here should shed light on what we might consider a new field of 'environmental immunology.' By combining demographic experiments with genetic tools we are better able to understand the interactions of species and perhaps develop new ways of controlling insect pests and vectors for disease.

INDEX WORDS: *Drosophila melanogaster*, *Pseudomonas aeruginosa*, *Lactococcus lactis*, temperature, environment, immunity, maternal effects, innate immune system, host-parasite interactions, age of infection.

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JODELL ELISE BOLI LINDER

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by

JODELL ELISE BOLI LINDER

Major Professor: Daniel Promislow

Committee: Sonia Altizer
Wyatt Anderson
Michael Strand
John Wares

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2009

DEDICATION

To Marty and Teri, who supported my love of science from the day I said my first word.

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CHAPTER 1

INTRODUCTION

From the beginning of its life an organism is faced with the struggle to survive. In order to transmit genetic information into the next generation, an individual has to grow, reproduce, and fight off all manner of predators and parasites who would like nothing better than to consume that individual in order to ensure their own survival. The struggle between host and parasite occurs in every organism on this planet, even parasites have parasites. The factors that determine if the parasite or host survives this interaction have intrigued many scientists over the years. Sometimes, a rapid co-evolution occurs between the two species, selection acting on mutations that confer any advantage. Complex immune systems have evolved in hosts in order to defend against parasites. However, the outcome of the host-parasite interaction is not that simple. Many factors, both genetic and environmental, can influence whether or not the host will survive the infection and live another day.

The main question of my dissertation deals with how extrinsic factors influence immunity in the fruit fly. This work advances research on insect immunity, in addition to informing research in many fields, including the biological controls of insect pests. In the next few paragraphs I give a brief review of the insect innate immune system, discuss what is known about extrinsic influences on fitness and immunity in insects, I examine how using *Drosophila* as a model has contributed to that research, as well as give a brief introduction to the three main chapters of my dissertation.

Drosophila immunity and pathogens

Drosophila melanogaster is an important model system used in many aspects of biology. Over the last few years, researchers have used *D. melanogaster* to obtain a comprehensive understanding of the molecular basis of the innate immune system in invertebrates (Hoffmann 2003; Hultmark 2003; Lemaitre and Hoffmann 2007). By using the genetic tools and gene knockouts that are available in *Drosophila*, researchers have distinguished several types of immune pathways that flies use in order to defend against a variety of pathogens. The humoral immune response is used to contain and clear bacterial and fungal infections. The two branches of this immune response are the IMD and Toll pathways, which up-regulates antimicrobial peptides. One of the main uses of the IMD pathway is for defense against gram-negative bacteria. The Toll pathway is mainly used for defense against gram-positive and fungal infections (Lemaitre et al. 1995; Belvin and Anderson 1996; Lemaitre et al. 1996; Hedengren et al. 1999). Cellular immunity consists of lamellocytes, plasmatocytes, and crystal cells that can wall off, encapsulate, engulf, and melanize a variety of different pathogens (for review see references Lemaitre and Hoffmann 2007 and Strand 2008). Another branch of the immune system, known as the phenoloxidase cascade, is used to melanize and encapsulate parasites, which is helpful against parasitic wasp infections (Ashida 1990; Rizki and Rizki 1990). Finally there are several other pathways that may defend against viral infection (JAK/STAT) and play a role in wound healing (JNK), but their exact functions have not been precisely determined (Ramet et al. 2002; Dostert et al. 2005).

Recent life history research on *Drosophila* has found that flies live with many types of bacteria in the wild (Lazzaro et al. 2006; Corby-Harris et al. 2007). I chose to use two types of bacteria throughout my experiments. *Pseudomonas aeruginosa* is gram-negative bacteria found

in natural populations of fruit flies (Corby-Harris et al. 2007), and we obtained the strain (PA01) from Brian Lazzaro's lab at Cornell University. I also used gram-positive bacteria, *Lactococcus lactis*, which was isolated from natural fly populations, and again obtained from Dr. Lazzaro's lab. By using both gram-negative and gram-positive bacteria in my experiments I was able to examine both pathways of the humoral immune response. In addition, bacteria grow quite well in the lab and we have developed procedures that allow us to inoculate the flies with bacteria, and still not observe mortality from the inoculation itself.

The ease of maintaining the flies as well as the bacterial pathogens allowed for large replications of the experiments as well as the ability to test many different experimental treatments at one time. In addition, I was able to use genetic mutant flies, which assisted me in answering several types of questions.

Many of the advances in insect immunity have come about through the wealth of genetic mutants available in *Drosophila*. A recent study compiled a list of 245 immune-related genes in *D. melanogaster* (Sackton et al. 2007). RNAi knockdown and knockout mutants are available for most of the genes that are related to the innate immune pathways, and can be ordered on several websites like the National Institute of Genetics Fly Stock Center and the Vienna Drosophila RNAi center. In addition, over sixty fly stocks are available on Flybase when the term 'immune' is searched. With these genetic tools available researchers have the power to tease apart many aspects of immunity and make significant advances in the field.

Although we have a solid understanding of the genetic basis of the insect immune system, we still lack an understanding of other factors that influence host-parasite interactions in insects. Decades of research have demonstrated that the environment can influence gene expression, regulation, evolution, and species interaction (see Parsons 2005; Reusch and Wood

2007; Lopez-Maury et al. 2008 for review). However, the study of the interaction between immunity and the environment in *Drosophila* is still in its infancy.

Environmental influences on fitness in *Drosophila*

Over the last few decades, flies have been used to determine how different types of environmental variables influence fitness in insects. In the following sections I discuss a few examples that demonstrate how using *Drosophila* to study environmental influences can lead to a greater understanding of the genes and genotypes behind these interactions.

Effects of larval density and nutrition on adult reproduction

Larval density is one of the more commonly studied environmental variables in fruit flies. Researchers have found that larvae in overcrowded vials can evolve and maintain genetic polymorphisms that allow some larva to have higher feeding rates but lower viability, and others to have slower feeding rates but higher survival (Santos et al. 1997; Borash et al. 1998). Larval nutrient ability and density have also been shown to influence reproduction. McGraw et al. (2007) found that nutrients available to larvae affected the number of sperm transferred by males and stored by females during mating. They also discovered that the level of nutrients altered transcription of an accessory gland protein gene, used in sperm competition and mating. Two recent studies found larval density affects sperm length. Morrow et al. (2008) discovered that higher larval density leads to smaller sperm, and that there are gene-by-environment interactions controlling sperm length. A previous study showed density and nutrient availability influenced both sperm length and sperm storage organ length in females, which influenced both female fitness and second male fertilization success (Amitin and Pitnick 2007). Clearly the

environmental conditions experienced by the larva have large effects on many aspects of adult fitness, and this can alter the evolution and maintenance of genotypes in a population.

Temperature effects on adult longevity and fecundity

Classic studies on *Drosophila* showed that cooler temperatures extend adult lifespan dramatically (Lobe and Northrop 1916; Northrop 1917; Strehler 1977). More recently, researchers found that inbreeding depression is more severe when flies are exposed to higher temperatures (Gebhardt and Stearns 1992). It was also shown that cooler temperatures select for flies with larger bodies and greater fecundity, potentially increasing fitness (McCabe and Partridge 1997). A recent study discovered that higher latitudes select for flies with greater cuticular melanization, which may be due to the cooler temperatures. Interestingly, these darker flies had greater desiccation resistance, longer copulations, and greater fecundity (Parkash et al. 2008). Since we know that melanization is an important component of the immune system, there may be an interaction between cuticular melanization and many different physiological systems in the fly.

Effects of Adult Diet Restriction

Nutrition provided to adults can also alter fitness components. Diet restriction has been shown to extend longevity in many animals (see Sinclair 2005; Bishop and Guarente 2007 for review) and recently the mechanisms behind this have been studied in the fruit fly. One study suggested that the life span extension is due to the fact that a reduction in calories reduces the amount of oxidative stress, which is thought to cause aging related damage (Sohal and Weindruch 1996). More recently, researchers examined the effect that diet restriction had on

gene expression in the fly, and found that genes associated with cell growth, metabolism, and reproduction were all down regulated when adults were placed on restricted diets (Pletcher et al. 2002). Burger et al. (2007) confirmed that dietary restriction reduced fecundity and extended longevity as previous work has shown, but they also found that it increased starvation resistance early in life but decreased it later in life. Work in *Drosophila* on diet restriction and adult resource availability is helping to determine the mechanisms behind the extension of life span as well as how this environmental factor affects other aspect of fitness in the fruit fly.

Collectively, this work illustrates several ways that environmental variables shape life-history traits in flies. As I discuss in the next section, one challenge now facing us is to determine the extent to which these and other environmental factors shape immune function and host-pathogen interactions.

Immunity and the environment

Over the last few years, researchers working on other insect systems—such as locusts and fungal pathogens—have found that environmental factors, like increases in temperature, can allow the locust to survive infection longer. However, these studies cannot take advantage of the immense number of molecular tools available to fly researchers. While we know environmental variables influence the outcome of host-parasite interactions, determining how the genes play a role in these interactions will allow researches to obtain a more comprehensive understanding of how the immune system functions. In addition, we will be able to use this knowledge to make advances in fields relevant to human health and agriculture.

Temperature effects on immunity

Some of the most well known studies on immunity and environmental temperatures were carried out with locusts, *Schistocerca gregaria*, and a parasitic fungus, *Metarhizum anisopliae*. Researchers discovered that the locusts could improve survival by relocating to warmer areas of their environment, effectively inducing a fever (Blanford and Thomas 2001; Elliot et al. 2002). This research had impacts on the use of fungus as a biological control, explaining why the fungus lost its effectiveness for controlling the locusts (Blanford and Thomas 2000; Thomas and Blanford 2003). However, it is still not clear if higher temperatures lowered pathogen development or up-regulated host immunity. We know that increases in temperature can cause changes in metabolism, the endocrine system, and nervous system in insects (Neven 2000), so it is possible that temperature also alters the immune system. If temperature is altering the immune system, then what genes are being affected and how are they being affected? These gene-by-environment interactions have yet to be identified. The interaction between temperature and the infection in flies has been observed in a few studies in *Drosophila*. Ballabeni et al. (1995) found that keeping mycophagous *Drosophila* infected with parasitic nematodes above 29 °Celsius (C) allowed them to maintain fertility compared to those flies kept below 27 °C. However, their study was limited by small sample sizes. Linder et al. (2008) examined survival after a bacterial infection at cool and warm temperatures. This study demonstrated that cooler temperatures were beneficial because they slowed bacterial growth. Interestingly, cooler temperatures were also shown to cause a small beneficial stress response in the fly, which may help fight the infection. Another study examined how flies from North America and Africa survived against bacterial infection at variable temperatures (Lazzaro et al. 2008). As in Linder et al. (2008), they found that temperature influenced the outcome of the host-parasite interaction. They also found that the

genotype of the fly (i.e., which population the fly was from) had an influence on the flies' ability to survive infection at the different temperatures. This suggests a more complex interaction than just the temperature altering pathogen growth.

Other environmental influences on immunity

A recent study in fruit flies examined nutrient availability and immunity. Researchers found that limited yeast caused an increase in the cost of immune system deployment (McKean et al. 2008). In another study, mating was found to effect survival after infection. Fedorka et al. (2007) demonstrated a decrease in ability to overcome bacterial infection three hours after mating when compared to virgin females. However, the effect went away 24 hours post mating. Spatial variation between fly populations may also influence immunity. One study found that flies from populations along the east coast of the United States varied in their survival against a bacterial pathogen. However, there was no rank order (i.e., latitudinal) effect on the differences in survival. In addition, this effect could have just been because the populations were genetically different and it was a genetic effect causing the differences in survival (Corby-Harris and Promislow 2008).

Studies on orthopterans have demonstrated that variables like population density can influence immunity. Mormon crickets from larger populations have stronger immune responses than those from smaller populations (Bailey et al. 2008). It has also been found that destruction of habitat and the stress caused by logging around their nests decreased the immune responses of a species of ant (Sorvari et al. 2008). It is clear that many different types of environmental factors can have profound impacts on the immune system of insects.

Finally, in two recent studies immune function in adult flies was examined in relation to diet restriction. Burger et al. (2007) examined immunity after diet restriction and found that late in life (but not early in life) there was a slight increase in immunity against gram-negative bacteria. Libert et al. (2008) did not find that diet restriction influenced lifespan in flies, even though two long-lived mutants (for knockouts in genes *puc* or *chico*) did increase pathogen resistance. Life extension may occur via separate pathways in these flies. It is unknown why these two studies had different outcomes but the differences may be due to the different genetic background of the flies. More work in this area would help to clarify these interactions and determine why nutrient availability sometimes alters immune function.

A growing number of studies are examining the effect of environmental variables on immunity, but more work needs to be done in order to understand out how these environmental influences affect the immune system and immunity related genes. *D. melanogaster* is a prime candidate with which to do this research. The ease of manipulation along with the vast array of genetic knowledge will allow us to examine gene-by-environmental interactions more closely. In fact, gene-by-environment interactions have already been studied in *Drosophila* in many situations, including how larval density and temperature influence metabolism (Santos et al. 1994; Santos et al. 1997; Kristensen et al. 2006). Conducting gene-by-environment studies, in combination with quantitative trait locus mapping, genetic knock outs (or knock downs), over-expression mutants, and the ease of searching the fly genome would allow researchers to clarify interactions between genes and environmental variables, and how these interactions alter the outcome of host-parasite interactions. By focusing *Drosophila* research not only on the function of the immune system but also on the variables that influence the expression and induction of

immunity, scientists will be able to move research in a direction that will be benefit human health and agriculture.

Summary of experiments

The following is a brief summary of the research I conducted during my PhD on how extrinsic influences shape immunity in *Drosophila*. Chapter two consists of work that I conducted on temperature and immunity. In this work, I determine how temperature alters the host-parasite interaction, and begin to determine the mechanisms behind these alterations. Briefly, we demonstrate that cooler temperatures enhance survival after infection with bacteria and alter expression of immune-related genes in flies. This effect appears to be due not only to the fact that colder temperatures slow down bacterial growth, but also to the beneficial effects of cooler temperature on immune function. We explore the possibility that heat-shock proteins, and in particular, *Hsp83*, may improve immune function at cool temperatures.

In chapter three I investigate the fitness consequences of immune challenge in female flies by examining both direct (within generation) and indirect (between generations) costs and benefits. In agreement with other studies, we found a direct cost to infection, where immune challenged females laid fewer eggs than unchallenged females. In addition, we found some evidence for indirect costs. Offspring from immune challenged mothers had shorter lifespans than those from unchallenged mothers. When we examined the offspring immune ability, which recently has been shown to be influenced by maternal infection in other insects (Sadd et al. 2005; Moret 2006), we did not see any effect of maternal immune challenge on offspring's ability to overcome an infection. In addition we did not see an effect on other fitness traits measured, including egg size, egg-adult viability, or offspring resistance to oxidative stress.

Finally, in chapter four, I examine how immunity differs in the early life of the fly. Theory predicts that although selection occurs throughout life, selection is strongest early in life (Hamilton 1966). Evidence suggests that early reproduction can have dramatic impacts on an individual's fitness. Even beginning to lay eggs one day earlier can increase the overall fitness of insects dramatically (Moore and Moore 2001; Fox et al. 2003). It is not unreasonable to suggest that other fitness components may also vary greatly within the first few days of life. Specifically, variation in immunity within the first few days of life might be important for the fitness of an individual. In flies, several studies have been conducted on how immunity changes between early and late life. However immunity during early life, specifically right after eclosion, has not been examined. In this chapter, I examine immune ability within the first five days of eclosion in *D. melanogaster* and discover that younger flies are not as able to defend against pathogens as well as older flies. We anticipated that such an effect might be due to physiological trade-offs in investment in egg production. However, we do not find that to be the case.

Over the next few years, studies teasing apart the effect that environmental influences have on the insect immune system are greatly needed. The few studies that have been done on environmental influences on immunity have raised more questions than they have solved. This research could help manage and control insects that affect humans as well. Insect pests not only attack agricultural crops, but can also be vectors that spread many diseases like Malaria, Chagas disease, and African Sleeping Sickness, just to name a few. By using *D. melanogaster*, researchers will have the tools available to tease apart the genetic and environmental effects that influence host-parasite interactions (Figure 1.1). This will help to move research forward and make advances relevant to society. If we focus on the genetics behind how environmental

factors alter host-parasite interactions we can use that information to determine how changes in the environment affect other insect species and influence host-parasite interactions in nature.

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Figure Legend

Figure 1.1. Environmental (E) and genotypic (G) effects that influence the host-parasite interactions between *Drosophila* and its pathogen. Varying environments can influence the immunity of the fly. By using *Drosophila* we are able to determine how these environmental factors influence gene expression and regulation in the host, and how different genotypes are affected by these environmental factors.

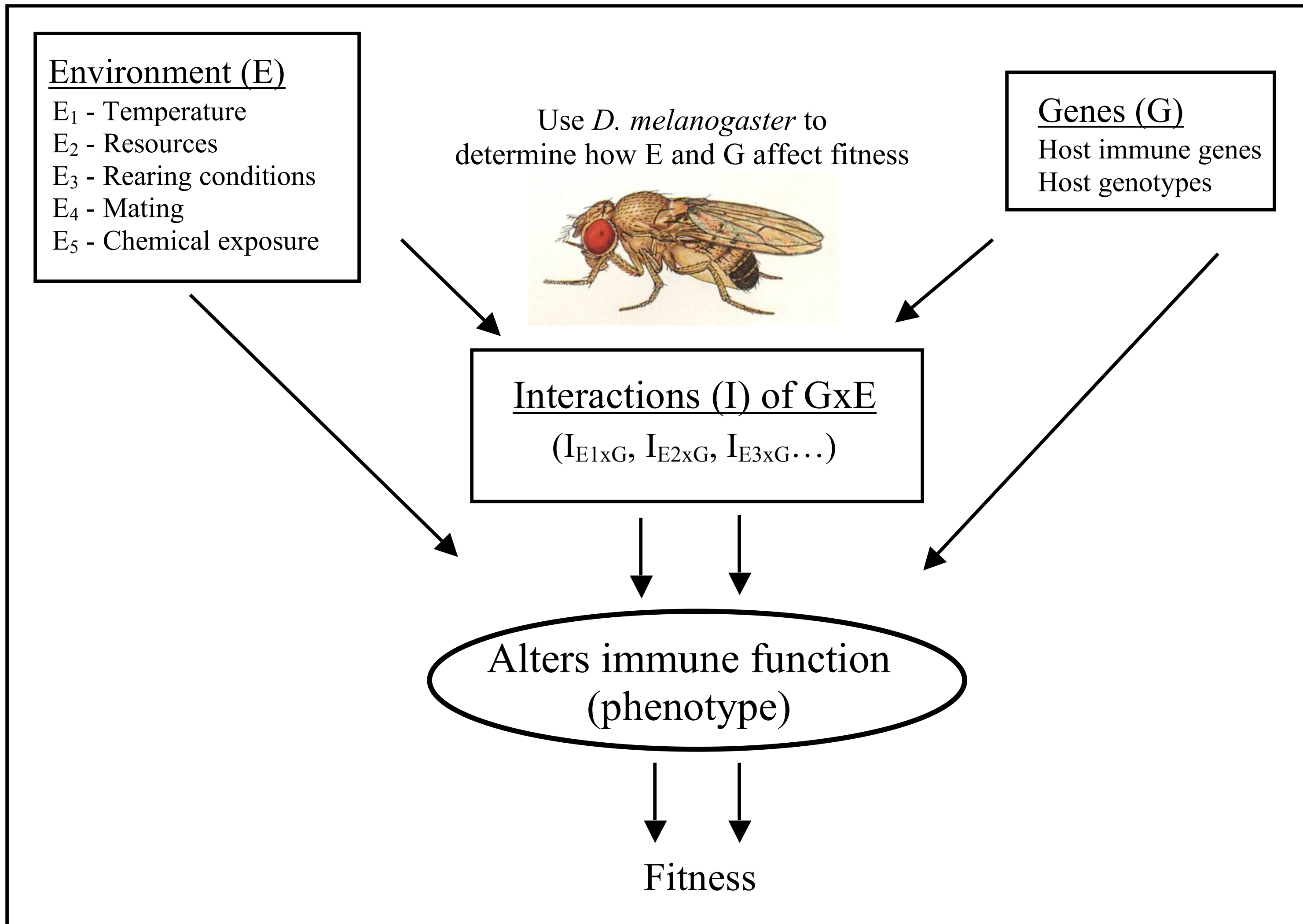

Figure 1.1

CHAPTER 2

THE EFFECTS OF TEMPERATURE ON HOST-PATHOGEN INTERACTIONS IN *D. MELANOGASTER*: WHO BENEFITS?[1]

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[1]Linder, J.E., Owers, K.A., and Promislow, D.E.L. 2008. *Journal of Insect Physiology*. 54:279-308. Reprinted here with permission of the publisher.

Abstract

Drosophila melanogaster is widely used to study immune system function in insects. However, little work has been done in *D. melanogaster* on the effect of temperature on the immune system. Here we describe experiments that demonstrate that cooler temperatures enhance survival after infection and alter expression of immune-related genes in flies. This effect appears to be due not only to the fact that colder temperatures slow down bacterial growth, but also to the beneficial effects of cooler temperature on immune function. We explore the possibility that heat-shock proteins, and in particular, Hsp83, may improve immune function at cool temperatures. We have long known that temperature can alter immune responses against microbial pathogens in insects. This approach described here allows us to determine whether this effect is due primarily to temperature-specific effects on the host or on its pathogen. These results suggest that both may be important.

Introduction

A half century of research has taught us much about the ways in which environmental factors influence immune function in insects (Steinhaus 1960; Kluger et al. 1975; Boorstein and Ewald 1987). In recent years, many researchers have focused on so-called ‘behavioral fever’, where insects alter their temperature through thermoregulatory behavior, and thereby increase their ability to fight off pathogens. Much of this work has been done on the locust-fungus model system. Elliot, Thomas, and Blanford, among others, discovered that when locusts were infected with a pathogenic fungus, placing them at warmer temperatures prolonged their survival. In fact, when given the choice, the infected locusts relocate themselves to warmer areas of their environment (Blanford et al. 1998; Elliot et al. 2002; Thomas and Blanford 2003). Similar

effects of temperature on fungal infections have also been seen in grasshoppers (Inglis et al. 1996, 1997) and houseflies (Watson et al. 1993). Though much of the work on insects, immunity, and temperature points to warmer temperatures being beneficial, there is some evidence that warmer temperatures are not always helpful. In the cockroach-acanthocephalan system, researchers found that warmer temperatures did not affect cockroach survival and in fact reduced fecundity in infected hosts (Guinnee and Moore 2004). Similarly, when the parasitic conopid fly infects bumblebees, colder temperatures are associated with higher bumblebee survival (Muller and Schmid-Hempel 1993).

Despite a substantial amount of work on the problem, we know little about how and why temperature affects the ability of insects to overcome infection. One possibility is that behavioral fever increases host survival by placing the infectious agent in a suboptimal environment (Kluger et al. 1975; Blanford and Thomas 2001). But the situation may be more complex. The efficacy of an insect's immune system may depend in part on temperature-dependent physiological changes in the host (Blanford et al. 1998; Elliot et al. 2002; Thomas and Blanford 2003). There are myriad changes in metabolism, the nervous system, endocrine system, and behavior that occur in insects as their temperature increases (Neven 2000). At a molecular level, temperature changes can lead to changes in the expression of various heat shock proteins (Lindquist and Craig 1988; Feder and Hofmann 1999). Studies of hemocyte counts and hemolymph proteins in thermoregulating locusts have found that warmer temperatures increase immunocompetence early in infection (Ouedraogo et al. 2002; Ouedraogo et al. 2003). It is not clear, however, why colder rather than warmer temperatures are beneficial in some interactions, nor do we understand the underlying mechanisms by which temperature might shape immunity.

Fortunately, we can begin to understand whether or how temperature affects the immune system thanks to recent advances in our understanding of the molecular basis of immunity in insects. Over the last decade, researchers working with *Drosophila* have described the many genes and gene interactions that lead from the initial encounter by an insect host with a particular pathogen, to the specific cellular and molecular mechanisms used to ward off the pathogen (see Hoffmann 2003; Hultmark 2003; Uvell and Engstrom 2007 for review). There are two primary genetic pathways that provide humoral immunity through the production of antimicrobial peptides. The *Imd* pathway defends against gram-negative bacteria, while the *Toll* pathway defends against gram-positive bacteria and fungi (Lemaitre et al. 1995; Hedengren et al. 1999; De Gregorio et al. 2001; Leclerc and Reichhart 2004). In addition, insects are also capable of mounting a cellular immune response, by which they can phagocytose a variety of pathogens (Wilson et al. 2001; Rolff and Siva-Jothy 2004).

At the same time that researchers have uncovered the molecular basis of immunity in flies, others have focused on the molecular basis of their thermal ecology. We have long known about the effects of temperature on survival and reproduction in insects (Crill et al. 1996; Huey and Berrigan 2001; Faurby et al. 2005; Mockett and Sohal 2006). Other work with *Drosophila* has looked at temperature fluctuations in the wild, and how flies can survive extreme temperatures. At both hot and cold extremes, flies produce heat shock proteins (hsps), which provide a variety of protective mechanisms (Lindquist and Craig 1988; Feder and Hofmann 1999; Qin et al. 2005). Hsps are also released in response to other stresses, including infection (Guedes et al. 2005). In humans, *Hsp60* assists in the activation of macrophages, part of the innate immune system (Kol et al. 1999; Kol et al. 2000). However, the direct or indirect role that hsps play in immunity in insects has not been well studied.

In light of the breadth of knowledge on both the molecular biology of immune function and of thermal ecology in *D. melanogaster*, flies offer an ideal system with which to test hypotheses about the effects of temperature on the ability of insects to survive infection. While most work on behavioral fever has been confined to the Orthoptera, there is some information on the effects of temperature on immunity in flies. For example, flies infected with a sterilizing nematode can recover their fertility when moved to higher temperatures. Unlike Orthopteran species, however, these infected flies do not appear to choose higher temperatures (Ballabeni et al. 1995). One study on *D. melanogaster* showed that temperature affects resistance of flies selected to overcome attack by a parasitic wasp. However, the effect was not consistent. Most selection lines showed an increased ability to encapsulate the wasp eggs at warmer temperatures, but some selection lines were less able to survive (encapsulate) in warmer environments (Fellowes et al. 1999).

Here we describe experiments that examine the effects of temperature on host survival in a *D. melanogaster*–bacteria model system. The initial experiment is used to resolve how temperature affects the ability of flies to survive bacterial infection. We then carry out a series of further experiments to determine whether the effects of temperature on survival are due to changes in bacterial growth or host immunocompetence. We first measure the effects of temperature on bacterial growth within the host. To examine the effect temperature has on host immune function, we take an indirect approach and examine levels of gene expression in immune-related genes after an immune challenge at different temperatures.

In the wild, flies experience a range of temperatures within and between days. In a final set of studies, we examine the potential impact of these rapid changes on immune function by switching flies, either before or after infection, between thermal regimes. Studies on the biology

of aging in flies have used switch experiments to great effect. It has long been known that rearing fruit flies on a calorically restricted diet can extend lifespan (Chippindale et al. 1993). By switching flies from restricted to standard diets, researchers learned that the life-extending effects of diet restriction (DR) are due to the immediate effects of DR on mortality, rather than long-term effects of reduced rates of damage accumulation under DR (Good and Tatar 2001; Mair et al. 2004). Here we use temperature switching to determine if the initial temperature at which a non-infected fly is held can confer costs or benefits once the fly is infected and placed at a standard temperature. We also determine whether early temperature experience in infected flies alters long-term dynamics of the host-bacteria interaction, once the host is placed at a standard temperature.

Taken together, these experiments help us to better understand not only the effects of temperature on immune function, but also who is in control of these effects—the host or its pathogen.

Materials and Methods

In the following section we describe four specific sets of experiment. The initial experiment is designed to determine directly the effect of temperature on the ability of flies to survive an intra-thoracic injection of bacteria. To determine the extent to which these results are due to temperature-dependent interactions either in the host or the pathogen, we then carry out three additional experiments. First, we measure the effect of host temperature on bacterial growth, using measures of bacterial growth in solid or liquid culture. Second, we measure the effect of temperature on expression levels of several host genes associated with immune function. And third, to determine when the temperature causes the change in survival rate as well

as how temperature affects gene expression in the absence of infection, we carry out a set of switch experiments in which animals are housed at one temperature initially, and then switched to a different temperature at a later time.

Fly Stocks

D. melanogaster flies were collected in August 2003 from the University of Georgia Horticulture farm in Athens, GA. Thirty-six isofemale, inbred lines were used to reconstitute a large outbred population in May of 2004. Flies were kept in a large population cage with overlapping generations at room temperature, approximately 22.5 °C, until September of 2005. The flies were then maintained in 15 vials, with non-overlapping generations until the completion of the experiment, June 2007. Each generation a subset of emerging adults, approximately 120 female-male pairs, were collected, randomized, and used to start the next generation (of 15 vials). All flies were raised at 25 °C with a 12-day generation time and a 12:12h L/D cycle. Flies were cultured in vials containing approximately 3 ml of a standard cornmeal-molasses-agar medium, supplemented with a pinch of live yeast in each vial to facilitate egg production. Eggs were trimmed to approximately 150 in each vial in order to ensure that larvae were not overcrowded. This provided approximately 2250 eggs to start the next generation. For the experiments, females were collect as virgins from the culture vials and then were aged for three days. This controlled for both age and mating status, which have both been shown to affect immune function (Lawniczak and Begun 2004; McGraw et al. 2004; Fedorka and Zuk 2005; Zerofsky et al. 2005; Fedorka et al. 2007).

Bacterial Strains

Flies were infected with two strains of bacteria that have been found in natural fly populations (Lazzaro et al. 2006; Corby-Haris et al. 2007), the gram-negative bacteria, *Pseudomonas aeruginosa* and the gram-positive bacteria, *Lactococcus lactis*. Both stocks were obtained from Dr. Brian Lazzaro at Cornell University. The *L. lactis* strain was isolated from a natural fly population (Lazzaro et al. 2006); the *P. aeruginosa* strain was derived from a laboratory culture (strain PA01). Bacteria were kept at 4 °C in liquid LB broth (Luria-Bertani, a standard medium for culturing bacteria) and then raised at 37 °C, their optimal growing temperature (Bhatti et al. 1976; Molina-Hoppner et al. 2003), for 24 hours prior to each experiment. A spectrophotometer was used to standardize the concentration of bacteria at 600 nm, and the bacterial solution was then diluted to an optical density of 0.01 Å. A 0.1 mm stainless steel pin mounted on the end of a plastic pipette tip was dipped into the bacterial solution, such that the needle contained between 10 and 15 bacteria (based on colony counts; data not shown). For each infection, the pin was dipped in 70% Ethanol, wiped dry, dipped in the bacteria solution, and inserted into the thorax of the fly (modified from (Lazzaro et al. 2004).

Temperature and Survival

To determine how temperature affects overall survival, females were infected with *P. aeruginosa, L. lactis*, or sterile broth (sham) as a control. After infection, females were placed in one of three treatments, 17, 25, or 29 °C. These temperatures represent a range the flies may encounter in nature (Feder et al. 2000), and are mild enough that flies can survive for extended periods of time. After infection, mortality was recorded every day for four days, by which time flies either succumbed to the infection or recovered at each of the temperatures. For each bacteria

× temperature treatment, we carried out five replicates using a total of 250 – 300 flies for each bacterial infection at each temperature (or 55 flies for the sham-infected flies).

Given that not all flies succumb to the infection, we compared survival in two ways. First, to compare the percentage of flies that died at different temperatures following infection, we conducted an ANOVA on arcsin square root transformed data. To test for the effect of infection on mortality rates among those flies that died, we used a Cox Proportional Hazards (PH) model (Parmar and Machin 1995; Therneau and Grambsch 2000), where temperature and replicate were the independent variables, and number dead at each time point was the dependent variable. In the PH model, between 15 and 18 percent of the flies survived the infection and were treated as censored data at the end of the four-day period. All statistics were carried out using JMP 5.1 (2007). Standard errors shown on graphs are for error between replicates.

Colony Counts

To determine how temperature affects bacterial growth, females were infected with *P. aeruginosa, L. lactis*, or a sterile broth and placed at 17, 25, or 29 °C. Flies were then removed at 1.5, 3, 8, or 13 hours post infection, anesthetized using CO_2, and ground up using a sterile pestle in individual 1.5 ml eppendorf tubes containing 25*ul* of Drosophila Ringer's Solution (Ashburner 1989). The mix was then plated out on *Pseudomonas* or *Lactococcus* selective agar plates (plates made in lab from powder concentrates, MP Biomedicals, LLC # 1008817; Fisher Scientific # 0XCM0817B, respectively). Plates were incubated at 37 °C for 24 hours in order to determine how many CFUs were present in the fly when they were ground up. Colonies were counted by hand, and if there were too many colonies to count on a plate, that plate was scored as 'lawn'. We conducted the whole experiment four times for each strain of bacteria. For *P. aeruginosa*

infections there were a total of 70 plates per temperature at 1.5 hours post infection, and 40 plates per temperature at 3, 8, and 13 hours post-infection. For *L. lactis* infections there were 70 plates per temperature at 1.5 and 3 hours, and 40 plates per temperature at 8 and 13 hours. Flies injected with sterile broth were ground up at 13 hours to confirm that the only bacteria present on the plates were those with which we infected the flies (N = 16 flies). Colony-count data are highly non-normal, and those plates with a solid lawn of bacteria could not be counted. Accordingly, within each temperature × time treatment, colony count data, including 'lawn' plates, were transformed to ordinal ranks, and the non-parametric Kruskal-Wallis test was used to test for differences among treatments. Lawn plates were given a value of 2200, which was a value higher than our highest count. Separate analyses were carried out for *Pseudomonas* and *Lactococcus* infections. A Nemenyi test (a non-parametric version of the Tukey test) was used to conduct post-hoc pair-wise comparisons between the temperature × time treatments on the ranked data. In addition, growth curves of the bacteria were conducted at 17, 25, and 29 °C in order to examine changes in growth of the bacteria in vitro at the three different temperatures.

In addition we also measured bacterial growth at 17, 25, 29, and 37 °C in vitro to confirm the results in the colony count experiment. We measured the optical density at 600 nm of the bacteria at 5, 10, 20, 30, 44 and 52 hours after being diluted to 0.01 Å from log phase growth. This allowed us to determine if the bacterial growth seen in the fly is similar to that seen in vitro for a given temperature.

Real Time rt-PCR

Two gene-expression studies were carried out. In the first, we used Real time rt-PCR to study expression levels in six immunity-related genes, including three in the *Imd* pathway (*Pgrp-*

LC, *Relish* (*Rel*), and *Diptericin* (*Dpt*)), for the gram-negative infections, and three in the Toll pathway (*Spatzle* (*Spz*), *Cactus* (*Cact*), and *Metchnikowin* (*Mtk*)), for the gram-positive infections. These genes represent the recognition, signaling, and antimicrobial peptides of their respective immune pathways. A second experiment (see Switch Experiments, below) measured *Spz*, *Mtk*, and a heat-shock protein, *Hsp83*, in uninfected flies. Primer sequences for all genes are provided in Table 2.1.

To examine gene expression at the three temperatures after infection, Real time rt-PCR was conducted on flies inoculated either with lipopolysaccharides (LPS) derived from *P. aeruginosa* (1 mg per 50*ul* sterile broth), or with *L. lactis* (1000 *ul*) grown to saturation, autoclaved for 15 minutes, spun down and the pellet re-suspended with 50 *ul* sterile broth. Septic injury was used to inoculate the flies, as in the previous infection experiments. Sham inoculations were also conducted with sterile broth and used as a calibrator for gene expression. Dead *L. lactis* or LPS were used in the inoculations in order to eliminate any confounding effects of bacterial growth. This insured that any temperature-dependent changes in gene expression reflected physiological changes in the host, and not temperature-related changes in pathogen levels.

Flies were inoculated, placed at 17, 25, or 29 °C for either 3 or 8 hours, and then groups of five flies were anesthetized under CO_2, and frozen at –80° C in 300 *ul* of Trizol. RNA was extracted from the flies using a standard protocol (Khodarev et al. 2002), treated with DNase (Promega, RQ1 RNase-free DNase), and 2.5 μg of RNA was converted to cDNA (Roche, Transcriptor First Strand cDNA Synthesis Kit). A 1:50 dilution of the cDNA was mixed with 12.5*ul* Sybr Green (Applied Biosystems), 3.5*ul* H_20, and 2*ul* of primer for each gene of interest. Controls using just the DNase treated RNA were carried out to confirm there was no left over

DNA. For the gene expression studies, three replicates, of five flies each, were analyzed for each temperature × infection × time point.

Real time rt-PCR measures gene expression levels (mRNA) by converting mRNA to cDNA, and then amplifying cDNA with PCR. The original amount of cDNA will determine the time it takes for the amplified DNA to reach a specific 'cycle threshold' (ct). In order to compare expression levels between genes, the ct has to first be normalized to control for variation in the quantity of cDNA added to each reaction. The observed ct is subtracted from that of an internal control, *Actin 5c*, to give us a Δct. We confirmed that *Act5c* expression did not differ due to the effect of temperature (ANOVA, $F_2 = 2.6515$, $P = 0.0911$). To compare genes between treatments, we further calibrated the Δct against the sham-inoculated control. This calibrated value, ΔΔct, is then transformed ($2^{\wedge -\Delta\Delta ct}$) in order to obtain the Relative Quantification (RQ), which is typically used to compare gene expression. RQ values were highly non-normal, however. Accordingly, for all statistical tests we used a natural log transformation, which greatly improved normality. To determine if there was a difference in levels of gene expression between LPS (or dead *L. lactis*) inoculated flies and sham-inoculated flies, we tested RQ values using a one-group, two-tailed t-test, asking if the average value of ln(RQ) was significantly different from 0. A Tukey-Kramer HSD test was used to determine if temperatures differed within genes. By examining gene expression at three different temperatures, we were able to determine how temperature affects the immune system in flies.

This experiment includes a large number of individual tests for changes in gene expression in infected versus sham flies (two bacterial species × three host genes measured per bacterial infection × two time points × three temperatures, for a total of 36 tests). With this large number of tests, we increase the risk of type-I error (falsely rejecting a true null hypothesis). To

correct for this effect of multiple comparisons, we used the Benjamini-Hochberg correction (Benjamini and Hochberg 1995). This test is less conservative than the more commonly used Bonferroni correction (see Biometry, Sokal 2000), which we consider overly conservative. To test for significance using the Benjamini-Hochberg correction, we first rank each P-value from largest to smallest. The corrected P-value is considered statistically significant if $P_{(i)} \leq \alpha m/i$, where α is the standard significance level, i is the ordinal rank of the ith P-value, and m is the number of samples being tested.

Temperature Switches

In order to understand how and when temperature affects survival rate we conducted several experiments in which temperature was changed before or after infection.

Three-hour switch treatment

We were interested in determining how host survival after infection would be affected by a brief period at a low, moderate or high temperature, followed by a more protracted incubation period at moderate temperature. For this experiment, we infected flies with *P. aeruginosa, L. lactis,* or sterile broth, and placed them immediately after infection at 17, 25, or 29 °C for 3 hours, and then returned them all to 25 °C. Flies kept at 25 °C for the whole experiment were labeled as controls (sham flies were inoculated with a sterile broth). In previous experiments, peak mortality occurred between 24 and 33 hours post infection at 25 °C. Accordingly, we recorded mortality every hour from 24 to 33 hours post infection. Differences in mortality rates between control and treatment flies were compared using the Proportional Hazards model. Three

replicates were conducted, with a total of N = 150 at each temperature (before moving to 25 °C) for each bacteria, and N = 30 for the sham infections at each temperature.

Preheat treatment

To examine the effects of temperature on immunity in the absence of infection, at two days after eclosion and prior to infection, flies were placed at 17, 25, or 29 °C for 24 h. After 24 h at this initial temperature, the flies were infected with *P. aeruginosa, L. lactis*, or a sterile broth and all the flies were placed at 25 °C. Mortality was recorded every hour from 24 to 33 hours post infection and the effect of temperature on age-specific mortality was tested using a Proportional Hazards model. Three replicates were conducted, with a total of N = 150 for each temperature for each bacteria, and N = 30 for the sham infections at each temperature.

Gene expression in preheated flies

For the preheat experiment, in addition to measuring survival, we also used Real time rt-PCR to measure gene expression in non-infected flies kept for 24 hours at 17, 25, or 29 °C. These flies were not infected and gene expression levels at 17 or 29 °C were measured relative to those in flies kept at 25 °C. Three genes were examined, including two immune genes from the *Toll* pathway (*Spz*, *Mtk*) and *Hsp83*, a heat shock protein that is induced both during cold stress (Qin et al. 2005) and in larvae infected with both gram+ and gram- bacteria (Guedes et al. 2005). Only *Toll* genes were examined (no *Imd* genes) due to limited resources, and due to the response that we see in gram+ infected flies after the preheat treatment. Three replicates of five flies at each temperature were analyzed. To compare expression levels at 17 or 29 °C relative to 25 °C, we used a two-tailed t-test on ln-transformed values of RQ.

Results

Temperature and Survival

The proportion of flies that died did not differ among the temperature treatments for either bacteria (*P. aeruginosa*: ANOVA, $F_2 = 0.3122$, $P = 0.7376$; *L. lactis:* $F_2 = 0.5199$, $P = 0.6191$). However, we found a significant difference in the rate at which flies died among the 17, 25, and 29 °C treatments for both *P. aeruginosa* and *L. lactis* (*P. aeruginosa*: $\chi_2^2 = 191.59$, $P < 0.0001$; *L. lactis*: $\chi_2^2 = 111.27$, $P < 0.0001$). Median time to death for *P. aeruginosa* flies was 3, 2, and 1 days post infection for 17, 25, and 29 °C respectively (Figure 2.1a). Median time to death for *L. lactis* flies was 4, 2, and 1 days for 17, 25, and 29 °C respectively (Figure 2.1b).

Overall survival rates were relatively low (15.9% of the flies survived infection with *P. aeruginosa* and 18.5% survived infection with *L. lactis*). Accordingly, we repeated the experiment with a lower dose (LD50, on average). Again, while rates of mortality increased with temperature, the proportion that survived the infection did not vary with temperature (data not shown). No sham-injected flies died during the mortality assay period for any of the experiments.

Colony Counts

At 8 and 13 h post-infection, bacterial concentration in flies, as measured by colony counts, was consistently greater at higher temperatures (Table 2.2). No differences were observed at 1.5 h post-infection, and in one case (*P. aeruginosa*, 3 h post-infection), flies held at 17 °C produced greater colony counts than flies held at 29 °C (Figure 2.2). No bacteria were observed on the agar plates for the sham-inoculated flies. Growth of the bacteria in vitro showed a similar pattern to that of the in vivo growth at 8 and 13 hours post infection, growth rate of bacteria increased with temperature (Figure 2.3).

Real time RT-PCR

After the Benjamini-Hochberg correction, in flies at 17 °C, we observed an up-regulation of *Cact* and *Mtk* at 3 hours (in dead *L. lactis* inoculations), and *Pgrp-LC* at 8 hours (in LPS inoculations) compared to sham flies (Figure 2.4b-c). At 25 °C at 3 hours *Mtk* and *Pgrp-LC* were up-regulated compared to sham. Finally at 29 °C, only *Mtk* at 8 hours was up-regulated (Figure 2.4d). When comparing between temperatures within one gene, at 17 °C gene expression was significantly higher than 25 and 29 °C in *Pgrp-LC* and *Cact*, and higher than 25° in *Spz*. We also found expression at 29 was higher than 25 °C in *Spz* (Table 2.3**)**.

Temperature Switches

<u>*Three hour switch treatment*</u>

In the three-hour Switch experiment, there was a significant difference among the temperature treatments (*P. aeruginosa* infections, $\chi_2^2 = 61.51$, P < 0.0001, *L. lactis*, $\chi_2^2 = 62.59$, $P < 0.0001$; Figure 2.5). Flies that were held at 17 °C for three hours after infection had higher survival rates than flies held at 29 °C for three hours (median time to death post infection for *P. aeruginosa*: 17 °C = 30 h, 25 °C = 28 h, and 29 °C = 27 h; *L. lactis* 17 °C = 30 h, 25 °C = 28 h, and 29 °C = 26 h hours. Final mortality was checked the next day, and no differences in overall mortality were seen between the three temperature treatments (data not shown).

<u>*Preheat treatment*</u>

In flies that were exposed to 17, 25 or 29 °C for 24 h prior to infection and then infected with *P. aeruginosa,* mortality rates were not affected by pre-infection treatment ($\chi_2^2 = 0.56$, $P = 0.75$; Figure 2.6a). Among flies infected with *L. lactis*, there was a significant difference among

pre-infection heat treatment ($\chi_2^2 = 12.53$, P = 0.0019; Figure 2.6b). Median time to death was 27 hours for all treatments, but a Proportional Hazards test between 17 and 29 °C shows 29 °C flies have a higher mortality rate than 17 °C flies ($\chi_1^2 = 9.421$, $P = 0.0021$). In both sets of infections, the proportion of flies that survived the infection did not differ among treatments (data not shown).

Gene expression in preheated flies

Flies showed an increase in expression of *Spz*, *Mtk* and *Hsp83* at both 17 °C and 29 °C relative to 25 °C. However, while the magnitude of the effects was often quite large (Figure 2.7), only two of the six comparisons were close to significance ($Hsp83_{17\text{ vs. }25}$: $t_2 = 4.135$, $P = 0.036$; $Mtk_{17\text{ vs. }25}$: $t_2 = 2.77$, P = 0.109).

Discussion

To fully understand how temperature affects immune function in insects, we teased apart the effects of temperature on host physiology from the effects of temperature on the pathogen. Our results showed that flies infected with bacteria survive for longer when held at lower temperatures. This effect appeared to be due to the influence of temperature on both bacterial growth and the physiological responses in the host.

Infected flies held at lower temperatures survived longer, but the benefit of cooler temperature was just an hour or two. Could this brief period of survival increase fitness in infected flies? While fecundity is reduced in many insects after infection (Elsawaf et al. 1994; Cole et al. 2003), females still continue to produce eggs. In fact, some bacteria, such as *Wolbachia*, can even lead to increased fecundity in flies (Fry et al. 2004). While we did not

examine fecundity in this experiment, flies held at cooler temperatures might lay a few more eggs while they are still alive. This effect could be further enhanced if, as our Switch experiment suggests, a period at cooler temperatures were to enhance immunocompetence, and then a period at warmer temperatures increased egg production (Partridge et al. 1995). Future studies should measure the specific fitness consequences of the interaction between temperature and infection.

We examined gene expression at various temperatures in the hope of more completely understanding how temperature affects immunity in insects. In our gene expression assays, we discovered that infected flies held at cooler temperatures tended to show a greater up regulation of immune genes than those held at warmer temperatures. This result was contrary to what we expected, since rates of other physiological systems such as metabolism and respiration generally increase at warmer temperatures (Imasheva et al. 1998; Neven 2000; Gilchrist and Huey 2001). One explanation for our data could be that in our gram-negative infected flies (LPS), genes at 29 °C were up regulated more quickly, the infection was cleared, and the genes returned to basal expression levels by 8 hours (Figure 2.4a-b). However, this explanation is not consistent with our data from flies injected with heat-killed gram-positive bacteria (*L. lactis*). In these flies, we saw greater expression at 17 than at 29 °C at 3 hours (Figure 2.4c) and there was still an up-regulation of *Mtk* at 29 °C at 8 hours (i.e. the immune system was still fighting off the infection; Figure 2.4d). Previous research has shown that up-regulation of genes in response to infection may last for 48 h or more, at least when flies are infected with live bacteria and held at 25 °C (De Gregorio et al. 2001). Although we cannot be certain that genes in the gram-negative pathway do not return to basal levels by 8 hours, our data do suggest that in the gram-positive pathway, immune genes in flies held at 17 °C show greater levels of expression after infection than those

in flies held at 29 °C. It remains to be seen whether these temperature-dependent effects on gene expression are also found in proteins involved in the immune system.

We hoped that the three-hour switch experiment would help us to determine when the effect of temperature on survival occurred, and whether the effect of temperature on survival was transient or permanent. Switch experiments have been used previously to better understand how a particular treatment affects mortality rate. For example, Good & Tatar as well as Mair et al. carried out switch experiments to determine how diet restriction (DR) extends life span in flies. They moved flies from a reduced diet to a full diet and examined age-specific mortality rates. These switch experiments enabled the authors distinguish between two hypotheses. If DR increases life expectancy by decreasing the immediate risk of death, then one would expect to see a transient effect, with lower mortality rates occurring only when animals were exposed to the DR regime. However, if DR worked by slowing the accumulation of irreversible damage, then any time spent on DR should decrease mortality rates, even after the organism has been switched to a standard diet. In both studies, when the flies were switched from one diet to the other, the mortality rate also switched. Based on these experiments, one can conclude that DR extends survival by decreasing the immediate risk of death (Good and Tatar 2001; Mair et al. 2004).

In light of these experiments, we were interested in determining whether the effect of temperature was due to an immediate and transient effect, or whether exposure prior to or early after infection would have long-term consequences on the ability to resist infection. In our switch experiment, we did not observe a change in the mortality rate of flies when they were switched between temperatures. In fact, and in marked contrast with the previous ‘switch’ studies on diet restriction, we saw that after only three hours at a given temperature, the mortality rate of the

flies appeared to be set permanently (Figure 2.5). This result may have been due to the lower temperature either slowing bacteria growth and effectively giving the immune system a head start, or causing some physiological change in flies that allowed them to fight off the pathogen more efficiently. Future studies on bacterial growth rates at different temperatures as well as more gene expression studies will help resolve this difference.

Finally, we found that low temperature led to an up-regulation of *Hsp83* as well as a trend of *Mtk* up-regulation. Given that cold stress induces *Hsp83* (Qin et al. 2005), our data suggest that flies may be undergoing cold stress at 17 °C. Other work has also shown that *Hsp83* is present in infected *D. melanogaster* (Guedes et al. 2005). The relationship between cold stress, immunity, and *Hsp83* is no doubt a complicated one. We also saw a slight up regulation of *Mtk*, an antimicrobial peptide normally activated by infection, at 17 °C, even in the absence of infection. It is possible that the cold stress activated some component of the immune system in our flies. Further work is needed to determine whether the heat-shock response system interacts with known immune pathways in insects, as has already been suggested by work with human cell lines (Kol et al. 1999; Kol et al. 2000).

Conclusions

We examined several aspects of temperature, immune function, and gene expression in *D. melanogaster*. In contrast with previous studies showing that warmer temperature helps insects fight fungal infections, we found that in flies infected with bacteria, colder temperatures increase the length of survival. Our data suggest that this effect is due not only to the fact that cooler temperatures slow the growth of the pathogen, but also that cooler temperatures enhance the ability of flies to fight off bacterial infections. Just how these effects influence fitness

remains to be determined. As we tease apart the role of temperature on immune function in insects, we may also gain a better understanding of a broad range of ecological problems, including biological control of pests, determinants of species distributions, and host-parasite interactions.

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Table 2.1. Primer sets for the Real time rt-PCR genes used in this study.

Gene	Sense	Antisense
Pgrp-LC	AAGATCCGGCGCAAACC	CCTTGCGTCCGACAGTGTT
Rel	CGGCCATACTCCCTTGGAA	GGACCACCCGGTCATTTG
Dipt	AAATTACGATGACAGGCGGTCT	TCGAAGGTTCTCTCGACAGTTTA
Cact	CAGGCAACTGTCATGGGATTG	GCTTTGGTGATCCTCGCTATTT
Spz	TGATGACGCCCATGTGGAT	GTGGCAAAGAAGGCGAACA
Mtk	GTGCTGGCAGAGCCTCATC	GCGACGGCCTCGTATCG
Hsp83	GCACCAAGATCGTGCTGTACA	CAAGGAGGACCAGACCG
Act5c	CCGAGCGCGGTTACTCTTT	CTCCTTGATGTCACGGACGAT

Table 2.2. Colony count comparisons for *P. aeruginosa* (*PA*) and *L. lactis* (*LL*) at different time points after infection[a].

Comparison	Hour	Bacteria	X^2-stat	DF	*P* value	Nemenyi
17-25-29	1.5	*PA*	1.312	2	0.519	ns
17-25-29	3	*PA*	6.249	2	0.044	17 > 29*
17-25-29	8	*PA*	62.66	2	< 0.0001	17 < 25*** 17 < 29*** 25 < 29*
17-25-29	13	*PA*	82.32	2	< 0.0001	17 < 25*** 17 < 29*** 25 < 29***
17-25-29	1.5	*LL*	1.753	2	0.416	ns
17-25-29	3	*LL*	29.08	2	0.011	25 < 29**
17-25-29	8	*LL*	77.86	2	< 0.0001	17 < 25*** 17 < 29*** 25 < 29**
17-25-29	13	*LL*	75.77	2	< 0.0001	17 < 25*** 17 < 29*** 25 < 29*

[a]Pair wise comparisons using the Nemenyi test were conducted on the ranked data to determine if there were significant differences between the treatments. *: *P* = 0.05; **: *P* = 0.01; ***: *P* = 0.001.

Table 2.3. Real time rt-PCR comparisons within a temperature between infected and sham inoculated flies[a].

Gene	Hour	Temp	t-stat	*P* value	Tukey-HSD
Pgrp-LC	3	17	1.46	ns	ns
		25	10.81	**0.0085**	
		29	0.99	ns	
Rel	3	17	3.05	0.093	ns
		25	0.83	ns	
		29	0.48	ns	
Dipt	3	17	1.32	ns	ns
		25	-0.75	ns	
		29	-0.29	ns	
Pgrp-LC	8	17	18.75	**0.0339**	17 > 25*
		25	0.80	ns	17 > 29*
		29	0.30	ns	
Rel	8	17	2.11	ns	ns
		25	-0.20	ns	
		29	-0.54	ns	
Dipt	8	17	5.06	ns	17 > 29†
		25	0.54	ns	
		29	-0.97	ns	
Spz	3	17	4.65	0.043	17 > 25*
		25	-0.82	ns	
		29	0.32	ns	
Cact	3	17	7.39	**0.018**	17 > 25*
		25	4.73	0.042	17 > 29**
		29	0.93	ns	
Mtk	3	17	6.43	**0.023**	ns
		25	5.73	**0.029**	
		29	2.98	ns	
Spz	8	17	-1.00	ns	25 > 29*
		25	-3.91	0.060	
		29	1.73	ns	
Cact	8	17	0.20	ns	ns
		25	-3.22	0.085	
		29	3.57	0.071	
Mtk	8	17	4.68	0.043	ns
		25	3.17	0.089	
		29	5.41	**0.033**	

[a]Results are based on two-tailed t-tests. *P*-values that are significant after the Benjamini-Hochberg correction are shown in bold. A Tukey-Kramer HSD test was conducted for the temperature comparisons within each gene. †: $P = 0.1$; *: $P = 0.05$; **: $P = 0.01$.

Figure Legends

Figure 2.1. Survival curves (± 1 standard error) for females infected with *P. aeruginosa* (1a) or *L. lactis* (1b) at 17, 25, and 29 °C. In both cases, there is a significant difference between the temperature treatments (Proportional Hazards Model, 1a: $\chi_2^2 = 191.59$, $P < 0.0001$; 1b: $\chi_2^2 = 111.27$, $P < 0.0001$). Median time to death (days post infection) for *P. aeruginosa*: 17 °C = 3, 25 °C = 2, 29 °C = 1 day; *L. lactis*: 17 °C = 4, 25 °C = 2, and 29 °C = 1.

Figure 2.2. Median colony counts for *P. aeruginosa* (solid bars) and *L. lactis* (hashed bars) infections, shown with first and third quartiles on a log scale. Early on in infection (1.5 and 3 hours) there is not a clear pattern with regards to the effect of temperature on bacterial growth. However, at eight and 13 hours after infection, flies held at warmer temperatures had higher colony counts than those at cooler temperatures for both bacteria. Lawn plates were given a value of 2200. In the 29 °C treatment at 13 hours, for *L. lactis* the majority of plates were lawns, and the median and quartiles were also lawns, so there are no quartile bars for that data point.

Figure 2.3. Bacterial growth of *P. aeruginosa* (a) and *L. lactis* (b) in vitro at 17, 25, 29, and 37 °C. We find that warmer temperatures lead to faster bacterial growth (higher optical density).

Figure 2.4. Measures of gene expression from Real time rt-PCR analysis of flies infected with LPS at (*a*) 3 hours; (*b*) 8 hours; and with flies infected with killed *L. lactis* at (*c*) 3 hours; and (*d*) 8 hours. Relative quantification (RQ) is on the y-axis (with 95% confidence intervals) and the immune genes are on the x-axis. An asterisk indicates a significant difference ($P < 0.05$) in gene expression between the infected and sham flies. An asterisk with a bracket indicates a significant

difference in gene expression between the two temperatures. We see an up regulation of *Cact, Mtk,* and *Pgrp-LC* genes at 17, an up regulation of *Pgrp-LC* and *Mtk* at 25, and only an up regulation of *Mtk* at 29 °C.

Figure 2.5. Survival curves (± 1 standard error) for the three-hour switch experiment for flies infected with (*a*) *P. aeruginosa* or (*b*) *L. lactis* at 17, 25, and 29 °C. After just three hours at the respective temperatures, we see a significant difference in subsequent survival rate (Proportional Hazards model: *P. aeruginosa*, $\chi_2^2 = 61.51$, $P < 0.0001$; *L. lactis*, $\chi_2^2 = 62.59$, $P < 0.0001$). Median time to death for *P. aeruginosa*: 17 °C = 30, 25 °C = 28, 29 °C = 26 hours; *L. lactis*: 17 °C = 30, 25 °C = 28, and 29 °C = 27 hours.

Figure 2.6. Survival curves (± 1 standard error) for flies maintained at 17, 25, or 29 °C before infection with (*a*) *P. aeruginosa* or (*b*) *L. lactis*. (*a*) There is no effect of pre-infection temperature regime on mortality when flies are infected with the gram-negative bacteria, (Proportional Hazards model $\chi_2^2 = 0.56$, $P = 0.756$). (*b*) However, in flies infected with the gram-positive bacteria, there is a significant difference in mortality rate, (Proportional Hazards model, $\chi_2^2 = 12.53$, $P = 0.0019$), with 29 °C flies having a greater mortality rate than 17 °C flies (Proportional Hazards model $\chi_1^2 = 9.421$, $P = 0.0021$).

Figure 2.7. Mean gene expression (RQ; with 95% confidence intervals) for flies kept at 17 or 29 °C for 24 hours, with no infection, relative to gene expression in 25 °C flies. *Hsp83* is significantly higher in flies held at 17 °C than in flies held at 25 °C ($t_2 = 5.13$, $P = 0.036$).

Though not statistically significant, *Mtk* also shows a trend of increased expression at 17 °C (t_2 = 2.77, P = 0.109).

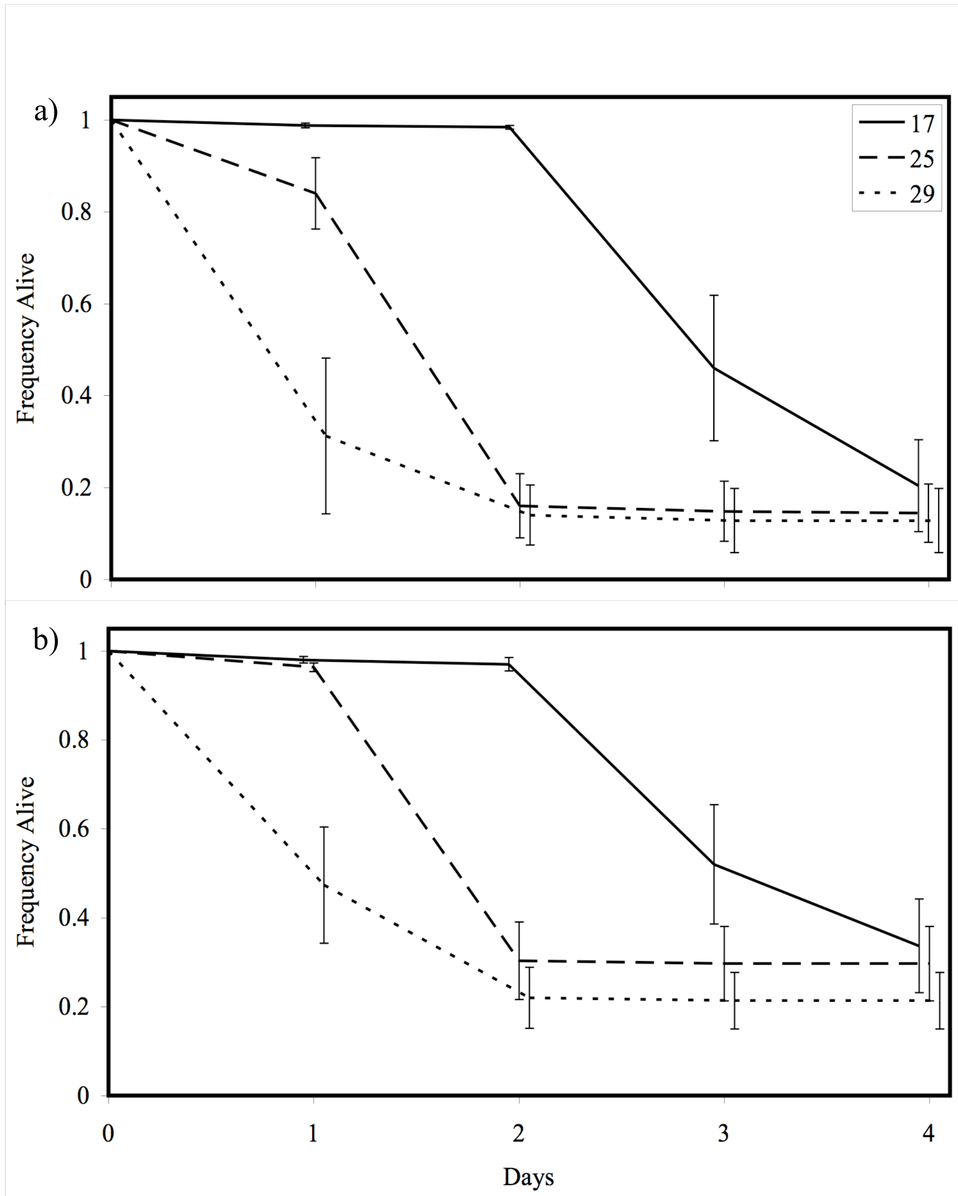

Figure 2.1

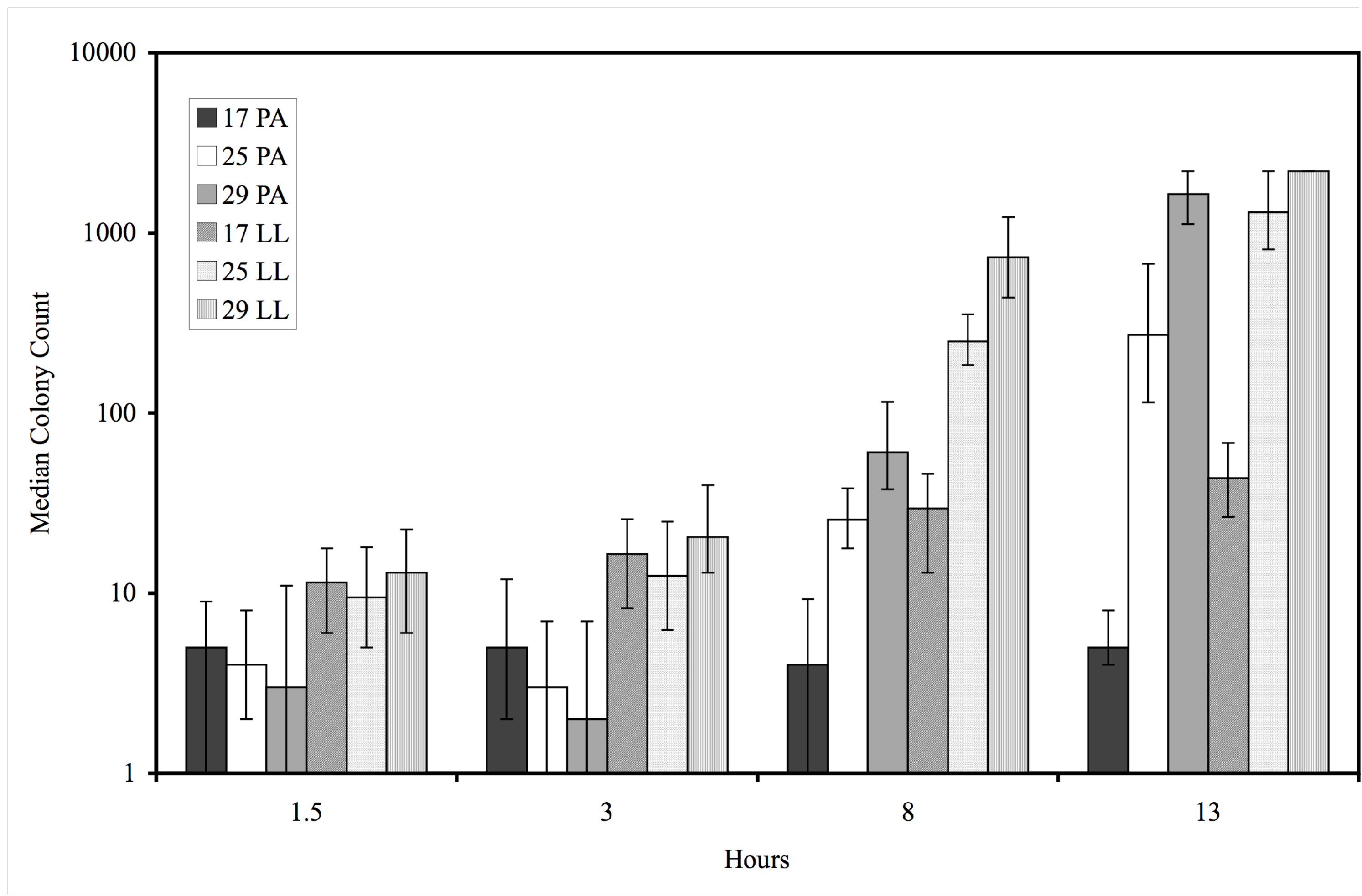

Figure 2.2

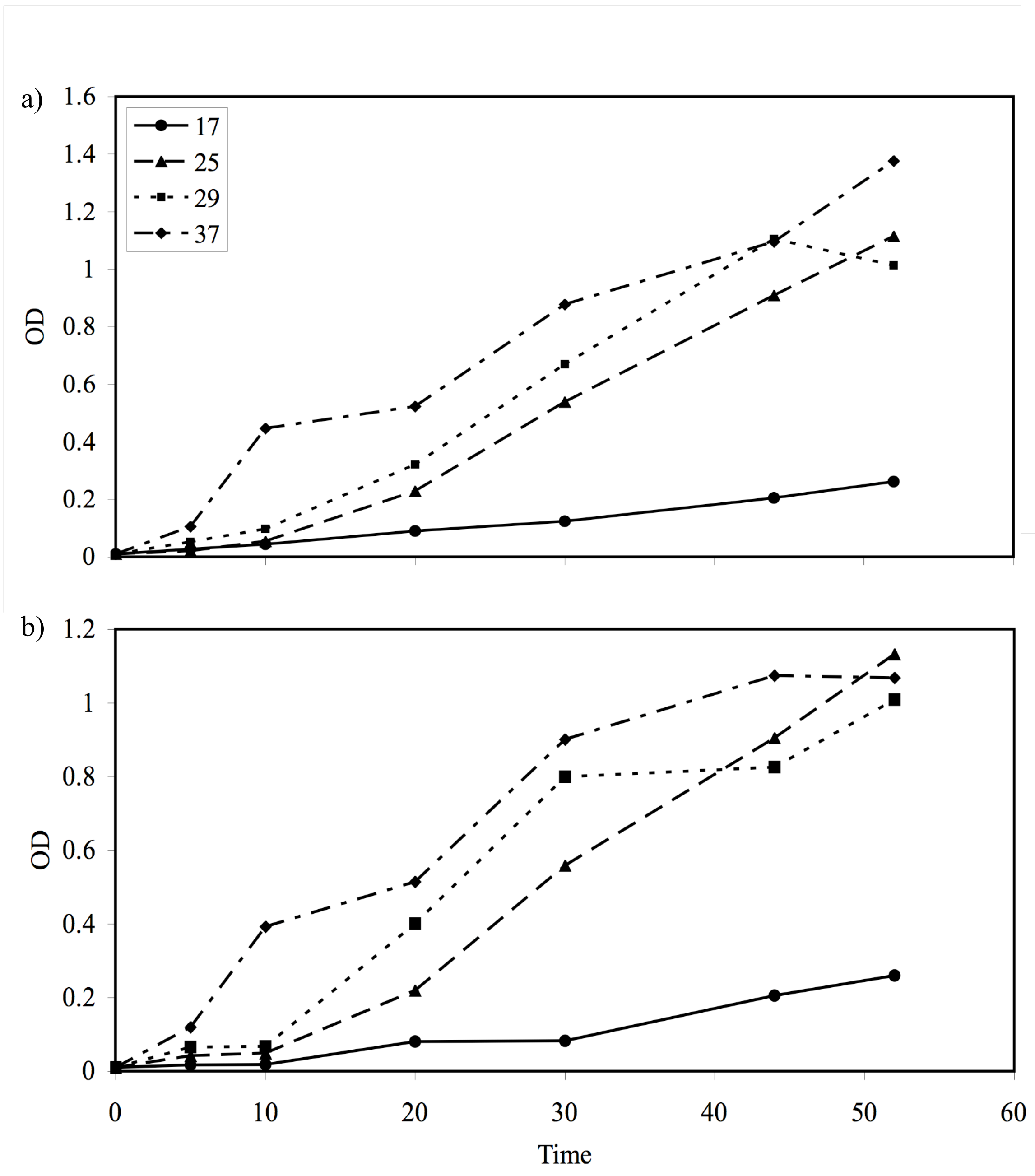

Figure 2.3

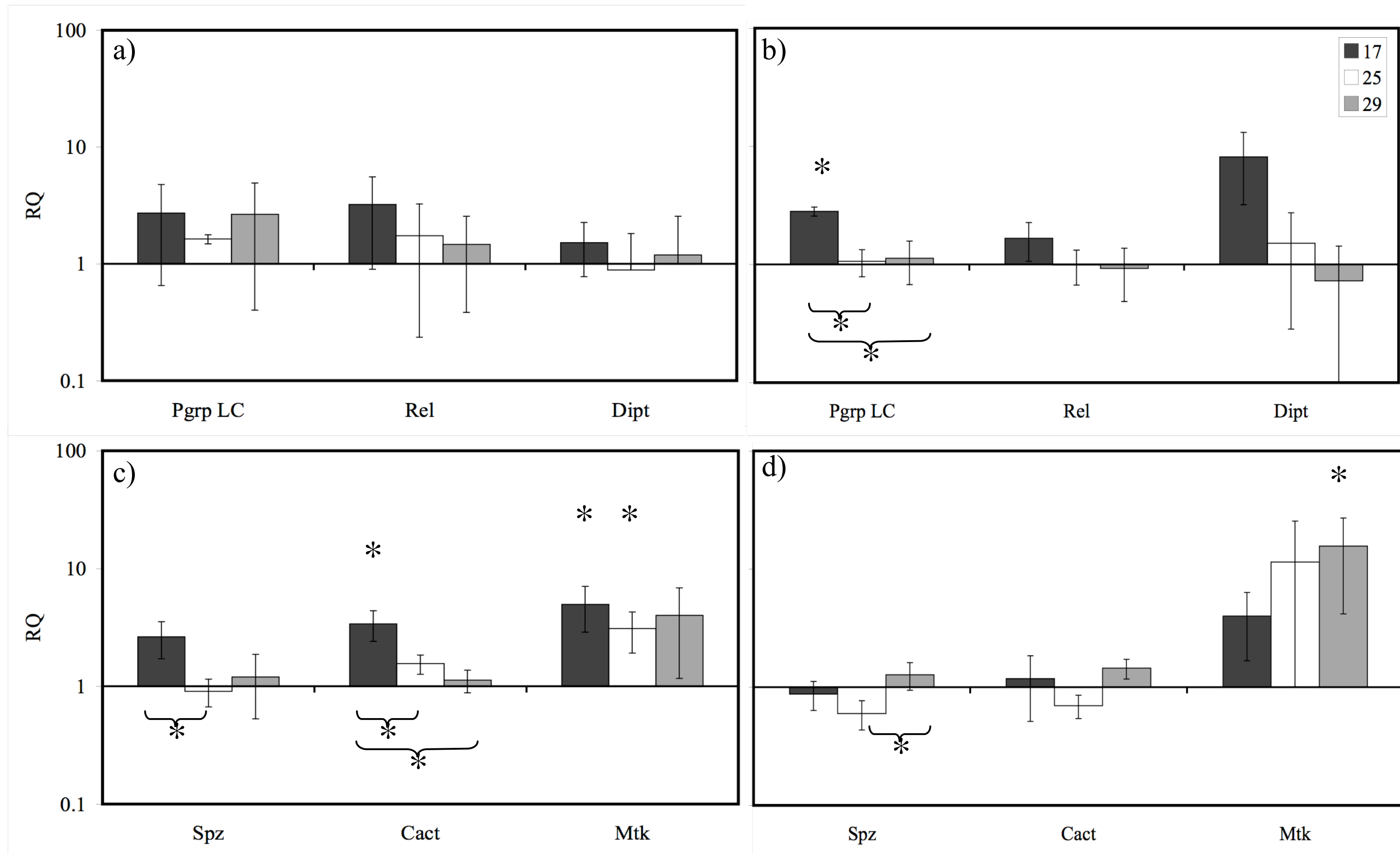

Figure 2.4

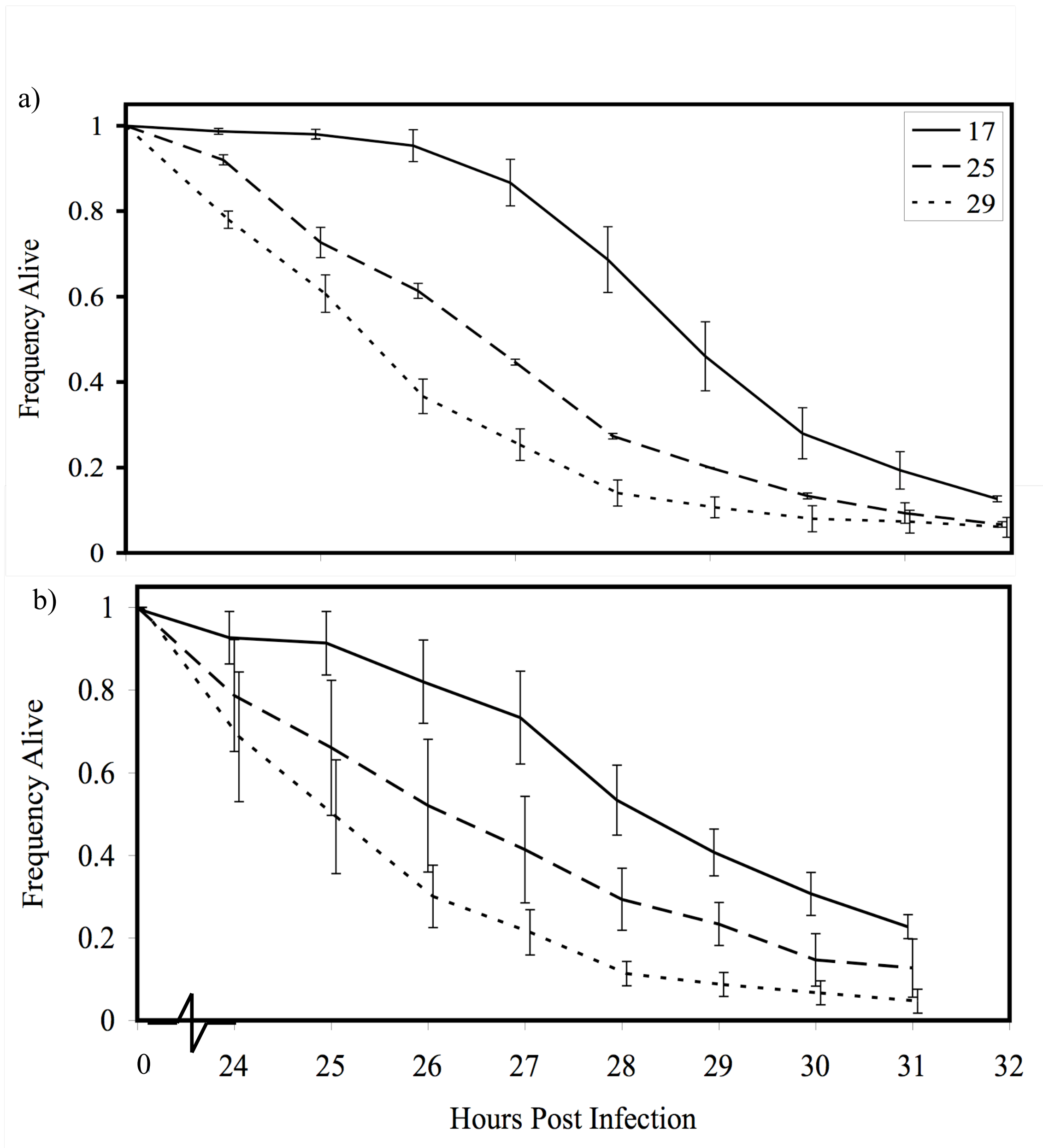

Figure 2.5

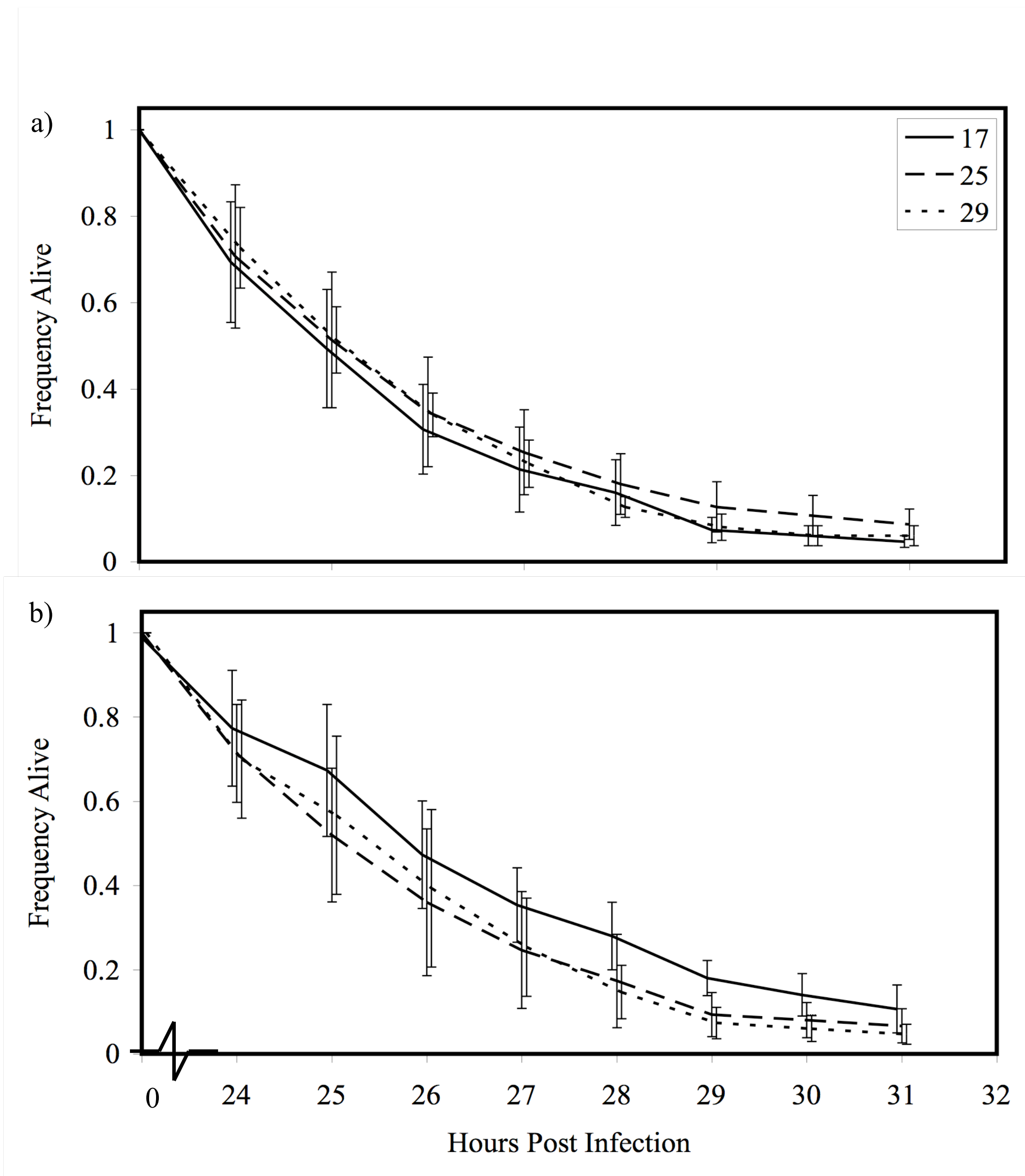

Figure 2.6

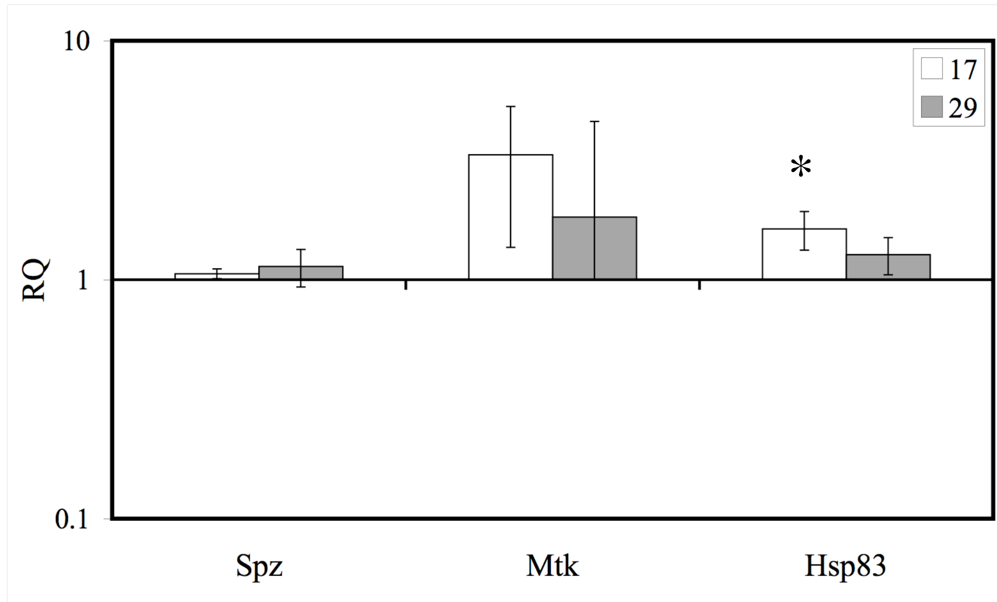

Figure 2.7

CHAPTER 3

CROSS-GENERATIONAL FITNESS EFFECTS OF INFECTION IN *DROSOPHILA MELANOGASTER*[2]

[2]Linder, J.E. and Promislow, D.E.L. 2008. *Fly*. 3(2): March/April 2009. In press. Reprinted here with permission of the publisher.

Abstract

Activation of the immune system is beneficial in defending against pathogens, but may also have costly side effects on an organism's fitness. In this study we examine the fitness consequences of immune challenge in female *Drosophila melanogaster* by examining both direct (within generation) and indirect (between generations) costs and benefits of immune challenge. Although passing immunity to offspring has been studied in mammals for many years, only recently have researchers found evidence for a cross-generational priming response in invertebrates. By examining both potential fitness costs and benefits in the next generation, we were able to determine what effect immune challenge has on fitness. In agreement with other studies, we found a direct cost to infection, where immune challenged females laid fewer eggs than unchallenged females in two of the three lines we examined. In addition, we found some evidence for indirect costs. Offspring from immune challenged mothers had shorter lifespans than those from unchallenged mothers in two of the three lines. Interestingly, we do not see any effect of maternal immune challenge on offspring's ability to overcome an infection, nor do we see an effect on other fitness traits measured, including egg size, egg-adult viability, and offspring resistance to oxidative stress. While previous studies in bumblebees and beetles have demonstrated cross-generation priming, our results suggest that cross-generational priming may not be a general phenomenon, and more work is needed to determine how widespread this priming is.

Introduction

Over the last few decades, dramatic advances have been made in the study of immunity in insects. We now know that insects have quite a sophisticated innate immune response, consisting

of humoral (IMD and Toll), cellular, and melanization defences (Hoffmann 2003; Hultmark 2003; Lemaitre and Hoffmann 2007; Strand 2008). Even though insects do not have what is typically thought of as an 'adaptive' immune response, there is some evidence that invertebrates challenged with a low dose of an infectious agent garner increased resistance to a subsequent infection with the same pathogen, a phenomenon known as 'priming'. This has been demonstrated in copepods (Kurtz and Franz 2003), *D. melanogaster* (Pham et al. 2006), and mealworm beetles initially challenged with lipopolysaccharides (LPS) (Moret and Siva-Jothy 2003). In addition, recent evidence suggests that there may also be 'cross-generational priming', where offspring from mothers that were immune challenged have a greater immune up-regulation in the next generation than offspring from un-challenged mothers. Cross-generational priming has been demonstrated in *Daphnia* (Little et al. 2003), bumblebees (Sadd et al. 2005), and beetles (Moret 2006). This evidence suggests there may be many factors that contribute to immunocompetence and ultimately to offspring fitness in the next generation.

The fitness of an organism is made up of several components including age-specific survival and reproduction (Charlesworth 2001). Reproductive fitness can be defined not only by the number of offspring produced, but also by their quality. Limited maternal resources may lead to changes in resource allocation between reproduction and other physiological traits. For example, if a female has a limited amount of resources available, investing more in reproduction can come at a cost to her survival (Tatar and Carey 1995). While this trade-off occurs within the mother, these costs can also have consequences for her offspring. The trade-off between egg number and size is an oft-cited example (Smith and Fretwell 1974; Fox and Czesak 2000), where the increased fecundity in the female may come at a cost of decreased per-offspring resources. So-called 'maternal effects' occur when factors that affect the mother influence offspring quality,

independent of the genes handed down from mother to offspring (Mousseau and Fox 1998). These factors could include the mother's physiological state. For example, the age of the mother has been found to alter longevity of the offspring (Kern et al. 2001; Priest et al. 2002), as well as pupal survival (Faurby et al. 2005). These factors can also be genetic. Allelic variation and gene expression patterns in the mother can influence offspring development independent of the offspring's genotype (e.g., maternal *bicoid* and *hunchback* expression influences the patterning of zygotes in *D. melanogaster*) (Driever and Nussleinvolhard 1988; Struhl et al. 1992). Finally, external environmental factors experienced by the mother can also alter offspring fitness. For example, temperature has been shown to affect egg size, viability, offspring longevity, and overall offspring fitness in flies (Gilchrist and Huey 2001).

Here we focus on the relationship between immune function and maternal effects, using the fruit fly, *D. melanogaster*, as a model system. While the innate immune system can provide rapid and often complete protection for a host, only recently have researchers begun to consider the impact of infection and immune gene expression on the host's reproduction and offspring. For example, recent work has suggested that survival from an infection is not straightforward as previously thought. Differences in an organism's ability to tolerate an infection versus actually resist an infection point to a complex relationship between immune system function and infection (Ayers and Schneider 2008; Read et al. 2008). This added complexity may be similar to the relationship between reproduction and immunity. In *D. melanogaster*, researchers have found that immune-challenged individuals exhibit a decrease in female fecundity (Zerofsky et al. 2005). In addition there seems to be a cost to immune system up-regulation and maintenance. For example, in fruit flies, male courtship of females leads to a reduction in immune function (McKean and Nunney 2001). It has also been found that the act of mating can induce immune

system up-regulation in female flies (Lawniczak and Begun 2004; McGraw et al. 2004), but the females have a decreased ability to overcome a bacterial infection after mating (Fedorka et al. 2007). This evidence suggests that reproduction and immunity are intertwined. Though much work has been done in *D. melanogaster* on immunity, we still do not know how immune up-regulation affects offspring fitness, either negatively (through reduced resources available to the offspring) or positively (through heightened offspring immune response).

Here we set out to determine if stimulation of the immune system in response to a bacterial infection has a fitness cost in the quantity or quality of the offspring produced. In addition, we wanted to determine if there were any benefits in the next generation, via cross-generational priming. We examined fecundity and egg size of infected versus non-infected mothers as well as egg to adult viability, longevity, oxidative stress resistance, and pathogen resistance of offspring derived from infected and non-infected mothers. Results from this study should provide further insight into the way in which maternal infection influences offspring fitness.

Two inbred strains (IR 56 and IR 57) and one outbred strain (GAo) of the fruit fly, *D. melanogaster*, were used in these experiments. Mothers were collected as virgins, aged for three days, and then mated for one hour in a ratio of five females to seven males to ensure females were only mated once (Linder and Rice 2005). Females were then anesthetized with CO_2, and placed 10 females per vial, where they recovered for 24 hours with a small pinch of live yeast. The next day, flies were pooled together and placed into one of the four treatments. One treatment was composed of mothers that were infected with the gram-positive bacteria *Lactococcus lactis* (**LL**), and a second treatment was infected with the gram-negative bacteria *Pseudomonas aeruginosa* (**PA**). Two control treatments were used, including a sterile broth

injection (**sham**), and a **naïve** treatment, in which there was no septic injury and the flies were anesthetized at the same time as the other treatments. Mothers were allowed to recover for one hour and then several different measurements were taken in order to determine the fitness consequences of immune challenge to the mothers as well as to the offspring.

For fecundity, viability, and egg size, ANOVAs were conducted on each replicate separately. We then calculated an overall *P*-value for each trait using Fisher's Combined Probability test. *Post-hoc* analyses were done using the False Discovery Rate method (Benjamini and Hochberg 1995), and *q*-values were reported. *A priori*, we had decided to make four comparisons among the treatments: Naïve ≠ PA, Naïve ≠ LL, Sham ≠ PA, and Sham ≠ LL. For treatment-by-host strain interactions, a standard ANOVA was carried out. All survival analyses were conducted using a proportional hazards model.

Results

Fecundity

Offspring number over 24 hours was measured in all four treatments. In two of the three strains, infection appeared to reduce fecundity, though the pattern differed slightly between strains. There were significant differences between treatment in both GAo females ($P < 0.0001$; Figure 3.1a) and IR 57 females ($P = 0.0014$; Figure 3.1c), but not IR 56 females ($P = 0.0851$; Figure 3.1b). Post hoc tests indicated that naïve females produced more offspring than both LL infected ($q = 0.0012$) and PA infected ($q = 0.0001$) females in the GAo flies. For the IR 57 flies we found that naïve females produced more offspring than LL ($q = 0.0262$), Sham produced more than LL ($q = 0.0134$) and more than PA mothers ($q = 0.0134$). There was a significant treatment-by-strain interaction: (treatment*strain: $F_{6, 1420} = 8.261$, $P < 0.0001$; treatment: $F_{3,1423}$ =

8.728, $P < 0.0001$; and strain: $F_{2, 1424} = 405.1$, $P < 0.0001$). Egg to adult viability was also measured. However, treatment had no significant effect on this trait (data not shown), with the average viability for each line being GAo = 87.2%, IR 56 = 86.3%, and IR 57 = 69.5%. In addition, treatment-by-strain interactions were not significant for viability (treatment*strain: $F_{6, 1420} = 0.407$, $P < 0.875$; treatment: $F_{3,1423} = 1.005$, $P = 0.3895$; and strain: $F_{2, 1424} = 100.74$, $P < 0.0001$).

Viability

Viability (egg to adult survival) was also measured at higher densities. In the 'Fecundity' measurement, egg density was very low (approximately 10-30 eggs per vial, depending on line, see Figure 3.1) and we wanted to determine viability at a density that may be more stressful for the larvae. In all of the following measurements, eggs were collected from egg-laying chambers. Each chamber housed 30 females for 18 hours. Egg chambers included a petri dish with a 30% molasses, 10% agar medium with a dab of 0.05 g of yeast paste on which the females could feed and lay eggs. After 18 hours females were discarded and eggs were collected. Females were placed into chambers one hour after exposure to the treatment. In this 'viability' measurement we placed 150 eggs from each treatment into a vial and measured the number of offspring that eclosed. We found that treatment did not have a significant effect on viability at a density of 150 eggs for GAo ($P = 0.284$), IR 56 ($P = 0.624$), or IR 57 ($P = 0.112$). Average viability and standard errors (SE) are given in Table 3.1. There were no treatment-by-strain interactions for viability (treatment*strain: $F_{(6,106)} = 0.4578$, $P = 0.8379$; treatment: $F_{(3,109)} = 3.825$, $P = 0.0122$; strain: $F_{(2,110)} = 53.83$, $P < 0.0001$).

Egg Size

Eggs were collected from egg-laying chambers, photographed and egg-length and egg-width were measured using NIH Image J software. We used the volume of a prolate spheroid, $\left(\frac{\pi}{6}\right) * w^2 * h$, to determine egg volume. Treatment had a significant effect on egg volume in all three lines (GAo, $P < 0.0001$; IR 56, $P < 0.0001$; and IR 57 $P < 0.0001$). Post hoc comparisons were significant in a few cases. For IR 56: Naïve > PA ($q = 0.022$), Sham > PA ($q < 0.0001$), and Sham > LL ($q < 0.0001$). In addition, in IR 57 we found a difference between Naïve > PA ($q < 0.0001$). Though there were a few significant post hoc comparisons, the treatments were not consistent between lines and do not suggest a pattern. This is demonstrated by the highly significant treatment-by-strain interactions for egg volume (treatment*strain: $F_{(6,106)} = 17.432$, $P < 0.0001$; treatment: $F_{(3,109)} = 14.723$, $P < 0.0001$; strain: $F_{(2,110)} = 43.355$, $P < 0.0001$). Average volume per replicate for each treatment in each line can be found in Table 3.2.

Longevity of offspring

Treatment of mothers had a significant effect on longevity of offspring (Figure 3.2). Overall, our data suggest that offspring from immune-challenged mothers may pay a survival cost compared to offspring from naïve or sham injected mothers in two of the three lines, while in the third line offspring of immune-challenged mothers seemed to benefit, at least early on in life. GAo offspring showed significant differences between treatments (Cox Proportional Hazards model $\chi^2_3 = 26.89$, $P < 0.0001$; Figure 3.2a). Post-hoc pair-wise tests showed that offspring derived from naïve mothers lived significantly longer than those derived from LL infected mothers ($q = 0.0004$) and a trend was seen where Naïve > PA, ($q = 0.07$). Treatment of mothers also had a significant effect on offspring longevity for IR 57 ($\chi^2_3 = 11.39$, $P = 0.019$;

Figure 3.2c). Pair-wise tests showed a significant effect for offspring derived from sham mothers, which lived longer than those derived from PA infected mothers (q = 0.038). Treatment of mothers also had a significant effect on offspring longevity for IR 56 (χ^2_3= 20.09, P = 0.0002; Figure 3.2b), and pair wise tests suggest a marginal trend that offspring from naïve mothers died off faster than offspring from LL infected mothers in early life, but the pattern (Figure 3.2b) appeared to switch later in life (q = 0.0856). In addition, there was a significant treatment-by-strain interaction (treatment*strain: χ^2_6 = 44.71, P < 0.0001; treatment: χ^2_3 = 7.60, P = 0.055; strain: χ^2_2 = 1074.6, P < 0.0001).

Oxidative Stress of Offspring

We also measured the offspring's ability to resist oxidative stress by exposing three-day old virgin offspring to hydrogen peroxide. Treatment of mothers was not found to have any significant effect on offspring's ability to resist hydrogen peroxide oxidative stress (Cox Proportional Hazards model, GAo: χ^2_3 = 1.364, P = 0.714; IR 56: χ^2_3 = 3.712, P = 0.294; IR 57: χ^2_3 = 6.225, P = 0.101). There was not a significant treatment-by-strain interaction (treatment*strain: χ^2_6 = 4.082, P = 0.665); treatment: χ^2_3 = 6.178, P = 0.103; strain: χ^2_2 = 1378.9, P < 0.0001).

Immune ability (priming) of offspring

Finally in order to determine whether offspring from immune challenged females had improved ability to defend against pathogens in the next generation, immune ability of the offspring was measured. Flies derived from *L. lactis* infected mothers were challenged with *L. lactis* (LL-LL), flies from *P. aeruginosa* mothers were challenged with *P. aeruginosa* (PA-PA),

and a subset of flies from naïve and sham mothers were challenged either with *L. lactis* (Naïve-LL, Sham-LL) or *P. aeruginosa* (Naïve-PA, Sham-PA). Treatment of mothers had no effect on offspring ability to overcome an immune challenge (survival curves not shown). When infected with PA, survival rate of offspring derived from PA infected mothers was not significantly different than offspring from sham or naïve mothers for GAo (χ^2_2 = 2.824, P = 0.244), IR 56 (χ^2_2 = 0.659, P = 0.720), or IR 57 (χ^2_2 = 0.277, P = 0.871). In addition, when infected with LL, survival rate of offspring derived from LL infected mothers also did not differ significantly from the survival rate of offspring from naïve or sham mothers for GAo (χ^2_2 = 1.003, P = 0.605), IR 56 (χ^2_2 = 1.243, P = 0.537), or IR 57 (χ^2_2 = 4.752, P = 0.093). There was not a significant treatment-by-strain effect for either LL (treatment*strain: χ^2_4 = 4.092, P = 0.394; treatment: χ^2_2 = 2.102, P = 0.349; strain: χ^2_2 = 23.85, $P < 0.0001$), or for PA (treatment*strain: χ^2_4 = 3.465, P = 0.483; treatment: χ^2_2 = 0.499, P = 0.779; strain: χ^2_2 = 86.42, $P < 0.0001$).

Discussion

Maternal effects can play an important role in determining offspring fitness of invertebrates (Azevedo et al. 1997; Gilchrist and Huey 2001; Priest et al. 2002; Faurby et al. 2005). However, only recently have researchers begun to use invertebrates to study how an immune challenge to the mother can affect the fitness of her offspring. In the last few years, researchers have found that offspring derived from mothers challenged with a particular pathogen may be better at resisting that pathogen in the next generation (Sadd et al. 2005; Moret 2006). In this study, we took a more comprehensive approach by examining both the potential costs as well as benefits that we might see in the next generation after maternal immune

challenge. This study allowed us to obtain a more complete understanding of the evolutionary forces acting on immunity in insects.

Our results point to both direct fitness costs of infection in the host mother as well as indirect fitness costs to the offspring through a maternal effect in two of the three lines examined. In terms of direct effects to the host, we see a fecundity cost of infection in immune challenged females (Figure 3.1), in agreement with previous studies (Fellowes et al. 1999; Zerofsky et al. 2005; Brandt and Schneider 2007). However, we only see this effect in two of the three lines. In the GAo line, we do not see a difference between infected and sham females (only between naïve and infected females). Previous studies have shown that piercing the fly causes the immune system to be up-regulated (Wigby et al. 2008). So even though it is only the sterile jab that is causing the effect in this line, it is still relevant to determining the effect that immune up-regulation has on the next generation. The variability of these results might also be due to genetic variation for immune-mediated maternal effects. Priest et al. (2002) found a similar pattern—genotype background influenced the role of parental age on offspring fitness. Previous studies have established that there is abundant variation for immune ability in flies ranging from Africa to the northern United States (Lazzaro et al. 2008). Moreover, researchers have also observed genotype-by-environment interactions in the cost of deploying the immune system in flies (McKean et al. 2008). In light of these studies and our own findings, if future studies find genetic variation for cross generational effects to be consistent and robust, subsequent studies on a larger scale could help us to identify specific loci that influence immune-mediated maternal effects.

At the same time, our data point to a small cost to survival in the next generation. Longevity is reduced in offspring derived from immune challenged females in both GAo and IR

57. This effect, along with the fact that we see higher fecundity in the control flies of these lines, suggests that mothers have to choose between allocating resources from a finite pool either to reproduction or to immune function. This trade-off between reproduction and immunity has been seen in several different studies on insects (Moret and Schmid-Hempel 2000; McKean and Nunney 2001; Fedorka and Mousseau 2007). These observations further support the hypothesis that infection is costly in the current generation, but can also have effects on offspring quality in the next generation. However, in the IR 56 line, we see a trend that offspring from LL infected mothers have lower early-age mortality but higher late-age mortality than offspring derived from naïve mothers, suggesting a potential trade-off between early-age and late-age fitness influenced by immune-mediated maternal effects. Interestingly, IR 56 did not show any direct costs of infection on number of eggs produced, so it might be that the fitness cost of infection is lowest in this line. The three genetic variants we used in this study gave us different results. Although the differences among genetic variants seen here complicates our interpretation, it is important to examine several different genetic lines when trying to answer both evolutionary and molecular based questions to determine the generality of any results. Even though we see different outcomes among lines, we can still observe a general pattern, namely that maternal condition influences offspring fitness traits. Thus, in any study that sets out to determine the overall fitness consequences of infection, one should consider effects both within and between generations.

Although our data point to costly maternal effects after infection, many aspects of fitness that we measured did not show an effect. For example, while egg volume varied among treatments, there was no consistent difference between infected and uninfected mothers across strains. There was evidence for eggs from sham mothers being larger than the LL or PA mothers in IR 56. However the pattern was not consistent between lines and naïve females did not

demonstrate a strong difference in egg size from infected mothers. We chose to measure egg volume due to the fact it has been used in previous studies to measure egg size. (Azevedo et al. 1997) However, a measurement like weight may be more accurate for examining differences in investment in egg size. Or perhaps there is no selection for differential investment in egg size while infected with a pathogen in our flies, and the differences we see are just due to random variation, or due to the fact most egg development occurs several days before they are laid.

We also failed to find an effect of maternal infection on viability of the eggs (Table 3.1). This may have been due in part to the fact that females in this study had abundant resources. In previous studies of trade-offs in invertebrates, researchers have often found that fitness costs are only apparent under conditions of limited resources and/or high levels of competition (Tatar and Carey 1995; Jenkins et al. 2004), and the costs of immune system deployment are exaggerated with limited resources (McKean et al. 2008). Alternatively, infection in mothers may simply not lead to maternal effects on egg viability. However, that seems unlikely, since viability costs are seen at later stages of development in offspring and in many other maternal effects (Azevedo et al. 1997; Kern et al. 2001; Faurby et al. 2005).

In this study, we examined many different aspects of fitness. It is perhaps not surprising that we only found an effect of maternal condition on a few of the offspring fitness components. Even if trade-offs constrain fitness optima, it is still possible that while some traits will correlate negatively with each other, others will show no correlation, or even positive correlations. As noted earlier, non-negative fitness correlations could arise if females had abundant resources, as they did in the laying chambers. While we did try to carry out experiments in which females in egg-laying chambers had limited yeast, the females would not then lay eggs. We note that females in the egg laying chambers were exposed to media with substantially higher sugar

content than in the normal media (30% molasses versus 10% molasses). Again, abundant (albeit temporary) resources may have mitigated against finding trade-offs between some fitness traits.

Finally, we did not see any maternal effects on resistance to infection in the next generation. This result is in contrast to Moret (2006) and Sadd et al. (2005), both of whom found that offspring of infected mothers had greater immune up-regulation in the next generation. This difference between our study and previous work may be due to the fact that our infection protocol did not stimulate the priming mechanism, or at least not to a detectible degree. A recent study by Pham et al. (2006) suggested that phagocytes may be the critical component of priming in insect immunity. If we were to challenge flies with another type of pathogen, such as the eggs of a parasitoid wasp, we might see an effect of priming. In our own study, we examined priming in *D. melanogaster* using a lethal pathogen. In previous studies that showed cross-generational priming, the authors used heat killed bacteria and LPS in bumblebees and beetles (Sadd et al. 2005; Moret 2006). In addition, these previous studies measured the offspring immune ability by examining phenoloxidase (PO) levels, which they obtained by extracting hemolymph. *D. melanogaster* is significantly smaller than both bumblebees and beetles, and it is extremely difficult to extract a significant amount of hemolymph out of adult flies. Since the authors used an inert immune stimulus, the functional ability to overcome an infection was not demonstrated. Previous studies have shown discrepancies in measures of immunocompetence. For example, pathogen load (number of colonies) did not correlate with ability to overcome a bacterial infection with *P. aeruginosa* in *D. melanogaster* (Corby-Harris et al. 2007). Furthermore, measuring actual survival in response to infection is arguably more directly relevant to fitness than measures of PO levels. In a series of pilot studies in our lab, we challenged mothers with LPS in order to see if that immune challenge would demonstrate a priming response as in Moret

(2006) and Sadd et al. (2005), or show costs of immune challenge, but were not able to find a concentration that effectively and consistently showed any effects (J. E. Linder, unpublished data).

It is unlikely that our negative results are due to insufficient statistical power. We used relatively large sample sizes, with 150 flies for each treatment and each type of bacteria, which were spread across three independent replicates. This size is adequate for *Drosophila* and larger than both of the prior studies that found cross-generational priming. In addition, neither Moret (2006) nor Sadd et al. (2005) considered the effect of genotypic variation. We examined the effect in three genetically distinct populations, whereas all of the bumblebees measured in each treatment from Sadd et al. (2005) were derived from one queen. And although Moret (2006) started with multiple cultures of beetles, they originated from the same original population. Perhaps some of our variation in results comes from looking at many different populations of flies, as is demonstrated by significant treatment-by-strain results in the fecundity, egg size, and longevity treatments.

Interestingly, a study was just published in the Yellow Fever mosquito, which also failed to find cross-generational priming. Voordouw et al. (2008) examined viability, age at emergence, body size, and melanization in offspring of mosquitoes that had been challenged with an injection of sephadex beads. They found no difference between offspring from challenged mothers and offspring from sham or naïve mothers. These results, taken together with our own findings, suggest that cross-generation priming is not universal among insects. In this light, to better understand the relative importance of cross-generational priming, it would be of benefit not only to replicate this and previous studies, but also to examine other types of insects for evidence of cross-generation priming.

Our results may also have been limited by the timing of our infections. We chose to measure fitness traits shortly after infection since females typically succumbed to infection within 48 hours. However, egg development in flies actually starts approximately seven days before the egg is laid, so even though we see an effect of treatment on some traits (like egg size and offspring longevity) we are not examining how infection influences the egg from the first stage of development. Future work should examine more closely the effect that infection has on the different stages of egg development, in order to better understand the potential impact of infection and maternal affects.

Overall, the most surprising result of our study is that we failed to find cross-generational priming. This suggests that the phenomenon may not be widespread. In addition, this study provides further support that there are reproductive costs of immunity in *D. melanogaster* (Zerofsky et al. 2005). Based on these findings, it would be appropriate to use molecular approaches to determine differences in gene expression in offspring derived from immune challenged versus naïve females. Investigating the genes involved in the differences in offspring survival could elucidate the relationship between longevity and immunity. It may also be worth developing models of host-parasite interactions that incorporate maternal effects. It would be of particular interest to determine whether maternal effects might influence the evolution of virulence (Elliot et al. 2003; Little et al. 2007). The tradeoffs that we observed here could also have important implications for the biological control of insects. Pathogens released to control insect pests might also have additional control effects by reducing fitness in the next generation (McMeniman et al. 2009).

Methods

Fly Stocks and Maintenance

Flies were cultured on standard cornmeal-molasses-agar media and maintained with 14-day non-overlapping generations, with a 12-hour L:D cycle, in a manner similar to Linder et al. (2008) The outbred strain of flies used in this experiment (GAo) was the same as in Linder et al. (2008) Two inbred lines (IR 56 and IR 57) were collected from the University of Georgia horticultural farm in Athens, GA in 2003. They were maintained with full sib mating until September of 2007, at which time they were expanded using random mating to approximately 75 male/female pairs (in each line) for approximately ten generations in order to create enough flies for the experiments. Flies were cultured in vials containing seven ml of food and five to seven male/female pairs. Eggs were trimmed to a density of 120 per vial in order to control for density effects during the experiment. Trimming was carried out on all lines for vials that were to give rise to the maternal generation in the experiment.

Bacterial stocks

To test for evidence of cross-generational priming, we exposed female flies to one of four treatments. **Naïve** flies received no immune challenge or injury of any kind, s**ham** flies received a sterile broth inoculation, **LL** flies were exposed to gram-positive bacteria, *Lactococcus lactis*, and finally **PA** flies were exposed to gram-negative bacteria, *Pseudomonas aeruginosa*. Both bacterial strains were originally obtained from Brian Lazzaro at Cornell University. *L. lactis* was derived from a natural fly population, and *P. aeruginosa* was derived from a laboratory culture (strain PA01). Bacteria were grown up overnight in liquid LB-broth at 37 degrees C. Bacteria where then standardized in a spectrophotometer to an optical density (OD) of 0.8 nm at 600 Å

and then diluted. An OD of 0.005 (for both bacteria) was used for the maternal infections, and an OD of 0.01 (for *P. aeruginosa*) and an OD of 0.06 (for *L. lactis*) was used for priming infections of the offspring.

Maternal Generation

Mothers were pooled together and randomly assigned to one of the four treatments (Naïve, Sham, LL, or PA). Flies were anesthetized under light CO_2 and then infected by piercing the thorax with the tip of a 0.1 mm stainless steel pin dipped in the bacterial suspension. To challenge the mothers, we used a dose of bacteria that led to the death of half of the flies over the course of three days (LD50), determined prior to the actual experiments. Immune gene activation has been shown to take place rapidly after infection (Lawniczak and Begun 2004; McGraw et al. 2004), so females were placed into egg laying chambers one hour after inoculation and allowed to lay for 18 hours. For the 'fecundity' measurement, females were placed into individual vials (see below). Separate mothers were used for each of the following measurements.

Measurements

Fecundity - Egg number and offspring number were counted for individual females. One hour after treatment, females were placed in individual vials for 24 hours. Eggs were then counted and the number of offspring that eclosed were counted 14 days later to determine egg to adult viability. Average number of eggs per vial ranged from 10-25 depending on the strain, so we also determined viability at a higher density (see 'viability' section below). In each of lines IR 56 and IR 57, we measured 145 females across three independent replicates for each treatment (so each

replicate had ~ 50 Naïve, Sham, LL AND PA females, and this was independently carried out three times), while 155 females across three replicates (again ~ 50 per replicate for EACH treatment) were measured for GAo.

Viability – We also determined egg to adult viability at a high density with three independent replicates. For each replicate two egg laying chambers were used for each maternal treatment. Each chamber had 30 females that had been exposed to their specific treatment. Females were placed in the chambers one hour after being exposed to the treatments. Eighteen hours later, one or two sets of 150 eggs were collected from each chamber (depending on how many eggs were laid) and placed in a vial (up to four vials of 150 eggs for each treatment could be collected for each replicate, depending on how many eggs were collected from the chambers). We recorded the number of offspring that eclosed after 14 days. Three independent experiments were carried out, in which two chambers were used for each treatment and each line. A total of 12 vials of 150 GAo eggs, 10 vials of 150 IR 57 eggs, and seven vials of 150 IR 56 eggs were measured for each treatment (Naïve, Sham, LL, and PA).

Egg Size – Three independent replicates were carried out. Eggs were collected as in the 'Viability' measurement, but with only one egg-laying chamber per independent replicate (for each treatment) and 50 eggs collected from each chamber. Approximately 150 eggs in total (spread across the three independent replicates) for each treatment and each line were photographed using a Q-Imaging photo-capture system on a dissection microscope. The length and width of each egg was measured using NIH Image J software. The entire set of eggs was

measured three times and the average for each egg was used to reduce the measurement error (we found a repeatability of $r^2 = 0.87$ for length and $r^2 = 0.40$ for width).

Longevity of offspring – After exposure to infection treatments, females from each treatment were placed in egg laying chambers for 18 hours (two chambers per replicate for each treatment; three independent replicates). Eggs were then collected and 120 eggs transferred to each vial. Virgin females were then collected from these vials nine days later and placed into survival cages (approximately 25-35 females per cage). In each replicate (three independent replicates total) there were two survival cages per treatment (to give a total of six survival cages per treatment). For the GAo line, each cage contained approximately 35 females, giving a total of approximately 200 females spread across the six cages. Approximately 130 females across six cages were measured for each treatment for IR 56 and IR 57 (in these lines there were only about 25 females per cage).

Oxidative stress of offspring – Offspring were collect as virgins as in the 'Longevity' measurement. Females were aged for three days and then exposed to a 3% H_20_2+5% sucrose solution. Two ml of the solution were added to a filter paper and 15 females were placed in each vial. Number of adults surviving was recorded approximately every eight hours over 120 hours, by which time > 90% of the flies had died. Approximately 200 females across three independent replicates (~50 to 75 per replicate) were analyzed in each treatment for GAo, 130 females for IR 56, and 160 females for IR 57. Control vials were filled with two ml of a 5% sucrose solution.

Immune ability (priming) of offspring – Female offspring were collected as virgins as in the 'Longevity' measurement and aged for three days, after which flies were infected with a lethal dose of bacteria. Survival was then measured for 48 hours by which time over 80% of the flies had died. Our previous work has shown that by 48 h, any fly that was going to succumb to infections at these concentrations would have done so (Fedorka et al. 2007). Fifty females were infected and measured for each treatment (Naïve-PA, Sham-PA, and PA-PA; or Naïve-LL, Sham-LL, and LL-LL) in three independent replicates, giving a total of approximately 150 females per treatment in each line. Infections with *P. aeruginosa* were conducted separately from those with *L. lactis*.

Statistics

We used a proportional hazards model to analyze survival data (Parmar and Machin 1995; Therneau and Grambsch 2000). If there was a significant effect of treatment on survival based on the proportional hazards model, pair-wise comparisons of the treatments were then carried out. *Post-hoc* analyses were done using a False Discovery Rate (FDR) method, and *q*-values are reported. *A priori*, we had decided make four comparisons among the treatments: Naïve ≠ PA, Naïve ≠ LL, Sham ≠ PA, and Sham ≠ LL. ANOVAs were conducted on treatment-by-strain interactions. For analysis within a strain ANOVAs were conducted on each replicate separately, and then post hoc comparisons were conducted using FDR. We then calculated an overall p-value using a Fisher's Combined Probability test. All data were analyzed using JMP 7.0 (Jmp 7.0 2007).

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Table 3.1. Egg to adult viability at a density of 150 eggs, ± SE.

Line	Naïve	Sham	LL	PA
GAo	0.697 ± 0.020	0.748 ± 0.026	0.664 ± 0.020	0.656 ± 0.027
IR 56	0.642 ± 0.030	0.646 ± 0.023	0.617 ± 0.023	0.581 ± 0.027
IR 57	0.536 ± 0.029	0.550 ± 0.023	0.511 ± 0.017	0.515 ± 0.014

Table 3.2. Mean egg volume for each replicate in mm^3, ± SE.

Line	Rep	Naïve	Sham	LL	PA
GAo	1	0.00839±0.00014	0.00798±0.00014	0.00860±0.00014	0.00797±0.00014
	2	0.00782±0.00016	0.00836±0.00016	0.00858±0.00016	0.00795±0.00016
	3	0.00772±0.00016	0.00831±0.00016	0.00842±0.00016	0.00783±0.00016
IR 56	1	0.00787±0.00014	0.00867±0.00014	0.00787±0.00014	0.00795±0.00014
	2	0.00808±0.00015	0.00880±0.00013	0.00769±0.00015	0.00752±0.00015
	3	0.00801±0.00015	0.00849±0.00015	0.00802±0.00015	0.00770±0.00015
IR 57	1	0.00888±0.00015	0.00817±0.00015	0.00883±0.00015	0.00897±0.00015
	2	0.00899±0.00016	0.00839±0.00016	0.00882±0.00016	0.00781±0.00016
	3	0.00926±0.00017	0.00825±0.00017	0.00882±0.00017	0.00824±0.00017

Figure Legends

Figure 3.1. Average fecundity (+ 1 S.E.) of singleton females after 24 hours, by replicate. Offspring number was significantly higher in naïve flies compared to LL and PA flies in the GAo lines (a), and fecundity was higher in sham-injected flies compared to PA and LL as well as higher in naïve compared to LL flies in IR 57 lines (c). No differences were seen among the treatments for the IR 56 (b) lines. Means of the three replicates are shown as open circles.

Figure 3.2. Survival curves for GAo (a), IR 56 (b), and IR 57 (c) offspring derived from females exposed to each of the four treatments. A proportional hazards model showed significant differences between treatments for all three lines. After pair-wise comparisons, we find that offspring from naïve females live significantly longer than offspring from LL infected females for GAo and a marginally significant effect for IR 57, where offspring derived from sham mothers survived longer than offspring derived form PA infected mothers. We find the opposite effect in IR 56, where flies from LL mothers live longer than those from naïve mothers.

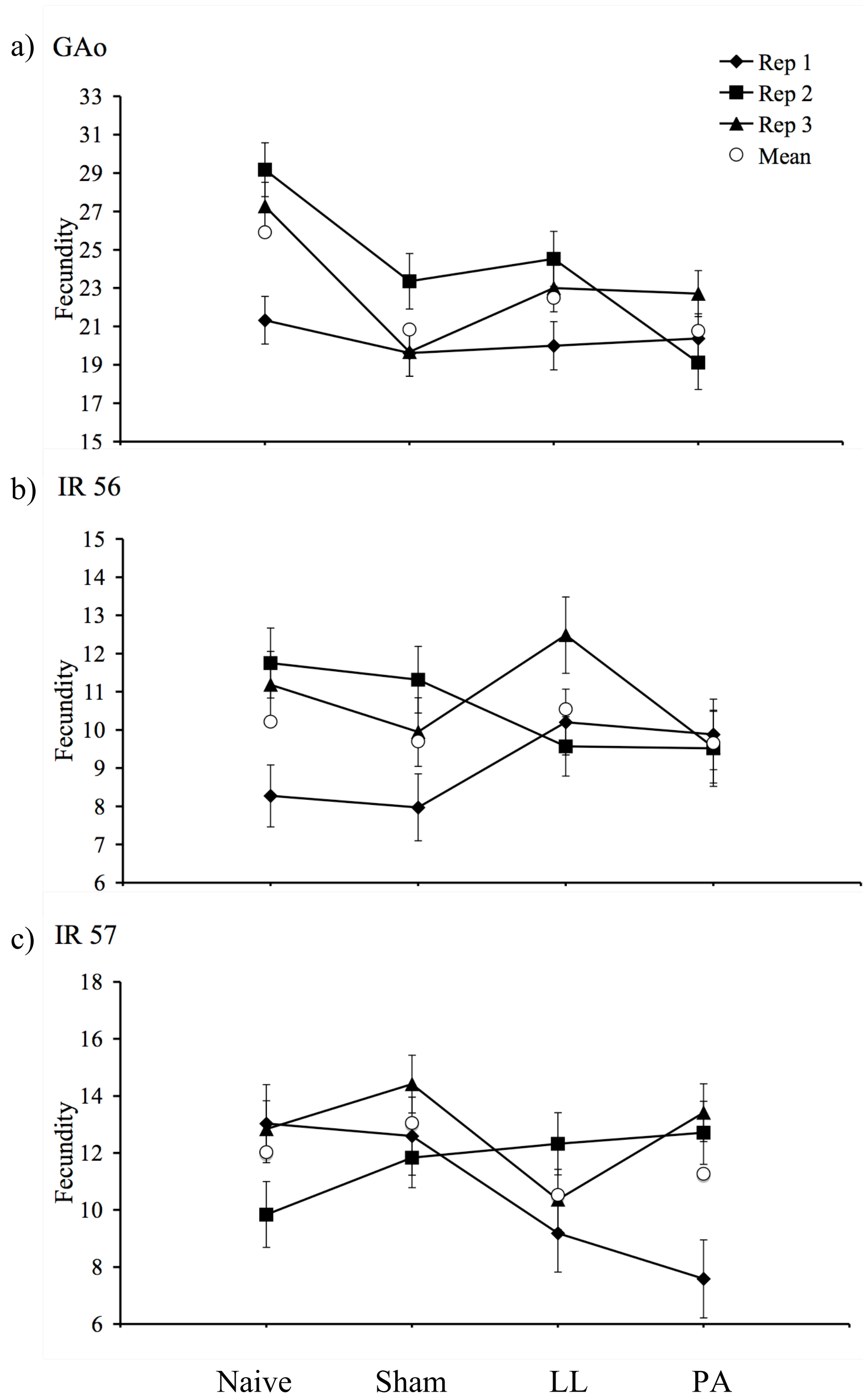

Figure 3.1

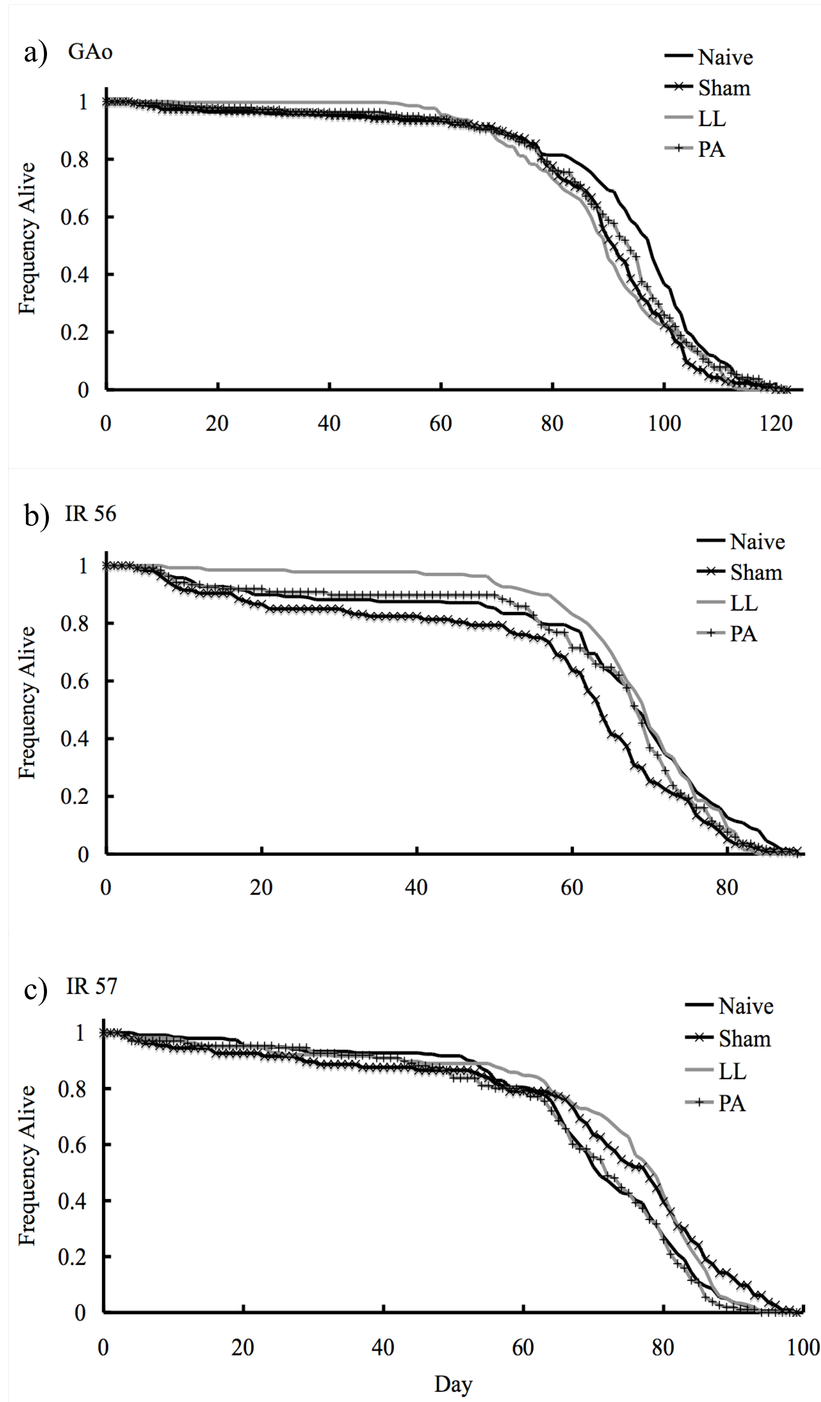

Figure 3.2

CHAPTER 4

VARIATION IN EARLY-AGE IMMUNITY IN *DROSOPHILA MELANOGASTER*[3]

[3]Linder, J.E. and Promislow, D.E.L. 2009. In review at *Journal of Evolutionary Biology*.

Abstract

Demographic models show that selection acts most strongly early in life, before and at reproduction, declining monotonically thereafter. Furthermore, the greatest rate of decline in the force of selection typically occurs during this early period. Recent studies on age-related changes in innate immunity in the fruit fly, *Drosophila melanogaster*, have focused on variation late in life. Little work has been conducted on changes early on in life, where selection should be strongest. In this article we examine variation in immunity in the first few days post eclosion in *D. melanogaster*. We find that flies within one and two days of eclosion cannot defend against a bacterial infection as well as older flies. We determine that this early deficit in immune function does not appear to be explained by a costly trade-off between investment in development of immunity and egg production. This study provides us with a first glimpse of the developmental trajectory of immunity in the early-adult fly, demonstrating that it may take several days for the immune system to become fully functional.

Introduction

Hamilton (1966) developed an explicit mathematical model for selection in age-structured populations. He showed that the strength of selection declines monotonically after the age of first reproduction. The intensity of selection is greatest just after an organism reaches reproductive maturity. While Hamilton's work has proved critical in understanding age-related changes in fitness traits that occur due to senescent decline, little attention has been paid to age-related changes during the earliest period immediately following reproductive maturity. While the theory predicts that fitness components should begin to decline as soon as an organism

reaches maturity, we might expect some fitness components to show age-related improvements if development of some systems continues into adult stages.

Small changes early in life, be they age-related increases or decreases in fitness components, can have dramatic consequences for fitness (e.g. Abrams 1991). For example, in the fruit fly, *Drosophila melanogaster*, a difference of a few hours in the timing of egg laying in laboratory culture made the difference between the success or failure of individuals to pass their genes on to the next generation (Houle and Rowe 2003). In the seed beetle, *Acanthoscelides obtecus*, when females aged 0 to 5 days old were mated to males, females mated at age zero or one day of age had a 12% drop in lifetime fecundity compared to older females (Maklakov et al. 2007). In a separate study on seed beetles, offspring from six-day-old seed beetle mothers had lower viability and higher larval death than eggs from younger mothers (Fox et al. 2003). Finally, researchers working on cockroaches found that females older than six days of age had fewer offspring per clutch and fewer clutches overall than females younger than six days of age (Moore and Moore 2001). These studies suggest that even subtle differences in early adult age can have profound effects on fitness. By understanding variation early in life we may be able to fully appreciate how selection shapes life history strategies.

Until now, no studies have examined early-age variation in immune function in insects. Insects are equipped with an innate immune response, a sophisticated system of distinct genetic modules that enable hosts to fight off pathogens. Insects have both humoral and cellular immune responses, which allow them to release antimicrobial peptides (AMPs) against gram-negative bacteria, gram-positive bacteria and fungal infections as well as to encapsulate and engulf pathogens (Hoffmann 2003; Hultmark 2003; Lemaitre and Hoffmann 2007; Strand 2008). Previous work has demonstrated that immune ability changes over the lifetime of fruit flies.

There is a large up-regulation of AMPs late in life in *D. melanogaster* even when the fly is not consistently challenged by pathogens (Pletcher et al. 2002). This up-regulation does not appear to necessarily confer increased immunity. Ramsden et al. (2008) found infection was more severe in 30 and 40 day old flies than three and 10 day old flies. Conversely, Burger et al. (2007) found that five and seven week old flies actually had an advantage defending against a gram-negative bacterial infection compared to seven-day old flies. However, these studies examine immunity late in life, not early on, when selection is potentially the strongest.

Given the importance of early-life events in determining fitness, here we examine how immune ability changes over the first five days of life in adult *D. melanogaster*. It is still relatively unknown as to when the immune system of an adult fruit fly is fully functional. Flies are exposed to parasites and pathogens throughout their life, including directly after eclosion. Since selection is potentially very strong early in life one would expect strong selection for a functional immune response early on. However, recent research suggests a cost to immune maintenance (McKean et al. 2008), so there may be variation in how quickly the immune system becomes functional. This variation could be explained by several factors. First, development of the immune system may not be complete; in this case we should see an increase in immune ability with age. Along those lines, there may be trade-offs between investment in the development of a functioning immune system and investment in other fitness components. For example, it may be more important for an individual to invest in egg production early on. Finally, if the onset of senescence of the immune system coincides with the onset of reproduction, we may see a decrease in immune ability even at an early age.

In this paper, we set out to determine if there is variation in early age immunity. We also test the hypothesis that some of this variation may be due to trade-offs in investment in egg

production. Previous work has suggested that there are important trade-offs between immunity and other physiological systems in insects (Sheldon and Verhulst 1996). Given that egg production is costly in females (Partridge et al. 1987; Salmon et al. 2001), we tested whether or not age-related changes in immune function might be shaped by trade-offs between investment in immunity and investment in egg-production early on in life. Our data suggests, that though we find that very young flies succumb more rapidly to infection against gram-negative bacteria than older flies, this is not due to a trade-off in investment in egg production.

Materials and Methods

Strains

This experiment was conducted on three sets of flies, including both males and females from a wild-derived Georgia population (GAo) crossed with w^{1118} (GAoW), and females from a mutant strain that could not produce eggs (Ovo^{D1}, stock #1309 from the Bloomington *Drosophila* Stock Center) crossed with GAo. The Ovo^{D1} mutant inhibits oogenesis prior to vitellogenesis (Mevelninio et al. 1991; Granadino et al. 1992), and was used to determine the influence of egg production on immunity.

The Georgia outbred line (GAo) was collected from a horticultural farm in Watkinsville, GA in 2003 and maintained as a large outbred stock (Linder et al. 2008). Female virgin GAo flies were collected, and at the age of three days were crossed to male w^{1118} flies to obtain the experimental flies (GAoW). While GAo was maintained as a large outbreeding population, to further reduce the risk of inbreeding, we crossed the wild-type GAo flies to w^{1118}, as well as crossing Ovo^{D1} with GAo females to generate the sterile Ovo^{D1} F_1 females. Thus, flies in each group should be roughly equivalent in their degree of heterozygosity. The sterile Ovo^{D1} females

were used to determine whether any age-related changes observed in immune function might be due to age-related changes in reproductive investment. There is no formal control for the Ovo^{D1} flies, as our relevant comparisons are between isogenic flies across ages, rather than between co-isogenic flies within ages. For all experiments, separate cultures of flies were used to collect each age-group of virgin females (or males). For example, one-day old virgin flies came from a distinct set of vials set up one day later than the vials that would eventually be used to collect two-day old virgins flies. Infections on all five age classes were conducted on a single day. However, replicates (three per line) were carried out on separate days, as were infections on each line (GAoW females and Ovo^{D1} females were infected on separate days).

For bacterial inoculations, we used a strain of *Pseudomonas aeruginosa* (PA 01) obtained from Brian Lazzaro's lab at Cornell University.

Infection protocol

To measure immunity, we inoculated flies with *P. aeruginosa* at ages 1, 2, 3, 4, and 5-days. *P. aeruginosa* liquid cultures were grown up over night, standardized at 600 nm to an optical density of 0.02, and administered to the fly. We inserted a 0.1 mm stainless steel pin (which had been dipped in this solution) into the thorax of the fly (see Linder *et al.* 2008 for further details). A subset of flies in each age class was inoculated with a sterile sham broth to confirm that death was not due to the insertion of the steel pin. The number of flies that survived was recorded every hour from 23 to 33 hours post infection and then every 24 hours for 3 more days. Previous work in our lab has found that if the fly is going to die from infection it typically happens within 72 hours.

We replicated the experiment three separate times for each line of flies, with 50 flies of each age group infected in each replicate, for a total of 150 flies infected for each age group in each line of flies.

Statistics

All survival estimates were analyzed using JMP 7.0 (2007). All lines were analyzed separately. We first determined if survival rates differed between the age groups using a Kaplan Meier test. This analysis was carried out separately for each replicate. Any flies that had not succumbed to infection were censored. To obtain an overall *P*-value among replicates, we applied Fisher's Combined Probability test. In addition, an ANOVA was conducted on the proportion of surviving flies in each age class for each line in order to determine if the total number of flies that died differed between treatments.

Based on our initial analysis, we carried out a Spearman-rank test to determine if there was a general trend among replicates for older flies to live longer than younger flies after infection. We used a Spearman rank correlation to test for a correlation between mean life span after infection (determined from the Kaplan-Meier test) and age. This was done on each line, and for each replicate, obtaining nine independent Spearman ρ-values. Next, a permutation test (Manly 1997) was done using the Spearman's ρ−values in order to determine the probability of obtaining our results by chance.

Results

The total proportion of surviving flies at 72 hours did not significantly differ between age groups for GAoW females, OvoD1 females, or GAoW males (ANOVA, $P > 0.05$ for all lines).

Less than 1% of sterile broth inoculated sham flies died in all the control experiments, so we can conclude that the vast majority of deaths that we observed were from bacterial infection.

Though all lines were analyzed separately, by examining trends in all the data we obtain a clearer idea of what is occurring in this system. In eight of nine cases (three lines with three replicates each), younger flies succumbed to infection at a higher rate than older flies, based on the Spearman rank test (Spearman ρ−values given in Table 4.1). A positive Spearman ρ-value suggests a positive correlation between age and survival after infection. Figure 4.1 represents trend lines in the data for each replicate in each line, demonstrating in eight out of nine cases there is a positive rank order to the data (one-day old flies die off the most quickly, then two-day old flies, then three-day, and so on; Figure 4.1).

GAoW females – The rate at which the flies succumbed to the bacterial infection differed significantly between age groups for GAoW females (Kaplan-Meier test, $P < 0.0006$). In general, younger flies died at a faster rate than older flies based on a Spearman rank test, (combined probability among replicates of $P = 0.0003$, Table 4.1, Figure 4.2a). Though survival data was collected for 72 hours post infection, graphs only show data till 33 hours for brevity.

Ovo^{D1} females – To eliminate the possibility that younger flies died faster than older flies due to investment in egg production, we carried out a similar experiment using sterile Ovo^{D1} flies. We found that age had a significant effect on ability to survive an infection in females that did not produce eggs (Kaplan-Meier test, $P = 0.0072$; Figure 4.2b). In particular, mean life span after infection increased with age (Spearman ρ test across replicates [Table 4.1], permutation test, $P = 0.0324$).

GAoW males – We also examined immune function in males at early age. As with females, survival rates differed between age groups (Kaplan-Meier test, $P = 0.0045$; Figure 4.2c). However, in this case the Spearman's ρ values between age and average life span were positive only in two out of the three replicates (Table 4.1), and a permutation test of significance among the three replicates was not significant ($P = 0.3219$).

Discussion

Here we set out to determine if there were differences in immune ability within the first five days of adult life in *D. melanogaster*. In all but one experiment, we found that younger flies were more likely to die after infection than older flies (Figure 4.1). There are several reasons why we may have observed this result. First, immediately following eclosion, *Drosophila* are still developing (Promislow and Bugbee 2000), and might not reach full reproductive maturity for 24-48 hours in *D. melanogaster*, and much longer in some other species within the genus (Pitnick et al. 1995). Thus, the patterns we observed may be due to gradual upregulation of immune function during this early-adult developmental period.

Second, there may be a trade-off between early immune function and upregulation of reproductive machinery. Such a trade-off would be further exacerbated by the fact that during the first 8 h after eclosion, adults do not feed but rely on fat body stores obtained from larval feeding for energy (Aguila et al. 2007), leading to limited resources. Previous work has established that there are tradeoffs between immunity and other physiological systems (Sheldon and Verhulst 1996), and that investment in reproduction can come at a cost to female survival (Tatar and Carey 1995). It is also been shown mounting an immune response can decrease egg production (Zerofsky et al. 2005), and there is a cost of immune system maintenance which is manifested in

a reduction in fecundity in flies (McKean et al. 2008). With this in mind, we hypothesized that the observed decrease in immune ability was due to the fact that females were investing in egg production. To test this, we carried out the same experiment in mutant females that did not produce eggs (Ovo^{D1}). We observed similar results, with younger individuals dying off more quickly after infection (Figure 4.2b). The Ovo^{D1} mutation arrests oogenesis before vitellogenesis (Mevelninio et al. 1991; Granadino et al. 1992). Though investment in egg production is limited, some investment in reproduction may still be occurring. Hormonal changes as well as other physiological changes occurring in the fly before egg production may still be taking place in the Ovo^{D1} mutants. However, by examining immunity in this line we were able to obtain an estimate of survival after infection when investment in egg production was reduced. We observed similar patterns in males, where there is obviously no cost of egg development, but costs of developing male reproductive physiological processes could influence immune function. Though we saw a positive correlation in both sterile and fertile females, the effect is weaker in the sterile females. It could be possible that some of the variation seen in the GAoW fertile females is explained by investment in egg production, due to the weaker correlation in the sterile line. Even though our data suggest that the majority of the differences in survival come from differences in age of the fly, further work on other egg-less mutants like *tudor* (Barnes et al. 2006), may help resolves these issues. It should also be noted we are comparing two genetically distinct lines of flies, and by conducting the experiment on co-isogenic lines, better resolution may be obtained.

The results presented here suggest that early-age changes in immune function may have important consequences for fitness. Future studies should examine the actual fitness consequences of these changes. Furthermore, if early-age increases in immunity reflect

underlying developmental processes, simple molecular studies may enable us to better understand the nature of these changes. We know that AMPs are activated very rapidly (within 90 minutes) after infection in *Drosophila*. However, most of this work has been done on adult flies that are three and four days old (De Gregorio et al. 2001). AMP production might be slower in younger flies. Research has demonstrated that cells that make up the fat body are different in larvae versus adult flies, and that the fat body is critical to immune function (Ferrandon et al. 2007). Also, Aguila et al. (2007) found that fat cells in the young adult are derived from larval fat cells. Perhaps, the fat body might not be fully developed or functional directly after eclosion. In addition, other components of the immune system, like phagocytes, may not be fully functional soon after the flies emerge. In order to resolve if development plays a roll in immunity, a simple selection experiment could be carried out. For example, Promislow and Bugbee (2000) conducted an experiment where they selected for differences in the period of time from eclosion to first egg laying in *D. melanogaster*. They found ample genetic variation for the trait and that females who had delayed egg production had faster larval development and longer adult lifespans compared to females who began egg laying sooner (Promislow and Bugbee 2000). It would be interesting to conduct a similar study on selection for increased immune ability within the first few days of eclosion and determine if increased immunity was involved with trade-offs in other fitness traits, like longevity.

While fruit flies can live for several months in the laboratory, they may not have as extended life spans in nature. Though immunity and lifespan has previously been examined in relationship to senescence and late age, little work has been conducted on immunity early in life. Interactions between flies and their pathogens within the first few days of life could have an enormous influence on lifetime fitness. Studies of variation in immune function during early

adulthood should inform our understanding of biologically relevant variation in natural populations.

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Table 4.1: Spearman's ρ values for age rank survival after infection.

Replicate	GAoW Female	Ovo^{D1} Female	GAoW Male
1	1	0.5	0.5
2	1	0.3	-0.5
3	0.7	0.9	0.9
Permutation test P-value	0.0003	0.0324	0.3219

Figure Legends

Figure 4.1. Mean life span by age (for each replicate and line) showed a positive trend in all cases except one. Short dashed lines = GAoW females, solid lines = Ovo^{D1} females, and long dashed = GAoW males.

Figure 4.2. Survival curves of 1, 2, 3, 4, and 5-day old flies infected with *P. aeruginosa*. We found a significant difference in (a) GAoW females ($P < 0.0006$), (b) sterile Ovo^{D1} females ($P = 0.0072$), and (c) GAoW males ($P = 0.0045$) in survival between the age groups.

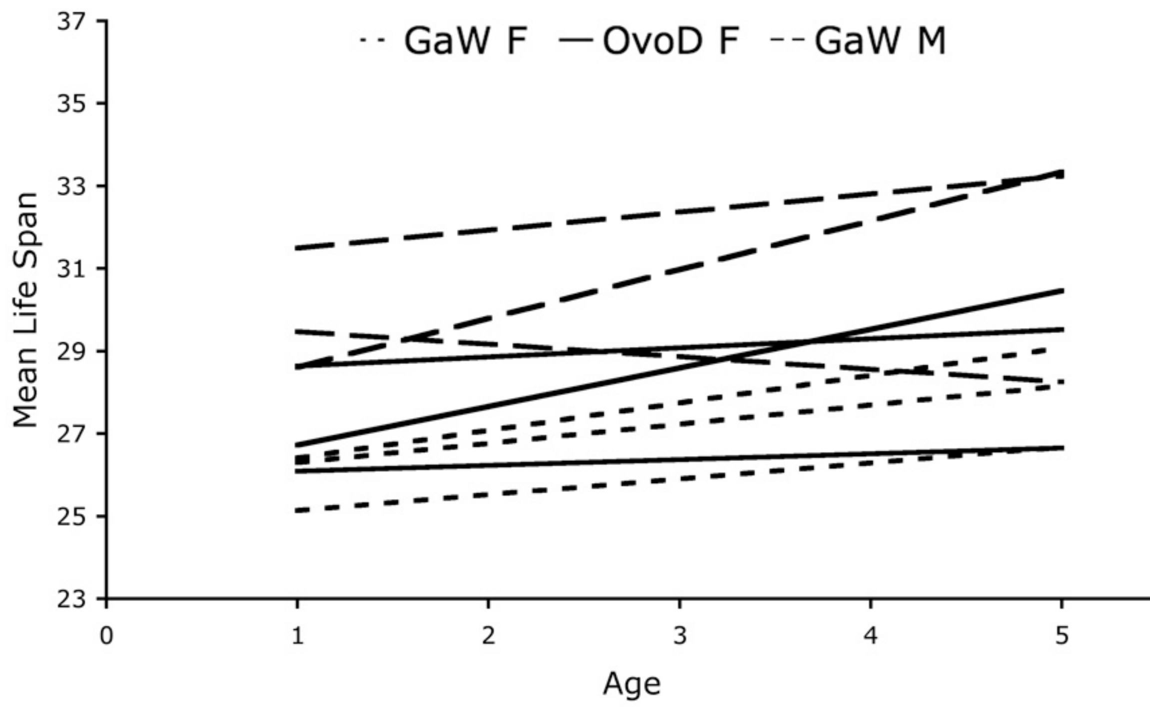

Figure 4.1

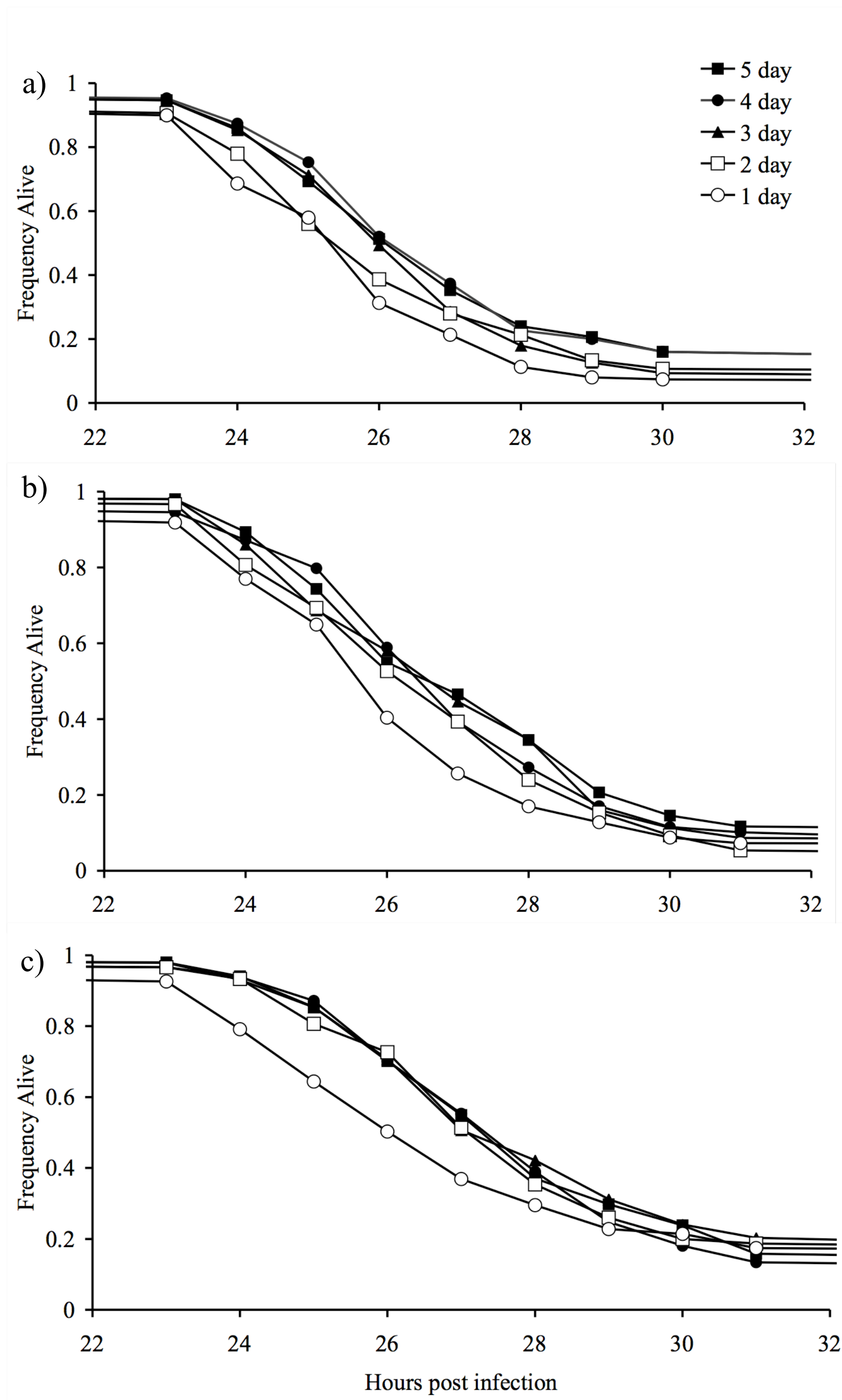

Figure 4.2

CHAPTER 5

CONCLUSIONS

The factors that contribute to the outcome of host-parasite interactions are quite complex. What determines if the host will recover or succumb to a parasitic infection has intrigued researchers for many years. More than just the genetic and molecular make up of the host's immune system, successful defense against a parasite also depends on many external factors.

In this thesis I have examined how extrinsic factors can alter the immune system of the fruit fly, *Drosophila melanogaster*. The fruit fly has been used for many years to develop models of how the innate immune system functions. I have added to this by examining how extrinsic factors influence immunity in the fly. I have demonstrated that temperature, resource availability, maternal condition, and age post eclosion can all contribute to variation in immunocompetence. In addition, I have begun to tease apart the molecular mechanisms that alter these host-parasite interactions in a few of these circumstances. By using an organism in which we have a great number of genetic tools and genetic mutants available, I have begun to uncover the mechanisms behind the differences in immunity that we see in nature.

Molecular genetics and biology are becoming more and more advanced every year. By using molecular tools to study genomes, gene expression, and proteins we can begin to uncover the mechanisms behind ecological phenomena. If we can determine how extrinsic factors alter the immune system, we will gain insight into the evolution of the complex immune system, and the co-evolution between hosts and its parasites.

As the great evolutionary biologist Theodosius Dobzhansky wrote in 1973, "Nothing in biology makes sense except in the light of evolution". By examining immunity not just in terms

of the pathways that make up the branches of the immune system, but also the extrinsic factors that shape the evolution of the immune system, we will be able to understand the process of host-parasite defense more completely. We are now entering a wonderful era of biological research. Advances in molecular biology allow us to decode genes and genomes and to examine the inner workings of a cell. By using that knowledge to study ecological interactions between species we can gain insight into evolution. Determining how organisms interact with their environment is one of the fundamental questions of biology. I hope that the research presented here has demonstrated that some of the variation seen in immunity can be attributed to extrinsic factors. In addition, I believe this research has raised new questions about how the immune system and environment interact. This is a burgeoning field in host-parasite interactions and it is hoped that more research will be conducted addressing these questions in the near future.