IMMUNOLOGICAL CONSEQUENCES OF MATING AND REPRODUCTIVE STATUS IN FEMALE *DROSOPHILA MELANOGASTER*

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Robin Allison Schwenke

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Robin Allison Schwenke, Ph. D

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Reproduction and immunity are critical for organismal fitness. However, organisms are physiologically constrained and are often unable to maximize both traits, resulting in a trade-off. In *Drosophila melanogaster*, females are more susceptible to an infection after mating, despite an apparent induction of antimicrobial peptides. Here, I examine the mechanisms promoting increased susceptibility in mated females and explore why mated females induce a less effective immune response.

First, I explored the molecular mechanism underlying mating-induced immunosuppression. I found that Sex Peptide, a male seminal fluid protein, induces the synthesis of juvenile hormone (JH), resulting in lower resistance to *Providencia rettgeri*. Although there are two nuclear receptors for JH, *germ cell-expressed* (*gce*) and *Methoprene-tolerant* (*Met*), I discovered that *gce* alone mediates the immunosuppressive effect of mating via JH. These results are consistent with a model of hormonal pleiotropy whereby JH promotes reproduction at the expense of host defense.

In a second study, I investigated the hypothesis that reproduction and immunity are resource-intensive processes and that the trade-off between the two traits is a consequence of resource allocation. I found that nutrient-rich diets increased

defense against *P. rettgeri* and eliminated post-mating immunosuppression, but the effect was genotype-dependent. I determined that supplementation with essential amino acids is responsible for increased host defense. Collectively, the findings are consistent with a model of resource allocation whereby immunity and reproduction compete for essential amino acids leading to both a physiological and evolutionary trade-off.

Lastly, I explored the fitness effects of a sexually transmitted infection and investigated whether antimicrobial peptides within the reproductive tract reduce susceptibility. I found that males can harbor bacteria on their genital plate which can be transferred to females during copulation. I detected severe fitness consequences associated with mating to bacteria-positive males. I revealed that immune defense is essential for survival after mating to a bacteria-positive male. While I detected significant immune induction after mating with bacteria-positive males, I did not find a stronger response within reproductive tissues. My findings are inconsistent with a model by which mating induces a localized immune response to increase host resistance to a sexually transmitted infection.

BIOGRAPHICAL SKETCH

Robin A. Schwenke was born on September 15, 1989 in Albany, New York. Her childhood fostered a curiosity for the natural world and a long-term passion for learning. After graduating from Colonie Central High School, Robin began classes at the University at Albany, SUNY, where she majored in Biology and minored in Chemistry.

As an undergrad at UAlbany, Robin participated in several volunteer organizations, honor societies, and research projects. She explored a variety of career pathways one of which resulted in her living on a horse farm for two months during a particularly brutal upstate New York winter. Robin finished her degree with a 3.98/4 and a yearning to remain in science and to pursue questions at the interface of evolution and genetics.

Despite living her entire life in New York, and briefly considering a move to the West Coast, Robin decided to stay in the beautiful state of New York where she pursued a doctoral degree at Cornell University in the graduate field of Genetics, Genomics, and Development. She completed her dissertation under the guidance of Dr. Brian Lazzaro. After the completion of her Ph.D., Robin relocated to Cape Town, South Africa to embark on many new adventures with her fiancé Bryan Maritz. Robin is hoping to begin a postdoc or an appointment at a nonprofit science organization in the coming months.

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Completing a Ph.D. is extraordinarily similar to training for a marathon. Both require a great deal of dedication, perseverance, mental stamina, confidence, and morale support. Both also come with inevitable rough patches, and having people who hold you accountable and cheer you on becomes indispensable for success. I am forever grateful to all of the people that have supported me through the finish line.

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More personally, I want to thank my parents for their unwavering support and for providing me with the privilege of a higher education. I am grateful for the curiosity that you both instilled within me and for the countless doors that you have opened.

Without the friendships that blossomed during my graduate work, the grind of a Ph.D. program would have worn me down beyond repair. Foremost, I must acknowledge the Tuesday night Chapter House crew. Frankly, the all too silly discussions and events that frequently occurred made Tuesday night the highlight of my week. Several people made my time at Cornell especially easier and far more entertaining including Ana Longo-Berrios, Nancy Chen, David Sannino, and Adam Dobson. I consider myself lucky to have your unwavering support, scientific input, and friendship. In particular, I would like to acknowledge Ginny Howick for being the best neighbor, labmate, and friend that I could have asked for. I am exceptionally appreciative for your encouragement and for your eagerness to take an adventure.

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ABSTRACT

Immune defense and reproduction are physiologically and energetically demanding processes and have been observed to trade off in a diversity of female insects. Increased reproductive effort results in reduced immunity, and reciprocally, infection and activation of the immune system reduce reproductive output. This trade-off can manifest at the physiological level (within an individual) and at the evolutionary level (genetic distinction among individuals in a population). The resource allocation model posits that the trade-off arises because of competition for one or more limiting resources, and we hypothesize that pleiotropic signaling mechanisms regulate allocation of that resource between reproductive and immune processes. We examine the role of juvenile hormone, 20-hydroxyecdysone, and insulin/insulin-like growth factor-like signaling in regulating both oogenesis and immune system activity, and propose a signaling network that may mechanistically regulate the trade-off. Finally, we discuss implications of the trade-off in an ecological and evolutionary context.

INTRODUCTION TO LIFE-HISTORY TRADE-OFFS

At its core, life-history evolution is a matter of optimization rather than maximization. Many traits that influence fitness are genetically and physiologically interrelated. Thus, increases in the fitness value of one trait may result in a corresponding decrease in the fitness value of another (Stearns, 1992, 2000).

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Reproduction and immune defense can be mutually constraining, with increased reproductive activity limiting immune performance and activation of the immune system resulting in decreased reproductive output. Both reproduction and immune responses are energetically costly, and the trade-off between them is likely due to alternative allocation of limiting energetic resources.

Trade-offs can occur at two discrete scales: physiological and evolutionary. At the level of an individual organism, trade-offs may arise as a consequence of direct physiological conflict between two traits or processes. For example, if both processes require the same limiting resource, allocation of the resource to one process inherently reduces the amount of that resource available to the other. We refer to these as physiological trade-offs. In an example that we discuss in more detail below, immunity and reproduction may trade off physiologically if, for example, both processes rely on dietary protein and protein nutrition is limiting. Physiological trade-offs are often plastic, meaning that they are responsive to environmental conditions, and an individual may shift allocations from one process to another as needed. Following the example above, a reproductively inactive insect may be able to devote fully sufficient protein resources to the immune response, but once the same individual becomes reproductively active, she may preferentially allocate that protein to egg provisioning and immune performance can become compromised.

At the population level, evolutionary trade-offs can occur if there is genetic variation among individuals for allocation between traits. In order for an evolutionary trade-off to exist in the example discussed above, there must be some individuals who are genetically predisposed to allocate protein preferentially to reproduction and others

who are genetically programmed for preferential allocation to immunity. If we were to examine the correlation between reproductive output and immune performance across individuals in the population, we might expect to find that individuals with better-than-average immunity tend to show reduced fecundity, and vice versa. We refer to these trade-offs as evolutionary because natural selection can effectively act on the underlying genetic variation. Genetic variation for evolutionary trade-offs is likely to be maintained as a consequence of fluctuating selection in spatially or temporally heterogeneous environments (Lazzaro and Little, 2009). Specifically, when pathogen prevalence is low, natural selection may favor increased allocation toward reproductive output. When infection pressure is high, however, selection may favor heightened immunity. It is important to appreciate that plastic physiological trade-offs can exist within individuals without a corresponding evolutionary trade-off at the population level (Flatt and Kawecki, 2007). Whether physiological and evolutionary trade-offs share their mechanistic bases remains an open question in life-history biology.

Reproduction and immune defense are intricately linked with other life-history traits (reviewed in Rose and Bradley, 1998; Schmid-Hempel, 2005), highlighting the complexity of life-history evolution. In this article, we review the literature on reproduction–immunity trade-offs in female insects. Although we focus on female insects, reflecting the preponderance of data, accumulating evidence suggests that male reproduction also has immunological costs (e.g., McKean and Nunney, 2001; Fedorka et al., 2004, but see Gupta et al., 2013) and that explicit differences in life-history strategies between the sexes can result in a sexually dimorphic immune system

(Rolff, 2002; Duneau and Ebert, 2012). For instance, premating sexual signals (e.g., horn length as in beetles, or pigmentation) can directly influence immune function and the evolution of host defense via sexual selection (reviewed in Lawniczak et al., 2007). Thus, many factors contribute to observed differences in life-history strategies. Here, we focus our discussion on the interactions between post-mating processes and immunity in female insects with special attention given to mechanisms governing the trade-offs.

The search for mechanisms underlying life-history trade-offs is challenging. It is comparatively easy to observe that two fitness-related traits are negatively correlated at the level of the whole organism. For example, we can readily observe that reproductively active insects have reduced resistance to infection (**Table 1.1**), and we may hypothesize that a resource reallocation is the basis for the observed trade-off. But it is much more difficult to determine the identity of the limiting resource or the cellular mechanism that specifies and regulates differential allocation. Yet the identification of these mechanisms is critical for understanding how traits trade off and how trade-offs evolve. In this review, we show how condition-dependence of physiological and evolutionary trade-offs can reveal the identity of limiting resources (e.g., McKean et al., 2008), and we discuss how pleiotropic hormones and signaling pathways may regulate resource allocation (e.g., Finch and Rose, 1995; Flatt and Kawecki, 2007). We incorporate findings from a variety of insect taxa that exhibit a diversity of reproductive strategies and experience distinct selective pressures into our discussion of organism-level traits, but the underlying molecular mechanisms have been ascertained primarily in genetically manipulable organisms such as *Drosophila*

melanogaster. Not all specific pathways and mechanisms established in *D. melanogaster* may operate the same way in all taxa. With recent advances in molecular techniques, such as CRISPR/Cas9 genome editing (reviewed in Sander and Joung, 2014), however, we are optimistic that mechanistic questions can soon be efficiently addressed in non-model organisms.

REPRODUCTIVE ACTIVITY INHIBITS IMMUNITY

There is widespread empirical support across multiple orders of insects for mutual constraint between immunity and reproduction (**Tables 1.1** and **1.2**), with a much smaller number of observed increases in female immunity as consequence of mating and reproduction (**Table 1.1**). Experiments to assess these trade-offs often involve genetic or physiological manipulation of reproductive capacity paired with assays of immunological capacity or, alternatively, manipulation of immune status followed by measurement of reproductive output. Immune traits that are commonly measured include survivorship of pathogenic infection, pathogen load sustained at various time points after infection, count or activity of circulating defensive blood cells (hemocytes), expression levels of genes encoding antimicrobial peptides, and phenoloxidase activity, because phenoloxidase is involved in defensive melanization and production of oxidative free radicals (Strand, 2002; González-Santoyo and Córdoba-Aguilar, 2012). The background levels of these traits can be measured in the absence of infection (constitutive immunity). Alternatively, the traits can be quantified after presenting a noninfectious immune elicitor or nonpathogenic microbe (induced immunity), or after infection with a bona fide pathogen (fighting infection). These

distinct but complementary approaches can give different results, providing further depth to our understanding of trade-offs. The cost of induced immunity may be higher than the cost of constitutive immunity because of the additional deployment of immune effector molecules, so trade-offs may be more readily observed under infection conditions. However, it may be impossible to distinguish costs of the immune response from consequences of pathogen virulence after a bona fide pathogenic infection. As in any experimental context, the data collected must be interpreted in terms of the design and assumptions of the experiment that was performed.

As a general rule, increased reproductive activity reduces constitutive and induced immunity across a diversity of female insects. Fedorka et al. (2004) showed that female ground crickets (*Allonemobius socius*) sustained progressively fewer circulating hemocytes with increasing copulation frequency. Mealworm beetles (*Tenebrio molitor*) and wood ants (*Formica paralugubris*) show a reduction in phenoloxidase activity after mating (Rolff and Siva-Jothy, 2002; Castella et al., 2009), although Fedorka et al. (2004) saw the opposite pattern with *A. socius*. Nevertheless, hemolymph samples from mated *A. socius* females were less bacteriolytic than hemolymph samples from virgin females (Fedorka et al., 2004), demonstrating reduced constitutive immunological effectiveness. Mating also reduces cellular encapsulation and melanization of implanted nylon filaments in a variety of insects (Siva-Jothy et al., 1998; Fedorka et al., 2004; Baer et al., 2006; Bascuñán-García et al., 2010), indicating reduced capacity to defend against macroparasites (Castillo et al., 2011). Mating reduces the probability that *Drosophila melanogaster* females will

survive a diverse array of pathogenic, bacterial infections, and mated female flies show higher pathogen loads and reduced inducibility of genes encoding antibacterial peptides after pathogenic infection (Fedorka et al., 2007; Short and Lazzaro, 2010; Short et al., 2012). Interestingly, however, mating does not decrease the ability of female *D. melanogaster* to clear nonpathogenic *Escherichia coli* infections (McKean and Nunney, 2005; Barnes et al., 2008). Therefore, the trade-off between mating and immunity is evident only when the infection is pathogenic.

The observation that mating reduces female *D. melanogaster* resistance to infection appears to be at odds with the recurrent observation that the expression of genes encoding antimicrobial peptides are modestly induced as a consequence of mating and the transfer of male seminal fluid proteins (Lawniczak and Begun, 2004; McGraw et al., 2004; Innocenti and Morrow, 2009). Upon closer inspection, however, this upregulation of antimicrobial peptide genes may be largely restricted to the reproductive tract (Mack et al., 2006; Domanitskaya et al., 2007). This tissue-specific induction may potentially be a local, prophylactic protection against sexually transmitted infection (Knell and Webberley, 2004; Miest and Bloch-Qazi, 2008) and may have little consequence in fighting a systemic infection.

INFECTION AND IMMUNE ACTIVATION REDUCE REPRODUCTIVE CAPACITY

Data from a diverse array of insects indicate that activation of immune responses decreases reproductive output and capacity (**Table 1.2**). In *D. melanogaster*, bacterial or fungal infections reduce fecundity (Zerofsky et al., 2005; McKean et al., 2008; Bashir-Tanoli and Tinsley, 2014; Howick and Lazzaro, 2014; Nystrand and

Table 1.1 Immunological consequences of mating and reproduction

Immune elicitor	Order	Species	Phenotype	Effect on immunity	Reference(s)
Unchallenged	Diptera	*Drosophila melanogaster*	Mating induces AMP expression in reproductive tissues	+	Lawniczak and Begun, 2004; McGraw et al., 2004; Peng, et al., 2005b; Domanitskaya et al., 2007
	Coleoptera	*Tenebrio molitor*	Mating reduces PO activity	−	Rolff and Siva-Jothy, 2002
	Orthoptera	*Allonemobius socius*	Mating reduces hemocyte number and bacterial lytic ability	−	Fedorka et al., 2004
			Mating increases PO activity	+	Fedorka et al., 2004
	Hymenoptera	*Formica paralugubris*	Mating reduces PO activity	−	Castella et al., 2009
Nonpathogenic	Diptera	*Drosophila melanogaster*	No difference in bacterial load between mated and virgin females (*Escherichia coli*)	=	Barnes et al., 2008; McKean et al., 2008
	Orthoptera	*Allonemobius socius*	Mating reduces encapsulation response (nylon filament)	−	Fedorka et al., 2004
		Acheta domesticus	Mating reduces encapsulation response (nylon filament)	−	Bascuñán-García et al., 2010
	Odonata	*Matrona basilaris*	Mating reduces encapsulation response (nylon filament)	−	Siva-Jothy et al., 1998
	Hymenoptera	*Atta colombica*	Mating and sperm storage reduce encapsulation response (nylon filament)	−	Baer et al., 2006

Table 1.1 continued

Immune elicitor	Order	Species	Phenotype	Effect on immunity	Reference(s)
Pathogenic	Diptera	*Drosophila melanogaster*	Mating reduces survivorship after infection and phenotype persists in absence of egg maturation (*Pseudomonas aeruginosa*)	−	Fellowes et al., 1999
			Mating reduces resistance to infection; effect is dependent on the presence of a germline and the receipt of Sex Peptide (*Providencia rettgeri, P. alcalifaciens*)	−	Short and Lazzaro, 2010; Short et al., 2012
			Mating has no effect on survivorship and bacterial load (*Enterococcus faecalis,Pseudomonas aeruginosa*)	=	Short and Lazzaro, 2010
	Coleoptera	*Tenebrio molitor*	Mating enhances resistance (*Beauveria bassiana*)	+	Valtonen et al., 2010
	Orthoptera	*Gryllus texensis*	Mating enhances resistance (*Serratia marcescens*)	+	Shoemaker et al., 2006
	Hemiptera	*Acyrthosiphon pisum*	Positive relationship between fecundity and susceptibility to parasitoid attack	−	Gwynn et al., 2005

Abbreviations: AMP, antimicrobial peptide; PO, phenoloxidase.

Table 1.2 Reproductive consequences of immunity and infection

Immune elicitor	Order	Species	Phenotype	Effect on reproduction	Reference(s)
Nonpathogenic	Diptera	*Drosophila melanogaster*	Reduced fecundity with increasing concentrations of immune elicitor (LPS)	−	Nystrand and Dowling, 2014
			Reduced fecundity (HK bacteria species)	−	Zerofsky et al., 2005; McKean and Lazzaro, 2010; Bashir-Tanoli and Tinsley, 2014
		Anopheles gambiae	Reduced ovarian protein and number of eggs laid (LPS)	−	Ahmed et al., 2002
			Increased number of apoptotic follicles (LPS or Sephadex beads)	−	Ahmed and Hurd, 2006
	Coleoptera	*Euoniticellus intermedius*	Fewer brood balls (LPS)	−	Reaney and Knell, 2010
	Orthoptera	*Acheta domesticus*	Fewer eggs later in life (nylon filament)	−	Bascuñán-García et al., 2010
		Gryllus texensis	Reduced oviposition rate (wounding; HK *Serratia marcescens*)	−	Stahlschmidt et al., 2013
			Reduced protein in egg (HK *Serratia marcescens*)	−	Stahlschmidt et al., 2013
		Hemideina crassidens	Repeated challenges reduced number of eggs laid and protein content per egg (LPS)	−	Kelly, 2011

Table 1.2 continued

Immune elicitor	Order	Species	Phenotype	Effect on reproduction	Reference(s)
Pathogenic	Diptera	*Drosophila melanogaster*	Reduced fecundity during acute phase of infection (*Providencia rettgeri*)	−	Lazzaro et al., 2008; McKean et al., 2008; Howick and Lazzaro, 2014
			Reduced fecundity during fungal infection (*Beauveria bassiana*)	−	Bashir-Tanoli and Tinsley, 2014
			Lower fecundity after surviving parasitoid wasp infection (*Asobara tabida*)	−	Fellowes et al., 1999
		Drosophila nigrospiracula	Negative relationship between ectoparasite load and egg number (*Macrocheles subbadius*)	−	Polak, 1996
		Anopheles gambiae	Increased number of apoptotic follicles (*Plasmodium yoelii nigeriensis*)	−	Ahmed and Hurd, 2006)
		Anopheles stephensi	Reduced fecundity during infection(*Plasmodium yoelii nigeriensis*)	−	Hogg and Hurd, 1995
		Armigeres subalbatus	Reduced ovarian protein and increased time to oviposition(*Brugia malayi*)	−	Ferdig et al., 1993
	Orthoptera	*Teleogryllus oceanicus*	Reduced viability of stored sperm (*Serratia marcescens*)	−	McNamara et al., 2014

Abbreviations: HK, heat-killed; LPS, lipopolysaccharide.

Dowling, 2014). Similar effects are seen in Orthoptera, where induction of the immune system with heat-killed bacteria or bacterial cell wall components reduces egg production in the house cricket (*Acheta domesticus*) (Bascuñán-García et al., 2010), the Wellington tree weta (*Hemideina crassidens*) (Kelly, 2011), and the Texas field cricket (*Gryllus texensis*) (Stahlschmidt et al., 2013). In *Anopheles* mosquitoes, challenge with bacterial cell wall components or infection by *Plasmodium* spp. significantly reduces the accumulation of protein in the ovaries, promotes the apoptosis of follicle cells, and reduces the number of eggs laid (Hogg and Hurd, 1995; Ahmed et al., 2002; Ahmed and Hurd, 2006). Immune signaling and/or the presence of pathogens may signal to degrade newly forming follicles, limiting egg production in Diptera (Drummond-Barbosa and Spradling, 2001; Terashima and Bownes, 2004; Clifton and Noriega, 2011). This could allow resources to be shunted back to immunity and recovery from infection (Hurd, 2001).

Whereas the above examples describe fecundity costs of immune deployment, constitutive maintenance of elevated immune potential can also reduce reproductive capacity (**Table 1.3**). *D. melanogaster* genotypes with high resistance to bacterial infection show low fecundity even when uninfected. However, this phenomenon is seen only when dietary yeast is limited, suggesting that nutrition is responsible for the constraint (McKean et al., 2008; Howick and Lazzaro, 2014). *D. melanogaster* that were artificially selected for increased resistance to a bacterial infection also evolved correlated reduction in egg viability (Ye et al., 2009), as did strains of Indian meal moth (*Plodia interpunctella*) that had been selected for resistance to a granulosis virus (Boots and Begon, 1993). In contrast, pigmentation in *T. molitor* females, a trait that

can be experimentally selected for and represents higher constitutive phenoloxidase activity (Armitage and Siva-Jothy, 2005), did not correlate with fecundity, suggesting that the constitutive expression of some immune modulators need not always have reproductive consequences (Armitage et al., 2003).

As shown by the examples above and in **Tables 1.2** and **1.3**, most published studies imply that immune induction and/or infection-responsive processes physiologically trade off with reproductive processes. Additional studies showing an evolutionary trade-off indicate constitutive costs of maintaining greater immunity even in the absence of infection (but see (Armitage et al., 2003)). This is largely a consequence of available experimental methodologies. Deployment costs, or the costs of mounting an immune response (McKean and Lazzaro, 2010; Kraaijeveld et al., 2011), can be experimentally measured in terms of alterations to physiological allocation before and after infectious challenge (e.g., Bajgar et al., 2015). Immunological maintenance costs (McKean and Lazzaro, 2010; Kraaijeveld et al., 2011), however, are experimentally revealed by comparing reproductive potential in the absence of infection among genetic strains with high resistance to infection with those with low resistance (e.g., Yan et al., 1997; Ye et al., 2009). Because of the experimental methodology employed, maintenance trade-offs are almost always revealed as evolutionary, although they must certainly have physiological basis. Correspondingly, the magnitude of physiological trade-offs and deployment costs can vary genetically within populations (e.g., Short and Lazzaro, 2010) and therefore can be evolutionarily subject to natural selection.

EGG PRODUCTION AND IMMUNITY DEMAND ALLOCATION OF RESOURCES

Both egg production and immunity are energetically costly processes, so a physiological trade-off between them could be mediated by the allocation of a mutually limiting resource. Although egg production is not the only energetic investment associated with reproduction [other post-mating changes include heightened activity and foraging (reviewed in Avila et al., 2011), we hypothesize that it is likely to be the largest cost endured and therefore it is our focus here.

To evaluate the resource allocation hypothesis, it is necessary to have some insight into what the limiting resource(s) might be and to have a mechanistic sense of how that resource(s) is allocated. Fisher articulated this need in 1930, when he wrote,

> *"It would be instructive to know not only by what physiological mechanism a just apportionment is made between the nutriment devoted to the gonads and that devoted to the rest of the parental organism, but also what circumstances in the life-history and environment would render profitable the diversion of a greater or lesser share of the available resources towards reproduction.*"

Since then, the resource allocation model has become a central dogma in life-history theory (Williams, 1966; Gadgil and Bossert, 1970; Law, 1979; Roff, 1992; Stearns, 1992). However, there are precious few examples in which the identity and the management of the resource are well understood. In the next three sections, we

Table 1.3 Evolutionary trade-offs between reproduction and immunity

Experiment	Order	Species	Findings	Relationship between reproduction and immunity	Reference(s)
Selection	Diptera	*Drosophila melanogaster*	Selection for resistance to *Pseudomonas aeruginosa* resulted in decreased egg viability	−	Ye et al., 2009
		Drosophila nigrospiracula	Selection for behavioral ectoparasite resistance resulted in a correlated reduction in fecundity	−	Luong and Polak, 2007
		Scathophaga stercoraria	Populations evolving in polyandry developed larger reproductive tissues and reduced PO activity	−	Hosken, 2001; Hosken et al., 2001
	Lepidoptera	*Plodia interpunctella*	Selection for resistance to the *Plodia interpunctella* granulovirus reduced egg viability	−	Boots and Begon, 1993
Genetic and phenotypic correlations	Diptera	*Drosophila melanogaster*	Negative correlation between uninfected fecundity and resistance to *Providencia rettgeri*	−	McKean et al., 2008
		Aedes aegypti	Negative correlation between uninfected fecundity/hatchability and resistance to *Plasmodium gallinaceum*	−	Yan et al., 1997
	Coleoptera	*Tenebrio molitor*	No correlation between pigmentation (PO activity) and fecundity	=	Armitage et al., 2003

Abbreviation: PO, phenoloxidase.

compile evidence from the published literature to support the hypothesis that both egg production and immunity in insects are nutritionally limited, and that the physiological trade-off between them may arise through resource allocation mediated by endocrine and metabolic signaling and joint reliance on critical and common tissues such as the fat body.

Although there are differences in the details among taxa (see Buning, 1994 for a comprehensive review), insect egg production generally begins in the stem cell niche (germarium) of an ovariole, where a cystoblast arises from the asymmetric division of a germline stem cell. The developing cyst undergoes a species-specific number of mitotic divisions and grows in size as it transits posteriorly. Critically, copious quantities of proteins (e.g., yolk proteins and vitellogenins), lipids, RNAs, ribosomes, and organelles are deposited into the growing oocyte to provide nutrients and patterning information for the future zygote (Cavaliere et al., 2008).

Provisioning of developing oocytes is energetically demanding (Wigglesworth, 1960; Wheeler, 1996; Awmack and Leather, 2002). Thus, the efficiency of egg production depends on the quality of dietary nutrition and a female's metabolic status (Dixon, 1963; Terashima and Bownes, 2004; du Plessis et al., 2012). Oogenesis can be arrested and, at least in *D. melanogaster*, partially developed oocytes can be resorbed during starvation conditions (Drummond-Barbosa and Spradling, 2001; Terashima and Bownes, 2004; Clifton and Noriega, 2011). The onset of reproduction drives females of many insects to ingest more food, frequently preferring protein-rich food sources (Carvalho et al., 2006; Ribeiro and Dickson, 2010; Tsukamoto et al., 2014). Notably, sanguivorous dipterans require a protein-rich blood meal to complete oogenesis

(reviewed in Attardo et al., 2005).

Immune defense is also energetically and metabolically costly (Clark et al., 2007; Simmons, 2011; Bajgar et al., 2015). Bumble bee (*Bombus terrestris*) workers are less resistant to starvation after immune system activation (Moret and Schmid-Hempel, 2000), and studies in multiple insects have established that resistance to infection is enhanced upon ingesting a protein-rich diet ((McKean and Nunney, 2005; Lee et al., 2008b; McKean et al., 2008; Povey et al., 2014) but see (Klemola et al., 2007)). Upon immune stimulation, *T. molitor* and *Spodoptera exempta* larvae shift their feeding preference toward protein-rich food sources (Catalán et al., 2011; Povey et al., 2014) and, at least in *Spodoptera* spp., ingestion of a high-protein diet permits increased production of antimicrobial molecules in the hemolymph (Lee et al., 2008b; Povey et al., 2014). In *D. melanogaster*, dietary L-arginine helps improve resistance to parasitoid wasps via lamellocyte proliferation and nitric oxide production (Kraaijeveld et al., 2011). Although a high-quality diet can enhance the host immune response, the net effect of diet on infection is complicated because infecting pathogens may also be able to access nutrients ingested by the host (Cressler et al., 2014). Indeed, one hypothesis to explain illness-induced anorexia is that cessation of host feeding deprives infecting pathogens of nutrients (Adamo et al., 2007; Ayres and Schneider, 2009; Povey et al., 2014), albeit with possible negative collateral consequences for reproduction (Becker et al., 2010). Finally, because different branches of the immune response may have different micronutritional requirements, it may be impossible to maximize all components of the immune system simultaneously (Cotter et al., 2011), giving rise to trade-offs between immune system components (e.g., Cotter et al.,

2003).

The energetic demands imposed by reproduction and immunity suggest that competition for nutritional resources could be at the center of the trade-off between them. In searching for the control center for the reproduction–immunity trade-off, our attention was drawn to the fat body. This tissue is the central metabolic control organ (reviewed in Arrese and Soulages, 2010). The fat body is critically important for oogenesis because it is a major site for yolk protein and vitellogenin production for oocytes (Kokoza et al., 2001; Isaac and Bownes, 2005). It is also the primary organ of systemic immunity (reviewed in Hoffmann, 2003).

Short et al. (2012) observed that *D. melanogaster* females lacking germ cells (that cannot form eggs) do not suffer from reduced immunity after mating, in contrast to females that produce eggs. These findings support the hypothesis that reproduction and immunity compete for nutritional resources. However, the cost of reproduction still persists in *D. melanogaster* females that arrest egg production at stage 4 of oogenesis, even though these females never produce mature oocytes (Fedorka et al., 2007). In corroboration of the persistence of the trade-off in the sterile females, evidence from a locust (*Locusta migratoria*) system suggests yolk protein is still produced in the absence of egg production (Chinzei and Wyatt, 1985). Therefore, the *D. melanogaster* mutants that arrest oogenesis in stage 4 may still invest resources into yolk protein production rather than into immunity. This could explain why post-mating immunosuppression occurs in these females despite the absence of fully formed eggs.

Further support for nutritional mediation of the trade-off between reproduction

and immunity comes from studies that showed that providing *D. melanogaster* females with dietary yeast ad libitum improved fecundity and resistance to infection and without any evolutionary trade-off between these phenomena (McKean et al., 2008). Yet access to food ad libitum did not fully rescue fecundity in chronically immune-stimulated *G. texensis* (Stahlschmidt et al., 2013). This disparity highlights the importance of future studies to identify the resource shared between the two processes, the required intake of the nutrient, and how ratios of dietary components can influence the two traits.

SIGNALING PLEIOTROPY MAY UNDERLIE RESOURCE ALLOCATION

If egg provisioning and immune defense rely on a common resource pool, the host organism should have a signaling mechanism to shunt those resources toward one process or the other. We propose that the physiological trade-off between reproduction and immunity in insects is regulated by endocrine signals, specifically the balance between juvenile hormone (JH) and 20-hydroxyecdysone (20E) and by altered metabolic status mediated by insulin/insulin-like growth factor-like signaling (IIS). Although we are specifically concerned here with the trade-off between reproduction and immunity, as a general principle pleiotropic signaling pathways are likely to be common switches for regulating life-history trade-offs (see Flatt et al., 2005; Harshman and Zera, 2007; Zera et al., 2007).

Juvenile Hormone and 20-Hydroxyecdysone

JH and 20E have been characterized in insects primarily for their role in

regulating metamorphosis (Nijhout, 1994). However, the balance between JH and 20E is important for activation of egg maturation and provisioning in a variety of phylogenetically diverse insects. Additionally, JH can be a direct or indirect antagonist of immune function in insects, and 20E is a known potentiator of insect immunity. We propose a model in which JH/20E signaling mediates the trade-off between reproduction and immunity, at least in part, by regulating the diversion of energetic resources from somatic maintenance to reproductive output (**Figure 1.1**).

The balance between JH and 20E dictates the progression of oogenesis, with JH promoting egg production and provisioning (reviewed in Gruntenko and Rauschenbach, 2008; Toivonen and Partridge, 2009). JH levels are responsive to diet and to mating in female insects. Increased JH levels promote the expression of vitellogenin or yolk protein genes in the fat body of female insects such as red flour beetles (*Tribolium castaneum*) (Sheng et al., 2011), cockroaches (*Leucophaea maderae*, *Blattella germanica*) (Brookes, 1969; Süren-Castillo et al., 2012), and Oriental fruit flies (*Bactrocera dorsalis*) (Chen et al., 2012). JH also promotes the uptake of vitellogenin or yolk protein into oocytes by creating intercellular spaces between the follicle cells (Abu-Hakima and Davey, 1975; Fleig, 1995) and aids in the progression of developing follicles (Soller et al., 1999). In contrast, high 20E titers typically result in the resorption of immature vitellogenic eggs (Soller et al., 1999). However, not all organisms may conform to these patterns of JH/20E signaling. For example, although both JH and 20E are essential for oogenesis in mosquitoes, 20E is more important for regulating vitellogenesis (reviewed in Hansen et al., 2014). Thus, reproductive aspects such as the requirement for blood meals may influence the

generality of molecular mechanisms.

JH and 20E also have opposite effects on immunity in most insects (**Figure 1.1**). Ectopic application of methoprene, a synthetic JH analog, reduces the activation of antimicrobial peptide genes in response to infection in *D. melanogaster* (Flatt et al., 2008). JH reduces phenoloxidase activity in *T. molitor* (Rolff and Siva-Jothy, 2002) and inhibits the spreading behavior of hemocytes in *Tribolium castaneum* and *Spodoptera exigua* (Kim et al., 2008; Hepat and Kim, 2014). In contrast, 20E potentiates the expression of antimicrobial peptide genes in *D. melanogaster* (Dimarcq et al., 1997; Meister and Lagueux, 2003; Flatt et al., 2008; Zhang and Palli, 2009) and signals through the ecdysone receptor (EcR) to drive expression of the peptidoglycan-recognition protein LC (PGRP-LC), a primary activator of the IMD humoral immune signaling pathway (Rus et al., 2013). 20E also regulates embryonic immunity in *D. melanogaster* (Tan et al., 2014) and ecdysone signaling is required for cellular immunity in *D. melanogaster* (Sorrentino et al., 2002; Regan et al., 2013).

The opposite effects of JH and 20E on reproduction and immunity raise the possibility that JH and 20E levels may mediate the physiological trade-off between the two processes. In light of this, it is intriguing that both JH and 20E can be transferred to female mosquitoes in the male seminal fluid (Clifton et al., 2014; Gabrieli et al., 2014). Most insects activate synthesis of JH in the corpora allata after mating. Transplantation of corpora allata from mated *T. molitor* females into virgins resulted in lower levels of phenoloxidase activity, similar to the mating-induced suppression of phenoloxidase observed in mated *T. molitor* females (Rolff and Siva-Jothy, 2002). In *D. melanogaster*, JH synthesis is activated when the protein Sex Peptide (Acp70A) is

transferred to the female in the male's seminal fluid (Fan et al., 2000). Short et al. (2012) demonstrated that females mated to males lacking Sex Peptide were as resistant as virgin females to a bacterial infection, whereas females mated to wildtype males showed depressed immunity.

In summary, strong evidence across a breadth of insects exists to support a model in which mating increases JH titers and suppresses 20E, promoting egg development and inhibiting immune capability. This provides perhaps the clearest example to date of widespread endocrinological regulation of a life-history trade-off through a pleiotropic signal (**Figure 1.1**). Although a general model is presented here, future comparisons among insect taxa are encouraged to test the universality of this mechanism.

Insulin/Insulin-Like Growth Factor-Like Signaling

As described above, both egg provisioning and immune response are sensitive to the insect's nutritional status and metabolism. As in vertebrates, IIS is a major regulator of metabolic status in response to dietary nutrition (reviewed in Wu and Brown, 2006), and both reproduction and immunity are responsive to IIS. Elevated IIS promotes oogenesis and inhibits immune responses. Thus, IIS serves as a potential control switch for regulating the physiological trade-off between reproduction and immunity.

Insects use flux through the insulin signaling pathway to indicate whether the female has sufficient nutrient stores to provision eggs (Badisco et al., 2013). High IIS activity promotes oogenesis, and under reduced IIS activity (indicative of nutritional

deprivation) egg production is reduced or halted (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005; Richard et al., 2005; Hsu and Drummond-Barbosa, 2009; Parthasarathy and Palli, 2011). In contrast, IIS and immune signaling are reciprocally antagonistic. In *D. melanogaster*, genetic activation of the Toll immune response pathway results in reduced IIS, even in the absence of infection (DiAngelo et al., 2009). IIS is inhibited also by the immune-responsive JNK pathway (Wang et al., 2005). Low IIS activity may enhance immunity through increased nuclear localization of the transcription factor FOXO, which can bind to the promoters of antimicrobial peptide genes and positively regulate their expression (Becker et al., 2010). Moreover, IIS pathway mutants survive bacterial infection better than wild-type *D. melanogaster* do (Libert et al., 2008), possibly through rescue from energetic loss associated with fighting infection (Dionne et al., 2006). Infection of skimmer dragonflies (*Libellula pulchella*) by protozoan parasites results in metabolic disruption and pathology, which can be reversed by blocking IIS (Schilder and Marden, 2006).

The effect of IIS signaling on reproduction and immunity may not be independent of JH and 20E; it may in fact partially act through JH and 20E. *D. melanogaster* insulin receptor (*dInR*) mutants exhibit low JH titers (Tu et al., 2005), indicating that IIS promotes JH synthesis. Nuclear FOXO—an indication of low IIS activity—also increases JH titers and vitellogenin production in the German cockroach (*B. germanica*) (Süren-Castillo et al., 2012; Abrisqueta et al., 2014). Moreover, JH and 20E can regulate IIS activity. 20E promotes FOXO activity in *Bombyx mori* (Hossain et al., 2013), and reduction of JH titers via ablation of the corpus allatum in *D.*

melanogaster reduces IIS signaling and increases 20E levels (Mirth et al., 2014). Thus, JH and IIS may form a positive feedback loop that promotes oogenesis and inhibits immunity in response to the nutritional environment and reproductive cues (**Figure 1.1**).

Most of this review has focused on how a trade-off can be generated by competition between two physiological processes for a limiting resource, with some thought to the mechanics of how allocation of that resource might be managed. We emphasize, though, that both reproduction and immune performance are critical to evolutionary fitness, and the trade-off between them is therefore likely subject to strong natural selection. Environmental fluctuations in resource availability or pathogen pressure could shift the selective pressure between reproduction and immunity, and a few examples exist in which genetically distinct individuals in a population vary in their hardwired bias, favoring one process or the other. However, at this time, the research community has virtually no understanding of the mechanistic basis for evolutionary trade-offs and we emphasize that they may or may not be the same as those underlying physiological trade-offs. More research is needed in this vein to understand how evolution shapes investment in and trade-offs between reproduction and immunity.

CONCLUDING REMARKS

Immune defense and reproduction are each central to organismal fitness, yet they trade off at both the physiological and evolutionary levels. Here we propose a

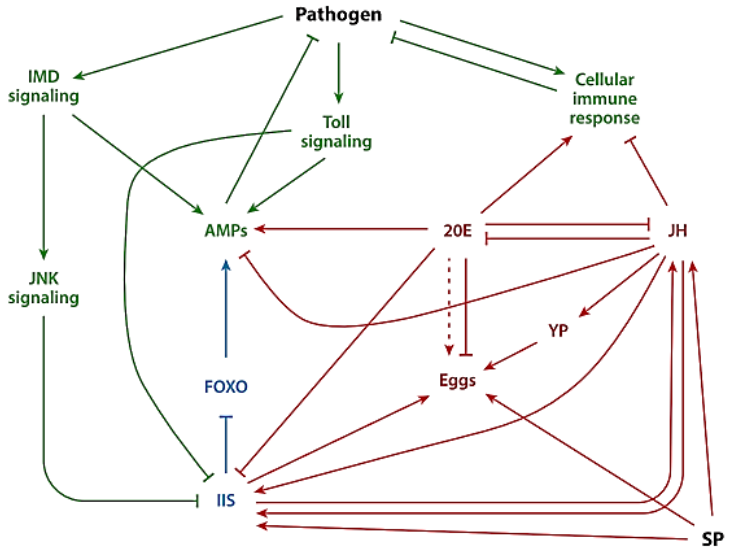

Figure 1.1 A generalized mechanism for the interactions between immunity and reproduction in females Reproductive pathways are red, metabolic signaling pathways are blue, immunity pathways are green, and exogenously supplied factors are in black. A female insect host responds to a pathogen via IMD and Toll pathways and cellular immune activation. Activation of the IMD pathway activates JNK signaling, which inhibits IIS signaling. The end result is the production of AMPs. The receipt of SP during mating alters the typical response to a pathogen. SP stimulates JH synthesis, which negatively regulates the cellular immune response and the production of AMPs. Abbreviations: 20E, 20-hydroxyecdysone; AMPs, antimicrobial peptides; FOXO, forkhead box, subgroup O; IIS, insulin/insulin-like growth factor-like signaling; IMD, immune deficiency; JH, juvenile hormone; JNK, c-Jun N-terminal kinase; SP, Sex Peptide; YP, yolk protein.

model (**Figure 1.1**) whereby endocrine and metabolic signaling may cooperatively mediate a physiological trade-off between reproduction and immunity via JH, 20E, and IIS. More research is needed to fully test this model and to determine its mechanistic detail. For example, although ample evidence exists that JH inhibits immune system activity in multiple insects, it is unknown whether this is a direct or indirect effect.

SUMMARY POINTS

1. The trade-off between female reproduction and immunity has been detected in a diverse range of insect species.
2. Physiological costs of immunity include reduced egg production and viability. Physiological costs of reproduction often include reduction in both basal and induced levels of immunity.
3. The energetic requirement of reproduction and immunity suggests that the reallocation of a common resource may be the basis for the trade-off between the traits.
4. Hormonal signaling, including JH and 20E, are critical for modulating egg production and immunity. Such signals may hold a central position in the allocation of resources required for both reproduction and immunity.

FUTURE ISSUES

1. How does reproduction suppress immunity? Are all components of immunity suppressed by reproduction? How does immunity suppress reproduction?
2. Are other post-mating changes (e.g., reduced siesta sleep or increased feeding) important for the trade-off? Is egg production the only point of conflict?
3. How do nutrition and metabolic state affect the physiological trade-off between reproduction and immunity? What limiting resources are shared between the two processes?
4. What is the mechanistic basis of evolutionary trade-offs between reproduction and immunity? Do physiological and evolutionary trade-offs have the same mechanistic basis? Are these mechanisms shared across insect taxa?

The following text was not included in the published work presented above.

SUMMARY OF DISSERTATION

This project aims to improve our understanding of the relationship between immunity and reproduction using the model organism *Drosophila melanogaster*. Specifically, it seeks to identify the underlying mechanism for impaired resistance to systemic bacterial infection in mated females and investigates why mating-induced antimicrobial peptides are seemingly ineffective during an infection. This work seeks to provide the first identified mechanism linking reproduction and immunity. With a basic understanding of the molecular mechanism uniting the two processes and the conditions under which the conflict occurs, scientists can more explicitly investigate specific signaling networks and explore how this conflict may evolve. Additionally, findings from this project will provide insight into a potential conflict between males and females and the causes of susceptibility to sexually transmitted infections, which is virtually unknown amongst insects. Collectively, this study will not only contribute to our basic understanding of reproductive biology and insect immunology but it will unravel the complexities which unite the two processes.

Homeostasis requires a complex signaling network to regulate the flux of nutrients to the appropriate tissues and physiological processes. It has been hypothesized that reproduction and immunity are antagonistically regulated in this fashion (see pp. 19-24). In Chapter 2, I investigate whether hormonal signaling, in particular juvenile hormone, mediates the trade-off between reproduction and

immunity in female *D. melanogaster*. Specifically, we investigate the inducers of juvenile hormone and the receptor that mediates juvenile hormone signaling.

Sex Peptide, a male seminal fluid protein, has prominent effects on female physiology (e.g., Peng et al., 2005a), including the ability to upregulate antimicrobial peptides in recipient females (Peng et al., 2005b) via a hydroxyproline motif (Domanitskaya et al., 2007). However, this upregulation is restricted to reproductive tissues (Mack et al., 2006; Domanitskaya et al., 2007; Kapelnikov et al., 2008), which may explain why mated females are more susceptible to a bacterial infection than virgin females despite expressing more antimicrobial peptides. Sex Peptide has other known functions including the ability to stimulate the production of juvenile hormone in the corpus allatum (in an *ex vivo* experiment), which requires an intact N-terminus (Fan et al., 2000). Thus, I chose to explore whether the N-terminus of Sex Peptide initiates a signaling cascade within the recipient female resulting in decreased resistance to a bacterial infection. After establishing a role for juvenile hormone, I investigated which nuclear receptor juvenile hormone signals through to suppress immunity after mating and show that the two known juvenile hormone receptors are not completely redundant this aspect. This investigation has solidified our understanding of the molecular basis for a well-documented life-history trade-off. The findings are highly consistent with a model of hormonal pleiotropy. This chapter has been submitted for publication.

Females require a considerable amount of energy and nutrients to support both oogenesis and immunity (see pp. 14-19). In Chapter 3, I evaluate the dietary conditions that enhance host defense during a bacterial infection and assess whether

ingesting a supplemented diet overrides the negative interaction between reproduction and immunity. While I would like to show the reallocation of molecules and nutrients from eggs to somatic tissue (or vice versa), it is an extremely challenging endeavor. Instead, I utilize dietary supplementation as a strategy to alter resource pools within females and investigate the reproductive and immunological consequences. Specifically, I examine whether additional resources alleviate the negative impact of mating on immunity across six genotypes and test for an evolutionary trade-off (i.e., a negative correlation) between fecundity and immunity. Upon closer examination of a high yeast diet, I find that the beneficial effect of supplementation is largely attributed to essential amino acids within the diet. Finally, I propose potential mechanisms for how nutrition may mediate both reproduction and immunity, and how dietary effects relate to hormone signaling.

Mating can impose direct risks to individual health due to sexually transmitted microbes. However, among insects, sexually transmitted bacterial infections are remarkably rare (Knell and Webberley, 2004). Some have hypothesized that this apparent absence of sexually transmitted bacteria is a consequences of mating-induced antimicrobial peptides (Knell and Webberley, 2004). In a population of susceptible hosts, pathogens can act as a remarkably strong selective pressure and after enough exposure resistance is expected to evolve. It is impossible to think *D. melanogaster* females have evolved a defense strategy that protects them from *all* sexually transmitted bacteria given the prevalence of trade-offs and varied pathogen pressure over time (Lazzaro et al., 2004; Lazzaro and Little, 2009; Unckless et al., 2016). However, it is reasonable to believe a combination of low-detectability, a small

number of investigations, and increased immune defense could contribute to the apparent patterns of sexually transmitted bacterial infections amongst insects. Thus, while mated females have higher antimicrobial peptide expression after mating (e.g., Peng et al., 2005b), this response may be localized (e.g., Mack et al., 2006) to prevent the procurement of a sexually transmitted infection.

In Chapter 4, I aim to establish whether mating with a bacteria-carrying male negatively affects female fitness and whether a mating-induced immune response prevents disease. Due to the lack of a naturally transmitted bacterium, I test for susceptibility in females by placing them with males who received application of *Serratia marcescens* to their genitalia, a technique established by Miest and Bloch-Qazi, (2008). My findings suggest that sexually-transferred bacteria impose severe fitness consequences. I investigate whether IMD signaling prevents the establishment of infection and examine patterns of *Diptericin* expression, an antimicrobial peptide, after mating to bacteria-carrying males. Lastly, I evaluate whether the receipt of Sex Peptide influences susceptibility to a sexually-acquired infection. My findings highlight the potential for surface bacteria to be transmitted to females and initiate an opportunistic infection in an insect system. Moreover, they provide novel insight into direct costs of mating, which could have implications for the evolution of male-female interactions. This study underscores the elusive nature of antimicrobial peptide induction in response to mating, remaining fertile territory for exploration. While this work offered several new insights, there are noteworthy shortcomings to this method and I conclude by discussing alternative strategies for addressing routes of infection and the effectiveness of immune induction in mated females.

CHAPTER 2
JUVENILE HORMONE MEDIATES RESISTANCE TO INFECTION IN FEMALE *DROSOPHILA MELANOGASTER*[1]

SUMMARY

Hormonal signaling provides metazoans with the ability to regulate development, growth, metabolism, immune defense, and reproduction in response to internal and external stimuli. The use of hormones as central regulators of physiology makes them prime candidates for mediating allocation of resources to competing biological functions (i.e., hormonal pleiotropy) (Flatt et al., 2005). In animals, reproductive effort often results in weaker immune response (e.g., McKean and Nunney, 2001; Ardia et al., 2003; Short and Lazzaro, 2010) and this reduction is sometimes linked to hormone signaling (see Ketterson and Nolan, 1999; Prall and Muehlenbein, 2014; Schwenke et al., 2016). In the fruit fly, *Drosophila melanogaster*, mating and the receipt of male seminal fluid proteins results in reduced resistance to systemic bacterial infection (Fedorka et al., 2007; Short et al., 2012). Here, we hypothesized that the immunosuppressive effect of reproduction in female *D. melanogaster* might be attributed to the endocrine signal juvenile hormone (JH)**,** which promotes the development of oocytes and the synthesis and deposition of yolk protein (Soller et al., 1999; Riddiford, 2012). Previous work has implicated JH as potentially immunosuppressive (Rolff and Siva-Jothy, 2002; Flatt et al., 2008), and transfer of the male seminal fluid protein Sex Peptide (SP) activates JH biosynthesis in female *D. melanogaster* (Fan et al., 2000). We find that post-mating immunosuppression in *D. melanogaster* females does indeed stem from SP-driven

[1] Submitted for publication

activation of JH synthesis, and depends specifically on the JH-receptor *germ cell-expressed* (*gce*). We argue that hormonal signaling is important for regulating immune system activity and, more generally, for governing trade-offs between physiological processes.

RESULTS AND DISCUSSION

Under a model of hormonal pleiotropy, reproduction and immunity are hypothesized to be interlinked by molecular cues that promote reproduction at the expense of immunity (Flatt et al., 2005; Schwenke et al., 2016). Thus, we tested whether the endocrine signaling molecule JH is responsible for post-mating immune suppression in females. We first tested whether application of a synthetic JH analogue, methoprene, blocks immune system activation. Methoprene exposure (10^{-2} ug) suppressed the induction of antimicrobial peptides (AMPs) by virgin females after an inoculation with heat-killed Gram-negative bacteria, *Providencia rettgeri*, (Tukey's HSD, $p < 0.0001$), rendering them similar to untreated mated, females (Tukey's test, p = ns; **Figure 2.1A**). Exposure to methoprene also significantly reduced the ability of virgin females to restrict the growth of live *P. rettgeri* (acetone vs methoprene across doses; t-test, $t_{37} = 6.54$, $p < 0.0001$; **Figure 2.1B**) and survive the infection (LR, X_1^2=13.9, $p < 0.0001$; **Figure 2.1C**). Application of methoprene to mated females increased the average number of eggs laid over the course of 5 days from 75.6 ± 24.3 to 95.4 ± 32.4 (t-test, $t_{42} = -2.37$, $p = 0.0225$). We conclude that JH facilitates reproduction but is immunosuppressive, and that its application can phenocopy the immunosuppression observed in mated females (**Figure 2.1A**).

Next, we tested whether the immunosuppressive effects of JH stem from the receipt of the male seminal fluid protein Sex Peptide (SP). SP drives a large number of physiological changes in mated *D. melanogaster* females (Avila et al., 2011) and is important in post-mating immunosuppression (Short et al., 2012). We used mRNA expression levels of *JH acid methyltransferase* (*jhamt*), which encodes a key regulatory enzyme in the JH biosynthesis cascade (Shinoda and Itoyama, 2003), as a

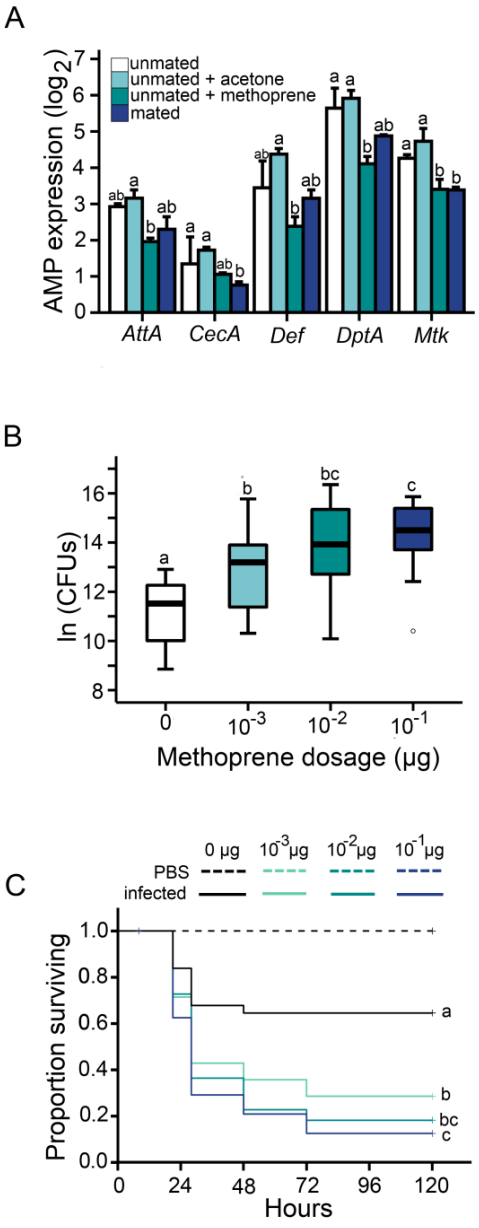

Figure 2.1
Juvenile hormone is immunosuppressive
(A) mRNA expression of antimicrobial peptide genes 8 hours after receiving heat-killed *P. rettgeri* relative to CO_2 controls. Unmated females were exposed to methoprene, acetone, or CO_2 and mated females were exposed to CO_2 (ANOVA, Gene: $F_{4,52}$=89.78, p<0.0001; Experimental class: $F_{3,52=}$20.48, p<0.0001). Tukey's HSD was performed for each gene. Means with the same letter are not significantly different (p>0.05).
(B) Bacterial load of individual unmated females that received acetone or methoprene (ANOVA, Treatment: $F_{3,\ 72}$=13.92, p<0.0001). Means with the same letter are not significantly different. N=20; two replicates.
(C) Survivorship of unmated females subsequent to methoprene exposure and injection with sterile medium (PBS) or *P. rettgeri* (Cox, Chemical (infected only): X_3^2=35.982, p<0.0001). N=50±6; two replicates.

proxy for JH level *in vivo*. We evaluated *jhamt* expression levels in females mated to: wildtype males, males lacking SP entirely, or males lacking the N-terminus of SP that is required for activation of JH synthesis (Moshitzky et al., 1996; Fan et al., 2000). We found that females who received wildtype SP in the seminal fluid expressed significantly higher levels of *jhamt* than females that did not receive SP and or that received SP missing the N-terminus (Tukey's HSD, WT-null, $p = 0.00977$; WT-Δ2-7, $p = 0.0158$) (**Figure 2.2A**). Thus, we confirm that the N-terminus is required for the stimulation of *jhamt* expression and infer that it is promoting synthesis of JH, consistent with *in vitro* results (Moshitzky et al., 1996; Fan et al., 2000). To test whether the inferred induction of JH leads to female immunosuppression, we mated females to several male genotypes expressing variant versions of SP (**Figure 2.2B**). Females mated to males whose seminal fluid lacked SP (SP^{null}) or whose SP was missing the N-terminus ($SP^{\Delta 2-7}$) exhibited virgin-levels of bacterial load and survivorship after mating, but females mated to SP^{QQ} or SP^{WT} males had significantly higher bacteria load and lower survivorship as a consequence of mating (**Figures 2.2C,D**). During copulation, SP and sperm are transmitted independently, but eventually SP adheres to the sperm tail via the N-terminus within the female (Peng et al., 2005a). Over time SP is cleaved from the sperm tail at a trypsin cleavage site, providing females with a continued source of C-terminus (Peng et al., 2005a). The trypsin cleavage site is disrupted in the SP^{QQ} males, yet females mated to these males show post-mating immunosuppression. We interpret that the immunosuppressive effect of SP is driven exclusively by the N-terminus via activation of JH synthesis.

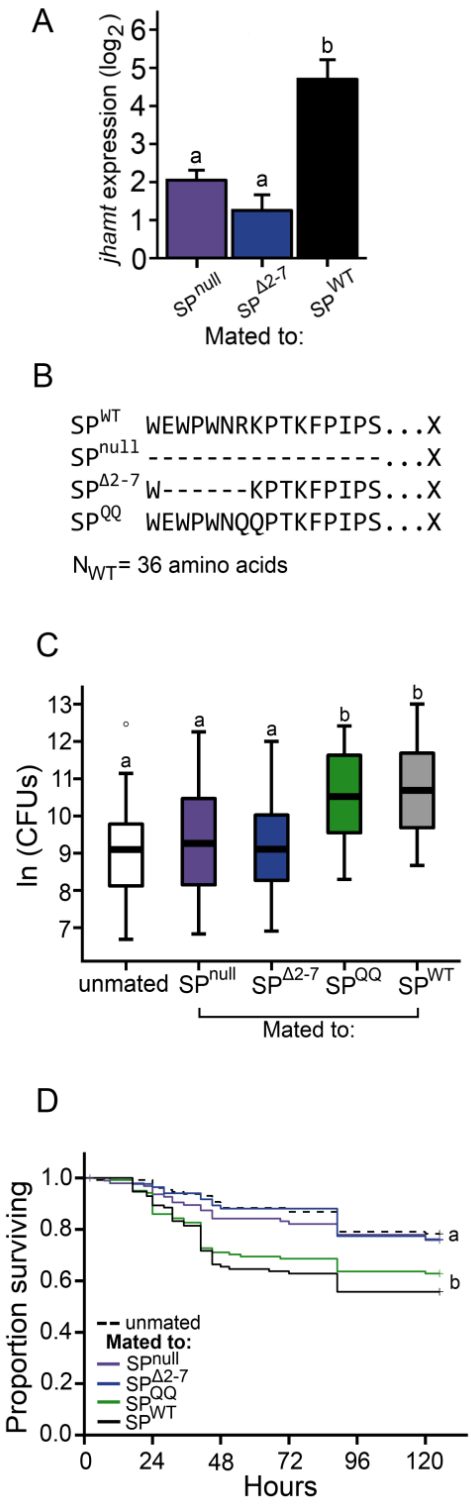

Figure 2.2.
Transgenic males lacking the N-terminus of Sex Peptide do not elicit immunosuppression in recipient females
(A) *jhamt* mRNA expression in females 10 hours after mating to SP genotypes relative to unmated females (ANOVA, Status: $F_{3,8} = 22.29$, $p = 0.00031$). Bars represent the mean (± SEM). Means with the same letter are not significantly different ($p>0.05$).
(B) Amino acid sequences of Sex Peptide; SP^{QQ}: the R_7K_8 trypsin cleavage site has been changed to Q_7Q_8; $SP^{\Delta 2\text{-}7}$: N-terminal amino acids (E_2-R_7) deleted; SP^{WT}: wildtype.
(C) Bacterial load of individual females that had been infected with *P. rettgeri* subsequent to mating with males of different SP genotypes (ANOVA, mating status: $F_{4,189} = 13.91$, $p<0.0001$). Letters indicate levels of significance. N = 40±6; three replicates.
(D) Infection survivorship subsequent to mating with males of different SP genotypes (Cox, mating status: $X_4^2 = 23.37$, $p = 0.00011$). Letters indicate levels of significance. N=110±15; three replicates.

To further substantiate the role of JH in post-mating immunosuppression, we tested whether blocking JH synthesis or receptor binding within the female would prevent the reduction in immunity after mating. JH is synthesized in the corpus allatum (CA). We used an inducible driver to overexpress either Diphtheria toxin (DTI) (Bilen et al., 2013) or *NIPP1* (Yamamoto et al., 2013) in the CA, ablating the tissue in late-stage pupae to avoid any early developmental defects caused by JH removal (Edgar, 2006). We found that females whose CA were ablated exhibited bacterial loads and infection survivorship that were no different than those of virgins (**Figure 2.3A-D**). Thus, removal of the CA was sufficient to prevent post-mating immunosuppression.

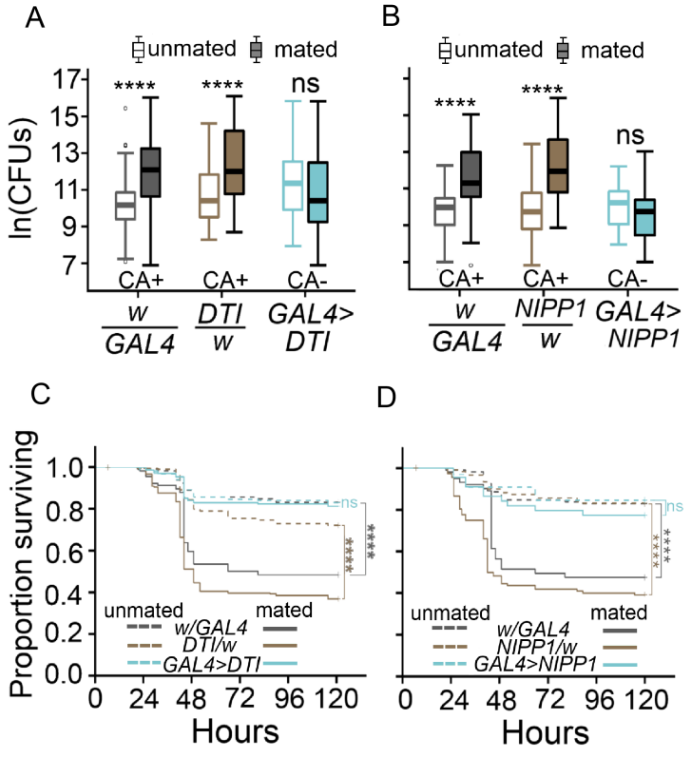

Figure 2.3 Genetic ablation of JH biosynthesis rescues virgin-levels of resistance
(A) (B) Bacterial load (CFU) of individual CA+ and CA-ablated females subsequent to mating and infection. Mean bacterial load of unmated and mated females within a genotype were compared with a Wilcoxon test. **(A)** *DTI*, N=60±10; four experimental replicates. **(B)** *NIPP1*, N=30±5; three replicates. **(C)(D)** Survivorship of CA+ and CA- females subsequent to mating and infection. Survivorship was compared within a genotype using a log-rank test. **(C)** *DTI*, N>120; five replicates. **(D)** *NIPP1*, N=48±15; three replicates. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

To confirm that CA ablation results in lower levels of JH activation after mating, we performed two separate validation experiments. First, we measured the expression of *jhamt* and two downstream targets of JH (*mnd* and *JHI-21* (Dubrovsky et al., 2002)) 10 hours after mating. Expression levels for all three genes were significantly reduced in CA-ablated females (t-test, $p < 0.001$ for each gene) and were less than 50% of the expression level of the control genotypes in all cases. Additionally, CA-ablated^{DTI} and CA-ablated^{NIPP1} laid significantly fewer eggs (33.3 ± 29.5 and 51.6 ± 35.0) than control genotypes (130.9 ± 53.0 and 167.5 ± 82.5) (Tukey's HSD comparisons, $p < 0.05$). Based on the full set of data, we conclude that CA-ablated females are deficient in JH synthesis and therefore exhibit reduced fecundity but virgin-levels of resistance to bacterial infection.

Finally, we sought to identify the receptor through which JH suppresses immunity in reproductively active females. Two recently-duplicated paralogs are thought to be responsible for mediating the JH signal during development (Baumann, et al., 2010a; Abdou et al., 2011). While *Methoprene-tolerant* (*Met*) and *gce* are partially redundant during development, it is unknown whether either or both of these receptors are required in post-mating immune suppression in adult females. We ubiquitously expressed an RNAi knockdown construct targeted against each gene in adult females. The independent knockdowns resulted in a 62% reduction in *gce* expression ($t_{2 =}$ -6.18, $p = 0.025$) and a 73.4% reduction in *Met* expression ($t_2 =$ -7.85, $p = 0.016$) relative to the controls.

RNAi knockdown of *gce* significantly improved resistance to infection and eliminated post-mating immunosuppression (**Figures 2.4B,D**), whereas knockdown of

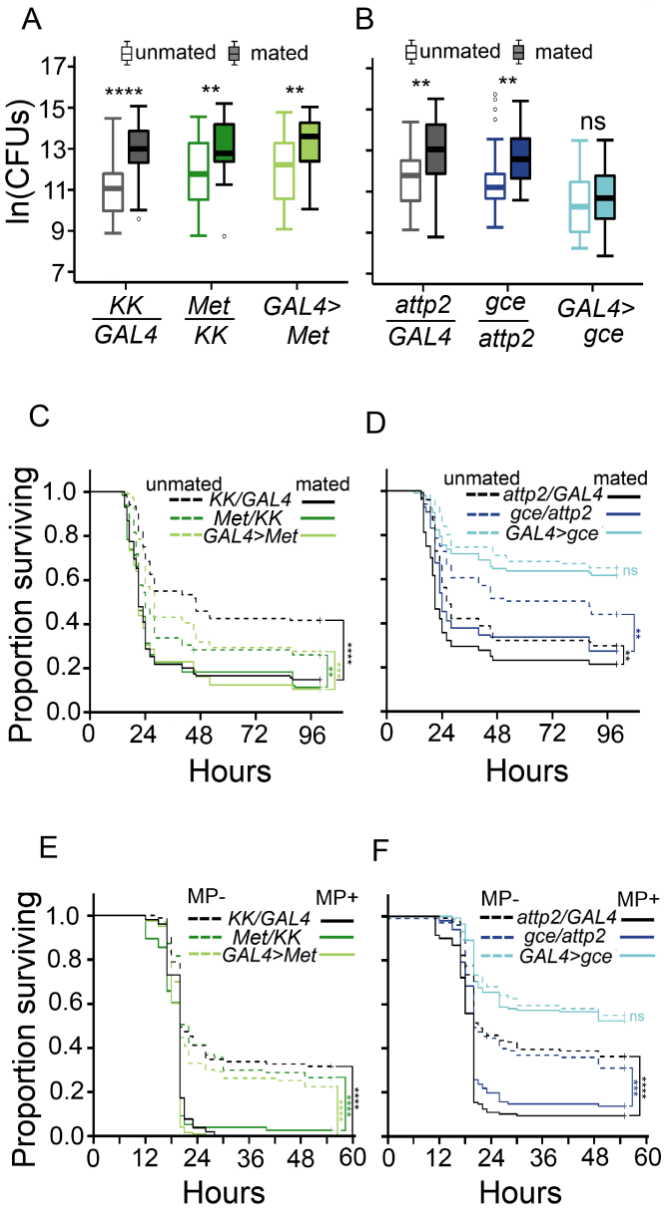

Figure 2.4 RNAi-mediated knockdown of *gce*, a JH receptor mediates the effect of JH on immunity

(A)(B) Bacterial load (CFU) within *Met*-RNAi or *gce*-RNAi females and their controls, respectively. Mean bacterial load of unmated and mated females within a genotype were compared with a Wilcoxon test. N=29±2; three replicates. **(C)(D)** Infection survivorship of *Met*-RNAi or *gce*-RNAi females and their controls, respectively. Survivorship was compared within a genotype using a log-rank test. N=105±15; four replicates. **(E)(F)** Infection survivorship of unmated *Met*-RNAi or *gce*-RNAi females exposed to methoprene (MP+) or acetone (MP-). Survivorship was compared within a genotype using a log-rank test. In the absence of infection, methoprene did not impact survivorship. **(E)** *Met*, N=80±15; three replicates. **(F)** *gce*, N = 120±20; three replicates. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

Met had no effect (**Figures 2.4A, C**). Specifically, bacterial loads within mated and unmated females were not significantly different in the absence of *gce* (Wilcoxon, W = 494, p = ns) (**Figure 2.4B**). In contrast, *Met* knockdown females suffered from significantly higher bacterial loads as a consequence of mating (Wilcoxon, W = 610.5, p = 0.00796) (**Figure 2.4A**). RNAi knockdown of *gce* improved female survivorship after mating and infection as well (**Figure 2.4D**), and mated and unmated females experienced similar rates of mortality (LR, X_1^2 = 0.5, p = ns). On the contrary, *Met* knockdown females remained sensitive to mating and experienced higher levels of infection-induced mortality as a consequence of mating (LR, X_1^2 = 23.7, p<0.0001), mirroring the patterns observed for control genotypes (**Figure 2.4C**). Interestingly, a reduction in *gce* expression significantly improved survivorship relative to background controls as well (Tukey's HSD, p<0.05), suggesting that even basal levels of JH in unmated females may negatively influence immune system induction. If *gce* expression mediates resistance to infection via JH signaling, then *gce* knockdown females should be resistant to the effect of methoprene and therefore not immunocompromised. We tested this, and found that methoprene application increased infection-induced mortality in all genotypes except for *gce*-RNAi (LR, X_1^2 = 11.3 – 37.6, p<0.0001) (**Figures 2.4E, F**). Thus, we conclude that GCE is the receptor that mediates the post-mating reduction in resistance driven by JH and SP and we have solidified a role for JH as a central mediator of the physiological trade-off between reproduction and immunity in *D. melanogaster*.

Our finding that *gce* alone regulates post-mating immune suppression highlights the intricate nature of the molecular action of JH. While MET and GCE

have apparent redundancies (Abdou et al., 2011), new evidence posits a divergence in the functionality of the two bHLH-PAS transcription factors (Baumann et al., 2013; Greb-Markiewicz et al., 2015; Reiff et al., 2015). For example, Reiff et al. (2015) demonstrated that the effect of JH on enterocyte growth and concomitant increases in reproduction are mediated largely by GCE. It is worth noting that the expansion of JH receptors is specific to Dipterans. *gce* is the ancestral gene (Baumann, et al., 2010b; Baumann et al., 2013) and JH-mediated immunosuppression may occur in other taxa (Rolff and Siva-Jothy, 2002).

Why has JH evolved an immunosuppressive function, and is post-mating immunosuppression adaptive? Under the immunopathology-avoidance hypothesis (Råberg et al., 1998), the risk of damage from autoimmunity is potentially greater than the risk associated with of immune system functionality (i.e., being immunocompromised). If immune activation disrupts reproductive tissues and output (Laird et al., 2003; Radhakrishnan and Fedorka, 2012), such processes would be strongly selected against due to their fitness consequences. Under this hypothesis, JH may act to suppress immune signaling to prevent instances of autoimmunity, especially in cases where reproductive tissues may be targeted. Thus, immunosuppression could occur to support reproductive output.

A perhaps more likely explanation is that the trade-off stems from a simple competition for resources. Both immune function and reproduction are resource-intensive (Owens and Wilson, 1999; Moret and Schmid-Hempel, 2000; Lee et al., 2008a). Under the resource-limitation hypothesis (Råberg et al., 1998), JH may operate as the molecular cue for the investment in reproduction rather than immunity.

While evidence for reallocation of a specific nutrient to immunity remains elusive, protein and specific amino acids strongly influence both reproduction and immunity (Lee et al., 2006; Grandison et al., 2009). Recently, JH was shown to increase reproductive output through enhanced lipid metabolism with sterile females storing more triacylglycerides (Reiff et al., 2015). Sterile females have also been shown to be resistant to the effects on mating on immunity (Short et al., 2012). The fat body is a tissue that drives systemic immunity, regulates central metabolism and allocation to egg provisioning, and stores lipid (Arrese and Soulages, 2010), and thus may be the organ regulating the trade-off.

Altogether, our work demonstrates an unambiguous role for JH in suppressing immunity in mated females, thus providing a mechanism for a classic life history trade-off and supporting the hormonal theory of pleiotropy. Whether this trade-off operates as a simple function of resource availability or whether there is a deeper adaptive explanation remains to be conclusively demonstrated.

EXPERIMENTAL PROCEDURES

Fly stocks and crosses Canton-S (CS) flies (Bloomington #1) were used as a generic wildtype genotype. SP^{null} males were created by crossing Δ130/*TM3, Sb ry* and 0325/*TM3, Sb ry* (Liu and Kubli, 2003). Males lacking the N-terminus of SP ($SP^{\Delta 2\text{-}7}$) were generated by crossing *w*; Cd/Cd; Δ130/*TM3, Sb ry* and 0325/*TM3, Sb ry* stocks (Peng et al., 2005a). Similarly, SP trypsin cleavage mutants (SP^{QQ}) were obtained by crossing *w*; QQ/QQ; Δ130/*TM3, Sb ry* and 0325/*TM3, Sb ry* (Peng et al., 2005a). To genetically ablate the corpus allatum, ablation construct lines, *w;* $GAL80^{ts}$; UAS-DTI

(Bilen et al., 2013) or *w;* UAS-*NIPP1*/*TM3* (Yamamoto et al., 2013), were crossed to the *Aug21*-GAL4, UAS-EGFP/CyO driver at 18°C; control genotypes were produced by crossing construct and driver lines to w^{1118}. Vials with white pupae were shifted to 29°C to activate tissue ablation and unmated females were housed at 29°C for 5 days and then shifted to 25°C for the remainder of the experiment. For receptor knockdowns, the *gce*-RNAi line (y^1 v^1; P{TRiP.JF02097}attP2) (Bloomington #26323) and the *Met*-RNAi line (P{KK104562}VIE-260B) (VDRC #10638) were crossed to GAL80^{ts}; *da*-GAL4, *dpt*-lacZ females at 18°C; control genotypes were produced by crossing RNAi construct and driver lines to the attP2 background (Bloomington #36303) for *gce*-RNAi or to the KK background (VDRC #60100) for *Met*-RNAi. White pupae were shifted to 29°C and remained there throughout the entire experiment.

Fly husbandry All flies were reared on a glucose medium (8.3% glucose, 8.3% Brewer's yeast, 1% agar, 0.04% phosphoric acid and 0.4% propionic acid). Except for the CA ablation and receptor RNAi crosses, all flies were housed continuously at 25°C.

Mating procedure Females were collected as virgins and housed in cohort sizes of 10-15 individuals. Virgin males were housed in groups of 30 individuals. Five days after eclosion (9 days in the RNAi experiments), males and females were combined or left as virgins. Vials were visually inspected to ensure copulation was occurring; within 5-30 minutes, all females were typically paired with males. Males were removed after 4-5 hours when injections began.

Bacterial infection Cultures of *Providencia rettgeri* (Juneja and Lazzaro, 2009) were

grown for 20 ± 2 hours in Luria Broth (LB) in a shaking incubator at 37°C. Cultures were spun down at 3000 RPM at 4°C and resuspended in sterile PBS. Cultures were diluted to OD_{600}=0.05 with PBS, which corresponds to 1000 (±200) viable cells per individual. CO_2-anesthetized females received an injection of 9.3nl of media in the thorax 3-4 hours after mating (or as virgins) using a pulled capillary needle mounted on a Nanoject II apparatus (Drummond Scientific). After injection, flies were placed into new food vials in a cohort size of 10-15.

Survival Assays Survival was recorded daily for 5 days. PBS-injection rarely resulted in mortality. Females that did not recover from the injection within 8 hours were censored from the dataset as their death was due to handling rather than infection.

Bacterial load 20 hours after infection, single flies were homogenized in 500 ul of sterile PBS using a linear motion homogenizer (OPS Diagnostics). A 1:100 dilution of the samples was performed and 50 ul of the homogenates were plated on LB agar plates using a WASP2 spiral plater (Microbiology International). Plates were incubated overnight at 37°C and colonies were counted using a ProtoCOL plate counting system (Microbiology International). Controls flies were injected with sterile PBS, and the plates from these individuals never yielded any colonies.

Hormonal treatment Methoprene (Sigma-Aldrich) was suspended in acetone and ectopically applied using a pulled glass capillary mounted on a Nanoject II apparatus. Females were anesthetized on CO_2 and received 50.6nl of the carrier or the methoprene suspension to their ventral, abdominal cuticle. All females were unmated except in the egg laying assay, in which females were had been mated recently before receiving a dose of methoprene.

Egg laying assays Females were housed as virgins in groups of 10-15 individuals and provided 30 CS males when 5 days old. Methoprene (or acetone) was applied to CS females within 5 hours of mating and females were placed singly into food vials. Eggs were counted daily for four days. N = 25.

In CA ablation egg-laying experiments, females were housed as virgins and provided 20 CS males. Males were removed and mated females remained together within a vial. Females were transferred to new vial every day for 4 days and eggs were counted daily. Egg counts represent the average number of eggs per female. Vials had 5-12 females and three biological replicates were collected. N = 3.

quantitative RT-PCR Flies were flash frozen and stored at -80°C until processing. Total RNA was isolated using a TRIZOL (Invitrogen)-chloroform extraction protocol and was resuspended in RNAase-free water. Purity was verified and RNA amounts were quantified using a NanoDrop 2000 (Thermo Scientific) spectrophotometer. Approximately 1000ng of nucleic acid from each sample was treated with DNase (Promega) and M-MLV reverse transcriptase (Promega). We used SsoAdvanced SYBR Green Supermix (Bio-Rad) and qRT-PCR reactions were performed using the CFX Connect Real-Time Detection System (Bio-Rad). Genes of interest were compared to the housekeeping gene *RpL32*. Primers were designed using the DRSC FlyPrimerBank (Hu et al., 2013). Gene expression analysis was performed using the ΔΔCt method. Each experiment consisted of three biological replicates and technical replicates.

Expression levels of five AMPs were measured in females that had received both the hormonal treatment (or acetone-carrier) and heat-killed *P. rettgeri*

(OD_{600}=1.0±0.2)**.** Flies were frozen 8 hours after injection with bacteria (i.e., 10 hours after hormonal treatment or mating). Expression of AMPs was compared to CO_2-exposed flies.

Unmated *da*-GAL4>*gce* and *da*-GAL4>*Met* females were collected and housed at 29°C for 9 days. Three pools of 20 females were collected and processed to validate the knockdown efficiency of the constructs.

Statistics All analyses were performed in R (R Core Team, 2015). All sample sizes refer to the total number of individuals within each treatment across experimental replicates. Survivorship data was fitted to a Cox proportional hazard model and treatments were tested pairwise with a Log-rank test. Multiple comparisons were tested with Tukey's HSD. When bacterial load data violated assumptions of normality, a Mann-Whitney-Wilcoxon test was performed. For AMP qRT-PCR, 'Treatment' and 'Gene' were loaded as fixed effects into an ANOVA model to test for differences in AMP gene expression, and Tukey's HSD was used to test for differences among experimental groups within a single gene.

CHAPTER 3
ASSESSING THE IMPACT OF DIETARY SUPPLEMENTATION ON REPRODUCTION AND IMMUNITY

ABSTRACT

Immunity and reproduction are energetically and nutritionally intensive processes. Under resource-limiting conditions, a conflict between phenotypic traits could arise when both processes require, and compete for, the same resource. The movement or investment of nutrients or energy in one trait versus another is the underlying basis for a trade-off driven by uneven resource allocation.

We investigated the costs of reproduction and immunity and tested whether dietary supplementation overrides this trade-off, as predicted by a model of resource allocation. Using *Drosophila melanogaster*, we found evidence that reproduction constrains immunity; the induction and the elimination of egg production had reciprocal effects on host defense. Therefore, we aimed to identify whether the costs associated with these traits are attributed to nutrition. We found that dietary yeast promotes resistance to infection and minimizes the effect of reproduction on immunity. Moreover, we detected genetic variation for the effect of diet on immunity. Across genotypes, we observed a weak negative correlation between immunity and reproduction, which strengthened under resource-limiting conditions, highlighting the potential for condition-dependent evolutionary trade-offs. Finally, we report that essential amino acids support both host defense and reproduction. We propose that immunity and reproduction require essential amino acids and that variation in molecular signaling may produce divergent allocation decisions among genotypes.

INTRODUCTION

Nutritional and energetic constraints are major determinants of life-history evolution (Reznick, 1985; Stearns, 1989; Rose and Bradley, 1998). The energetic and nutritional requirements of a physiological process are hypothesized to be limited by an internal pool of resources that is shared by multiple organismal traits (Zera and Harshman, 2001; Boggs, 2009). As a result, when internal resources are limiting, physiological trade-offs are hypothesized to arise. These can constrain organismal performance and the evolution of the physiological processes involved (Min et al., 2006).

Immunity, or the ability of a host to respond to an infectious agent, is critical for organismal fitness. However, the metabolic costs associated with maintaining and deploying the immune system are significant (Lochmiller and Deerenberg, 2000). In vertebrates, inoculation with either inactive microbes or living pathogens induces higher metabolic rates (i.e., increased energy expenditure) (Cooper et al., 1992; Martin et al., 2003). For instance, immune system induction leads to a 16% increase in resting metabolic rate in humans (Cooper et al., 1992) and can exceed 30% during sepsis (Kreymann et al., 1993). Similarly, immune activation promotes higher metabolic activity in a variety of insect species (Freitak et al., 2003; Ardia et al., 2012; but see Arnold et al., 2013; Bashir-Tanoli and Tinsley, 2014). Therefore, immune activity appears to be a metabolically demanding process that requires substantial investment from the host.

To fully understand the costs associated with immunity, it is important to identify the nutrients essential for defense. A substantial body of literature suggests

that protein is the most important macronutrient required for host defense (Beisel, 1977). Although macronutrients such as glucose are required by cellular defenses as metabolic fuel (Calder, 1995), protein is required by nearly every branch of immunity and a successful response requires protein-dependent tissue repair and the production of an array of enzymes and antimicrobial peptides (Beisel, 1977). Consistently, studies from vertebrates to invertebrates find that higher protein regimens support stronger immune system activation during both innocuous and highly pathogenic challenges (Lochmiller et al., 1993; Lee et al., 2008b; Povey et al., 2009; Venesky et al., 2012; Brunner et al., 2014).

The metabolic costs associated with immune system activity can constrain the expression of other life history traits, thus leading to a physiological trade-off (Sheldon and Verhulst, 1996; Schmid-Hempel, 2003; Van Der Most et al., 2011). Theory suggests that a trade-off arises when two physiological traits that are both metabolically expensive draw resources from a shared pool of resources within a resource-limited system (Zera and Harshman, 2001). Consequently, the hypothesized resources are expected to be internally reallocated between the two traits resulting in the trade-off. By extension, the negative functional interaction between two costly traits should be reduced or eradicated in resource-rich environments (Zera and Harshman, 2001; Boggs, 2009). The relationship between immunity and other energetically demanding traits, such as reproduction, can have substantial fitness consequences for the host, and allocation decisions are likely to be highly dependent on host environment and physiological condition and the underlying mechanisms should be subject to natural selection.

Reproduction is one the most physiologically demanding processes that female animals experience, due to the high energetic requirements of producing and (where relevant) caring for offspring. Across animal species and reproductive strategies, metabolic rate increases during bouts of reproduction (e.g., McLean and Speakman, 2000; Johnson et al., 2001; Krockenberger, 2003; Foucart et al., 2014). For instance, mammals require extra food and meals that are more protein-rich in order to support the large costs associated with pregnancy and lactation (Godfrey et al., 1996; Speakman, 2008). Similar nutritional requirements have been noted for a wide range of insects (Wheeler, 1996) including *Drosophila* where females consume more food after mating (Carvalho et al., 2006) and require a protein-rich diet to support vitellogenesis (i.e., the deposition of yolk protein into developing oocytes) (Drummond-Barbosa and Spradling, 2001).

A substantial number of studies suggest that reproduction and immunity are highly coupled processes and trade-offs between the two are common (reviewed in Sheldon and Verhulst, 1996; Harshman and Zera, 2007; Schwenke et al., 2016). Among insects, both immune stimulation with inactive microbes and live pathogens can diminish egg production (McKean et al., 2008; Bashir-Tanoli and Tinsley, 2014; Howick and Lazzaro, 2014; Nystrand and Dowling, 2014). Reciprocally, reproduction can negatively affect the induction of host defenses (Fedorka et al., 2004; Baer et al., 2006; Short and Lazzaro, 2010). For instance, mating and the receipt of seminal fluid proteins cause female *Drosophila melanogaster* to exhibit a lower level of immune system induction relative to unmated females (Short et al., 2012). Thus, the activation of both reproductive and immunological processes often clash, resulting in a

physiological trade-off within an individual female.

We have evidence that immunity trades off against reproduction, but how the availability of nutrients and the identity of these different nutrients that affects the interplay between these traits remains unknown. Here, we investigated whether mated females are immunosuppressed after mating due to a nutritional limitation that favors reproductive processes over host defense. Specifically, we tested: (1) the costliness of immune responses and reproductive investment within and across genotypes, (2) whether costs of immunity and reproduction are explained by rates of nutritional acquisition, (3) whether nutritional supplementation improves host defense, and (4) whether there are specific dietary components necessary for immunity.

RESULTS

Reproduction and immunity negatively interact

Under a trade-off model, an increase in female immunity should result in a concomitant reduction in fecundity (i.e., a physiological trade-off) within individuals. We found that immune activation significantly reduced female fecundity (Kruskal-Wallis, $X_2^2 = 10.89$, $p = 0.0043$) (**Figure 3.1A**). Immune stimulation using heat-killed or live *Providencia rettgeri* resulted in a significant reduction in the number of eggs laid relative to the number of eggs laid by sterilely injected females over the course of five days (*post-hoc* multiple comparisons $p < 0.05$). Reciprocally, the removal of immune activation through genetic mutation of the IMD signaling pathway, an innate immune signaling pathway that targets Gram-negative bacteria, resulted in a significant increase in female fecundity relative to Oregon-R (genetic background of

mutant) (Welch's t-test, t_{100} = -4.67, $p < 0.0001$) (**Figure 3.1B**). We measured fecundity in axenic females (i.e., without gut and surface microbes), with and without IMD signaling, to investigate whether increased fecundity in IMD mutants was attributed to a dysregulated microbiome. In the absence of a microbiome, *imd* mutants were still more fecund than the control females (Welch's t-test, $t_{21.6}$ = 2.39, p = 0.026) (**Figure 3.1C**).

We investigated the immunological costs associated with egg production by eliminating oocyte development either at stage 4 of oogenesis (Ovo^{D1}) or earlier during germline stem cell differentiation ($bam^{\Delta 59}/ bam^{\Delta 86}$); both mutations prevent the formation of any vitellogenic (i.e., protein- and lipid-filled) oocytes. In the absence of complete oogenesis, females survived an infection with *P. rettgeri* better than wildtype controls (**Figures 3.2A, B**). Ovo^{D1} females survived an infection better than controls (CS) (Cox prop. hazards, Genotype: X_1^2 = 19.46, $p < 0.0001$) and mating compromised survivorship in the egg-producing genotype only (Cox prop. hazards, Genotype*Status: X_1^2 = 8.21, p = 0.00416) (**Figure 3.2A**). Similarly, *bam* females survived an infection better than controls (w^{1118}; e^1) (Cox prop. hazards, Genotype: X_1^2 = 39.77, $p < 0.0001$) and mating compromised survivorship in the egg-producing genotype only (Cox prop. hazards, Genotype*Status: X_1^2= 4.03, p = 0.0448). The comparison between egg mutants and their control genotypes should be evaluated cautiously because the genetic backgrounds were not fully controlled for here (see Experimental Procedures).

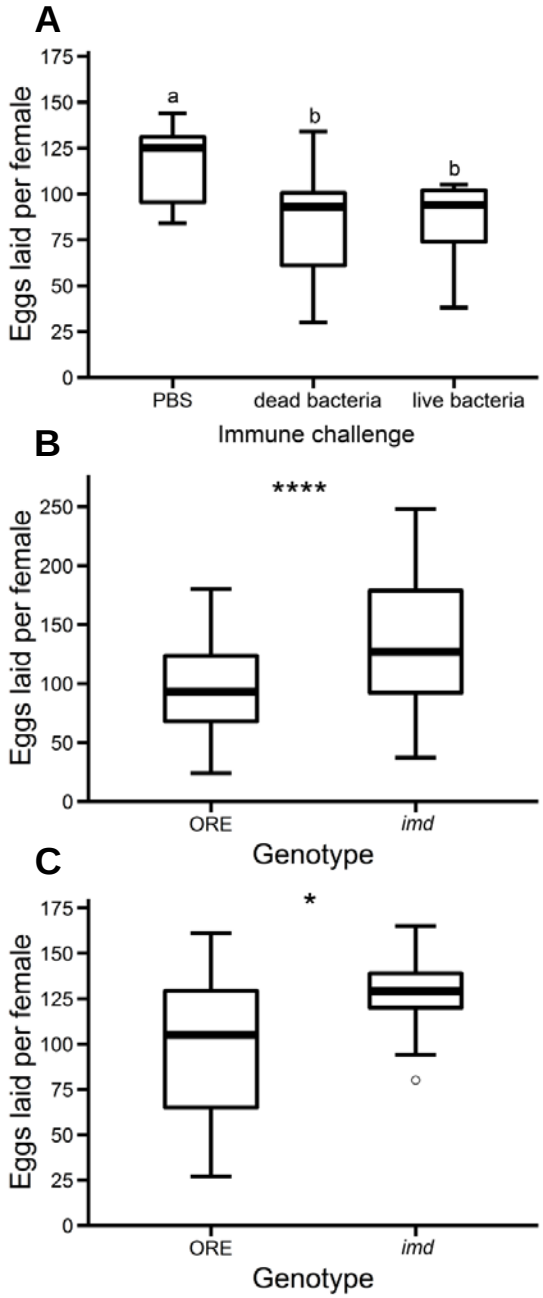

Figure 3.1
Effect of immune defense and activation on female fecundity
(A) Females were injected with sterile PBS, heat-killed *P. rettgeri*, or live *P. rettgeri*. Treatments sharing a letter are not significantly different. N = 16 ± 3, one replicate. **(B)** Uninfected fecundity in *imd* null and Oregon-R (ORE) females. N = 71 ± 8, four replicates.**(C)** Uninfected fecundity in the absence of gut bacteria. Both genotypes were reared and maintained axenically. N = 19, one experimental trial.'*' $p<0.05$; '**' $p<0.01$; '***' $p<0.001$; '****' $p<0.0001$

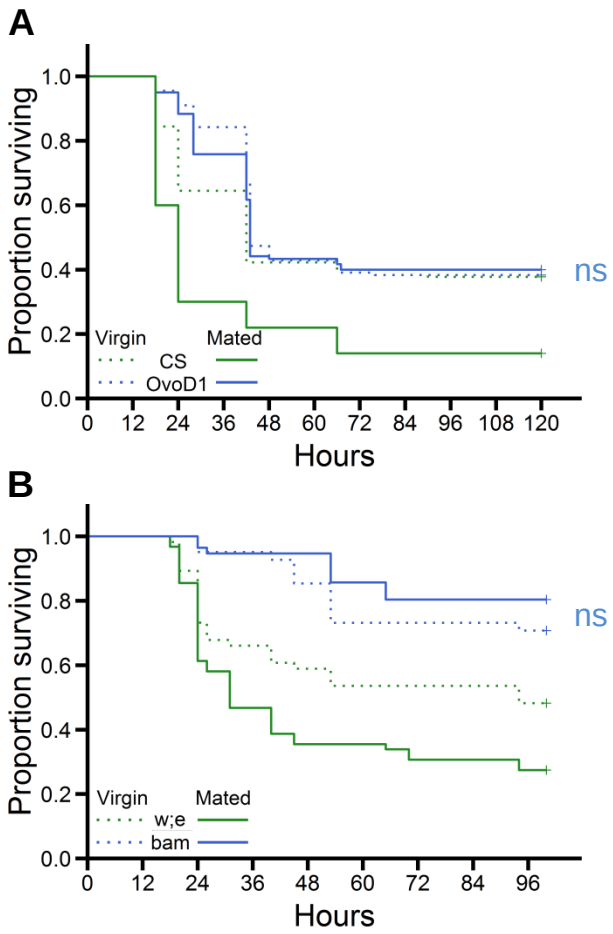

Figure 3.2
Elimination of egg production improves infection survivorship
(A) CS = Canton-S; OvoD1 = *Ovo*^{D1}/CS. N_{CS} = 50, N_{OvoD1} = 120, two replicates. **(B)** bam = w^{1118}; e^{1}, *bam;* w;e = w^{1118}; e^{1}. N = 52 ± 10, two replicates.

Neither wounding nor infection affects nutrient acquisition

Given the costliness of reproduction and immune activity, trade-offs could arise if an individual fails to acquire resources. Therefore, we tested whether rates of nutrient acquisition could explain the disparity between mated and virgin females. Mated and unmated females were placed onto a food dyed with Bromophenol blue and Xylene cyanol for 18 hours after receiving an injection with *P. rettgeri*, sterile PBS, or

no injection (CO_2) (**Figure 3.3**). A homogenate of the female and the frass produced was then collected, thus representing all food ingested and passaged during the time spent on the dyed food. In support of previous findings (Carvalho et al., 2006), mated females consistently consumed more food, suggesting this methodology is effective at detecting differences in food consumption (ANOVA, Mating Status: $F_{1,256} = 17.24$, $p<0.0001$) (**Figure 3.4**). Feeding was not significantly affected by wounding (PBS) or infection (Live bacteria) in either mated or unmated females (ANOVA, Treatment: $F_{2,255} = 0.419$, p = ns; Treatment*Mating Status: $F_{2,255} = 2.086$, p = ns) (**Figure 3.4**).

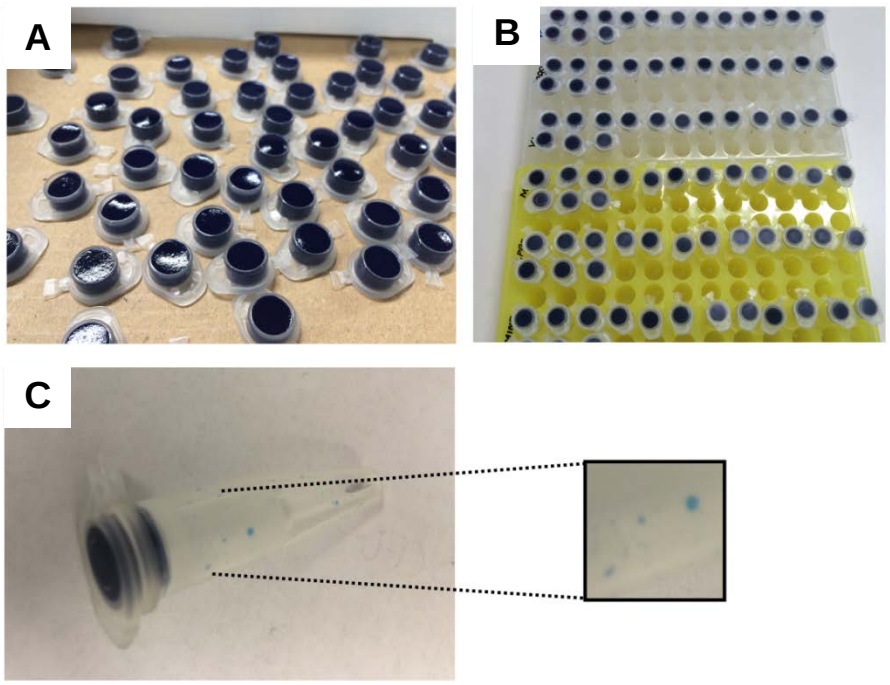

Figure 3.3
Feeding assay setup (A) 1.5ml Eppendorf tube caps were filled with a mixture of: 8% glucose, 8% Brewer's yeast, 1% agar, 0.1% Bromophenol blue and 0.5% Xylene cyanol. Females were placed into an Eppendorf tube without a cap after the experimental treatment and provided with a dyed food cap. **(B)** Tubes were kept in Eppendorf racks throughout the experiment. **(C)** Dyed frass is visible on tube walls and also within a female's digestive tract (not shown).

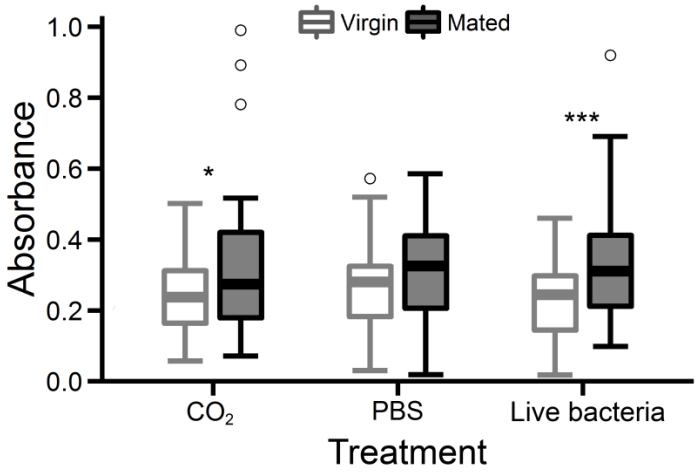

Figure 3.4
Nutrient intake and output during infection Absorbance was measured at 614nm 18 hours after receiving one of three inoculation treatments: no injection – CO_2, sterile injection – PBS, or an injection with live bacteria. N = 40-49, three replicates. '*' $p<0.05$; '***' $p<0.001$

A high-yeast diet improves immunity but the effect is genotype-dependent

We hypothesized that additional nutrients in the diet would provide females with more resources to fight a bacterial infection and eliminate the effect of mating on immunity (i.e., block allocation decisions). We assayed survivorship and bacterial load after an inoculation with *P. rettgeri* in females fed a low-yeast (5% yeast, 5% glucose) or a high-yeast (10% yeast, 5% glucose) diet (**Figures 3.5, 3.6**). Phenotypes were measured across six genotypes: a laboratory strain (Canton-S) and five isofemale lines from a single population near Ithaca, NY (Lazzaro et al., 2008). We utilized Ithaca isofemale because they were established more recently, and we aimed to avoid any artifacts of selection caused by a laboratory diet, which likely exists in more established lines like the Drosophila Genetic Research Panel. We detected significant differences in survivorship across genotypes (**Figure 3.5**, **Table 3.1**; Genotype: $p <$

0.0001). Females were significantly more likely to survive an infection on the yeast-enriched diet but the beneficial effect of yeast was genotype-dependent (**Table 3.1**; Diet: $p < 0.0001$; Genotype*Diet: $p = 0.000123$). Across all six genotypes, there was a significant effect of mating status on the low-yeast diet (**Figure 3.5**, LR test, $p < 0.05$). However, reduced survivorship in mated females was only statistically detectable in 3 of the 6 genotypes on the high-yeast diet, although we were unable to detect a significant three-way interaction between 'Genotype', 'Status', and 'Diet' (**Figure 3.5**, **Table 3.1**; $p = 0.625$).

A high-yeast diet significantly improved resistance to infection as measured by bacterial load per female (**Figure 3.6**, **Table 3.2**; Diet: $p = 0.0001$). Consistently, we also observed that the beneficial effect of diet was genotype-dependent (**Table 3.2**; Genotype*Diet: $p = 0.0012$). We were unable to detect a significant effect of mating 'Status' nor an interaction between mating 'Status' and 'Diet' on bacterial number (**Table 3.2**; Status: $p = 0.653$; Status*Diet: $p = 0.221$), presumably due to the high variability in the pathogen load data (**Figure 3.6**). As in the survivorship analysis, we were unable to detect a significant three-way interaction between 'Genotype', 'Status', and 'Diet' (**Table 3.2**; $p = 0.201$).

Table 3.1
ANOVA results from Cox proportional hazards model:
Survivorship ~ Genotype*Status*Diet + (1|Replicate).
Fixed effects: 'Status' (mated or unmated), 'Diet' (high- or low-yeast), and 'Genotype'. Random effects: 'Replicate' for the five experimental replicates of the experiment. All flies considered in this model are infected.

	log-likelihood	Chi sq	Df	p-value
Null	-7996.8			
Genotype	-7745.1	503.3	5	< 0.0001
Status	-7704.0	82.24	1	< 0.0001
Diet	-7669.3	69.35	1	< 0.0001
Genotype:Status	-7667.9	2.911	5	0.714
Genotype:Diet	-7655.2	25.27	5	0.000123
Status:Diet	-7652.4	7.770	1	0.00531
Genotype:Status:Diet	7649.6	3.492	5	0.625

Table 3.2
ANOVA results linear mixed-effects model:
Bacterial load ~ Genotype*Status*Diet + (1|Replicate)
Fixed effects: 'Status' (mated or unmated), 'Diet' (high- or low-yeast), and 'Genotype'. Random effects: 'Replicate' for the four experimental replicates of the experiment.

	Df	F-value	p-value
Intercept	1	437.2	< 0.0001
Genotype	5	34.21	< 0.0001
Status	1	0.202	0.6530
Diet	1	16.47	0.0001
Genotype:Status	5	1.073	0.3740
Genotype:Diet	5	4.069	0.0012
Status:Diet	1	1.504	0.2205
Genotype:Status:Diet	5	1.453	0.2013

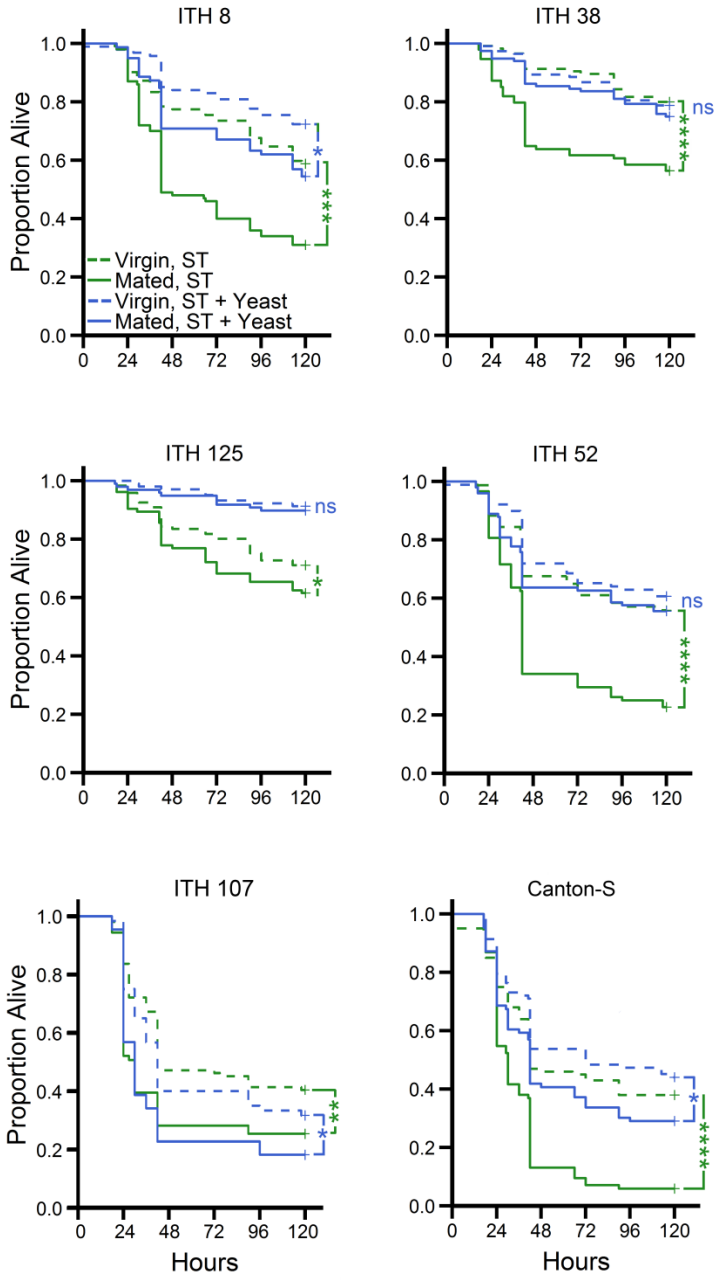

Figure 3.5
Infection survivorship is mediated by genotype, diet, and mating status Females were placed onto a low yeast (green lines) or high yeast (blue lines) diet after eclosing and remained on the medium throughout the experiment. Mated females were provided with Oregon-R males. See Table 3.1 for full model statistics. N = 90-125, five replicates
'*' $p<0.05$; '**' $p<0.01$; '***' $p<0.001$; '****' $p<0.0001$

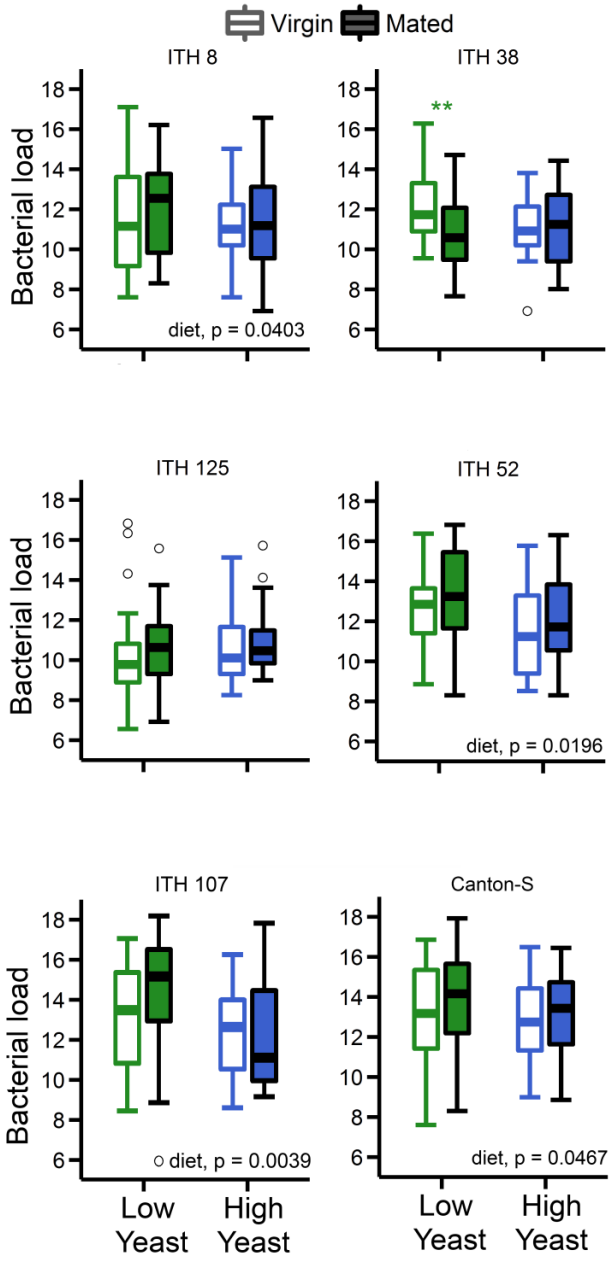

Figure 3.6
Bacterial load of individual females across genotypes, diets, and mating status Bacterial load is the natural log of the number of viable bacteria (colony forming units, or CFU) obtained from each individual female 20 hours after infection. Females were placed onto a low yeast (green) or high yeast (blue) diet after eclosing and remained on the medium throughout the experiment. Mated females were mated to Oregon-R males. Within-genotype comparisons were tested using a Wilcox test and significant results are indicated; no significant effect of mating status was detectable. See Table 3.2 for full model statistics. N = 40 ± 10, four replicates. '**' $p<0.01$

Testing for a diet-dependent evolutionary trade-off

We tested for an evolutionary trade-off between reproduction and immunity, in the form of a negative correlation between the traits across several genotypes, and we evaluated whether resource supplementation eliminated the trade-off. Yeast supplementation uniformly improved fecundity across the six genotypes (ANOVA, Diet: $F_{1, 163} = 135.6$, $p < 0.0001$; Genotype: $F_{5, 163} = 1.32$, p = ns; Genotype*Diet, $F_{5,163} = 1.22$, p = ns) (**Figure 3.7**). Infection survivorship presented more obvious patterns than bacterial load data and therefore survivorship was utilized as the immune metric to test against fecundity. Average values for uninfected fecundity within a genotype were not significantly correlated with infection survivorship on the low-yeast diet (Spearman's correlation, $r = -0.26$, $p = 0.62$) or on the high-yeast diet (Spearman's correlation, $r = -0.31$, $p = 0.54$) (**Figures 3.7**). However, if CS is excluded, the correlation on the low-yeast diet strengthens to $r = -0.7$ but the correlation is still not significant ($p = 0.19$). Removing CS from the high-yeast dataset does not improve the strength of the correlation.

Essential amino acids improve infection survivorship

Dietary yeast is the primary source of proteins, lipids, and trace minerals, and we reasoned that the benefit might come from increased dietary provisioning of any of these. We investigated which macro- or micronutrient within yeast supports a stronger host response during infection. We assumed that the beneficial effect of yeast observed in the previous section (**Figure 3.5**) was not attributed to carbohydrates, given that higher dietary glucose levels typically have a negative effect on host

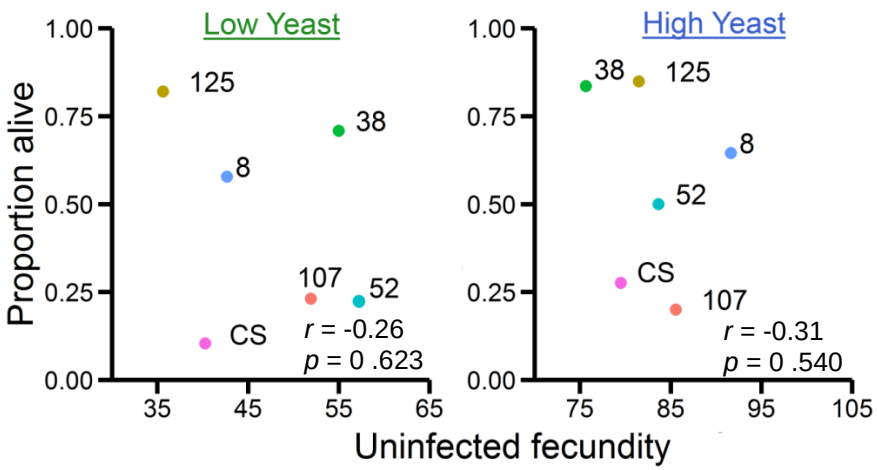

Figure 3.7
Relationship between infection survivorship and uninfected fecundity
Values for proportion alive are from the data in Figure 3.5. Labels next to points refer to the genotype name. These values are plotted against the average number of eggs laid for each genotype. Spearman's correlation is presented. N = 15-25, one trial for egg laying assay.

defense (Howick and Lazzaro, 2014; Unckless et al., 2015). Based on the work of Lee and Micchelli (2013), we supplemented a low-yeast diet with a pre-defined mixture of amino acids or micronutrients (**Tables 3.3, 3.4**); the nutrient mixtures (Harlan Laboratories) and have predefined proportions of nutrients. On these diets, we tested whether females have improved infection survivorship (**Figures 3.8A, B, 3.9A)** and resistance to infection (**Figure 3.9B**).

We found that supplementing the diet with a mix of vitamins and minerals (**Figures 3.8A, Table 3.3**) or with a mix of non-essential amino acids (NAA; **Figure 3.8B**) failed to improve infection survivorship in Canton-S females. Females that received a micronutrient-enriched diet were as susceptible to infection as females on a low-yeast diet and there was no significant effect of varying the concentration of micronutrient mix in the diet (Cox prop. hazard, Diet: X_3^2 = 3.25, p = ns).

Micronutrient supplementation affected mated and virgins females similarly and did not remove the immunosuppressive effect of mating (Cox prop. hazard, Status*Diet: X_3^2 =0.653, p = ns). Similarly, NAA supplementation did not improve infection survivorship relative to the unsupplemented diet (Cox prop. hazards, Diet: X_1^2 = 0.218, p = ns) (**Figure 3.8B**). The addition of NAA to the diet did not alleviate the effect of mating on immunity (Status: X_1^2 = 9.41, p = 0.00216; Status*Diet: X_1^2 = 0.0025, p=ns).

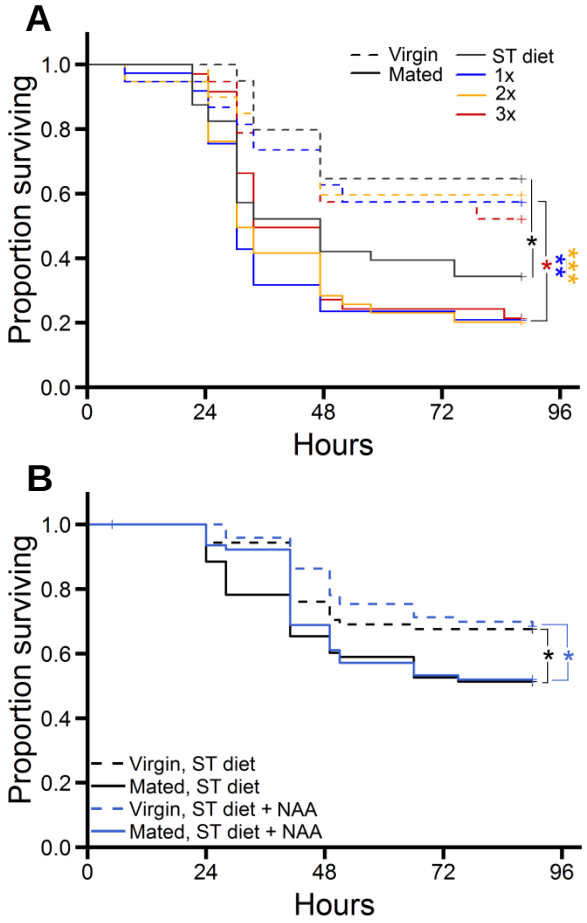

Figure 3.8
Effect of dietary supplementation on infection survivorship
(A) Micronutrient supplementation. See Table 3.3 for vitamins and minerals added to the low-yeast diet (ST). Increasing the concentration of micronutrients 1X (blue), 2X (yellow), 3X (red) did not significantly affect female survivorship. N = 30 ± 10, one experimental trial. **(B)** Non-essential amino acid supplementation. See Table 3.4 for non-essential amino acids (NAA) added to the low-yeast diet (ST). There was no significant effect of diet on survivorship. N = 76 ± 3, one experimental trial.
'*' p<0.05 '**'; p<0.01;
'***' p<0.001

We found that supplementing a low-yeast diet with essential amino acids (EAAs) significantly improved infection survivorship (Cox prop. hazards, Diet: X_1^2 = 10.8, p = 0.00103), and the immunosuppressive effect of mating persisted on the EAA diet (LR, X_1^2, p = 0.00217) (Cox prop. hazards, Status: X_1^2 = 32.4, p<0.0001; Status*Diet: X_1^2 = 0.870, p = ns) (**Figure 3.9A**), similar to the pattern observed for Canton-S females on a high-yeast diet. EAA-supplementation did not affect bacterial load (ANOVA, Diet: $F_{1, 780}$ = 1.296, p = ns; **Figure 3.9B**). Additionally, unmated females were more resistant to infection on both diets (ANOVA, Status: $F_{1, 780}$ = 10.64, p = 0.00128; Status*Diet: $F_{1, 780}$ = 0.330, p = ns). Moreover, females that consumed an EAA-supplemented diet before and after mating were more fecund than females on the low-yeast diet (Welch's t-test, $t_{23.21}$ = 3.51, p = 0.00187) (**Figure 3.9C**).

Table 3.3 Micronutrients included in prepackaged vitamin and mineral mix Harlan Laboratories, TD.10475

Vitamin & Mineral Mix	
Biotin	Potassium Phosphate, dibasic
Calcium Chloride	Potassium Phosphate, monobasic
Calcium Pantothenate	Pyridoxine HCl
Choline Bitartrate	Riboflavin
Chromium Potassium Sulfate, dodecahydrate	RNA
Cupric Carbonate	Sodium Chloride
DNA	Thiamin HCl
Ferrous Sulfate, heptahydrate	Vitamin A Palmitate
Folic Acid	Vitamin B12
Inositol	Vitamin D3, cholecalciferol
Magnesium Sulfate, heptahydrate	Vitamin E, DL-alpha tocopheryl acetate
Manganese Sulfate, monohydrate	Vitamin K, MSB complex
Niacin	Zinc Carbonate
p-Aminobenzoic Acid	

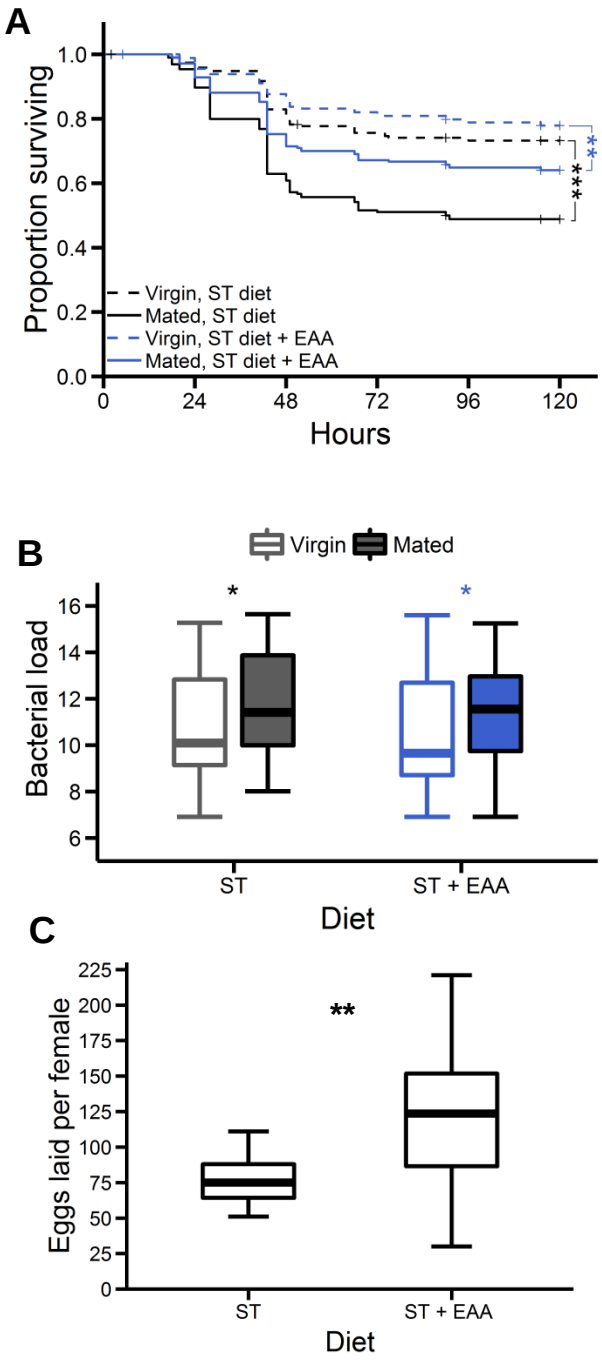

Figure 3.9
Essential amino acids improve immunity and fecundity
Effect of essential amino acid (EAA) supplementation versus standard diet (ST) on: **(A)** Infection survivorship; N = 195 ± 15, three replicates. **(B)** Bacterial load is the natural log of colonies within an individual female 20 hours after injection; N = 56 ± 14, two replicates. **(C)** Uninfected fecundity. Eggs laid per female over the course of five days; N = 20, one trial. '*' $p<0.05$ '**' $p<0.01$; '***' $p<0.001$

To investigate whether any single amino acid is responsible for the beneficial effect of EAAs, we mated and infected females who had fed on diets supplemented with one of the ten essential amino acids. Supplementation with specific EAAs significantly altered patterns of female infection survivorship (**Figure 3.10**). Survivorship curves of females on an EAA-supplemented diet were compared to females on a diet containing an excess of all ten EAAs and to a low-yeast diet without EAA supplementation. Arginine, lysine, and threonine, methionine, and histidine did not improve survivorship relative to females that did not receive EAAs ($p > 0.05$) whereas tryptophan, phenylalanine, leucine, isoleucine, and valine significantly improved survivorship relative to females on the supplemented diet ($p < 0.05$).

Table 3.4 Essential and non-essential amino acids included prepackaged mix
Harlan Laboratories, (TD.10473, TD.110036)

Essential Amino Acid Mix	Non-essential Amino Acid Mix
L-arginine HCl	L-alanine
L-histidine HCl-H_2O	L-asparagine
L-isoleucine	L-aspartic acid
L-leucine	L-cystine
L-lysine HCl	L-glutamic acid
L-methionine	L-glutamine
L-phenylalanine	Glycine
L-threonine	L-proline
L-tryptophan	L-serine
L-valine	L-tyrosine

DISCUSSION

We investigated whether dietary supplementation alleviates costs of reproduction and immunity in female *D. melanogaster*. We detected significant costs

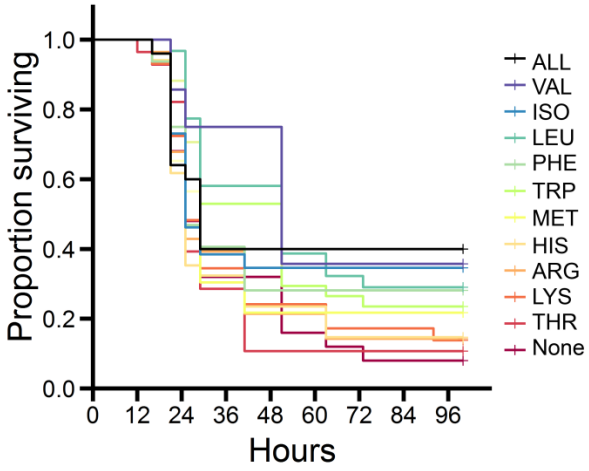

Figure 3.10
Individual essential amino acids have varying effects on infection survivorship Tryptophan (TRP), Phenylalanine (PHE), Leucine (LEU), Isoleucine (ISO), and Valine (VAL) significantly improved survivorship relative to females on standard food without amino acid supplementation (p<0.05). All females were mated. N = 25-30, one trial.

associated with immune activation and reproduction. Across genotypes, we found a stronger correlation between resistance to infection and fecundity on a resource-limited diet. Moreover, we found strong evidence that dietary supplementation can improve host defense and even eliminate the immunosuppressive effect of mating, although both effects are genotype-dependent. Lastly, we found that essential amino acids within the diet significantly benefit host immunity.

Our findings revealed that both constitutive and inducible immunity come at a cost to reproduction. Our results supplement a growing body of literature demonstrating that immunity can impact other life-history traits, (e.g., Moret and Schmid-Hempel, 2000; Armitage et al., 2003). The disruption of immune signaling promoted higher fecundity while the induction of the immune response had severe consequences for egg production, thus demonstrating an inherent and antagonistic

interaction between the two processes.

We provide further evidence that mating and the production of eggs has severe consequences for host immune defense. When egg production was arrested via mutations in *ovo* or *bam*, females survived an infection with *P. rettgeri* significantly better than controls and the effect of mating on immunity was eliminated (**Figure 3.2**). Although we did not detect a significant difference in immune response between mated and unmated Ovo^{D1} females, Fedorka et al. (2007) observed that a mutation in *ovo* did not eliminate the immunosuppressive effect of mating when an infection occurred shortly after mating. The inconsistencies are somewhat puzzling, but the fact that our studies did not use the same fly strains (Bloomington, #1309 (this study) vs. #2121), which differ in their genetic background and the nature of the *ovo* mutation (chemically-induced vs P-element-induced) may explain the observed differences between the two studies. Moreover, it has been noted that the P-element within the stock used by Fedorka et al. (2007) is unstable and that sterility is not fully penetrant (Bloomington Drosophila Flystock Center, 2016).

A previous study from our lab used germline-less females (daughters of *tudor* mothers) to test immunocompetency in the absence of egg production, and also observed that eliminating egg production prevented mating-induced immune suppression (Short et al., 2012). Taken together with my results, the data indicate that eliminating mature oocytes through mutation in three different genes strongly supports a model in which egg production negatively impacts immunity, potentially underlying the immunosuppressive effect that mating has on immune induction.

Trade-offs can arise from both resource allocation and inadequate resource

acquisition (Boggs, 2009). Based on previously developed techniques that have utilized a traceable dye to measure food intake (amount of food in the gut) or output (amount excreted) (Ayres and Schneider, 2009; Cognigni et al., 2011; Apger-McGlaughon and Wolfner, 2013), we developed an experimental setup that uses the amount of a traceable dye within an individual's digestive tract and within their excreta as a proxy for the amount of food ingested during a given period (**Figure 3.3**). As confirmation of the technique, we consistently detected a positive effect of mating on food consumption, consistent with previous work (Carvalho et al., 2006). Our findings suggest that neither an infection with *P. rettgeri* nor wound repair alter nutrient acquisition. Moreover, we consistently found that mated females are consuming more food relative to unmated females. Collectively, these findings imply that reproduction does not result in immune suppression through a failure to acquire nutrients.

Despite consistently consuming more food than virgins, mated females remain more susceptible to infection. Previous studies that have examined the impact of infection on food intake often observe significant reductions during an immune response (Adamo et al., 2007; Ayres and Schneider, 2009; Povey et al., 2009, 2014; Bashir-Tanoli and Tinsley, 2014). While this effect may seem counterintuitive given the metabolic requirements required for immunity, illness-induced anorexia is now considered a host response, suggesting it may be adaptive (Exton, 1997). Although it is plausible that the stimulatory effect of mating on food ingestion overrides any anorexia-induced immune responses, it seems unlikely that this underlies our observed pattern of infection susceptibility given that anorexia does not make hosts more

resistant to infection (Adamo et al., 2007; Ayres and Schneider, 2009).

Patterns of illness-induced anorexia are highly variable across studies (see Adamo et al., 2007; Ayres and Schneider, 2009; Povey et al., 2014; Bashir-Tanoli and Tinsley, 2014). The variable pattern observed may be a consequence which insect species, pathogen, dietary medium, or methodology was used within the study. For instance, illness-induced anorexia, despite being a host response, is not uniformly observed across all bacterial infections in *Drosophila* (Ayres and Schneider, 2009). Thus, the variability observed among studies may be attributed to different pathogen pressures. Second, several studies have established that the quantity of food consumed depends on the ratio of protein-to-carbohydrate (e.g., Povey et al., 2009; Mason et al., 2014; Rho and Lee, 2014), and this pattern changes during infection (Povey, et al., 2014). We only tested 50:50 ratio protein-to-carbohydrates and the provided ratio or the concentration of nutrients that we provided may not have permitted a decrease in food consumption during infection. Finally, our method quantified the amount of food consumed during 18 hours, which is likely to mask any effects that occur over a small temporal scale. Feeding behavior is often measured for less than 30 minutes (e.g., Adamo et al., 2007; Ayres and Schneider, 2009, but see Bashir-Tanoli and Tinsley, 2014), and observed reductions in food intake may be behaviorally-driven (e.g., slow to initiate eating), thus missing long-term pattern. An experiment that evaluates food consumption at several time points during an infection, across a spectrum of diets, and with a panel of bacteria will be required to ascertain the importance of illness-induced anorexia and to determine whether the absence of it in our study is meaningful.

While the results from our feeding assay suggests that allocation of nutrients,

rather than acquisition, is the strongest candidate for observed immunological costs of mating, there is one final way that acquisition could mediate the trade-off between reproduction and immunity. Neither reproduction nor infection impaired a female's ability to behaviorally acquire nutrients. However, despite consuming more food, mated females may not have been physically able to acquire enough resources to support both an immune response and reproduction (i.e., allocation based-tradeoffs can arise when individuals fail to obtain enough nutrients). A failure to acquire enough resources could occur if the diet that we provided did not offer an appropriate concentration of protein (or ratio of protein-to-carbohydrate) and females are limited in their ability to ingest nutrients. Finally, an increase in the acquisition of a beneficial nutrient, such as protein, requires a higher consumption of carbohydrates because the two resources are mixed within one diet, which has been noted to differentially alter phenotypic traits (Lee et al., 2008a). Therefore, a diet that is not heavily supplemented with protein may promote higher food intake and the ingestion of more sugar, thus negatively affecting resistance to infection (Howick and Lazzaro, 2014; Unckless et al., 2015).

Dietary supplementation with yeast promotes immune defense and reproduction, consistent with a resource-mediated trade-off model. When females received a supplemented diet, we detected significant improvements in resistance to *P. rettgeri* and infection survivorship. Moreover, females on the supplemented diet were less likely to succumb to infection after mating. Patterns within our bacterial load data were much more difficult to identify. While a significant effect of diet and genotype emerged from the data (**Table 3.2**), we failed to detect a significant effect of mating

status in the pooled data and within a single genotype. While previous findings indicate that mating lowers resistance to *P. rettgeri* (Short and Lazzaro, 2010; Short et al., 2012; Chapter 2), we cannot fully assess whether dietary supplementation improves resistance in mated females without a significant effect of mating. Diet can differentially affect resistance (the ability to reduce pathogen growth) and tolerance (the ability to curb the negative fitness effects of a pathogen) (Howick and Lazzaro, 2014; Kutzer and Armitage 2016). Thus, while dietary supplementation can improve resistance to infection irrespective of mating status, our findings suggest that diet may disproportionately affect infection survivorship. In other words, diet appears to have a stronger impact on host tolerance in mated females. However, it is apparent that the data are excessively noisy which prevents us from confidently drawing any conclusions (**Figure 3.6**).

It is worth highlighting a recent study by Kutzer and Armitage (2016), who also evaluated how dietary protein affects resistance and tolerance in *D. melanogaster*. The authors found that protein limitation did not significantly affect resistance to infection, but that it did affect fecundity. Moreover, a lower yeast diet improved tolerance relative to a standard yeast diet, which is in sharp contrast to the present study. Pathogen-type is likely to explain the apparent differences, given they observed a beneficial effect of yeast restriction on tolerance during an infection with *Escherichia coli* but not *Lactococcus lactis*. Interestingly, the authors utilized fecundity tolerance for which they detected a significant effect of diet whereas others found largely no impact of diet on fecundity tolerance (Howick and Lazzaro, 2014). These findings in combination with ours highlight that the relationship between

resistance, tolerance, and diet is less than straightforward and is dependent upon the phase of infection and the metric used to describe tolerance (Howick and Lazzaro, 2014; Kutzer and Armitage, 2016; this study). Despite these complications, our results still suggest a resource-based trade-off model whereby reproduction and immunity are energetically demanding and the addition of more resources to an individual's resource pool improves overall defense. Additionally, we found genetic variation for both resistance and the ability for diet to alter resistance. Finally, we find that dietary supplementation can override costs of reproduction and improve immune defense through both resistance and tolerance mechanisms in a genotype-dependent manner.

Across genotypes, we failed to detect a significant interaction between reproduction and immunity; however this was likely attributable to the small number of lines evaluated in this study. Notably, eliminating Canton-S from the dataset increased the correlation between uninfected fecundity and infection survivorship on the resource-limited diet. The detection of a stronger correlation between traits on a nutrient-limited diet is consistent with previous findings and highlights how competition between traits may be resource-based (McKean et al., 2008). Canton-S has been utilized in laboratory experiments for a significant number of generations and may be atypical in many physiological traits, including metabolic indices (Reed et al., 2010) making it justifiable to consider the correlation between the traits independently of this genotype. Our findings highlight how trade-offs may be hard to detect in a laboratory environment where resources are rarely limiting. Moreover, we find that not all genotypes can benefit from changes in resource availability, thus highlighting the potential for co-dependent trait evolution.

We aimed to identify the component within yeast that explains how dietary supplementation benefits immunity and reproduction. Closer inspection revealed that essential amino acids (EAAs) rather than non-essential amino acids, vitamins, or minerals may restrict immunity and reproduction. Under EAA-enriched conditions, females produced more eggs and were more likely to survive an infection with *P. rettgeri*. However, we were unable to detect a significant improvement in resistance. Interestingly, the amino acids that promoted the greatest increase in survivorship are all branched-chain amino acids. Branched-chain amino acids are important due to their stimulatory effect on protein synthesis (Kimball and Jefferson, 2006) and because they provide the alpha-amino group necessary for the production of glutamine, which is a major source of energy for immune cells (Wu et al., 1991). Notably, a number of studies in mammalian systems find a strong link between branched chain amino acids and immune function (reviewed in Li et al., 2007). For instance, mice fed a diet lacking branched-chain amino acids were more susceptible to *Salmonella typhimurium* (Petro and Bhattacharjee, 1981) and human patients who received branched-chain amino acids during sepsis were more likely to survive (Freund et al., 1978).

At the molecular level, branched-chain amino acids, particularly leucine, are stimulators of TOR (Target of Rapamycin) signaling across animal species (Dann and Thomas, 2006). TOR signaling integrates internal and external cues in order to assess how the organism should respond (Katewa and Kapahi, 2011), and is thus the most critical regulator of homeostasis. TOR signaling has been implicated in immune signaling since the initial discovery of the pathway (Heitman et al., 1991). In *D. melanogaster*, infection and the activation of the immune system alters both TOR and

Insulin/Insulin-like Signaling (IIS), which the authors hypothesize is a way to reallocate resources toward immunity (DiAngelo et al., 2009). Moreover, expression of *mos*, a positive regulator of TOR, is positively correlated with resistance to infection (Felix et al., 2012). Thus, branched amino acids may be positive regulators of immunity through the TOR signaling pathway.

Recently, Williams et al. (2015) revealed that while genotype is the most significant factor contributing to observed differences in nearly all metabolic indices in *D. melanogaster*, both leucine and isoleucine levels show significant genotype-by-diet effects. Given the aforementioned link between amino acids and TOR signaling, this finding suggests that both genotype and diet could strongly influence how organismal status is perceived and maintained. Thus, variation in internal resource pools among females could explain the significant genotype-by-diet effects that we detected in our study.

Resource allocation arises when two traits compete for energy or a shared nutrient, and because the resource is limited, the organism responds by allocating resources into one or the other (van Noordwijk and de Jong, 1986). Our data support a resource-allocation based trade-off between reproduction and immunity because they suggest that an increased resource pool (specifically essential amino acids) will promote a stronger immune response and greater fecundity. Thus, amino acids could be the shared resource that links the two traits and when essential amino acids are not limiting immunity and reproduction do not trade off. However, there are important caveats that suggest a model of resource allocation does not fully explain the trade-off between reproduction and immunity. First, while egg production in insects is strongly

stimulated by essential amino acids (O'Brien et al., 2002), evidence thus far suggests that methionine is the most critical amino acid for egg production (Lee et al., 2014). While methionine-fed flies were more likely to survive an infection than unsupplemented females in our experiments, this difference was not statistically significant (**Figure 3.10**). While the present study cannot address whether a single amino acid restores both immunity and reproduction, it is an essential piece of information that will be required to convincingly demonstrate that the trade-off operates via resource allocation. Future studies would benefit from the use of isotope-labeled amino acids for an analysis of metabolic flux between the two processes (see Zamboni et al., 2009).

Second, although supplementation with yeast or EAAs improves overall host performance the effect of mating is not erased in several genotypes including Canton-S. Thus, individuals may vary in their resource pools and may require different amounts of supplementation to overcome resource-based trade-offs between traits. However, mechanisms that are more complex may also be at play. For instance, in Chapter 2, I showed that females are immunocompromised after mating via the endocrine signaling molecule, juvenile hormone (JH). JH is an important component of the insulin/insulin-like signaling pathway (Tu et al., 2005; Mirth et al., 2014) and thus it may be molecularly well-placed to act as the regulator that dictates where and when nutrients should be reallocated. Thus, there could be variation in the effectiveness of reallocation via a JH-mediated mechanism. Alternatively, JH could negatively impact immunity in a resource-independent manner (i.e. diet sufficiently benefits immune signaling in a way that is not driven by the actual movement of

acquired nutrients into reproduction versus immune signaling). As highlighted above, our inability to pinpoint a specific molecular mechanism that unites reproduction and immunity is a consequence of not knowing the exact movement of resources between the two processes.

Finally, while females have a strong investment in reproduction after mating, our results and a number of other studies (reviewed in Schwenke et al., 2016) suggest that immune induction and infection uniformly suppresses reproductive activity. Therefore, the 'mated' state could make females more prone to infection which in turn compromises their ability to produce offspring. The model is as follows: mating increases reproduction which suppresses constitutive levels of immunity or the inducibility of the immune response, thus allowing an infection to take over when present. This then causes a female to enter a state of stress which shuts down reproductive activity leading to subpar levels of immunity and reproduction. Thus, if a female were to survive the infection one would hypothesize that she has higher levels of constitutive immunity or a more inducible immune response which would come at the expense of fecundity. However, under nutritionally-rich conditions females may be able to avoid this cost if they are able to respond to the additional surplus of nutrients, which is detectable in some genotypes.

CONCLUDING REMARKS

Here we demonstrate significant and clear costs of immunity and reproduction as well as the ability for dietary environment to override these apparent costs in a genotype-dependent manner. We reveal that the ability to acquire resources is unlikely

to explain the interaction between the traits, but instead propose a model of resource allocation. By altering individual components of the diet, we revealed that essential amino acids benefit both reproduction and immunity and could be the limiting-resource that constrains the expression of the two traits. Collectively, the results highlight how trade-offs are context-specific and provide the basis to explore how metabolic signaling produces allocation decisions within and among individuals.

EXPERIMENTAL PROCEDURES

***D. melanogaster* strains and husbandry** Unless otherwise stated, Canton-S flies were utilized for the experiment. Oregon-R and IMD-null (OreR; IMD-null) were obtained from David Schneider. Isofemale lines were founded from wild-collected females in Newfield, New York in 2004 (Lazzaro et al., 2008). In experiments using isofemale lines, Oregon-R males were used to produce the 'Mated' females. Ovo^{D1} females are the progeny of Canton-S females and Ovo^{D1}, v^{24}/Y males (#1309, Bloomington). *bam* females are the progeny of *w;* $bam^{\Delta 59}$, e^1/*TM3*, e^1 and *w;* $bam^{\Delta 86}$, e^1/*TM3*, e^1 (stocks from H. Flores 2013). w^{1118}; e^1 background was created by crossing w^{1118} females to e^1 males. The resulting progeny were crossed and w^{1118}; e^1 siblings were propagated for five generations prior to being utilized in the experiment; the stock is unlikely to be isogenic. All flies were reared on a glucose medium (8.3% glucose, 8.3% Brewer's yeast, 1% agar, 0.04% phosphoric acid and 0.4% propionic acid) and maintained at 25°C. All flies were 5-7 days old at the time of the experiment.

We found that females of all genotypes used in this study mated very rapidly, and thus we opted to conduct all mating experiments in mass by placing 10-15 females

with ~30 males for 6 hours. Afterwards, males and females were anesthetized briefly, males were discarded, and females were injected (or left untouched for egg-laying experiments) and placed into a new food vial.

Immune phenotyping A strain of the Gram-negative bacterium *Providencia rettgeri* that was isolated from a wild-caught fly (Juneja and Lazzaro, 2009) was used for all infection assays. Cultures of *P. rettgeri* were grown overnight in liquid Luria Broth (LB) while shaking at 37°C. Cultures were spun down at 4000 rpm, the supernatant was removed, and then the bacteria were resuspended in PBS. Bacterial suspensions were diluted to an OD_{600} = 0.05, which corresponds to 600 - 1200 CFUs per fly. Heat-killed bacterial suspensions were prepared similarly, but were heated to 75°C for 1 hour and then diluted to an OD_{600} = 1.0.

All injections were performed using a pulled capillary needle mounted on a Nanoject II apparatus (Drummond Scientific) and each female received 9.2nl of medium into the thorax. Females were placed into fresh food vials after receiving a treatment and allowed to recover from injection and CO_2 exposure. Females were housed in groups of ~15 individuals and placed at 25°C.

Individuals that died within 8 hours were removed from dataset given that the death of these flies preceded the onset of a full-blown bacterial infection (i.e., their death was due to handling). Survivorship was noted 2-3 times per day for five days. Bacterial load was assayed at 20 hours which precedes the first bout of death caused by infection. Females were anesthetized on CO_2 and placed singly into 1.5ml microcentrifuge tubes with 500 ul of sterile PBS and a small metal ball. Tubes were placed in a linear motion homogenizer (OPS Diagnostics), and flies were

homogenized to release the bacteria into the solution. A 1:100 dilution of the samples was performed and 50 ul of the homogenates were plated on standard LB agar plates using a WASP 2 spiral plater (Microbiology International, Bethesda, MD, USA). Plates were incubated overnight at 37°C and the resulting colonies were counted using a ProtoCOL plate counting system (Microbiology International). Controls flies were injected with sterile PBS, and the plates from these individuals never yielded any colonies.

Dietary medium Fresh food was prepared for each experimental replicate. Glucose (5%), agar (1%) and acid mix (0.04% phosphoric acid and 0.4% propionic acid) remained constant across the following experimental diets. The 'Low Yeast' diet consisted of 5% Brewer's yeast and 'High Yeast' diet contained 10% Brewer's yeast. We altered specific components in the diet based on the work of Lee and Micchelli, 2013.Vitamin and mineral-enriched media contained 5% Brewer's yeast with the addition of 3.2 g/L (1X), 6.4 g/L (2X), or 9.6 g/L (3X) (**Table 3.3**). Essential amino acid media contained 5% Brewer's yeast with the addition of 1% EAA mix (**Table 3.4**). Non-essential amino acid media contained 5% Brewer's yeast with the addition of 1% NAA mix (**Table 3.4**). Diets containing one of ten essential amino acids were prepared by dissolving each amino acid in distilled water and adding the solution to food that had been cooled to 55°C with a final concentration of 1g/L. Media containing all ten EAAs had 1g/L of each amino acid.

Food intake and processing The dietary medium contained 8% glucose, 8% Brewer's yeast, 1% agar, 0.1% Bromophenol blue and 0.5% Xylene cyanol (added from a 20X solution). Caps of 1.5ml microcentrifuge caps were cut off of tubes and

dyed food was dispensed into the cap until flush with the top (~400ul). Caps were stored flat and in a plastic bag at 4°C for no more than 24 hours to prevent desiccation.

Unmated females were housed in groups of 15 individuals until they were 5 days old. Mated and unmated females were exposed to CO_2 only, injected with PBS, or injected with bacteria. After treatment, females were placed singly into a 1.5 ml microcentrifuge tube missing the cap and provided a dyed-food cap. Tubes with females were kept in microcentrifuge racks at 25°C. After 18 hours, flies were kept in their tube and anesthetized on ice (dead flies were excluded from the study). Dyed-food caps were discarded, and one metal ball and 100ul of PBS were added to each tube. Each tube received a clean, unfilled cap and tubes were homogenized for 3 minutes. After homogenization, tubes were spun at 10,000 rpm for 5 minutes and 80 ul of supernatant were added to a 96-well plate. Absorbance readings were measured with at 614nm.

Fecundity assays To measure uninfected fecundity, females were mated then placed singly into a vial with food after a brief exposure to CO_2. Females were transferred into a new food vial every 24 hours and eggs were counted daily for five days.

To measure how the immune system influences egg output, recently mated females were either injected with 9.2nl of sterile PBS, heat-killed *Providencia rettgeri*, or live *P. rettgeri*. Immediately after receiving an injection treatment, females were placed singly into a vial with food. Females were transferred into a new food vial every 24 hours and eggs were counted daily for five days. Females that died from the live infection were censored from the study to separate costs of infection from costs associated with death (N = 3).

Axenic fecundity assay Axenic flies were created by suspending eggs in a 10% hypochlorite solution for 10 minutes and transferring eggs to sterile food in a laminar flow hood. Unmated females were collected under CO_2 exposure on a pad that was bleached and treated with 95% ethanol, and housed in sterilized glass vials with sterile food. Females were mated to Oregon-R males that had been reared axenically. After mating, females were placed singly into sterile food vials and transferred daily to new vials in the laminar flow hood. Eggs were counted for five days. Homogenates from axenic flies did not produce any bacterial colonies on MRS plates (N = 5).

Statistical analysis All statistical analyses were conducted in R (R Core Team, 2015). The 'survival' package was used for survival analyses, and the 'coxme' package was used to model survivorship with 'Replicate' as a random variable using Cox proportional hazard model. Analysis of variation was then performed on the mixed-effects model. The 'survdiff' function was used to test for significant differences between survivorship curves with a log-rank test (LR).

Effects of treatment within egg laying and bacterial load assays was tested with Welch's t-test. A Kruskal-Wallis test was used to test for a significant effect of inoculation regime given that the data were not normally distributed within each treatment. Bacterial load data from the panel of genotypes on 'High Yeast' vs 'Low Yeast' diets Type I ANOVA was modeled with 'Replicate' as a random variable ('nlme' package), and an analysis of variation was then performed on the mixed-effects model.

CHAPTER 4
SEXUALLY TRANSMITTED INFECTIONS: FITNESS CONSEQUENCES AND MEDIATORS OF SUSCEPTIBILITY

ABSTRACT

Copulation provides an opportunity for close contact with potentially infected individuals. Similar to vertebrate taxa, insects host a diverse assortment of sexually transmitted parasites. During the evolutionary history of a species, repeated exposure to pathogens places a strong selective pressure on host defense. In female *Drosophila melanogaster*, mating induces the expression of antimicrobial peptides; yet, mating promotes susceptible to a systemic bacterial infection. Prior findings suggest mating-induced antimicrobial peptide expression is strongest in reproductive tissues. Thus, we hypothesized that the local induction of an immune response may be an evolved response which could prevent the establishment of a sexually transmitted infection.

There are no known naturally occurring, sexually transmitted bacterial infections described in *D. melanogaster*. Thus, we explored the fitness consequences of a bacterial infection and the reasons for susceptibility by applying *Serratia marcescens* to male genitalia to provide an artificial sexually transmitted infection to females, a technique that has proven successful by Miest and Bloch-Qazi (2008). Females who mated with contaminated males had reduced fecundity and greater mortality. Although female immunity reduced susceptibility to infection, we did not find strong evidence to support a protective effect of mating-induced antimicrobial peptides within reproductive tissues. Our work highlights how opportunistic pathogens may influence host fitness and provides new evidence for direct costs of reproduction.

INTRODUCTION

Animals harbor a diversity of microbes many of which can impose a risk to host health. In situations where individuals physically interact, the likelihood of pathogen transmission increases significantly. As such, the spatial proximity of individuals, the transfer of fluids, and the potential for epithelial injuries during copulation provides an opportune moment for disease transmission.

Disease transmission through sexual contact occurs in all major taxonomic groups, and infectious agents include a range of viruses, bacteria, protozoa, fungi, helminths, and arthropods (Lockhart et al., 1996; Knell and Webberley, 2004). Among humans, there are at least eight sexually transmitted infections (STIs) caused by bacteria (Lockhart et al., 1996). However, there are a strikingly low number of described STIs caused by bacteria among insects despite evidence for transmission of other infectious agents during copulation (Knell and Webberley, 2004).

Despite the apparent scarcity of bacterial STIs among arthropods, two lines of evidence suggest that sexual transmission of bacteria can occur. First, laboratory experiments utilizing an artificially applied STI to male *Drosophila melanogaster* demonstrated that bacteria are transferrable to females during copulation (Miest and Bloch-Qazi, 2008). Second, several studies have found bacteria on the genitalia of male arthropods (reviewed in Otti, 2015), which could elicit an infection in females during mating events (Reinhardt et al., 2003). Therefore, opportunistic pathogens incidentally acquired by females during copulation can pose as a severe health risk.

Sexually transmitted pathogens are responsible for both localized infections in reproductive tissues and systemic infections (Lockhart et al., 1996). For instance, in

humans, gonococcal infections generally localize to reproductive tissues, but in some instances, bacterial cells spread to the blood and form a systemic infection (Ross, 1996). Pathogen virulence and immune activity are predictive of the eventual spread of infection throughout a host's tissues, but infection may spread more easily when epidermal barriers are breached (e.g. mucosal microtraumas during mating in mammals; see Norvell et al., 1984).

Traumatic mating events that make the female hemocoel (circulatory system) easily accessible are well-document in arthropods (Lange et al., 2013). In some arthropod taxa, sclerotized spines cover the male insemination organ (aedeagus), which can pierce the epithelial surface of the genital tract within a female during mating (i.e., 'traumatic penetration'; terminology used by Lange et al., 2013) (Crudgington and Siva-Jothy, 2000; Kamimura, 2007; Kamimura and Polak, 2010; Mattei et al., 2015). In other taxa, including *Drosophila parabictinata* and *Cimex lectularius*, males traumatically inseminate females, a process in which a male pierces a female's abdomen injecting sperm and seminal fluids directly into the hemocoel (Morrow and Arnqvist, 2003; Kamimura, 2007). Lastly, in some arthropods like *D. melanogaster*, the male aedeagus occasionally injures female tissues, however non-inseminating genital structures that are used to grasp and retain females also create extragenital wounds ('traumatic penetration') (Kamimura, 2007). Thus, traumatic mating events can impose significant costs to females due to the risk of infection and resources required for wound healing (Lange et al., 2013).

Among insects, STIs negatively impact fertility and fecundity in all instances where a significant effect has been identified (reviewed in Knell and Webberley,

2004). While the impact of a naturally occurring sexually transmitted bacterial infections, remains untested in insects, evidence from mammalian systems (e.g., *Chlamydia trachomatis* and *Neisseria gonorrhoeae*) suggests that bacterial infections, too, can negatively affect female fertility (Apari et al., 2014). Transmission mode aside, it is well-established that a pathogenic bacterial infection can negatively impact female fecundity and infertility (McKean et al., 2008; Bashir-Tanoli and Tinsley, 2014; Howick and Lazzaro, 2014; Chapter 3). Therefore, the localization of a microbe within the reproductive tract or its spread to hemocoel may have dire consequences for the female fitness.

Insects deploy a suite of antimicrobial peptides to defend themselves against invading bacteria (Lemaitre and Hoffmann, 2007). Intriguingly, mating induces the expression of antimicrobial peptides, in the absence of bacteria, via the male seminal fluid protein, Sex Peptide (Lawniczak and Begun, 2004; Peng et al., 2005b; Mack et al., 2006; McGraw et al., 2008). Despite this immune induction, mated females are less resistant to a systemic bacterial infection (Wigby et al., 2008; Short and Lazzaro, 2010; Short et al., 2012; Chapters 2, 3). However, a localized induction of antimicrobial peptides after mating may explain this apparent disparity. In other words, the expression of several antimicrobial peptides expressed by female reproductive tissues (Ferrandon et al., 1998; Tzou et al., 2000; Mack et al., 2006; Domanitskaya et al., 2007; Kapelnikov et al., 2008) could prevent the establishment of an infection by newly introduced microbes. Besides initiating an immune response within a female, males may take a more direct role in preventing STIs by transferring bactericidal molecules within the seminal fluid to recipient females (Lung et al., 2001;

Otti et al., 2009; Peng et al., 2016). The detection of bactericidal molecules within the reproductive tissues of males and females has led some to hypothesize that the apparent lack of sexually transmitted bacterial infections is a product of host defenses (Knell and Webberley, 2004). Moreover, the findings suggest that females may be primed by mating to respond to STIs.

Despite a wealth of knowledge existing for STIs in mammals, especially humans given concerns for public health, the transmission, fitness consequences, and prevention of STIs in insect systems remains poorly explored. Such infection pressures have the potential to shape the evolution of male-female interactions, immune defense, and reproductive strategies. Using *D. melanogaster*, we investigated whether contaminated males can transmit a bacterial infection to females during mating and we tested for any associated fitness consequences. Additionally, we explored the mechanisms underlying susceptibility in females including how males impact a female's response to the establishment of an acquired infection.

RESULTS

Males can transfer bacteria to females during copulation

We utilized the method of Miest and Bloch-Qazi (2008), and we tested whether bacteria on the male genitalia are transferrable to females during mating. We used a highly virulent strain of *Serratia marcescens,* originally acquired from a wild-caught fly, to assess the potential for bacteria transmission. Infections with *S. marcescens* are highly traceable due to the intense red pigment that the bacterium produces (**Figure 4.1A**). Male flies received an artificial STI composed of a

concentrated dose of bacterial cells ($3.02x10^4 \pm 3.2x10^3$) (**Figure 4.1B**). After mating with bacteria-carrying males $1.86x10^3 \pm 1.6x10^3$ bacterial cells were detectable in or on recipient females (**Figures 4.1C**).

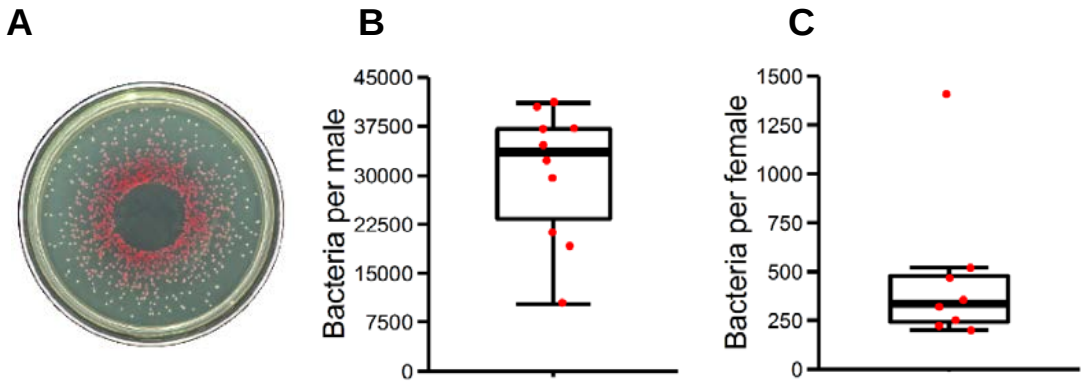

Figure 4.1
Sexual transmission of *Serratia marcescens*
(A) *S. marcescens* colonies produce a red pigment making for easy detectability of STI events **(B)** Quantity of bacteria on male genitalia at Time = 0. N = 10; two replicates. **(C)** Amount of bacteria transferred to females during mating. N = 8; one trial. Points represent the number of bacteria within or on an individual fly.

Sexually transmitted infections have fitness consequences for the female

Finding that *S. marcescens* is transferable during mating, we evaluated the risk of copulating with a bacteria-carrying male. We found that bacteria-carrying males pose a significant risk for females (**Figures 4.2A, B**). Females who mated with bacteria-carrying males had higher mortality relative to females who copulated with bacteria-free males (**Figure 4.2A**; Log-rank, $X_1^2 = 4.6$, $p = 0.0315$). In the absence of mortality, copulating with bacteria-carrying males significantly reduced the number of eggs a female laid relative to females that were not exposed to an STI (**Figure 4.2 B**; Wilcox, W = 1455 $p = 0.00195$).

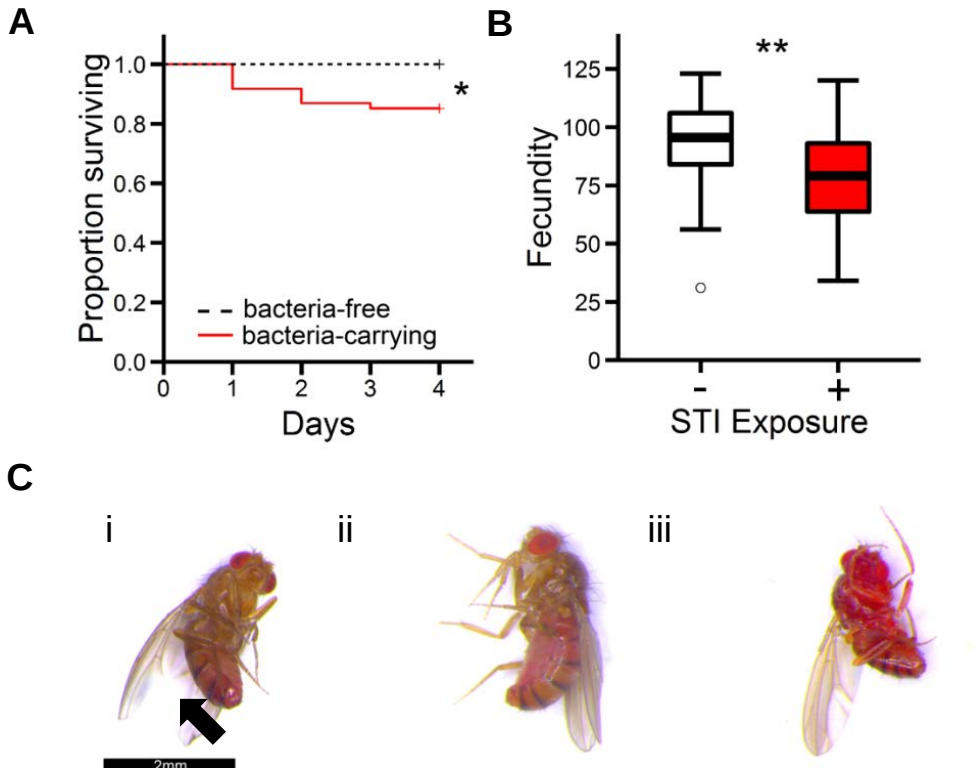

Figure 4.2
Sexually transmitted bacteria negatively affect fitness
(A) Copulation events between females and bacteria-carrying males (red line) or bacteria-free males (PBS, black dotted line). N = 45 ± 15; 3 replicates. **(B)** Female fecundity after copulating with bacteria-carrying males (+ STI, red) or bacteria-free males (- STI, white). The dataset excludes dead females. N = 46; 2 replicates. **(C)** Examples of infection mortality events in three different dead females: (i) evidence for infection beginning at the posterior region (arrow) (ii) infection greatest in the abdomen (iii) eventual progression of infection '*' $p<0.05$; '**' $p<0.01$

As a female succumbs to an infection with *S. marcescens*, the bacterial cells proliferate exponentially, and eventually the red pigment becomes visible in cells where the infection reaches a high density (**Figure 4.2C**). Our attempts to find the

localization of *S. marcescens* within live females using light microscopy were unsuccessful because pigmentation was not visible until death had occurred. We dissected females who had died recently and were showing early stages of red pigmentation. We observed that the bacteria had already spread among tissues in the abdomen and tissue integrity was poor, which resulted in the inability to distinguish between tissue types at a fine scale. Consequently, we monitored dead females for signs of an infection origin at the broad-scale. Infections tended to spread from the posterior region of the female (**Figure 4.2C(i)**) and the abdomen (**Figure 4.2C(ii)**) before spreading throughout the entire fly (**Figure 4.2C(iii)**), consistent with an infection origin that is caused by copulation.

Systemic immunity mediates susceptibility to infection

We tested whether a localized immune response within reproductive tissues protects a female from a sexually transmitted infection. Using the *OAMB*-GAL4 driver, we knocked down the expression of *imd,* a component of the humoral immune system that targets Gram-negative bacteria, within the oviducts of females, a region that is known to express antimicrobial peptides after mating (Domitskaya et al., 2007; Kapelnikov et al., 2008) (**Figure 4.3A**). We found no significant effect on infection survivorship when *imd* was knocked down within the oviduct (Chi-square test with Hoch correction, $p > 0.05$) (**Figure 4.3B**). However, ubiquitous RNAi knockdown of *imd* with *tub*-GAL4 significantly increased the risk of acquiring a lethal infection (Chi-square tests with Hoch correction, $p < 0.05$) (**Figure 4.3B**).

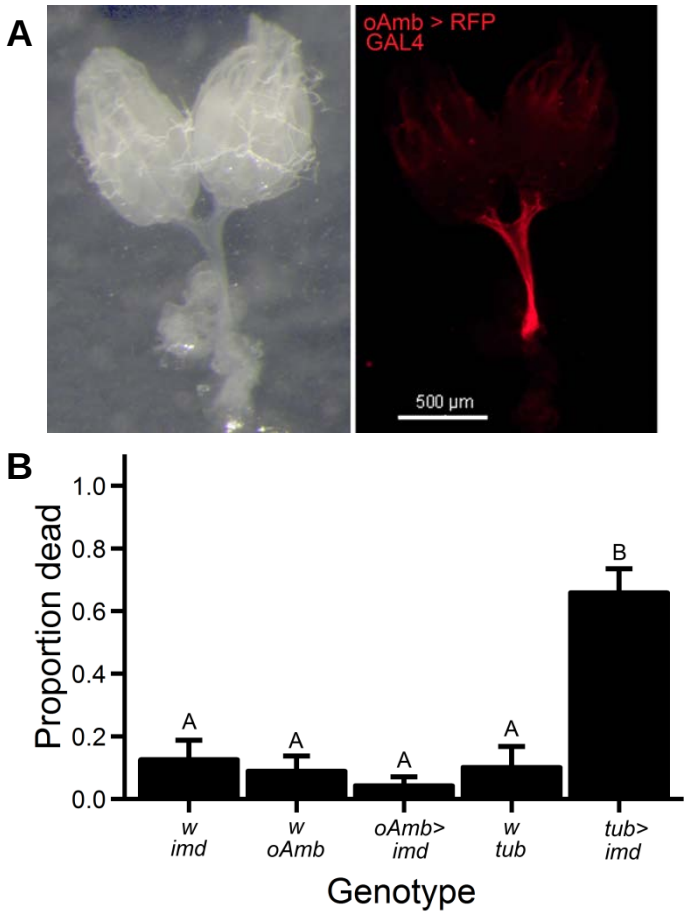

Figure 4.3
IMD signaling reduces susceptibility to STIs
(A) *OAMB*-GAL4 drives expression of UAS-RFP within the central and lateral oviducts. RFP: Red Fluorescent Protein **(B)** Proportion of females that died after mating to a bacteria-carrying male (Proportion dead ± SE). *OAMB*>*imd*: RNAi knockdown of *imd* within the oviducts; *tub*>*imd*: ubiquitous RNAi knockdown of *imd*; Other genotypes are the progeny of RNAi and GAL4 drivers crossed to w^{1118}. Groups with different letters are significantly different from each other.
N = 34 ± 14; two replicates.

The effect of mating and STI on antimicrobial peptide induction

Several studies have shown that mating induces the expression of antimicrobial peptides in *D. melanogaster* (e.g., Lawniczak and Begun, 2004; Peng et al., 2005b; McGraw et al., 2008). We tested whether mating-induced antimicrobial peptide

(AMP) expression after mating is specific to the reproductive tract and assists in fighting a sexually transmitted bacterial infection. We utilized flies that express GFP (Green Fluorescent Protein) when *DptA*, an AMP in the IMD pathway, is expressed to assess immune activity in reproductive tissues. We failed to detect a GFP signal after mating to either bacteria-free or bacteria-carrying males at 10 and 24 hours after mating within reproductive tissues (**Figure 4.4A**). *Dpt*-GFP flies fluoresced strongly when given a systemic infection (not shown). To increase the likelihood of detecting small differences in gene expression, we performed a quantitative RT-PCR experiment on whole flies, reproductive tissues, and carcasses lacking the reproductive tissues to determine if *DptA* expression is localized, responsive to mating, and affected by STI (**Figure 4.4B**). Females that were mated to bacteria-free males had higher *DptA* expression relative to unmated females, but this was only statistically significant in the whole fly samples (t-test, $t_2 = 5.591$, $p = 0.03054$). RNA pools from bacteria-exposed carcasses were highly variable in their expression of *DptA*, and with a small sample size (N = 2) we failed to detect any statistical significance despite an apparent large effect (**Figure 4.4B**). Females that copulated with bacteria-carrying males had significantly higher *DptA* expression levels relative to females that mated with bacteria-free males (Whole fly: t-test, $t_3 = -3.83$ $p = 0.0266$). Mating to a bacteria-carrying male resulted in higher *DptA* expression across all tissue categories (ANOVA, Infection: $F_{1, 13} = 14.749$, $p = 0.00494$; Tissues: $F_{2, 12} = 2.441$, $p = 0.1488$; Interaction: $F_{2, 12} = 2.0572$, $p = 0.1901$).

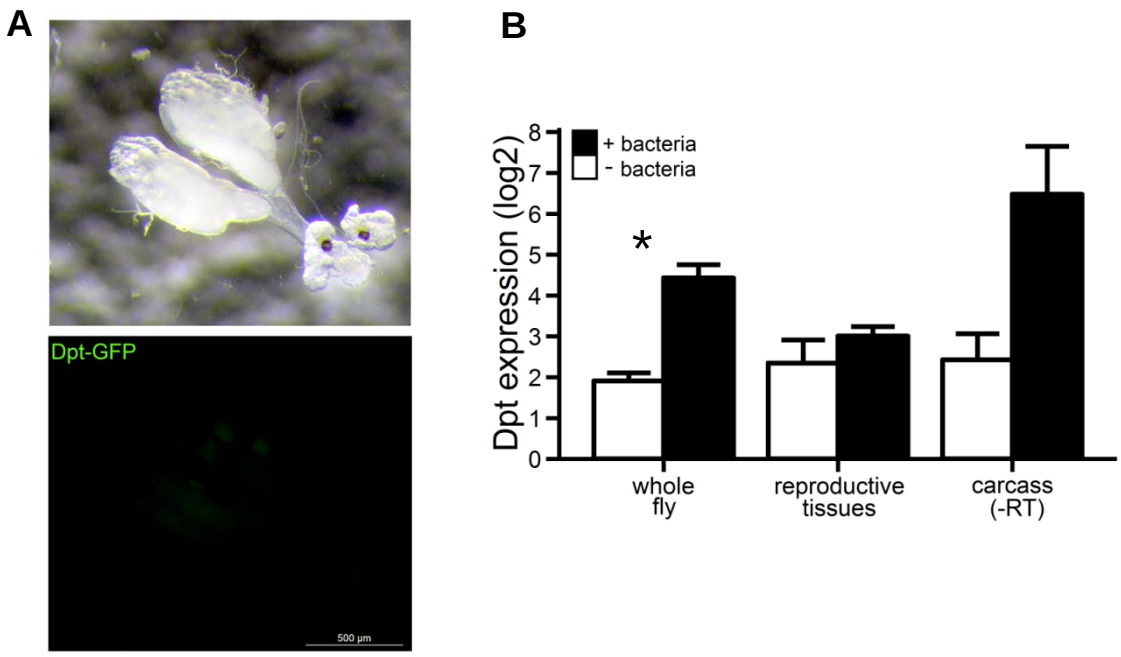

Figure 4.4
The effect of mating and STI on immune system induction
(A) Light microscopy and fluorescent microscopy of reproductive tissues in females expressing GFP under a *diptericin* promotor. Similar results observed for females mated to bacteria-carrying, bacteria-free, and unmated females.
(B) *Diptericin* (*DptA*) mRNA expression relative to unmated females (mean expression ± SE). 'whole fly' includes mRNA from entire, undissected flies; 'reproductive tissues' and 'carcass – reproductive tissues (-RT) tissues originated from the same pool of flies. Females in the '+ bacteria' treatment were mated to males carrying *S. marcescens*. Females in the '- bacteria' treatment were mated to males carrying sterile PBS.
'*' $p<0.05$

The effect of Sex Peptide on susceptibility to an STI

Male seminal fluid proteins can induce AMP expression in females (McGraw et al., 2004; Peng et al., 2005b), especially within their reproductive tissues (Mack et al., 2006; Domanitskaya et al., 2007; Kapelnikov et al., 2008). Moreover, when

females fail to receive Sex Peptide, a male seminal fluid protein, they do not exhibit a mating-induced immune response (Peng et al., 2004; Domanitskaya et al., 2007). Thus, if AMP induction in reproductive tissues protects against STIs, then females who mate with Sex Peptide null males should be more susceptible to a sexually transmitted bacterial infection. We found that females who copulated with bacteria-carrying Sex Peptide null males had an increased risk of infection relative to females who mated with wildtype males (Experiments 1 & 2: Cochran-Mantel-Haenszel, S = 5 $p = 0.00209$) but in subsequent experimental setups this effect was completely reversed (Experiments 3 & 4: Cochran-Mantel-Haenszel, S = 40, $p = 0.0495$) presenting a challenge for the interpretation of the full data (**Figure 4.5**). Notably, in 'Experiments 1 & 2' females and males were left together for 24 hours, whereas females and males were separated after 3 hours in 'Experiments 3 & 4'.

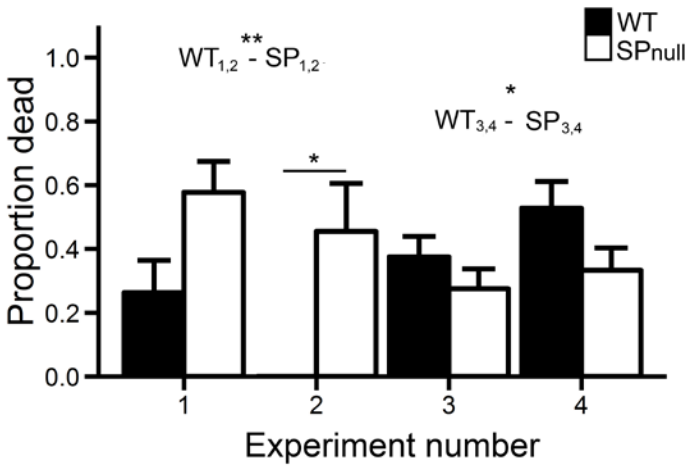

Figure 4.5
The effect of Sex Peptide on susceptibility to STI
Females were mated to bacteria-carrying Sex Peptide null (SPnull, white bars) or wildtype (WT, black bars) males. Bars represent the proportion surviving ± SE. '*' $p<0.05$; '**' $p<0.01$

DISCUSSION

We investigated the ability of males to carry and transmit an opportunistic bacterium to females in *Drosophila melanogaster*. Once we confirmed that males can transfer *Serratia marcescens* to females during mating, leading to infection and death, we evaluated the factors that dictate susceptibility to the sexually acquired bacterial infection. This works strongly suggests that female immunity is required to combat a sexually transmitted pathogen. We discuss our findings in the context of costs of reproduction and discuss the function of mating-induced antimicrobial peptides.

Mating with bacteria-carrying males not only increased the risk of mortality but also reduced female fecundity. In our experiment, females that mated with bacteria-carrying males laid fewer eggs and this effect remained detectable after excluding females that died from infection. Female fecundity is mediated by both female and male inputs, and both can be influenced by an immune challenge (e.g., Radhakrishnan and Fedorka, 2012; Chapter 3). While the presence of bacteria on the male genital plate can promote immune activation (Gendrin et al., 2009) and impair male reproductive traits like sperm viability (Radhakrishnan and Fedorka, 2012), males in these experiments were exposed to the pathogen for no longer than 60 minutes prior to mating. Thus, it is unlikely that the observed reduction in female fecundity was a consequence of reduced male fitness. Alternatively, the observed reduction in fecundity is attributed to female physiology. Both systemic bacterial infection and immune system activation are known to reduce female fecundity in insects (Reinhardt et al., 2003; McKean et al., 2008; Bashir-Tanoli and Tinsley, 2014; Howick and Lazzaro, 2014; Nystrand and Dowling, 2014; Chapter 3). However, when

S. marcescens is injected directly into the hemocoel, 100% mortality occurs (*pers. obs*.) suggesting that reduced fecundity in surviving females is not attributed to a systemic infection. Rather, our finding suggests that females experience a cost to having either a localized infection and/or inducing an immune response.

We identified that female immune defense mediates susceptibility to a sexually transmitted bacterium. In the absence of IMD signaling, females experienced a greater risk of infection after mating with bacteria-carrying males. Both systemic and localized immune responses are mediated by IMD (Lemaitre and Hoffmann, 2007), and we examined whether a localized immune response within reproductive tissues is required for infection resistance. Although the lateral and central oviduct have been identified previously as a site of AMP expression (Domanitskaya et al., 2007; Kapelnikov et al., 2008), RNAi against *imd* in these tissues did not affect susceptibility to an STI.

Recently, Zhong et al. (2013) revealed that sexually transmitted fungal spores are limited by *TotM*, a stress-response gene that is induced by mating, but not by Toll signaling (i.e., the immune pathway that responds to Gram-positive bacteria and fungi). While their result highlight that the genes induced by mating that are most beneficial to preventing a STI may lie outside of the canonical immune pathway, the strong effect of *imd* knockdown in our system suggests transmission of *S. marcescens* is limited by canonical immune signaling. This discrepancy between our studies highlights that pathogen and pathogen entry route are likely important factors to consider when evaluating how hosts respond to an STI challenge.

Observed patterns of immune induction after mating and within reproductive

tissues are thought to be the evolutionary products of STI pressures (Knell and Webberley, 2004; Zhong et al., 2013). *DptA* is a remarkably important antimicrobial peptide that defends against Gram-negative bacteria (Unckless et al., 2016). Despite its potential importance, we found no strong evidence for *DptA* expression in reproductive tissues using GFP reporters, similar to Tzou et al. (2000). In a subsequent experiment, we detected a significant increase in *DptA* expression after mating using qRT-PCR. However, the degree of induction was modest in comparison to previous findings (Peng et al., 2005b). The expression of *DptA* was greatest when females mated with bacteria-carrying males, thus, acquiring *S. marcescens* or bacterial by-products stimulates a host response.

Peng et al. (2005b) demonstrate that Sex Peptide mediates AMP expression through IMD. Moreover, the authors suggest that AMP signals are restricted to the female abdomen and likely come from the reproductive tract. Upon investigation, we found no reproducible effect of Sex Peptide on susceptibility to an STI. Although our first two replicates suggest Sex Peptide reduces susceptibility to infection, we were unable to repeat this subsequently. One potential reason for this discrepancy is male behavior. In the first two experiments, we left females and bacteria-carrying males together for 24 hours whereas we kept males and females together for three hours in the second set of replicates. Consequently, because the receipt of Sex Peptide reduces a female's likelihood to remate within 24 hours (Peng et al., 2005a), Sex Peptide null males may have remated multiple times, which put females at a greater risk of infection relative to females paired with wildtype males whom are likely to mate only once. In the last two experiments, the pattern reverses and female mated to Sex

Peptide null males were less susceptible to infection. This result is consistent with our understanding of post-mating immune suppression in which females are more susceptible to a systemic infection due to the receipt of Sex Peptide (Short et al., 2012; Chapter 2).

The purpose and function of AMP induction after mating has been elusive. Our study is in line with previous reports (Fedorka et al., 2007; Short et al., 2012; Chapters 2, 3), which suggest AMP induction after mating may be too minor to benefit host health or in tissues that are not located at the primary contact zone during the initial infection. First, inspection of AMP localization indicates that expression is relatively limited to the oviduct, spermathecae, and seminal fluid receptacle (Ferrandon et al., 1998; Tzou et al., 2000; Domanitskaya et al., 2007). Bacteria on the male aedeagus are more likely to make contact with the uterus and reproductive tract prior to reaching the AMP expressing reproductive tissues. Therefore, in instances when an infection is not delivered directly within the male ejaculate, AMP expression may not be beneficial. However, a sexually transmitted bacterium has not been identified within *Drosophila* seminal fluid (Knell and Webberley, 2004). Second, while the findings from our final experiment should be interpreted cautiously (**Figure 4.5**), the second experimental block indicates that mating to wildtype males suppresses a female's ability to fight an infection despite having elevated AMP expression, a finding that has been observed several times (Fedorka et al., 2007; Short and Lazzaro, 2010; Short et al., 2012; Chapters 2, 3). Collectively, although mating can induce AMPs, there is a paucity of strong evidence to suggest it benefits host defense. However, this may be a limitation of laboratory experiments and mating-induced AMPs could protect against a

bacterium transmitted within the seminal fluid, which studies have yet to evaluate in an insect system.

Consistent with Miest and Qazi-Bloch (2008), we demonstrated that males can transfer bacteria to females during copulation. This finding suggests copulatory events, especially those that traumatically penetrate the cuticle and make contact with the hemocoel, as seen in bed bugs (Reinhardt et al., 2005), could have severe consequences for the female. Although *D. melanogaster* does not exhibit traumatic insemination, we propose two routes of entry that produce the observed infection patterns. In *D. melanogaster*, the male inserts external genitalia (posterior lobes) between abdominal segments of the female during copulation (Masly and Kamimura, 2014). In doing so, males occasionally pierce a female's cuticle (Kamimura, 2012), thus granting any surface bacteria direct access to the hemocoel. In support of this route of entry, we find that most females die from infection within 24 hours, which matches the rate of *S. marcescens*-induced mortality after receiving an injection directly into the hemocoel. Subsequent mortality events may be explained by bacteria that were deposited within the reproductive tract that eventually gain access to the hemocoel either across the intima of the reproductive tract or through the anal opening (Miest and Bloch-Qazi, 2008). It is unlikely that bacteria gained access to the hemocoel via the gut given that the consumption of *S. marcescens* never resulted in mortality (*pers. obs.*).

CONCLUDING REMARKS

Sexually transmitted infections are pervasive in all animal taxa and the present

results demonstrate that males may can acquire pathogens from the environment and transmit them to females. Our findings highlight how bacteria need not be tissue-specific to infect females during sexual contact. Female immunity dictates susceptibility to STI, and we propose that systemic immunity may be most important due to the entry of bacteria into the hemocoel through wounds associated with traumatic penetration. The ineffectiveness of mating-induced AMPs during a systemic and localized infection highlights that the correct ecological pressure, which favors a prophylactic response after mating, is not yet identified. Our understanding of female immune defense and the evolution of male-female interaction will be greatly improved with the discovery of a seminally-transmitted bacterium.

EXPERIMENTAL PROCEDURES

***D. melanogaster* strains and husbandry** All flies were reared on a glucose medium (8.3% glucose, 8.3% Brewer's yeast, 1% agar, 0.04% phosphoric acid and 0.4% propionic acid) and maintained at 25°C. All flies were 5-7 days old at the time of the experiment.

Unless otherwise stated, Canton-S flies were utilized for the experiment. Except for Sex Peptide null males, all other males used were Canton-S. Sex peptide null males were generated from a cross between the SP deficiency line (Δ130/*TM3, Sb ry*) and the SP null line (0325/*TM3, Sb ry*) (Liu and Kubli, 2003). *imd* knockdown flies were generated by crossing w; UAS-*imd*-RNAi to a ubiquitous driver (*w, tub*-GAL4) or an oviduct-specific driver (*OAMB*-GAL4). Control flies were generated by crossing the UAS or GAL4 line to *w1118*. AMP visualization was performed with

GFP expression lines under either a *Diptericin A* promotor (Tzou et al., 2000).

Bacterial strain and application A highly virulent strain of the Gram-negative bacterium *Serratia marcescens* ('Unckless') was used for all infection assays. Cultures of *S. marcescens* were grown overnight in liquid Luria Broth (LB) while shaking at 37°C. Cultures were spun down at 4000 rpm, the supernatant was removed, and then the bacteria were resuspended in PBS. Bacterial suspensions used were an OD_{600} = 2.0 ± 0.2, which corresponds to 30,000 CFUs per male fly.

Males were anesthetized on CO_2 and received 30 nl to the genital plate using a pulled glass capillary mounted on a Nanoject II apparatus (Drummond Scientific). Males remained on the CO_2 pad until the droplet mostly dried (1-2 minutes) and then were placed into a fresh food vial and allowed to recover from CO_2 exposure for 30 minutes before providing unmated females to the vial. Extended CO_2 exposure severely limits the probability of copulation events.

Mating procedure Females were collected as virgins and housed in cohort sizes of 10-15 individuals. Virgin males were housed in groups of ~15 individuals. Five days after eclosion, males were received an application of bacteria and two vials of males were combined with females (15 females/30 males). Vials were visually inspected to ensure copulation was occurring; within 5-30 minutes, all females were typically paired with males. Except for two replicates of SP-null matings, males were removed after 4-5 hours.

Egg laying assays Females were housed as virgins in groups of 10-15 individuals and provided 'infected' or PBS-treated males when five days old. Females were placed singly into vials and transferred daily. Eggs were counted for four days. Females that

died were removed from the analysis.

quantitative RT-PCR Females were unmated, mated to 'infected' or mated to control males. 10 hours after treatment, females were collected for RNA. Reproductive tissues (uterus, oviduct, spermathecae seminal receptacle, ovaries) were dissected out in 1X PBS. Samples included whole fly (undissected), reproductive tissues, and carcasses (*sans* reproductive tissues). Pools consisted of 15 females; n = 3 for whole fly, n = 2 for dissected samples. Dissected reproductive tissues and carcasses are from the same flies. Samples were frozen stored at -80°C until processing. Total RNA was isolated using a TRIZOL (Invitrogen)-chloroform extraction protocol according to the manufacturer's recommendations and was resuspended in RNAase-free water. Purity was verified and RNA amounts were quantified using a NanoDrop 2000 (Thermo Scientific) spectrophotometer. Approximately 1000ng of nucleic acid from each sample was treated with DNase (Promega) and M-MLV reverse transcriptase (Promega). We used SsoAdvanced SYBR Green Supermix (Bio-Rad) and qRT-PCR reactions were performed using the CFX Connect Real-Time Detection System (Bio-Rad). *DptA* expression was normalized with *RpL32*. Gene expression of samples are relative to virgin controls.

Imaging *Dpt*-GFP and *OAMB*>RFPwas observed under epifluorescent illumination with Leica M165 FC microscope fitted with a Leica DFC 450 camera. Images were captured using the Leica Application Suite V4.0.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Mating and reproduction have recurrently been associated with reductions in life history traits, including immune defense (Chapter 1). Despite these observations, the molecular mechanisms mediating the link between these two processes and the physiological conditions that affect the relationships have been largely undefined. Previous findings in the Lazzaro lab revealed that mated *Drosophila melanogaster* females are less resistant to a bacterial infection and that the effect is mediated by the male seminal fluid Sex Peptide and in part by egg production (Short and Lazzaro, 2010; Short et al., 2012). Moreover, we consistently observe that mating reduces a female's ability to deal with an acquired bacterial infection despite detectable increases in antimicrobial peptides after mating (Lawniczak and Begun, 2004; McGraw et al., 2004; Peng et al., 2005b; Mack et al., 2006). I explored the mechanisms and conditions that govern female susceptibility to a bacterial infection.

In this thesis, I described how mating and reproduction dictate an immune response and alter the risk of infection in *D. melanogaster* females. In this work, I explored two leading hypotheses embedded within the study of trade-offs: hormonal pleiotropy (Chapter 2) and resource allocation (Chapter 3). I also evaluated whether the induction of locally-expressed immune genes protect against sexually transmitted pathogens as an explanation for why mating-induced antimicrobial peptides occurs with seemingly little benefit during a systemic infection (Chapter 4). Here, I describe the broader context for my findings and provide suggestions for future work on reproduction and immunity.

Juvenile hormone and hormonal pleiotropy

Hormone signaling molecules are essential for the integration of internal and external cues and their interconnectedness among several signaling pathways results in hormonal regulation of several physiologies at once (Flatt et al., 2005). Juvenile hormone (JH) regulates numerous processes within insects and its centrality makes it a prime candidate for mediating trade-offs between phenotypic traits. In Chapter 2, I tested the hypothesis that the signaling molecule, JH, co-regulates immunity and reproduction to produce a trade-off between the two processes.

I revealed that the immunosuppressive effect of JH acts through the transcription factor *germ cell-expressed* (*gce*). Unfortunately, we do not know how *gce* regulates immune expression and why *Methoprene-tolerant* (*Met*), a known paralog, does not influence immune activity. Accruing phenotypic evidence suggests that *gce* and *Met* could have divergent functions and tissue specificity (Abdou et al., 2011; Greb-Markiewicz et al., 2015; Reiff et al., 2015). Results from the FlyAtlas Project suggest *gce* is more highly expressed than *Met* with some minor differences in tissue specificity, including stronger *gce* expression in the midgut relative to *Met* (Chintapalli et al., 2007). Interestingly, evidence from the FlyFactorSurvey suggests *Met* and *gce* bind to the same DNA sequence motifs (Zhu et al., 2011). However, this activity may not be present during all physiological conditions and may depend on various co-factors that promote the translocation of transcription factors into the nucleus (i.e., transcriptional activity) (Greb- Markiweicz et al., 2015). Thus, the sequence divergence between *gce* and *Met* could alter JH responsiveness in a tissue-specific manner and produce a pattern whereby *gce* negatively regulates immunity but

Met does not. Now that we have an understanding of the molecular drivers that mediate reproduction and immunity, several follow-up experiments could address how *Met* and *gce* differ in their activities and what targets of *gce* contribute to immunosuppression. One could perform an RNAi screen using tissue-specific drivers to knockdown *Met* and *gce* to identify the tissue responsible for *gce*-mediated reductions in immunity. Moreover, because we now know the transcription factor that mediates susceptibility to infection, one could perform a ChIP-seq experiment using a factorial design that tests the influence of JH and infection on DNA binding capacity and location. Together these would provide the most comprehensive understanding of a physiological trade-off to date.

Dietary supplementation and resource allocation

Nutrient limiting conditions often reveal trade-offs between physiological processes (e.g., McKean et al., 2008). Under a model of resource allocation, phenotypic traits that require a shared resource should be constrained by nutrient-limiting conditions. Under these circumstances, an organism diverts resources into the more favorable outcome based on molecular signaling cues. In Chapter 3, I revealed that dietary supplementation positively influenced both female immunity and reproduction, with essential amino acids providing the greatest benefit to overall fitness. Under a model of resource allocation, providing a surplus of a shared resource (i.e., essential amino acids) should eliminate any costs of reproduction that come at the expense of immunity. While I found evidence that supports a model of resource allocation, we still do not know how nutrients are directly responsible for the observed

trade-off. For instance, a surplus of nutrients could provide more amino acids for either the production of immune defense and egg proteins or the influx of nutrients could promote signaling cascades that strengthen host defense and reproductive output independent of absolute resource levels, both of which are not mutually exclusive.

Ultimately, we do not know the precise movement of nutrients within a female over the course of an infection, which should be addressed in future studies. One method for evaluating an investment in somatic tissue versus reproductive tissue requires feeding flies stable isotopes and measuring the abundance of the labeled molecules within the tissues of choice (O'Brien et al., 2008; Zamboni et al., 2009). While this method provides the best opportunity to assess nutrient movement, there are obvious shortcomings that make it a highly challenging experiment. First, any signal that denotes an allocation to the soma (e.g., cuticle or fat body) does not imply an investment in immune defense molecules. Second, molecules that enter the hemolymph like yolk protein and antimicrobial peptides will be difficult to assess due to the small volumes of hemolymph in *Drosophila*. Moreover, the potential for antimicrobial peptides and yolk protein to be found jointly in the hemolymph disallows one to distinguish between an investment in reproduction and immune defense. To conclusively demonstrate that reproduction and immunity interact under a model of resource allocation, future work must examine internal amino acid resource pools and investigate how both mating and infection affects the allocation to antimicrobial peptides or yolk proteins.

Juvenile hormone and nutrient supplementation: what's the connection?

In Chapters 2 and 3, I describe how blocking JH signaling and increasing the amount of essential amino acids in the diet improves immune defense in mated females, respectively. Nutrient sensing and hormonal signaling exhibit a high-degree of interconnectedness through Insulin/Insulin-like Signaling (IIS) and Target of rapamycin (TOR) signaling pathways, which control host homeostasis. Therefore, it is possible that JH and diet converges through shared signaling pathways, in a way that effects both reproduction and immunity.

Several studies suggest a tight coupling between JH and IIS which could explain JH-induced immunosuppression (Mirth et al., 2014; Perez-Hedo et al., 2014). For instance, increased expression of FOXO, a downstream transcription factor in the IIS pathways, corresponds to low levels of JH (Mirth et al., 2014). Activation of FOXO (i.e., translocation into the nucleus) leads to the induction of several lines of immune defense (Becker et al., 2010; Spellberg and Marr, 2015). Incidentally, higher levels of JH and IIS positively affect fecundity (Soller et al., 1999; Ikeya et al., 2002). Therefore, the induction of JH could reduce FOXO activity and prevent the induction of genes that support host defense contributing to decreased resistance to infection after mating. Unfortunately, we still do not know how JH signals to the IIS pathway including the involvement of *gce*, further emphasizing how it will be critical to investigate the targets of *gce*. One potential mechanism is through increased midgut proliferation and nutrient absorption which are both JH and *gce*-dependent (Reiff et al., 2015). Thus, increased feeding and nutrient absorption could contribute to heightened IIS activity at the expense of FOXO-induced stress and immune responses.

TOR signaling detects and responds to ingested amino acids through at least two amino-acid transporters on the surface of fat body cells (Colombani et al., 2003; Reynolds et al., 2009; Boudko et al., 2015). During digestion, amino acids transcend the epithelium of the gut and enter the hemolymph before finally reaching the adjacent fat body cells (Boudko et al., 2015). While we do not fully understand how internalized amino acids activate TOR, the final step in the signaling cascade promotes translation and nutrient uptake (Teleman, 2010). The importance of TOR signaling for mammalian adaptive immunity has been long recognized (Thomson et al., 2009); however, there has been a scarcity of studies evaluating the relationship between TOR signaling and resistance to infection within insect systems. Recently, Allen et al. (2016) demonstrated that the suppression of TOR signaling activity, through the application of rapamycin and by overexpressing TSC1/TSC2, significantly decreased infection resistance. Thus, supplementation with essential amino acids and the resulting increase in TOR signaling could positively influence host defense by either directly or indirectly overriding the immunosuppressive effect of JH.

The interdependence of JH, IIS, TOR, and immune signaling emphasizes the complex level of signaling that is required to maintain homeostasis (Grewal, 2009; Teleman, 2010). Ultimately, an intricate series of epistasis experiments will be essential to understand the signaling network that results in diet improving immunity and JH suppressing it. There are too many uncertainties in the signaling pathways to proceed with epistasis analyses within adult females. Future studies should utilize S2 cells to evaluate how altered signaling in adjacent pathways affects the desired phenotype before scaling up to the whole organism. Furthermore, S2 cells are more

amenable to double RNAi knockdown experiments (Clemens et al., 2000; Xu et al., 2009) and single cells avoids the complexity associated with multicellular signaling, and importantly pre-pupal lethality (Teleman, 2010).

S2 cells have proven successful in elucidating how signal transduction occurs between pathways (Miron et al., 2003; Flatt et al., 2008; Willis et al., 2010; Tsokanos et al., 2016). Using this methodology, one could easily administer JH, amino acids, and an immune stimulant (e.g., lipopolysaccharides) after RNAi knockdown and measure the concomitant activity of S6K (TOR pathway), FOXO (IIS pathway), REL (IMD pathway), and several antimicrobial peptides. A finding that RNAi knockdown of *gce* contributes to increased antimicrobial peptide expression in the absence of REL would suggest JH signaling through GCE suppresses immunity in an IMD-independent manner. One could then evaluate if immune induction persists in GCE(-), REL(-), FOXO(-) cells. A failure to find antimicrobial peptide expression within these cells would strongly support a model whereby JH signaling through GCE suppresses immunity by inactivating the transcription factor FOXO. Lastly, determining the DNA sequences that GCE binds to will provide more gene candidates to evaluate within the epistasis experiments which will ultimately clarify how GCE relays a hormonal signal and fits into the model. Together, the combination of S2 RNAi experiments and tissue-specific RNAi will provide a better understanding of the signaling network that interlinks TOR, IIS, JH, and immune signaling pathways.

Sexually transmitted infections and female antimicrobial peptides

Mating can impose direct risks to individual health and when faced with

repeated pathogen pressure, defense strategies are likely to evolve to prevent severe fitness effects. As highlighted in Chapter 4, I found strong evidence that female immunity prevents the onset of a sexually transmitted infection (STI) and that females experience fitness costs due to mating with bacteria-carrying males. However, the data did not convincingly support a model whereby mating induced antimicrobial peptides protect against STIs.

The ectopic application of bacteria provides a useful model for understanding the sexual transmission of bacteria in *Drosophila* (Miest and Bloch-Qazi, 2008), including the identification of fitness costs. However, this methodology does not provide enough resolution to test among hypotheses for when and why immune induction occurs as well as where and how pathogens gain entry. I have several suggestions for future experiments that would permit a greater understanding of the mechanics underlying female susceptibility and immune system regulation.

First, we would greatly benefit from the discovery of a bacterium that is sexually transmitted within the seminal fluid of males to test whether the induction of antimicrobial peptides within reproductive tissues after mating is protective. Therefore, experiments that find a naturally occurring STI or a better mimic are essential. Males harboring a systemic infection with either *Serratia marcescens* or *Providencia rettgeri* never transferred their infection to females during mating. However, one could test if a systemic infection with other bacteria can reach male reproductive tissues and be passaged to recipient females. Lastly, several methodological advances demonstrate that female insects can be artificially inseminated (Takemura et al., 2000; den Boer et al., 2013) and these techniques could

be utilized to artificially administer bacteria into the reproductive canal, thus mimicking a seminally dispensed bacterium.

Second, experiments that distinguish between bacteria gaining entry through the reproductive tract, through external copulatory wounds, or both internal and external routes are essential to understand female susceptibility to sexually acquired disease. One could laser ablate the posterior lobes of males (see Polak and Rashed, 2010), a structure which causes abdominal wounds on females (Kamimura, 2007; Masly and Kamimura, 2014), and subsequently test whether females contract an STI. If females still succumb to infection when males lack posterior lobes, the result would suggest bacteria in the reproductive tract can cause an infection independent of any copulatory wounds. Because the laser-ablation technique can be performed on all genotypes, one could easily test the influence of Sex Peptide without the formation of copulatory wounds.

Third, Sex Peptide stimulates the induction of antimicrobial peptides through the central hydroxyproline-rich region of the molecule (Domanitskaya et al., 2007). To easily test whether the stimulatory effect of Sex Peptide reduces female susceptibility to STIs, one could utilize transgenic lines that have already been establish (SP^{NDT}) which fail to elicit an immune response in the reproductive tract (Domanitskaya et al., 2007). Moreover, the JH stimulatory region (i.e., the N-terminus) of Sex Peptide is unaffected in SP^{NDT} males and thus the receipt of SP ^{NDT} will reduce female receptivity to mating and prevent any complications associated with multiple matings. Together, if a seminally transmitted bacterium is unobtainable, then an SP^{NDT} male who lacks posterior lobes (via laser ablation) would provide the clearest test to assess

the protectiveness of mating-induced AMPs against an STI in the absence of copulatory wounding.

Finally, the low prevalence of bacterially-caused STIs is a fascinating observation. Are sexually transmitted bacteria truly absent in insects? Or, can low detectability explain the apparent patterns? Future studies should investigate these questions by performing a large ecological survey of potential bacterial pathogens in wild-caught males. One could mate wild-caught males to laboratory-reared females and utilize 16S sequencing to determine if there are bacteria in the reproductive tissues common to both the male and his mating partner. Additionally, one could aim to culture identified pathogens for use in subsequent experiments. This study, if successful, would be the first to identify a bacterial STI in insects.

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