ABSTRACT

Title of Document: COMBINING THE *DROSOPHILA* AND *METARHIZIUM* MODEL SYSTEMS TO INVESTIGATE HOST-PATHOGEN INTERACTIONS

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Even closely related individuals vary in their response to infection. In this dissertation, I combined the fruit fly model system with multiple pathogens, including 16 strains of the fungus *Metarhizium,* to dissect how pathogens with different virulence strategies interact with variable host resistance and tolerance mechanisms. I began by infecting 188 sequenced *Drosophila melanogaster* lines [the Genetic Reference Panel (DGRP)] with broad host range *Metarhizium anisopliae* (Ma549) or the bacterium *Pseudomonas aeruginosa* (Pa14, originally from a human host). Resistance to the two pathogens was correlated (suggestive of general multipurpose defense mechanisms) and associated with oxidative stress sensitivity, starvation resistance, and in particular sleep indices (flies that take a lot of naps are particularly resistant to disease). I followed up by showing that this nonspecific defense extends to the specialist co-evolved *Drosophila* pathogen, *Entomophthora muscae*. A genome wide association study implicated several metabolic pathways and physiological processes in individual variation to disease, but not the canonical antifungal Toll immune pathway.

Indeed, *Metarhizium* strains that killed faster induced a stronger and earlier Toll immune response, indicating virulence does not depend on suppressing immunity.

Disrupting the Toll pathway component Dif only increased susceptibility to the early diverged broad host range *Metarhizium frigidum*, whereas flies disrupted in Persephone (a sensor of pathogen proteases) succumbed quickly to all *Metarhizium* strains. Microarray analysis of mutants revealed a suppressed transcriptomic response to infection when either Persephone or Dif were disrupted, with overlap with GWAS-implicated pathways.

COMBINING THE *DROSOPHILA* AND *METARHIZIUM* MODEL SYSTEMS TO INVESTIGATE HOST-PATHOGEN INTERACTIONS

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Table of Contents **Page Number**

List of Tables **Page Number**

Chapter 1

Chapter 2

Chapter 3

List of Figures **Page Number**

Chapter 1

Chapter 2

Chapter 3

Introduction

Insect pests are perhaps humanities' greatest adversaries. Insects, primarily dipterans, are responsible for a wide range of vectored diseases [1]. Mosquitoes by themselves have been estimated to have killed half of all humans who have ever lived [2]. At the same time, it is estimated that close to 15% of all annual crop production is lost to insect damage and herbivory [3]. As a consequence of the societal impact of these organisms, most entomological research has been channeled into pest management.

Insects themselves are prey to entomopathogenic fungi, which are major re regulators of insect populations [4]. Perhaps because of their ease of use in the laboratory, the best well studied are in the genera *Beauveria* and *Metarhizium,* and much of what we know about the biochemistry of insect-fungal interactions was worked out using these two genera. *Metarhizium* species are being used as environmentally friendly alternatives to chemical insecticides. *Metarhizium anisopliae* (Metschn.) Sorokin was one of the first organisms seriously investigated for use against agricultural pests. The pioneering immunologist Elie Metchnikoff initiated trials of this fungus against the wheat cockchafer *Anisoplia austriaca* in 1879 [5]. Products formulated with *Metarhizium* are currently used world-wide; one of the most successful biological control programs anywhere involves treating two million hectares of sugar cane in Brazil each year with *M. anisopliae* to control spittlebugs [6]. *Metarhizium acridum* is used to control locust populations, including across ~1 million hectares in China, and a transgenic *M. pingshaense* strain is being developed as a biocontrol agent against malaria vectoring *Anopheles* spp. [7]. Entomopathogenic fungi are particularly well suited for development as biopesticides because unlike bacteria and viruses that must be ingested to cause

diseases, fungi typically infect insects by direct penetration of the cuticle. These fungi combine turgor pressure and cuticle degrading enzymes to bypass and assimilate insect cuticle [8]. When they reach the hemocoel, the fungi bud off as yeast-like blastospores to disperse, multiply, and compete for nutrients, all while avoiding the humoral and cellular response of the insect's innate immune system [9]. Upon the death of the host, the fungus re-emerges from the insect and produces conidia to start the process all over again.

The finding that *Metarhizium* spp. form plant associations and can boost plant growth has implications for their dual use as agricultural protectants [10]. Clearly there is practical utility in studying the interactions between insects and *Metarhizium*. Technologies being developed for sustainable pest control utilizing pathogens will benefit from knowledge of how pathogens infect and kill their hosts, and conversely how the hosts defend themselves from pathogens.

Besides their prominence as pests, insects, primarily *Drosophila*, have been used as a reductionist model for uncovering the secrets of innate immunity, bypassing ethical concerns with vertebrate models, and increasing statistical power because of the large numbers that can be tested. *Drosophila* as a model organism has many genetic tools available to query a wide variety of questions including those involving host-pathogen interactions. Most famously, the Toll pathway uncovered in *Drosophila* was found to be evolutionarily conserved in a wide range of organisms from Porifera to humans [11,12]. A Gram-positive or fungal infection triggers the activation of the Toll pathway in *Drosophila*, which leads to the systemic production of antimicrobial peptides (AMPs) [13]. Advancements in sequencing technologies has allowed for understanding of the

genetic basis of disease resistance and other phenotypes through genome-wide association studies (GWAS). Such advancements signal the coming of a new age of personalized medicine, whereby we treat the individual and not the disease [14]. *Drosophila* share 70% of disease related alleles with humans and have proven to be a powerful, genetically tractable model for cancer and diabetes [15,16]. From a biocontrol standpoint, genes associated with resistance offer the promise of genetic targets for pathogen improvement. The large *Drosophila* community and its genetic tools combined with the versatility of *Metarhizium* offers a powerful system for understanding host-pathogen interactions.

Chapter 1: The genetic basis for variation in resistance to infection in the *Drosophila melanogaster* genetic reference panel

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1.1 Abstract

Individuals vary extensively in the way they respond to disease but the genetic basis of this variation is not fully understood. We found substantial individual variation in resistance and tolerance to the fungal pathogen *Metarhizium anisopliae* Ma549 using the *Drosophila melanogaster* Genetic Reference Panel (DGRP). In addition, we found that host defense to Ma549 was correlated with defense to the bacterium *Pseudomonas aeruginosa* Pa14, and several previously published DGRP phenotypes including oxidative stress sensitivity, starvation stress resistance, hemolymph glucose levels, and sleep indices. We identified polymorphisms associated with differences between lines in both their mean survival times and microenvironmental plasticity, suggesting that lines differ in their ability to adapt to variable pathogen exposures. The majority of polymorphisms increasing resistance to Ma549 were sex biased, located in non-coding regions, had moderately large effect and were rare, suggesting that there is a general cost to defense. Nevertheless, host defense was not negatively correlated with overall longevity and fecundity. In contrast to Ma549, minor alleles were concentrated in the most Pa14-susceptible as well as the most Pa14-resistant lines. A pathway based analysis revealed a network of Pa14 and Ma549-resistance genes that are functionally connected through processes that encompass phagocytosis and engulfment, cell mobility, intermediary metabolism, protein phosphorylation, axon guidance, response to DNA damage, and drug metabolism. Functional testing with insertional mutagenesis lines

indicates that 12/13 candidate genes tested influence susceptibility to Ma549. Many candidate genes have homologs identified in studies of human disease, suggesting that genes affecting variation in susceptibility are conserved across species.

1.2 Author Summary

We have shown that there is significant genetic variation for host defenses against the fungus *M. anisopliae* (Ma549) in a set of 188 *Drosophila* lines derived from nature that have been completely sequenced. This manifested as differences between lines in mean survival times, how they balanced resistance and tolerance to disease, and their microenvironmental plasticity. Despite having very different modes of infection, resistance to Ma549 is positively correlated with resistance to *Pseudomonas aeruginosa*, and resistance to the two pathogens is correlated jointly with phenotypes (sleep, oxidative stress resistance) that have been measured by other researchers. We identify a host of candidate genes associated with variation in disease resistance, many of which are known to interact physically and/or genetically enabling us to place them in a biologically informative genetic network. Overall, our results suggest that natural lines differ in their ability to control and tolerate replicating fungi during infection, which is achieved through the coordinated interplay of morphological and physiological restraints, and different immune system effectors that function in subtly different ways in different lines. Generally speaking, the results presented here can provide a starting point for further research on these important traits.

1.3 Introduction

Fungal pathogens of insects are major regulators of insect populations, and are being developed for biocontrol of insect pests [17]. Beyond insects, fungal pathogens

have an enormous influence on plant and animal life, leading to species extinctions, food security issues, and ecosystem disturbances [18]. The increased prevalence of fungal infections has stimulated investigations into antifungal immune responses in humans. A defining moment was the discovery of innate-immune Toll-like receptors in antimicrobial host defense. These were originally identified in *Drosophila* as essential components for the development of resistance to infection with *Aspergillus* (and later, other opportunistic insect pathogens) [19].

Fungi, such as *Metarhizium anisopliae* cause the majority of insect disease and play a crucial role in natural ecosystems [20]; *M. anisopliae* is also being developed as a biocontrol agent against fruit fly pests [21]. As most *M. anisopliae* strains, including the one used in this study, have a broad host range, they are unlikely to be engaging in a strict coevolutionary arms race with a particular *Drosophila* population. Using *M. anisopliae* in infection experiments gives us the possibility to study how hosts respond to a generalist fungal pathogen and to assess if variability among host populations is present, possibly due to divergent life histories [9]. Unlike viruses and bacteria that normally infect through the oral route, *M. anisopliae* breaches the cuticle reaching directly into the hemocoel using a combination of mechanical pressure and an array of cuticle-degrading enzymes [22]. We previously screened 2,613 insertional mutant *Drosophila* lines for their effects on resistance to *M. anisopliae* ARSEF strain 549 (Ma549) [23]. Overall, 9% of the lines had altered resistance to Ma549 indicating a large mutational target for disease resistance, and approximately 13% of these where in genes encoding immune responses including coagulation, phagocytosis, encapsulation, and melanization [23]. The non-

immune genes impacted a wide variety of biological functions, including behavioral traits and nutrition.

It is generally agreed that complex traits such as disease resistance are caused by interactions between multiple gene variations and environmental factors [9]. Natural selection would weed out many of the highly deleterious mutations in the insertional mutant lines that affected disease resistance. Thus, the genetic changes with the biggest impact on disease risk are likely to occur infrequently in natural populations. A complementary approach to mutagenesis is to identify loci at which alleles with subtler effects segregate in natural populations [24]. Here, we use a community resource, the *Drosophila* Genetic Reference Panel (DGRP) [25,26], to identify mutations associated with natural variation in disease resistance. The DGRP is a panel of inbred lines with fully sequenced genomes that was created by mating full siblings of wild-caught isofemale lines for 20 generations [26]. As experimental surrogates for individual variation, DGRP lines collectively deliver much higher statistical power compared to outbred individuals, and the lack of heterozygotes means that more extreme phenotypes may be represented in the population because rare recessives of large effect are exposed [26].

Using the DGRP, we show that wild-derived populations of *Drosophila* have substantial differences in susceptibility to Ma549, a natural fungal pathogen, and that this variation correlates with resistance to a clinical isolate of *Pseudomonas aeruginosa* (Pa14). *P. aeruginosa* is a quintessential opportunistic pathogen that infects a broad range of hosts, including plants and insects [27], and causes the highest human case fatality rate

of all Gram-negative infections [28]. We additionally found correlations between susceptibility to Ma549 or Pa14 and several previously published DGRP phenotypes [26], [29–31]. We used single nucleotide polymorphisms (SNPs) and indels (hereafter collectively called polymorphisms), associated with natural variation in resistance in the DGRP to identify candidate genes. In contrast to variation in resistance to viruses [32], the majority of alleles associated with variation in susceptibility to Ma549 and Pa14 were rare. We used insertional mutagenesis lines to validate a subset of candidate genes at a high rate. Combining tagged genes from Ma549 and Pa14 GWA analyses revealed a statistically enriched network of genes involved in phagocytosis and engulfment, cell mobility, intermediary metabolism, protein phosphorylation, axon guidance, response to DNA damage, and drug metabolism.

1.4 Results

1.4.1 Quantitative genetics of disease resistance to Ma549 in the DGRP

To characterize natural variation, we quantified susceptibility to *M. anisopliae* (Ma549) using ~71,974 flies from 188 lines of the DGRP Freeze 2, which includes documentation of insertion—deletion polymorphisms and chromosomal inversions in addition to SNPs [25]. Age-matched flies from each line were infected topically with spores of Ma549, and survival time was monitored using three replicates (~20 flies each), per sex per line. Each line was screened this way at least twice, and ~30 lines with similar survival times were screened >3 times to validate small differences. A list of the lines used, along with LT_{50} data and *Wolbachia* status, can be found in S1 Table.

ANOVAs showed highly significant genetic variation in disease resistance (P<0.0001) between lines, with a broad sense heritability of $H^2 = 0.23$ ($H^2 = 0.27$) for males (females) from the pooled data (Table 1). This compares with H^2 values of 0.47 (0.38) males (females) for resistance to *P. aeruginosa* Pa14. Disease resistance by males (females) to Ma549 was significantly [P = 0.036 (0.003)] associated with only one of the 5 major chromosomal inversions (*In_3R_K*) (S2 Table), indicative of localized LD effects. The same inversion, *In_3R_K,* also impacts disease resistance by females to Pa14 (P = 0.044). *Wolbachia pipientis* is a natural intracellular symbiont of many arthropods [33], and *Wolbachia* may confer protection against the fungus *Beauveria bassiana* in one *D. melanogaster* line [34]. *Wolbachia* status in the DGRP lines was without significant effect on the susceptibility of either males (P = 0.7332) or females (P = 0.8070) to Ma549, but this does not preclude an impact by *Wolbachia* on a line-by-line basis, i.e., to an individual *D. melanogaster* line.

Trait	Analysis	Source of Variation	df	MS	*F*	P-value	Variance
Ma549 Survival	Sexes Pooled	Sex	1	477.59	39.56	<0.0001	0.30
		Line	187	102.77	6.62	<0.0001	0.10
		Sex*Line	186	15.57	12.52	<0.0001	1.24
		Error	55216	1.24			
	Females	Line	187	52.06	56.55	<0.0001	0.33
		Error	28852	0.92			0.92
	Males	Line	186	67.68	42.37	<0.0001	0.47
		Error	26364	1.6			1.60
PA14 Survival	Sexes Pooled	Sex	1	370.74	8	0.0059	2.86
		Line	80	623.17	10.98	<0.0001	0.53
		Sex*Line	80	56.78	12.46	<0.0001	4.56
		Error	16219	4.56			
	Females	Line	80	304.05	62.2	<0.0001	3.03
		Error	7984	4.89			4.89
	Males	Line	80	385.48	90.95	<0.0001	3.75
		Error	8235	4.24			4.24

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Table 1. Analysis of variance of survival times of flies treated with Ma549 and Pa14.

The average LT_{50} for males (females) with Ma549 was 5.3 (5.1) days with a range of 3.73 (3.55) to 7.05 (6.81) days i.e. a range of 3.32 (3.26) days (Fig 1a). The mean natural lifespan in the DGRP is 55 days [35].

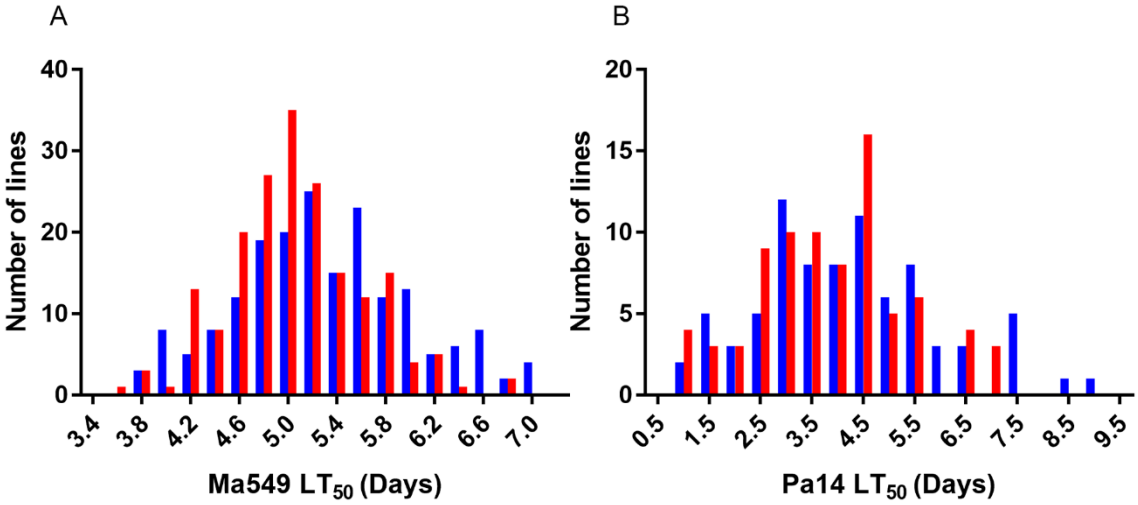

Fig 1. Distribution of male (blue bars) or female (red bars) lifespans among DGRP lines infected with Ma549 (A) or Pa14 (B).
Lifespans were measured as the time required for half the flies to die (LT_{50}).

To identify sexual dimorphism, we measured disease resistance separately for males and females infected with Ma549 (Fig 2). Cross-sex genetic correlations were high ($r = 0.74$), indicating that many of the same variants affect disease resistance in males and females; but that some alleles will have sex-specific effects. As observed previously [23], males were typically more resistant than females ($t = 7.026$, $P < 0.0001$), however in 57 of the 188 lines (30.3%) females were more resistant (Fig 3). Of the 57 female-resistant lines, 45 (78.9%) were in the 94 most susceptible lines in the DGRP collection. Thus, females were more resistant than males in 47.9% of susceptible lines and only 12.8% of resistant lines.

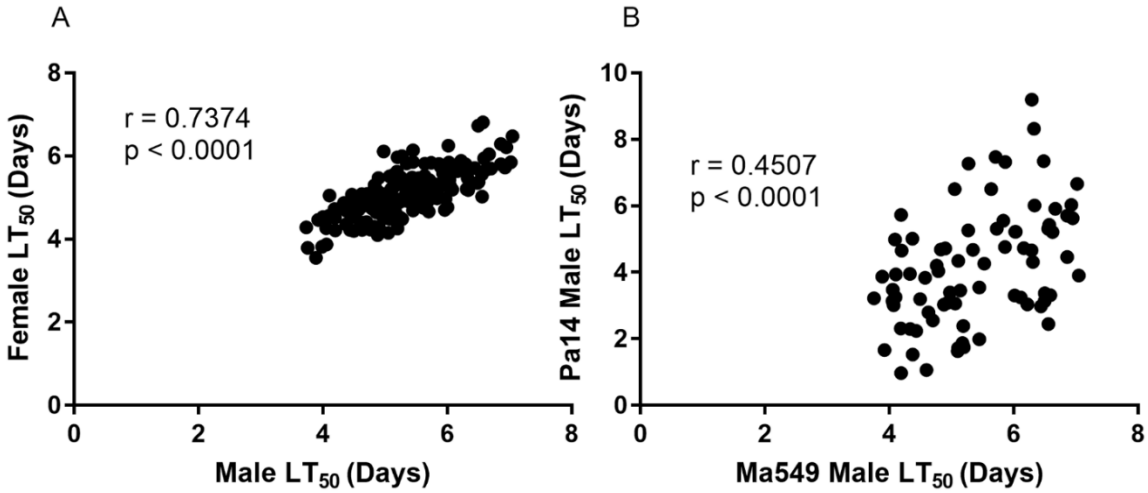

Fig 2. Correlation analyses of LT_{50} values among the DGRP lines between male and female flies infected with Ma549 (A), and between male flies infected with Pa14 or Ma549 (B).

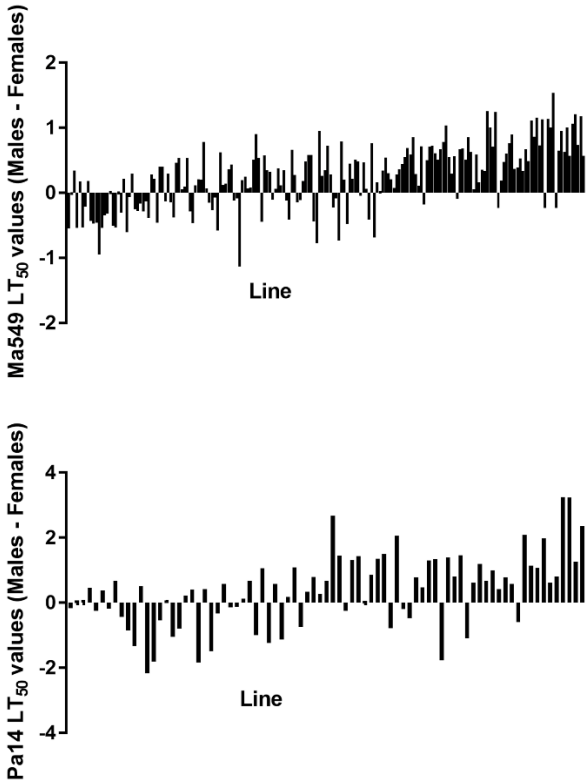

Fig 3. LT_{50} differences between male and female flies in DGRP lines infected by Ma549 and Pa14.

LT_{50} values for females were subtracted from those of males so negative values indicate lines where female flies are more resistant than males. Lines are ranked from most to least susceptible males.

1.4.2 Resistance to infection by Ma549 is correlated with other phenotypes

We asked to what extent disease resistance responses to fungi and bacteria were correlated by determining LT_{50} values for a subset of 81 randomly chosen *Drosophila* lines fed food contaminated with PA14 (Fig 1b). The average LT_{50} for males (females) was 4.2 (3.8) days with a range of 0.97 (1.07) to 9.2 (6.99) days. This 8.23 (5.92) day range in variation in LT_{50} values for males (females) infected by PA14 was ~ 2.48 (1.82)-fold greater than the range for Ma549. Nevertheless, LT_{50} values for *Metarhizium* Ma549 and Pa14 were moderately correlated for both males ($r = 0.45$) and females ($r = 0.40$) ($p<0.0001$) consistent with *Drosophila* only partially discriminating between these pathogens (Fig 2b). Phenotypic correlations between sexes were greater than correlations between the pathogens, with $r = 0.74$ for Ma549 ($p < 0.0001$)) and $r = 0.77$ for PA14 ($p<0.0001$). The average LT_{50}'s of Pa14 infected male flies (4.2 days) was significantly higher than females (3.8 days) ($t = 2.96$, $p = 0.004$). The distribution of sexual dimorphism to Pa14 was also similar to Ma549 infected lines, with females being more resistant than males to Pa14 in 30 of the 81 lines (37%) with the majority (22, 73.3%) of these being in the 40 most susceptible lines. However, the correlation of the magnitude of divergence in LT_{50}'s between male and female flies infected with Ma549 or Pa14 fell short of significance ($r = 0.19$, $P = 0.1043$).

To identify trade-offs associated with disease resistance, we measured correlations between our disease resistance phenotypes and several other traits that have been measured in the DGRP and for which the data are publicly available (longevity, fecundity, courtship behavior, starvation stress resistance, nutritional stores, chill coma recovery, startle response, aggression, oxidative stress response, endoplasmic reticulum

stress, sleep indices) [24,26,29,31,36–40]. Table 2 contains the correlation coefficient for each trait combination. Correlations between disease resistance and broad ecological measures of health such as longevity or several measures of fecundity [36], were not significantly different than zero, indicating that in a pathogen-free environment disease resistance would not be associated or traded off against general robustness or lifetime fitness. Some weak but significant associations did not pass a Holm-Bonferroni correction for multiple testing e.g., male courtship behavior and the startle response (Table 2).

Negative geotaxis (a measure of innate escape response and general stress resistance) as determined by Jordan et al., [29] is positively correlated with resistance to Ma549 in both males ($r = 0.2$) and females ($r = 0.2$) ($P < 0.01$), but was only correlated with the resistance of female flies to Pa14 ($r = 0.26$, $P < 0.05$). Negative geotaxis has been shown to be sensitive to oxidative stress [29]. Sensitivity to oxidative stress, induced by paraquat but not menadione sodium bisulfate (MSB) [40], was positively correlated with the resistance of female flies to Ma549 ($r = 0.31$ $P < 0.001$) and male flies to Pa14 ($r = 0.36$, $P < 0.001$).

	Ma549		Pa14	
PHENOTYPES	**Male**	**Female**	**Male**	**Female**
Male flies vs. Ma549 LT50	1	**0.74**[c]*	**0.45**[c]*	**0.48**[c]*
Female flies vs Ma549 LT50	**0.74**[c]*	1	**0.31**[b]*	**0.4**[c]*
Male flies vs Pa14 LT50	**0.45**[c]*	**0.31**[b]	1	**0.77**[c]*
Female flies vs Pa14 LT50	**0.48**[c]*	**0.4**[c]*	**0.77**[c]*	1
Overall Fitness				
Lifespan LS Mean	n/a	0.03	n/a	0.17
Wk1 Fecundity LS Mean	n/a	-0.07	n/a	0.11
Wk3 Fecundity LS Mean	n/a	0.07	n/a	0.03
Wk5 Fecundity LS Mean	n/a	0.06	n/a	0.08
Wk7 Fecundity LS Mean	n/a	0.09	n/a	-0.06
Life Time Fecundity LS Mean	n/a	-0.01	n/a	0.11
Courtship Behavior	**0.17**[a]	n/a	0.04	n/a
Response to Stimuli				
Startle Response	0.1	**0.17**[a]	0.07	0.03
Startle Response (MSB)	0.14	**0.18**[a]	0	0.03
Negative Geotaxis	**0.2**[b]	**0.2**[b]	0.06	**0.26**[a]*
Aggression	0.04	n/a	0.11	n/a
Response to Stress				
Paraquat resistance	0.13	**0.31**[c]*	**0.36**[b]*	0.17
MSB resistance	-0.06	0.07	0.18	0.01
ER Stress Hazard Ratio	0.15	n/a	0.2	n/a
ER Stress T50	0.05	n/a	0.07	n/a
Chill Coma Recovery	0.08	-0.05	0.13	0.21
Nutritional Status				
Starvation Resistance	0.1	**0.16**[a]	**0.27**[a]*	**0.28**[a]*
High Glucose Diet				
Blood Glucose	0.07	n/a	0.02	n/a
Weight High Glucose Diet	0.05	n/a	0.04	n/a
Low Glucose Diet				
Blood Glucose	**0.17**[a]	n/a	**0.31**[a]*	n/a
Triglyceride Low Glucose Diet	**-0.18**[a]	n/a	-0.12	n/a
Weight Low Glucose	0.08	n/a	0.17	n/a
Sleep indices				
Night Sleep Duration (Min)	**-0.32**[c]*	**-0.28**[c]*	-0.01	-0.14
Day Sleep Duration (Min)	**-0.2**[a]	0.04	-0.05	-0.05
Night Bout Number	**0.25**[b]*	**0.24**[b]*	-0.04	**0.27**[a]*
Day Bout Number	0.12	**0.16**[a]	-0.03	0
Night Avg. Bout Length (Min)	**-0.2**[a]	**-0.21**[b]	-0.02	**-0.29**[a]*
Day Avg. Bout Length (Min)	**-0.19**[a]	-0.05	0	-0.14
Waking Activity	-0.12	**-0.2**[a]	0.09	0.07

[a]P < 0.05,
[b]P < 0.01,
[c]P < 0.001.
* Passes Holm-Bonferroni method
n/a = trait not studied in that sex

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Table 2. Correlations of LT_{50} values among the DGRP lines for *M. anisopliae* Ma549 and *P. aeruginosa* Pa14 (this study) with traits previously measured by other groups.

Resistance to starvation [10] is positively correlated with resistance to PA14 in both males (r = 0.27) and females (r = 0.28) (p< 0.05), but was only correlated with the

resistance of female flies to Ma549 ($r = 0.16$, $P < 0.05$), indicating that Pa14 causes greater nutrient stress to *Drosophila* than Ma549. However, disease resistance was not associated with wet weight of the fly lines so larger flies are not necessarily more resistant. Various measurements of energy reserves by Unckless et al., [31], such as glycogen stores, total triglycerides and soluble proteins in flies showed no correlation with disease resistance, suggesting that there is no straightforward association between these traits. Unckless et al., [41] found that bacterial (*Providencia rettgeri*) loads were negatively correlated with blood glucose levels. Conversely, we found resistance to Pa14 in male flies (Unckless et al., [31] only tested males) was positively correlated ($r = 0.31$, $P < 0.05$) with glucose levels in flies fed a low glucose diet.

Resistance to Ma549 was negatively correlated with sleep duration, particularly at night in males ($r = -0.32$) and females ($r = -0.28$) ($P < 0.001$) and to a lesser extent, and then only in males, during the day ($r = -0.2$, $P < 0.05$). Conversely, there was a positive association between resistance and the number of sleep bouts in males (females) of 0.25 (0.24) ($P < 0.01$). Similarly, resistance of female flies to Pa14 was positively correlated with number of nocturnal sleep bouts ($r = 0.27$, $P< 0.05$) and negatively correlated with average bout length ($r = -0.29$, $P< 0.05$). Hence there is a trend for resistant flies to have more sleep bouts than susceptible flies, but these bouts are shorter and total sleep time is less. This may be related to findings that the phagocytic activity of *Drosophila* immune cells is circadian-regulated and peaks at night during the night rest [42]. However, our data suggests that the number of sleep bouts has more effect than sleep duration on resisting infections with Ma549.

Our measurement of longevity in 20 lines was moderately ($r = 0.52$, $P < 0.05$) correlated with that of Durham et al., [36], indicating the genetic robustness of phenotypes across lab groups and different assay conditions (we used batches of flies grown on cornmeal-molasses-yeast-agar medium with Tegosept and propionic acid, whereas Durham et al., [36] used pairs of flies kept separately and grown on sucrose-yeast agar). However, resistance to Pa14 was not significantly correlated ($r = -0.21$, $P = 0.12$, $n = 58$) with resistance of female flies to *Pseudomonas entomophila*, even when we expressed our data as % killed in 3 days ($r = 0.22$, $p = 0.10$, $n = 58$) following Sleiman et al [43]. Using this metric (% killed) for our data we lost correlations with sleep indices. As the specialized entomopathogen *P. entomophila* relies on novel secondary metabolites and toxins to kill insects [44], we speculate that the Sleiman et al., [43] analysis may have included measuring variation in resistance to these.

The absence of overall positive or negative correlations between resistance and most metabolic indices does not exclude trade-offs in individual fly lines, as all these parameters are complex traits and the product of pleiotropic genes. Thus, polymorphisms associated with increases (decreases) in disease resistance are not consistently associated with increases (decreases) of resistance to oxidative stress, starvation stress, nutrient levels, fecundity etc. S3 Table shows a subset of the 10 most Ma549 resistant and 10 most Ma549 susceptible DGRP lines (hereafter called the "divergent subset"), and their life cycle parameters and rankings in correlated data from other groups. RAL-38, the most resistant line to Ma549, ranked 154 out of 167 for resistance to paraquat, whereas the 3rd and 5th most resistant lines ranked 33rd and third, respectively. Thus, resistance to oxidative stress may be a factor in resistance of some fly lines but not others. While there

is no significant correlation between MSB resistance and resistance to Ma549 in female flies, a t-test comparison of the absolute rankings of the divergent subset for MSB resistance reveals significant differences in survival time to MSB. Similarly, there are lines with increased levels of resistance to Ma549 and starvation stress, sleep duration or nutrient levels, but there are also resistant lines with moderate or low rankings for these indices. Consequently, overall correlations could be non-significant for some indices if there are pleiotropic effects of polymorphisms affecting disease resistance on other traits, but the effects are not in the same direction.

1.4.3 The impact of natural host variation on fungal fitness

To further investigate the impact of natural host variation on Ma549 fitness we compared a subset of 20 divergent lines (S3 Table), for differences in impact on four key Ma549 life history traits at different steps of the infection process; within-host growth (fungal load, measured as CFU's), host life span (LT_{50} values), latent period (the lag time between inoculation and sporulation), and sporulation capacity (the total number of spores per *Drosophila* cadaver).

A time course of CFU counts showed that resistant flies delayed fungal growth compared to susceptible flies (Fig 4). Absolute numbers of viable fungi recovered after infection from hemolymph differed substantially between different lines and did not necessarily correlate directly with lethality (LT_{50}). However, in all lines, except for the susceptible line RAL_439, fungal loads increased in the 36 hours preceding death. Consequentially, there was a strong association between LT_{50} values and the time points

at which flies carried fungal loads of >10 CFUs (r = 0.61, P = 0.0086) or >100 CFUs/fly (r = 0.82, P = 0.002).

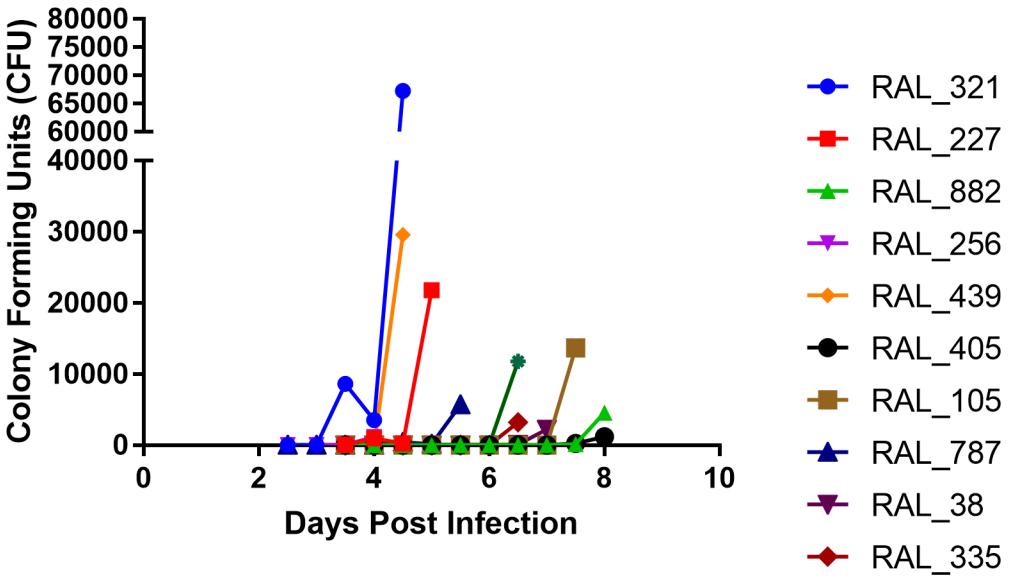

Fig 4. Time course of CFU production in male flies from DGRP lines.
Flies were homogenized and plated at 12 h intervals post-infection until death. Ten lines are shown as representative examples. CFUs were averaged from ten individual flies per fly line per time point.

We also used a Ma549 transformant expressing GFP (Ma549-GFP) to track infections in whole insects and hemolymph in the 20 different lines (Fig 5 for exemplar images). Ma549-GFP is sufficiently bright as to be clearly visible from outside the infected insect's abdomen, which confirmed that blastospores and hyphal bodies accumulated in the body cavity in the day preceding death. Consistent with CFU counts, the timing of colonization and the fungal load in the hemocoel are affected by the fly's genetic background (Fig 5), indicative of micro-environmental plasticity. Fluorescence showed blastospores (yeast-like budded cells thought to be important for dissemination of the pathogen) appearing in the hemolymph ~day three in most susceptible lines, as illustrated by RAL_321 (Fig 5). In contrast, proliferation of blastospores and

subsequently elongated ellipsoid cells only occurred 4 to 4.5 days post-infection in resistant fly lines, demonstrating a longer time lag between penetration and proliferation than occurred in susceptible lines. However, we also noted differences in fungal behavior in different fly lines, even where these had very similar LT50's. Ma549 produced very few (<5) blastopores in the susceptible line RAL_439 three to 3.5 days post infection when flies were already dying. In contrast, the slightly less susceptible line RAL_321 contained abundant blastospore's 3.5 days post infection (average 8,600 CFU counts/fly), and at time of death, these had differentiated into hyphal filaments with simple branching (Fig 5). These filaments consisted of chains of budding cells marked by constrictions rather than septa at the junctions, and thus fit the definition of pseudohyphae [45]. The proliferation of hyphal chains before fly death would result in CFUs underestimating the number of fungal cells in hemolymph. In most lines, long hyphal lengths accumulated in the body at or after death. This probably reflects different environments in line RAL_321 and the other lines in the day preceding death but the nature of the environmental signals that control the ability of Ma549 to form blastospores or pseudohyphae is unknown.

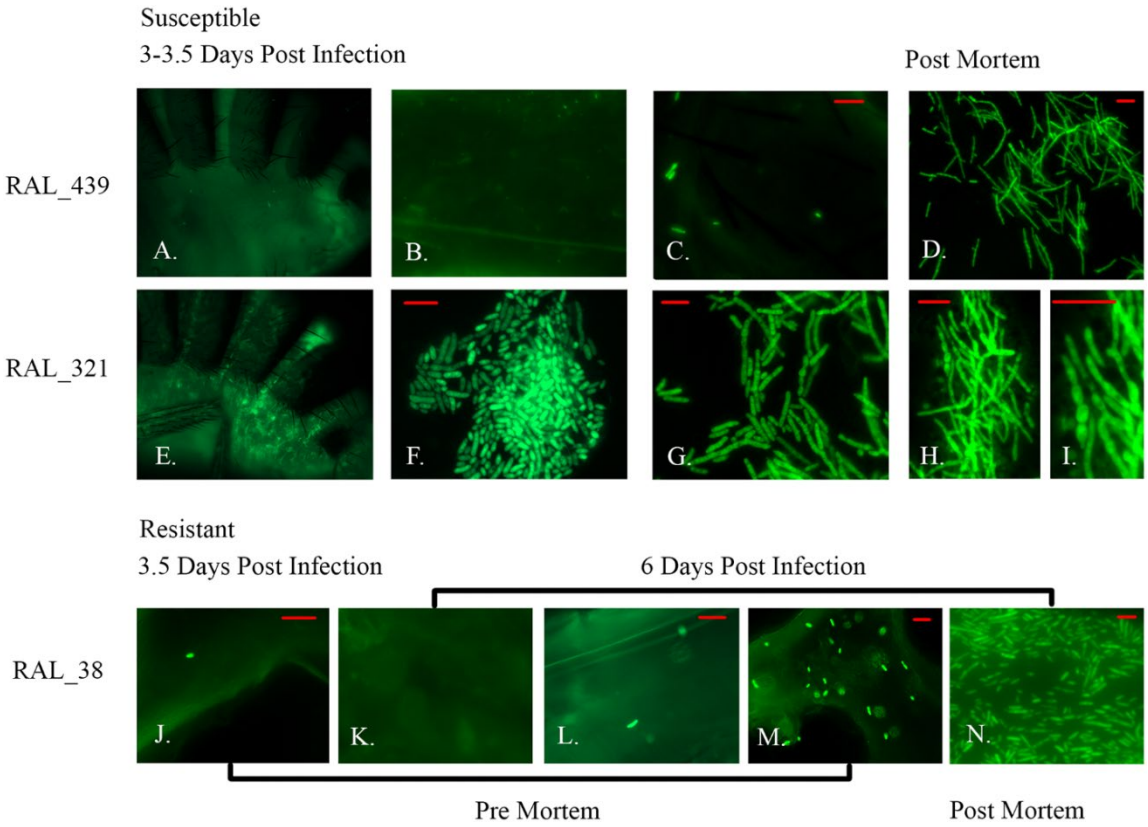

Fig 5. Growth of GFP-expressing Ma549 can be visualized in DGRP flies. DGRP fly lines RAL_439, RAL_321, and RAL_38 photographed at different time points post infection with Ma549-GFP. A) No visible growth of Ma549-GFP in a living RAL_439 fly's abdomen, and there are very few pre-mortem fungal propagules in squashed RAL_439 flies (B-C). Ma549-GFP only proliferates in RAL_430 post mortem (D). In contrast, Ma549-GFP blastospores and short hyphal lengths are visible in the hemocoele from outside a still living RAL_321 fly's abdomen (E), and in pre- and post-mortem squash preparations (F to I). Variation among individual flies in resistant line RAL_38 in number of Ma549-GFP propagules per fly 6 days' post-infection (J-N). At death, Ma549-GFP blastospores and short hyphal lengths are found in hemocoele of all DGRP lines (D, H, I, and N). The pictures are representative of the 10 flies per line per time point examined for the experiment. Bars in images represent 20 μm.

Spore production is a measure of pathogen transmission potential and therefore pathogen fitness [23]. Host genotype impacted the onset of Ma549 sporulation (latent period) which moderately correlated ($r = 0.51$, $P < 0.01$) with life span. This is readily explained by sporulation only commencing on cadavers within 60 hours' post-mortem. However latent period was not associated with total spore production. Indeed, we found

no significant difference (P = 0.26) in spore production per cadaver between the 10 most resistant ($1.86 \times 10^7 \pm 1.94 \times 10^6$) and 10 most susceptible ($1.67 \times 10^7 \pm 1.48 \times 10^6$) lines in the divergent subset (S3 Table), indicating that rapid kill of susceptible hosts will not be disfavored by natural selection because it is traded off against reduced time to exploit host nutrients for substantial pathogen reproduction.

1.4.4 Micro-environmental plasticity in GWA lines

To quantify micro-environmental plasticity (variation among individuals of the same genotype reared in a common environment), for mean times to death values we used the within-line standard deviation (σ_E, and its natural log $\ln(\sigma_E)$, and the within-line coefficient of environmental variation (CV_E, $\ln(CV_E)$) (Figs 6 and 7). The number of segregating sites and standard deviation per fly line were not correlated ($r = 0.07$, $p = 0.354$) which suggests residual heterozygosity does not contribute to within line standard deviation. CV_E is often used to remove any relationship between mean and variance, but $\ln(\sigma_E)$ has other advantages [46], so we used both metrics. The correlations between $\ln(\sigma_E)$ and CV_E are high in Ma549 infected flies ($r = 0.94$, $p < 0.001$) (Fig 6). Likewise, LT_{50} values and mean survival times were highly correlated ($r = 0.99$, $p < 0.0001$) (Fig 6a). With either metric, disease resistance was highly variable among flies with identical genotypes (Fig 7), suggesting that some lines are relatively more canalized and others more phenotypically plastic in response to the same random environmental effects [47]. Genetic correlations show that the micro-environmental plasticity (σ_E or CV_E), was most variable in lines having the highest LT50 or mean survival times (Fig 6B and 6C) suggesting that some of the variants affecting the mean also affect the micro-

environmental variance. As reported for stress responses [46], the magnitude of the genetic variance affecting micro-environmental plasticity is high, with broad sense heritability's (H^2) of ln(σ_E) of $H^2 = 0.5$ (Ma549) and $H^2 = 0.52$ (Pa14). Thus, the broad sense heritability at the variance level for resistance to Pa14 is of the same magnitude as that at the level of the mean and, for Ma549, the heritability of micro-environmental variance is twice as large as that of the mean.

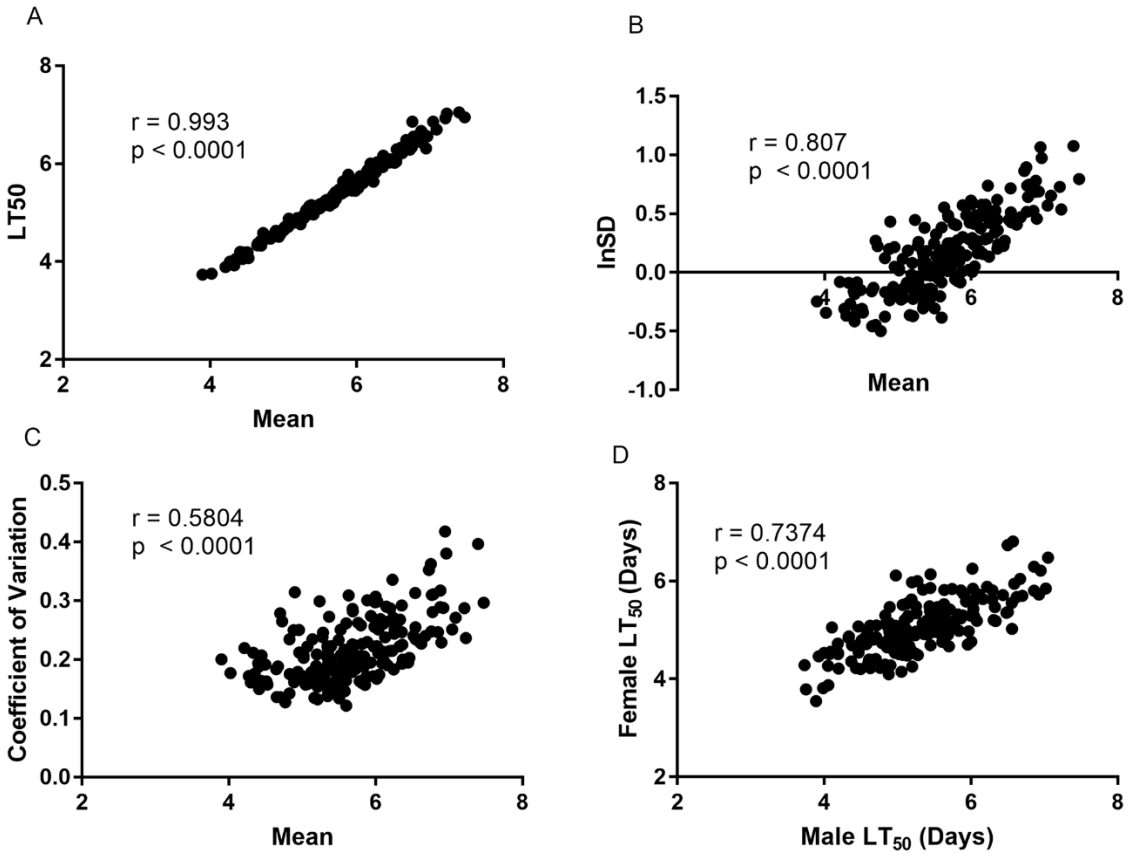

Fig 6. Correlation analyses in DGRP lines challenged with Ma549: (A) LT_{50} versus mean survival times; (B) ln(SD) versus mean survival times; (C) coefficient of variation (CV_E) versus mean survival time, and (D) CV_E versus ln(SD).

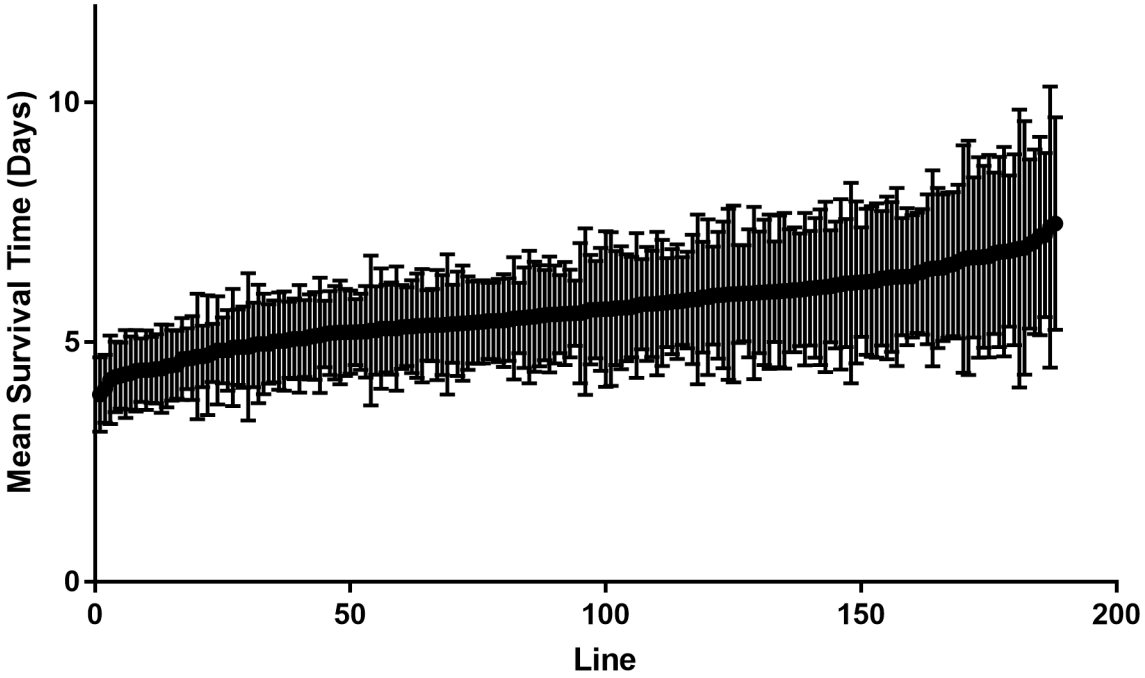

Fig 7. Male mean survival times of DGRP lines and their standard deviations when treated with Ma549.

1.4.5 Genotype-phenotype associations

To identify genes that harbor alleles that confer altered susceptibility, Ma549 and Pa14 mean LT50s were plugged into the DGRP pipeline with a discovery *P* value $<10^{-5}$. Most polymorphisms associated with mean time to death were at the low range of the allele frequency spectrum, with frequencies below 0.2 for 44% (41%) of Pa14 (Ma549) alleles (Fig 8). These lower frequency alleles had larger effects on LT50 values than common alleles (Fig 8), consistent with GWA studies on some other complex traits in the DGRP population [40,48]. Negative effects (where flies homozygous for the minor low frequency allele live longer following infection than do flies homozygous for the major allele), greatly outnumbered positive effects. A corollary of this is that the most Ma549 resistant DGRP lines had a preponderance of low-frequency alleles ($r = 0.23$, $P < 0.0012$) (Fig 9A). The effect was more complicated for Pa14 [where there was no overall

correlation (r = 0.05, P = 0.67)] and the distribution of minor alleles traced a parabola (Fig 9B). The heritability of Pa14-induced mortality was analyzed on 81 lines only, which means there may be a higher level of false associations [14 (>20%) of the polymorphisms have a minor allele count of 5 or less (S4 Table)]. However, if susc susceptible lines (LT50's < 4 days) and resistant lines (LT50's > 4 days) were considered separately the associations were r = -0.65 (P < 0.0001) and r = 0.45 (P 0.0034), respectively, consistent with minor alleles being concentrated in the most susceptible as well as the most resistant lines.

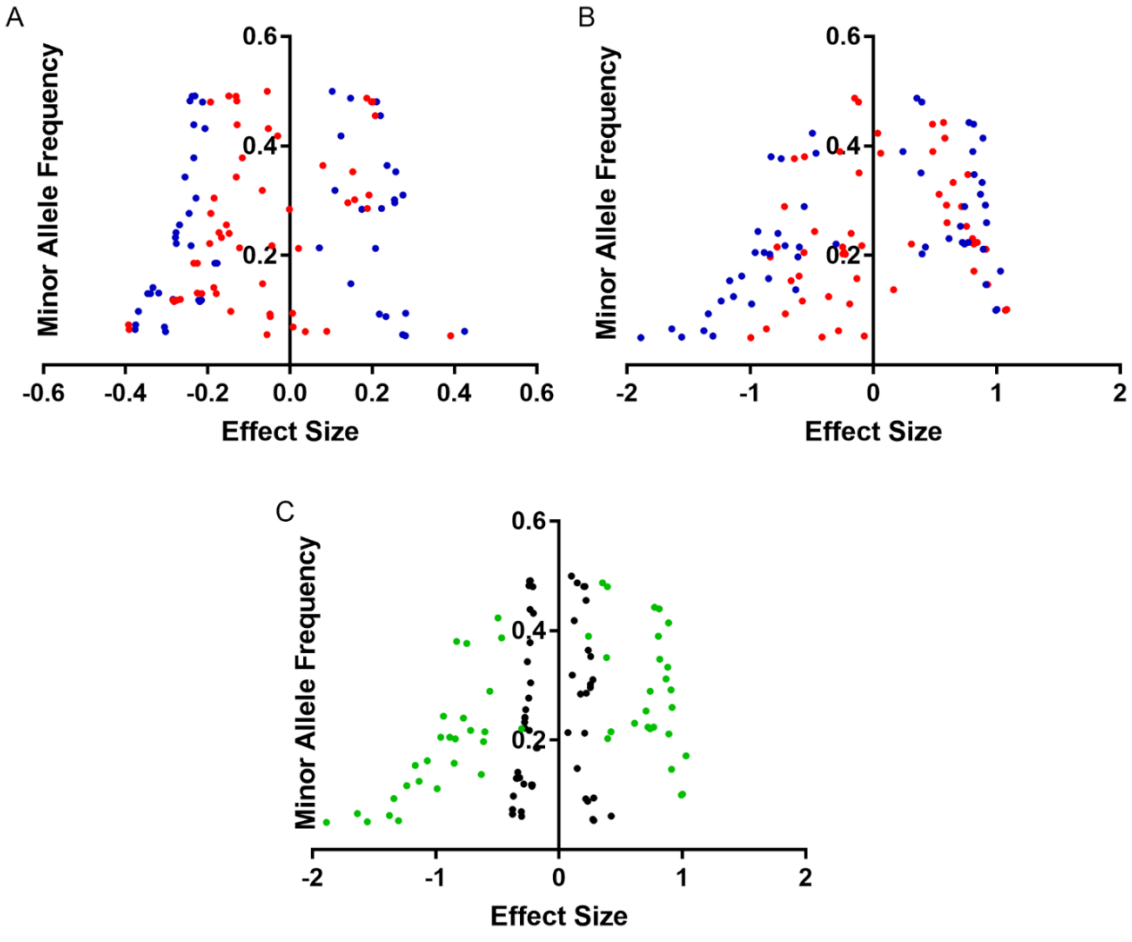

Fig 8. Minor allele frequency versus effect size for females (red) and males (blue) infected by Ma549 (A) or Pa14 (B), and comparing Ma549 (black) and Pa14 (green) infected male flies (C).

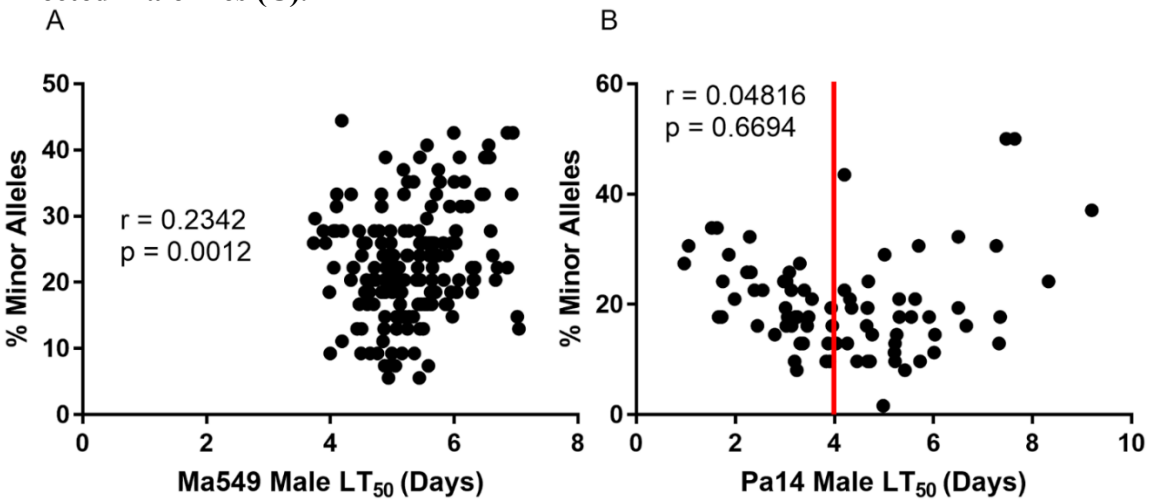

Fig 9. Mean LT_{50} per line plotted against number of minor alleles per line for Ma549 (A) and Pa14 (B).

Pa14 polymorphism effects were much larger than those observed in Ma549 (Fig 8C), consistent with the much greater variation between lines observed in Pa14 LT_{50} values (Fig 1). Likewise, male polymorphism effects with Ma549 or Pa14 were larger than those observed in females (Fig 8a and 8b). The majority (63%) of Ma549 tagged polymorphisms had sex-specific effects, with the greater number (20) effecting survival of males as compared to 14 for females. This is consistent with the mean differences in male and female survival (Fig 1), but contrasts with alleles conferring genetic risk to oxidative stress where SNP effects were larger in females than males [40]. For Pa14, 14 mutants had female-specific effects, and 17 mutants had male-specific effects.

1.4.6 Polymorphisms associated with resistance to Ma549

SNPs/indels that are significantly associated with variation in LT50's to Ma549 and Pa14 ($P < 10^{-5}$) are presented in S4 Table. We found 50 SNPs and 4 indels associated with Ma549's speed of kill with a discovery $P<10^{-5}$ (45 total associated genes). With a

more stringent cut off of $P<10^{-6}$, there were four top-candidate genes (*hig*, *Cyp4p2*, *msn*, and *Rab26*). Overall, polymorphisms significantly associated with variation in disease resistance are disproportionately found in introns and UTRs, as opposed to synonymous substitutions or positions more than 1000 bp from known genes. For example, of the 54 candidate polymorphisms, five were indels (three in introns, two within 1000 bps downstream of a gene) and 49 were unique SNPs. Of these SNPs, four were synonymous, five mapped to within 1000 bps downstream of a gene, 9 were intergenic (more than 1000 bp from known genes), three were non-synonymous, two were in a 5'UTR and the remaining 26 were intronic. Fourteen polymorphisms are located in overlapping genes that could affect either or both genes. Thus, for Ma549 resistance, 34 out of 54 (63%) total significantly associated polymorphisms are found in introns, UTRs, or as nonsynonymous SNPs, and 45 (83.33%) overall were genic. Given the percentage composition of the *Drosophila* genome (48.2% genic (including 18.3% exonic and 30% intronic) and 51.8% intergenic [49], this enrichment for putatively functional polymorphisms is significant ($\chi 2 = 4.714$, df = 1, $P < 0.03$). Each polymorphism that associates significantly with variation in a measured phenotype is given in S4 Table, including significance level, estimated effect size, minor allele frequency and type of polymorphism.

Of the 45 candidate genes, 34 (75.6%) have human counterparts, and 22 (48.9%) have human counterparts associated with disease (S5 Table). *hig*, *Cyp4p2*, *jhamt*, *Mctp*, *tRNA:CR30229*, *sickie*, *CG12344*, *CG13229*, *CG33172*, *CG17209* had multiple significant polymorphisms affecting resistance to Ma549. With the singular exception of one of the four polymorphisms in *CG13229*, the effects of these polymorphisms in each

gene were in the same direction implying LD in variable genes. All four synonymous and intron polymorphisms in *hig* had a positive effect (lines homozygous for the major allele survived infection longer than flies homozygous for the minor allele), and the best-supported SNP had a nominal P-value of $P = 3.18x\ 10^{-7}$. Aside this synonymous SNP, the other three SNPs were in the same intron and between 411–442 base pairs upstream of the nested gene *Cyp4p2*. None of the SNPs outside the *hig* gene localized to genes surrounding *hig* so this area of strong linkage disequilibrium only extends across the *hig* gene. *Cyp4p2* is involved in resilience to sleep deprivation and wakefulness [50]. Being involved in the functioning of cholinergic synapses, *hig* is also required for wakefulness, and deficiency mutations show severely reduced activity and longevity [51,52]. Both polymorphisms in *jhamt* (hormone secretion) had a negative effect. Two polymorphisms in *Mctp* (calcium ion binding) had negative effects; the *Drosophila* gene has no reported function but its human homolog is implicated in oxidative stress and disorders in eating [53].

Most of the genes affecting response to Ma549 have not been previously implicated in interactions with *Drosophila* pathogens. Overall, they fall into 11 ontological categories with reported roles in defense, metabolism, morphogenesis and development, stress responses, cellular communication, behavior, and gene expression. Immune genes include *sickie* required for activation of Relish, an Imd signaling component involved in antibacterial and antifungal polypeptide induction [54], and *CG5794/puffeye* (*puf*), a ubiquitin-specific protease that is a negative regulator of Imd and Toll innate immune defenses; its human homolog also plays regulatory roles in immune signaling [55].

Many of the candidate genes are pleiotropic with functions in cell adhesion and epithelial wound repair plausibly linked with infection, and some may link the immune system, nervous system and nutrition e.g., *Neuroglian* (*Nrg*) is involved in neuron cell-cell adhesion as well as melanotic encapsulation of parasites [56]. Likewise, *Lar* is involved in multiple processes involving cadherin and adhesive interactions [57]. The cadherin *Dystroglycan* (*Dg*) is associated with stress responses in *Drosophila* and humans [58]. *forked* (f) regulates the differentiation of epidermal cells and cuticle formation [59]. The kinase *misshapen* (*msn*) regulates cell migration and the epithelial response to wounding [60], and *Mks1* is also involved in epithelial repair [61].

Schnurri (*shn*) and Star (*S*) also have roles in regulating tissue differentiation, and both antagonize Notch signaling [62–64]. Notch signaling is highly conserved and plays critical roles in cell fate specification. In *Drosophila* it is key to differentiation of crystal cells as vehicles for the prophenoloxidase-activating cascade [65], previously implicated as one of *Drosophila*'s most effective defenses against Ma549 [23].

We found a number of genes involved in hormonal regulation of development, including *taiman* (*tai*), co-activator of the ecdysone regulator [66], *juvenile hormone acid methyltransferase* (*jhmat*), and *Sik3*, a hormone dependent regulator of blood glucose metabolism and starvation responses [67,68]. Ecdysone mediates the development of immunity in the *Drosophila* embryo [69], and promotes induction of antimicrobial peptides, whereas juvenile hormone is an immuno-suppressor [70]. Juvenile hormone stimulates reproduction at the expense of shorter life span in *Drosophila* [71], opening up

the possibility of hormones being regulators of trade-offs between disease resistance and other aspects of fitness at the evolutionary level.

As LT_{50} values correlate with sleep cycles we would expect to find genes that are known to regulate *Drosophila* circadian rhythms. The GABA receptor *Rdl* is a key gene regulating sleep and wake transitions in *Drosophila* [72,73]. In addition, we found considerable overlap between Ma549 tagged genes and sleep tagged genes from a previous GWAS with the DGRP [24], including, not surprisingly *Rdl* and *hig*, as well as *CG12344*, *CG17209*, *CG32061, CG33172*, *CG9990*, *f*, *gem3*, *Nrg*, *S*, *tai* (day average bout length), *Rbp6* (night sleep) and *jhamt*, *msn* (waking activity). *Sickie* and *Rbp6* were also tagged in a screen for parquet-induced oxidative stress [40].

Lastly, several genes had inferred activities (i.e., no experimental evidence) in FlyBase including *Rab26*, a GTPase of no reported function in *Drosophila*, but its mammalian homolog regulates secretion by highly active secretory cells [74]. *Rab26* harbored an SNP with a nominal P-value of $P = 1.66 \times 10^{-7}$. Other inferred activities included G-coupled protein receptors (*CG13229*, *CG44153*), a regulator of cell proliferation (*CG33172*), an RNA-binding protein (*Rbp6*), an extracellular-glycine-gated chloride channel (*CG12344*), and an ABC transporter (*CG9990*). Several genes had no known function: *CG13313*, *CG33136*, *CG5111*, *CG8508*, and non-protein coding gene *CR43259*.

To identify genes that confer micro-environmental plasticity to susceptibility to Ma549 infection, we also performed GWA analyses to associate CV_E values with allelic variation. We found 39 SNPs and 7 indels (26 candidate genes) that were associated with

micro-environmental plasticity in response to Ma549 at $P<10^{-5}$. A total of five SNPs remained significant when the significance threshold was $P<10^{-6}$. A single gene, *f*, was identified in both this analyses and the GWAS using LT_{50} values.

The screen tagged several cell adhesion molecules involved in the axon guidance system and/or phagocytosis including *Con* [75], *DSCAM* and *DSCAM4* [76], *gukh* [77], and *hdc* [78]. Interestingly, *DSCAM* and *Mhcl* have been studied for their ability to express multiple isoforms suggesting molecular complexity of the systems they influence. Consistent with complexity, *wmd* (muscle morphology), and *Mhcl1* (myosin) are associated with multiple developmental defects in *Drosophila* and have human homologs linked with multisystem diseases [79–81]. Likewise, *bab1*, *Xpd* and *qless* have human homologs linked to multiple syndromes [82–85].

Another notable feature was the number of tagged genes previously implicated in regulating circadian rhythms (*bab1*, *CCKLR-17D3*, *CG10953*, *CG9705*, *Con*, *Ddr*, *DSCAM*, *f*, *gukh*, *CG33687*, and *CG8664*) [86–88]. Most of these genes as well as *CG13917*, *CG13983*, *hdc*, and *Mhcl* were tagged in a screen of genes affecting CV_E of DGRP lines to sleep [24]. As with the LT50 screen, some tagged genes had inferred activities (i.e., no experimental evidence) in FlyBase including *CG14204* (acetyltransferase) and *CG4901* (helicase), and other genes had no known function *CG10953*, *CG13917*, *CG33687*.

1.4.7 *Pseudomonas aeruginosa* GWAS

Because we terminated the Pa14 bioassay at 14 days, when some of the most resistant fly lines had residual survivors, only the Pa14 mean LT50s were plugged into

the DGRP pipeline. Of 62 polymorphisms ($P<10^{-5}$) (51 total associated genes), 12 were indels (7 introns, 4 intergenic and one codon deletion) and 50 were unique SNPs (2 non-synonymous, 7 synonymous, one within 1000 bps downstream, 10 intergenic, two in a 5'UTR, 1 in an exon, and the remaining 27 were intronic). CG42343, a protein coding immunoglobulin-like gene with no known function had 9 significant intronic polymorphisms (4 SNPs, 5 deletions) affecting resistance to Pa14, and all had a negative effect. Each polymorphism that associates significantly with variation in resistance to PA14 is given in S4 Table, including significance level, estimated effect size, minor allele frequency and type of polymorphism.

Surprisingly, given the correlation between Ma549 and Pa14 virulence to DGRP lines, only one tagged gene, *CG44153*, was in common. A similar lack of overlap has been reported in other DGRP studies and this is often attributed to epistasis [29]. Notwithstanding this, Pa14 and Ma549 responsive genes effected many of the same pathways and functions, including Notch signaling and secretion e.g., *CrebA* activates expression of every secretory pathway component gene [89]. Several genes are involved in developmental processes, morphogenesis and tissue maintenance including *Osi1* [90], *Zasp52* [91], G-protein coupled receptor *Mthl1* [92], and *Usp10* (*CG32479*); an ubiquitin specific protease that functions as a positive regulator of notch signaling [93]. Several genes may relate to Pa14's mode of *per os* infection, including *Mnt* involved in gut cell differentiation and body size [94], and *cert*, which is required for a normal oxidative stress response in the gut [95].

As with Ma549 tagged genes, many of the Pa14 candidates were highly pleiotropic. *Pura* (*CG33275*) is a positive regulator of Rho protein signaling involved in circadian rhythms, perception of pain, and regulation of locomotion [96]. *Ca-alpha1T* is also involved in neural pathways and behavior [97]. *Pde9* has no reported function in *Drosophila*, but its human homolog (63% sequence similarity) is involved in cGMP signaling, hyperglycemia, diabetes, learning, differentiation of stem cells, and neurodegenerative disease [98].

1.4.8 Gene enrichment and network analysis

We used the DAVID algorithm [99,100] to perform GO enrichment analysis to assess to what extent the entire suite of candidate genes associated with variation in response to Ma549 and PA14 were functionally related. Using a Benjamini-corrected $P < 0.05$; GO categories that were significantly enriched for Ma549 resistance (S6 Table) included biological process terms for metamorphosis, morphogenesis, and neuron differentiation indicating that early developmental processes effect subsequent responses to disease. Resistance to PA14 was not associated with significant GO enrichment. We also ran an exploratory GO analysis on genes tagged by polymorphisms using a relaxed p-value of $1x10^{-4}$ as described [31,101]. The top Ma549 categories were analogous to GO categories identified at $1x10^{-5}$ including developmental and morphogenesis genes, but also included cell motion, chemotaxis, cell recognition and cell adhesion, and signaling (S6 Table). Protein domain analysis for either the stringent or the relaxed GO categories displayed an over-representation of immunoglobulin-like genes, fibronectins, and epidermal growth factor-like domains. GO analysis on Pa14 genes tagged by

polymorphisms with a p-value of $1x10^{-4}$ also included morphogenesis, development, adhesion and signaling, with an over representation of immunoglobulin-like genes and fibronectins. These domain analyses suggest that candidate gene with polymorphisms associated with disease resistance include an over representation of extracellular matrix proteins associated with cell adhesion and immunity.

We also used the relaxed p-value of $1x10^{-4}$ to examine whether these polymorphisms where enriched for true positive associations and cellular networks. To accomplish this, we used the R spider program [102], which organizes gene products into cellular pathways based on the Reactome signaling network and the KEGG metabolic network to determine if interactions are over-represented more than expected by chance. Using Ma549 GWA alone did not produce a significantly enriched network. We therefore performed a network enrichment analysis by pooling all GWA candidate genes associated with resistance to Ma549 (including those that confer micro-environmental plasticity) and Pa14. Using a model that allows for no more than one missing gene or compound, we found a network ($P<0.005$), comprising 55 candidate genes associated with variation in resistance to Pa14 and Ma549 (Fig 10). The network revealed that genes that harbor alternative natural variation (alleles) associated with susceptibility/resistance are functionally connected through processes that encompass phagocytosis and engulfment, cell mobility, intermediary metabolism (arginine and proline, purine, ether lipids and glycerolipid), protein phosphorylation, axon guidance, response to DNA damage, and cyp450 drug metabolism, which may play a role in detoxification. Many of these genes are well connected in the network, but not all potential connections are included. Thus, transcription factor *FOXO* (4 SNPs tagged in the Ma549 CV_E GWA

screen (6.98 x 10^{-5}, 2.46 x 10^{-5}, 5.67 x 10^{-5}, 7.31 x 10^{-5}), included in the "Response to DNA damage" domain (Fig 10), is also involved in cross regulation of metabolism and innate immunity [103], and transcriptional regulation for nutrient-stressed flies during resource allocation [104]. Functional validation of the FOXO mutant, showed significantly decreased resistance of the mutant when compared to isogenic control flies (S7 Table). The implication of axon guidance shows that individual variation in susceptibility to pathogens may at least in part be determined by polymorphisms that affect subtle variation in neural function.

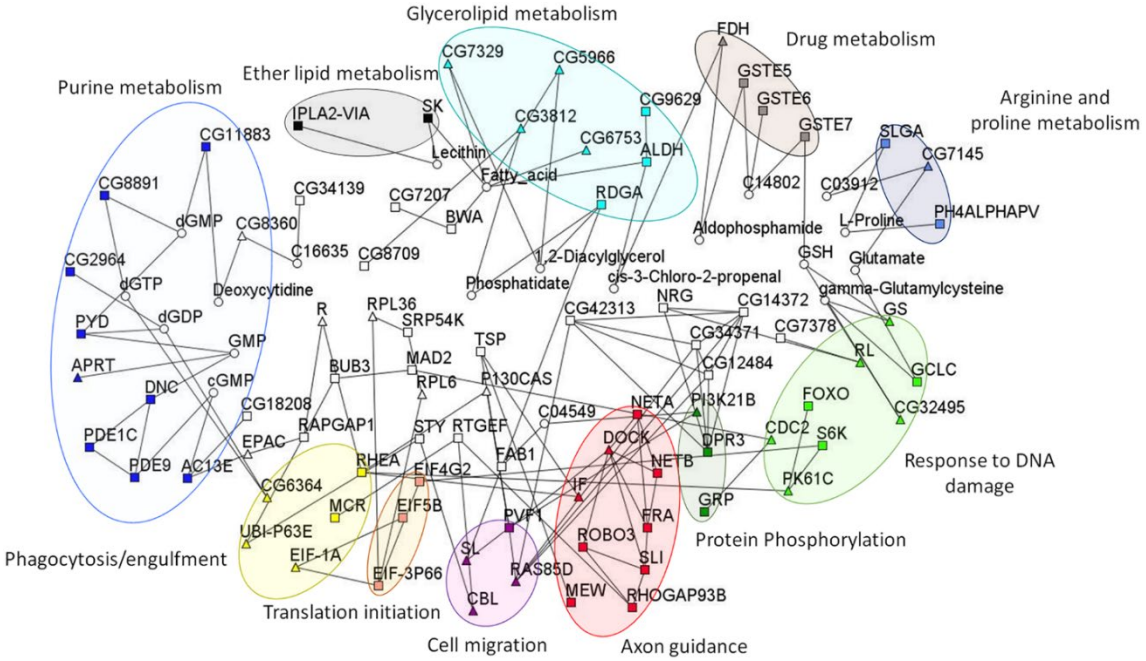

Fig 10. Cellular networks of candidate genes.
Enriched cellular genetic pathway for candidate genes from all genome wide association analyses (squares), allowing one missing gene (white triangles) or compound (white circles). The border colors indicate the over-represented gene ontology categories (P<0.005): axon guidance (red), translation initiation (orange), protein phosphorylation (dark green), cell migration (magenta), phagocytosis and engulfment (yellow), arginine and proline metabolism (dark blue), purine metabolism (light blue), response to DNA damage (light green), ether lipid metabolism (gray), glycerolipid metabolism (teal), P450 related drug metabolism (brown).

1.4.9 Functional validation of candidate genes

Ten of the random insertional mutations screened previously [23] were in genes tagged in the current Ma549 GWAS screen. Of these six (*Lar, msn, CG14304, CG44153, CG14995, Rbp6*) had insertional mutations with significant effects on disease resistance, a greater proportion than the 9% expected from random insertional mutations [23].

We additionally used the publicly available toolkit of P-element mutants to confirm the influence of a subset of 13 candidate genes [*S* (Notch signaling), *msn* (response to wounding), *shn* and *CG33172* (cell proliferation), *tai* (ecdysone regulation), *Sik3* (response to starvation), *Rdl* (circadian rhythms), *f* (cuticle formation), *CG9990* (ABC transporter), *CG32066* (unknown function), *CG33111* (unknown function), *puf* (negative regulation of innate immune responses), and *FOXO* (cross regulation of metabolism and innate immunity). These genes were chosen based on the significance level of their association with Ma549, or in the case of FOXO, its detection in a network enrichment analysis. To exclude mutations with generally deleterious effects on fitness we excluded from study genes in which lethal mutations are known. All 13 genes and corresponding controls were tested for both sexes with Ma549.

Nine mutants had significant effects on resistance to Ma549 in both sexes, two mutants (*Sik3* and *CG32066*) had a female-specific effect, one mutant (*puf*) had a male-specific effect, and a mutation in *shn* had no significant effects (S7 Table). In total, 21 out of 26 tests were significant, a much greater proportion than the 9% [23] expected from random insertional mutations (Fisher's exact test; $P = 0.0001$), supporting the contention that the top polymorphisms were enriched for true positive associations.

1.5 Discussion

We have utilized two complementary strategies for studies on fungal disease resistance in the fruit fly model system: a mutant screen approach aimed at the characterization of individual candidate genes [23], and in this paper a systems genetic approach to identify natural variation associated with disease resistance. The DGRP has lines that harbor most common variants and a representative sample of rare variants that have survived natural selection, and are unlikely to be produced by mutagenesis screens [24]. Our current study aimed at both quantifying levels of host genetic variation for resistance against different diseases and identifying the specific physiological and genetic factors that influence these traits.

If resistance is defined as an individual's ability to limit infection by reducing pathogen replication, then disease tolerance can be defined as the ability to limit the impact of infection on a host [105]. To elucidate the underlying mechanisms of variation in LT50 values we determined the fungal loads in hemolymph after infection. Although CFU counts from Ma549-infected insects are clearly affected by the genetic background, flies that succumb to Ma549 only carry high fungal loads in the 36 hours preceding death, irrespective of when this is, suggesting that flies in the more susceptible lines are less able to restrain *Metarhizium* growth. RAL_439 was exceptional in that there was very little fungal proliferation preceding death, suggesting that this line has a physiology that makes it less able to tolerate fungal colonization. Many *Drosophila* mutants succumb to bacterial infections because of defects in tolerance rather than resistance [106], whereas RAL_439 is evidence for genetic variation for tolerance in natural lines. Previously, we suggested based on work with *Drosophila* mutants, that it may be more difficult to evolve

tolerance traits to a filamentous fungal pathogen because unlike bacteria they actively penetrate and colonize infected tissues [23]. The present study suggests that most *Drosophila* lines have high tolerance to Ma549, but this is only put to the test in advanced infections when resistance breaks down. The implications of selection for resistance acting in concert with tolerance will need to be considered. Presumably, resistance could lead to selection pressure for higher virulence in the pathogen, whereas selection for tolerance could plausibly result in co-existence of pathogen and host [107].

Susceptibility to Ma549 within the DGRP is sexually dimorphic (Table 1; Fig 1), with males demonstrating higher resistance than females for most lines consistent with our previous mutant screen [23]. This finding is contrary to what is known from most pathogenicity studies in mammals, where females are the more resistant sex. However, exceptions include female mice being more susceptible to *P. aeruginosa* infection, showing higher bacterial loads in the lungs [108], so our finding may be part of a broader biological phenomenon. In addition to sexual dimorphism in susceptibility to Ma549 averaged over all DGRP lines, there is also genetic variation in the magnitude and direction of the difference in disease resistance, with females being more resistant than males in about half of the 94 lines that overall are most susceptible to Ma549. More work will be needed in order to understand the molecular mechanisms of these predispositions but they are presumably attributable to multifactorial sex-specific differences in genetics, immune processes, behavior and physiology. We previously demonstrated that virgin and mated females of laboratory *Drosophila* lines have similar susceptibility to Ma549 [23], but that would not necessarily carry over to wild strains.

By studying micro-environmental plasticity, we determined that even a single genotype allows for the production of flies with different susceptibilities to disease, and that this plasticity itself varies depending on genotype. That plasticity is to a large extent under genetic control is shown by its broad sense heritability being twice as high as that of the trait mean for Ma549-induced lethality using the same data set. A similar discordance in heritability values between trait plasticity and mean was obtained for chill-coma recovery time in *Drosophila* [46]. Genetic variation for plasticity will provide the genetic basis of evolution of phenotypic plasticity, making plasticity a heritable trait in its own right and subject to evolutionary mechanisms. Phenotypic plasticity is beneficial in predictably changing environments. The DGRP lines are all derived from an out-crossed population in Raleigh, North Carolina [26], but *Drosophila*'s relatively high migration rate [109], means they may not all have come from the same habitat and be locally adapted to the same pathogens. In these circumstances, phenotypic plasticity to variable pathogen exposures could allow a population to shift from one environment to another without genetic changes, buffering the strength of selection and preventing loss of genetic variation (a "bet-hedging" strategy) [110]. The trait mean and micro-environmental plasticity were highly correlated. Thus, directional selection for an increase in the mean disease resistance will result in more phenotypic variation. If applicable to breeding programs for disease resistance in domestic animals [111] these programs will result in high environmental variance rather than the desired uniformity.

Our results suggest that few of the polymorphisms that contribute to natural variation in resistance to bacteria and fungi in *Drosophila* populations affect canonical immune genes, but rather they cause variation in genes affecting many different aspects

of host physiology. These observations are in line with insertional mutagenesis techniques to document resistance genes [23]. Lu [23] reported that 87% of mutated genes in more susceptible lines are involved in a broad spectrum of biological functions not connected with canonical immune systems including basic cellular processes, early development, muscle and nervous system development and function, the senses, and metabolism. Those results are broadly recapitulated in the current GWAS analysis, although there was little overlap in the individual genes implicated by random mutagenesis and natural polymorphisms. The large number of candidate genes involved in development and function of the nervous system is potentially an artifact of the observation that neurological genes tend to be large and therefore provide a larger target for association studies [26], but neurological terms were enriched in our GO analysis that controlled for gene size.

The absence of many canonical immunity genes or immunity related gene ontology terms is of interest as it indicates that these have not been targets of pathogen-dependent selection in the DGRP. Of particular interest, neither Lu [23], nor this study implicate the antifungal peptide genes, although Lu [23] report that drosomyocin is induced by Ma549. One potential explanation is that there is little natural variation in canonical immune genes. However, the DGRP contains 838 variants in the *Toll* gene, 4 in the *Drosomyocin* gene, and 6 in the *Metchnikowin* gene. In contrast, Lu [23] found several indications for octopamine having an immune effect, but that was not replicated in our current GWAS analysis. Octopamine is the key hormone involved in the acute stress response and prepares the insect for flight or fight behaviors, as well as mediating a connection between the nervous system and the immune system [112]. There are several

possible and nonmutally exclusive explanations for these observations [113]. In the context of our current study: 1) Naturally occurring polymorphisms may not result in individual variation in resistance responses. This could occur if these genes are under purifying selection and hence functionally invariant. 2) Our GWAS necessarily did not take into account natural selection directed by other pathogens, and specialist pathogens evolving under pairwise co-evolution with *Drosophila* may be more likely to produce signatures of positive selection in immune genes. 3) Effect sizes of causal polymorphisms at these loci are too small to be detected given the resolution of the infection assay and the sample size. 4) Rare alleles at these loci, not interrogated in our analyses, affect natural variation in responses. 5) Functional redundancy in disease resistance genes may obscure the effects of individual polymorphisms on phenotypic variation.

Overall, our results suggest that natural lines differ in their ability to control replicating fungi during infection through the coordinated interplay of morphological restraints and different physiological and immune system effectors. Changes in physiological state likely alter immune-system function via neural/neuroendocrine/immune connections that adapt the immune system to changing needs [9], in subtly different ways in different lines. These results are consistent with studies on domestic animals where the proportion of the total variation in survival explicable by immune variables is too low to be useful as a selection criterion [111]. This was explained by the complexity of the mechanisms involved in the immune response and the large number of factors that may be involved in disease resistance [111]. A majority of polymorphisms associated with disease resistance were intronic, suggesting that gene expression variation may play a major role in determining variability in disease

phenotypes. Most were rare suggesting that mutations that increase resistance to Ma549 and PA14 may tend to be deleterious, so are either removed from the population or kept at a low frequency by purifying selection. Our results contrast with a GWAS study which showed that a small number of common polymorphisms have a major effect on resistance to viruses [32]. This may be because there are relatively few genetic changes that can cause viral resistance [32]. Major effect polymorphisms that protect hosts against infection have also been identified in humans [114–116], although the majority of human GWAS studies on non-communicable diseases have identified many rare alleles often with small effects [117]. Our association study, like a similar study on sleep in *Drosophila* [24], found that the lower frequency variants had the largest effects (Fig 8), supporting the rare variants hypothesis.

Previous studies have suggested that there is no clear-cut relationship between genetic resistance of *Drosophila* to different bacteria, so a given host genotype does not have a universal effect on a range of bacterial pathogens [118]. Sleiman [43] in their GWAS study found little correlation between enteric infection with *P. entomophila* and stab inoculated *Erwinia carotovora*, and concluded that the determinants of gut immunocompetence are distinct from those that govern systemic immunity. Martins [119] also conclude that *Drosophila* adaptation is contingent upon the infection route taken by the pathogen [119]. It was not axiomatic therefore that resistance to a clinical isolate of *P. aeruginosa* and Ma549 would be correlated, particularly as bacteria and fungi evoke the IMD and Toll pathways, respectively. Furthermore, we previously found an apparent trade-off in genes affecting resistance to bacterial and fungal infection [23], that was not supported by this finding. The correlation of resistance to PA14 and Ma549

is suggestive of general (multipurpose) defense mechanisms. *Metarhizium* species are abundant in the same soil and plant locations as *Pseudomonas* spp, [120], so local adaptation to these environments will be associated with heightened risk of contracting pathogens.

Depending on the way a pathogen interacts with its host, it may encounter specific or less specific defense barriers. Some of these may also be encountered by other pathogens depending on their routes of entry, host tissues infected and other factors. Fungi infect via the cuticle and bacteria through the gut so these components of the defense machinery will be specific to these pathogens, the unspecific generalized defense components are presumably in host tissues and hemolymph. The host responses triggered by *P. aeruginosa* remain poorly understood [121]. However, like *Metarhizium* [122], *P. aeruginosa* has means of limiting or resisting antimicrobial peptide gene expression [123]. Our current study shows that resistance to both Ma549 and PA14 correlates with survival times on paraquat but not menadione sodium bisulfite (MSB). Toxicity of paraquat is primarily due to production of superoxides whereas MSB toxicity is due to electrophilic attack [124], suggesting that the ability to alleviate or tolerate superoxide stress is a feature of a generalized defense response to multiple pathogens. *Drosophila* shows circadian regulation of response to oxidative stress [125], so between-line variation in these rhythms may influence how they respond to ROS produced during infection. Circadian control of the immune system is well established in humans [126], and circadian mutants in mice and flies have immune phenotypes [127]. Our data suggests that naturally occurring variation in sleep patterns also impact bacterial and fungal pathogenesis. Given the conservation of both circadian and innate immune

signaling between flies and vertebrates, this could have significant implications for vertebrate immunity. It has recently been established that insects can anticipate infections by up-regulating immune genes when they find themselves in scenarios associated with increased disease risk. Zhong et al., [128] raise the interesting possibility that control of immune genes by circadian clock genes might reflect "anticipation" of predictable fluctuations of disease risk over the course of 24 hours. Thus, if frequent naps were associated with pre-emptive up-regulation of immune genes this might be representative of a general pattern of immune anticipation in insects.

Resistance to multiple pathogens should have a selective advantage unless this general defense is traded off against other (pathogen-independent) fitness components [129]. In the absence of such a trade-off, directional selection should lead to fixation of genotypes showing general resistance [130]. However, the most resistant lines to Ma549 were enriched in minor (rare alleles), suggesting that these alleles have negative correlations with other fitness related traits. A trade-off of the cumulative cost of defense could have been reflected by a negative association with longevity and fecundity, which we did not observe. We found some weak positive correlations with blood sugar levels and resistance to starvation stress, consistent with nutritional status altering the quality of immune defense [31]. However, measurements of energy reserves (glycogen stores, total triglycerides and soluble proteins) showed no correlation with disease resistance. This was surprising since Ma549 would compete with hosts for resources, and it makes intuitive sense that overall genotypes that store more nutrients would have better tolerance to disease. Our previous insertional mutagenesis screen showed that half of the mutant lines with altered disease resistance had significant effects on starvation

resistance, but there was no simple association between disease and starvation resistance as networks of pleiotropic genes regulate complex traits [23]. Clearly being more or less tolerant to starvation does not by itself alter resistance to Ma549. However, many of the polymorphisms associated with variation in susceptibility to Ma549 are in genes affecting cellular processes and metabolism, and it is plausible that alterations in these processes could specifically change expenditure of energy on immune responses [131].

We found no negative genetic correlations between resistance to Ma549 and several other physiological variables and metabolic indices. Likewise, there were no correlations with measures of ability to cope with important abiotic stresses such as chill-coma recovery time. Southern *Drosophila* populations tend to have higher starvation resistance whereas northern populations tend to have fast chill coma recovery time [132], but our data suggest that this would not be traded off against resistance to Ma549. We did however find examples of genetic variation in the magnitude and direction of associations, such as DGRP lines RAL_399 and RAL_440 that were both highly resistant to Ma549 but demonstrating low and high life time fecundity, respectively. The absence of overall positive or negative correlations between resistance and metabolic indices does not exclude trade-offs as all these parameters were taken by other researchers from uninfected flies, and are complex traits that may not obey simple, single factor models [133]. The lack of a common pattern of correlations among the most resistant or the most susceptible lines i.e., some resistant lines were also particularly resistant to oxidative stress and some were not, suggests that there are multiple mechanisms by which the complex trait of disease resistance can be altered. Consequentially, a GWAS study will identify common trends in populations and not idiosyncratic differences between lines.

With that proviso, our single polymorphism association analyses using the DGRP provided insight into the genetic architecture of susceptibility/resistance to Ma549 associated with variation in this complex trait, and identified novel candidate genes outside the conventional immune system that may be selected for in determining susceptibility to infection. We performed secondary screens using mutations to confirm the reliability of the GWAs in predicting genes that indeed affect disease resistance toward different pathogens. The high validation rate engenders confidence that functional tests of other candidate genes involved in metabolism, development, oxidative stress and function of the nervous system will identify new components of genetic networks affecting disease resistance. The GWA studies presented here are a hypothesis-generating paradigm that lays the foundation for a detailed dissection of allelic effects of candidate genes in future endeavors.

The ecological features that might function as good predictors of host immune investment in *Drosophila* are unknown, but environmental variables, such us parasite species richness, could be informative. For example, fly populations coming from locations with a rich bacterial community have been found to be less susceptible to the bacteria *Lactococcus lactis* [134]. Tinsley [135] found regional differentiation in *Drosophila* susceptibility to the fungus *Beauveria bassiana*, although Paparazzo [136] suggested these differences in susceptibility could be due to general stress resistance. Clearly, much would be gained by being able to integrate our GWA data with studies of ecological genetics in wild *Drosophila* systems that evaluated the process of adaptation to different environments and pathogens [137]. Our current study assessing patterns of variation in host-pathogen interactions improves understanding of the relationship

between genetic variation and phenotypic variation for disease resistance, which is necessary for predicting responses to selection. This could have implications for estimating disease risk in humans as several studies have shown the *Drosophila* DGRP can be used to identify functionally similar homologous human genes [24]. It also has implications for development of wild type and genetically engineered entomopathogenic fungi as biocontrol agents of agricultural pests and mosquito vectors of human disease [138–140]. Extensive genetic variation in individual resistance from the same geographical population could set the stage for the evolution of resistance with implications for their sustainability. Future studies should also take account of the time of day when applying pathogens to insects in experimental settings or as biocontrol agents, as circadian rhythms may introduce considerable variability.

1.6 Methods

1.6.1 Growth conditions

The *Drosophila* Genetics Reference Panel (DGRP) [25,26], and transposon (P-element and Minos insertion) lines were obtained from Bloomington *Drosophila* Stock Center, IN USA. Candidate genes were tested for resistance to fungal infections using insertional mutant fly lines (Bloomington stock number in parenthesis): *S* (20272), *msn* (22796), *shn* (22518), *CG33172* (15945), *tai* (13204), *Sik3* (20921), *Rdl* (26404), *f* (14224), *CG9990* (24814), *CG32066* (16746), *CG33111* (24046), *puf* (15697), and *FOXO*. We received permission to use the *FOXO* mutant from Linda Partridge (University College of London) and the mutant and its control (wDAH) were generously provided by Michael Marr (Brandeis University) [141]. Flies were reared under standard

culture conditions (cornmeal-molasses—yeast-agar-medium with Tegosept and propionic acid, 25°C, 12-hr light-dark cycle).

M. anisopliae (ARSEF 549) was obtained from the USDA Entomopathogenic Fungus Collection (Ithaca, N.Y.). Ma549 is the active ingredient of Metabiol; a commercial product effective against hemipterans, lepidopterans and dipterans, and is a frequently used as a vehicle for genetic engineering projects [139]. Fungal cultures were moved from -80°C stock tubes 10 days before each bioassay and grown on potato dextrose agar at 27°C. Plasmid construction and transformation for the GFP fluorescent Ma549 strain was described previously [138]. *P. aeruginosa* (Pa14) was obtained from Vincent Lee (University of Maryland). Bacterial cultures were moved from -80°C stock tubes and plated on LB plates at 37°C two days before each bioassay. Single colonies were moved the next day for overnight growth into flasks containing 25 ml of brain heart infusion (BHI) broth at 37°C and placed on a shaker at 200 rpm.

1.6.2 Bioassays

Ma549 was used in an infection bioassay as described previously [23]. Flies were maintained at 27°C, 85% humidity, on food made without Tegosept and propionic acid. We bioassayed 3 tubes of ~20 flies (aged 2–4 days) per DGRP line, per sex with a spore suspension ($2.5x10^4$ spores/ml of water) produced from 10 day old Ma549 plates. Replicates were run on different days to randomize environmental variation. Control flies were treated with water alone as a control for the bioassay process. Fly mortality was monitored every 12 hrs. We found that in many vials one or two flies died in

experimental and control tubes in the first day but subsequently we found that as before [23], there were no differences in longevity between untreated and water treated flies.

A total of 81 randomly chosen DGRP lines were orally infected with PA14 as described in Lutter [142]. Approximately twenty 2–4 day old flies per line, per sex were put into vials and starved without food or water for 5 hrs. During this time, bacterial cultures were normalized to 3.0 at OD600, and 2 ml aliquots centrifuged at 7000 rpm for 5 minutes. The bacterial pellet was suspended in 175 ul of sterile 5% sucrose and then added to 2.3-cm Whatman filter disks placed inside vials containing 6 ml of 5% sucrose agar. Flies were then transferred into the vials and incubated at 27°C and ~85% humidity. Fly mortality was monitored every 12 hrs for 14 days. As *Drosophila* survive night-time infections with *Pseudomonas* significantly better than day-time ones [143], all infections with Ma549 and *Pseudomonas* Pa14 took place within an hour of 6pm.

Time to die was calculated for each replicate tube and expressed as LT_{50}. The standard deviation and coefficient of variation for each line were calculated using Kaplan-Meier standard errors. All calculations were done using SPSSv23.

1.6.3 Quantitative genetic analyses

To assess the effect of *Wolbachia* infection status on survival time to Ma549 and PA14, we used a factorial, type III mixed model ANOVA. The model used was $Y = \mu+S+I+S\times I+L(I)+S\times L(I)+\varepsilon$, where I denotes the effect of infection status, S is the fixed effect of sex, L is the random effect of the DGRP line, and ε is the error variance.

We partitioned phenotypic variance with the ANOVA model $Y = \mu + S + L + S \times L + \varepsilon$ to partition variance among lines (L, random), sex (S, fixed), line by sex interaction

($L \times S$, random), and within-line variance (ε). Broad-sense heritabilities for disease resistance were estimated from the variance components as $H^2 = (\sigma_L^2 + \sigma_{SL}^2)/(\sigma_L^2 + \sigma_{SL}^2 + \sigma_E^2)$.

To assess the degree of sensitivity of disease resistance to the environment, we first tested the heterogeneity of within line variance among lines using Levene's test. We then estimated the error mean square separately for each line and replicate by fitting a linear model which only included the intercept ($Y = \mu + \varepsilon$, where Y is the phenotypic value of the trait, μ is the overall mean and ε is the within-replicate random error). We estimated the micro-environmental standard deviation, σ_E as the square root of the mean square errors. We then assessed the genetic variance of $\ln(\sigma_E)$ using a mixed model factorial ANOVA model of form $Y = u + L + \varepsilon$, where Y is $\ln(\sigma_E)$, μ is the overall mean, and L is the random effect of the line. Broad sense heritability's for micro-environmental heterogeneity was calculated as $H^2 = (\sigma_L^2)/(\sigma_L^2 + \sigma_E^2)$. All calculations were done using SAS University edition.

1.6.4 Fungal growth, latent period, and sporulation capacity

Twenty lines were used to survey the impact of fly genetics on Ma549 life history traits. For epifluorescence imaging, ~40 individuals of each line were infected with Ma549-GFP. Fly images were taken starting 12 hours preceding the estimated LT50 for each line, using a Zeiss Axioimager M1. Intact flies were placed on microscope slides underneath a coverslip and viewed at 100x. To view the hemolymph, flies were squashed with the coverslip.

Ten of the lines were selected for a time course bioassay of fungal growth in the hemolymph, using previously described protocols [23]. At each time point, 10 flies per sex were individually homogenized with 45 μl of 0.1% Tween 80. The homogenate was spread onto Rose Bengal Agar plates supplemented with oxbile, CTAP, oxytetracycline, streptomycin, penicillin, chloramphenicol, and cycloheximide. Colony forming units (CFUs) were counted after 7–10 days' incubation at 25°C.

For sporulation analysis, ten flies per sex were harvested within 12 hours of death and individually transferred into tubes containing a damp cotton ball. The first appearance of spores (latent period) was recorded, and after 20 days, 500 μl of 0.1% Tween 80 was added to each tube and the tubes were vortexed (1 minute). Spore counts per individual fly were made using a hemocytometer, and results are the average of 10 flies per line.

1.6.5 Phenotypic correlations with other traits

We examined correlations among our measured traits, and between our disease-related phenotypes and independent traits that have been measured in the DGRP lines by other research groups. Correlation analyses were performed in R (R Core Team 2012) using rcorr and our line mean estimates, and we report both correlation coefficient and P value. We used the Holm-Bonferroni method for significant correlations to control for the familywise error rate [144]. For significantly correlated traits, we queried whether a single gene or a few genes might drive the correlation by determining whether the same SNPs were significantly associated with variation in both traits with a P value threshold of 10^{-5}.

1.6.6 GWA analyses

Associations were computed for Ma549 and PA14 separately using line LT50's and coefficient of variation for phenotypic scores, using ~2 million polymorphic markers [26]. These GWA analyses adjust for the effects of *Wolbachia* infection and 5 major chromosomal inversions (*In(2L)t*, *In(2R)NS*, *In(3R)P*, *In(3R)K*, *In(3R)Mo*), and were implemented using the DGRP website (dgrp2.gnets.ncsu.edu/). The same analysis was performed for each sex separately and for sex average and sex difference of the adjusted phenotypes.

1.6.7 GO and bioinformatics analysis

Annotation of SNPs was based on Flybase release 5.49 [26]. SNPs were considered in a gene if they were located in or within 1 kb upstream and downstream of a gene model. GO analyses were performed using the DAVID algorithm [99,100], with the Benjamini correction for multiple tests. To identify ensembles of interacting gene products, we used the R-spider program in the BioProfiling.de web portal [102]). This analysis tool incorporates data for ~2,000 genes and combines signaling and metabolic pathways from Reactome and Kyoto Encyclopedia of Genes and genomes (KEGG) databases to determine whether interactions between the input genes are greater than expected by chance using a permutation test. The network is built by connecting genes with known interactions in the two databases, allowing zero, one, or two missing nodes.

1.6.8 Functional tests

P-element insertions in 13 candidate genes were selected for functional assessment, using the criteria that the corresponding polymorphisms had high statistical

significance in the GWA analyses, and the mutant alleles were available from *Drosophila* stock collections with co-isogenic controls. We tested *P*-element insertions for their effects on resistance to Ma549 with three to five replicates of approximately 20 flies per line and sex. The *puf* mutant (15697) was originally created using the p-element construct P{EPgy2} which contains a Scer\UAS binding site, inserted into the gene *ash2* [145]. We therefore crossed this line and its isogenic control with a fly line expressing GAL4 (4414) to validate the effect of the *puf* gene. Statistically significant differences in responses to Ma549 between mutants and their coisogenic controls were determined using the log-rank test.

(See attached for supplementary tables)

S1 Table. Mean LT_{50} values for Ma549 and PA14, and *Wolbachia* infection status (WI).

S2 Table. ANOVA table for *Wolbachia* and common inversions.

S3 Table. Absolute rankings for fly lines for different phenotypic traits.

S4 Table. GWA analysis results.

S5 Table. Ma549 LT50 top association human orthologs

S6 Table. Gene ontologies.

S7 Table. Mutant validation data.

Chapter 2: Genetic variation for resistance to the specific fly pathogen *Entomophthora muscae*

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2.1 Abstract

We found substantial variation in resistance to the fly-specific pathogen *Entomophthora muscae* 'Berkeley' (Entomophthoromycota), in 20 lines from the *Drosophila melanogaster* Genetic Reference Panel (DGRP). Resistance to *E. muscae* is positively (r = 0.55) correlated with resistance to the broad host range ascomycete entomopathogen *Metarhizium anisopliae* (Ma549), indicative of generalist (non-specific) defenses. Most of the lines showing above average resistance to Ma549 showed cross-resistance to *E. muscae*. However, lines that succumbed quickly to Ma549 exhibited the full range of resistance to *E. muscae*. This suggests fly populations differ in *E. muscae*-specific resistance mechanisms as well as generic defences effective against both Ma549 and *E. muscae*. We looked for trade-offs that could account for inter-line variation, but increases (decreases) in disease resistance to *E. muscae* are not consistently associated with increases (decreases) of resistance to oxidative stress, starvation stress and sleep indices. That these pathogens are dynamic agents of selection on hosts is reflected in this genetic variation for resistance in lines derived from wild populations.

2.2 Introduction

Considerable genetic variation in resistance and tolerance to infection can exist within populations [115,146]. This variation determines the burden of disease, and represents the raw material from which populations can evolve resistance either naturally

or artificially (i.e. through selective breeding by humans) [147]. Insects are no exception to this pattern, and the same population of *Drosophila melanogaster* can contain resistant and susceptible genotypes to viruses, fungi and bacteria [32,148].

Many arthropod pathogenic fungi belong to the phylum Entomophthoromycotina and most of the remainder are distantly related ascomycetes [149]. We previously demonstrated significant variation in the life-span of 188 *Drosophila* Genetic Reference Panel (DGRP) lines infected with the ascomycete fungus *Metarhizium anisopliae* ARSEF 549 (Ma549) [148]. In addition, we found that resistance to Ma549 was correlated with resistance to the bacterium *Pseudomonas aeruginosa* (Pa14), and several previously published DGRP phenotypes including oxidative stress sensitivity, starvation stress resistance, hemolymph glucose levels, and sleep indices. As bacteria infect per os and fungi through the cuticle, the cross-resistance to Pa14 and Ma549 is suggestive of general (multipurpose) humoral defense mechanisms that do not involve cuticle or gut immunocompetence. Consistent with this, a genome-wide association study revealed a network of Pa14 and Ma549-resistance genes that are functionally connected through many different aspects of host physiology [148]. These observations are in line with insertional mutagenesis results: Lu [23] reported that 87% of mutated genes in more susceptible *Drosophila* lines are involved in a broad spectrum of biological functions not connected with canonical immune systems. The large numbers of pleiotropic genes involved in resistance to Ma549 and Pa14 contrasts with the small number of common polymorphisms associated with resistance to viruses [32]. Interestingly, each viral resistance SNP was associated with resistance to only one virus, which suggests that viral immunity is mediated by a suite of specific factors [32].

Most models of hosts and pathogens assume there is a tight relationship of co-evolved interactions between species pairs [150]. Such hosts and parasites are thought to engage in antagonistic coevolution, where a newly evolved parasite virulence mechanism is negated over time by a newly evolved host immune mechanism and vice versa [151]. The broad host range of *Metarhizium* and *Beauveria* spp. used in several previous studies on *Drosophila* [135,152–154] suggest that these pathogens have not engaged in a strict coevolutionary arms race with *Drosophila* [155]. As study systems, these microbes will not, therefore, tell us about how specialist parasites suppress host immunity, or about any secondary immune mechanisms hosts deploy against specialist parasites [9,156].

Given the importance of the *Drosophila* model system to our understanding of immunity, it is surprising that very little is known about its natural parasites. There may be 5.5 million insect species [157], and if every metazoan species has at least one host-specific parasite as some studies suggest [158], narrow host range entomopathogenic fungi may exist by the millions as well. However, an ecologically relevant specialist fungal pathogen of *Drosophila* pathogen that would facilitate understanding of host pathogen evolution and identify specialized immune mechanisms has only recently been identified [159]. Behavior-manipulating fungal pathogens in the *Entomophthora muscae* (Entomophthoromycota) species complex are best known for causing epizootic outbreaks in house flies. However, Elya et al. [159] identified an epizootic in Californian *Drosophila* caused by a single strain of *E. muscae* (*E. muscae* 'Berkeley'). It remains unclear if *E. muscae* 'Berkeley' is a distinct lineage (or even species) from those that infect other fly species, and how specific it may be for *Drosophila* spp. over other dipterans is also unknown [159]. However, contrary to other fungal infections

(e.g., *Metarhizium*), and consistent with previous *E. muscae* infections described in house flies [160], *E. muscae* 'Berkeley' invaded the *Drosophila's* nervous system and caused a characteristic set of behaviors: on their final day of life, a few hours before sunset, the flies climb upward, extend their proboscides, affixing them to a substrate, then raise their wings, clearing a path for infectious spores to launch from their abdomens [159]. This robust control of behavior by *E. muscae* 'Berkeley' indicates a high level of adaptation of the pathogen to the host. However, many aspects of this disease (e.g., the climbing behavior of critically ill hosts), are typical for narrow host range pathogens of arthropods, and probably involve the pathogens taking advantage of sleep behavior in insects, as these behaviors are highly conserved [161]. Many of the best characterized and most commonly witnessed epizootics are caused by behavior-modifying entomophthoralean species infecting flies, ants, grasshoppers, caterpillars and cicadas [162,163].

In this study we bioassayed *E. muscae* 'Berkeley' (hereafter referred to as *E. muscae*) against a subset of 20 DGRP lines selected because they represent the genotypes that are the most resistant or susceptible to Ma549 from the DGRP collection. Using this divergent subset, we show that wild-derived populations of *Drosophila* have substantial differences in susceptibility to *E. muscae*, and that this variation correlates with resistance to Ma549, and, to a lesser extent, with starvation resistance and sleep indices. However, lines that succumbed quickly to Ma549 covered the whole spectrum of resistance to *E. muscae* from low to high. This suggests there are additional mechanisms by which disease resistance to *E. muscae* can be altered, besides those effective against Ma549.

2.3 Methods

2.3.1 Divergent DGRP Lines

DGRP Freeze 2 lines, originally derived from an out-crossed population in Raleigh, North Carolina, by the Mackay laboratory [26] were obtained from the Bloomington Stock center. To characterize natural variation in susceptibility to *E. muscae,* we used a subset of the 188 DGRP lines deployed in Wang et al. [148], comprising the 10 most Ma549 resistant and 10 most Ma549 susceptible DGRP lines. Called the "divergent subset" in Wang et al. [148], they represent the most extreme disease phenotypes to Ma549 in the DGRP. Ma549 and Pa14 LT_{50} data for the divergent subset was previously published in Wang et al. [148].

2.3.2 *E. muscae* exposure of divergent DGRP lines

All flies were reared on cornmeal-based diet (3% weight per volume (w/v) cornmeal, 11% w/v dextrose, 2.3% w/v yeast, 0.64% w/v agar and 0.125% w/v tegosept) at 21 °C on a 12:12 light:dark cycle. For infection, we followed a modified version of the protocol described in Elya et al. [159] using *E. muscae* that has been propagated in *Drosophila* in vivo since 2015. Briefly, 21 "exposure vials" were prepared, each by embedding six Canton-S *Drosophila* cadavers freshly killed by *E. muscae* headfirst into minimal media containing 5% sucrose and 1.5% agar in wide fly vials (Genesee Scientific). For each of the 20 DGRP lines and Canton-S, fifty flies (25 male and 25 female) aged < 5 days post eclosion were transferred to a fresh vial and the plug of the vial was pushed down to confine the flies within 2 cm to improve the likelihood that they would encounter infectious spores. Vials were housed for the first 24 h under high

humidity at 21 °C with a 12:12 light:dark cycle, at which point the plug was raised to relieve fly confinement. Flies were housed at 21 °C with ~ 40% humidity for the remainder of the experiment. Each vial was monitored twice daily (once before subjective sunset, once after) for deaths and subsequent sporulation, to confirm death by *E. muscae*. All experiments were replicated five times, the raw data is provided in Supplemental Table S1.

2.3.3 Data analysis

All statistics were done using R version 3.6.1. To determine the relationship between different phenotypes, we performed Pearson correlations using the package Hmisc. We tested for normality using Shapiro-Wilks test. Comparisons between sexes and *Wolbachia* infection statuses were done using the non-parametric two-sided Mann–Whitney test.

2.4 Results and Discussion

To characterize natural variation in pathogen resistance, we quantified susceptibility to *E. muscae* using the divergent subset of the 10 most and the 10 least Ma549 resistant DGRP lines (selected out of 188 DGRP lines). Age-matched flies from each line were exposed to *E. muscae*, and survival time was monitored using five replicates (25 flies each), per sex per line. Elya et al. [159] report that the Wolbachia-free CantonS *Drosophila* developed a strong immune response one day after infection with *E. muscae* but by the third day the fungus had spread throughout the body, and most flies died around four to five days following infection. Unlike Ma549 and Pa14, *E. muscae* consistently kills hosts at the same zeitgeber time every day (always in the hours leading

to subjective sunset), therefore, we used the percentage surviving at five days post-exposure to *E. muscae* (referred to as a PS_5; daily deaths only rarely peak after this time point) as our metric to compare to Ma549 and Pa14. In contrast to *E. muscae*, DGRP lines die from Ma549 or Pa14 at different rates over a broader range of day's post-exposure, so post-infection survival for these pathogens is better measured using LT_{50} values.

Using the DGRP lines, we show that wild-derived populations of *Drosophila* have substantial differences in susceptibility with mortalities ranging from 1.6 to 94%, and a mean survival of males (females) of 70% (62%) (Fig. 1). Less than 25% of flies in the most *E. muscae*-resistant DGRP lines had died seven days post exposure, and most of those that succumbed did so after 4 to 5 days. At the other extreme, almost 100% of RAL 227 flies were dead at five days post exposure. After five days the death rate plateaued off for most lines, and approached that of uninfected flies, suggesting that the survivors had cleared the infection. Thus, variable host susceptibility is illustrated by some DGRP lines dying more than others four to five days post infection with *E. muscae*.

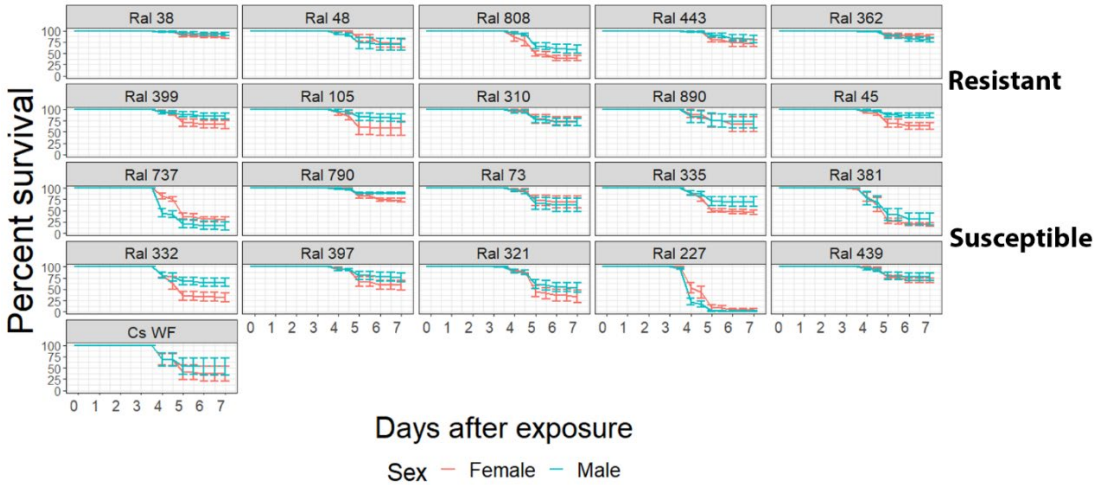


Figure 1: Percent survival of DGRP lines tested with *E. muscae.* Flies of the divergent

subset were broken up into two groups, those resistant to *Metarhizium anisopliae* (top two rows) and those susceptible to *M. anisopliae* (middle two rows). Canton-S flies (CS WF) (bottom) used previously to establish that *E. muscae* 'Berkeley' is a *Drosophila* pathogen [159] were used as a positive control. Percentages are an average of five replicates and error bars reflect standard errors.

To identify general (multipurpose) defense, the PS_5 for males and females exposed to *E. muscae* was plotted against average LT_{50} for males (females) infected with Ma549 (Fig. 2b). The data on Ma549 and Pa14 is derived from our earlier publication which used replicates run on different days to randomize environmental variation [148]. Correlations were moderate but highly significant ($r = 0.54, 0.57$, $p = 0.0143, 0.0084$ for males, females respectively), consistent with *Drosophila* utilizing unspecific generalized defense components against *E. muscae* and Ma549 (Fig. 2b). We previously reported that LT_{50} values for Ma549 and *P. aeruginosa* Pa14 were correlated for both males ($r = 0.45$, $n = 78$) and females ($r = 0.40$, $n = 78$) [148]. Correlations between Ma549 and Pa14 in the divergent subset used to assay *E. muscae* were greater, with $r = 0.7$ for males ($p = 0.0024$, $n = 16$) and $r = 0.55$ for females ($p = 0.0262$, $n = 16$) (Fig S1b). Although correlations were still positive between *E. muscae* and *P. aeruginosa*, they were not significant for males ($r = 0.09$, $p = 0.7295$, $n = 16$) or for females ($r = 0.39$, $p = 0.138$, $n = 16$) (Fig S1a). Ma549 is a broad host range generalist insect pathogen, while Pa14 is a human clinical isolate, and so represents a novel association that will have no history of coevolution. Our results suggest that the genetic basis for resistance to a non-coevolved bacterium (Pa14) and an opportunistic broad host range fungus, share more genetic causes than Pa14 and *E. muscae*.

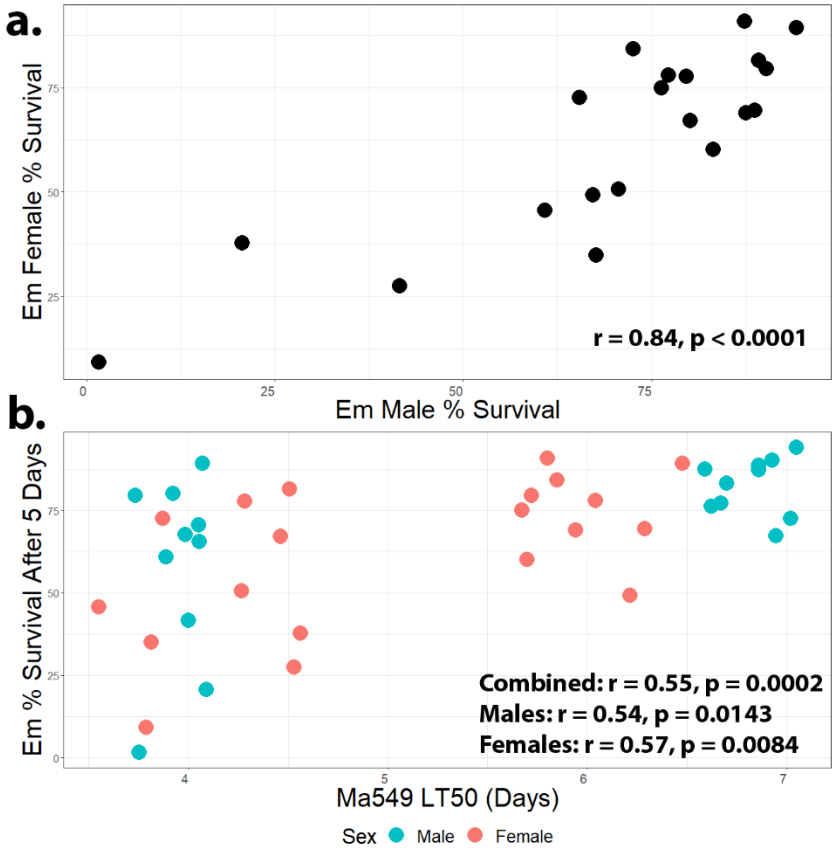


Figure 2: Correlation graphs. Positive correlations of (**a**) % survival of male and female DGRP flies 5 days post-infection with *E. muscae*, and (**b**) between flies infected with *E. muscae* (% survival) or *M. anisopliae* (Ma549 LT_{50} values). Ma549 LT_{50} values were obtained from Wang et al. 2017 [148].

The weak correlations indicate that *Drosophila* has alleles with pathogen-specific effects to *E. muscae*. This is consistent with variation in the magnitude and direction of association between Ma549 and *E. muscae*. We found greater variation in susceptibility to *E. muscae* among the lines susceptible to Ma549 (male range: 1.6–76.28%, female range: 9.18–84.23%) compared to lines resistant to Ma549 (male range: 77.25–94.2%, female range: 60.13–90.83%) (Fig. 1). Except for RAL 808, the lines resistant to Ma549 were also resistant to *E. muscae*, while lines that succumbed quickly to Ma549 covered the spectrum of resistance to *E. muscae* from low to high (Fig. 1). The exception, RAL

808, is the third most resistant line to Ma549, but is moderately susceptible to *E. muscae* ranking fifteenth out of the twenty lines ($PS_5 \sim 50\%$). These results suggest that there are multiple mechanisms by which disease resistance to *E. muscae* can be altered besides those effective against Ma549. The shared history of *E. muscae* and *D. melanogaster* could have resulted in a co-evolutionary process that altered the diversity of resistance genes compared to naïve pairs of hosts and pathogens. Similarly, host–pathogen coevolution increases genetic variation in susceptibility to viruses [164]. Thus, heritable variation for host resistance was detectable for two natural viruses of *D. melanogaster*, but not for two non-natural viruses [32].

To identify sexual dimorphism, we measured disease resistance separately for males and females infected with *E. muscae* (Fig. 2a). Cross-sex genetic correlations were high ($r = 0.84$, $p < 0.0001$, $n = 20$), indicating that many of the same variants affect *E. muscae* resistance in males and females. Females flies died more quickly than males when infected with Ma549 [148] ($p = 0.00039$, $n = 188$). This difference is not significant overall for females of the divergent set ($p = 0.37$, $n = 20$), but females group separately from males in the most resistant DGRP lines (Fig. 2b). Females were also slightly more susceptible than males to *E. muscae* though this fell short of significance ($p = 0.2$, $n = 20$). As observed previously for Ma5495, RAL 737 was exceptional, as females of this fly line were more resistant to *E. muscae* than males (Fig S2).

Wolbachia pipientis is a natural intracellular symbiont of many arthropods, and *Wolbachia* may confer protection against the fungus *Beauveria bassiana* in one *D. melanogaster* line [34]. *Wolbachia* status in the DGRP lines was without significant

effect on susceptibility to Ma549 [148]. Eleven of the twenty divergent lines were positive for Wolbachia, seven of these eleven were present in the ten most susceptible lines producing no significant effect on the susceptibility to *E. muscae* for males ($p = 0.15$, n = 20 or females $p = 0.71$, n = 20).

Resistance to multiple pathogens should have a selective advantage unless this general defense is traded off against other (pathogen-independent) fitness components [129]. In the absence of such a trade-off, directional selection would presumably lead to fixation of genotypes showing general resistance. Table S2 shows the divergent subset, and their life cycle parameters and rankings in publicly available data from other publications, including our data for Ma549 and Pa14. Figure S1 presents correlations between the disease resistance phenotypes in our studies and these other traits. The small sample size (n = 20) of the divergent set reduces the discriminatory power of correlation analysis. However, r values for the divergent set and the total population (188 lines) are similar for many phenotypes. For example, correlations between female resistance to Ma549 and paraquat (a source of oxidative stress) are r = 0.46, $p = 0.0541$ (divergent set, n = 18) and r = 0.31, $p < 0.0001$, n = 156 (total population), and correlations between female resistance to Ma549 and negative geotaxis are r = 0.29, $p = 0.2411$ (divergent set, n = 18) and r = 0.2, $p < 0.0079$, n = 171 (total population). The corresponding values for *E. muscae* are r = 0.25, $p = 0.325$, n = 18 (paraquat resistance) and r -0.04, $p = 0.861$, n = 18 (negative geotaxis).

We previously reported that resistance to Ma549 among 188 DGRP lines was negatively correlated with sleep duration at night in males (r = − 0.32, $p < 0.0001$, n =

156) and females (r = − 0.28, p = 0.0004, n = 156) [148]. Conversely, there was a positive association between resistance and the number of sleep bouts in males (r = 0.25, p = 0.0018, n = 156) and females (r = 0.24, p = 0.0028, n = 156) [148]. Compared to the total population, the resistance of the 20 divergent subset to Ma549 was even more closely associated with the number of nocturnal sleep bouts (males r = 0.67, p = 0.0026, n = 18; females r = 0.67, p = 0.0021, n = 18) and negatively correlated with night sleep duration (males r = − 0.71., p = 0.0009, n = 18; females r = −0.74, p = 0.0005, n = 18). Hence, compared to the general population, there is a stronger trend for the 10 most resistant DGRP flies to have more sleep bouts than the 10 most susceptible DGRP flies, but these bouts are shorter and total sleep time is less. This trend was retained for *E. muscae,* but to a lesser degree, and falling short of significance, with the number of nocturnal sleep bouts (males r = 0.29., p = 0.2352, n = 18; females r = 0.35, p = 0.1483, n = 18) and negatively correlated with night sleep duration (males r = − 0.36, p = 0.1402, n = 18; females r = − 0.32, p = 0.1919, n = 18).

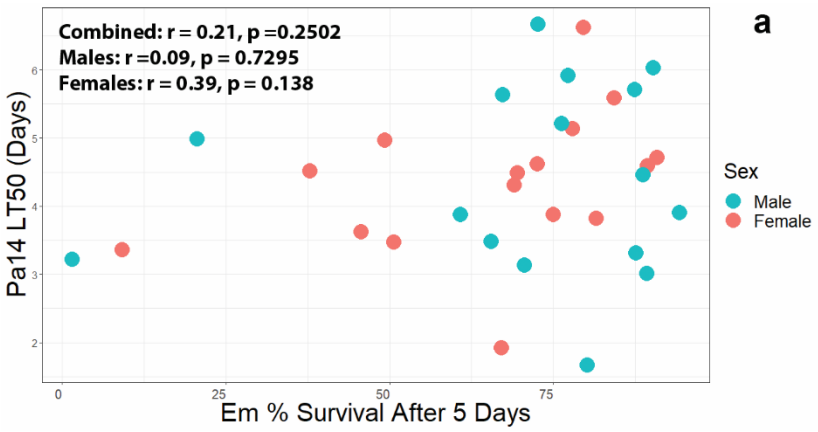
Looking at the data on a line-by-line basis shows why the associations are so weak. There are lines with increased levels of resistance to *E. muscae* and negative geotaxis, oxidative stress or sleep duration, but there are also resistant lines with moderate or low rankings for these indices, suggesting that there are no straightforward associations or trade-offs. Taking starvation stress as an example, as *E. muscae* colonizes the host's body it will compete with it for resources [159], so it makes intuitive sense that genotypes better able to tolerate starvation would have better tolerance to disease. Resistance to starvation is positively correlated with resistance to *E. muscae* in both males (r = 0.21, n = 20) and females (r = 0.34, n = 20). Although these values fall short of

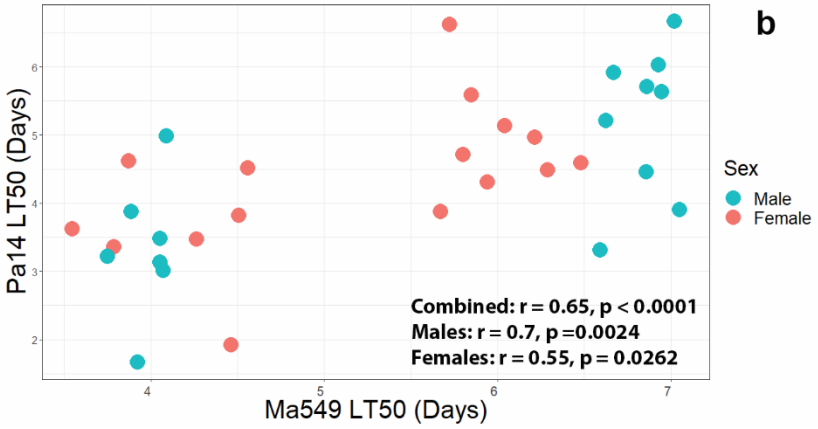
significance ($p > 0.05$) (Supplementary Fig S1p), they are higher than the correlations between resistance to Ma549 and starvation in males of ($r = 0.17$, $n = 20$) and females ($r = -0.03$, $n = 20$). Resistance to starvation in the total population was only weakly correlated with the resistance of female flies to Ma549 ($r = 0.16$, $p = 0.0335$) [148], indicating that *E. muscae* may cause greater nutrient stress to *Drosophila* than Ma549. However, on a line-by-line basis, DGRP lines RAL 38, RAL 48, RAL 443 and RAL 362 (highly resistant to both Ma549 and *E. muscae*), ranked 159, 28, 62 and 8 out of 203 DGRP lines for resistance to starvation. RAL 808 (Ma549 resistant, *E. muscae* susceptible), RAL 439 (Ma549 susceptible, *E. muscae* resistant) and RAL 227 (susceptible to both Ma549 and *E. muscae*) ranked 152, 162 and 149, respectively.

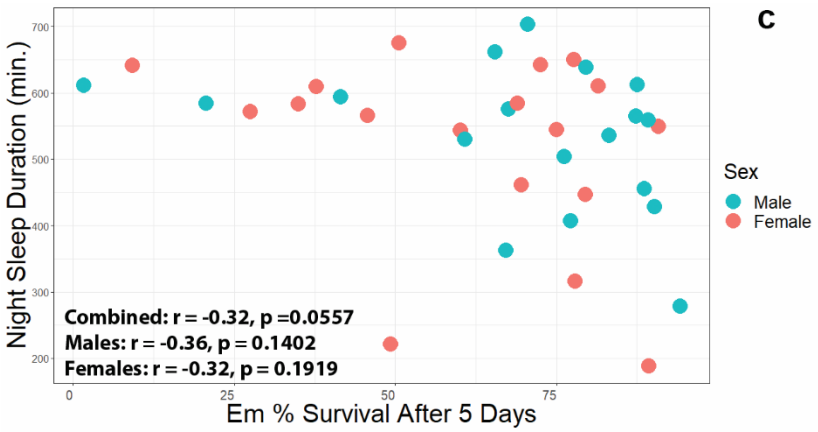
2.5 Conclusion

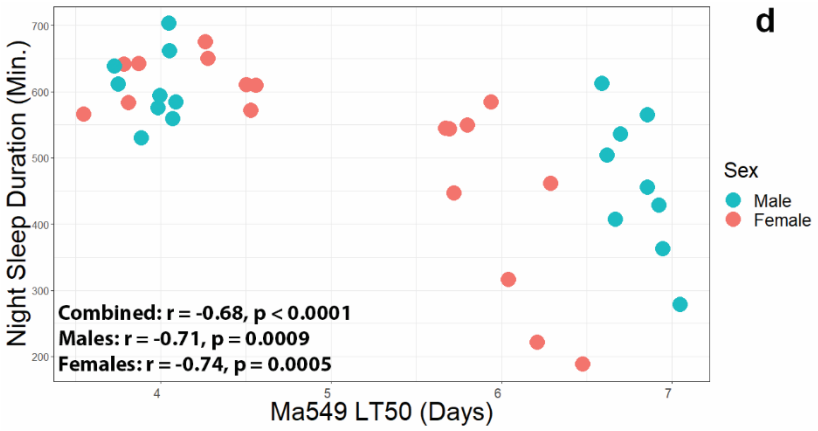
Fungal-host interactions include both general broad host range and narrow host range pathogens. *E. muscae* is a dipteran specialist that naturally causes epizooitic outbreaks in *D. melanogaster*. Similar to broad host range ascomycete fungi, we identified considerable host genetic variation in resistance to *E. muscae* infection. However, we found that this variation is unlike ascomycetes, which kills different host genotypes with varying rapidity, but instead reflected considerable differences in the number of flies that died in a narrow window of time four to five days post-exposure. This reflects the unique *E. muscae* behavioral trait of killing flies in the hours leading to subjective sunset to ultimately maximize fungal dispersal. Despite differences in the co-evolutionary dynamics between *D. melanogaster* and *E. muscae*, versus other fungal pathogens such as *M. anisopliae*, flies showed cross-resistance to both fungi, indicative of generic anti-fungal defences. However, cross-resistance was greater between *M.*

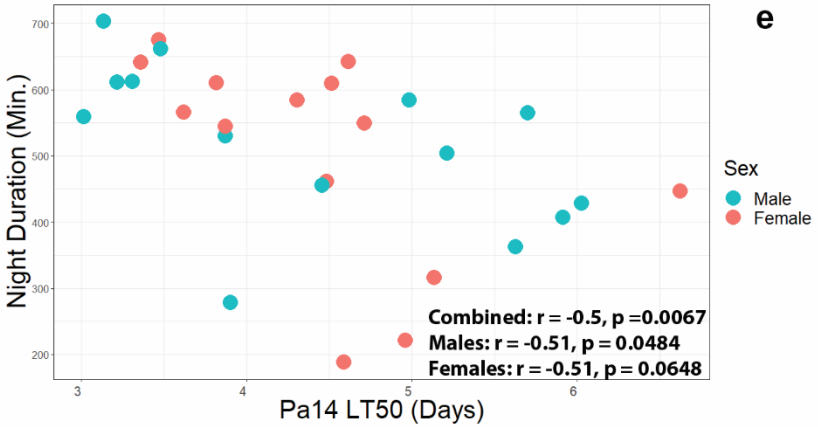
anisopliae and an opportunistic bacterial pathogen, *P. aeriginosa*, than between *M. anisopliae* and *E. muscae*. Also, *D. melanogaster* lines killed quickly by *M. anisopliae* Ma549 show greater variation in susceptibility to *E. muscae*, indicating that Ma549-susceptible individuals vary in evolution or retention of narrow anti-*E. muscae* mechanisms. Also, with the notable exception of starvation resistance, resistance to Ma549 and Pa14 correlated with non-specific physiological features such as sleep indices, to a greater extent than *E. muscae* infected flies, consistent with specific defenses being more important. This study demonstrates the continued utility of *Drosophila* for understanding host-fungus interactions, the clear potential for *Drosophila* to become a powerful in vivo comparative system to study the diversity of antifungal responses, and supports the utility of *E. muscae* as a model for studying varied aspects of host–pathogen interactions in the fly.

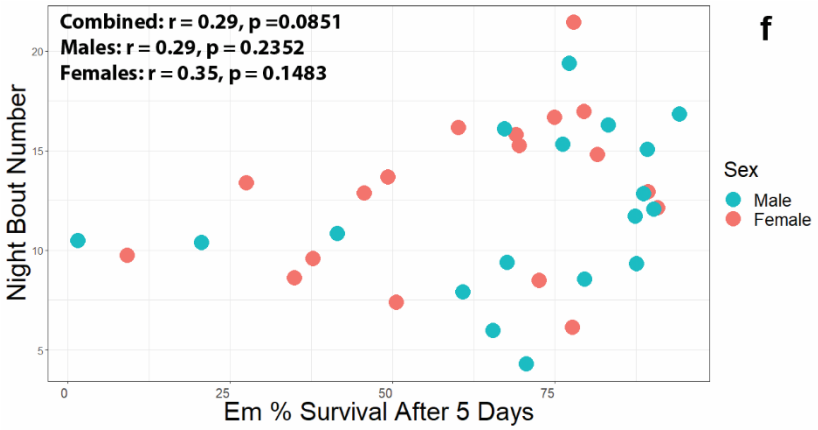


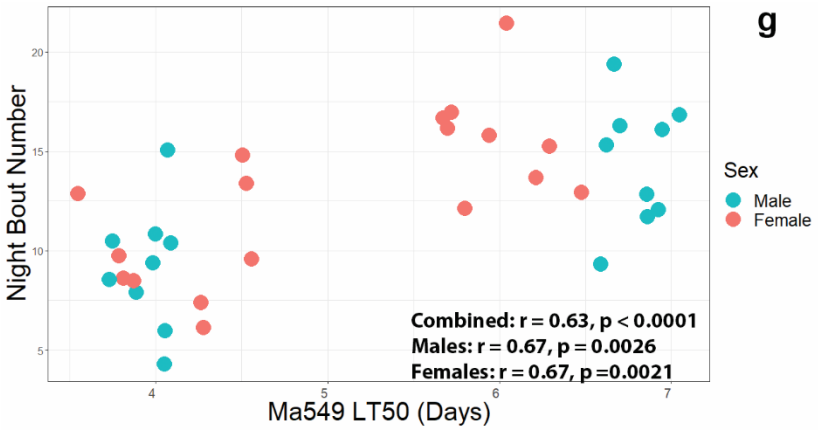

b
Pa14 LT50 (Days)
Ma549 LT50 (Days)
Combined: r = 0.65, p < 0.0001
Males: r = 0.7, p =0.0024
Females: r = 0.55, p = 0.0262
Sex
Male
Female

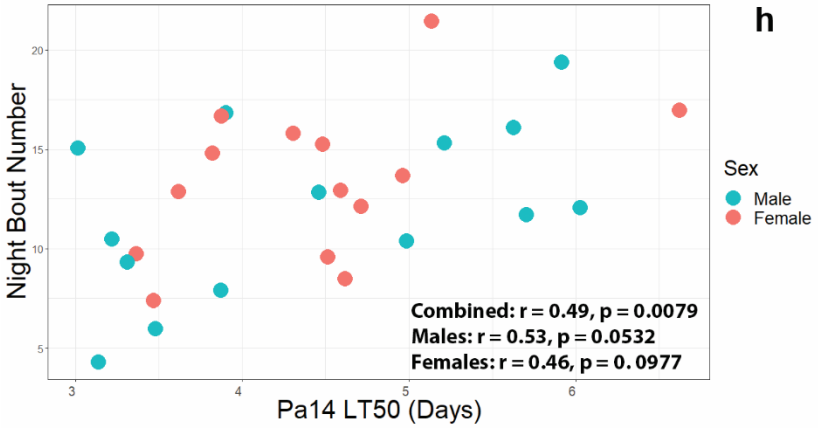

c
Night Sleep Duration (min.)
Em % Survival After 5 Days
Combined: r = -0.32, p =0.0557
Males: r = -0.36, p = 0.1402
Females: r = -0.32, p = 0.1919
Sex
Male
Female

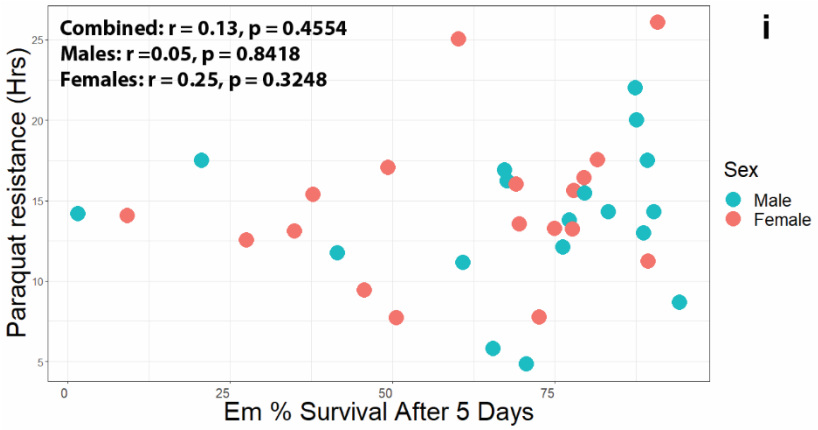

d
Night Sleep Duration (Min.)
Ma549 LT50 (Days)
Combined: r = -0.68, p < 0.0001
Males: r = -0.71, p = 0.0009
Females: r = -0.74, p = 0.0005
Sex
Male
Female

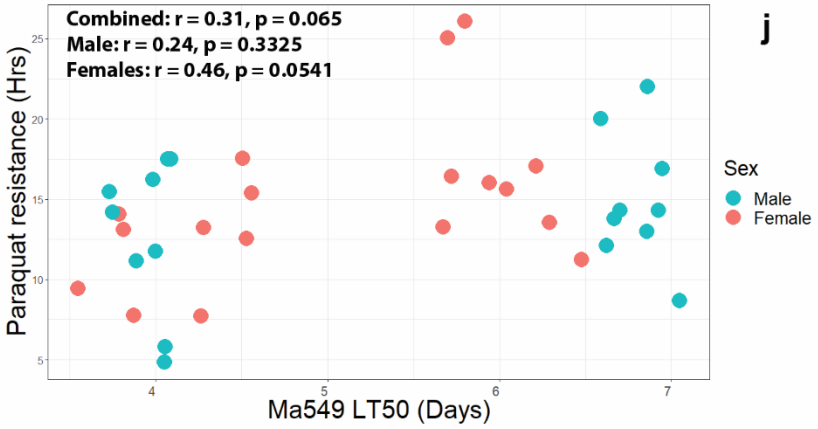

e
Night Duration (Min.)
Pa14 LT50 (Days)
Combined: r = -0.5, p =0.0067
Males: r = -0.51, p = 0.0484
Females: r = -0.51, p = 0.0648
Sex
Male
Female

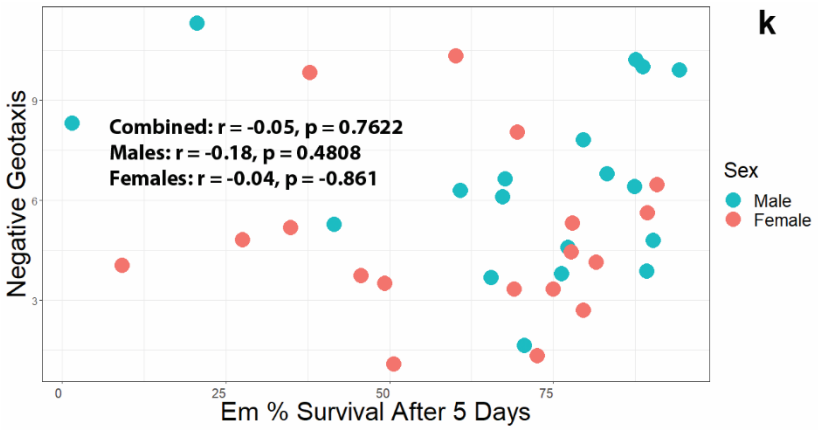

f
Night Bout Number
Em % Survival After 5 Days
Combined: r = 0.29, p =0.0851
Males: r = 0.29, p = 0.2352
Females: r = 0.35, p = 0.1483
Sex
Male
Female

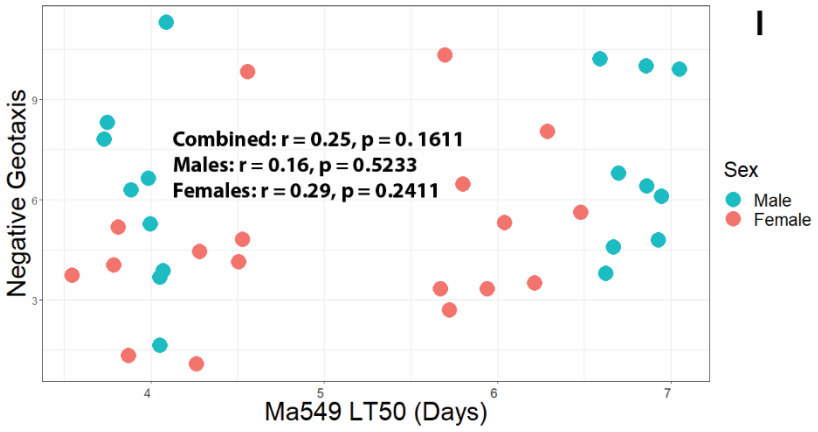

g
Night Bout Number
Ma549 LT50 (Days)
Combined: r = 0.63, p < 0.0001
Males: r = 0.67, p = 0.0026
Females: r = 0.67, p =0.0021
Sex
Male
Female

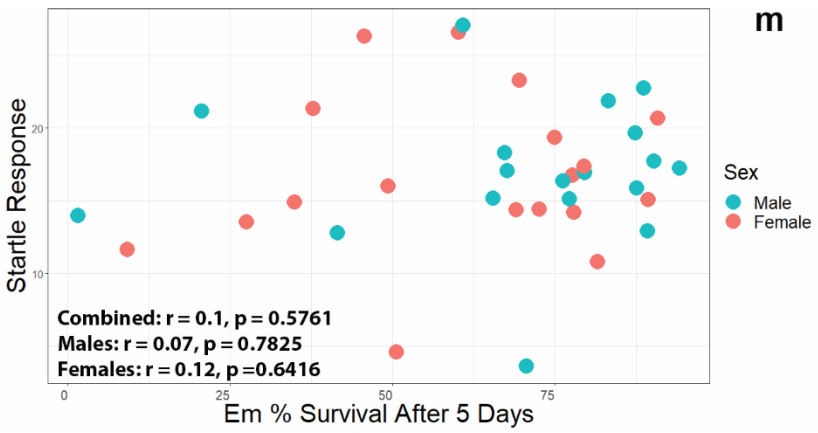

h
Night Bout Number
Pa14 LT50 (Days)
Combined: r = 0.49, p = 0.0079
Males: r = 0.53, p = 0.0532
Females: r = 0.46, p = 0. 0977
Sex
Male
Female

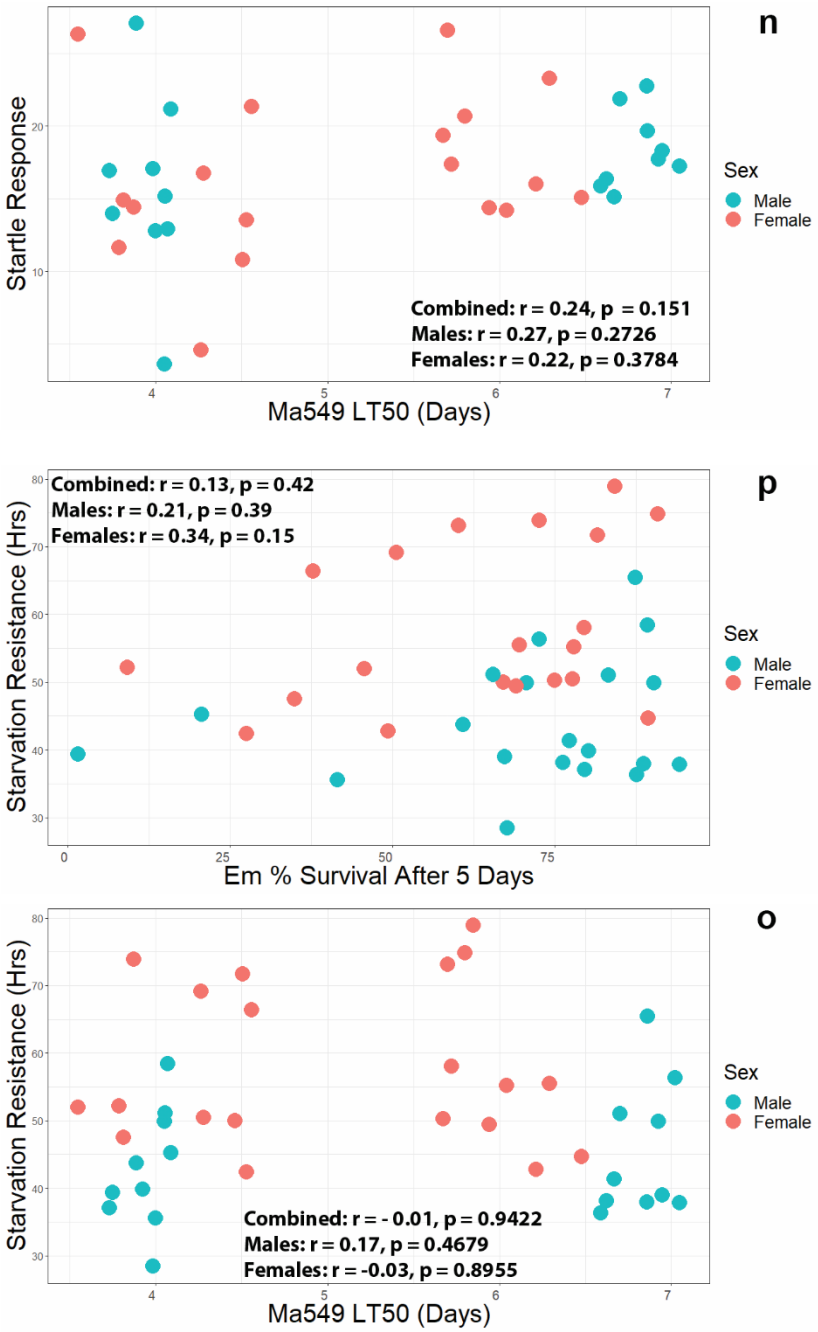

i
Paraquat resistance (Hrs)
Em % Survival After 5 Days
Combined: r = 0.13, p = 0.4554
Males: r =0.05, p = 0.8418
Females: r = 0.25, p = 0.3248
Sex
Male
Female


j
Paraquat resistance (Hrs)
Ma549 LT50 (Days)
Combined: r = 0.31, p = 0.065
Male: r = 0.24, p = 0.3325
Females: r = 0.46, p = 0.0541
Sex
Male
Female

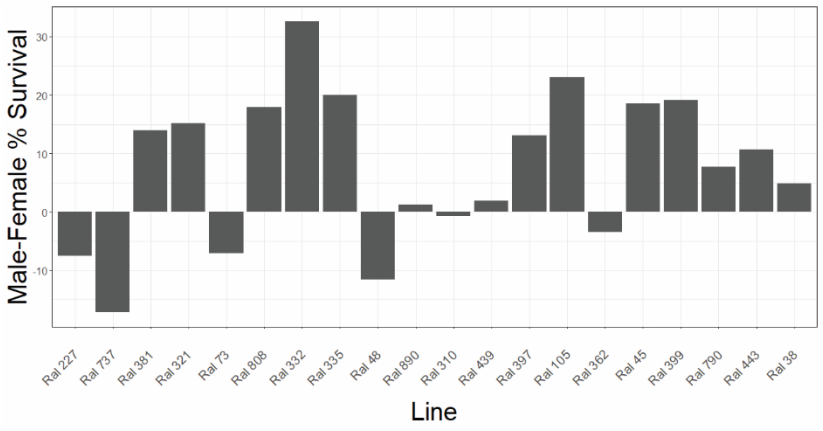

k
Negative Geotaxis
Combined: r = -0.05, p = 0.7622
Males: r = -0.18, p = 0.4808
Females: r = -0.04, p = -0.861
Em % Survival After 5 Days
Sex
Male
Female


l
Negative Geotaxis
Combined: r = 0.25, p = 0. 1611
Males: r = 0.16, p = 0.5233
Females: r = 0.29, p = 0.2411
Ma549 LT50 (Days)
Sex
Male
Female


m
Startle Response
Combined: r = 0.1, p = 0.5761
Males: r = 0.07, p = 0.7825
Females: r = 0.12, p =0.6416
Em % Survival After 5 Days
Sex
Male
Female



Supplementary Figure S1: Correlation graphs of phenotypes. (a) Positive correlation of % survival (5 days) among the DGRP lines between male and female flies infected with *E. muscae* and diverse phenotypes (b-p). *Pseudomonas aeruginosa* (Pa14) and Ma549 LT50 values were obtained from Wang et al., 2017 [148], sleep data from Harbison et al., 2014 [24], paraquat resistance from Weber et al., 2012 [40], negative geotaxis and startle response data from Jordan et al., 2012 [29], and starvation data from Mackay et al., 2012 [26].



Supplementary Figure S2: Percent survival differences between male and female flies in DGRP lines infected by *E. muscae*. Flies were ordered (based on male percent survival) from the most susceptible (left) to the most resistant (right). Female percent survival was subtracted from male percent survival so positive values indicate lines where males are more resistant.

Chapter 3: Interactions between different pathogen strategies and innate immunity

(Being prepared for publication)

3.1 Abstract

We used *B. bassiana* and 16 strains of *Metarhizium* spp. that differ in infection strategies, with LT_{50}'s ranging from 3 to 10 days, to study how individual pathogen genotypes interact with the fruit fly *Drosophila melanogaster*. The fastest killing strains had the greatest reproductive capacity on cadavers, irrespective of whether they produce toxins, and induced the earliest and largest Toll immune response, so their greater virulence does not depend on suppressing immunity. Consistent with this, *Drosophila*'s antifungal peptide drosomycin does not inhibit *Metarhizium* strains. Disrupting the Toll component *Dif* (Dorsal-related immunity factor) only increased susceptibility to *Beauveria bassiana* and *Metarhizium frigidum*, whereas flies lacking the immune sensor Persephone succumbed quickly to all pathogen strains, including the very weakly pathogenic *M. album*. Females of most *Drosophila* lines are more susceptible to *Metarhizium* than males, but this sexual dimorphism was lost or reversed with some combinations of pathogens and immune-deficient flies. Using the Agilent Array platform, we showed that many Ma549-responsive genes, affecting immune, physiological and homeostatic responses, require Dif or Persephone. This study emphasizes that the outcome of an infection depends on factors specific to each pathogen interacting with diverse aspects of host immunity.

3.2 Author Summary

The expansive radiation within both *Drosophila* and *Metarhizium* spp. provides an excellent opportunity to examine the interrelationship between natural variation in host resistance and pathogen infectivity, and the factors that may act as selective forces to shape such variation. To understand how differences in pathogen strategies and host range influence the outcome of infection, we have studied interactions between diverse *Metarhizium* strains, and the fly immune response to fungal invasion. We found that the fastest killing *Metarhizium* strains induce the earliest and largest immune response, so fungal virulence does not depend on suppressing immunity. Disrupting a gene (*Dif*) that mediates Toll immune responses produces a greatly reduced gene expression response to *Metarhizium*, although for most *Metarhizium* strains the effect on longevity was slight. This shows that there are no simple extrapolations between changes to gene expression and duration of survival. Conversely, flies succumb quickly if disrupted in the *psh* gene that encodes a sensor of fungal virulence factors. Persephone also mediates immunological differences between males and females in response to some *Metarhizium* strains, consistent with the outcome of an infection depending on the specific combination of host and parasite genotypes.

3.3 Introduction

Most models of interactions between hosts and pathogens are based around a concept of tightly coupled co-evolved interactions between species pairs [150]. This is despite the fact that most pathogens of plants and animals are generalists that infect multiple host species, and evidence that many emerging diseases are caused by generalists, of which fungal diseases make up the majority [18]. The outcomes of

pathogen infection in nature vary widely because hosts differ in resistance and tolerance to infection, while pathogens vary in their ability to grow on or within hosts [146]. This variation determines the burden of disease, and represents the raw material from which populations can evolve resistance [147]. Insects are continually exposed to a vast number of potential pathogens with widely varying life histories and they have consequently evolved a series of intricate mechanisms to resist pathogen attack [9]. That these pathogens are dynamic agents of selection on hosts is reflected in genetic variation for resistance in wild insect populations [148,165,166].

Fungi cause a large proportion of insect disease [20], and include the ascomycete genus *Metarhizium*; a radiating lineage of insect pathogens. Besides their crucial role in natural ecosystems, *Metarhizium* spp. are frequently used as biological insecticides [167,168], and for genomic studies into the nature of adaptive differences underlying pathogen speciation [169]. While many *Metarhizium* strains are specialized pathogens of a narrow range of hosts, others attack a broad range of species providing a model for studying the basis of generalism and specificity, and the potential of pathogens to cross the species barrier and infect new hosts. Thus, *Metarhizium album, Metarhizium acridum* and *Metarhizium majus* have specialized to hemipteran, orthopteran and coleopteran insects, respectively [170,171]. *Metarhizium frigidum* split early from other *Metarhizium* species and evolved independently as a generalist, whereas *Metarhizium brunneum*, *Metarhizium robertsii* and *Metarhizium anisopliae* are recently diverged generalists (the "PARB clade"), that parasitize many insect orders including dipterans [172,173].

Infection by *Metarhizium* typically starts by conidial adhesion to the insect integument, followed by germination that is triggered by topographical and chemical

signals from insect cuticles, and environmental cues such as relative humidity [122,174]. Germlings produce adhesive infection structures (appressoria) and hyphal penetration through the host cuticle occurs by a combination of mechanical pressure and cuticle degrading enzymes, including many proteases [8,175]. Penetrating multicellular hyphae respond to factors present in the host hemolymph by switching to growth as single-celled blastospores which facilitate dissemination and have mechanisms for evading the insect immune system [122,176]. Once the host is dead, the fungus breaches the cuticle from the inside outwards, allowing the formation of conidia that disperse and start new infections [8]. Thus, onward transmission of *Metarhizium* requires the death of the host. Relating to host specificity, *Metarhizium* species differ in the host-related factors required to induce appressoria [177], and in their infection strategies. For example, both *M. anisopliae* ARSEF strain 549 (Ma549) and *M. robertsii* ARSEF strain 2575 (Mr2575) have broad host ranges, but Ma549 is biotrophic (grows through the living host) and produces no destruxins (toxins) whereas *M. robertsii* ARSEF strain 2575 (Mr2575) kills with toxins and is subsequently necrotrophic [178].

Fungal infection processes encounter a dedicated immune response that includes melanization and anti-microbial peptides (AMPs) [5], generated by the highly conserved Toll pathway, the chief *Drosophila melanogaster* anti-fungal pathway described in the literature [179,180]. In this species, the circulating protease persephone (psh), an immune sensor of pathogen proteases, and GNBP3, which detects fungal wall components, act exclusively to detect infection. They link their activation into proteolytic serine protease cascades, which induces Toll-mediated AMP transcription through the nuclear translocation of *Drosophila* Dif, an NF-κB homolog [181,182]. Insects disrupted

in *Dif* or *psh,* succumb quickly to the entomopathogenic fungus *Beauveria bassiana* [183]. We found previously that despite fungal recognition and Toll immune elicitation by the fly, it is unable to successfully eliminate infection with Ma549 [23]. A genome-wide association (GWAS) study deploying the *Drosophila* Genetic Reference Panel (DGRP) found considerable genetic variation in the susceptibility of *D. melanogaster* to Ma549, but how long DGRP lines took to succumb to Ma549 was not associated with canonical immune processes [148]. These studies revealed a complex genetic architecture for disease resistance, with large numbers of pleiotropic genes and alleles with sex-, environment- and genetic background-specific effects.

In this paper, we used generalist strains of *Metarhizium* spp. with diverse pathogenic strategies, and specialists not adapted to *Drosophila*, to identify barriers than must be overcome in order to transition to a new host species. We found, unexpectedly, that the most pathogenic strains elicit the largest immune response. To obtain a broader understanding of the effects of the Toll pathway on *Metarhizium* virulence in the context of general *Drosophila* physiology we compared the transcriptomes of flies disrupted in the intracellular component Dif and the circulating protease psh. Overall, our analysis indicated that many Ma549-responsive genes, affecting immune, physiological and homeostatic responses require Dif and psh, but psh up regulated additional genes involved in many processes including melanization, host recognition and shutting down the immune response.

3.4 Results:

3.4.1 Infection protocols

Table 1 lists the *Metarhizium* strains we deployed in this study, their USDA ARSEF collection accession numbers, their original hosts, and their infection parameters including differences in LT_{50}'s. Infection involved immersing flies in a spore suspension, typically 2.5 x 10^4 per ml for the studies with mutant flies, which results in ~200 spores/fly [23]. At a high spore concentration (1 x 10^6 per ml), LT_{50}'s ranged from 2.92 (*M. robertsii* 2575) to 10.67 (*M. album* 1941) days. At lower spore concentration (1 x 10^4 or 1 x 10^5 per ml), more than 50% of flies infected with *M. album* 1941 or *M. pingshaense* 443 where still alive 12 days post infection when the experiments were terminated.

Laboratory infection of *Drosophila* by the "natural route" rather than injection typically involves rolling flies on a plate of sporulating fungi so the insects are covered in a layer of spores. Taylor and Kimbrell [184] reported that after infection with *B. bassiana* spores all parts of the body are groomed and cleaned as much as possible, leaving only the areas hard to reach, mainly the back of the thorax, with any visible fungal spores. Our alternative procedure of immersing flies in a spore suspension does not produce a visible layer of spores on the insect. Using GFP-expressing spores of various *Metarhizium* strains we found that grooming removed most spores from the sclerites (smooth and hard portions of the fly's body), but they were frequently trapped in loose aggregations in the intersegmental regions of the abdomen (Fig 1). These interscleral regions, being unsclerotized and soft to allow flexibility, could represent a zone of weakness in the cuticle for penetrant hyphae [185].

3.4.2 How does environmental humidity affect the lethality of *Metarhizium* strains with different virulence?

Of the environmental factors that influence epizootic qualities of a mycopathogen, humidity is particularly critical for sporulation, germination and invasion of the host [186]. We used Toll activity readout (*Drs-GFP*) flies to check if there is a time difference for immune-response fluorescence at 100%, 96%, and 80% RH when infected with virulent *M anisopliae* (Ma2105), intermediate virulence *M. acridum* (Mac324) and low virulence *M. pingshaense* (Mp443). All three fungal strains killed significantly faster at 100%>96%>80% RH ($p<0.05$) (Figure 2). At each RH, Ma2105 is significantly more virulent to flies than Ma324 or Mp443 (At 100% RH, Ma2105 vs. Ma324, $p=0.0403$; Ma2105 vs Mp443, $p=0.0016$; At 96% RH, Ma2105 vs. Ma324, $p=0.0006$; Ma2015 vs Mp443, $p=0.0003$. At 80% RH, Ma2105 vs. Ma324, $p=0.0008$; Ma2105 vs Mp443, $p<0.0001$). Increased spore germination at high RH produced an earlier immune response consistent with more rapid penetration into the insect and faster kills; time to kill was negatively correlated ($r=-0.782$, $p=0.0128$) with higher Drs-GFP immunofluorescence. Although 100% humidity dramatically increases both mortality and Drs-GFP immunofluorescence for the three strains, this did not make Mp443 or Mac324 as virulent to flies as Ma2105. Thus, the favorable humidity for pathogenicity and spore germination is not a key factor in the differential lethality of generalists and specialists.

Using a high (1×10^6 per ml) spore concentration to provide sufficient number of spores to count, we also monitored germination of GFP-fluorescent Mp443 or Ma2105 on different parts of *Drosophila* bodies at different RH (Table 1). At 80% RH, germination over 48 hrs took place almost exclusively in intersegmental abdominal membranes. At 96% RH, early (16 hr.) germination was localized to intersegmental membranes. By 48 hrs., a minority (< 15%) of spores on abdominal segments were

germinating. At 100% humidity, by 16 hrs. more than 50% of spores had germinated on all parts of the fly's body. Bidochka et al. [187] writing about locusts noted that the cuticle contains numerous microenvironments within its folds and cleavages where humidity may be conducive to fungal germination. We conclude that *Drosophila* intersegmental membranes possess a suitable microclimate for *Metarhizium* germination.

3.4.3 How do pathogen genotypes with different infection strategies interact with *Drosophila melanogaster*?

To characterize variation in pathogen properties, we previously separated *Metarhizium* strains into four metabolic flexibility categories ranging from group A (good germination in yeast extract media (YEM) and production of appressoria *in vitro* against a hard hydrophobic surface) to group B (good germination in YEM but requires cuticle for production of appressoria) to group D (little to no germination in YEM) [177]. None of the strains deployed in the study were group C (poor germination in YEM). Two D strains, *M. robertsii* 1046 and *M. pingshaense* 2162, kill flies rapidly, while D strains Mp443 and two *M. majus* are weakly virulent (Table 1). However, overall, *Metarhizium* isolates in-group A had the fastest average LT_{50} (3.95±0.45 days), followed by group B (4.95±0.43 days), and group D (5.99±1.27 days) when infected with $1x10^6$ spores/ml (Fig 3). A one-way ANOVA of the mean LT_{50}'s shows no significant differences between these groups. *M. album* 1941 (D) and Mp443 (D) killed less than 50% of the population at spore concentrations less than $1x10^6$ spores/mL and so was left out of the LT_{50} analyses at lower doses**.**

The differences in LT_{50}'s were linked to host range but not necessarily to metabolic flexibility. Group A contains six species that are considered generalists

because of their broad host ranges, Group B contains two generalists and two specialists, and Group D contains two generalists and four specialists [22]. There is a significant difference in LT_{50} values (t=3.47, df=14, p=0.0038) between generalist (μ=3.87±0.278 days) and specialist (μ=6.79±1.00 days) strains. The hemipteran specialist, *M. album* 1941 (Mal1941), did not immobilize flies, nor produce fungal growth on any cadavers, and was excluded from calculations for immobilization time, emergent period, and latent period. Flies infected with specialists *M. majus* (297 and 1946, D group), *M. acridum* (Mac324 and 5736 B group) and *M. pingshaense* (Mp443, D group) had an extended immobilization time. Group A fungal strains immobilize flies before death for significantly less time (μ=7.36±0.08 hrs.) than those in Group D (μ=18.24±4.5 hrs.) (Dunn's Test Holm-Bonferroni correction p=0.014). Consistent with this, Welch's two sample t-test comparisons between specialists (μ=23.32±1.80 hrs.) and generalists (μ=7.48±0.07 hrs.) revealed that generalists immobilize flies for a shorter period (t=8.78, df=4.01, p=0.0009). The ~15-hour difference in pre-mortem immobilization time between generalists and specialists accounted for 11% (*M. pingshaense* 443) to 27% (*M. majus* 297) of the longer LT_{50} values delivered by specialists.

We previously reported that lines of the *Drosophila melanogaster* Genetic Reference Panel (DGRP) showed significant genetic variation in their ability to tolerate Ma549 colonizing their hemolymphs [148]. Conversely, it is conceivable that the $w[1118]^{DrosDel}$ line used in this study could be better able to tolerate some *Metarhizium* strains colonizing the hemolymph than others. We performed a time course for detection of GFP-tagged blastospores in the hemolymph of 10 flies per selected *Metarhizium* strain (*M. robertsii* Mr2575, *M. anisopliae* Ma2105, *M. robertsii* Mr1046 and *M. majus*

Mm1946). We did not detect blastospores of 2575 until postmortem. Blastospores were visible in *Drosophila* infected with 1946 (26 ± 9.22 per fly), Mr2105 (26 ± 4.84 per fly) and Mr1046 (47± 34.25 per fly) coincident with the onset of immobilization, which ranged from 7.4±0.52 (Ma2105) to 22±7 hours (Mm1946). Generalist strains are often toxigenic whilst nontoxigenic *Metarhizium* spp. (e.g., *M. acridum, M. majus, M. album*) usually have narrow host ranges and kill by growing within the host [171]. Destruxins (dtxs) may suppress both cellular and humoral immune responses, including melanization, to facilitate fungal colonization in insects [171,188]. *M. robertsii* Mr2575 produces high levels of dtxs so we used a Mr2575 null mutant of dtxs [189] to test whether dtxs contribute to a short immobilization period. A Kruskal-Wallis test showed no significant differences ($p=0.91$) in the immobilization period in Drs-GFP flies infected with Ma549 (does not produce destruxins), 2575ΔDtx and Mr2575.

While there was no significant difference between groups A, B and D ($F > 0.05$), for average emergent period, latent period, and sporulation capacity, t-test comparisons showed generalists ($\mu=36.07\pm3.64$ hrs) take less time to emerge (emergent period) from *Drosophila* cadavers than specialists ($\mu=63.95\pm4.98$ hrs) ($t= 4.47$, $df=13$, $p=0.0006$). Generalists ($\mu=62.59\pm2.93$ hrs) have a shorter latent period than specialists ($\mu=96.12\pm8.05$hrs, $t=4.86$, $df=13$, $p=0.0003$) and the mean sporulation capacity of generalists ($\mu=3.30\times10^{6}\pm6.96\times10^{5}$ spores) is significantly greater than specialists ($\mu=5.18\times10^{5}\pm2.36\times10^{5}$ spores) (Welch's two sample t-test $t=3.79$, $df=10.9$ $p=0.003$), with *M. album* 1941 producing no spores at all on cadavers. Thus, pathogenicity is more closely related to the strain's classification as a generalist or specialist than its metabolic flexibility i.e., whether it has broad (group A) or narrow (group D) nutrient requirements

for germination and growth. This data suggests generalist strains take less time than specialists to infect the insect and convert the host into fungal biomass more rapidly and with greater reproductive potential. This is consistent with specialists being ill adapted for utilizing novel hosts.

3.4.4 *Drosophila* immune response to different *Metarhizium* strains

Comparing all *Metarhizium* strains, LT_{50} and LC_{50} (the lethal spore concentration required to kill 50% of the population) values are positively correlated (spore doses per mL: $1x10^6$, $r=0.93$, $p<0.0001$; $1x10^5$, $r=0.86$, $p<0.0001$; $1x10^4$: $r=0.8$, $p=0.0006$). Drs-GFP flies were used to measure immune activation. The more virulent the *Metarhizium* strain (i.e., the lower it's lethal spore dose, LC_{50}), the stronger the induction of drosomycin five days post-infection ($r= -0.69$, $p=0.02$). This is also illustrated by a time course of GFP immunofluorescence (Fig. 4). Virulent strains cause immunofluorescence by day two indicating early penetration and detection by the host immune system, although there was some plasticity within strains. Thus, ~80% of flies infected with Ma549 were fluorescing by day two and the remaining flies commenced fluorescing at 2.5 days, but we found no significant differences in longevity between the early and late fluorescing flies. Less virulent strains do not induce fluorescence until later, and the least pathogenic strain, *M. album* 1941 did not induce fluorescence until day nine, indicating delayed penetration and activation of the immune system. There is a negative correlation between the onset of fluorescence (time point where fluorescence is significantly different compared to uninfected controls) and the max fluorescence achieved during an infection ($r=-0.53$, $p=0.0497$) (Fig 5). However, max fluorescence fell short of being

significantly negatively correlated with LT_{50}'s (r=-0.39, p=0.1916). Three strains were outliers: *M. frigidum* 7436, that evolved to be a generalist independently of the PARB clade induces high fluorescence but kills only moderately quickly (~5 days), and the least pathogenic strains, pathogenic *M. pingshaense* 443 and *M. album* 1941, produce late and weak fluorescence (Fig. 5). Removing these three strains improves the correlation (r= -0.69, p=0.0278). Collectively, our results are consistent with virulent strains inducing an earlier immune response and of greater magnitude than less virulent strains, suggesting that virulence does not depend on suppressing a strong immune response.

In terms of physiological groupings: group A strains produce significantly higher max fluorescence ($\mu=1.16x10^6\pm1.15x10^5$) than either group B ($\mu=8.80x10^5\pm7.30x10^4$) or group D ($\mu=8.20x10^5\pm7.71x10^4$) (Dunn's Test Holm-Bonferroni correction p=0.013 and p=0.0023). As expected therefore, generalist strains, which are mostly in group A and usually kill faster, exhibit stronger max immunofluorescence ($\mu=1.07x10^6\pm8.38x10^4$) compared to specialists ($\mu=8.01x10^5\pm6.32x10^4$) (p=0.04).

3.4.5 psh-dependent processes confer resistance to *Metarhizium* strains post cuticle penetration.

Flies, either wild type or immune deficient mutants, were challenged with *B. bassiana* 80.2 and the panel of generalist and specialist *Metarhizium* strains (Table 2) or a subset of it. A model showing the potential interconnections of components of Toll, Imd and melanization responses represented by the mutants is shown in Fig 6. Flies lacking persephone (psh^4), a serine protease implicated in the recognition of pathogen proteases [152], succumbed to generalist and specialist *Metarhizium* strains ~50% more rapidly than their isogenic backgrounds (Fig. 7).

Persephone activates a serine protease cascade which induces Toll-mediated AMP transcription through the nuclear translocation of *Drosophila* Dif, a NF-κB homolog that activates production of AMPs such as drosomycin [181]. However, flies lacking active Dif (Dif^1), succumbed to most *Metarhizium* strains about the same time as their background controls (Fig 7). An exception was *M. frigidum* 7436 where the survival time of male (female) Dif^1 flies was 85% (72%) of that of WT flies (Fig 6), t=3.36, p=0.014 (t=7.88, p=0.0006). Similar results were obtained with *B. bassiana* 80.2: the survival time of male (female) Dif^1 flies was 72% (68%) of that of WT flies (Fig 8). These results were in agreement with earlier studies with Bb 80.2, but according to Le Bourg [190], the appearance of high mortality in infected Dif^1 may partly be an artifact of this mutant's low longevity. The lifespan of uninfected Dif^1 flies in our study was >3 weeks, so longevity should not have constrained our results.

Persephone activates späetzle processing enzyme (SPE) which processes the extracellular Toll ligand, späetzle (spz) [13] (Fig. 6). We analyzed the role of these genes in resistance to *Beauveria bassiana* 80.2 (Bb80.2), *M. frigidum* (Mf7436), and *M. anisopliae* (Ma549). Flies lacking *Grass* (functions upstream of SPE), *SPE* or *spz* were significantly more susceptible to *Metarhizium* spp. and Bb80.2 than their backgrounds. The survival time of spz^{rm7} mutants was reduced by 50 to 60%, similar to psh^4 flies. The lack of a large effect of mutating intracellular *Dif*, and reduced resistance of psh^{Δ}, SPE^{SK6} and spz^{rm7}, suggests that a group of extracellular components affect *Metarhizium* due to functions outside their established role in the Toll pathway. The antifungal peptide drosomycin is reported to be the principal product of *Dif* [13]. *Metarhizium* spores (Mf7436, Ma2105, Ma549 and Mac324) germinated at a higher frequency in an aqueous

solution of drosomycin than in water alone (Fig 9), suggesting that *Metarhizium* spp. can use drosomycin as a nutrient source. Combining the antimicrobial peptides metchnikowin and cecropin with drosomycin had no additional impact on *Metarhizium* spores compared to drosomycin alone. The saprophytic nonpathogenic fungus *Neurospora crassa* used as a control was strongly inhibited by drosomycin as expected (Fig 9). Besides drosomycin, the Toll pathway is believed to be responsible for the induction of the *Drosophila* specific Bomanin (Boms) and Daisho peptides [191,192]. Double knockouts of the *Daisho* gene pair show comparable survivability to the wild-type (~90%) when infected with *Metarhizium* and only had a large significant impact when treated with Bb80.2. The 10 gene deletion $Bom^{\Delta 55C}$ mutant succumbed ~50% more rapidly when exposed to Bb80.2, Mf7436, or Ma549.

Sp7 is a serine protease which activates the two prophenoloxidases (PPO1 and PPO2) that produce the bulk of hemolymph phenoloxidase activity after immune challenge [193]. *Hayan* ^{SK3} and *Sp7* ^{SK6} are reported to have specific phenotypes with *Hayan* being responsible for cuticular melanization in adults after clean injury [194]. $PPO1^{\Delta},2^{\Delta}$ and *Hayan* ^{SK3} mutant flies succumbed to Mf7436 and *B. bassiana* 3% to 25% more rapidly than their isogenic controls, but only some of these differences were significant (Fig 8). In contrast, *Hayan* ^{SK3} and *Sp7* ^{SK6} were more resistant than wild type when challenged with Ma549. *Sp7* ^{SK6} was also more resistant to Mf7436 and *B. bassiana*. Double mutant flies, *Hayan-psh* and *psh;;Sp7*, were more susceptible to all fungal strains similar to the *psh* single mutant.

Toll is reported to be responsible for sexual dimorphism in longevity for flies infected with bacteria and *Beauveria bassiana* [195,196]. We found that directions of

dimorphism in infection outcome are difficult to predict, being dependent on both the immune mutation and the pathogen. Males in most DGRP lines are more resistant to Ma549 than their female counterparts [148], and that was true for 4 of the 5 isogenic background strains deployed in this study. Female w[1118]^{DrosDel} flies, the background for *Sp7*^{SK6}, *SPE*^{SK6}, *Relish*^{E20}, *spz*^{rm7} and *Hayan-psh* mutants, lived longer than males following infection with Ma549, Mf7436 and *B. bassiana* ($p<0.05$). Most of these mutations reversed the direction of dimorphism with males becoming more resistant than females. Flies lacking peptidoglycan recognition protein SA (PGRP-SA) implicated in recognition of Gram-positive bacteria, did not affect the susceptibility of females to fungi, but male *PGRP-SA*^{seml} were significantly more resistant to Ma549 ($t=3.48$, $p=0.0084$) and *B. bassiana* ($t=3.75$, $p=0.0057$). *psh*^{4} flies infected with *M. frigidum* retained significant sexual dimorphism, unlike *psh*^{4} flies infected with Ma549.

The Imd pathway is not involved in the detection of a fungal infection, but downstream crosstalk with the Toll pathway has been suggested previously, and in particular, survival of Imd pathway mutants against *B. bassiana* is less than wild-type [184,197]. Flies mutated in *Relish*, the terminal transcription factor in the Imd pathway, but not *Imd* itself showed variably reduced survival ranging from 15% (females infected with Ma549) to 37% (females infected with Mf7436). As reported by Shahrestani et al., [195] we also found that disrupting *Relish* eliminated sexual dimorphism in survival to *B. bassiana* infection. However, it reversed sexual dimorphism to infection with *M. frigidum* (Fig 8). These results suggest that the immune sexual dimorphism common in *Drosophila* lines is dependent on specific interactions of each pathogen with immune pathways.

3.4.6 Analysis of *Drosophila* gene expression patterns in response to Ma549.

Next, we used Drosophila Agilent GeneChips to assess which genes in adult males cease to be regulated in Dif^1 and psh^4 mutants after infection with Ma549, thereby identifying genes dependent on *psh* and *Dif*. We picked a time of 46 hours post-infection for the assessment as this coincides with onset of visible *Drs-GFP* expression, signposting involvement of the Toll pathway. To identify differentially expressed genes, we performed Volcano Plot filtering between treated and untreated groups. As it is less likely that genes with small expression changes are of biological significance, we initially focused on those genes induced by at least a factor of two. The two *Drosophila* WT backgrounds for psh^4 ($w[1118]^{6326}$) and Dif^1 (cn bw) mutations are not isogenic and responded to Ma549 by up-regulating 2919 and 2540 genes, respectively ($p \leq 0.05$), with 57.2% overlap. We then looked at all genes and set a p-value threshold of 0.1 where at least one WT has significant differential expression ($p < 0.05$) and the other has a p-value of 0-0.1 (n=8196). Using these criteria, the WT's are more similar with 97.9% of all genes (n=8023) regulated in the same direction (regardless of magnitude of expression). Of these 8023 genes, 7101 genes were significant ($p \leq 0.05$) and expressed in the same direction (regardless of magnitude) in both WT lines. A minority of criteria genes (1.6%, n = 173) are regulated in different directions (regardless of magnitude) by the two backgrounds during infection. Following infection with Ma549, psh^4 and Dif^1 mutants up-regulated 147 and 339 genes, respectively, compared to uninfected mutants, with 17.7% overlap.

We performed GO enrichment analysis to assess to what extent the entire suite of Ma549 response genes were functionally related. Using a Benjamini-corrected $P < 0.05$,

GO categories that were significantly enriched in both background lines infected with Ma549 (Table S2) included many biological process terms for regulation, cell communication, signaling, development and morphogenesis. As reported in previous microarray studies [198], Relish (Imd signaling pathway) and many genes of the Toll signaling cascade were induced by fungal challenge. The cactus gene was induced by *B. bassiana* [198] and Ma549 (this study), and it ultimately contributes to shutting-off expression of the immune-responsive genes. The *necrotic* gene is also induced by both *B. bassiana* [198] and Ma549. As the physiological inhibitor of psh, necrotic may be involved in shutting down the hemolymph protease cascade that activates Toll [199]. In contrast to de Gregorio et al., [200], we found up-regulation of peptidoglycan recognition GNBP and PGRP proteins and these are also expressed at relatively high levels in naive adults. Previous microarray studies have deployed *B. bassiana* isolates and different fly lines than us, which may contribute to these differences. It is also likely that expression of these genes peaked at earlier or later time points than the ones used in our studies. Like previous studies, the flies used in the experiments were not raised in aseptic conditions and base-line levels of the immune-inducible family members in naive WTs may indicate the presence of minor microbial infections or stress within the naive population.

In 2001, Irving et al., [198] identified genes up-regulated by infection processes as "actin-associated, calcium binding, cell adhesion, chaperones, heat-shock proteins, enzyme inhibitors, growth factors, carrier proteins, motor proteins, nucleic acid-binding factors, structural proteins (cytoskeleton, cuticle, and muscle), transcription factors, and others". These authors noted "that nearly half of the immune-induced genes in *Drosophila* have not yet been ascribed a putative function and do not show obvious

similarities to any known genes." It remains true that many immune induced genes are of unknown function but with systems like KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg) we have an improved knowledge base for integration and interpretation of large-scale datasets. We used the KEGG database to examine whether differentially expressed genes were enriched for true positive associations and cellular networks (Fig 10a). Ma549 response genes in both w[1118]^{6326} and cn bw are functionally connected through processes involving multiple signaling pathways including the Toll, FoxO, AGE-RAGE. mTOR, MAPK, Hippo and phosphatidylinositol signaling systems. Endocytosis and apoptosis genes were also enriched in the analysis. FOXO is involved in cross regulation of metabolism and innate immunity [103], and transcriptional regulation for nutrient-stressed flies during resource allocation [104]. Validating the functional significance of these results, we found previously that alleles of the transcription factor FOXO cause variation between individual DGRP lines in susceptibility to Ma549, and a *foxo* mutant succumbed quickly to Ma549 [148]. Phosphoinositides (PtdInsPs) are lipids that mediate a range of conserved cellular processes in eukaryotes. These include the transduction of ligand binding to cell surface receptors, vesicular transport and cytoskeletal function [201]. PtdInsPs are involved in promoting autophagy and endocytosis [202], and regulating TOR signaling [203]. The conserved mTOR signaling pathway integrates both intracellular and extracellular signals (including nutrient signals), and serves as a central regulator of cell metabolism, growth, proliferation and survival; in *Drosophila,* it is required for stem cells to rapidly proliferate in response to damage [204]. Yorkie (Yki) was one of 26 Hippo pathway genes up regulated during Ma549 infection. Hippo

signaling integrates multiple upstream inputs, including energy status and disruptions of the cytoskeleton, in order to regulate Yki and thereby promote cell proliferation in response to injury [205]. The 42 up-regulated genes in the MAPK signal transduction pathway overlap with the 32 up-regulated genes listed in the Jun N-terminal kinase (JNK) cascade (GO:0007254); both pathways are involved in orchestrating wound repair and tissue regeneration. Yki overcomes the tumor-suppressive role of JNK [206], showing the potential for cross-talk between these pathways. JNK activation is also sufficient to activate Toll signaling [207], and by a mechanism involving the cytoskeleton, Toll activation leads to Yki activation [208]. A highly conserved apico-basal polarity gene scribble (*scrib*) that can cause tumors, aberrant epithelial architecture, and invasive cell behaviors [208], is up-regulated in Ma549-infected flies. Elevated Toll signaling in *scrib* mutants results in Yki-mediated tumorous overgrowth through JNK activation and F-actin accumulation [208], indicating more cross talk between pathways. Before being implicated in survival to fungal infection, the Toll pathway was known to be involved in dorsal-ventral patterning, and many observations point to the reuse of developmental programs and genetic reprogramming to drive regeneration [209]. Sixteen genes identified as involved in dorso-ventral axis formation and cytoskeletal organization were up regulated during infection; these genes also overlap with those in the MAPK signaling pathway and are involved in cell shape change during wound healing [210]. Advanced glycation end products (AGEs), via their chief signaling receptor—the AGE-specific receptor (RAGE)—generate reactive oxygen species and activate inflammatory signaling cascades [211]. However, many of the genes in this pathway are highly pleiotropic e.g., Signal-transducer and activator of transcription protein at 92E (Stat92E) is involved in

biological processes described with 16 unique terms many of which group under proliferation, growth control, organismal metabolism, cell competition, stem cell self-renewal, immunity and developmental patterning.

To identify *Drosophila* response genes controlled by Dif and/or psh, we compared the gene expression profiles of Dif^{1} and psh^{4} mutants with their genetic backgrounds. Using the stringent 2-fold change cut off point, most Ma549 responsive genes are completely or partially dependent on either or both *psh* and *Dif*, but the Psh-dependent transcriptome was even larger (Fig. 10). *Drosomycin* was up regulated following Ma549 infection in $w[1118]^{6326}$, cn bw, psh^{4} and Dif^{1} by 7.4, 4.4, 2.9 and 6.5-fold respectively. However, the level of *Drosomycin* expression in naive WT adults was more than 3-fold that of naive Dif^{1}, so the responsiveness of infected Dif^{1} flies was relative to a low baseline level. Likewise, compared to WT and psh^{4}, naive Dif^{1} expressed lower average baseline levels of Attacin A, Cecropin A1, Defensin, Diptericin A and Metchnikowin implying that active Dif maintains defensive levels of AMPs even in the absence of infection. In contrast to *Drosomycin*, the level of expression of *CecA1*, *CecA2*, *Def* and *Mtk* in infected Dif^{1} flies was several fold above levels in infected WT.

Both Dif and psh are required to up-regulate large categories of important genes, including FOXO and most other signaling pathways that respond to challenge with Ma549 in the WT. However, since Dif^{1} shows WT resistance to Ma549 while psh^{4} are highly susceptible, we focused on the minority of genes uniquely regulated by *psh* to identify the source of the anti-Ma549 response (Table S3). For psh controlled genes, we looked at genes upregulated in both Dif^{1} and its WT (cn bw) during infection and *vice*

versa with *psh*^{4} and its WT for Dif controlled genes. We identified 161 genes of which psh exclusively controls 79 and Dif exclusively controls 39. In total, 90 of these genes (55.9%) had variable transcripts compared to only 14.3% (n=1891) of all the genes profiled by the microarray. A minority of those 1891 genes (8.1%) with variable transcripts showed significant differential expression patterns between Dif and psh when using a 1.5x fold cut-off. Among immune category genes, psh but not Dif was required for induction of *Relish* and *Hayan*, identified as effectors of Ma549 success, but Dif regulated *spätzle,* and *spz*^{rm7} flies are also highly susceptible. A notable feature of the psh-dependent transcriptome was that it included a couple of circulating polypeptides involved in recognition of non-self (PGRP-SA and PGRP-SB2), indicating that psh, itself providing a signal of danger, increases the degree of "readiness" of antimicrobial systems to be able to respond to additional infections. Both psh and PGRP-SA are implicated in immunological sexual dimorphism (Fig 8). The fungal recognition protein GNBP-like3 was co-regulated by psh and Dif. The induction of *cactus* and *necrotic* were also dependent on psh, indicating that this upstream activator of the Toll pathway is also involved indirectly in shutting it down. Perhaps related to the danger of a hyperimmune response, psh regulates *Stress Induced DNase* (*Sid*) previously shown to be highly induced by bacterial infection and oxidative stress. Other genes specifically regulated by psh were involved in epigenetic regulation of gene expression (*Skeletor*), response to stress (*Thor*), stimulus and behavior (*stim, svr*) and regulation of signaling pathways (*Socs36E, spg*). *Socs36E* is a transcriptional target of the product of *Stat92E*. Two of the three tailed *Bomanins* (the other is absent from the microarray), CG16836 and CG5778, described in Clemmons et al. [191] also depend on psh. The authors note with regard to

the partial resistance of a partial Bomanin knockout to the ascomycete fungus *Fusarium oxysporum* that "resistance to some pathogens require more than one Bom peptide" [191].

Although many of the genes regulated by either psh or Dif listed in Sup Table 3 are uncharacterized, some were previously implicated in host resistance using random insertional mutations [23]. These include three trypsin-like peptidases, CG11843, CG5909 and CG9733 [29]. CG5909 and CG9733 are also up regulated in flies infected with *Beauveria* [70]; CG9733 is of particular interest as one of the few remaining uncharacterized CLIP-domain containing serine proteases. The non-peptidases implicated in host resistance are CG34114 with immunoglobulin and fibronectin-like domains that imply an involvement in ligand recognition and cell adhesion and CG16772 and CG12880 with no predicted function in FlyBase.

3.5 Discussion

Specialization to particular hosts can be qualitative, characterized by the inability of a pathogenic isolate to infect many hosts, or quantitative, where the pathogens have lower performance. The specialization of most *Metarhizium* strains appears to be quantitative, as they killed fruit flies, albeit slowly. Most *Metarhizium* species that readily kill multiple orders of insects produce toxins, whereas the nontoxigenic *Metarhizium* spp. (e.g., *M. acridum, M. majus, M. album*) have narrow host ranges [171]. There are exceptions to this: the broad host range Ma549 does not produce destruxins (Dtx) *in insecta* [178]. Dtxs may suppress both cellular and humoral immune responses, including melanization, to facilitate fungal colonization in insects [171]. The specialists had a notably long "immobilized time" compared to the generalists, which commenced with the

appearance of fungal propagules in the hemolymph. However, disrupting Dtx in Mr2575 did not significantly lengthen immobilized time or longevity, suggesting that Dtx production is not the time limiting factor for either pathogenic parameter.

We evaluated the impact of Ma549 on gene regulation in fly's deficient for components of the Toll pathway, the major immune signaling pathway for fungal pathogens. Pathway analysis and GO Analysis were applied to determine the roles differentially expressed genes played in biological pathways or GO terms. Our analysis revealed a large impact of Ma549 infection, and consequentially by Dif^{1} and psh^{4} mutants, on non-classically categorized immune response genes, particularly aspects of metabolism and multiple signaling pathways. Knocking out either *Dif* or *psh* blocked Ma549 induced regulation of FOXO, MAPK signaling and Hippo signaling genes. Surprisingly in this context, some antimicrobial peptides and Toll pathway components were still produced, showing they did not require both active *Dif* and *psh*. It has been reported that significant crosstalk between the Toll pathway, melanization and other immune responses could coordinate immune attack against invaders [212], and is consistent with events following septic injury, where multiple signaling events and pathways contribute in addition to the Toll and Imd pathways [213]. We previously found that 9% of insertional mutant *Drosophila* lines had altered resistance to Ma549, indicating a large mutational target for disease resistance, and approximately 13% of these were in genes encoding immune responses including coagulation, phagocytosis, encapsulation, and melanization [23].

A complicating factor in our study, as in many before it, is that the WTs for the different mutations were not isogenic. An RNA-Seq study found that an American

Drosophila line was transcriptionally diverged from African and European fly lines in its response to *B. bassiana* [136], and a GWAS found significant differences in resistance to Ma549 within lines from North Carolina [148]. The survival time of male w[1118]^{6326} was 92.13% of cnbw flies (the background fly lines for psh^4 and Dif^1, respectively) and so transcriptional divergence of individual genes may in part be because a single time point did not precisely capture the same stage in infection for both fly lines. Nevertheless, there was extensive overlap in the gene families induced in response to Ma549, and the impact of both Dif^1 and psh^4 on the magnitude of expression was dramatic. It was surprising therefore, that disrupting *Dif* resulted in only a small, statistically insignificant increase in susceptibility. According to Bourg [190], the appearance of increased Dif^1 susceptibility to *B. bassiana* may be in part an artifact of the weakness of the Dif^1 line. We found that the impact of Dif^1 on *B. bassiana* was significant, but much less than disrupting psh. *B. bassiana* kills more slowly than the PARB clade, but it's unlikely that the apparent lack of impact of Dif^1 on Ma549 was due to the rapid lethality of *M. anisopliae,* as Dif^1 did not significantly increase susceptibility to low-virulence isolates either, unlike psh^4. Furthermore, Dif^1 flies survived, in apparent good health, for >12 days (the duration of the experiment) following infection with *M. album*. An interesting exception was provided by the increased susceptibility of Dif^1 to *M. frigidum* 7437, a broad host range strain which induces an unusually strong immune response in Drs-GFP flies, and that resembled *B. bassiana* in its comparatively slow kill (Fig 8).

The lack of impact of Dif^1 on survival with Ma549 shows that the extent of gene expression reduction does not necessarily correlate with death rates. A knock-out of a specific gene such as *FOXO* may dramatically effect survival, and a change in expression

of many genes, as caused by *Dif*^{1} may elicit little change in survival. There are several, not mutually exclusive, potential explanations for this. In contrast to induced gene expression, a knockout of a physiological regulator such as *FOXO* will affect growth and development of the fly, contributing to the state of the host at the time of infection. More profoundly, the changes to gene expression during infection occur within the context of a complex highly interconnected genetic architecture which may preclude simple extrapolations to predict the extent and direction of the effect on survival. At its simplest, there might be a threshold effect of gene expression level on the flies' survival caused by small reductions in gene expression throughout several pathways in *psh*^{4} relative to *Dif*^{1} (Fig. 10). As well as quantitative differences, some genes were independently regulated in *psh*^{4} and *Dif*^{1} that might also contribute to phenotype variability in survival. Further research will identify any complicated phenotypes *Dif*^{1} may have on fungal loads, tolerance and resistance, which do not change overall survival. These studies will also need to consider whether some *Metarhizium* strains may have evolved strategies to evade the effects of *Dif* activation. *Dif* is part of an ancient and highly conserved cascade, and Gottar *et al.,* 2006 [152] speculated that the *psh*-dependent system evolved in response to selective pressure exerted by entomopathogenic fungi after the *GNBP*-3-based fungal cell wall sensing system. *Metarhizium* may have evolved ways to escape the effects of *Dif* activation, but not *psh*.

Beauveria spp. evolved into insect pathogens independently of the *Metarhizium* lineage, and similar expansion of protease, chitinase families etc. reflects convergent evolution of an "entomopathogenicity toolkit" associated with functions necessary for insect pathogenesis [214]. As *Metarhizium* and *Beauveria* inevitably confronted the

insect immune system, they independently evolved a series of strategies under long-term selection to evade or overcome these immune responses. There are features unique to *Metarhizium* spp., that include blastospores producing a collagen coat (MCL1) to mask antigenic cell wall β-glucans from phagocytes [122], and destruxins (dtxs) that suppress both cellular and humoral immune responses, including melanization [171,188,215]. Despite these, several species of generalist *Metarhizium* strains evoke a rapid and robust innate immune response, showing that they do not escape recognition or block activation. A cost of infecting multiple host species may be the degree to which generalist *Metarhizium* spp. can adapt to a host's immune system. Generalists may only have adapted to ancient widely distributed defenses. *Metarhizium* like *Beauveria* [216], has evolved resistance to the defensin-like peptide drosomycin, presumably under strong selective pressure. Defensins are ubiquitous in arthropods, and previously we found that *Metarhizium* can be engineered to express large quantities of scorpine (from the scorpion *Pandinus imperator*), a structurally similar but more potent anti-fungal/protozoan than drosomycin, at no harm to itself [140]. Our results contrast with *Drosophila* parasitoids where immune suppression is critically important in determining the outcome of infection [217].

Neither an insertional mutagenesis study [23], nor a GWAS investigation [148] implicated the antifungal peptide genes, despite their being induced by Ma549, indicating that these have not been targets of pathogen-dependent selection. In contrast, the GWAS highlighted *FOXO* dependent processes, as did the current gene expression study, consistent with host survival to infection requiring reallocation of resources and potential tradeoffs with other vital processes. The other signaling pathways included many

biological process terms for the cytoskeleton, cell proliferation and tissue repair and regeneration. The importance of stress and repair programs as factors that contribute to survival is an emerging theme in disease across the animal kingdom, for example, the activation of growth-promoting developmental pathways via JNK mediated stress-responsive signaling [218]. Many of these non-canonical clusters were down regulated by disrupting either *psh* or *Dif*, suggesting that these pathways are cross-linked directly or indirectly with Toll. It is possible that they are coupled through a common upstream signaling pathway involving psh. Co-regulation of pathways effecting resource allocation and tissue repair processes through psh implies a close linkage of directed anti-fungal activity with a robust ability to repair damaged structures.

One of the genes specifically up regulated by psh was that for necrotic protein (nec), the physiological inhibitor of psh *in vivo*. Conversely, a small subset of antibacterial peptides, including Cecropin A2, and the antibacterial/antifungal metchnikowin were highly induced by Ma549 in Dif^{1} but not psh^{4} suggesting that the Dif-dependent transcriptome includes molecular selectors specifying the choice between subsets of NF-κB (Relish and Dif) target genes. In contrast to *drosomycin*, induction of *metchnikowin* gene expression can be mediated either by the Toll or the Imd pathways [219]. The melanization response is mostly mediated by prophenoloxidases (PPOs) 1 and 2, which are activated by extracellular serine protease (SP) cascades involving Hayan and Sp7. Our results agree with previous studies [200] that transcription of PPO genes is not induced by infection consistent with PO activity being largely regulated at the post-transcriptional level. New protein synthesis of Hayan occurred in the presence of *psh*

which suggests a sustained response rather than a process that targets fungi immediately at the interception of infection, as proposed for GNBP3 [153].

According to Dudzic et al., 2019 [194], *Hayan* and *psh* arose from a recent duplication, and Hayan like psh may be activated by pathogen proteases [194]. Hayan provides an immediate source of phenoloxidase that blackens wound sites whereas Sp7 regulates a slower melanization reaction in the hemolymph that is itself regulated by Toll pathway activator Grass [194]. Our microarray data suggests that psh plays a role in regulating *Hayan* in response to fungal attack, and $Grass^{Herrade}$ dies at a similarly fast rate as psh^{4}. Disrupting *Hayan* had less impact on longevity than disrupting *psh* or *Grass*. It was a surprise to find that $Sp7^{SK6}$ is more resistant to *B. bassiana* and Ma549. However, Ayres and Schneider [220] reported that a Sp7 mutant was more resistant to some bacteria, and proposed that in the absence of melanization, other, more effective immune responses may show increased activity and/or that microbes may benefit from autoimmune damage to the host. Consistent with this, a *Metarhizium* strain engineered to activate melanization in the hemocoel killed more quickly [221]. Double mutant flies, *psh;;Sp7*, were more susceptible to all fungal strains similar to the *psh* single mutant, suggesting that the longevity of infected $Sp7^{SK\,6}$ flies is dependent on an active *psh*.

Dudzic et al [194] concluded that resistance to *Staphylococcus aureus* introduced through septic injury correlates with the melanization response, but not the deposition of melanin itself. *Metarhizium* strains in our study were allowed to naturally infect flies by penetration of the cuticle, and deposition of melanin in cuticle is usually the first sign of fungal infection [9]. We previously demonstrated that while growth and Pr1 (a chymoelastase subtilisin) production by *M. robertsii* was reduced on melanized *Manduca*

sexta cuticle, this was mainly due to toxic effects from catechol and L-DOPA oxidation products [222]. *M. robertsii* has adaptations to resist this toxicity including producing a metalloprotease that degrades host phenoloxidases [223], and secreting its own phenoloxidase in growth-limiting conditions to oxidize soluble phenolics to less toxic insoluble melanin [224]. Also, (in contrast to proteases from non-pathogens), *Metarhizium* proteases are resistant to melanizing mixtures. In fact, *Metarhizium* Pr1 subtilisin was used in the original publication demonstrating the action of the circulating psh in insect hemolymph [152]. *Metarhizium* itself rapidly represses expression of most proteases during transition through the cuticle [225], and these proteases bind tightly to the cuticle [226,227] to limit activation of the host PPO system [221]. The collagen capsule around blastospores and precise regulation of proteases are mechanisms that enable *Metarhizium* to reduce the generation of host responses during hemolymph infection. It appears that *M. robertsii* attempts to manipulate the melanization response to promote its own growth and survival although our understanding of these processes is incomplete [222]. There is a time lag during cuticle penetration before drosomycin is expressed consistent with psh being activated in the hemolymph, but the failure of several pathogen adaptations to prevent immune activation in the hemolymph suggests such activation may occur earlier in the cuticle. Further study should be devoted to determining where and by what mechanism does *Drosophila* first detect it is under attack from a fungal invader.

3.6 Materials and methods

3.6.1 Fungal strains

Beauveria bassiana 80.2 (Bb80.2) was kindly donated by George Dimopoulos (Johns Hopkins Bloomberg School of Public Health). This *B. bassiana* strain has been used as a representative fungal pathogen in previous *Drosophila* studies [152]. We validated the species identification as *B. bassiana* by BLASTing the sequencing results of the *Tef*-1α region after PCR amplification using the primers F:ATGGGTAAGGACGACAAGAC and R:GGAAGTACCAGTGATCATGTT. Other fungal strains were obtained from the USDA Entomopathogenic Fungus Collection (Ithaca, N.Y). The strains used were *M. anisopliae* (generalists 2105, 7427 and 549), *M. robertsii* (generalists 2575, 1046), *M. brunneum* (generalist 346), *M. frigidum* (generalists 4124, 7436), *M. pingshaense* (generalists 538, 2162, gryllid specialist 443), *M. majus* (scarab specialists 297, 1946), *M. acridum* (acridid specialists 5736, 324), and *M. album* (hemipteran specialist 1941) (see Table 2 for origin of strains). These fungal cultures were moved from −80˚C stock tubes 10-14 days before each bioassay and grown on potato dextrose agar (PDA) at 27 °C. To facilitate studies on strain differences, we transformed several *Metarhizium* strains to express green fluorescent protein (GFP). Plasmid construction and transformation for GFP fluorescence were as described previously [138].

3.6.2 Fly strains and infection protocols

Drs-GFP, $Imd^{EY08573}$, Dif^{1}, psh^{4} and their isogenic control lines were kindly donated by Dominique Ferrandon (University of Strasbourg, Strasbourg, France) [228]. The $PGRP\text{-}SA^{seml}$, SPE^{SK6}, $Sp7^{SK6}$, $Hayan^{SK3}$, $PPO1^{\Delta}$, $PPO2^{\Delta}$, $Grass^{Herrade}$, $Relish^{E20}$, $spätzle^{rm7}$, *Hayan-psh, psh;;Sp7* mutants and their isogenic control lines were kindly

donated by Bruno Lemaitre (École Polytechnique Fédérale de Lausanne, Lausanne, France). The Δ*daisho* and Bom$^{\Delta 55C}$ fly lines were kindly donated by Steven Wasserman (University of California San Diego, San Diego, CA, USA). Most of the mutants had w[1118] backgrounds, but we found that these differed slightly but consistently in susceptibility to *Metarhizium* spp. and *B. bassiana*, and are distinguished here as w[1118]^{6326}, w[1118]^{VDRC} and w[1118]^{DrosDel} (S1 Table). The IMD (17474) mutant line was obtained from the Bloomington *Drosophila* Stock Center (flystocks.bio.indiana.edu/). *Metarhizium* strains were used in infection bioassays as described previously for Ma549 [23,148]. Following infection, flies were examined at six-hour intervals to determine time to immobilization (flies not walking but still responding to touch) and time to death (completely moribund). To determine the effect of destruxin (Dtx) on immobilization period ~10 w[1118]^{DrosDel} flies infected with either Ma549, Mr2575, or Mr2575ΔDtx were collected without anesthesia, placed into food vials, and monitored at 3 hr. intervals until time to death. Germination rates on cuticles and fungal growth in the hemolymph by GFP-tagged strains infecting w1118^{DrosDel} *Drosophila* were monitored as described [23]. For each fly, we evaluated conidia on the tergum, wings, in four abdominal intersegmental regions, and six dorsal and ventral areas on abdominal segments.

We tested the effects of different (100%, 96%, and 80%) relative humidity (RH) levels on infection parameters of a virulent *Metarhizium anisopliae* strain (Ma2105) isolated from *Hydrellia sp.* [Ephydridae; close relation of *Drosophila*], and a *M. pingshaense* strain (Mp443) with a strong host preference for crickets (Gryllidae) and low virulence to *Drosophila*. Flies (w[1118]^{DrosDel}) were infected with GFP-fluorescent

Mp443-GFP or Ma2105-GFP, and spore germination rates and hyphal lengths were monitored microscopically post infection at different RH. RH was measured with a Traceable™ Digital Humidity/Temperature Meter (Fisher Scientific™). Fly tubes containing 3-5 ml fly food had 96% RH i.e., this is the RH at which we routinely bioassayed the *Metarhizium* strains in an incubator set at 85% RH.

Drosomycin reporter Drs-GFP flies were used to check for temporal differences in immune-response fluorescence. Fluorescence of 10 individual flies per time interval per infection with a 1 or 5 $x10^6$ spore suspension of each *Metarhizium* strain was quantified using a FilterMax F5 microplate reader. Data was collected for up to 10 days post-infection for less virulent strains (Mp443, Ma324, *M. album* 1941).

3.6.3 Post-mortem analysis

The ability of different fungal strains to colonize and exploit *Drosophila* cadavers was measured. For emergent period, latent period and sporulation capacity, ten female flies, harvested within six hours of death, were individually transferred into tubes containing a damp cotton ball. At six-hour intervals, we recorded the interval between death and emergent hyphae covering at least half of the fly cadaver (emergent period) and the appearance of spores (latent period). After 20 days, 500 µl of 0.1% Tween 80 was added to each tube, the tubes were vortexed (1 minute), and spore counts per individual fly were made using a hemocytometer (sporulation capacity). Results are the average of 10 flies per fungal strain. Correlations between LT_{50} survival values, emergent period, sporulation capacity, and immobilization time were analyzed using GraphPad Prism 7 (GraphPad Software, Inc.) or R.

3.6.4 Mutant analysis

To quantify the role that individual components of the insect immune system play in resisting fungal infection, fly lines disrupted in known immune genes were challenged with Bb80.2 and seven different strains of *Metarhizium*. Flies were infected with high (Mr2575, Ma549, Ma2105) and low virulence strains (Mp443, Ma324, Mm1946, Mal1941, Bb80.2) ($2.5x10^4$ spores/mL). We sequenced both Dif^1 and its isogenic control (cn bw) to confirm that guanine 1104 (found in the cn bw) was point-mutated into an adenine resulting in a radical missense change from glycine to aspartic acid in Dif^1.

3.6.5 Analysis of immune peptides

The expression and purification of drosomycin were carried out as described previously [229]. The sequence encoding the mature drosomycin (Drs) was amplified via PCR from *D. melanogaster* genomic DNA and cloned into the NcoI and BamHI sites of a pET-32b expression vector derivative used for transformation of *Escherichia coli* strain Rosetta-gami (Novagen). The recombinant drosomycin, fused to a His_6 tag, was purified on a HisTrap® affinity column (GE Healthcare), and the tag was cleaved with thrombin. Drosomycin was purified using a Resource® 3-ml reverse phase high-pressure liquid chromatography column. The molecular mass of the recombinant Drs was confirmed by mass spectroscopy. Cecropin A was purchased from Sigma-Aldrich (St. Louis, MO). Metchnikowin was synthesized as a service by Peptide 2.0 Inc (Chantilly, VA). The effect of peptides on different fungi was determined by adding 50 µl of peptide (0.5 mg/ml) to 60 µl of water or 0.2% yeast-extract containing ~$1x10^5$ fungal spores/mL and calculating the percent of germinated spores at 16 and 24 hours.

3.6.6 Microarray analysis

To investigate the roles of psh and Dif during infection with Ma549, 10 male flies of each deficient mutant (and unmutated backgrounds) 46 hours post infection with Ma549 were homogenized using a hand homogenizer in 1 mL of TRIzol per replicate (total 3 replicates per fly line). The homogenates were shipped to Arraystar (Rockville, MD) for RNA extraction and subsequent microarray analysis using Oligo Microarray 4x44K - V2 (4 x 44K, Agilent Technologies). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software (Agilent Technologies). After quantile normalization of the raw data, genes that at least 3 out of 25 samples have flags in Detected ("All Targets Value") were chosen for further data analysis. Differentially expressed genes were identified through Volcano filtering (P-value < 0.05, Fold Change >=2.0). Hierarchical Clustering was performed using the R software package. GO analysis and Pathway analyses were performed by the standard enrichment computation method.

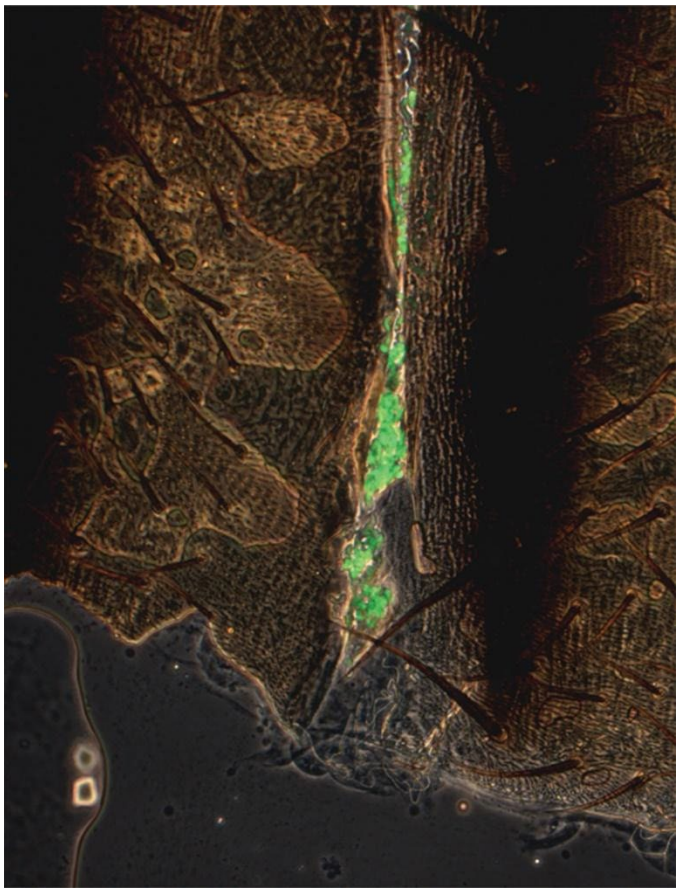


Fig 1. The folds of the intersegmental membranes provide the best growth environment. GFP-expressing *M. robertsii* strain 2105 photographed on the abdomen of a fly 48 hours post-infection.

Strain	RH	LT_{50}
Ma2105	100%	1.31±0.12
Mac324	100%	1.79±0.07
Mp443	100%	3.24±0.22
Ma2105	96%	2.64±0.19
Mac324	96%	4.73±0.08
Mp443	96%	5.64±0.17
Ma2105	80%	3.36±0.12
Mac324	80%	6.28±0.30
Mp443	80%	7.80±0.18

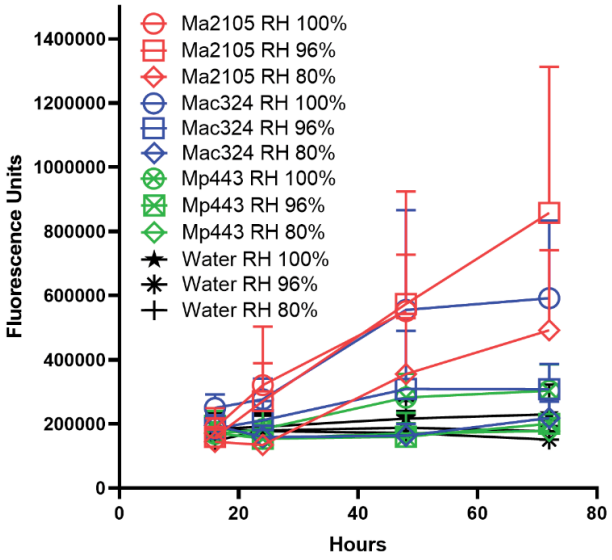


Fig 2. The effect of humidity on *Metarhizium* spp. Fly immune system responses to infection with *M. anisopliae* 2105, *M. acridum* 324 and *M. pingshaense* 443 at different relative humidity's (100%, 96%, and 80%) was studied by measuring Drosomycin expression and calculating LT_{50} values. A) Increasing RH leads to increased mortality and a decline in LT_{50} times. B) Faster kills at higher RH elicits earlier and higher Drs-GFP immunofluorescence in infected insects. Fluorescence data was collected 16, 24, 48, and 72 hours post infection. Points represent the means of 10 individual flies $\pm$ SE. Control flies were dipped in water instead of spore suspensions and then incubated at different RH in parallel with infected flies.

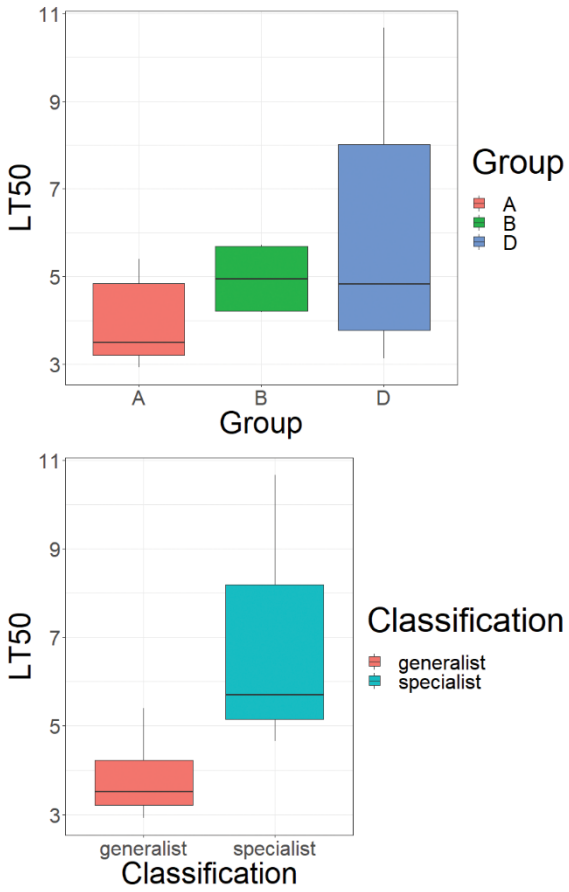


Fig 3. Boxplots showing the LT_{50}'s (inoculum load $1x10^6$ spores ml^{-1}) of *Metarhizium* groups A, B and D, and generalist/specialist classifications.

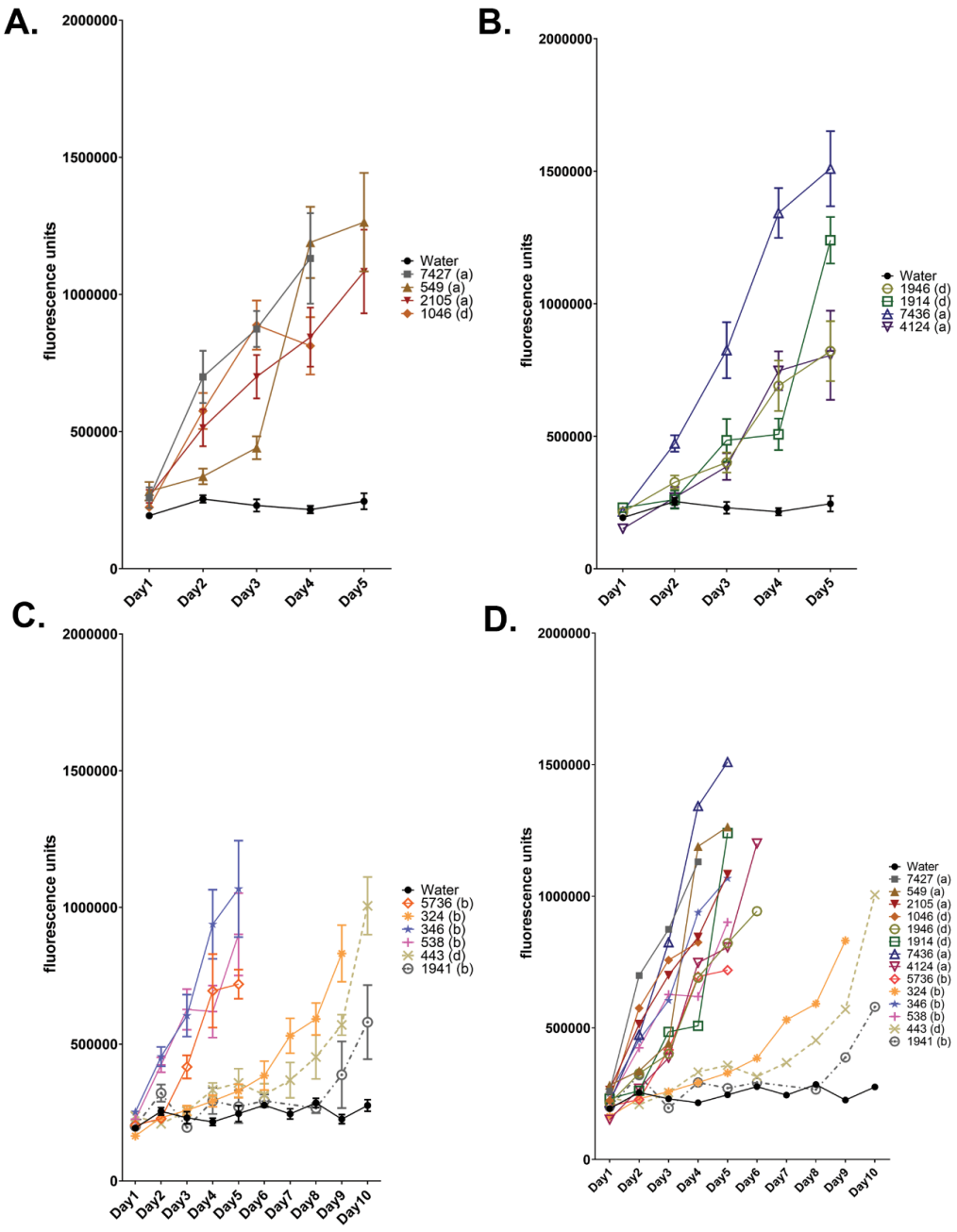


Fig 4. Fly immune system responses to infection with *Metarhizium* strains of high, medium and low virulence to *Drosophila* (A) Immunofluorescence results from Drs-GFP flies infected with *M. anisopliae* (2015, 7427 and 549) and *M. robertsii* (1046). **(B)** Drs-GFP immunofluorescence results from *M. majus* (1946 and 1914), and *M. frigidum* (7436 and 4124) infections. **(C)** Immunofluorescence results from *M. brunneum* (346), *M. pingshaense* (538 and Mp443), *M. acridum* (5736 and Ma324) and *M. album* (1941) infections. **(D)** Combined data from A-C.

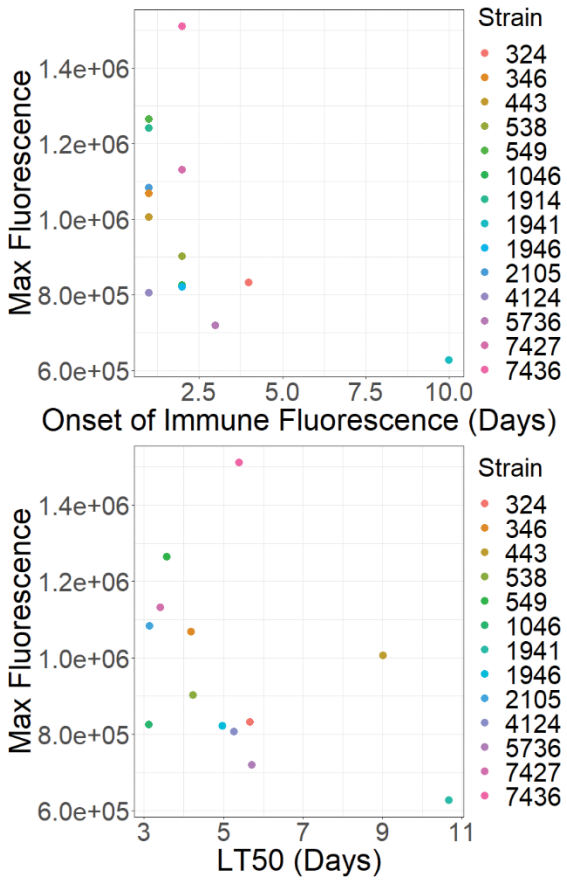


Fig 5. Max Fluorescence correlations for *Metarhizium* panel. (**Top**) Negative correlation (r=-0.53, p=0.0497) between max fluorescence achieved over the course of an infection, and the day flies infected with each *Metarhizium* strain show fluorescence significantly greater than controls (treated with water instead of a spore suspension). (**Bottom**) Negative correlation (r=-0.39, p=0.1916) between max fluorescence and LT_{50} for each *Metarhizium* strain. *M. frigidum* 7436 induces high fluorescence but kills only moderately quickly (~5 days), and the poorly pathogenic *M. pingshaeaense* 443 and *M. album* 1941 produce late or extremely weak fluorescence. Removing these three strains improves the correlation (r= -0.69, p=0.0278).

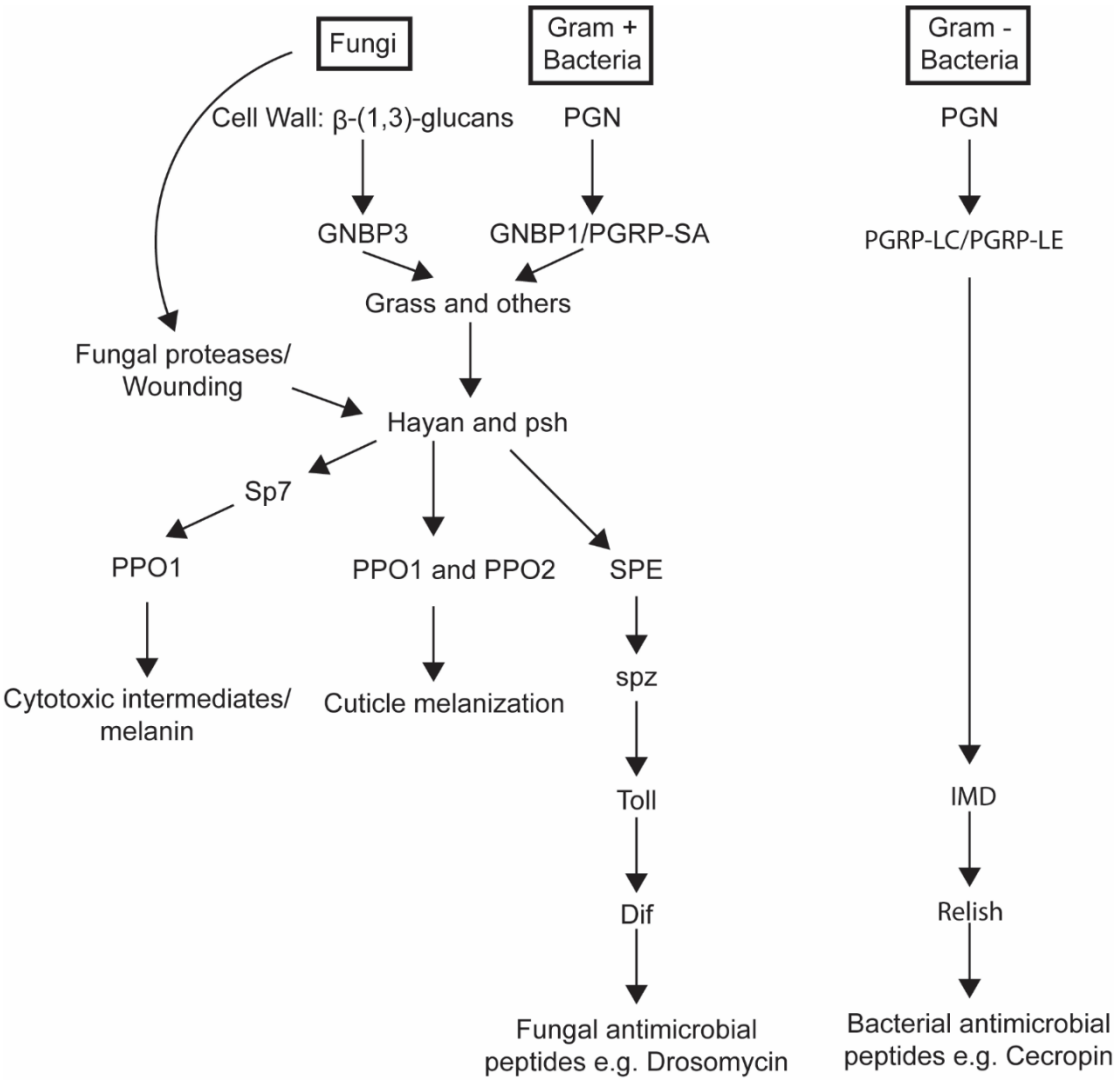


Fig 6. A simplified model of the immune (Toll and Imd) pathways and the melanization reaction, principally based on Dudzic et al. [194]**.**

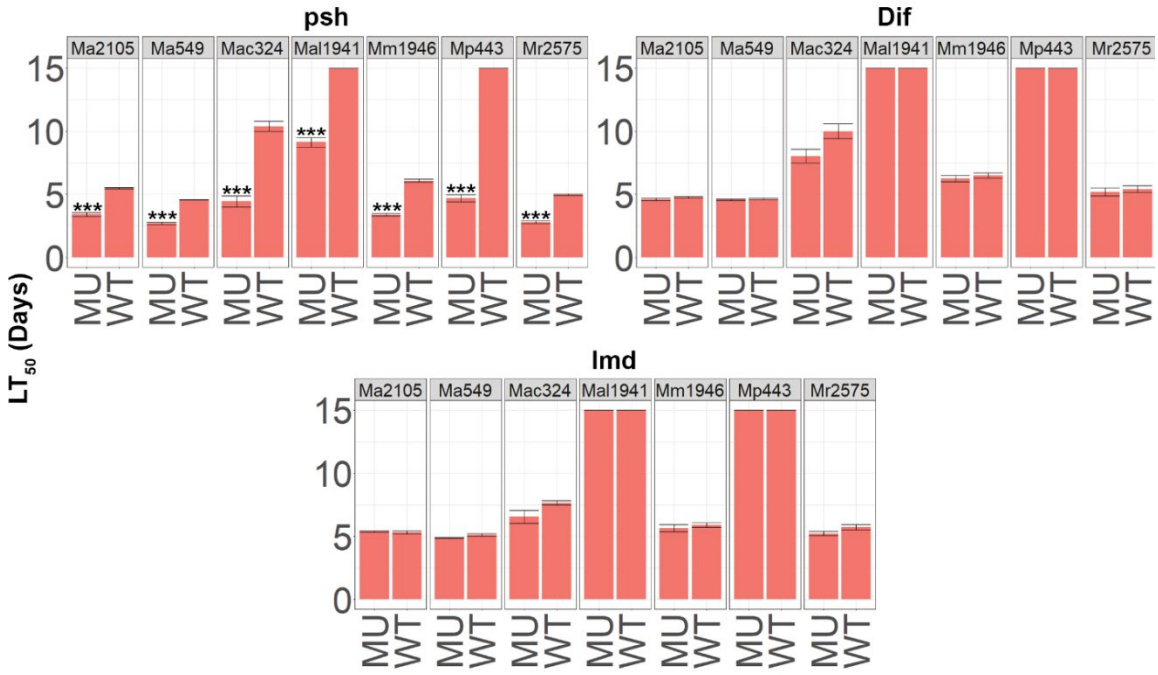


Fig 7. Quantifying the role individual components of the insect immune system play in resisting infection to the *Metarhizium* panel. Survival measured as LT_{50}'s of fly lines disrupted in known immune genes and their isogenic WT backgrounds against a panel of different *Metarhizium* spp. Bars represent the mean of three replicates with ~20 flies per genotype. Significance was evaluated using t-tests and is shown relative to the WT (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). *M. album* Ma1941 was not lethal to most fly lines during the 12 day duration of the experiment.

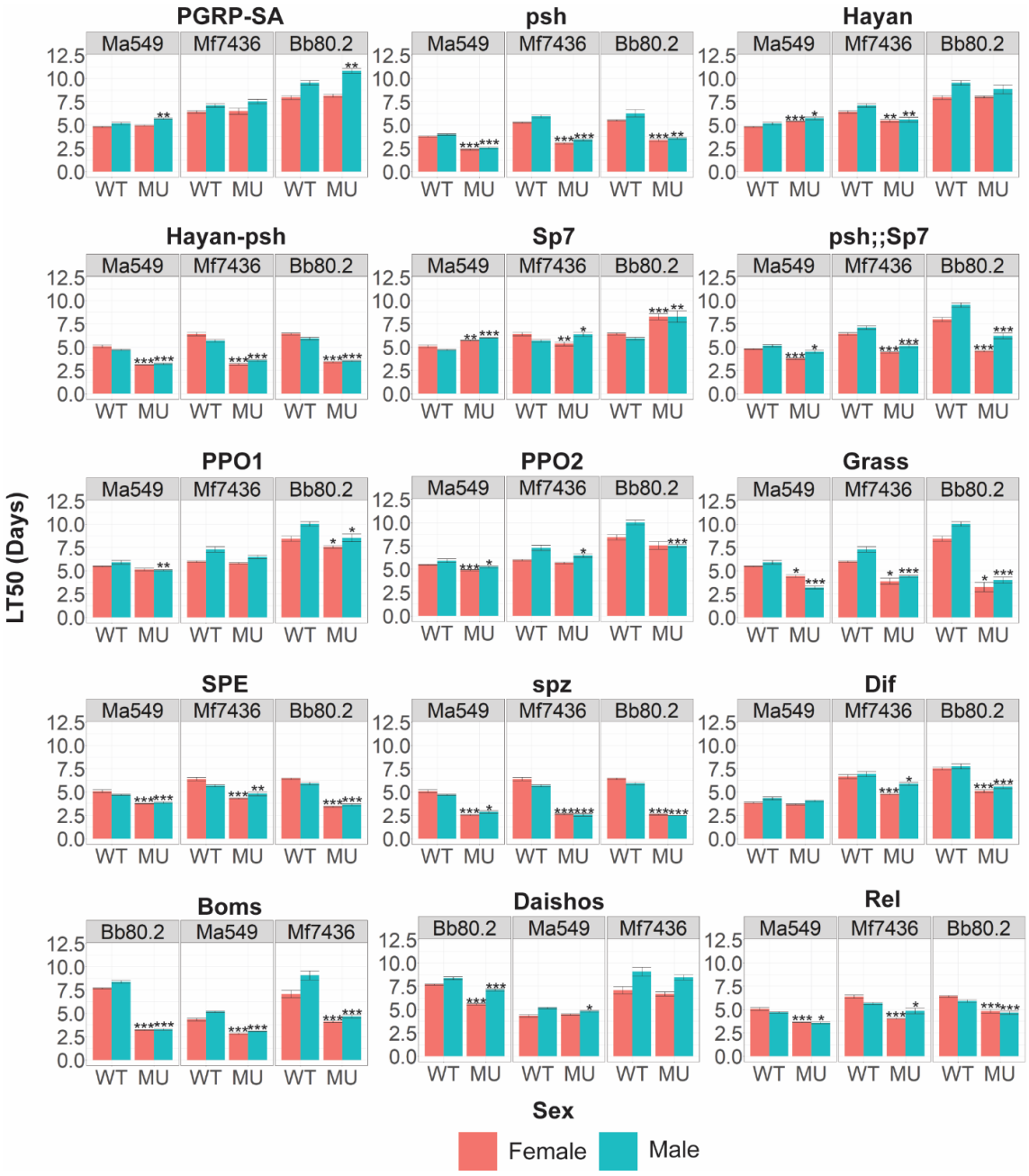


Fig 8. Quantifying the role individual components of the insect immune system play in resisting infection. Survival measured as LT_{50}'s of fly lines disrupted in known immune genes and their isogenic WT backgrounds against *M. anisopliae* (Ma549), *M. frigidum* (Mf7436) and *B. bassiana* (Bb80.2). Shown is the combination of three independent experiments for each pathogen with ~20 flies per genotype per experiment. Significance was evaluated using t-tests and is shown relative to the WT (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

Strain	Germination Rates							
	Water		Water + Drs		Yeast extract		Yeast extract + Drs	
	16 hrs	24 hrs	16 hrs	24 hrs	16 hrs	24 hrs	16 hrs	24 hrs
M. frigidum 7436	3.2±1.3%	15±1.7%	30±8.6%	42±1.2%	91±3.8%	<100%	91±3.9%	<100%
M. anisopliae 2105	8±2.8%	14±4%	51±3.4%	63±4.9%	100%	100%	100%	100%
M. anisopliae 549	0%	<1%	13±3.1%	18±0.9%	100%	100%	100%	100%
M. acridium 324	<1%	<1%	1.4±1.2%	6±0.5%	47±5.4%	75±1.3%	34±7.8%	65±7.7%
N. crassa	51±6.1%	52±11.8%	0%	0%	60±5.9%	60±16%	0%	0%

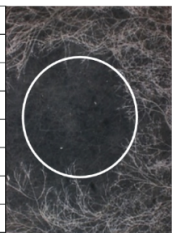


Fig 9. The effect of Drosomycin on *Metarhizium.* (A) Germination percentages of spores of *Metarhizium* spp in water, yeast extract and water or yeast extract plus or minus drosomycin. (B) *N. crassa* on agar showing growth inhibition by 0.01µg drosomycin applied to center of plate.

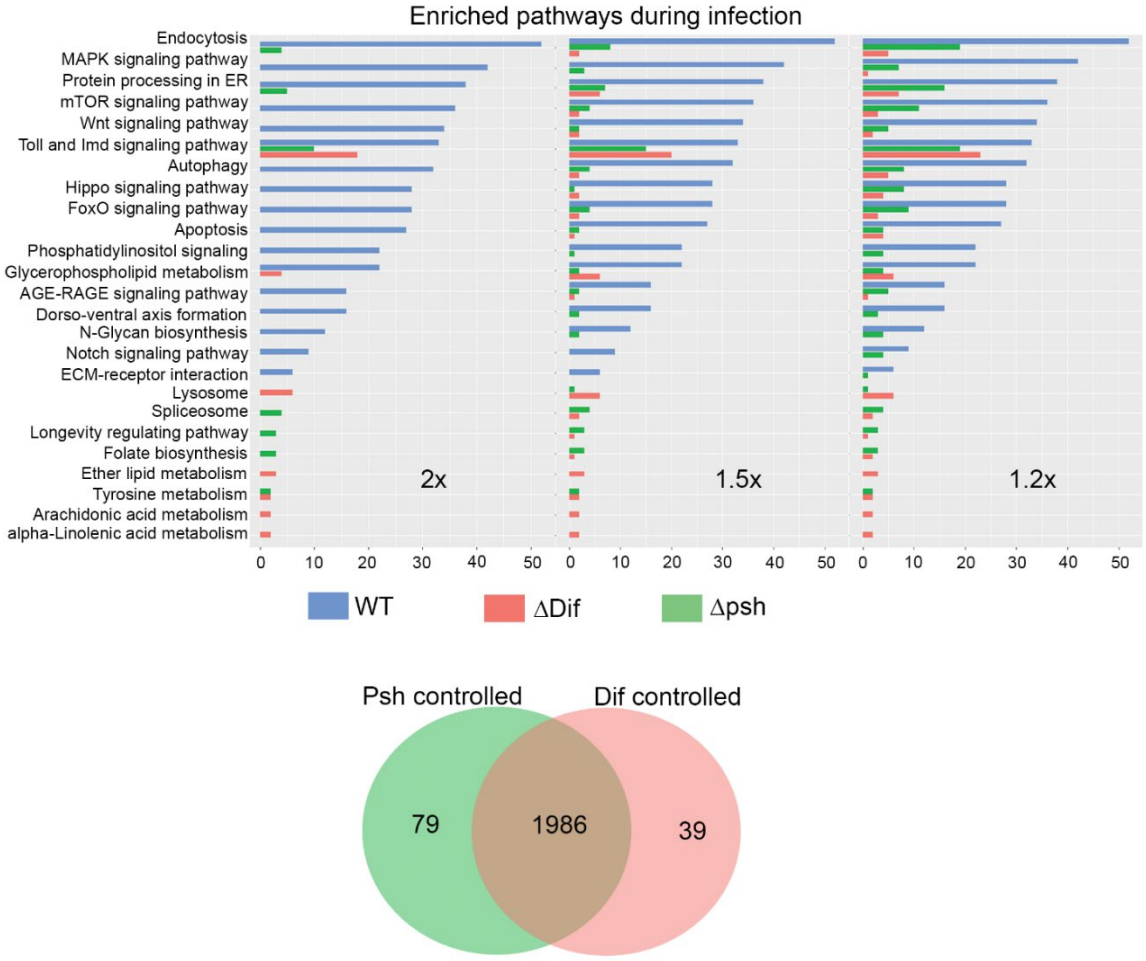


Fig 10. Genes involved in pathways upregulated during infection for WT, *psh*$^{\Delta}$ and *dif*^{1} mutants. The three graphs represent the number of significant genes for different levels of upregulation (Left: >2-fold upregulated, Middle: >1.5-fold upregulated, Right: > 1.2-fold upregulated). Controls are combined. **(Bottom)** A Venn diagram representing the number of genes controlled by Dif or psh from Table S3.

Species and ARSEF ID	Group	Isolated From	LT50 ($1x10^6$ spores/mL)	LT50 ($1x10^5$ spores/mL)	LT50 ($1x10^4$ spores/mL)	Day 5 LC50	Immobilization time in hrs (mean+SEM)	Emergent period in hrs (mean+SEM)	Latent period in hrs (mean+SEM)	Sporulation capacity (mean+SEM)
M. anisopliae Ma2105	A	*Hydrellia* sp. [Diptera: Ephydridae]	3.14±0.16	4.11±0.02	4.98±0.14	1.11x10^4	7.40±0.52	42.20±0.95	70.60±0.80	6.88±0.66 x10^6
M. anisopliae Ma7427	A	unknown species [Diptera: Stratiomyidae]	3.41±0.05	4.24±0.04	5.40±0.11	1.73x10^4	7.20±0.44	25.20±0.49	55.00±2.00	4.18±0.65 x10^6
M. anisopliae Ma549	A	[Homopteran: Cercopidae]	3.57±0.09	4.18±0.03	5.16±0.02	1.28x10^4	7.20±0.44	47.60±0.87	74.60±0.99	6.95±0.59 x10^6
M. robertsii Mr2575	A	*Curculio caryae* [Coleoptera: Curculionidae]	2.92±0.01	4.05±0.07	5.14±0.10	1.52x10^4	7.60±0.50	59.33±2.13	76.20±1.31	1.13±0.15 x10^5
M. robertsii Mr1046	D	*Popillia japonica* [Coleoptera: Scarabaeidae]	3.13±0.06	3.92±0.05	4.82±0.04	1.27x10^4	7.80±0.55	29.10±1.97	57.70±0.30	1.93±0.18 x10^6
M. brunneum Mb346	B	*Aphodius tasmaniae* [Coleoptera: Scarabaeidae]	4.19±0.09	4.75±0.08	6.04±0.28	8.93x10^4	7.80±0.55	26.00±1.57	55.90±1.66	3.83±0.53 x10^6
M. frigidum Mf4124	A	*Adoryphorus* sp. [Coleoptera: Scarabaeidae]	5.26±0.32	7.46±0.11	9.57±0.33	1.78x10^6	7.60±0.65	34.00±1.87	62.50±1.44	2.22±0.23 x10^6
M. frigidum Mf7436	A	*Coptotermes lacteus* [Isoptera: Rhinotermitidae]	5.39±0.15	7.21±0.09	8.42±0.18	2.23x10^6	7.20±0.61	42.90±1.39	66.60±2.15	2.23±0.47 x10^6
M. pingshaense Mp538	B	*Oryctes rhinoceros* [Coleoptera: Scarabaeidae]	4.23±0.05	4.93±0.11	5.83±0.18	1.07x10^5	7.40±0.52	28.40±1.66	59.20±1.58	2.73±0.20 x10^6
M. pingshaense Mp2162	D	Unknown species [Coleoptera: Scarabaeidae]	3.47±0.06	4.22±0.03	6.26±0.22	4.49x10^4	7.60±0.58	26.00±0.89	47.60±0.43	2.00±0.19 x10^6
M. pingshaense Mp443	D	*Teleogryllus commodus* [Orthoptera: Gryllidae]	9.02±0.17	>12	>12	3.78x10^7	23.80±2.79	55.33±2.67	108.00±4.68	3.38±0.96 x10^5
M. majus Mm 297	D	*Xyloryctes jamaicensis* [Coleoptera: Scarabaeidae]	4.66±0.02	6.74±0.51	9.13±0.21	6.81x10^5	30.00±8.00	58.60±1.97	81.70±1.98	3.18±0.63 x10^5
M. majus Mm1946	D	*Oryctes rhinoceros* [Coleoptera: Scarabaeidae]	4.98±0.22	5.31±0.09	6.98±0.24	2.22x10^6	22.00±6.93	54.00±1.58	71.90±1.52	6.00±1.70 x10^5
M. acridum Mac5736	B	*Locusta migratoria capito* [Orthoptera: Acrididae]	5.72±0.05	7.12±0.17	8.48±0.25	3.58x10^6	19.60±1.51	78±7.9	109.8±11.73	1.63±0.12 x10^6
M. acridum Mac324	B	*Austracris guttulosa* [Orthoptera: Acrididae]	5.67±0.08	8.28±0.35	10.01±0.28	4.70x10^6	21.20±0.90	73.8±11.63	109.2±11.41	2.23±0.43 x10^5
M. album Ma1941	D	*Nephotettix virescens* [Homoptera: Cicadellidae]	10.67±0.29	>12	>12	4.64x10^7	NA	NA	NA	NA

Table 1: *Metarhizium* spp. phenotypes

Strain Mp443		100% RH	96% RH	80% RH
Hours post infection	Location	Germination rates (%) (mean±SEM)	Germination rates (%) (mean±SEM)	Germination rates (%) (mean±SEM)
16 hr	Ventral abdomen	59.9±8.06	0	0
	Dorsal abdomen intersegmental	75.1±4.35	70.9±2.76	19.6±7.12
	Dorsal abdomen segments	66.8±3.56	0	0
24 hr	Ventral abdomen	85.2±5.93	0	0
	Dorsal abdomen intersegmental	88.1±2.83	68.7±6.07	17.4±1.81
	Dorsal abdomen segments	68.6±5.12	0	0
48 hr	Ventral abdomen	62.1±0.30	4.4±1.90	0
	Dorsal abdomen intersegmental	overgrown	84.2±3.14	81.1±3.85
	Dorsal abdomen segments	50.8±10.77	0	0

Strain Mr2105		100% RH	96% RH	80% RH
Hours post infection	Location	Germination rates (%) (mean±SEM)	Germination rates (mean±SEM)	Germination rates (mean±SEM)
16 hr	Ventral abdomen	65.3±7.88	0	0
	Dorsal abdomen intersegmental	90.9±1.17	76.3±4.07	29.2±4.19
	Dorsal abdomen segments	62.4±3.61	0	0
24 hr	Ventral abdomen	81.3±5.20	0.3±0.33	0
	Dorsal abdomen intersegmental	91.7±2.91	87.3±6.92	35.7±8.63
	Dorsal abdomen segments	66.9±12.05	3.7±1.97	0
48 hr	Ventral abdomen	overgrown	13.3±7.62	2.5±2.50
	Dorsal abdomen intersegmental	overgrown	overgrown	overgrown
	Dorsal abdomen segments	overgrown	9.7±9.74	0

Table 2: The germination rates of two *Metarhizium* strains on *Drosophila melanogaster*

		Ma549		Mf7436		Bb80.2		Control for:
Line	Source	Male	Female	Male	Female	Male	Female	
w[1118]^{6326}	Bloomington Stock center (BDSC)	3.98±0.06	3.77±0.05	5.95±0.13	5.26±0.05	6.26±0.39	5.49±0.07	psh
w[1118]^{DrosDel}	Originally from BDSC (donated by Bruno Lemaitre)	4.70±0.07	5.09±0.16	5.67±0.14	6.39±0.17	5.93±0.14	6.43±0.09	SP7, SPE, Rel, spz, Hayan-psh
w[1118]^{VDRC}	Originally from Vienna Stock Center (donated by Bruno Lemaitre)	5.90±0.20	5.47±0.05	7.29+0.29	6.41+0.13	10.02±0.25	8.42±0.28	Grass, PPO1, PPO2

Table S1: Differing susceptibilities of W1118 wild-type controls. LT50s and SE are given separately for male and female flies infected with three different fungi.

(See attached)

Table S2: Gene ontology biological process categories comparing infected flies to non-infected flies for the four lines used in the microarray study.

(See attached)

Table S3: Volcano plot filtered genes controlled by psh or Dif.

Conclusion

This dissertation provides a foundation for the *Drosophila-Metarhizium* host-pathogen interactions system. Conservation of many processes between *Drosophila* and other animals suggests that my research may be generally useful in understanding the genetic basis of disease resistance. In addition, the information herein may be useful for developing *Metarhiizum* spp. as biocontrol agents.

In the first two chapters, based on the DGRP, we found there is there is significant variation in resistance to *Metarhiziuim anisopliae* (Ma549) [LT_{50}: 3.5-7 days], *Pseudomonas aeruginosa* (Pa14) [LT_{50}: 1-9 days], and *Entomophthora muscae* (EMB) [PS_5: 1.6-95%]. Variation is the fuel for natural selection to act upon and therefore directional selection towards resistance to applied biocontrol agents can be expected. The three pathogens have very different strategies of infection and yet resistance in the DGRP to these pathogens was correlated. Ma549 is a generalist entomopathogenic fungus that infects via the cuticle and colonizes the insect using blastospores eventually depleting the resources of the host. Pa14 is virulent gram-negative bacteria isolate originally from a human patient and must infect *per os*. In *C. elegans,* Pa14 can kill via toxins such as pycoanin (fast) and colonization (slow) kill methods; this is mediated by richness of the growth media [92]. In our study, we used bacteria cultured on rich media (blood heart infusion broth). EMB is a specific fly pathogen with a very high level of co-evolved adaptation to the host that includes modifying the behavior of the insect. Doomed flies exhibit summit disease prior to sunset on the final day of their life [93]. It appears therefore that *Drosophila* possesses a generalized resistance to very different types of

pathogens. This complicates the paradigm of using multiple pathogens to control insect populations in the hope that this will delay evolution of resistance. Or perhaps more positively, beneficial insects such as silkworms and bees can be developed (bred or engineered) that have resistance to a wide swath of pathogens. The network of correlations between disease resistance, sleep indices, starvation resistance, negative geotaxis, and oxidative stress resistance associated with disease resistance links together and implicates different biological phenomena in disease resistance. The correlations between many different processes and disease resistance, and the many candidate genes with pleiotropic effects suggest that resistance differences between individuals is due to genes of multiple effects involved in a variety of processes.

In our GWAS, we found that polymorphisms most strongly associated with resistance are rare. If there was no cost to these alleles, we would expect them to sweep through the population given the importance of entomopathogenic fungi and disease in insect populations. Their scarcity points to a yet to be determined trade-off. This trade-off can be a general one across all fly lines or alternatively vary on a line by line basis. The pathogenesis of *Metarhizium* is informative. The variation in how quickly flies of the same genotype succumb to infection in the same environment (micro-environmental plasticity) and the nature of the fly's response to infection (variation in tolerance and resistance) affect the disease dynamics of the host-pathogen system. Combined with the fact that sporulation doesn't vary significantly across fly lines, we can develop ideas about how individual genotypes affect epidemiology. Thus, for example, tolerant, highly resistant, and highly plastic lines may allow more opportunity for dispersal.

The final chapter investigates how *Drosophila melanogaster* responds to *Metarhizium* spp. We found specialists strains of *Metarhizium* were delayed in various life history traits and were less reproductively fit. The lower fitness and the potential for knock-out of genes previously demonstrated by the St. Leger lab to be involved in sustainability, such as *Mad*2 or *Hsp25*, can form the basis of a containment strategy limiting the environmental impact of specialist strains used in biocontrol [94–96]. The GWAS in chapter one and a previous mutant library study showed alleles associated with resistance did not include components of the Toll pathway [36]. We found that more virulent *Metarhizium* strains induced a stronger and earlier Toll immune response and pathogenesis proceeded without apparent suppression of the Toll response. We assayed mutants defective in components of the Toll response to clarify and determine their effect, if any, on *Metarhizium* resistance. Disrupting *Dif* only increased susceptibility to *M. frigidum* (and *B. bassiana*) whereas flies disrupted in the pathogen protease sensor Persephone succumbed quickly to all *Metarhizium* tested. Microarray analysis revealed that these mutants had a suppressed transcriptomic response to infection in many different pathways. This suggests that most Ma549 responsive genes are completely or partially dependent on *psh* and *Dif.*

Studies of these two systems began more than 100 years ago, with Elie Metchnikoff's proposal to use *Metarhizum anisopliae* to control the wheat cockchafer in 1879 and Thomas Hunt Morgan's patient wait for his famous white-eyed mutant fly. Their combination has proved fruitful. Collectively these studies advance our knowledge of insect-*Metarhizium* interactions, and in doing so pave the way forward for better

understanding of the use of *Metarhizium* as a biocontrol agent and of how individuals respond to disease.

References

1. Coetzee M. How important are dipteran vectors of disease in Africa? Transactions of the Royal Society of Tropical Medicine and Hygiene. 2014. doi:10.1093/trstmh/tru020
2. Snyder T. The Mosquito: A Human History of Our Deadliest Predator. Emerg Infect Dis. 2020. doi:10.3201/eid2610.202806
3. Van Der Meijden E. Herbivorous insects—a threat for crop production. Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture. 2015. doi:10.1007/978-3-319-08575-3_12
4. Goettel MS, Eilenberg J, Glare T. Entomopathogenic Fungi and their Role in Regulation of Insect Populations. Comprehensive Molecular Insect Science. 2005. doi:10.1016/B0-44-451924-6/00088-0
5. Lord JC. From Metchnikoff to Monsanto and beyond: The path of microbial control. Journal of Invertebrate Pathology. 2005. doi:10.1016/j.jip.2005.04.006
6. Iwanicki NS, Pereira AA, Botelho ABRZ, Rezende JM, Moral R de A, Zucchi MI, et al. Monitoring of the field application of Metarhizium anisopliae in Brazil revealed high molecular diversity of Metarhizium spp in insects, soil and sugarcane roots. Sci Rep. 2019. doi:10.1038/s41598-019-38594-8
7. Lovett B, Bilgo E, Millogo SA, Ouattarra AK, Sare I, Gnambani EJ, et al. Transgenic Metarhizium rapidly kills mosquitoes in a malaria-endemic region of Burkina Faso. Science (80-). 2019. doi:10.1126/science.aaw8737
8. Wang J, Lovett B, St. Leger RJ. The secretome and chemistry of Metarhizium; a genus of entomopathogenic fungi. Fungal Ecol. 2019;38. doi:10.1016/j.funeco.2018.04.001
9. Lu H-L, St. Leger RJ. Insect Immunity to Entomopathogenic Fungi. In: Lovett B, St. Leger RJ, editors. Advances in Genetics. 2016. doi:10.1016/bs.adgen.2015.11.002
10. St. Leger RJ, Wang JB. Metarhizium : jack of all trades , master of many. Open Biol. 2020;10.
11. Imler J-L, Zheng L. Biology of Toll receptors: lessons from insects and mammals. J Leukoc Biol. 2004. doi:10.1189/jlb.0403160
12. Janeway CA. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor Symposia on Quantitative Biology. 1989. doi:10.1101/sqb.1989.054.01.003
13. Valanne S, Wang J-H, Ramet M, Rämet M. The Drosophila Toll Signaling Pathway. J Immunol. 2011. doi:10.4049/jimmunol.1002302
14. Kasai Y, Cagan R. Drosophila as a tool for personalized medicine: A primer. Personalized Medicine. 2010. doi:10.2217/pme.10.65
15. Mirzoyan Z, Sollazzo M, Allocca M, Valenza AM, Grifoni D, Bellosta P. Drosophila melanogaster: A model organism to study cancer. Frontiers in Genetics. 2019. doi:10.3389/fgene.2019.00051
16. Graham P, Pick L. Drosophila as a Model for Diabetes and Diseases of Insulin Resistance. Current Topics in Developmental Biology. 2017. doi:10.1016/bs.ctdb.2016.07.011

17. Fang W, Azimzadeh P, St. Leger RJ. Strain improvement of fungal insecticides for controlling insect pests and vector-borne diseases. Current Opinion in Microbiology. 2012. pp. 232–238. doi:10.1016/j.mib.2011.12.012
18. Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, et al. Emerging fungal threats to animal, plant and ecosystem health. Nature. 2012. pp. 186–194. doi:10.1038/nature10947
19. Buchon N, Silverman N, Cherry S. Immunity in Drosophila melanogaster-from microbial recognition to whole-organism physiology. Nature Reviews Immunology. 2014. pp. 796–810. doi:10.1038/nri3763
20. Roberts DW, St. Leger RJ. Metarhizium spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. Adv Appl Microbiol. 2004;54: 1–70. doi:10.1016/S0065-2164(04)54001-7
21. Toledo J, Liedo P, Flores S. Use of Beauveria bassiana and Metarhizium anisopliae for fruit fly control: a novel approach. Proceedings of the 7th International Symposium on Fruit Flies of Economic Importance. 2006.
22. Wang JB, St. Leger RJ, Wang C. Advances in genomics of insect pathogenic fungi. Lovett B, St. Leger RJ, editors. Adv Genet. 2016;94: 67–105. doi:10.1016/bs.adgen.2016.01.002
23. Lu H-L, Wang JB, Brown MA, Euerle C, St. Leger RJ. Identification of Drosophila Mutants Affecting Defense to an Entomopathogenic Fungus. Sci Rep. 2015;5: 12350. doi:10.1038/srep12350
24. Harbison ST, McCoy LJ, Mackay TFC. Genome-wide association study of sleep in Drosophila melanogaster. BMC Genomics. 2013. doi:10.1186/1471-2164-14-281
25. Huang W, Massouras A, Inoue Y, Peiffer J, Rámia M, Tarone A, et al. Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines. Genome Res. 2014; gr.171546.113. doi:10.1101/gr.171546.113
26. Mackay TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, et al. The Drosophila melanogaster Genetic Reference Panel. Nature. 2012;482: 173–178. doi:10.1038/nature10811
27. Mulcahy H, Sibley CD, Surette MG, Lewenza S. Drosophila melanogaster as an animal model for the study of Pseudomonas aeruginosa biofilm infections in Vivo. PLoS Pathog. 2011. doi:10.1371/journal.ppat.1002299
28. Aliaga L, Mediavilla JD, Cobo F. A clinical index predicting mortality with Pseudomonas aeruginosa bacteraemia. J Med Microbiol. 2002. doi:10.1099/0022-1317-51-7-615
29. Jordan KW, Craver KL, Magwire MM, Cubilla CE, Mackay TFC, Anholt RRH. Genome-wide association for sensitivity to chronic oxidative stress in Drosophila melanogaster. PLoS One. 2012. doi:10.1371/journal.pone.0038722
30. Ayroles JF, Carbone MA, Stone EA, Jordan KW, Lyman RF, Magwire MM, et al. Systems genetics of complex traits in Drosophila melanogaster. Nat Genet. 2009;41: 299–307. doi:10.1038/ng.332
31. Unckless RL, Rottschaefer SM, Lazzaro BP. A Genome-Wide Association Study for Nutritional Indices in Drosophila. G3 Genes|Genomes|Genetics. 2015; g3.114.016477. doi:10.1534/g3.114.016477

32. Magwire MM, Fabian DK, Schweyen H, Cao C, Longdon B, Bayer F, et al. Genome-Wide Association Studies Reveal a Simple Genetic Basis of Resistance to Naturally Coevolving Viruses in Drosophila melanogaster. PLoS Genet. 2012. doi:10.1371/journal.pgen.1003057
33. Werren JH, Baldo L, Clark ME. Wolbachia: Master manipulators of invertebrate biology. Nature Reviews Microbiology. 2008. doi:10.1038/nrmicro1969
34. Panteleev DI, Goriacheva II, Andrianov B V., Reznik NL, Lazebnyĭ OE, Kulikov AM. The endosymbiotic bacterium Wolbachia enhances the nonspecific resistance to insect pathogens and alters behavior of Drosophila melanogaster. Genetika. 2007.
35. Ivanov DK, Escott-Price V, Ziehm M, Magwire MM, Mackay TFC, Partridge L, et al. Longevity GWAS using the drosophila genetic reference panel. Journals Gerontol - Ser A Biol Sci Med Sci. 2015. doi:10.1093/gerona/glv047
36. Durham MF, Magwire MM, Stone EA, Leips J. Genome-wide analysis in Drosophila reveals age-specific effects of SNPs on fitness traits. Nat Commun. 2014;5. doi:10.1038/ncomms5338
37. Shorter J, Couch C, Huang W, Carbone MA, Peiffer J, Anholt RH, et al. Genetic architecture of natural variation in Drosophila melanogaster aggressive behavior. Proc Natl Acad Sci U S A. 2015. doi:10.1073/pnas.1510104112
38. Chow CY, Wolfner MF, Clark AG. Using natural variation in Drosophila to discover previously unknown endoplasmic reticulum stress genes. Proc Natl Acad Sci U S A. 2013. doi:10.1073/pnas.1307125110
39. Gaertner BE, Ruedi EA, McCoy LJ, Moore JM, Wolfner MF, Mackay TFC. Heritable variation in courtship patterns in drosophila melanogaster. G3 Genes, Genomes, Genet. 2015. doi:10.1534/g3.114.014811
40. Weber AL, Khan GF, Magwire MM, Tabor CL, Mackay TFC, Anholt RH. Genome-wide association analysis of oxidative stress resistance in drosophila melanogaster. PLoS One. 2012;7: e34745. doi:10.1371/journal.pone.0034745
41. Unckless RL, Rottschaefer SM, Lazzaro BP. The Complex Contributions of Genetics and Nutrition to Immunity in Drosophila melanogaster. PLoS Genet. 2015. doi:10.1371/journal.pgen.1005030
42. Stone EF, Fulton BO, Ayres JS, Pham LN, Ziauddin J, Shirasu-Hiza MM. The Circadian Clock Protein Timeless Regulates Phagocytosis of Bacteria in Drosophila. Wessels MR, editor. PLoS Pathog. 2012;8: e1002445. doi:10.1371/journal.ppat.1002445
43. Bou Sleiman MS, Osman D, Massouras A, Hoffmann AA, Lemaitre B, Deplancke B. Genetic, molecular and physiological basis of variation in Drosophila gut immunocompetence. Nat Commun. 2015. doi:10.1038/ncomms8829
44. Vodovar N, Vallenet D, Cruveiller S, Rouy Z, Barbe V, Acosta C, et al. Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium Pseudomonas entomophila. Nat Biotechnol. 2006. doi:10.1038/nbt1212
45. Berman J, Sudbery PE. Candida albicans: A molecular revolution built on lessons from budding yeast. Nature Reviews Genetics. 2002. doi:10.1038/nrg948
46. Morgante F, Sørensen P, Sorensen DA, Maltecca C, Mackay TFC, Falconer DS, et al. Genetic Architecture of Micro-Environmental Plasticity in Drosophila

melanogaster. Sci Rep. 2015;5: 9785. doi:10.1038/srep09785
47. Hill WG, Mulder HA. Genetic analysis of environmental variation. Genet Res (Camb). 2010. doi:10.1017/S0016672310000546
48. Swarup S, Harbison ST, Hahn LE, Morozova T V., Yamamoto A, MacKay TFC, et al. Extensive epistasis for olfactory behaviour, sleep and waking activity in Drosophila melanogaster. Genet Res (Camb). 2012. doi:10.1017/S001667231200002X
49. Alexander RP, Fang G, Rozowsky J, Snyder M, Gerstein MB. Annotating non-coding regions of the genome. Nature Reviews Genetics. 2010. doi:10.1038/nrg2814
50. Thimgan MS, Seugnet L, Turk J, Shaw PJ. Identification of genes associated with resilience/vulnerability to sleep deprivation and starvation in Drosophila. Sleep. 2015. doi:10.5665/sleep.4680
51. Nakayama M, Suzuki E, Tsunoda SI, Hama C. The matrix proteins hasp and hig exhibit segregated distribution within synaptic clefts and play distinct roles in synaptogenesis. J Neurosci. 2016. doi:10.1523/JNEUROSCI.2300-15.2016
52. Hut RA, Van der Zee EA. The cholinergic system, circadian rhythmicity, and time memory. Behavioural Brain Research. 2011. doi:10.1016/j.bbr.2010.11.039
53. Qiu L, Yu H, Liang F. Multiple C2 domains transmembrane protein 1 is expressed in CNS neurons and possibly regulates cellular vesicle retrieval and oxidative stress. J Neurochem. 2015. doi:10.1111/jnc.13251
54. Foley E, O'Farrell PH. Functional dissection of an innate immune response by a genome-wide RNAi screen. PLoS Biol. 2004. doi:10.1371/journal.pbio.0020203
55. Engel E, Viargues P, Mortier M, Taillebourg E, Couté Y, Thevenon D, et al. Identifying USPs regulating immune signals in Drosophila: USP2 deubiquitinates Imd and promotes its degradation by interacting with the proteasome. Cell Commun Signal. 2014. doi:10.1186/s12964-014-0041-2
56. Williams MJ. The Drosophila cell adhesion molecule Neuroglian regulates Lissencephaly-1 localisation in circulating immunosurveillance cells. BMC Immunol. 2009. doi:10.1186/1471-2172-10-17
57. Prakash S, McLendon HM, Dubreuil CI, Ghose A, Hwa J, Dennehy KA, et al. Complex interactions amongst N-cadherin, DLAR, and Liprin-α regulate Drosophila photoreceptor axon targeting. Dev Biol. 2009. doi:10.1016/j.ydbio.2009.09.016
58. Kucherenko MM, Marrone AK, Rishko VM, Magliarelli H de F, Shcherbata HR. Stress and muscular dystrophy: A genetic screen for Dystroglycan and Dystrophin interactors in Drosophila identifies cellular stress response components. Dev Biol. 2011. doi:10.1016/j.ydbio.2011.01.013
59. Chanut-Delalande H, Fernandes I, Roch F, Payre F, Plaza S. Shavenbaby couples patterning to epidermal cell shape control. PLoS Biol. 2006. doi:10.1371/journal.pbio.0040290
60. Lewellyn L, Cetera M, Horne-Badovinac S. Misshapen decreases integrin levels to promote epithelial motility and planar polarity in Drosophila. J Cell Biol. 2013. doi:10.1083/jcb.201209129
61. Weber U, Gault WJ, Olguin P, Serysheva E, Mlodzik M. Novel regulators of

planar cell polarity: A genetic analysis in Drosophila. Genetics. 2012. doi:10.1534/genetics.111.137190
62. Torres-Vazquez J, Warrior R, Arora K. Schnurri is required for Dpp-dependent patterning of the Drosophila wing. Dev Biol. 2000. doi:10.1006/dbio.2000.9900
63. Iyer EPR, Iyer SC, Sullivan L, Wang D, Meduri R, Graybeal LL, et al. Functional Genomic Analyses of Two Morphologically Distinct Classes of Drosophila Sensory Neurons: Post-Mitotic Roles of Transcription Factors in Dendritic Patterning. PLoS One. 2013. doi:10.1371/journal.pone.0072434
64. Figeac N, Jagla T, Aradhya R, Da Ponte JP, Jagla K. Drosophila adult muscle precursors form a network of interconnected cells and are specified by the rhomboidtriggered EGF pathway. Development. 2010. doi:10.1242/dev.049080
65. Lebestky T, Jung SH, Banerjee U. A Serrate-expressing signaling center controls Drosophila hematopoiesis. Genes Dev. 2003. doi:10.1101/gad.1052803
66. König A, Yatsenko AS, Weiss M, Shcherbata HR. Ecdysteroids affect Drosophila ovarian stem cell niche formation and early germline differentiation. EMBO J. 2011. doi:10.1038/emboj.2011.73
67. Wang B, Moya N, Niessen S, Hoover H, Mihaylova MM, Shaw RJ, et al. A hormone-dependent module regulating energy balance. Cell. 2011. doi:10.1016/j.cell.2011.04.013
68. Ugrankar R, Berglund E, Akdemir F, Tran C, Kim MS, Noh J, et al. Drosophila glucome screening identifies Ck1alpha as a regulator of mammalian glucose metabolism. Nat Commun. 2015. doi:10.1038/ncomms8102
69. Tan KL, Vlisidou I, Wood W. Ecdysone mediates the development of immunity in the drosophila embryo. Curr Biol. 2014. doi:10.1016/j.cub.2014.03.062
70. Flatt T, Heyland A, Rus F, Porpiglia E, Sherlock C, Yamamoto R, et al. Hormonal regulation of the humoral innate immune response in Drosophila melanogaster. J Exp Biol. 2008. doi:10.1242/jeb.014878
71. Flatt T, Kawecki TJ. Juvenile hormone as a regulator of the trade-off between reproduction and life span in Drosophila melanogaster. Evolution (N Y). 2007. doi:10.1111/j.1558-5646.2007.00151.x
72. Chung BY, Kilman VL, Keath JR, Pitman JL, Allada R. The GABAA Receptor RDL Acts in Peptidergic PDF Neurons to Promote Sleep in Drosophila. Curr Biol. 2009. doi:10.1016/j.cub.2009.01.040
73. Haynes PR, Christmann BL, Griffith LC. A single pair of neurons links sleep to memory consolidation in Drosophila melanogaster. Elife. 2015. doi:10.7554/eLife.03868
74. Yoshie S, Imai A, Nashida T, Shimomura H. Expression, characterization, and localization of Rab26, a low molecular weight GTP-binding protein, in the rat parotid gland. Histochem Cell Biol. 2000. doi:10.1007/s004180000130
75. Nose A, Mahajan VB, Goodman CS. Connectin: A homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in Drosophila. Cell. 1992. doi:10.1016/0092-8674(92)90426-D
76. Watson FL, Püttmann-Holgado R, Thomas F, Lamar DL, Hughes M, Kondo M, et al. Immunology: Extensive diversity of Ig-superfamily proteins in the immune system of insects. Science (80-). 2005. doi:10.1126/science.1116887

77. Berger J, Senti KA, Senti G, Newsome TP, Åsling B, Dickson BJ, et al. Systematic identification of genes that regulate neuronal wiring in the Drosophila visual system. PLoS Genet. 2008. doi:10.1371/journal.pgen.1000085
78. Avet-Rochex A, Carvajal N, Christoforou CP, Yeung K, Maierbrugger KT, Hobbs C, et al. Unkempt Is Negatively Regulated by mTOR and Uncouples Neuronal Differentiation from Growth Control. PLoS Genet. 2014. doi:10.1371/journal.pgen.1004624
79. Dworkin I, Gibson G. Epidermal growth factor receptor and transforming growth factor-β signaling contributes to variation for wing shape in Drosophila melanogaster. Genetics. 2006. doi:10.1534/genetics.105.053868
80. Guzik-Lendrum S, Nagy A, Takagi Y, Houdusse A, Sellers JR. Drosophila melanogaster myosin-18 represents a highly divergent motor with actin tethering properties. J Biol Chem. 2011. doi:10.1074/jbc.M111.218669
81. Kronert WA, Edwards KA, Roche ES, Wells L, Bernstein SI. Muscle-specific accumulation of Drosophila myosin heavy chains: A splicing mutation in an alternative exon results in an isoform substitution. EMBO J. 1991. doi:10.1002/j.1460-2075.1991.tb07787.x
82. Pointud JC, Larsson J, Dastugue B, Couderc JL. The BTB/POZ domain of the regulatory proteins Bric à brac 1 (BAB1) and Bric à brac 2 (BAB2) interacts with the novel Drosophila TAFII factor BIP2/dTAFII155. Dev Biol. 2001. doi:10.1006/dbio.2001.0358
83. Aguilar-Fuentes J, Fregoso M, Herrera M, Reynaud E, Braun C, Egly JM, et al. p8/TTDA overexpression enhances UV-irradiation resistance and suppresses TFIIH mutations in a Drosophila trichothiodystrophy model. PLoS Genet. 2008. doi:10.1371/journal.pgen.1000253
84. Li X, Urwyler O, Suter B. Drosophila Xpd regulates Cdk7 localization, mitotic kinase activity, spindle dynamics, and chromosome segregation. PLoS Genet. 2010. doi:10.1371/journal.pgen.1000876
85. Grant J, Saldanha JW, Gould AP. A Drosophila model for primary coenzyme Q deficiency and dietary rescue in the developing nervous system. DMM Dis Model Mech. 2010. doi:10.1242/dmm.005579
86. Claridge-Chang A, Wijnen H, Naef F, Boothroyd C, Rajewsky N, Young MW. Circadian regulation of gene expression systems in the Drosophila head. Neuron. 2001. doi:10.1016/S0896-6273(01)00515-3
87. Helfrich C. Role of the optic lobes in the regulation of the locomotor activity rhythm of drosophila melanogaster: Behavioral analysis of neural mutants. J Neurogenet. 1986. doi:10.3109/01677068609106857
88. Shi M, Yue Z, Kuryatov A, Lindstrom JM, Sehgal A. Identification of Redeye, a new sleepregulating protein whose expression is modulated by sleep amount. Elife. 2014. doi:10.7554/eLife.01473
89. Fox RM, Hanlon CD, Andrew DJ. The CrebA/Creb3-like transcription factors are major and direct regulators of secretory capacity. J Cell Biol. 2010. doi:10.1083/jcb.201004062
90. Shah N, Dorer DR, Moriyama EN, Christensen AC. Evolution of a large, conserved, and syntenic gene family in insects. G3 Genes, Genomes, Genet. 2012.

doi:10.1534/g3.111.001412
91. Katzemich A, Liao KA, Czerniecki S, Schöck F. Alp/Enigma Family Proteins Cooperate in Z-Disc Formation and Myofibril Assembly. PLoS Genet. 2013. doi:10.1371/journal.pgen.1003342
92. Manning AJ, Peters KA, Peifer M, Rogers SL. Regulation of epithelial morphogenesis by the gprotein-coupled receptor mist and its ligand fog. Sci Signal. 2013. doi:10.1126/scisignal.2004427
93. Zhang J, Liu M, Su Y, Du J, Zhu AJ. A targeted in vivo RNAi screen reveals deubiquitinases as new regulators of notch signaling. G3 Genes, Genomes, Genet. 2012. doi:10.1534/g3.112.003780
94. Loo LWM, Secombe J, Little JT, Carlos L-S, Yost C, Cheng P-F, et al. The Transcriptional Repressor dMnt Is a Regulator of Growth in Drosophila melanogaster. Mol Cell Biol. 2005. doi:10.1128/mcb.25.16.7078-7091.2005
95. Rao RP, Yuan C, Allegood JC, Rawat SS, Edwards MB, Wang X, et al. Ceramide transfer protein function is essential for normal oxidative stress response and lifespan. Proc Natl Acad Sci U S A. 2007. doi:10.1073/pnas.0705049104
96. Petsakou A, Sapsis TP, Blau J. Circadian Rhythms in Rho1 Activity Regulate Neuronal Plasticity and Network Hierarchy. Cell. 2015. doi:10.1016/j.cell.2015.07.010
97. Ryglewski S, Lance K, Levine RB, Duch C. Ca v2 channels mediate low and high voltage-activated calcium currents in Drosophila motoneurons. J Physiol. 2012. doi:10.1113/jphysiol.2011.222836
98. Singh N, Patra S. Phosphodiesterase 9: Insights from protein structure and role in therapeutics. Life Sciences. 2014. doi:10.1016/j.lfs.2014.04.007
99. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009. doi:10.1093/nar/gkn923
100. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009. doi:10.1038/nprot.2008.211
101. Gerke O, Høilund-Carlsen PF, Vach W. [Different meaning of the p-value in exploratory and confirmatory hypothesis testing]. Ugeskr Laeger. 2011.
102. Antonov A V., Schmidt EE, Dietmann S, Krestyaninova M, Hermjakob H. R spider: A network-based analysis of gene lists by combining signaling and metabolic pathways from Reactome and KEGG databases. Nucleic Acids Res. 2010. doi:10.1093/nar/gkq482
103. Becker T, Loch G, Beyer M, Zinke I, Aschenbrenner AC, Carrera P, et al. FOXO-dependent regulation of innate immune homeostasis. Nature. 2010. doi:10.1038/nature08698
104. Bauer M, Katzenberger JD, Hamm AC, Bonaus M, Zinke I, Jaekel J, et al. Purine and folate metabolism as a potential target of sex-specific nutrient allocation in Drosophila and its implication for lifespan-reproduction tradeoff. Physiol Genomics. 2006. doi:10.1152/physiolgenomics.00009.2006
105. Doeschl-Wilson AB, Bishop SC, Kyriazakis I, Villanueva B. Novel methods for quantifying individual host response to infectious pathogens for genetic analyses.

Front Genet. 2012;3. doi:10.3389/fgene.2012.00266
106. Ayres JS, Freitag N, Schneider DS. Identification of drosophila mutants altering defense of and endurance to Listeria monocytogenes infection. Genetics. 2008. doi:10.1534/genetics.107.083782
107. Howick VM, Lazzaro BP. Genotype and diet shape resistance and tolerance across distinct phases of bacterial infection. BMC Evol Biol. 2014. doi:10.1186/1471-2148-14-56
108. Guilbault C, Stotland P, Lachance C, Tam M, Keller A, Thompson-Snipes L, et al. Influence of gender and interleukin-10 deficiency on the inflammatory response during lung infection with Pseudomonas aeruginosa in mice. Immunology. 2002. doi:10.1046/j.1365-2567.2002.01508.x
109. Lange BW, Langley CH, Stephan W. Molecular evolution of Drosophila metallothionein genes. Genetics. 1990.
110. Bull JJ. Evolution of Phenotypic Variance. Evolution (N Y). 2006. doi:10.2307/2409140
111. Yáñez JM, Houston RD, Newman S. Genetics and genomics of disease resistance in salmonid species. Frontiers in Genetics. 2014. doi:10.3389/fgene.2014.00415
112. Burke CJ, Huetteroth W, Owald D, Perisse E, Krashes MJ, Das G, et al. Layered reward signalling through octopamine and dopamine in Drosophila. Nature. 2012. doi:10.1038/nature11614
113. Arya GH, Magwire MM, Huang W, Serrano-Negron YL, Mackay TFC, Anholt RRH. The genetic basis for variation in olfactory behavior in drosophila melanogaster. Chem Senses. 2015. doi:10.1093/chemse/bjv001
114. Cooke GS, Hill AVS. Genetics of susceptibility to human infectious disease. Nature Reviews Genetics. 2001. doi:10.1038/35103577
115. Hill AVS. Evolution, revolution and heresy in the genetics of infectious disease susceptibility. Philosophical Transactions of the Royal Society B: Biological Sciences. 2012. doi:10.1098/rstb.2011.0275
116. Chapman SJ, Hill AVS. Human genetic susceptibility to infectious disease. Nature Reviews Genetics. 2012. doi:10.1038/nrg3114
117. Pritchard JK. Are rare variants responsible for susceptibility to complex diseases? Am J Hum Genet. 2001. doi:10.1086/321272
118. Lazzaro BP, Sackton TB, Clark AG. Genetic variation in Drosophila melanogaster resistance to infection: A comparison across bacteria. Genetics. 2006;174: 1539–1554. doi:10.1534/genetics.105.054593
119. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É. Host Adaptation Is Contingent upon the Infection Route Taken by Pathogens. PLoS Pathog. 2013. doi:10.1371/journal.ppat.1003601
120. Fang W, St. Leger RJ. RNA binding proteins mediate the ability of a fungus to adapt to the cold. Environ Microbiol. 2010. doi:10.1111/j.1462-2920.2009.02127.x
121. Apidianakis Y, Rahme LG. Drosophila melanogaster as a model host for studying Pseudomonas aeruginosa infection. Nat Protoc. 2009;4: 1285–1294. doi:10.1038/nprot.2009.124
122. Wang C, St. Leger RJ. A collagenous protective coat enables Metarhizium anisopliae to evade insect immune responses. Proc Natl Acad Sci U S A.

2006;103: 6647–6652. doi:10.1073/pnas.0601951103
123. Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, Davis RW, et al. Profiling early infection responses: Pseudomonas aeruginosa eludes host defenses by suppressing antimicrobial peptide gene expression. Proc Natl Acad Sci. 2005. doi:10.1073/pnas.0409588102
124. Wallace MA, Bailey S, Fukuto JM, Valentine JS, Gralla EB. Induction of phenotypes resembling CuZn-superoxide dismutase deletion in wild-type yeast cells: An in vivo assay for the role of superoxide in the toxicity of redox-cycling compounds. Chem Res Toxicol. 2005. doi:10.1021/tx050050n
125. Krishnan N, Davis AJ, Giebultowicz JM. Circadian regulation of response to oxidative stress in Drosophila melanogaster. Biochem Biophys Res Commun. 2008. doi:10.1016/j.bbrc.2008.07.011
126. Scheiermann C, Kunisaki Y, Frenette PS. Circadian control of the immune system. Nature Reviews Immunology. 2013. doi:10.1038/nri3386
127. Liu J, Mankani G, Shi X, Meyer M, Cunningham-Runddles S, Ma X, et al. The circadian clock Period 2 gene regulates gamma interferon production of NK cells in host response to lipopolysaccharide-induced endotoxic shock. Infect Immun. 2006. doi:10.1128/IAI.00287-06
128. Zhong W, McClure CD, Evans CR, Mlynski DT, Immonen E, Ritchie MG, et al. Immune anticipation of mating in Drosophila: Turandot M promotes immunity against sexually transmitted fungal infections. Proc R Soc B Biol Sci. 2013;280: 20132018. doi:10.1098/rspb.2013.2018
129. Mitchell-Olds T, Bradley D. Genetics of Brassica rapa. 3. Costs of Disease Resistance to Three Fungal Pathogens. Evolution (N Y). 1996. doi:10.2307/2410743
130. Little TJ, Carius HJ, Sakwinska O, Ebert D. Competitiveness and life-history characteristics of Daphnia with respect to susceptibility to a bacterial pathogen. J Evol Biol. 2002. doi:10.1046/j.1420-9101.2002.00436.x
131. Felix TM, Hughes KA, Stone EA, Drnevich JM, Leips J. Age-specific variation in immune response in Drosophila melanogaster has a genetic basis. Genetics. 2012. doi:10.1534/genetics.112.140640
132. Overgaard J, Kristensen TN, Mitchell KA, Hoffmann AA. Thermal tolerance in widespread and tropical Drosophila species: Does phenotypic plasticity increase with latitude? Am Nat. 2011. doi:10.1086/661780
133. Levin BR, Antia R. Why we don't get sick: The within-host population dynamics of bacterial infections. Science. 2001. doi:10.1126/science.1058879
134. Corby-Harris V, Promislow DEL. Host ecology shapes geographical variation for resistance to bacterial infection in Drosophila melanogaster. J Anim Ecol. 2008. doi:10.1111/j.1365-2656.2008.01399.x
135. Tinsley MC, Blanford S, Jiggins FM. Genetic variation in Drosophila melanogaster pathogen susceptibility. Parasitology. 2006. doi:10.1017/S0031182006009929
136. Paparazzo F, Tellier A, Stephan W, Hutter S. Survival rate and transcriptional response upon infection with the generalist parasite Beauveria bassiana in a world-wide sample of Drosophila melanogaster. PLoS One. 2015;10: 1–20.

doi:10.1371/journal.pone.0132129
137. Kaltz O, Shykoff JA. Local adaptation in host–parasite systems. Heredity (Edinb). 1998. doi:10.1046/j.1365-2540.1998.00435.x
138. Fang W, Lu HL, King GF, St. Leger RJ. Construction of a hypervirulent and specific mycoinsecticide for locust control. Sci Rep. 2014. doi:10.1038/srep07345
139. Pava-Ripoll M, Posada FJ, Momen B, Wang C, St. Leger RJ. Increased pathogenicity against coffee berry borer, Hypothenemus hampei (Coleoptera: Curculionidae) by Metarhizium anisopliae expressing the scorpion toxin (AaIT) gene. J Invertebr Pathol. 2008. doi:10.1016/j.jip.2008.05.004
140. Fang W, Vega-Rodríguez J, Ghosh AK, Jacobs-Lorena M, Kang A, St. Leger RJ. Development of transgenic fungi that kill human malaria parasites in mosquitoes. Science (80-). 2011. doi:10.1126/science.1199115
141. Giannakou ME, Goss M, Jünger MA, Hafen E, Leevers SJ, Partridge L. Long-lived Drosophila with over-expressed dFOXO in adult fat body. Science (80-). 2004. doi:10.1126/science.1098219
142. Lutter EI, Faria MMP, Rabin HR, Storey DG. Pseudomonas aeruginosa Cystic Fibrosis Isolates from Individual Patients Demonstrate a Range of Levels of Lethality in Two Drosophila melanogaster Infection Models. Infect Immun. 2008;76: 1877–1888. doi:10.1128/IAI.01165-07
143. Lee JE, Edery I. Circadian Regulation in the Ability of Drosophila to Combat Pathogenic Infections. Curr Biol. 2008. doi:10.1016/j.cub.2007.12.054
144. Holm S. A simple sequentially rejective multiple test procedure. Scand J Stat. 1979.
145. Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, Tsang G, et al. The BDGP gene disruption project: Single transposon insertions associated with 40% of Drosophila genes. Genetics. 2004. doi:10.1534/genetics.104.026427
146. Kniskern JM, Barrett LG, Bergelson J. Maladaptation in wild populations of the generalist plant pathogen pseudomonas syringae. Evolution (N Y). 2011. doi:10.1111/j.1558-5646.2010.01157.x
147. McGonigle JE, Leitão AB, Ommeslag S, Smith S, Day JP, Jiggins FM. Parallel and costly changes to cellular immunity underlie the evolution of parasitoid resistance in three Drosophila species. PLoS Pathog. 2017. doi:10.1371/journal.ppat.1006683
148. Wang JB, Lu HL, St. Leger RJ. The genetic basis for variation in resistance to infection in the Drosophila melanogaster genetic reference panel. PLoS Pathog. 2017;13. doi:10.1371/journal.ppat.1006260
149. Araújo JPM, Hughes DP. Diversity of Entomopathogenic Fungi. Which Groups Conquered the Insect Body? In: Lovett B, St. Leger RJ, editors. Advances in Genetics. 2016. pp. 1–39. doi:10.1016/bs.adgen.2016.01.001
150. Barrett LG, Kniskern JM, Bodenhausen N, Zhang W, Bergelson J. Continua of specificity and virulence in plant host-pathogen interactions: Causes and consequences. New Phytologist. 2009. doi:10.1111/j.1469-8137.2009.02927.x
151. Dawkins R, Krebs JR. Arms races between and within species. Proc R Soc London - Biol Sci. 1979. doi:10.1098/rspb.1979.0081
152. Gottar M, Gobert V, Matskevich AA, Reichhart JM, Wang C, Butt TM, et al. Dual

Detection of Fungal Infections in Drosophila via Recognition of Glucans and Sensing of Virulence Factors. Cell. 2006. doi:10.1016/j.cell.2006.10.046
153. Matskevich AA, Quintin J, Ferrandon D. The Drosophila PRR GNBP3 assembles effector complexes involved in antifungal defenses independently of its Toll-pathway activation function. European Journal of Immunology. 2010. doi:10.1002/eji.200940164
154. Roxström-Lindquist K, Terenius O, Faye I. Parasite-specific immune response in adult Drosophila melanogaster: A genomic study. EMBO Rep. 2004. doi:10.1038/sj.embor.7400073
155. Kawecki TJ. Red queen meets Santa Rosalia: Arms races and the evolution of host specialization in organisms with parasitic lifestyles. Am Nat. 1998. doi:10.1086/286195
156. Keebaugh ES, Schlenke TA. Insights from natural host-parasite interactions: the Drosophila model. Dev Comp Immunol. 2014;42: 111–23. doi:10.1016/j.dci.2013.06.001
157. Stork NE. How Many Species of Insects and Other Terrestrial Arthropods Are There on Earth? Annu Rev Entomol. 2018. doi:10.1146/annurev-ento-020117-043348
158. Rohde K. Ecology and Biogeography, Future Perspectives: Example Marine Parasites. Geoinformatics Geostatistics An Overv. 2016. doi:10.4172/2327-4581.1000140
159. Elya C, Lok TC, Spencer QE, McCausland H, Martinez CC, Eisen M. Robust manipulation of the behavior of drosophila melanogaster by a fungal pathogen in the laboratory. Elife. 2018. doi:10.7554/eLife.34414
160. Brobyn PJ, Wilding N. Invasive and developmental processes of Entomophthora muscae infecting houseflies (Musca domestica). Trans Br Mycol Soc. 1983. doi:10.1016/s0007-1536(83)80157-0
161. Lovett B, St. Leger RJ, de Fine Licht HH. Going gentle into that pathogen-induced goodnight. J Invertebr Pathol. 2020. doi:10.1016/j.jip.2020.107398
162. Hughes DP, Araújo JPM, Loreto RG, Quevillon L, de Bekker C, Evans HC. From So Simple a Beginning. The Evolution of Behavioral Manipulation by Fungi. Adv Genet. 2016. doi:10.1016/bs.adgen.2016.01.004
163. Roy HE, Steinkraus DC, Eilenberg J, Hajek AE, Pell JK. BIZARRE INTERACTIONS AND ENDGAMES: Entomopathogenic Fungi and Their Arthropod Hosts. Annu Rev Entomol. 2006;51: 331–357. doi:10.1146/annurev.ento.51.110104.150941
164. Duxbury EM, Day JP, Maria Vespasiani D, Thüringer Y, Tolosana I, Smith SC, et al. Host-pathogen coevolution increases genetic variation in susceptibility to infection. Elife. 2019. doi:10.7554/eLife.46440
165. Lazzaro BP, Little TJ. Immunity in a variable world. Philos Trans R Soc B Biol Sci. 2009. doi:10.1098/rstb.2008.0141
166. Wang JB, Elya C, St. Leger RJ. Genetic variation for resistance to the specific fly pathogen Entomophtora muscae. Sci Rep. 2020. doi:10.1038/s41598-020-71262-w
167. St. Leger RJ, Wang C. Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests. Appl Microbiol Biotechnol. 2010;85: 901–

907. doi:10.1007/s00253-009-2306-z
168. Wang C, Feng MG. Advances in fundamental and applied studies in China of fungal biocontrol agents for use against arthropod pests. Biol Control. 2014. doi:10.1016/j.biocontrol.2013.06.017
169. Hu X, Xiao G, Zheng P, Shang Y, Su Y, Zhang X, et al. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc Natl Acad Sci. 2014;111: 16796–16801. doi:10.1073/pnas.1412662111
170. Rombach MC, Humber RA, Evans HC. Metarhizium album, a fungal pathogen of leaf- and planthoppers of rice. Trans Br Mycol Soc. 1987. doi:10.1016/s0007-1536(87)80028-1
171. Wang B, Kang Q, Lu Y, Bai L, Wang C. Unveiling the biosynthetic puzzle of destruxins in Metarhizium species. Proc Natl Acad Sci U S A. 2012. doi:10.1073/pnas.1115983109
172. Wang S, Fang W, Wang C, St. Leger RJ. Insertion of an esterase gene into a specific locust pathogen (Metarhizium acridum) enables it to infect caterpillars. PLoS Pathog. 2011. doi:10.1371/journal.ppat.1002097
173. Wang C, St. Leger RJ. Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen Metarhizium anisopliae var. acridum. Eukaryot Cell. 2005;4: 937–947. doi:10.1128/EC.4.5.937-947.2005
174. St. Leger RJ, Roberts DW, Staples RC. A model to explain differentiation of appressoria by germlings of Metarhizium anisopliae. J Invertebr Pathol. 1991;57: 299–310. doi:10.1016/0022-2011(91)90134-C
175. Butt TM, Coates CJ, Dubovskiy IM, Ratcliffe NA. Entomopathogenic Fungi: New Insights into Host-Pathogen Interactions. Adv Genet. 2016. doi:10.1016/bs.adgen.2016.01.006
176. Gao Q, Jin K, Ying S-HH, Zhang Y, Xiao G, Shang Y, et al. Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi Metarhizium anisopliae and M. acridum. PLoS Genet. 2011;7: e1001264. doi:10.1371/journal.pgen.1001264
177. St. Leger RJ, May B, Allee LL, Frank DC, Staples RC, Roberts DW. Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus Metarhizium anisopliae. J Invertebr Pathol. 1992. doi:10.1016/0022-2011(92)90159-2
178. Samuels RI, Charnley AK, Reynolds SE. The role of destruxins in the pathogenicity of 3 strains of Metarhizium anisopliae for the tobacco hornworm Manduca sexta. Mycopathologia. 1988. doi:10.1007/BF00437924
179. Igboin CO, Griffen AL, Leys EJ. The Drosophila melanogaster host model. J Oral Microbiol. 2012. doi:10.3402/jom.v4i0.10368
180. Govind S. Innate immunity in Drosophila: Pathogens and pathways. Insect Science. 2008. doi:10.1111/j.1744-7917.2008.00185.x
181. Stokes BA, Yadav S, Shokal U, Smith LC, Eleftherianos I. Bacterial and fungal pattern recognition receptors in homologous innate signaling pathways of insects and mammals. Front Microbiol. 2015. doi:10.3389/fmicb.2015.00019
182. Issa N, Guillaumot N, Lauret E, Matt N, Schaeffer-Reiss C, Van Dorsselaer A, et al. The Circulating Protease Persephone Is an Immune Sensor for Microbial

Proteolytic Activities Upstream of the Drosophila Toll Pathway. Mol Cell. 2018. doi:10.1016/j.molcel.2018.01.029
183. Ligoxygakis P, Pelte N, Hoffmann JA, Reichhart JM. Activation of Drosophila toll during fungal infection by a blood serine protease. Science (80-). 2002. doi:10.1126/science.1072391
184. Taylor K, Kimbrell DA. Host immune response and differential survival of the sexes in Drosophila. Fly (Austin). 2007. doi:10.4161/fly.5082
185. St. Leger RJ. The integument as a barrier to microbial infections. In "The Physiology of Insect Epidermis"(A. Retnakaran, and K. Binnington, Eds.). Inkata Press, North Clayton, Victoria, Australia; 1991.
186. Sharififard M, Mossadegh MS, Vazirianzadeh B. Effects of temperature and humidity on the pathogenicity of the entomopathogenic fungi in control of the house fly, Musca domestica L. (Diptera: Muscidae) under laboratory conditions. J Entomol. 2012. doi:10.3923/je.2012.282.288
187. Bidochka MJ, St. Leger RJ, Roberts DW. Mechanisms of deuteromycete fungal infections in grasshoppers and locusts: An overview. Memoirs of the Entomological Society of Canada. 1997.
188. Pal S, St. Leger RJ, Wu LP. Fungal peptide destruxin a plays a specific role in suppressing the innate immune response in Drosophila melanogaster. J Biol Chem. 2007;282: 8969–8977. doi:10.1074/jbc.M605927200
189. Donzelli BGG, Krasnoff SB, Sun-Moon Y, Churchill ACL, Gibson DM. Genetic basis of destruxin production in the entomopathogen Metarhizium robertsii. Curr Genet. 2012. doi:10.1007/s00294-012-0368-4
190. Le Bourg É. The NF-kB like factor DIF has weaker effects on Drosophila melanogaster immune defenses than previously thought. J Comp Physiol B Biochem Syst Environ Physiol. 2011. doi:10.1007/s00360-011-0567-1
191. Clemmons AW, Lindsay SA, Wasserman SA. An Effector Peptide Family Required for Drosophila Toll-Mediated Immunity. PLoS Pathog. 2015. doi:10.1371/journal.ppat.1004876
192. Cohen LB, Lindsay SA, Xu Y, Lin SJH, Wasserman SA. The Daisho Peptides Mediate Drosophila Defense Against a Subset of Filamentous Fungi. Front Immunol. 2020. doi:10.3389/fimmu.2020.00009
193. Castillejo-López C, Häcker U. The serine protease Sp7 is expressed in blood cells and regulates the melanization reaction in Drosophila. Biochem Biophys Res Commun. 2005. doi:10.1016/j.bbrc.2005.10.042
194. Dudzic JP, Hanson MA, Iatsenko I, Kondo S, Lemaitre B. More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in Drosophila. Cell Rep. 2019. doi:10.1016/j.celrep.2019.03.101
195. Shahrestani P, Chambers M, Vandenberg J, Garcia K, Malaret G, Chowdhury P, et al. Sexual dimorphism in Drosophila melanogaster survival of Beauveria bassiana infection depends on core immune signaling. Sci Rep. 2018. doi:10.1038/s41598-018-30527-1
196. Duneau DF, Kondolf HC, Im JH, Ortiz GA, Chow C, Fox MA, et al. The Toll pathway underlies host sexual dimorphism in resistance to both Gram-negative and Gram-positive bacteria in mated Drosophila. BMC Biol. 2017.

doi:10.1186/s12915-017-0466-3
197. De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. EMBO J. 2002. doi:10.1093/emboj/21.11.2568
198. Irving P, Troxler L, Heuer TS, Belvin M, Kopczynski C, Reichhart JM, et al. A genome-wide analysis of immune responses in Drosophila. Proc Natl Acad Sci U S A. 2001. doi:10.1073/pnas.261573998
199. Pelte N, Robertson AS, Zou Z, Belorgey D, Dafforn TR, Jiang H, et al. Immune challenge induces N-terminal cleavage of the Drosophila serpin Necrotic. Insect Biochem Mol Biol. 2006;36: 37–46. doi:10.1016/j.ibmb.2005.10.004
200. De Gregorio E, Spellman PT, Rubin GM, Lemaitre B. Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc Natl Acad Sci U S A. 2001. doi:10.1073/pnas.221458698
201. Balakrishnan SS, Basu U, Raghu P. Phosphoinositide signalling in Drosophila. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. 2015. doi:10.1016/j.bbalip.2014.10.010
202. Juhász G, Hill JH, Yan Y, Sass M, Baehrecke EH, Backer JM, et al. The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. J Cell Biol. 2008. doi:10.1083/jcb.200712051
203. Gupta A, Toscano S, Trivedi D, Jones DR, Mathre S, Clarke JH, et al. Phosphatidylinositol 5-phosphate 4-kinase (PIP4K) regulates TOR signaling and cell growth during Drosophila development. Proc Natl Acad Sci U S A. 2013. doi:10.1073/pnas.1219333110
204. Haller S, Kapuria S, Riley RR, O'Leary MN, Schreiber KH, Andersen JK, et al. mTORC1 Activation during Repeated Regeneration Impairs Somatic Stem Cell Maintenance. Cell Stem Cell. 2017. doi:10.1016/j.stem.2017.11.008
205. Ma S, Meng Z, Chen R, Guan K-L. The Hippo Pathway: Biology and Pathophysiology. Annu Rev Biochem. 2019;88: 577–604. doi:10.1146/annurev-biochem-013118-111829
206. Gerlach SU, Eichenlaub T, Herranz H. Yorkie and JNK Control Tumorigenesis in Drosophila Cells with Cytokinesis Failure. Cell Rep. 2018. doi:10.1016/j.celrep.2018.04.006
207. Wu C, Chen C, Dai J, Zhang F, Chen Y, Li W, et al. Toll pathway modulates TNF-induced JNK-dependent cell death in Drosophila. Open Biol. 2015. doi:10.1098/rsob.140171
208. Katsukawa M, Ohsawa S, Zhang L, Yan Y, Igaki T. Serpin Facilitates Tumor-Suppressive Cell Competition by Blocking Toll-Mediated Yki Activation in Drosophila. Curr Biol. 2018. doi:10.1016/j.cub.2018.04.022
209. Repiso A, Bergantiños C, Corominas M, Serras F. Tissue repair and regeneration in Drosophila imaginal discs. Development Growth and Differentiation. 2011. doi:10.1111/j.1440-169X.2010.01247.x
210. Kiehart DP, Galbraith CG, Edwards KA, Rickoll WL, Montague RA. Multiple forces contribute to cell sheet morphogenesis for dorsal closure in Drosophila. J Cell Biol. 2000. doi:10.1083/jcb.149.2.471
211. Tsakiri EN, Iliaki KK, Höhn A, Grimm S, Papassideri IS, Grune T, et al. Diet-

derived advanced glycation end products or lipofuscin disrupts proteostasis and reduces life span in Drosophila melanogaster. Free Radic Biol Med. 2013. doi:10.1016/j.freeradbiomed.2013.08.186
212. Nakhleh J, El Moussawi L, Osta MA. The Melanization Response in Insect Immunity. Advances in Insect Physiology. 2017. doi:10.1016/bs.aiip.2016.11.002
213. Boutros M, Agaisse H, Perrimon N. Sequential activation of signaling pathways during innate immune responses in Drosophila. Dev Cell. 2002. doi:10.1016/S1534-5807(02)00325-8
214. Xiao G, Ying S-HH, Zheng P, Wang Z-LL, Zhang S, Xie X-QQ, et al. Genomic perspectives on the evolution of fungal entomopathogenicity in Beauveria bassiana. Sci Rep. 2012;2: 483. doi:10.1038/srep00483
215. Chen X-RR, Hu Q-BB, Yu X-QQ, Ren S-XX. Effects of destruxins on free calcium and hydrogen ions in insect hemocytes. Insect Sci. 2014;21: 31–38. doi:10.1111/1744-7917.12028
216. Tzou P, Reichhart JM, Lemaitre B. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proc Natl Acad Sci U S A. 2002. doi:10.1073/pnas.042411999
217. Leitão AB, Bian X, Day JP, Pitton S, Demir E, Jiggins FM. Independent effects on cellular and humoral immune responses underlie genotype-by-genotype interactions between Drosophila and parasitoids. PLoS Pathog. 2019. doi:10.1371/journal.ppat.1008084
218. Abdullah C, Duronio RJ. The many fates of tissue regeneration. PLoS Genet. 2018. doi:10.1371/journal.pgen.1007728
219. Levashina EA, Ohresser S, Lemaitre B, Imler JL. Two distinct pathways can control expression of the gene encoding the Drosophila antimicrobial peptide metchnikowin. J Mol Biol. 1998. doi:10.1006/jmbi.1998.1705
220. Ayres JS, Schneider DS. A signaling protease required for melanization in Drosophila affects resistance and tolerance of infections. PLoS Biol. 2008. doi:10.1371/journal.pbio.0060305
221. St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW. Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc Natl Acad Sci U S A. 1996;93: 6349–6354. doi:10.1073/pnas.93.13.6349
222. St. Leger RJ, Cooper RM, Charnley AK. The effect of melanization of Manduca sexta cuticle on growth and infection by Metarhizium anisopliae. J Invertebr Pathol. 1988. doi:10.1016/0022-2011(88)90059-6
223. Huang A, Lu M, Ling E, Li P, Wang C. A M35 family metalloprotease is required for fungal virulence against insects by inactivating host prophenoloxidases and beyond. Virulence. 2020. doi:10.1080/21505594.2020.1731126
224. St. Leger RJ, Charnley AK, Cooper RM. Production of polyphenol pigments and phenoloxidase by the entomopathogen, Metarhizium anisopliae. J Invertebr Pathol. 1988. doi:10.1016/0022-2011(88)90128-0
225. Wang C, Hu G, St. Leger RJ. Differential gene expression by Metarhizium anisopliae growing in root exudate and host (Manduca sexta) cuticle or hemolymph reveals mechanisms of physiological adaptation. Fungal Genet Biol.

2005;42: 704–718. doi:10.1016/j.fgb.2005.04.006
226. St. Leger RJ, Charnley AK, Cooper RM. Cuticle-degrading enzymes of entomopathogenic fungi: Mechanisms of interaction between pathogen enzymes and insect cuticle. J Invertebr Pathol. 1986. doi:10.1016/0022-2011(86)90099-6
227. St. Leger RJ, Charnley AK, Cooper RM. Kinetics of the digestion of insect cuticles by a protease (Pr1) from Metarhizium anisopliae. J Invertebr Pathol. 1991. doi:10.1016/0022-2011(91)90057-W
228. Ferrandon D, Jung AC, Criqui MC, Lemaitre B, Uttenweiler-Joseph S, Michaut L, et al. A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. EMBO J. 1998. doi:10.1093/emboj/17.5.1217
229. Cohen L, Moran Y, Sharon A, Segal D, Gordon D, Gurevitz M. Drosomycin, an innate immunity peptide of Drosophila melanogaster, interacts with the fly voltage-gated sodium channel. J Biol Chem. 2009. doi:10.1074/jbc.M109.023358