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# The role of pathogens in sexual selection

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# The role of pathogens in sexual selection

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*献给我挚爱的爸爸，妈妈，婷婷，和在另一个世界守护我的二伯父。*

# Abstract

"good genes" sexual selection stipulates that a positive additive genetic correlation exists between sexual success and non-sexual success. Despite the solid theoretical framework, findings from empirical research remain ambiguous. This thesis explores the "good genes" hypothesis and investigates the role of pathogens in sexual selection with an emphasis on including various epidemiological contexts for sexual selection and the realization of the potential "good genes", using a trio system of *Drosophila melanogaster*, the fungus *Metarhizium brunneum,* and the gram-negative bacterium *Pseudomonas entomophila*.

Chapter 1 looks into the impacts of fungal infection on male reproductive potential in non-competing settings to evaluate the potential role of sexual selection in promoting resistant genes. Despite the strongly aroused immune responses and high mortality, little impact was revealed. Chapter 2 shifts focus to investigate how much offspring fitness can be gained through female pathogen avoidance. Females actively avoided infectious oviposition sites, but no fitness penalty was found for laying eggs on fungi-contaminated sites.

Subsequent chapters explore whether and how pathogens mediate sexual selection in promoting offspring fitness. Chapter 3 asks how the presence of *M.brunneum* in the sexual selection context affects the link between sire sexual success (sire paternity share) and offspring pathogen resistance (survival post infection), showing that the sign of the correlation was mediated by the pathogen occurrence and was sex-specific. Infected sires had reduced sexual success, yet those of higher resistance achieved greater sexual success when competing for mates, suggesting a potential for sexual selection in promoting pathogen resistance. However, no positive link was found between sire sexual success and offspring pathogen resistance, challenging the "good genes" hypothesis. Chapter 4 broadens the scope by introducing *P.entomophila* to the offspring generation and assessing the offspring reproductive fitness (relative contribution to the grand-offspring generation) across various epidemiological contexts. Weak evidence was found showing the sex-specific/biased selection on offspring fitness-related traits. Although no correlation was found between sire sexual success and offspring reproductive fitness within any specific pathogenic context, weak evidence suggests a difference in the observed sire-offspring relationship depending on whether the environment of the sire and the offspring align or not.

In summary, findings from this thesis challenge the "good genes" hypothesis and underscore the necessity of including various contexts (e.g., occurrence of pathogens, offspring sex) in experimental design.

## Abstract (In French)[1]

La sélection sexuelle lié à l'hypothèse des "bons gènes" stipule qu'il existe une corrélation génétique additive positive entre le succès sexuel et le succès non sexuel. Malgré le solide cadre théorique, les résultats de la recherche empirique restent ambigus. Cette thèse explore l'hypothèse des "bons gènes" et examine le rôle des agents pathogènes dans la sélection sexuelle. L'accent a été mis sur l'inclusion de divers contextes épidémiologiques pour la sélection sexuelle et pour la réalisation du potentiel des "bons gènes", en utilisant un système à trois composantes : la mouche *Drosophila melanogaster*, le champignon *Metarhizium brunneum* et la bactérie gram-négatif *Pseudomonas entomophila*.

Le chapitre 1 examine l'impact de l'infection fongique sur les potentiels de reproduction masculine dans des environnements non compétitifs afin d'évaluer si la sélection sexuelle joue un rôle dans la promotion des gènes de résistance. Malgré les fortes réponses immunitaires suscitées et le taux de mortalité élevé, l'impact a été minime. Le chapitre 2 est axé sur la question de savoir dans quelle mesure la fitness des descendants peut être augmentée grâce à la capacité à éviter les agents pathogènes par les femelles. Les femelles ont activement évité les sites de ponte infectieux, mais aucune pénalité en termes de fitness n'a été constatée pour la ponte d'œufs sur des sites contaminés par des agents pathogènes. Les chapitres suivants explorent si et comment les agents pathogènes interviennent dans la sélection sexuelle pour favoriser la fitness des descendants. Le chapitre 3 examine comment la présence de *M. brunneum* affecte le lien entre le succès sexuel du mâle (part de paternité du mâle) et la résistance des descendants aux agents pathogènes (survie après l'infection), montrant que la corrélation était médiée par l'occurrence de l'agent pathogène et que le lien était spécifique au sexe. Les mâles infectés avaient un succès sexuel réduit, mais ceux qui avaient une résistance plus élevée étaient capables de rivaliser avec succès pour les partenaires, ce qui suggère que la sélection sexuelle peut contribuer à la résistance aux agents pathogènes. Cependant, aucun lien positif n'a été trouvé entre le succès sexuel du mâle et la résistance des descendants aux agents pathogènes, remettant en question l'hypothèse des "bons gènes". Le chapitre 4 élargit la portée en introduisant *P. entomophila* à la génération des descendants et en évaluant le fitness reproductif des descendants (contribution relative à la génération des arrière-

[1] This abstract in French was translated from English by my dear officemate/close friend Dr. Christian du Guttry. I greatly admire his language skills. This translation is both elegant and precise.

petits-enfants) dans divers contextes épidémiologiques. De faibles indications ont été trouvées montrant la sélection spécifique/biaisée du sexe sur les traits liés à la condition physique de la progéniture. Bien qu'aucune corrélation n'ait été trouvée entre le succès sexuel du père et l'aptitude à la reproduction de la progéniture dans un contexte pathogène spécifique, nous avons trouvé des preuves faibles indiquant que la relation observée entre le père et la progéniture était plus robuste lorsque les environnements du père et de la progéniture différaient que lorsqu'ils ne différaient pas.

En résumé, les conclusions de cette thèse remettent en question l'hypothèse des "bons gènes" et soulignent la nécessité d'inclure différents contextes (par exemple, la présence d'agents pathogènes, le sexe des descendants) dans la conception expérimentale.

# The role of pathogens in sexual selection

# General Introduction

## Sexual selection and "good genes"

First proposed in Darwin's seminal work *The Descent of Man* (Darwin, 1871), the concept of "sexual selection" has been under continuous debate. What is sexual selection? How is it different from natural selection? What is its relationship with natural selection? Researchers have not yet reached a consensus on these questions after nearly two centuries of working on the topic. Very often, the debate starts with the definition of sexual selection (Alonzo and Servedio, 2019). In this thesis, I am using the definition proposed by Shuker and Kvarnemo (2021): *sexual selection is any selection that arises from fitness differences associated with nonrandom success in the competition for access to gametes for fertilization.* A clear definition of sexual selection will facilitate us to better understand the process and the consequences of sexual selection, which will in return facilitate our understanding of the relationship between sexual selection and natural selection. Though sexual selection affects both sexes, the strength of sexual selection is suggested to be higher on males than on females because males compared to females invest more on traits that enhance sexual success (Bateman, 1948, Wade, 1979, Singh et al., 2002, Andersson, 1994, Singh and Punzalan, 2018, Janicke et al., 2016, Davies et al., 2023). Therefore, in most cases, as I discuss here, males are competitive, females are choosy and how males become sexually successful is of interest. To understand the relationship between sexual selection and natural selection, one needs to first figure out what traits give males advantages in the competition for access to gametes.

Males can enhance their mating success by exhibiting traits that appeal to females. Female preference could be simply the by-product of sensory bias or perceptual bias (Kirkpatrick, 1987). Any traits that trigger the right stimuli for females would be favored. The contribution of sexual selection in this process is negligible (Fuller et al., 2005). However, female preferences for specific male phenotypes can also arise due to the properties of these traits. For instance, in some species, females favor males with good resources because breeding is costly and parental care for the young is needed (Price et al., 1993, Iwasa and Pomiankowski, 1999). Likewise, in species where males compete against each other, weapons like antlers, horns, or large body sizes will earn the male victory in the battles and gain access to the gametes. However, traits making a male sexually successful are not in all cases of concrete usage and are often costly to survival, such as bright

feathers of birds of paradise, red spots on three-spined sticklebacks, courtship of fruit flies, and calling of frogs (Andersson, 1994). Among many proposed mechanisms on why males carrying such traits are favored[2], two sets of theories involving additive genetic covariance stand out: Fisherian run-away model and the "good genes" hypothesis.

Fisher (1930) proposed that males with an arbitrary trait would be sexually successful if there is a mean preference of females for such a trait. Exaggerated sexual traits then evolve through a run-away process in which the existence of additive genetic variation for both the preference and the preferred trait leads to additive genetic correlation between the two, and further exaggeration of female preference results in intensifying sexual trait expression. Under Fisher's theory, male traits will be more and more extravagant until the point natural selection puts a halt on them (Lande, 1981). The benefit of this form of female mate choice is attributed mainly to "sexy sons" and females benefit from a greater number of grand-offspring through sons' higher mating rate. The "good genes" hypothesis, on the other hand, proposes that males carry costly sexual displays that signal their high genetic quality (or more precise, high breeding value (Neff and Pitcher, 2005, Hunt et al., 2004, Tomkins et al., 2004)), making them attractive to females (Kokko et al., 2002, Kokko et al., 2003, Hamilton and Zuk, 1982, Zahavi, 1975). Despite the many similarities shared by these two sets of theories, the "good genes" model requires an additional element, that is heritable variation in quality which promotes the viability and reproductive value of the offspring. Yet, the requirement on genetic covariance is often overlooked in "good genes" studies (reviewed in (Achorn and Rosenthal, 2019)). The good genes version of sexual selection facilitates natural selection to promote adaptation to the environment where the individuals live. Among the empirical evidence for the "good genes" hypothesis, Moore (1994) has shown that female cockroach *Nauphoeta cinerea* mated with attractive males had offspring of higher fitness, for instance, faster developmental time. An experiment using Trinidadian guppies, *Poecilia reticulata* has shown that larger body size was correlated with higher offspring growth rate (Reynolds and Gross, 1992). Song repertoire size is positively correlated with offspring's post-fledging survival (Hasselquist et al., 1996). In pronghorns (*Antilocapra americana*), the offspring of the preferred

[2] Compatible genes are not discussed within the scope of this thesis. For more information on the benefits of mate choice, see Achorn and Rosenthal (2019) and therein Figure 1&2.
ACHORN, A. M. & ROSENTHAL, G. G. 2019. It's not about him: Mismeasuring 'good genes' in sexual selection. *Trends in Ecology & Evolution.*

sire had a higher chance of surviving in the field (Byers and Waits, 2006). However, a meta-analysis, spanning a broad range of taxa, investigates the relationship between sire attractiveness and offspring fitness and reveals that only a few studies testing these predictions involved measuring non-sexual performance and that most studies testing the "good genes" hypothesis did not find improved offspring fitness (Prokop et al., 2012). This highlights the prevalence of finding "good genes" in empirical studies is low.

Early research on "good genes" focused on the consequences arising from female mate choice, while more recently, sexual selection studies have broadened their scope to post-copulatory processes including cryptic female choice (Eberhard, 1996) and sperm competition (Parker, 1970). The basic principle of "good genes" developed to explain the evolution of female choice can be applied to all sexual selection modalities, yet the nature of the traits conferring sexual success becomes more complex. The relative contribution of pre-copulatory and post-copulatory sexual success to overall fitness varies across species and environments, with a notable dependence on post-copulatory sexual selection in some species (Collet et al., 2012, Pelissie et al., 2014). Understanding yielded from these early studies may not represent the true effects of sexual selection, especially when post-copulatory sexual selection does not reinforce pre-copulatory sexual selection. For instance, female preference can be the result of sexual conflict (Gavrilets et al., 2001). Increased sexual success can be achieved through "male harms" like armed genitalia (Rönn et al., 2007), coercion or harassment (Sakurai and Kasuya, 2008), and insertion of harmful seminal fluid proteins (Wigby and Chapman, 2005). Even so, the same episodes of sexual selection may still contribute to promoting good genes (Vuarin et al., 2019). These developments contribute to the increasing difficulties of finding "good genes" in empirical studies.

Whether females, the choosing party in most cases, can distinguish males of high genetic quality among the many available underlies the basic principle of "good genes". This act often involves evaluating sexual signals, which like many life history traits, are condition-dependent (Zahavi, 1975, Jennions et al., 2001). Condition can be ecologically relevant (e.g., better food, better shelter, more resources, etc.) or genetically encoded (e.g., the number of deleterious genes). Here, I use the definition proposed by Hill (2011): *Condition is the relative capacity to maintain optimal functionality of essential cellular processes*, and it reflects a large part of the overall genetic variation (i.e. genetic quality, the genetic potential of the individual) (Lorch et al., 2003, Rowe and

Houle, 1996, Tomkins et al., 2004). Such common condition dependency provides a solution to the paradox that directional preference should in theory deplete the genetic variance of males, yet in reality, genetic variation persists. It designates that sexual selection acts on the underlying condition. Therefore, it's unlikely to deplete genetic variation because numerous genes are involved and molecular processes like mutations slow the journey even more (Tomkins et al., 2004). Sexual selection can then improve the overall population fitness by acting more intensely on males to purge deleterious mutation load and by favoring high-condition males (Whitlock and Agrawal, 2009, Grieshop et al., 2021, Grieshop et al., 2016, Hollis et al., 2009, Cally et al., 2019). Within this mechanism, the allele frequency of genes conferring high condition or sexual success increases, and via the pleiotropic effects of genes, genetic variation related to other fitness components is also selected (e.g., viability, pathogen resistance, etc.).

Nonetheless, "good genes" or any benefits raised by sexual selection is itself a relative term indicating context dependency. Good genes in one context may be bad genes in another. Globally, the best condition in one environment may not be the most favorable one in another. One notable gap in the current body of "good genes" research is underemphasizing the genotype × environment interactions (Hunt et al., 2004, Ingleby et al., 2010). If sexual signals and offspring fitness (e.g., viability, pathogen resistance) are expressed in different environments and there are G×Es for these traits, then the sexual signals and the "promised" high offspring fitness may become uncoupled in the offspring environment, disrupting the realization of "good genes". However, when the environment in which sexual selection happens aligns with the environment where offspring will live and reproduce, and if the "good genes" hypothesis stands, sire sexual success will be positively correlated with offspring fitness as if there is no G×E. These two scenarios are not restricted to female mate choice or male sexual signaling, but other modalities of sexual selection. Moreover, the strength of sexual selection is also affected by the G×Es on gene expression (Huang et al., 2020) and life-history traits (Svensson et al., 2020), which is rather critical for non-sexual fitness components. Thus, it is important to consider the environmental contexts for both sire and offspring when testing the net effect of sexual selection.

In sexual selection, environmental context also includes sex. Gene expression differs in males and females (Innocenti and Morrow, 2010, Lopes-Ramos et al., 2020, Ellegren and Parsch, 2007). The interests of males and females on shared traits do not always align and in the case of sexual conflict

(Arnqvist, 2004, Arnqvist and Rowe, 2005), traits favored in one sex may prove maladaptive when expressed in the other. Moreover, males' fitness may be also more sensitive to environmental changes compared to females' fitness, leading to unbalanced benefits for the two sexes even when there are "good genes". The alignment of natural selection and sexual selection through "good genes" requires that the net effect of sexual selection should still promote offspring fitness despite the potential sex-specific selection or sexually antagonistic selection. To address this gap, the heart of this thesis lies in incorporating various contexts for sexual selection and for the realization of "good genes" (i.e., offspring sex, environment where offspring inhabit) in empirical experiments, which I experimentally tested and discussed extensively in Chapter 3 and Chapter 4.

## The role of pathogens in sexual selection

### Direct host-pathogen interactions

Male's condition could be strongly affected by pathogens, as infection induces allocating resources into immune responses (Sheldon and Verhulst, 1996), which may lead to reduced sexual signals and reduced reproductive potential (Hasik and Siepielski, 2022, Córdoba-Aguilar and Munguía-Steyer, 2013, Vergara et al., 2012). However, infection can also trigger terminal investment where males increase current reproductive efforts given the uncertainty of future opportunities (Duffield et al., 2017, Foo et al., 2023), which will make their sexual traits expression a dishonest signal of their genetic quality (Kivleniece et al., 2010). Further complication arises when females, instead of avoiding infected males (Borgia, 1986, Beltran-Bech and Richard, 2014), are drawn towards infected males due to pathogen manipulation (Dass et al., 2011). The direct host-pathogen interactions vary from one (type of) pathogen to another and such complexity can in some cases mask the true effects of sexual selection on promoting beneficial genes. Thus, before using a certain pathogen in sexual selection studies, one should take a look at the direct host-pathogen interactions (e.g., immune response, behavior, reproductive efforts, mortality, etc.) to evaluate potential confounding factors.

### Pathogen as the mediator/ environment

Hamilton and Zuk (1982) proposed that females distinguish between males that differ genetically in their pathogen loads, and hence in their condition. This observation kicks off the era for understanding the role of pathogens as the mediator in sexual selection or more precisely, its role

in mediating the cost-benefit balance of pathogen resistance. Previous work testing the Hamilton-Zuk hypothesis mainly focuses on using immunity as the measure of pathogen resistance (Lawniczak et al., 2007) and such measure overlooks the cost of immune responses on host performance (Jacobs et al., 2015). Here I define pathogen resistance as a generic term that includes the ability to avoid pathogens, evoke immune responses upon infection, tolerate infections, and also to mitigate the cost of immune responses. Pathogen resistance, though essential, is a rather costly trait to maintain (Kraaijeveld and Godfray, 1997, Koella and Boëte, 2002).

Hamilton-Zuk hypothesis, as an extended version of "good genes", is built upon the "Red Queen" hypothesis (Papkou et al., 2019, Woolhouse et al., 2002, Van Valen, 1973), which emphasizes the host-parasite coevolution and the potential for fluctuating selection. This setting applies equally well to an environment with several pathogens being common at different times. In an environment with a prevalent virulent pathogen, pathogen resistance should in theory be favored by natural selection because the benefits outweigh the costs. If the Hamilton-Zuk hypothesis is fulfilled, individuals with higher pathogen resistance should be more sexually successful as resistant males are able to keep a good condition and still provide good performance in the presence of pathogens. If females choose males of higher resistance against one specific pathogen, once the pathogen changes, the potential genetic benefits given by a higher level of defense will vanish. Carrying such specific pathogen resistance is then a burden to the host when the corresponding pathogen is absent. Thus, the presence of the pathogen mediates the sign of the additive genetic correlation between sexual success and pathogen resistance: positive when the pathogen is present and becomes negative or no longer exists when the pathogen is absent. Hereafter, I will refer to this scenario as the "Specific Resistance" hypothesis. On the other hand, if the pathogen resistance favored by sexual selection is a general one (effective against a broad range of pathogens), correlations between the sire sexual success and offspring pathogen resistance will be consistently positive regardless of the epidemiological context due to the common condition dependency (hereafter, "General Immunocompetence" hypothesis). The fundamental difference between the "Specific Resistance" hypothesis and the "General Immunocompetence" hypothesis is that in the latter, the same genetic variants conferring pathogen resistance are favored in any pathogenic environment (summarized in (Westneat and Birkhead, 1998)). Nonetheless, the two scenarios are not mutually exclusive, but their relative contributions to sexual selection remained unresolved.

The ultimate goal of "good genes" sexual selection is to have high offspring reproductive value. Pathogen resistance is only one fitness component among many. Trade-offs, typically described as negative phenotypic or genetic correlations between life history traits, are commonly seen (Flatt and Heyland, 2011, Stearns, 1989). Pursuing certain resistant genes may be at the cost of the expression of other vital life functions, like fecundity and longevity. Pathogen as a mediator does not only affect the value of a single trait but also the correlations between traits. Therefore, one should also look at the link between pathogen resistance and reproductive fitness to better understand the link between sexual success and offspring reproductive values under different pathogenic contexts. Yet, such effort is scarce in the current body of "good genes" studies.

### Sexual dimorphism in immunity

Sexually dimorphism in shared life history traits between the two sexes, especially sex differences in immunity affect the role of pathogens in sexual selection. Sexual dimorphism in pathogen resistance can be generated by the differences between females' and males' explorative behaviors which affects the rate of pathogen encounter and infection (Duneau and Ebert, 2012). But when facing generalist pathogens, differences in behaviors are not the major drivers as these pathogens are not specialized in one sex and should be encountered by both sexes at similar rates. On the physiological level, each sex is often pushed toward different evolutionary optima on shared traits, which suggests that females and males may evolve distinct immune strategies that are optimal for their specific reproductive roles and challenges. For example, females prioritize somatic maintenance to have high fecundity while males prioritize mating efforts and sexual success (Rolff, 2002). It has been shown that females and males display a diverged gene expression pattern upon pathogen infection (Duneau et al., 2017) and that in general, sexual dimorphism has an impact on host-pathogen coevolution (Gipson and Hall, 2016, Ahlawat et al., 2022). Altogether, it is important to address these intrinsic differences between females and males when looking at the net effects of sexual selection.

## Study System

In this thesis, I used the fruit fly *Drosophila melanogaster* as the host for pathogens. It is a powerful model organism widely used in evolutionary biology and sexual selection studies (Roberts, 2006, Taylor and Kekic, 1988, Promislow et al., 1998, Mahdjoub et al., 2023). Despite the lack of

extravagant sexual ornaments, *D.melanogaster* has energetically costly secondary sexual traits like courtship songs (Talyn and Dowse, 2004). When facing rivals, they will also aggressively compete for mates (Saltz and Foley, 2011). But the combat among males does not end here. When the Colosseum moves inside the females, males are subjected to cryptic female choice (Firman et al., 2017) and sperm competition (Parker, 1970) also comes into action, during which the production of seminal fluid protein plays a vital role in paternity assurance and fecundity (Hopkins et al., 2019, Wigby et al., 2020). Such complex dynamics of sexual selection make *D.melanogaster* an excellent experimental system for sexual selection study. Moreover, sexual dimorphism in the immunity of *D.melanogaster* manifests as distinct differences in immune responses and differences in susceptibilities and efficiencies in combating pathogens (Belmonte et al., 2020, Duneau et al., 2017), which makes it an excellent system to investigate potential sex-specific or sex-biased "good genes" effects.

*Pseudomonas entomophila* is a gram-negative bacterium, isolated from wild *D.melanogaster* (Dieppois et al., 2015). Natural *P.entomophila* infection is through oral uptake. *P.entomophila* "attacks" *Drosophila* by generating perturbations to the gut epithelium, disrupting the integrity of the gut (Buchon et al., 2009), and it is highly pathogenic to both *Drosophila* larvae and adults with a sufficiently high dose (Vodovar et al., 2005). *Metarhizium brunneum* is a fungal pathogen to many insects (St. Leger and Wang, 2020) and is also virulent to *D.melanogaster* (Ramírez-Camejo et al., 2022). Fungal spores attach to the host's cuticles. After penetration through the skin into the hemocoel, spores transform into yeast-like cells. In such form, they can successfully avoid host defense, swindle nutrients from the host, and proliferate. When the host is dead, they will sporulate from the host and spread to the new host. Infection of *Metarhizium* also causes substantial tissue damage to the host due to the multiple toxins and lytic enzymes excreted by the fungi (Lu and St. Leger, 2016). To combat these infections, multiple cellular and humoral responses are activated in *Drosophila*. It has been shown that the Toll pathway is mainly involved in defenses against Gran-positive bacterial and fungal infections, while the Imd pathway is mainly effective against Gram-negative bacterial infections (Vodovar et al., 2004). The two pathogens and their interactions with the host differ in many ways (Abro et al., 2019, Butt et al., 2016, Vodovar et al., 2005, St. Leger and Wang, 2020), which makes them good pathogen candidates for our study in generating different contexts for sexual selection and the realization of potential "good genes".

## Thesis outline

Previous work has presented that good-gene sexual selection promotes pathogen resistance, indicating the alignment of sexual selection and natural selection in various aspects. Nonetheless, it remains ambiguous in what way and to what extent, pathogen affects the relationship between sexual success and offspring fitness. This thesis particularly builds on the theoretic framework proposed by Westneat and Birkhead (1998) and explores the role of pathogens in sexual selection and the link between sexual success and non-sexual success under different epidemiological contexts, emphasizing the importance of including sexual selection context in experiments testing "good genes". A brief outline of the thesis is given below.

Chapter 1 investigates the effects of fungal infection on males' reproductive potential. Pathogen virulence is often measured with post-infection mortality and potential impacts on reproductive fitness before mortality occurs are seldom studied, especially in males. To do so, we[3] infected *D.melanogaster* males with *M.brunneum* and measured multiple traits relevant to the reproductive potential in non-competing setups during the course of infection. We found that although the fungal infection aroused strong immune responses and caused high mortality in the infected males, it had rather small negative effects on the males' reproduction before any mortality occurred. This finding indicates that in a non-competing setting, the selection for resistant genes was mainly through mortality and sexual selection added little scope in promoting pathogen resistance.

Chapter 2 looks into females' pathogen avoidance against the fungal pathogen and its fitness consequences. This chapter is the fruit of the *Experimental Design* course 2021 and 2022 from the University of Lausanne. Pathogen avoidance is an important topic to explore to understand the impact of pathogens on females and understand how much offspring fitness can be gained through pathogen avoidance, which is important background information when investigating the role of sexual selection in improving offspring fitness. We performed multiple two-choice assays to investigate whether *D.melanogaster* females avoid infectious food and then measured the viability of eggs laid on infectious food to understand the fitness consequences of females' egg-laying decisions. We found that females actively avoided infectious food, signs of avoidance during

[3] Some of the experiments reported in Chapter 1 and 2 were done with the help of colleagues and students, so I used the pronoun "we". Experiments reported in Chapter 3 and 4 were carried out by me. For writing consistency, I will use "we" for the subsequent chapters.

offspring development were also detected but no fitness penalty for females who laid eggs in potentially infectious environments.

Findings from the first half of the thesis suggest that while *D. melanogaster* exhibits adaptive behaviors and immune responses to *M. brunneum* infection, the influence on reproductive success and offspring viability appears more complex than initially anticipated.

Chapter 3 moves on to investigate whether sexual selection happens with and without pathogen exposure would affect the links between sire sexual success and offspring pathogen resistance. We found that fungal infection significantly reduced male's sexual success in a competing environment. The link between sire sexual success and offspring pathogen resistance was sex-specific and the sign of the link depended on the epidemiological context of the sexual selection. This finding partially supports the "specific resistance" hypothesis. Signs of sexually antagonistic selection on pathogen resistance were detected, indicating that the benefits of "good genes" may be offset by the costs of sexual conflict.

Chapter 4 continues to explore the sire-offspring relationship in more complex sire-offspring pathogenic environment combinations. We introduced the bacterial pathogen *P.entomophila* to the offspring generation and in addition to offspring pathogen resistance, we also looked into offspring reproductive fitness to provide answers for whether the net outcome of sexual selection aligns with natural selection. We could not reach a definite conclusion on whether specific resistance or general immunocompetence was favored by sexual selection as in most cases, we failed to find a significant sire-offspring correlation. However interestingly, sire of higher sexual success from the environment with *M.brunneum* sired offspring of higher pathogen resistance to *P.entomophila*. There was weak evidence suggesting that the link between sire sexual success and offspring pathogen resistance to *M.brunneum* was sex-specific. Daughters' pathogen resistance was positively linked to their reproductive fitness when pathogens were present, a pattern not seen in sons. We also found weak evidence showing that the correlation between sire sexual success and offspring reproductive fitness differed when the pathogenic environments of sire and offspring aligned compared to when they were not. These results demonstrate the prevalence of sex-specific/biased selection and the context-dependent relationship between sexual success and non-sexual fitness and highlight the intricacies of testing "good genes" in empirical studies.

Finally, this thesis concludes with a general discussion of major findings and some commonly asked questions on the topics.

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# Chapter 1 Effects of an entomopathogenic fungus on the reproductive potential of *Drosophila* males

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**Abstract**

While mortality is the primary focus of pathogen virulence, non-lethal consequences, particularly for male reproductive fitness, are less understood; yet, they are essential for understanding how sexual selection contributes to promoting resistance. We investigated how the fungal pathogen *Metarhizium brunneum* affects mating ability, fertility, and seminal fluid protein (SFP) expression of male *Drosophila melanogaster* paired with highly receptive virgin females in non-competitive settings. Depending on sex and dose, there is a general 3-6-day incubation period after infection, followed by an abrupt onset of mortality. Meanwhile, the immune response was strongly induced merely 38 h after infection and continued to increase as infection progressed. Latency to mate somewhat increased during the incubation period compared to sham-treated males, but even on day 5 post infection >90% of infected males mated within 2h. During the incubation period, *M. brunneum* infection reduced male reproductive potential (the number of offspring sired without mate limitation) by 11%, with no clear increase over time. Approaching the end of the incubation period, infected males had a lower ability to convert the number of mating opportunities into the number of offspring. After repeated mating, infected males had lower SFP expression than sham controls, more so in males that mated with few mates 24 hours earlier. Overall, despite strong activation of the immune response, males' mating ability and fertility remained surprisingly little affected by the fungal infection, even shortly before the onset of mortality. This suggests that the selection for resistance acts mainly through mortality, and the scope for fertility selection to enhance resistance in non-competing settings is rather limited.

**Keywords**: Pathogen virulence, Fungal infection, Mating ability, Male fertility, Seminal fluid protein

## Introduction

Pathogens are a ubiquitous challenge to individual health and population stability and persistence. The negative impact of a pathogen on host fitness (i.e., pathogen virulence), determines the strength of selection for resistance. In ecological and evolutionary contexts, pathogen virulence is most commonly quantified by the mortality rate of infected hosts. Although mortality puts an immediate halt on the host's reproduction, it is only part of the story when it comes to the pathogen's impact on host fitness. Even in the absence of mortality, or well before it occurs, pathogens can have severe impacts on host fitness by reducing its reproductive capacity. This can be a result of direct damage to reproductive tissues and other traits mediating reproduction (Wilson and Denison, 1980, Sadd and Siva-Jothy, 2006, Polak, 1998), consumption of host resources by the pathogen, interference with the host's ability to acquire resources, or diversion of resources from reproduction to maintain somatic health (Gupta et al., 2022, Stahlschmidt et al., 2013). The impact of pathogens on the host's reproductive potential varies depending on pathogen types (Lower et al., 2023), host condition (Chambers et al., 2014, Lower et al., 2023), and the environment where the infection occurs (Bedhomme et al., 2004). Moreover, an infected host facing the prospect of impending mortality may increase its immediate reproductive effort, a phenomenon referred to as terminal investment (Duffield et al., 2017, Zurowski et al., 2020, An and Waldman, 2016). Thus, the negative effects of pathogens on host reproduction are not universally expected; counterintuitively, pathogen exposure may even enhance reproduction in the short term.

Studies on pathogen virulence on host reproduction have primarily focused on females (Chadwick and Little, 2005, Hurd, 2009, Hudson et al., 2020, Rose et al., 2022). Some studies have looked into the effects of infection on various aspects of male's reproductive biology (e.g., courtship (Kennedy et al., 1987, Pélabon et al., 2005), sexual ornaments (Longo et al., 2020, Dougherty et al., 2023), sperm quality (Pham et al., 2022)). Still, we know little about how infection affects males' overall reproductive success in the absence of mortality (Simmons, 1993, Lehmann and Lehmann, 2000, Worden et al., 2000) and to what extent sexual selection contributes to the selection for resistance. Male's reproductive success is often primarily determined by access to females and their gametes (Bateman, 1948, Trivers, 1972); thus, consequences of infection for male reproductive success will largely be mediated by responses of females to infected males. In

other words, the effect of non-lethal (or not-yet-lethal) infections on male reproductive success would to a large degree mediated by sexual selection. Indeed, sexual selection is often postulated to favor males that are more resistant to pathogens (Hamilton and Zuk, 1982, Adamo and Spiteri, 2005, Andersson and Simmons, 2006), and this prediction rests on the assumption that infection impairs sexual competitiveness and attractiveness of males, at least of those that are more susceptible. Furthermore, the variance of reproductive success among males is often higher than among females (Janicke et al., 2016); hence, in contrast to selection mediated by mortality, selection for pathogen resistance mediated by reproduction can potentially be much stronger in males than females. However, the potential strength of this selection is limited by the degree to which the pathogen actually reduces male reproductive success prior to or without any mortality.

In this study, we used the fungal pathogen *Metarhizium brunneum* and *Drosophila melanogaster* as our experimental system to examine the impact of infection on traits contributing to male reproductive success. *Metarhizium* spores attach to the *Drosophila* cuticle, penetrate it and reach the hemolymph, proliferate within the host, and eventually kill the host when the life cycle of the fungus is completed, typically within 7-10 days (St. Leger and Wang, 2020, Lu et al., 2015). During proliferation, the fungus exploits the host for nutrients and energy and causes tissue damage through toxins or filamentous growth (Castrillo et al., 2005, St. Leger and Wang, 2020). Additionally, the activation of the immune system in response to the fungal infection (e.g. the production of antimicrobial peptides (AMPs)) disrupts cellular and organismal homeostasis (Tzou et al., 2002a). Thus, both the fungal development within the host and the host's immune response impose an increasing physiological burden on the host well before death. Mating is an expensive endeavor for *Drosophila* males, involving complex and energetically costly courtship and the production of seminal fluid proteins (SFPs). While courtship is important for convincing the female to mate, SFPs transferred from male to female during mating are important for securing the post-copulatory sexual success and the outcome of fertilization (Wigby et al., 2020, Avila et al., 2011).

Here we test how early, and to what degree the burden of immune response translates into a lower male reproductive success, and which of the multiple traits that contribute to pre- and post-copulatory aspects of male reproductive success are affected. First, we conducted a survival assay to establish the timeline of pathogen-induced mortality and measured the expression of AMPs

following infection to examine the time course of the immune response. This also allowed us to test if any effects of infection on male sexual performance coincide with the activation of the immune system, as would be expected if such effects were mediated by the costs of the immune response. Then to evaluate how the progression of infection affects male sexual and reproductive potential in the absence of rival males and under high availability of potential mates (competitive success will be the subject of another study), we quantified number of mates, number of offspring per mated female, and the total number of offspring at different time points post infection but before the onset of infection-induced mortality. While the total number of offspring represents each male's overall reproductive success, number of mates indicates male's attractiveness or its ability to convince females to mate, and number of offspring per mated females demonstrates the male's ability to fertilize eggs and to promote egg production and laying by the female. Lastly, we looked into the replenishment of five well-characterized SFPs (*SP*, *Acp26Aa*, *Acp29AB*, *Acp62F,* and *Acp36DE*) after repeated mating. SFPs are transferred to female along with sperm during mating, their stock in accessory glands eventually becomes depleted after repeated mating (Sirot et al., 2009, Hihara, 1981), and they are costly to produce (both time- and energy-wise). Along with other >200 SFPs, they have important effects on post-mating processes such as female receptivity (*SP*), ovulation (*SP*, *Acp26Aa*), oogenesis (*Acp62F*), sperm storage (*Acp29AB*), sperm competition (*Acp29AB*, *Acp62F*, *Acp36DE*), etc. (Avila et al., 2011, Chapman, 2001). Quantifying SFP replenishment allowed us to investigate the impact of infection on males' non-behavioral component of reproductive effort. We hypothesized that the progression of infection would negatively affect various components contributing to male's reproductive success but only when the infection is established within the host and when the immune system has been fully activated.

## Materials and Methods

### *Fly Stock*

Flies used in the experiments originated from a lab-adapted outbred population of *Drosophila melanogaster*, originally collected in Valais (Switzerland) in 2007. All flies were raised at a controlled density (~200 eggs on 40 ml food) and maintained at 25°C, 55% relative humidity, and 12L:12D photoperiod on standard yeast-sugar-cornmeal-agar media with Nipagin. When needed for experiments, virgin flies were collected 6-8h post-emergence and maintained in single-sex groups until used in the experiment. Female virgin status was further confirmed by the absence of larvae in the food media. All fly transfers were done under light $CO_2$ anesthesia.

### *Pathogen Origin and Infection Protocol*

The pathogen used in this experiment is *Metarhizium brunneum* KVL 03-143 (Ma275, previously known as *M. anisopliae*, but now separated as a sister species (Bischoff et al., 2009); a generous gift from Nicolai Vitt Meyling, University of Copenhagen). The fungus was grown on Sabouraud dextrose agar (SDA) for 10 days at 26 °C, after which spores were harvested and suspended in 0.05% Triton X-100 (#9036-19-5, Sigma-Aldrich). The concentration of spores was determined using a Neubauer hemocytometer. For the infection treatment, adult flies were dipped in groups of 10-15 for 30 seconds in 2ml spore suspension of the desired concentration. Males assigned to sham treatment were treated the same way but with spore-free 0.05% Triton X-100 (protocol adapted from (Ugelvig and Cremer, 2007)). Infection and sham treatment were done between 18:00-18:30 on the day before the experiment (i.e., day 0). Measures on any day post-treatment were done at 8:00 on the day of experiment, meaning that measures for day 1 post-treatment correspond to around 14 hours post-treatment, and subsequent measures were conducted every 24 hours.

### *Post-infection Mortality*

To establish the timeline of infection-induced mortality and to investigate whether it differs between the sexes, we conducted a post-infection survival assay. Three spore concentrations in the infective suspension ($10^6$, $10^7$, $10^8$ spores/ml) were used to understand how the dose affects fly mortality. Non-virgin flies were subject to infection or sham treatment at the age of 3-4 days post-emergence. They were then kept in groups of 10 in vials at 25°C and mortality was recorded daily

until day 16 post-treatment. Any deaths of flies observed within the first 2 hours were attributed to handling rather than infection; these individuals were therefore removed from the analysis (less than 1% of the treated flies). Mortality data were analyzed with a generalized linear mixed model (GLMM; binomial distribution, logit link) with the number of flies remaining alive (out of the initial number) as the response variable, day post-treatment (DPT), dose (i.e., spore concentration), sex and their interactions as fixed factors and replicate vial identity as a random factor. DPT used in the model as a continuous variable was center-scaled by subtracting the mean DPT value. This approach was chosen over typical survival analysis because a GLMM can better handle complex data structures and allow us to effectively find factors affecting mortality.

*Activation of the Immune System*

The immune response mechanism in *Drosophila* has been extensively studied (Lemaitre and Hoffmann, 2007, Vodovar et al., 2004). In *Drosophila*, one can easily track the immune response against *M. brunneum* by monitoring the expression of AMPs. To investigate the dynamics of the immune response post infection, we subjected 3-4 days old males to either *M. brunneum* infection ($10^7$ spores/ml) or sham treatment (Immune Assay 1). Treated flies were then kept in groups of 16 and 14 vials per treatment were set up. We randomly selected 2 vials from each treatment pool and then collected 4 samples of 8 flies on each day post treatment until day 5 post treatment. These samples were used to measure the expression of *Drosomycin*, an AMP regulated by the Toll pathway and active against fungi and Gram-positive bacteria. Considering that fungal infection might disturb the host homeostasis and facilitate the proliferation of other microbes within the host, we also quantified the expression of another AMP, *Diptericin A*, which is regulated by the IMD pathway and targets Gram-negative bacteria.

We then carried out another immune response assay (Immune Assay 2) to investigate if the activation of the immune system is affected by the dosage of *M. brunneum* spores. We collected 3-4 samples of 2-3 flies from three concentration treatments ($10^6$, $10^7$, $10^8$ spores/ml) on each day post infection until day 5 post infection.

Total RNA of samples from both immune response assays was extracted with the Total RNA Purification Plus Kit, following the manufacturer's protocol (#48400, Norgen Biotek). 100ng RNA was converted into cDNA using the PrimeScript RT$^{TM}$ reagent kit with gDNA Eraser (#RR047B,

TaKaRa Bio). Each cDNA sample was diluted 10-fold prior to the RT-qPCR. RT-qPCR was performed in 10µl reaction volumes, containing 5µl of SsoAdvanced Universal SYBRGreen Supermix (#1725272, BioRad, Switzerland), 0.3µM of each forward primer and reverse primer, and 2µl of cDNA templates. Cycling conditions consisted of 30 seconds of initial activation of the polymerase at 95 °C, followed by 40 cycles with 15s denaturation at 95 °C, 30s annealing, and extension at 60 °C. Following amplification, a melting curve analysis was performed ranging from 60 °C to 95 °C with 0.5 °C increments for 1s each. qPCR amplifications were performed in duplicate for each sample using the QuantStudio 6 Flex system equipped with a 384-well block. We repeated the qPCR for samples with a ΔCt SD between the two technical replicates more than 0.3. We performed qPCR for *Drosomycin* and *Diptericin A*, and three reference genes (*αTub84B*, *eEF1α2,* and *RpL32*). All primers used in the experiment are listed in Table S1. The expression of target genes relative to the reference genes was calculated using Pfaffl *et al* (2001) method but without a calibrator group.

To analyze the $\log_2$-transformed relative expression of the immune genes, we used a linear mixed model (LMM) with treatment (infected vs. sham-treated for immune assay 1 data and three doses for immune assay 2 data), day post treatment (DPT; a continuous variable), the quadratic effect of day post treatment, and their interactions as fixed factors. DPT used in the model was center-scaled. Additionally, vial was included as a random factor to account for possible vial effects. Pairwise comparison was done using the *contrast()* function in the *emmeans* package (v.1.7.1-1) and *p* values were adjusted with the Holm-Bonferroni method.

*Mating Ability and Latency*

To study how developing infection affects male physiological and behavioral capability to mate, we performed a mating latency assay with receptive 3-day-old virgin females in a non-competing setup. 2-3 days old virgin males were infected with *M. brunneum* ($10^7$ spores/ml) or sham-treated as described above, and subsequently kept in groups of 10 until used in the mating trials. These mating trials were performed at five time points (day 1-5 post treatment). On the day before mating, we randomly selected $N = 50$ infected males and 50 sham-treated males (i.e., 5 vials each) for the mating trials (any male was only used once). Then one virgin male and one virgin female were put into the mating vial but kept separated by a paper separator (Hollis and Kawecki, 2014). The

observation started the next morning with the removal of the separator at lights-on time (8:00) and lasted for two hours (flies are most active during this period). The time elapsed between the separator removal and the start of the first observed mating in the vial was noted as mating latency. No fly mortality was observed during the experiment. This experiment was done in two experimental blocks.

We compared the mating latency of infected and sham-treated males on each day post treatment with a mixed-effects Cox's proportional hazards regression model with package *coxme* (v.2.2-16). The model included the treatment (infected vs. sham-treated), day post-treatment (DPT; a continuous variable), and their interaction as fixed factors. Experiment block (n = 2) and day of experiment were included as random factors. DPT used in the model was center-scaled. Males that did not mate within the 2 h observation period were included as right-censored observations in the model. The estimated ratio of mating rate ("hazard ratio") and the 95% confidence intervals were then acquired with the *emmeans()* function in the *emmeans* package.

*Reproductive Potential*

To investigate how *M. brunneum* infection affects the male's reproductive potential (i.e., the maximum number of viable offspring a male can sire within a given timeframe), we coupled each male (infected or sham-treated) with 10 4-5 days old virgin females and gave them 3.5h to mate. This assay was done at three time points (day 1, 3, and 5) post treatment. Each male was only used once. After the mating period, we transferred females into individual food vials, and each female was given 48 hours to lay eggs before being removed from the food vial. On day 12 following the female removal (the usual emergence time for this population is ~10 days after the eggs are laid), we counted the number of vials with offspring, which we took as a measure of the number of females successfully inseminated by each male (referred to as number of mates in the analysis). We also counted the number of offspring that emerged from each vial, thus obtaining the total number of each male's offspring. Two experimental blocks were done consecutively within two weeks, with $N$ = 23-27 males per treatment and time point.

As we only collected data at three time points post-treatment in this experiment, DPT was included in the analysis as a categorical variable. Number of mates of each male was the outcome of 10 binary events (female mates with the male or not), so we analyzed it with a GLMM (binomial

distribution, logit link) with treatment, DPT (a categorical variable), and their interaction as fixed factors and experiment block and male identity (nested in block) as random factors. Number of offspring per mated female was calculated by dividing the total number of offspring by the number of mates. We then analyzed this measure using a LMM with treatment, DPT (a categorical variable), and their interaction as fixed factors and experiment block as the random factor. Then, total number of offspring was analyzed with a LMM including treatment, DPT (a categorical variable), and their interactions as fixed factors, and experiment block as a random factor. To test how number of mates affects overall male reproductive success, we modified the LMM analyzing the total number of offspring to include the number of mates and interaction terms involving the number of mates along with other variables included in the previous model as fixed factors. The relationship between number of mates and total number of offspring in the two treatments was compared with the *lstrends()* function in the *emmeans* package and *p* values were adjusted with the Holm-Bonferroni method.

*Replenishment of Seminal Fluid Proteins*

To investigate if the fungal infection affects the SFP replenishment rate after repeated mating, we compared the gene expression level of the SFPs in infected and sham-treated males from the assay described in the *Reproductive Potential* subsection, i.e., after they have mated with multiple females. At the end of the mating period, each male was transferred to a fresh food vial and kept for 24 h before being collected, snap-frozen in liquid nitrogen, and transferred to –80°C until RNA extraction. As each sample only contained a single fly (small biomass), in this assay, the total RNA was extracted using the RNeasy Micro Kit (#74034, Qiagen GmbH) following the manufacturer's protocol. RNA sample was reverse-transcribed into cDNA using the PrimeScript RT$^{TM}$ reagent kit with gDNA Eraser (#RR047B, TaKaRa Bio). Ideally, 100ng RNA would have been used for the cDNA conversion but in some samples, this amount was not obtained, so the maximum amount of RNA was taken (range = [51ng,100ng], mean = 91.52 ng). Each cDNA sample was then diluted 10-fold. RT-qPCR was performed and relative expression was calculated in the same way as described in *Activation of the Immune System.* Primers for the reference genes and SFPs used in the experiment are listed in Table S 1-1.

Reflecting the small amount of material obtained from single males and the individual variation, the SFP expression estimates were quite variable, with several apparent outliers. To identify the outliers, we fitted a LMM to $\log_2$ expression levels of each SFP, with treatment, DPT, and their interaction as fixed effects, and block as a random effect. From this model we obtained externally Studentized residuals with the *rstudent()* function of the *stats* package (v.4.1.2). Across the five SFPs, we removed six data points (out of 755) with Studentized residuals of an absolute value greater than 3.7. Under Student's *t* distribution with the number of degrees of freedom of the model ($df = 143$) and sample size ($N = 151$), the likelihood of obtaining one or more values above this threshold is $p < 0.05$.

To test whether *M. brunneum* infection affects SFP replenishment and whether different SFPs respond differently, we analyzed the relative gene expression of all five SFPs jointly. We fitted a LMM to $\log_2$-transformed relative gene expression, with the identity of the SFP, treatment (infected vs. sham-treated), day post treatment (i.e., day of mating; a categorical variable), and their interactions as fixed effects; male identity nested in experiment block was included as a random effect.

Males used in this assay had mated a variable number of times, and the number of matings should affect SFP depletion and thus likely the investment in SFP replenishment. This may not only add variation to the SFP gene expression data but could also cause systemic differences between infected and sham-treated males without infection affecting the capacity to invest in SFP investment if these two groups mated with a different number of females. We thus tested the relationship between a male's investment in SFP replenishment and the number of females it had mated with 24 hours prior to being collected. To facilitate this analysis, we combined the expression of all five SFPs into a single index. To obtain the index, log2 expression values of each SFP were zero-centered (by subtracting the mean) and scaled by dividing by the residual standard deviation from each SFP-specific model (which included relative expression of each SFP as the response variable, number of mates, DPT, treatment and their interactions as fixed factors and experiment block as the random factor). The index was then calculated by averaging these scaled values across the SFPs. By using the residual standard deviation from the SFP-specific models, we took into account the different characteristics of different SFPs while using a combined index. We fitted a LMM with the combined SFP expression index as the response variable, number of mates

(centered on the mean), DPT, treatment, and their interactions as fixed factors, and experiment block as a random factor. The relationship between number of mates and SFP replenishment of the two treatments was compared with the *lstrends()* function in the *emmeans* package and *p* values were adjusted with the Holm-Bonferroni method.

*Statistical Analysis*

All statistical analyses described above were done with R (v. 4.1.2) and R studio as IDE. Visualization of the results was conducted with package *ggplot2* (v. 3.4.1). Statistics of the (generalized) linear mixed models were attained using the *mixed()* function within the *afex* package (v.1.0-1) and *p* values were calculated using the likelihood ratio test.

## Results

### Post-infection Mortality

For both females and males, there was a pathogen incubation period of about 3-6 days following the infection treatment (i.e., a period when fungal proliferation has not yet caused any mortality; Figure 1-1). Mortality was dose-dependent, increasing with the concentration of *M. brunneum* spores (Figure 1-1; LRT, dose, $\chi^2_2 = 99.8$, $p < 0.001$; Table S 1-2). At any given dosage, males had a lower mortality rate than females (Figure 1-1; sex, $\chi^2_1 = 33.4$, $p < 0.001$; Table S 1-2), suggesting that males were less susceptible to *M. brunneum* infection than females.

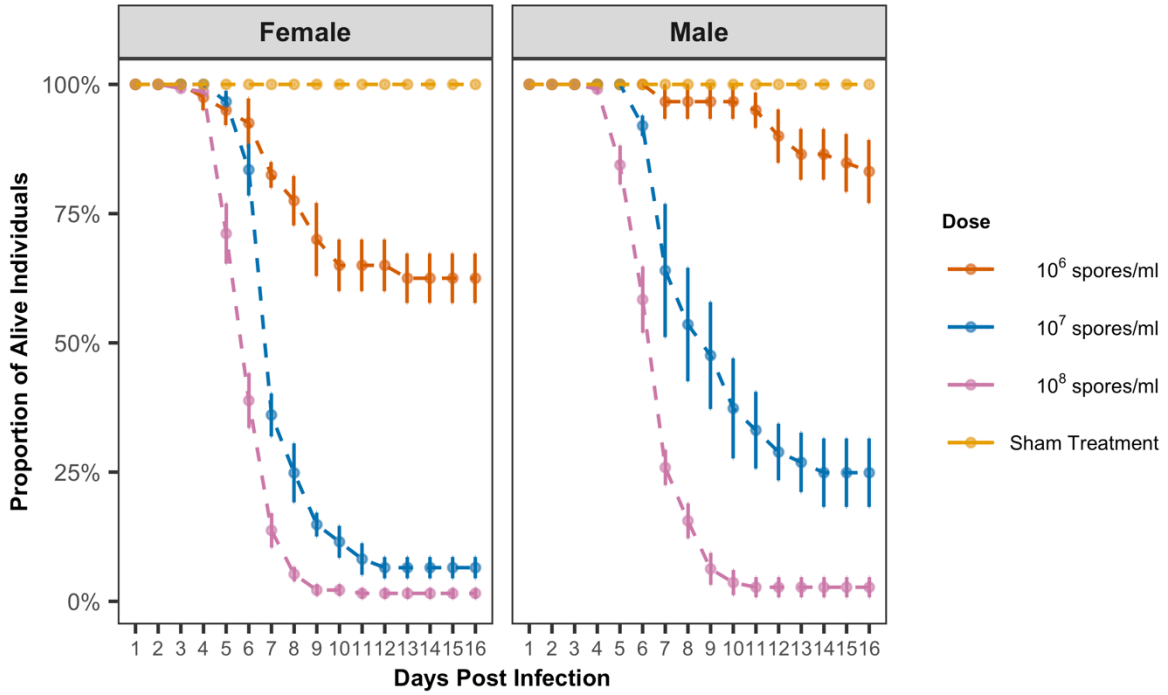


*Figure 1-1 Post infection survival of flies following infection with different pathogen doses (concentrations of spore suspension). Symbols are means ± SE.*

## Activation of the Immune System

We examined the expression of *Drosomycin* (active against fungi and Gram-positive bacteria) and *Diptericin A* (primarily induced by Gram-negative bacteria) following the *M. brunneum* infection. Following treatment, the level of *Drosomycin* expression within infected males increased as the infection progressed (treatment, $\chi^2_1 = 80.5$, $p = 0.002$, treatment × day post treatment, $\chi^2_1 = 59.2$, $p < 0.001$; Table S 1-3) and became significantly higher than that of the sham-treated males starting from day 2 post infection (Figure 1-2A). The expression of *Diptericin A* also increased over time (treatment, $\chi^2_1 = 4.3$, $p = 0.039$, treatment × day post treatment, $\chi^2_1 = 7.8$, $p = 0.005$; Table S 1-3) and infected males had a higher level of the *Diptericin A* expression starting from DPT 4 (Figure 1-2B). Different dosages of *M. brunneum* spores activated the AMP expression to a similar magnitude (Figure S1-1A, Figure S1-1B; Table S1-4).

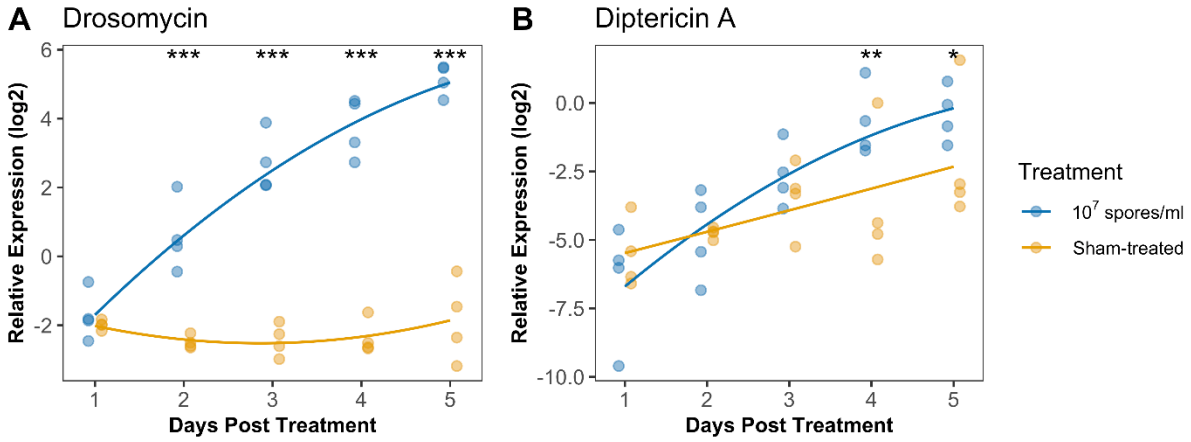


*Figure 1-2 Relative expression of **A**: Drosomycin and **B**: Diptericin A after Metarhizium infection ($10^7$ spores/ml) or sham treatment. Data are from **Immune Assay 1**. Each dot represents a sample of 8 males. Solid lines demonstrate the predicted values from the linear mixed models; significance levels from pairwise comparisons are shown: ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$.*

## Mating Ability and Latency

No mortality due to infection was observed during the mating trials, which was consistent with the mortality of males infected with $10^7$ spores/ml not starting before day 6 post infection (Figure 1-1). Nearly all infected males (≥ 90%) mated within the 2 h observation period; even on day 5 post infection ~90% of the infected males mated, implying that infection has little effect on males' ability to mate during pathogen proliferation (Figure 1-3; Figure S1-2).

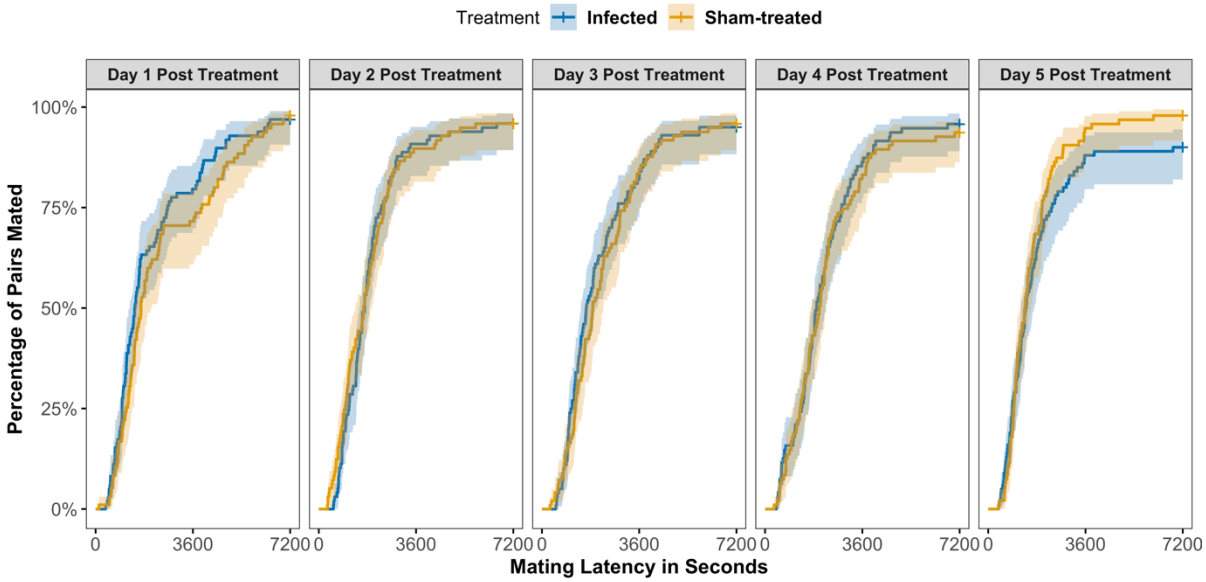


*Figure 1-3 Mating latency of females (a proxy for male sexual performance) when paired with an infected or sham-treated male. Flies not mated within the 2h observation are censored and labeled as crosses (+) in the plot. Shadow indicates the 95% confidence interval.*

Nonetheless, we detected a significant interaction between day post treatment (DPT) and treatment on the mating latency (Cox proportional hazard model with mixed effects, day post treatment × treatment, $p = 0.034$; Table S 1-5), implying that the progression of the infection did have a slightly negative effect on this aspect of male sexual performance. Although the ratios of mating rate were not statistically different from 1 on any day (Figure 1-4), there is a noticeable trend suggesting that on day 1 post treatment infected males may have performed better than the sham-treated males (ratio of mating rate: 1.237, 95%CI: 0.927 – 1.649, $p = 0.148$), but on day 5 post treatment the trend was inversed (ratio of mating rate: 0.794, 95%CI: 0.593-1.064, $p = 0.122$).

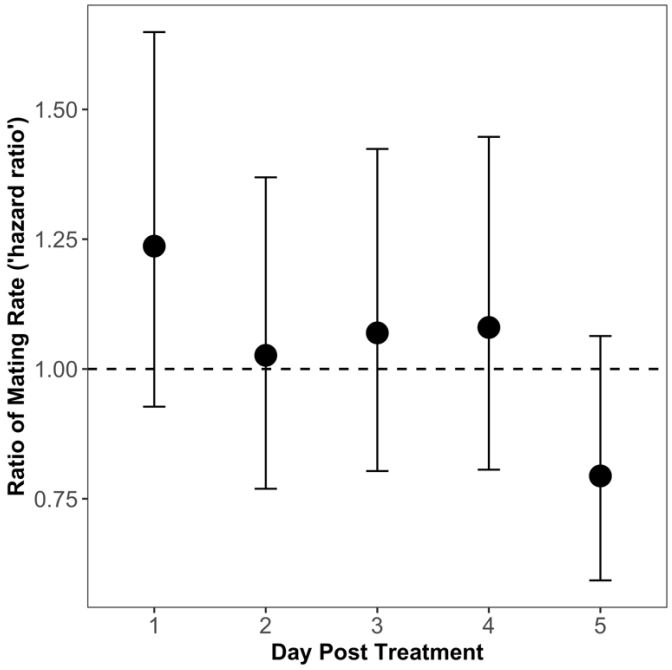


*Figure 1-4 Estimated ratio of mating rate (contrasting Infected over Sham-treated on each day post treatment) and its 95% confidence interval from the mixed effects Cox's proportional hazards regression model.*

## Reproductive Potential

No male mated with all 10 virgin females within the mating period, implying that this number of available mates was sufficient to assess the males' maximum mating potential. Most males in the experiment mated with 4 to 8 females; only one male mated with 3 females and two mated with 9 females. Number of females successfully inseminated by the male (i.e., those that produced at least one offspring, referred to as number of mates) and the number of offspring per mated female (Figure 1-5A,B) are two key factors contributing to male overall reproductive output. Although both components showed a trend for lower means in infected males (Estimated Marginal Means (EMM) ± SE, proportion of mated females: infected 59.5 ± 1.8%, sham-treated 63.3 ± 1.8%; number of offspring per mated female, infected 37.4 ± 0.681, sham-treated 39.1 ± 0.696), neither difference was statistically significant (Table S 1-6). Nonetheless, when the two components were combined in a measure of total offspring production, infected males sired on average 10.6% fewer offspring compared to sham-treated males (EMM ± SE, infected, 220 ± 5.40, sham-treated, 246 ± 5.51; treatment, $\chi^2_1 = 11.4$, $p < 0.001$; Figure 1-5C). We did not detect any significant interaction between treatment and DPT (Table S 1-6), indicating that the effects of infection did not change significantly as infection advanced. Although we did not find significant effects of the three-way

interaction between DPT, treatment, and number of mates on number of offspring (Table S 1-6), the relationship between the number of mates and the number of offspring (i.e., the Bateman gradient) appeared to differ between infected and sham-treated males on day 5 post-treatment (Figure 1-5D; pairwise comparison, $p = 0.039$, adjusted $p = 0.117$). Specifically, we observed that infected males had a significantly flatter slope than sham-treated males, suggesting a decrease in the efficiency of male translation of mating opportunities into actual offspring as the number of mating increases.

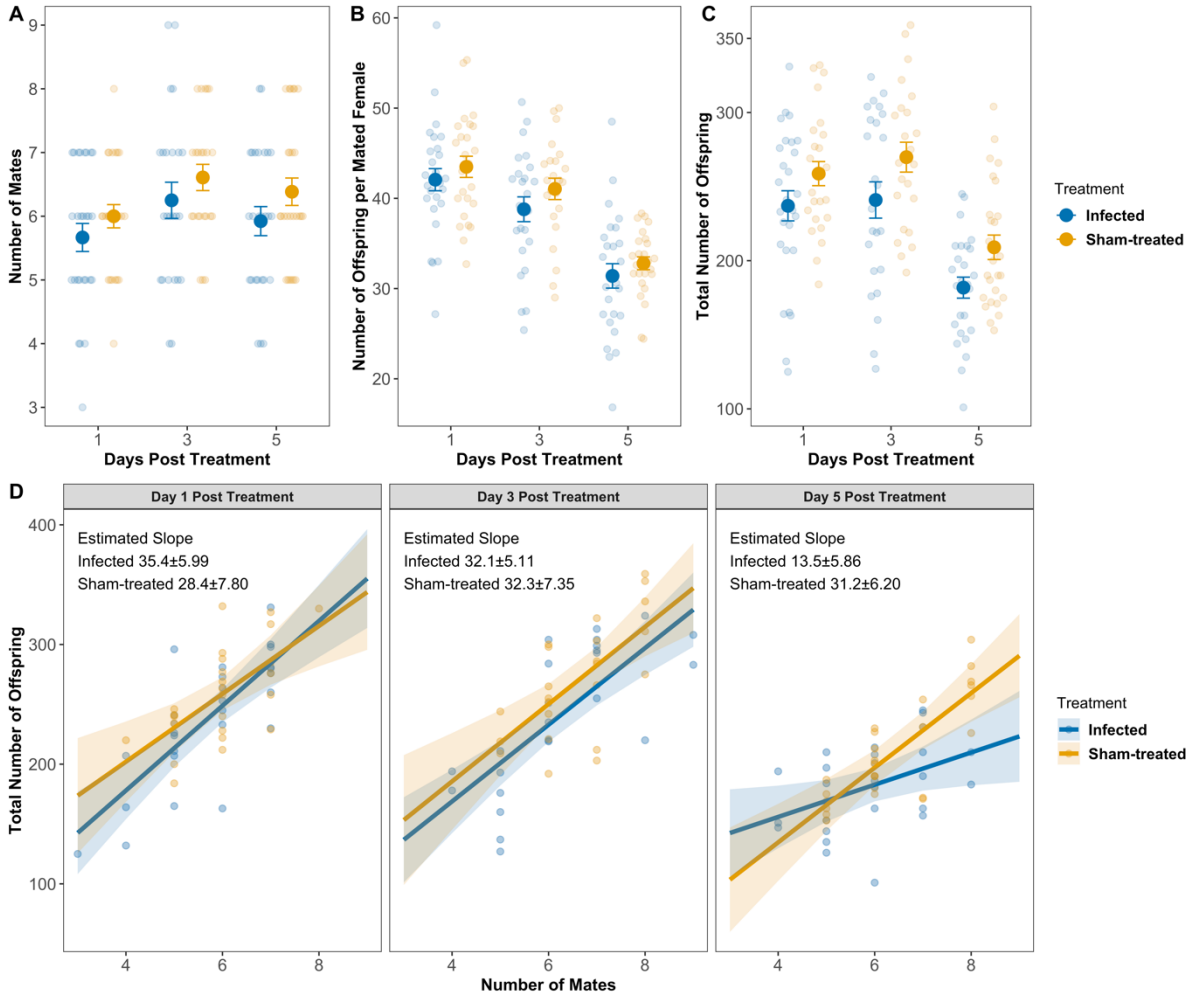


*Figure 1-5 Reproductive potential of infected males compared to the sham-treated males on day 1, 3, 5 post treatment. **A**: Number of females successfully inseminated by infected and sham-treated males (i.e., number of mates); **B**: Number of offspring per mated female; **C**: Total number of offspring sired by infected and sham-treated males; **D**: Relationship between number of mates and the total number of offspring sired by each male. Each transparent dot represents one male. In **A**-**C**, symbols demonstrate the mean ± SE. In **D**, the solid line demonstrates the estimated marginal means, and its 95% confidence interval is indicated by shadow. The slope of the relationship for infected males is significantly lower than that for the sham-treated males on day 5 post treatment ($p = 0.039$, adjusted $p = 0.117$).*

## Replenishment of Seminal Fluid Proteins

Despite their different overall expression levels (*SP* > *Acp36DE* > *Acp26Aa* > *Acp62F* > *Acp29AB*), the five seminal fluid proteins (SFPs) demonstrated consistent gene expression differences between infected and sham-treated males (treatment × SFP, $\chi^2_4 = 2.1$, $p = 0.71$, treatment × SFP × day post treatment, $\chi^2_8 = 3.9$, $p = 0.86$; Table S 1-7). In general, infected males had a lower level of SFP expression compared to sham-treated males (treatment $\chi^2_1 = 4.1$, $p = 0.043$; Figure 1-6). However, the impact of infection on SFP expression did not seem to increase with time since infection as indicated by the insignificant two-way interaction between day post treatment and treatment (day post treatment × treatment, $\chi^2_2 = 2.3$, $p = 0.32$; Figure 1-6).

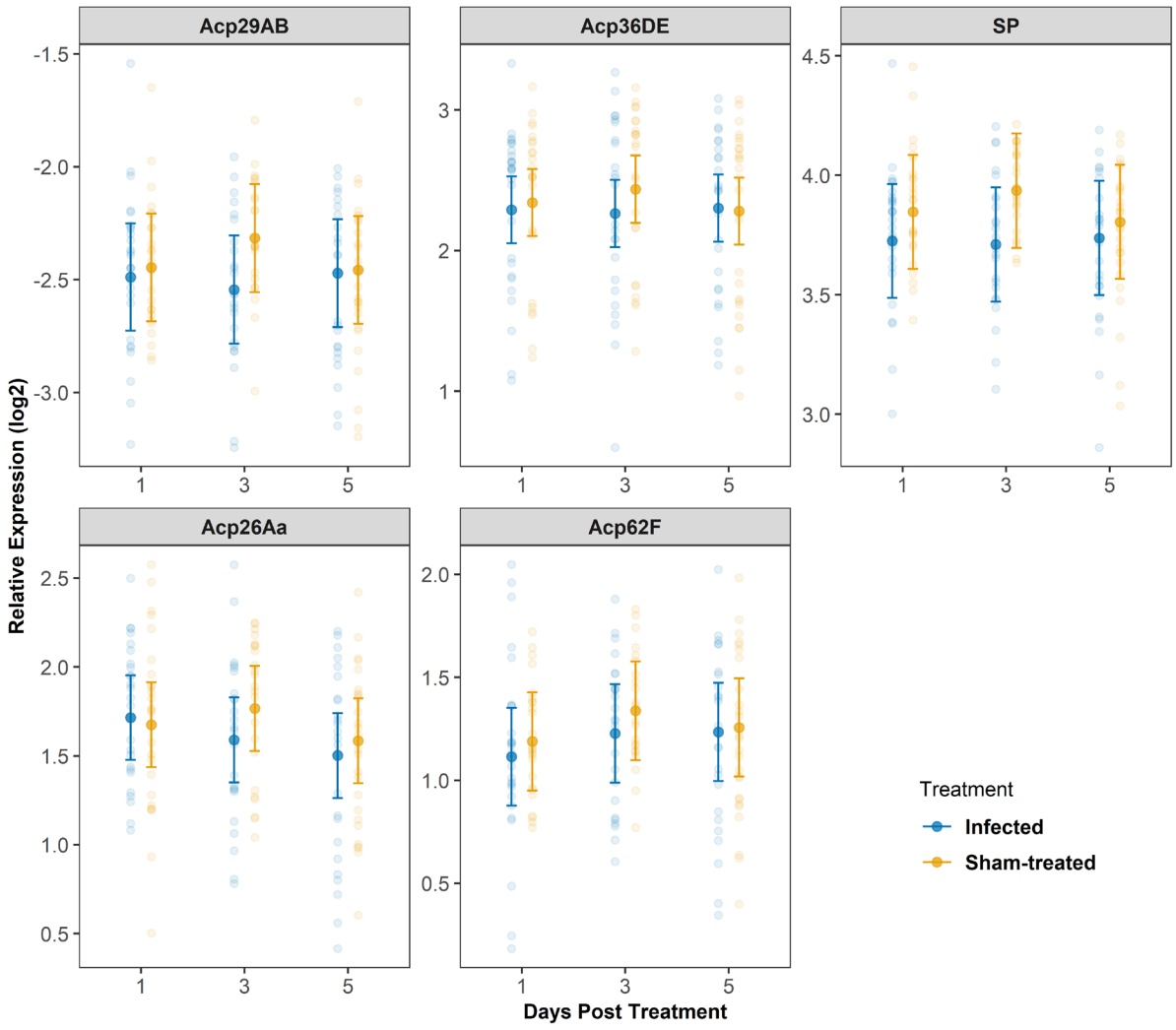


*Figure 1-6 Relative expression of seminal fluid protein genes in infected and sham-treated males after repeated mating (a proxy for SFP replenishment). Each transparent dot represents one male. Symbols are estimated marginal means ± SE.*

When the number of mates the male had mated with was taken into account, the overall investment in SFP replenishment (quantified by a combined SFP expression index) increased with the number of mates the male had mated with 24 h earlier (number of mates, $\chi^2_1 = 6.3$, $p = 0.012$; see Table S1-8 for full model output). However, this relationship was different between the treatments (treatment × number of mates, $\chi^2_1 = 4.3$, $p = 0.038$; Figure 1-7, Figure S1-3): infected males had higher overall SFP expression as number of mates increased ($t_{74} = 3.7$, $p < 0.001$; Table S1-9), while no consistent pattern was detected for sham-treated males ($t_{71} = 0.8$, $p = 0.45$; Table S1-9). Even when the number of mates was taken into account, the predicted SFP expression index value at the point corresponding to the mean mating success (mean number of mates = 6.1) was lower

for infected than sham-treated males (treatment, $\chi^2_1 = 5.4$, $p = 0.020$; Table S1-8). It demonstrates that, among males that achieved few matings, the infected males had lower SFP expression index than sham-treated males, but the difference vanished among males that were more sexually successful.

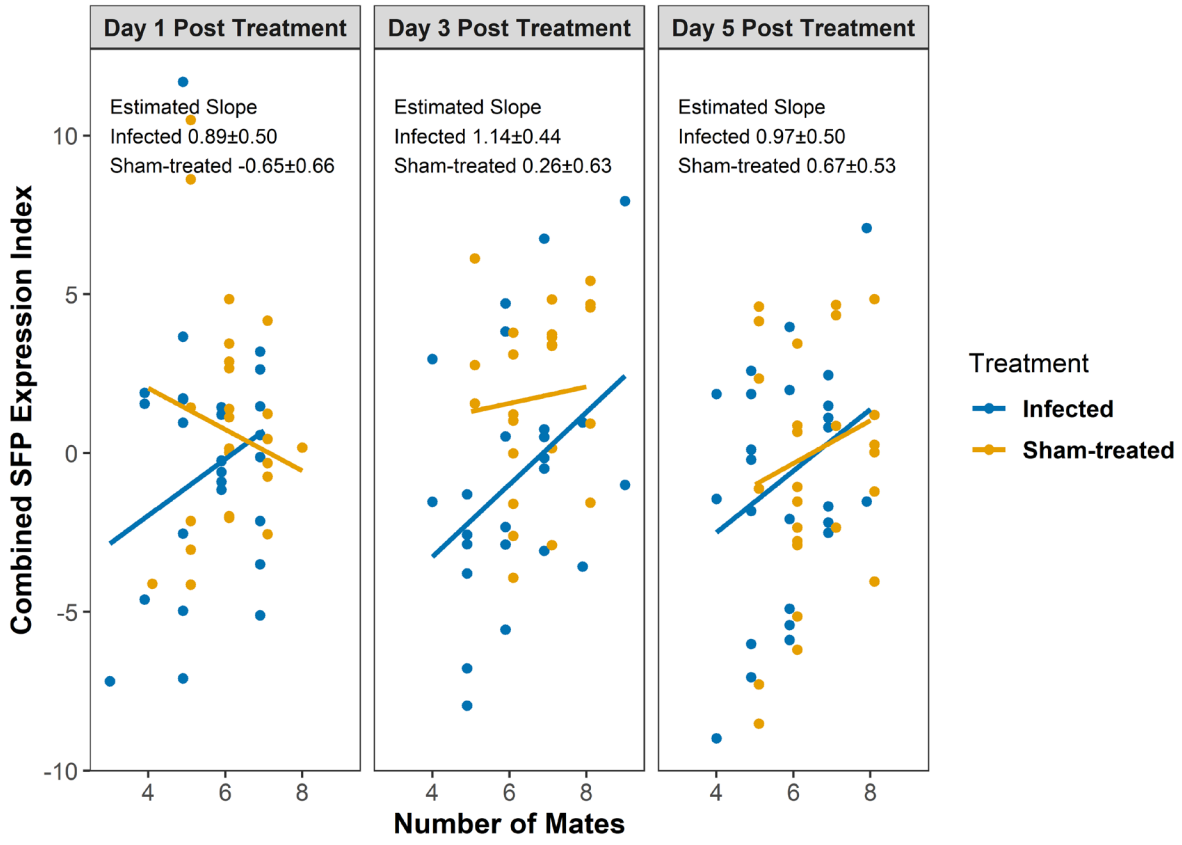


*Figure 1-7 Relationship between number of mates and combined SFP expression index for the infected and sham-treated males. Each dot represents one male. Solid lines are plotted with the fitted values from the linear mixed models and estimated slopes ± SE are indicated.*

## Discussion

Consistent with reported lethality in a broad range of insects (St. Leger and Wang, 2020), infection by *Metarhizium brunneum* induced high adult morality in our *D. melanogaster* population. Females were more susceptible than males, a finding that aligns with the male-biased survival observed in previous studies involving fungal infection of *Drosophila* with *Beauveria bassiana* (Taylor and Kimbrell, 2007) and *M. anisopliae* (Lu et al., 2015). Mortality did not occur until several days after infection, as has been shown for other *D. melanogaster* populations (Wang et al., 2017) and other insect species (Clifton et al., 2019, Cossentine et al., 2016).

Yet, within 38 hours of infection, the host immune system was already strongly activated and continued to mount an increasing response, as indicated by the increasing level of *Drosomycin* expression, reaching more than 100-fold the level of sham-treated flies. This increasing level of immune responses over time is consistent with continuing fungal proliferation within the host. While the course of mortality following fungal infection was dose-dependent, the degree of the immune response – at least in terms of AMP gene expression – appeared not to be. This implies that the spore concentration ($10^7$ spores/ml, $LT_{50}$ for males 9 days post infection) used in the remaining experiments was sufficient to induce the maximum level of host immune response against fungal infection. Previous studies looking at the *Diptericin A* expression (active against gram-negative bacteria) after either injection or natural infection with fungi have shown that *Diptericin A* is also strongly induced by the fungal challenge (Lemaitre et al., 1997, Hedengren-Olcott et al., 2004), even if *Diptericin A* does not appear to contribute any antifungal activity (Tzou et al., 2002b). Yet, in our experiment, the increase of *Diptericin A* was only seen at a later stage of the infection and was relatively small (about 2-fold that of sham controls). This suggests that the immune response to *M. brunneum* in our *D. melanogaster* population was largely confined to the Toll pathway, with little activation of the IMD pathway (Lemaitre and Hoffmann, 2007).

The fungal infection significantly reduced male total reproductive output in the absence of rival males and with more potential mates than appeared possible to mate with within the time available. This may be a result of less available resources after allocating to immune responses and being exploited by the fungus (Cressler et al., 2014). However, the reduction of reproduction success (~11%) reported here was rather small compared to other studies showing the negative relationship between parasitism and male reproductive success, for instance, about 56% reduction reported for

Taiwan field mice infested by mites (Lin et al., 2014) and about 42% for tapeworm-infected grain beetles (Worden et al., 2000). The two components of the overall reproductive success, number of mates and number of offspring per mated females, both tended to be lower in the infected males, but neither trend was significant, suggesting that they may have contributed to a similar degree to the reduced overall reproductive output. As indicated by the similar mating latency and the comparable number of mates, females did not discriminate strongly against infected males as potential mates, at least in the absence of alternatives. This implies that despite investing in a strong immune response, infected males still managed to provide a satisfying courtship display (Rose et al., 2022) and did not emit any aversive sensory (e.g., olfactory) cues.

The fungus growing within the host not only consumes host resources but also inflicts host damage by releasing metabolites like toxins (St. Leger and Wang, 2020, Butt et al., 2016). Particularly approaching the end of the fungal proliferation, filamentous growth starts and causes serious tissue damage to the host (Castrillo et al., 2005, Hajek and St. Leger, 1994). One would expect that if there were negative impacts of infection on males, the effects would appear several days before death and be more profound at the later stage of the infection. Contrary to this prediction, we found no evidence of increasing negative effects of the infection over time, affecting neither total reproductive output nor its two key components. Nevertheless, we still detected some signs of declining performance of infected males appearing progressively as the infection advanced. The average time taken for infected males to convince females to mate somewhat increased, suggesting a lower sexual performance over the days. Moreover, compared to the sham-treated males, infected males exhibited a shallower increase in the number of offspring sired as the number of mates increased at the end of the incubation period. This less efficient conversion of mating success to offspring may be a result of faster depletion of sperm or more likely seminal fluid proteins in infected males: SFPs are typically depleted before sperm in *Drosophila* (Hihara, 1981, Hopkins et al., 2019). A previous study has shown that approximately 30-35% of the SFPs are transferred to females at the first mating (Ravi Ram et al., 2005), and Sirot *et al* (2009) have demonstrated a significant decrease in SFP transfer during three successive matings. Traits like the ability to restock SFPs are important in keeping the reproduction machine functioning effectively as SFP depletion will lead to substantially decreased male fertility and paternity assurance (Linklater et al., 2007, Hihara, 1981). Thus, upon repeated mating observed in our experiment (some males

mated with up to 9 females), males must replenish their supply of SFPs during and after repeated mating to maintain a high level of fertility.

In general, infected males had lower SFP expression than the sham-treated males after repeated mating. The five SFPs examined in our study were of different functions and abundance, but they changed in the same direction and at a similar magnitude after repeated mating, which is consistent with the fact that SFPs have coordinated gene expression (Mohorianu et al., 2018). Although the advancement of infection (represented by day post treatment in the analysis) did not affect the relationship between number of mates and overall SFP expression, we found a significant difference in this relationship when comparing infected males and sham-treated males. SFP expression was positively associated with the number of mates in infected males. While this is consistent with males who mated more having to invest more in SFP replenishment, this relationship was not observed in sham-treated males. A more parsimonious explanation is that infected males vary in the degree they are affected by the infection: those that can buffer the physiological cost of infection better are in better conditions and thus, they manage to obtain more mates and can afford to express more SFPs than males that are suffering more. To our knowledge, how infection affects SFP gene expression after repeated mating over the course of infection has never been reported in *Drosophila*. However, changes in the quantity and quality of SFPs have been reported in other stressful scenarios. For example, prolonged mite infestation leads to reduced SFP expression, a pattern not evident after brief exposure or in uninfected controls (Benoit et al., 2020). Additionally, it has been shown that as age advances, gene expression of the five representative SFPs decreases, and functions (and potentially quality) of SFPs also decline, both of which were accompanied by decreased male reproductive success (Koppik and Fricke, 2017, Sepil et al., 2020). Likewise, the reduced levels of SFP expression in infected males observed in our study may hinder their ability to stimulate female egg production and impair their competitiveness in sperm competition against other males, ultimately leading to lower reproductive success. (Perry et al., 2013, Wigby et al., 2020, Hopkins et al., 2019).

Altogether, the negative effects of fungal infection on male fertility and associated traits in our study were rather mild to undetectable compared to the level of mortality induced by the infection, and they did not markedly increase from day 1 to day 5 post infection – even though by day 7-8 many males would be dead. There are two potential explanations: (1) the infection initially

develops slowly and the physiological burden of the disease remains low until shortly before death, as shown in Lu *et al* (2015), and/or (2) the males compensate by sacrificing other potential future function, as predicted by terminal investment hypothesis. While our data do not allow us to distinguish between these explanations, the course of AMP expression indicates that the infection is a burden from early on, if not in terms of damage by the fungus itself, then at least in terms of costs of activation of immune defense, whether due to costs of synthesis of antimicrobial peptides (Gupta et al., 2022) or collateral damage (Bou Sleiman et al., 2015). It has been reported that virgin *D. melanogaster* females strongly upregulate the production of antimicrobial peptides in response to infection with a gram-negative bacterium (*Providencia*), while this is not seen in reproductively active females, which leads to their much faster mortality (Gupta et al., 2022). This response, seeming to be pathological in this infection context (Gupta et al., 2022), may represent an overreaction of a system evolved to balance the needs of immune defense and current reproduction, as opposed to the maximum activation of the immune system in virgin females. It is tempting to speculate that in the case of *M. brunneum* infection, during the early phases, infected males may also largely compensate for the negative effects of the pathogen infection to maintain mating ability and fertility, at the cost of precipitous mortality once a threshold is reached. If so, there would be little additional loss of reproductive fitness during the early stages of infection, suggesting that selection for resistance is in this case almost entirely mediated by mortality. However, while this result was unexpected, it still leaves scope for sexual selection to contribute to selection for resistance, particularly if the mild effects we observed become magnified in scenarios where multiple males compete for and are chosen by females.

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**Data Accessibility**

Data and R scripts will be made available on Zenodo (DOI: 10.5281/zenodo.10132327) upon acceptance. For peer review, these materials are provided as supplementary materials.

**Competing Interest**

The authors have declared no competing interests.

## Supplementary Materials

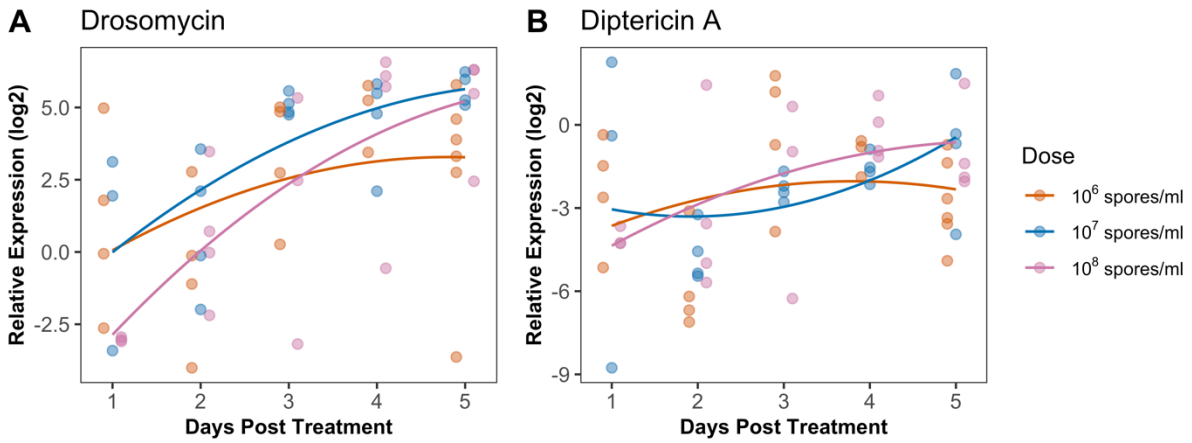


*Figure S1-1* ***Immune gene expression is not dose-dependent****. Relative expression (log$_2$ transformed) of* ***A:*** *Drosomycin and* ***B****: Diptericin A. Plotted data were from* ***Immune Assay 2*** *described in Activation of Immune System. Each dot represents a sample with 2-3 males. Solid lines demonstrate the predicted values from the linear mixed models.*

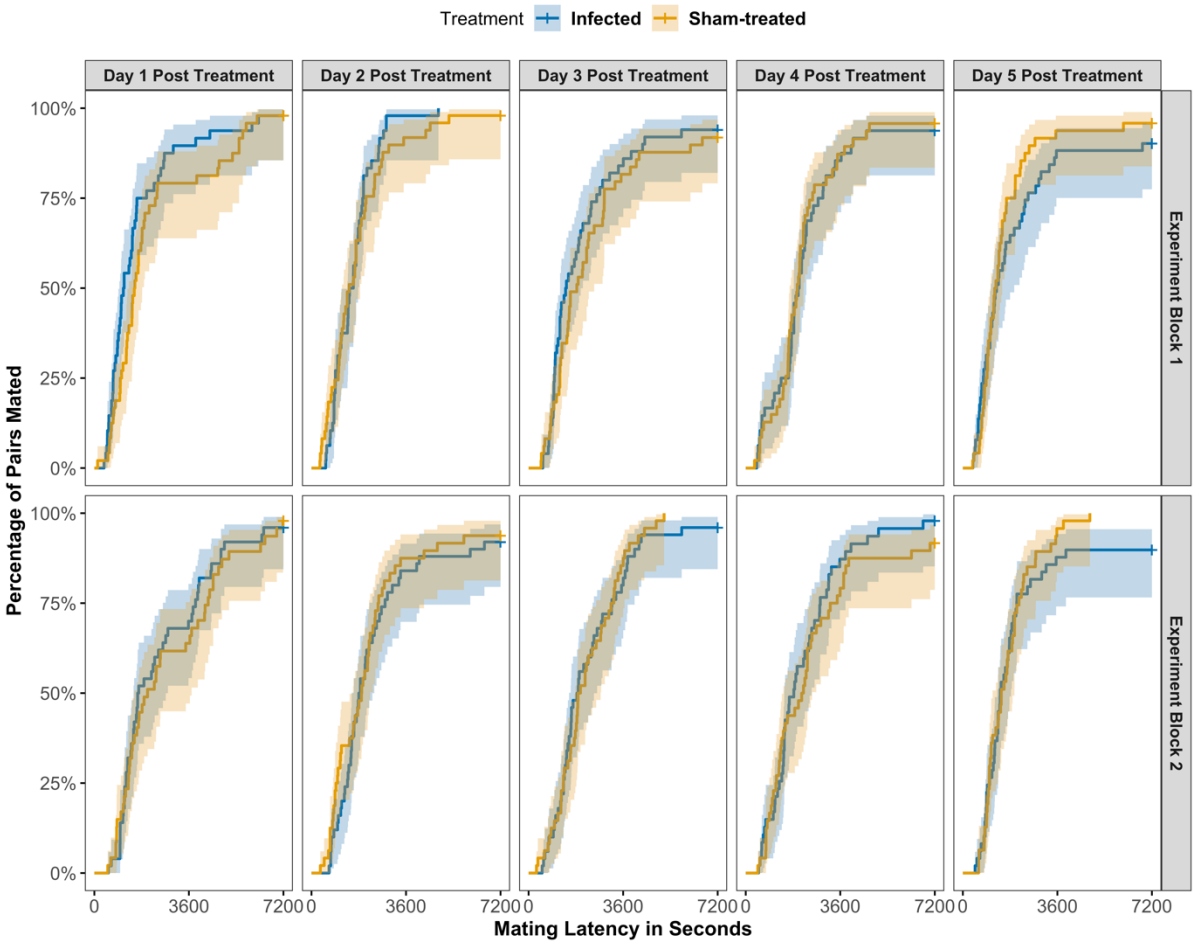


*Figure S1-2 Mating latency of females (a proxy for male sexual performance) when paired with an infected or sham-treated male. Flies not mated within the 2h observation are censored and labeled as crosses (+) in the plot. Shadow indicates the 95% confidence interval predicted by the Kaplan-Meier model.*

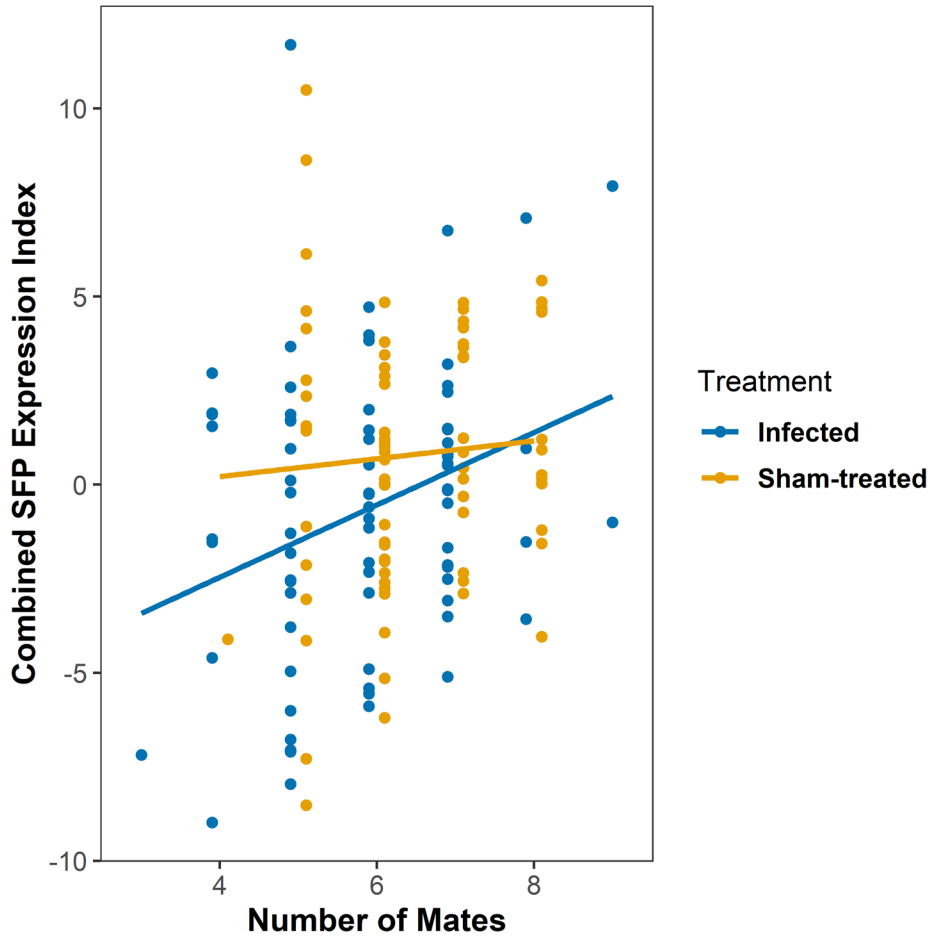


*Figure S1-3 Relationship between number of mates and combined SFP expression index for the infected and sham-treated males. Each dot represents one male and plotted data are from all three time points. Solid lines are plotted with the fitted values from the linear mixed models. See Table S9 for estimated coefficients from the linear mixed model.*

*Table S 1-1 Primer overview*

| | FlyBase ID | Sequence Forward Primer (5' -3') | Sequence Reverse Primer (5' -3') | Reference |
|---|---|---|---|---|
| **Reference Gene** | | | | |
| *RpL32* | FBgn0002626 | ATGCTAAGCTGTCGCACAAATG | GTTCGATCCGTAACCGATGT | |
| *eEF1α2* | FBgn0000557 | GCGTGGGTTTGTGATCAGTT | GATCTTCTCCTTGCCCATCC | |
| *αTub84B* | FBgn0003884 | TGTCGCGTGTGAAACACTTC | AGCAGGCGTTTCCAATCTG | Ponton et al. 2011 |
| **Immune Gene** | | | | |
| *Drosomycin* | FBgn0283461 | CGTGAGAACCTTTTCCAATATGAT | TCCCAGGACCACCAGCAT | |
| *Diptericin A* | FBgn0004240 | GCTGCGCAATCGCTTCTACT | TGGTGGAGTGGGCTTCATG | Leulier et al, 2003 |
| **Seminal Fluid Protein Gene** | | | | |
| *Acp29AB* | FBgn0015583 | CCACAAACGCCGCAAAATAC | AACGGCTGAAGCTGGATTTC | |
| SP | FBgn0003034 | TTCTTGGTTCTCGTTTGCGT | CTTATCACGAGGATTGGGGC | |
| *Acp26Aa* | FBgn0002855 | GCTCTCCAATTTTACTGCTGC | TCGCCCTTTTTCGCATCTTT | |
| *Acp36DE* | FBgn0011559 | TGGTGCCCAGTGAGTCTTTT | TGTGAAGACTCGGGCTTTGG | |
| *Acp62F* | FBgn0020509 | GACGGAGTGTCCTGTAGCAT | TATCCCGGCTTACACACACA | Koppok and Fricke, 2017 |

*Table S 1-2 Effects of the fixed factors based on the Likelihood Ratio Test. The generalized linear mixed model (binomial distribution, logit link) analyzing the post infection survival includes day post infection, dose, sex, and their interactions as fixed factors and vial identity as the random factor. Significant effects ($p \leq 0.05$) are highlighted in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| **Day Post Infection** | **2245.5** | **1** | **< 0.001** |
| **Dose** | **99.8** | **2** | **< 0.001** |
| **Sex** | **33.4** | **1** | **< 0.001** |
| **Day Post Infection × Dose** | **238.3** | **2** | **< 0.001** |
| **Day Post Infection × Sex** | **8.5** | **1** | **0.004** |
| Dose × Sex | 5.3 | 2 | 0.071 |
| **Day Post Infection × Dose × Sex** | **23.6** | **2** | **< 0.001** |

*Table S 1-3 Effects of the fixed factors on the expression of AMPs based on the Likelihood Ratio Test. The linear mixed model predicting the relative gene expression of Drosomycin and Diptericin A (Infected males were treated with **$10^7$** spores/ml M.brunneum; Data from **Immune Assay 1**). Significant effects ($p \leq 0.05$) are highlighted in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| ***Diptericin A*** | | | |
| **Treatment** | **4.3** | **1** | **0.039** |
| **Day Post Treatment** | **39.8** | **1** | **< 0.001** |
| I(Day Post Treatment^2) | 0.8 | 1 | 0.383 |
| **Treatment × Day Post Treatment** | **7.8** | **1** | **0.005** |
| Treatment × I(Day Post Treatment^2) | 0.8 | 1 | 0.364 |
| **Drosomycin** | | | |
| **Treatment** | **80.5** | **1** | **< 0.001** |
| **Day Post Treatment** | **62.1** | **1** | **< 0.001** |
| I(Day Post Treatment^2) | 0.3 | 1 | 0.612 |
| **Treatment × Day Post Treatment** | **59.2** | **1** | **< 0.001** |
| **Treatment × I(Day Post Treatment^2)** | **7.8** | **1** | **0.005** |

*Table S1-4 Effects of the fixed factors based on the Likelihood Ratio Test. The linear mixed model predicting the relative gene expression of Drosomycin and Diptericin A (Infected males were treated with **$10^6$, $10^7$, $10^8$** spores/ml M.brunneum; Data from **Immune Assay 2**). Significant effects ($p \leq 0.05$) are highlighted in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| ***Diptericin A*** | | | |
| Dose | 1.2 | 2 | 0.550 |
| **Day Post Infection** | **8.6** | **1** | **0.003** |
| I(Day Post Infection^2) | 0.0 | 1 | 0.869 |
| Dose × Day Post Infection | 1.6 | 2 | 0.442 |
| Dose × I(Day Post Infection^2) | 1.7 | 2 | 0.423 |
| ***Drosomycin*** | | | |
| Dose | 1.7 | 2 | 0.429 |
| **Day Post Infection** | **28.0** | **1** | **< 0.001** |
| I(Day Post Infection^2) | 1.8 | 1 | 0.181 |
| Dose × Day Post Infection | 4.9 | 2 | 0.087 |
| Dose × I(Day Post Infection^2) | 0.0 | 2 | 0.987 |

*Table S 1-5 Effects of the fixed factors of Cox's proportional hazard mixed effect model. Significant effects ($p \leq 0.05$) are highlighted in bold.*

| Term | Chi Df | Chisq | Pr(>Chisq) |
|---|---|---|---|
| **Treatment** | **1** | **4.0** | **0.046** |
| Day Post Treatment | 1 | 0.5 | 0.494 |
| **Treatment × Day Post Treatment** | **1** | **4.5** | **0.034** |

*Table S 1-6 Effects of the fixed factors in (generalized) linear mixed models analyzing different components of the male's reproductive success based on the Likelihood Ratio Test. Significant effects (p <= 0.05) are highlighted in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| **Number of Offspring** | | | |
| **Day Post Treatment** | **17.3** | **2** | **< 0.001** |
| **Treatment** | **11.4** | **1** | **0.001** |
| Day Post Treatment × Treatment | 0.2 | 2 | 0.920 |
| **Number of Mates** | | | |
| Day Post Treatment | 3.7 | 2 | 0.156 |
| Treatment | 2.4 | 1 | 0.125 |
| Day Post Treatment × Treatment | 0.1 | 2 | 0.973 |
| **Number of Offspring per Mated Female** | | | |
| **Day Post Treatment** | **20.0** | **2** | **<0.001** |
| Treatment | 3.1 | 1 | 0.077 |
| Day Post Treatment × Treatment | 0.2 | 2 | 0.918 |
| **Number of Offspring (model including Number of Mates)** | | | |
| **DPT** | **81.9** | **2** | **<0.001** |
| **Treatment** | **6.4** | **1** | **0.011** |
| **Number of Mates** | **95.2** | **1** | **<0.001** |
| Day Post Treatment × Treatment | 0.4 | 2 | 0.799 |
| Day Post Treatment × Number of Mates | 3.5 | 2 | 0.173 |
| Treatment × Number of Mates | 0.5 | 1 | 0.467 |
| Day Post Treatment × Treatment × Number of Mates | 4.4 | 2 | 0.112 |

*Table S 1-7 Effects of the fixed factors based on the Likelihood Ratio Test. The linear mixed model predicting the relative gene expression of the seminal fluid protein (SFP) is with the following independent variables: day post treatment (DPT), identity of SFPs, male treatment (infected vs. sham-treated) and all the possible two-way and three-way interactions of three variables. Significant effects (p <= 0.05) are highlighted in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| DPT | 1.4 | 2 | 0.487 |
| **Treatment** | **4.1** | **1** | **0.043** |
| **SFP** | **2924.4** | **4** | **<0.001** |
| DPT × Treatment | 2.3 | 2 | 0.320 |
| DPT × SFP | 13.4 | 8 | 0.099 |
| Treatment × SFP | 2.1 | 4 | 0.711 |
| DPT × Treatment × SFP | 3.9 | 8 | 0.863 |

*Table S1-8 Effects of the fixed factors on the combined SFP expression index based on the Likelihood Ratio Test. The linear mixed model predicting values of the combined SFP expression index is with the following independent variables: day post treatment (DPT), number of mates, treatment (infected vs. sham-treated), and their interactions as fixed factors and experiment block as the random factor. Significant effects (p <= 0.05) are highlighted in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| **Model with Number of Mates** | | | |
| DPT | 1.8 | 2 | 0.414 |
| **Treatment** | **5.4** | **1** | **0.020** |
| **Number of Mates** | **6.3** | **1** | **0.012** |
| DPT×Treatment | 3.8 | 2 | 0.151 |
| DPT×Number of Mates | 1.9 | 2 | 0.383 |
| **Treatment×Number of Mates** | **4.3** | **1** | **0.038** |
| DPT×Treatment×Number of Mates | 1.4 | 2 | 0.509 |

*Table S1-9 Estimated slopes of the relationship between number of mates and the combined SFP expression index for infected and sham-treated males (supplementary to Figure S3). Data are from males of all three days post treatment. Linear mixed models used for extracting the estimated coefficients are with combined SFP expression index as the response variable, number of mates as the fixed factor, and experiment block as the random factor. Intercepts represent the estimates when the number of mates is at the mean level (mean number of mates=6.1). Significant terms (except intercept) are highlighted in bold.*

**Estimates of Coefficients**

| Male Treatment | | Estimate | Std. Error | df | t value | Pr(>\|t\|) |
|---|---|---|---|---|---|---|
| Infected | (Intercept) | -0.41 | 1.67 | 2.00 | -0.25 | 0.830 |
| | **Number of Mates** | **0.97** | **0.27** | **74.06** | **3.68** | **<0.001** |
| Sham-treated | (Intercept) | 0.71 | 1.39 | 2.00 | 0.51 | 0.661 |
| | Number of Mates | 0.26 | 0.34 | 71.08 | 0.77 | 0.445 |

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# Chapter 2 Navigating through the pathogen jungle: Pathogen avoidance of *Drosophila melanogaster* females and its consequences on offspring fitness

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Abstract

Pathogen avoidance acts as the first-line host defense. Avoiding pathogens during oviposition not only reduces female's own infection risk but also increases offspring's survival probability. Yet, environmental constraints sometimes make avoidance infeasible. The fitness consequences of raising young in pathogen-contaminated environments and whether potential fitness penalty could be mitigated if offspring also present a certain level of pathogen avoidance, remains an open question. We tested whether *Drosophila melanogaster* females actively avoid sites with the fungal pathogen *Metarhizium brunneum* during oviposition, and whether failing to avoid pathogens induces a fitness cost. We carried out multiple two-choice assays varying the source of infection risk (fungal spores or infection-killed conspecifics) and independently measured the viability of eggs laid on potentially infectious surfaces. We also tested whether third-instar larvae exhibit pathogen avoidance behaviors. We found that females had a stronger preference for pathogen-free oviposition sites but did not differentiate between sites with high doses of pathogens and low doses of pathogens. Interestingly, offspring raised on *M.brunneum*-contaminated food did not suffer from reduced viability but exhibited behaviors like higher pupation height and faster emergence, possibly indicating avoidance strategies. Yet, third-instar larvae did not actively avoid *M.brunneum*-contaminated food as seen in adult females. Our findings underline the need to consider multiple life stages to fully understand the consequences of pathogen avoidance.

## Introduction

The ability to perceive and avoid what could decrease fitness, like highly virulent pathogens, is important for living organisms. Pathogen avoidance, also referred to as behavioral immunity, serves as the first line of defense and is seen across different species (de Roode and Lefèvre, 2012, Curtis, 2014, Gibson and Amoroso, 2022). Individuals can enhance their fitness by actively avoiding pathogens, environments of infection risk, or infected conspecifics, especially when the infection cost outweighs the behavioral cost (Parker et al., 2011, Schaller and Park, 2011, Curtis, 2014, Sarabian et al., 2023). The level of pathogen avoidance varies with the mode of infection and the severity of infection. For example, female mandrills avoid feeding on any feces-contaminated food to reduce exposure to orofecally transmitted pathogens but show no avoidance of contaminated environment when they are not feeding (Poirotte et al., 2019). To be able to successfully avoid pathogens or anything that presents infection risk, individuals learn to utilize possible cues, both visible (e.g., presence of wounds, parasites) and invisible (e.g., chemical cues) (Kavaliers et al., 2022). However, pathogens are not always under the radar. The ectoparasitoid *Cephalonomia tarsalis* is not able to detect *Beauveria bassiana*-infected grain beetle larvae or free conidia, leading to a high larval mortality (Lord, 2001). *Drosophila melanogaster* is susceptible to a gram-negative bacterium *Pseudomonas entomophila* but does not show aversion towards *P.entomophila*-contaminated food (Babin et al., 2014). Moreover, some pathogens evolve to emit semiochemicals to attract hosts to facilitate their transmissions (Leroy et al., 2011). The pathogenic fungus *Entomophthora muscae* can lure healthy male houseflies to mate with a female corpse by releasing a volatile compound (Naundrup et al., 2022). Therefore, pathogen avoidance is not a universal phenomenon but rather pathogen-specific.

Successfully avoiding pathogens does not only benefit the current generation but also has transgenerational effects, with choice by the parents affecting the pathogen exposure of offspring. In many insect species, the oviposition decision of females decides the offspring's performance (Jeffries and Lawton, 2008, Thompson, 1988). Therefore, there should be a stronger selection for females laying eggs in an “enemy-free” environment in order to achieve higher offspring fitness. However, under environmental constraints and resource limitations, females may also lay eggs in pathogen-contaminated sites as a strategic move to maximize their reproductive output. Offspring performance in these unfavorable environments is also a factor influencing the level of pathogen

avoidance displayed by females during oviposition. Yet, to predict the fate of the offspring is not a straightforward task. First, pathogen avoidance is not restricted to a single life stage but can also be seen across various stages of development (Lopes et al., 2022). The cost of females not finding a pathogen-free environment for oviposition could be mitigated, for instance, when offspring also present pathogen avoidance during development. Second, the susceptibility to pathogens varies across life stages. Larvae can be much more susceptible than adults, as has been shown in grasshoppers (Kistner and Belovsky, 2013), or in some cases, larvae tolerate the infection until the adult stage (Keehnen et al., 2020) and larvae are more resistant than adults (Ramoutar et al., 2009). Many other factors contribute to offspring survival, like competition, food availability, etc. Therefore, reduced performance of offspring raised in pathogen-contaminated environments is also not always the case but underemphasized.

In this study, we used the fruit fly *Drosophila melanogaster* and the fungal pathogen *Metarhizium brunneum* as our experiment system to address the question of maternal pathogen avoidance and its fitness consequences. *M.brunneum* is a natural pathogen to many insects, including *D.melanogaster*, and it causes high mortality (St. Leger and Wang, 2020). It infects insects by attaching the spores to their surface, which then germinate, penetrate the cuticle with mechanical and enzymatic action, proliferate inside by consuming host nutrients, and eventually kill the host. The fungus sporulates from the corpse and the infection-killed individuals then become infectious and infect other flies in the proximity (Butt et al., 2016). Using *D.melanogaster* and *M.robertsii*, Keiser et al. (2020) find that females avoid infectious corpses but not spore-contaminated food patches, which demonstrates the ability of *D. melanogaster* females to detect the presence of *Metarhizium*. Larvae of *D.melanogaster* have the capacity to avoid certain pathogens like nematodes (Kunc et al., 2017), while they display no sign of avoidance against the *Drosophila C virus* (Siva-Jothy et al., 2018). However, it's not clear whether larvae would display avoidance behaviors when facing a fungal pathogen. In this study, we aim to investigate the following questions: (1) Do females actively avoid *M.brunneum*-contaminated sites (food with spores or infection-killed conspecifics) for oviposition? (2) Can females distinguish between sites of different infection risk (i.e., different pathogen abundance)? (3) What are the fitness consequences of females failing to avoid pathogen-contaminated sites? (4) Can pathogen avoidance also be observed during the larval stage?

## Materials and Methods

### Fly origin, pathogen origin, and infection protocols

We used flies from a lab-adapted outbred population (as previously described in Chapter 1). All flies were raised at a controlled density (~200 eggs per 40ml food) and maintained at 25°C, 55% relative humidity, and 12L:12D photoperiod on standard cornmeal-yeast-agar media. When virgin flies were required for the experiment, they were collected 6-8 hours post emergence and maintained in food vials until used in the experiment. Female virginity was further confirmed by the absence of larvae in the vials. All fly transfers were done under light $CO_2$ anesthesia.

The pathogen used in this experiment is *Metarhizium brunneum*. Pathogen origin and the infection protocols were previously described in Chapter 1.

### Pathogen avoidance in *Drosophila melanogaster* females (egg-laying decision)

To investigate whether females actively avoid pathogen-contaminated sites when deciding where to lay eggs, we performed a two-choice assay, allowing females to choose between pathogen-free sites and pathogen-contaminated sites. We put 10 3-day-old virgin females and 10 3-day-old virgin males into each oviposition chamber (Figure S 2-1). Each chamber had two small petri dishes (i.e. oviposition sites). The base of both petri dishes was orange juice agar and a sprinkle of baker's yeast was added to stimulate female egg laying.

First, females were given a choice between a pathogen-free oviposition site and an oviposition site with a high dose of spores ($10^8$ spores/ml), and between a pathogen-free oviposition site and that with a lower dose of spores ($10^6$ spores/ml). We evenly spread either 40µl spore-free 0.05% Triton X-100 or spore suspension (higher dose or lower dose) depending on the assigned treatment onto the food surface. The position of the petri dishes (left or right) in the chamber was also randomized. Flies in the chamber were allowed to mate and lay eggs for 24 hours, after which we counted the number of eggs on each petri dish. For each spore concentration treatment, we had 10 replicates.

Since there were two petri dishes in each chamber, our data was naturally paired. To understand whether females avoid laying eggs on pathogen-contaminated food, we calculated an avoidance index for each replicate. The avoidance index is calculated by first dividing the number of eggs

laid on pathogen-free food by the number of eggs laid on pathogen-contaminated food, and then log2-transforming the ratio. A zero logarithmic ratio (avoidance index) means that there is no pathogen avoidance by females when deciding where to lay eggs and an avoidance index >0 means that more eggs are laid on a pathogen-free site. To investigate whether females generally display avoidance against infectious sites, we pooled the two concentration treatments and only looked at the avoidance index of pathogen-contaminated sites versus pathogen-free sites. It has been shown that in *D.melanogaster*, females examine the environment and lay eggs in an egg-by-egg manner (Yang et al., 2008), so where to lay each egg is an independent event and has a binary outcome (pathogen-free environment versus pathogen-contaminated environment). Then to examine whether females lay more eggs on pathogen-free sites when choosing between a pathogen-free site and a site of a high dose of spores compared to when choosing between a pathogen-free site and a site of a lower dose of spores, we fitted the data to a generalized linear mixed model (GLMM, binomial distribution, logit link) with the number of eggs laid on pathogen-free site versus the number of eggs laid on pathogen-contaminated site as the response variable, concentration treatment as the fixed factor, and the chamber ID and the position of the pathogen-free site (left versus right) as the random factors.

To investigate whether females have stronger avoidance when choosing directly between a more-contaminated site and a less-contaminated site, we additionally set up 20 replicates with one petri dish brushed with a high dose of spores ($10^9$ spores/ml) and the other one brushed with a lower dose of spores ($10^6$ spores/ml). Then we compared the avoidance index of each replicate, which in this case, is calculated by first dividing the number of eggs laid on site of lower dose by the number of eggs laid on site of high dose, and then log2-transforming the ratio.

In a separate experiment, we also tested whether females avoid infection-killed conspecifics. As a pathogen-free choice, we used freeze-killed flies. Inside each oviposition chamber, we had one plate with freeze-killed flies and one plate with infection-killed flies. The position of the plate was randomized. As for the spore treatment, we also varied the number of dead flies (2 or 5) placed on the plate to see if females demonstrate stronger avoidance against oviposition sites of higher infection risk (i.e. plates with higher number of infection-killed flies). We had 10 replicates for each fly treatment. We first compared the avoidance index of each chamber, and the avoidance index is calculated by first dividing the number of eggs laid on site with freeze-killed flies by the

number of eggs laid on site with infection-killed flies, and then log2-transforming the ratio. Then to investigate whether females display a stronger avoidance when the number of flies on the plate increases, we performed a GLMM (binomial distribution, logit link) with the number of eggs on the site with freeze-killed flies versus the number of eggs on the site with infection-killed flies as the response variable, number of dead flies per plate as the fixed factor and the chamber ID and position of the site with freeze-killed flies as the random factors.

One-sample t test was done to check if each avoidance index was significantly different from 0. Effect of the fixed factor in the GLMMs was from the likelihood ratio test (LRT) with the *mixed()* function of the *afex* package (v.1.0-1). Estimated marginal means and their 95% confidence intervals were estimated by the *emmeans()* function of the *emmeans* package (v.1.7.1-1). *p* values were adjusted with the Holm-Bonferroni method.

**Fitness consequences of females failing to avoid *Metarhizium brunneum***

To understand the potential fitness consequence of females failing to avoid pathogens, we investigated the offspring's susceptibility to pathogen exposure. Maternal investments in offspring or simply the presence of moms will affect the offspring's performance (Wertheim et al., 2002). To investigate the direct effects of pathogens on offspring without the interference of maternal effects, here we started the experiment by placing 100 eggs directly from the outbred population on the center of the food surface. There were four types of *M.brunneum*-contaminated food: 400µl spore suspension of high concentration ($10^8$ spores/ml; referred to as Spore High), 400µl spore suspension of low concentration ($10^6$ spores/ml; Spore Low), 8 infection-killed conspecifics evenly distributed on the food surface (Infectious Fly High) and 3 infection-killed conspecifics evenly distributed on the food surface (Infectious Fly Low). Additionally, two types of controls were used: food with 400µl 0.05% Triton X-100 pipetted onto the surface (Triton X Control) as a control for the spore-contaminated food and 8 freeze-killed flies (Frozen Fly High) or 3 freeze-killed flies (Frozen Fly Low) evenly distributed on the surface as a control for infection-killed-fly-contaminated food. For each of the 6 treatment, we had 7 replicates. On day 6 after placing the eggs, we counted the number of pupae attached to the wall of the food bottle, and on day 9, 10, and 11, we counted the emerged flies. Egg-to-pupae viability is calculated by dividing the number of pupae by the number of eggs (i.e., 100) and egg-to-adult viability is calculated by dividing the

total number of emerged flies by the number of eggs. To investigate whether the egg-to-pupae and egg-to-adult viability are reduced when eggs are raised on contaminated food, we fitted the data to a GLMM (binomial distribution, logit link) with the number of pupae or the number of adults versus number of eggs that did not reach pupae or adult stage as the response variable, treatment as the fixed factor and food bottle ID as the random factor. This was done for the two sources of infection separately.

To understand whether offspring also present some level of pathogen avoidance during development by moving away from contaminated food surfaces, we also recorded the pupation height while counting the number of pupae. We divided the food bottle into 8 zones, where the food surface is level 0 and a new level every centimeter until level 7. Pupae found standing on the borders between two zones were grouped into the lower level. Mean pupation height is calculated by dividing total pupation height by the number of pupae. Counting emerged flies on three consecutive days allowed us to also examine the mean time to emergence (the normal emergence time of the original population is about 10 days) to investigate whether offspring raised on pathogen-contaminated food emerge sooner than those on pathogen-free food. We then fitted the data to a linear model (assumption on normal distribution is fulfilled) with either average pupation height or mean time to emerge as the response variable, and treatment as the explanatory variable. This analysis was done for the two sources of infection separately.

**Pathogen avoidance of third instar larvae**

To further investigate the avoidance behaviors at the larval stage, we performed a two-choice assay with early third-instar larvae as their brains are relatively developed at this stage and are able to make choices (Khurana and Siddiqi, 2013). We started the experiment by placing 16 third instar larvae on the center of a petri dish (diameter: 10cm), and they were allowed to move freely for 20 minutes to choose between the two available food blocks (normal fly food, 1cm×1cm×1cm). By the end of the 20-minute free-moving period, we counted the number of larvae around each food block. In total, we prepared 13 replicates, with the food block previously soaked in spore suspension on the center of one side and the food block soaked in pathogen-free 0.05% Triton X-100 on the other side. We then calculated an avoidance index for each replicate, which is calculated by first dividing the number of larvae on the side with pathogen-free food by the number of larvae

on the side with pathogen-contaminated food, and then log2-transforming the ratio. We did a one-sample t test to check if the avoidance index was significantly different from 0.

## Results and Discussions

### Females actively avoided pathogen-contaminated sites but did not have stronger avoidance against sites of higher infection risk.

The number of eggs on food with fungal spores (pooling both concentration treatments as sites of infection risk) was significantly less than that on the pathogen-free site (Figure 2-1A; One Sample t test, $t_{19} = 2.55$, $p = 0.020$). This result demonstrates that adult females actively avoid laying eggs on sites presenting infection risk and prioritize sites that are not, which is in line with studies on different host-pathogen pairs (Siva-Jothy et al., 2018, Meyling and Pell, 2006, Sadek et al., 2010). However, given the large number of eggs laid by females (average=719 eggs), females may present a certain level of aversion towards pathogen-free sites due to the perceived high potential of offspring competition on these sites, which blurs the actual magnitude of avoidance.

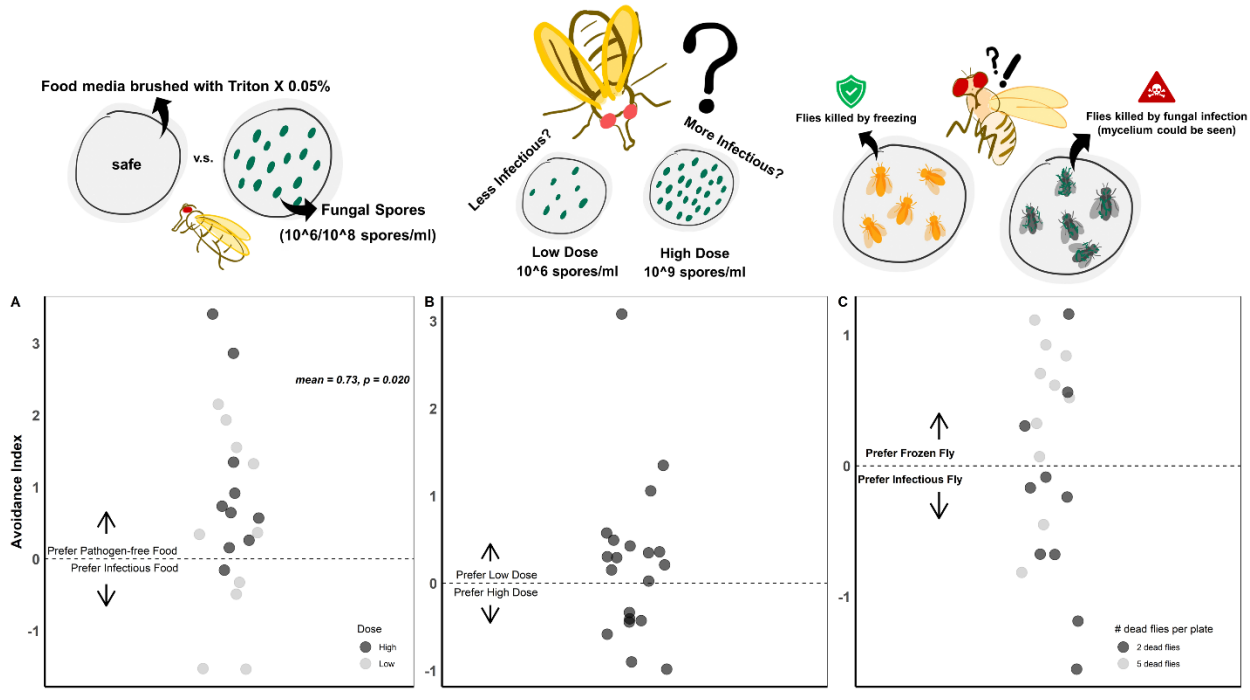


*Figure 2-1 Avoidance index of Drosophila females in the oviposition chamber. **A**: when they were given a choice between plates brushed with 0.05% Triton X-100 (pathogen-free) and plates with fungal spores ($10^6$ or $10^8$ spores/ml; also see Figure S 2-2); **B**: when they chose between a plate with high dose of spores and a plate with low dose of spores; **C**: when they were given a choice between a plate with infection-killed conspecifics and a plate with flies killed by freezing. Each dot represents an oviposition chamber.*

Fungal infection is initiated by physical contact with spores and the virulence of *M.brunneum* like other entomopathogenic fungi is dose-dependent (Hughes et al., 2004). It's likely that the female perception of risk is also dose-dependent. Although females presented a tendency to avoid the plate with a high dose of spore in a stronger way, compared to the avoidance of the plate with a lower dose of spore versus the non-infectious plate, this difference was not statistically significant (Figure S 2-2). Similarly, when females were given a direct choice between a highly infectious environment ($10^9$ spores/ml) and a relatively less infectious environment ($10^6$ spores/ml), there was no significant difference between the number of eggs laid on a highly infectious plate and that on a less infectious plate (Figure 1B; $t_{19} = 1.14$, $p = 0.267$). Overall, females did not have a stronger avoidance against the oviposition site of higher risk of infection.

When we changed the infectious object to infection-killed conspecifics, the number of eggs found on plates with infection-killed flies was similar to that on plates with flies killed by freezing (Figure 2-1C; $t_{19} = 0.37$, $p = 0.713$). Yet, stronger avoidance was detected when the number of dead flies on the plate increased (Figure 2-2; Likelihood Ratio Test, Number of dead flies per plate (2 versus 5), $\chi^2_1 = 3.91$, $p = 0.048$). It is possible that a certain threshold exists to trigger females' avoidance against the pathogen-contaminated media and it differs for different sources of infection risk (Romano et al., 2022, Keiser et al., 2020).

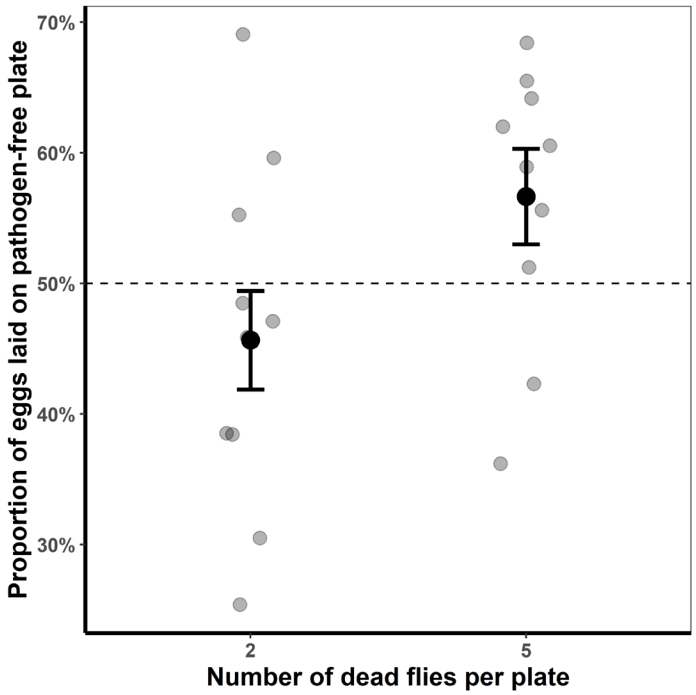


*Figure 2-2 Proportion of eggs laid on the non-infectious plate when females were choosing between a plate with infection-killed flies and a plate with flies killed by freezing. Each transparent dot indicates an oviposition chamber. The dark dots and the error bars demonstrate the estimated marginal means and the 95% confidence interval.*

## Offspring raised on infectious media did not suffer from reduced viability but showed signs of pathogen avoidance.

To understand whether there is a fitness penalty for females laying eggs on pathogen-contaminated food, we compared the offspring viability between offspring raised on pathogen-free food and pathogen-contaminated food. When the infectious source was spores, treatment had a significant effect on egg-to-pupae viability (LRT, Treatment, $\chi^2_2$ =7.1, $p$ = 0.029) and egg-to-adult viability (Treatment, $\chi^2_2$ =9.4, $p$ = 0.008). However, contrary to our prediction, offspring reared in food with a low dose of spores had increased viability compared to those in pathogen-free food (Figure 2-3A,B). When offspring were exposed to infection-killed or freeze-killed conspecifics, we did not find a significant difference between the viability of the two groups of offspring (Figure 2-3C,D; for egg-to-pupae viability, Treatment, $\chi^2_3$ =0.02, $p$ = 0.971, for egg-to-adult viability, Treatment, $\chi^2_3$ =1.3, $p$ = 0.723). Therefore, we conclude that there was no major fitness penalty for oviposition on infectious sites in terms of egg viability.

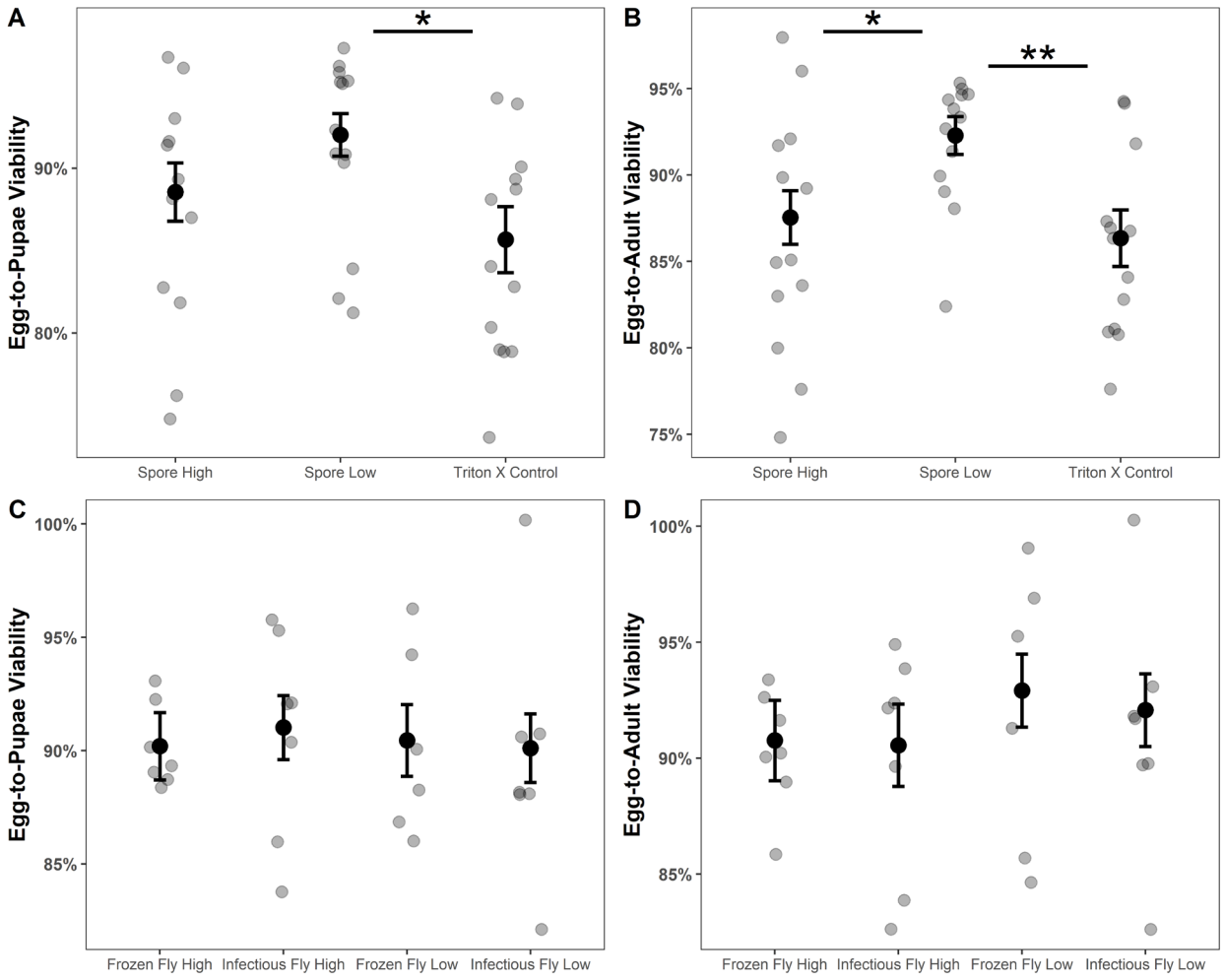


*Figure 2-3 Egg-to-pupae viability and egg-to-adult viability **A** and **B**: when the food was contaminated by fungal spores; **C** and **D**: when the food was contaminated by infection-killed flies. Each transparent dot represents a food bottle where eggs were incubated. The dark dots and the error bars demonstrate the estimated marginal means and the 95% confidence interval. The significance level of pairwise comparison is also indicated: p≤0.05 *, p≤0.01 **.*

There are two possible explanations for this lack of fitness loss: (a) The actual effective spore concentration is low, and (b) fitness consequences may only be revealed at later life stages. During the larval stage, offspring are continuously foraging for food, feeding, and getting in and out of the food ("tunneling"). The actual spore concentration may have been diluted through this process as the same quantity of spores is now mixed with more volume of food. Such physical movement also effectively reduces the germination of spores due to very brief attachment of the cuticles (Wang and Leger, 2007, Greenfield et al., 2014). Moreover, although larvae were feeding on the fungal spores or infected conspecifics, thanks to the peritrophic matrix of the gut, these infectious sources were capsuled and were not in direct contact with the host (Butt et al., 2016). Fungal spores

and the corpse (filled with fungal mycelium, easier to consume compared to freeze-killed flies) in this case may provide extra nutrients to the larvae, which improves viability. It is also possible that the fungal proliferation is on halt until the adult stage. Vijendravarma et al. (2008) have shown that *Tubulinosema kingi*, a microsporidian pathogen to *D.melanogaster*, barely proliferates in the larval stage but significantly increases in the adult stage, likely a strategy to maximize the chance of dispersion through infected adults. If that is also the case for *M.brunneum*, the fitness cost of females laying eggs on pathogen-contaminated media would only become obvious in the adult stage either in forms of reduced survival or reduced sexual success.

Despite the similar viability during development, we still detected some differences between the offspring raised on pathogen-free media and contaminated media. The mean pupation height was higher when the pathogen was present compared to when it was absent (Figure 2-4A, C; Spore, $F_{2,18}$=8.0, $p$=0.003, Fly, $F_{3,23}$=5.1, $p$=0.008). The mean pupation height in food with a high dose of spores was 3.38cm, which is 17.2% higher (~0.50cm higher; pairwise comparison, adjusted $p$ = 0.014) than the value recorded in the clean food and 22% (~0.61cm; adjusted $p$ = 0.014) than in the food with a low dose of spores. There was no significant difference between the mean pupation height recorded in clean food and the food with low doses of spores (adjusted $p$ =0.498). There was a tendency for an increase in the pupation height in the environment with infection-killed conspecifics than in the environment with flies killed by freezing (Figure 2-4C). However, increasing the number of flies on the food surface did not increase the pupation height (Infectious Fly High vs. Frozen Fly High, ~0.48cm, adjusted $p$= 0.116; Infectious Fly Low vs. Frozen Fly Low, ~0.49cm, adjusted $p$= 0.081). The higher pupation height observed here could be a sign of pathogen avoidance. When larvae are raised at high larval densities, an adverse environment for development due to high levels of competition and toxicity, a higher pupation height is also reported (Henry et al., 2020).

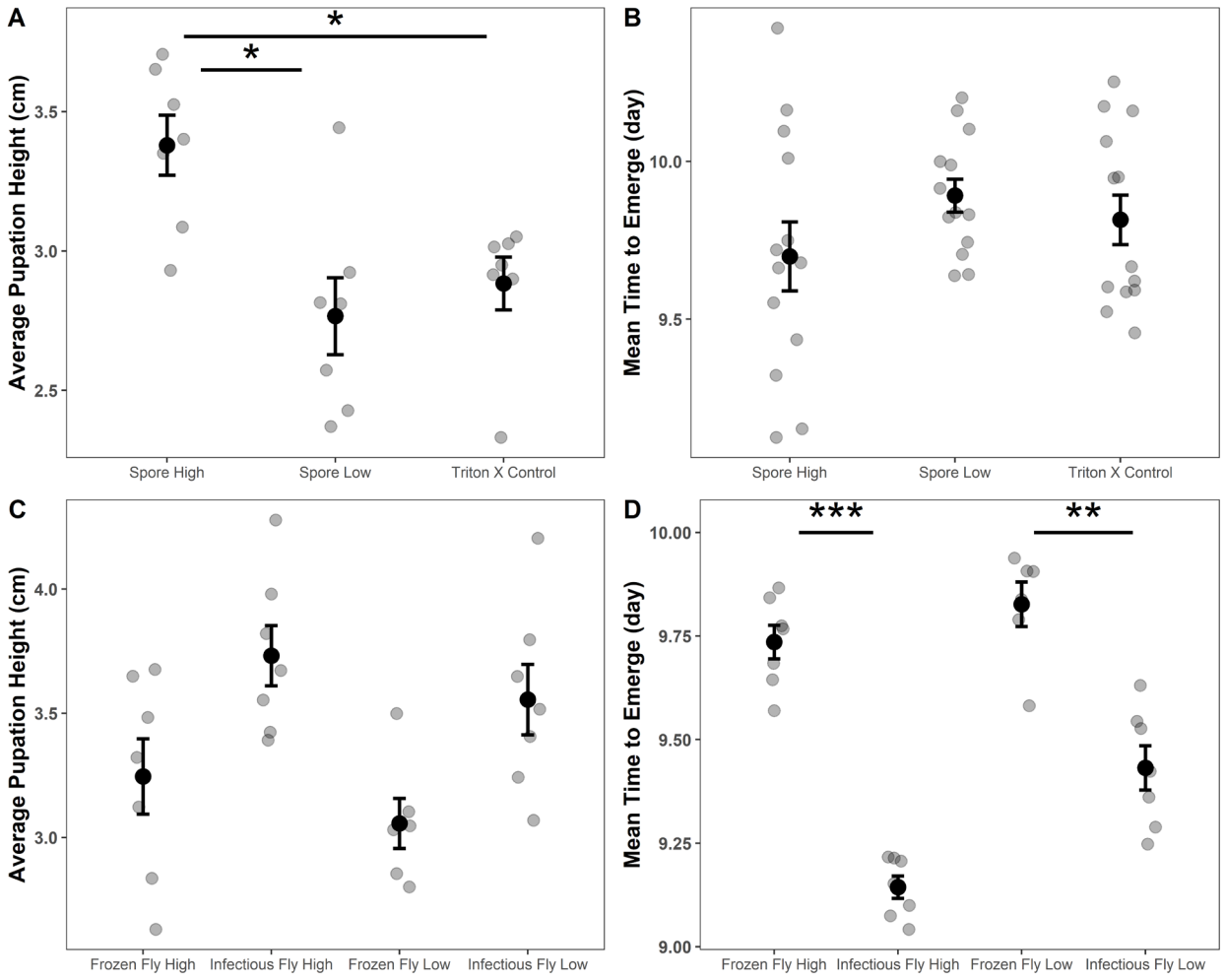


*Figure 2-4 Average pupation height and mean time to emerge. **A** and **B**: when the food was contaminated by fungal spores; **C** and **D**: when the food was contaminated by infection-killed flies. Each transparent dot represents a food bottle where eggs were incubated. The dark dots and the error bars demonstrate the estimated marginal means and the 95% confidence interval. The significance level of pairwise comparison is also indicated: p≤0.01 **, p<0.001 ***.*

As for mean time to emerge, we did not detect any significant differences among groups in the spore treatment (Figure 2-4B), but flies in the environment with infection-killed flies emerged sooner than those in the non-infectious environment (Figure 2-4D; Infectious Fly High vs. Frozen Fly High, ~0.6day earlier, adjusted $p$<0.001; Infectious Fly Low vs. Frozen Fly Low, ~0.4day, adjusted $p$= 0.001). The difference in the average emergence time between the control and the infectious group increased when we increased the number of dead flies on the surface.

Entomopathogenic fungi are known to release different volatile organic compounds (Hussain et al., 2010), which can be perceived by insects and trigger avoidance (Pereira and Detrain, 2020). The higher pupation height and shorter time to emerge could be the response of *D.melanogaster*

larvae to such cues. Meanwhile, the presence of pathogens may also alter the microenvironment (Keebaugh et al., 2018), which affects larval development (Casares and Carracedo, 1987). Faster development may be in trade-off with other fitness traits, for example, longevity (Modak et al., 2009). This result also supports our hypothesis that the fitness consequences of raising offspring on infectious food would become evident in later life stages. Our results demonstrate the intricate interplay between insect behavior and environmental cues shaped by the presence of pathogens, which calls for further investigation.

### Pathogen avoidance was not seen during larval foraging.

We did not detect any preference for non-infectious food of the third instar larvae during foraging (Figure 2-5; $t_{12}$ =0.00, $p$=1.0). While our result is similar to findings from a previous study where pathogen avoidance by larvae against the *Drosophila C virus* is not observed (Siva-Jothy et al., 2018), it has also been shown that larvae are able to sense bacterial infections in the gut through the central nervous system and develop disgust towards the infectious food (Surendran et al., 2017). However, in the previous experiments, we did not find any fitness penalty in terms of viability when growing up in food contaminated by fungal spores, which may indirectly indicate that the selective pressure at the larval stage is rather small and does not trigger any avoidance. If spores are indeed an extra food source, infectious food may be even more attractive than non-infectious food. One should also keep in mind that the results of this experiment do not have enough statistical power to reach a convincing conclusion due to the small number of replicates and high variation in the data. Therefore, a further experiment should be done with more replicates to investigate pathogen avoidance at the larval stage.

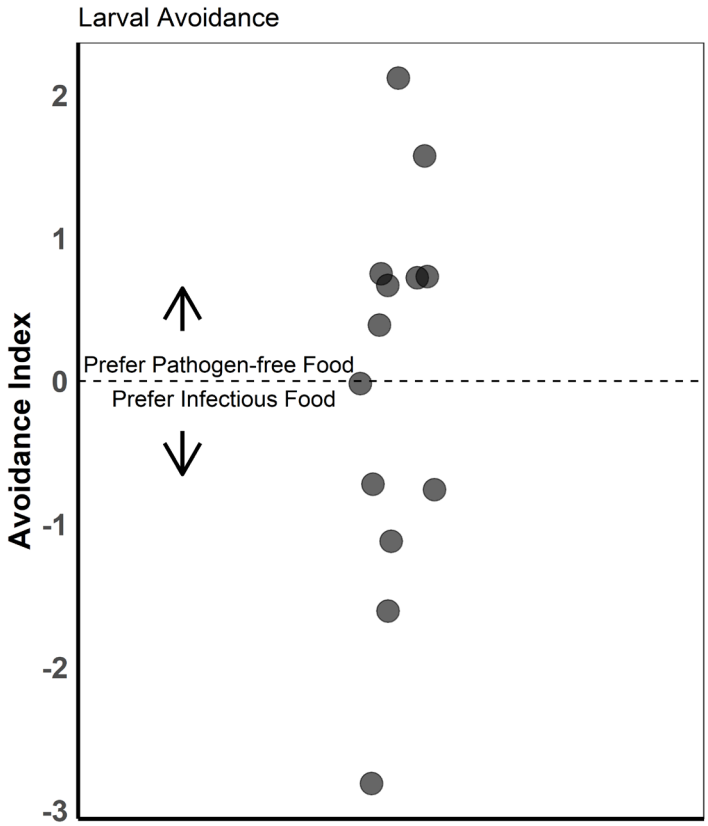


*Figure 2-5 Avoidance index when third instar larvae were choosing between food contaminated with fungal spores (infectious) and food soaked with spore-free 0.05% Triton X-100 (pathogen-free). Each dot is an oviposition chamber.*

## Conclusions

In this study, we were able to find evidence of pathogen avoidance in adult females and during the development of offspring. Yet, we did not detect any fitness loss in offspring raised in the environment of infection risk in terms of egg viability. The observed changes during larval development may have further consequences in the later life stages. Host-pathogen interaction is rather pathogen-specific. Further studies on pathogen avoidance in multiple host-pathogen pairs will help shed light on understanding whether female preference for healthy males is pure pathogen avoidance which supports the pathogen transmission avoidance theory of sexual selection (Loehle, 1997) or is on purposely selecting for good genes.

**Acknowledgment**

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**Author Contributions**

A.L. and T.J.K. designed the experiments; A.L. supervised the project and wrote the first draft of the manuscript; E.B., T.B., L.K., M.M., V.V., M.B., G.K., A.S., N.P., and L.R., equally contributed to conducting the experiment and the manuscript writing.

## Supplementary Materials

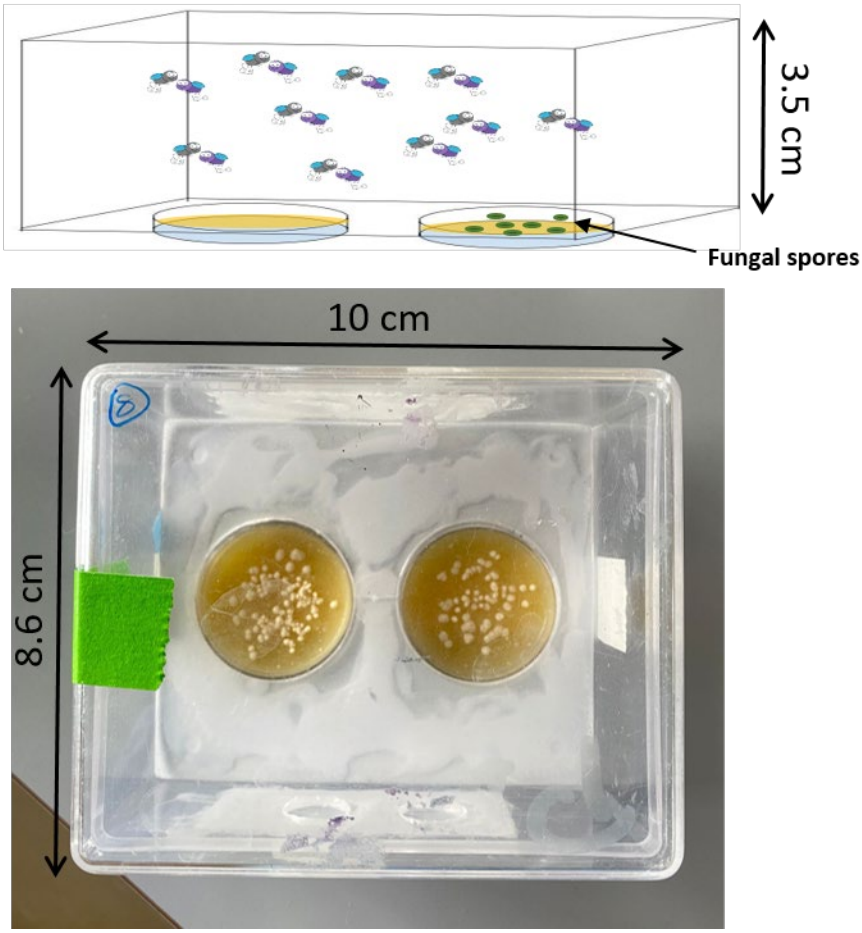


*Figure S 2-1 Oviposition chamber used in the experiment. Each has two replaceable petri dishes which were filled with orange juice agar. Sprinkles of yeasts were also added to stimulate females' egg laying.*

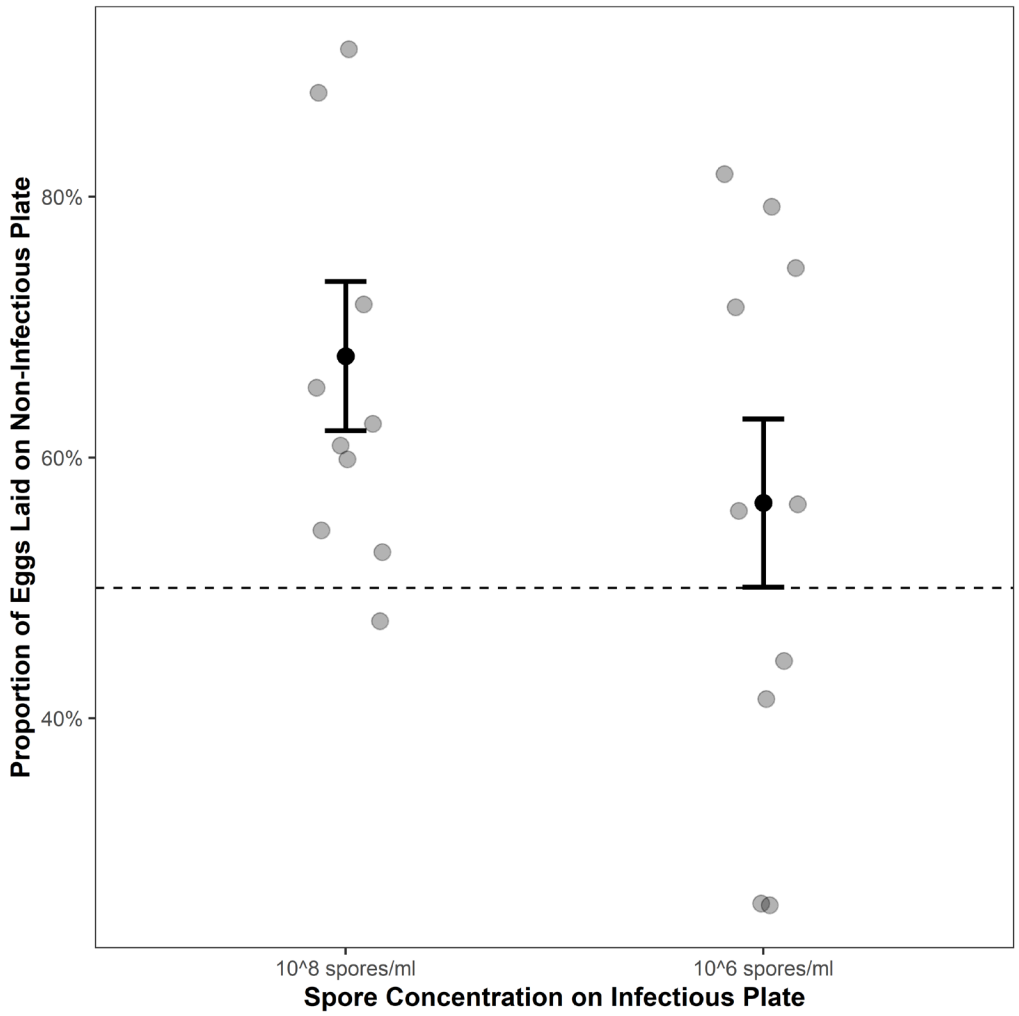


*Figure S 2-2 Proportion of eggs laid on the non-infectious plate when females were choosing between a plate with infectious fungal spores and a plate brushed with spore-free Triton X 0.05%. The dashed line is 50%. Each transparent dot indicates an oviposition chamber and the dark dots and the error bars demonstrate the estimated marginal means and the 95% confidence interval.*

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# Chapter 3 Context- and sex-dependent links between sire sexual success and offspring pathogen resistance

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Abstract

"good genes" sexual selection posits that traits conferring sexual success are honest signals of genetic quality, often reflected in the individual's general health ("condition"). Such a mechanism joins forces with natural selection in promoting pathogen resistance. However, previous studies have yielded equivocal results. Few studies have differentiated general immunocompetence and specific pathogen resistance, let alone explored their different links with condition in the design. Here, we used *Drosophila melanogaster* and the fungal pathogen *Metarhizium brunneum* as our experiment system to investigate the link between sire sexual success and offspring pathogen resistance when the pathogen is present or absent in sexual selection. We measured sire paternity share in a competing mating trial as a proxy for sexual success and assessed offspring survival post infection as an indicator of pathogen resistance. Our results provide further evidence supporting the "specific resistance" scenario as the sign of sire-son correlation was mediated by the occurrence of the pathogen: when sires were not infected before the mating trial, sires of higher sexual success had less-resistant sons, but when sires were infected, such negative correlation was no longer detected. Pathogen resistance of sons and daughters was positively correlated, yet the link between sire's paternity share and offspring pathogen resistance was sex-specific: sire paternity share was negatively correlated with daughters' survival post infection regardless of sire's exposure to pathogen, suggesting the presence of sexually antagonistic selection on pathogen resistance. Overall, we found no support for "good genes". Our findings stress the importance of including different contexts of sexual selection in the "good genes" study and demonstrate that the correlation between sire sexual success and offspring pathogen resistance can be highly sex-specific or even sexually antagonistic, which may offset the benefits of "good genes".

## Introduction

Sexual selection, initially proposed by Darwin (Darwin, 1871), has been a topic of ongoing debate over the years, especially its relationship with natural selection. The triumph of the sexiest is often observed going against the survival of the fittest. For example, calling in frogs decreases their ability to avoid predators, elongated tails in birds reduce their foraging rates, and so on (Andersson, 1994). However, sexual selection can also reinforce natural selection (Parrott et al., 2019, Kokko et al., 2002, Hollis et al., 2009). One mechanism to achieve such alignment would be through the positive genetic covariance between sexual success and non-sexual fitness, referred to as the "good genes" hypothesis (Houle and Kondrashov, 2002, Møller and Alatalo, 1999, Zahavi, 1975). It suggests that alleles favored by sexual selection also improve offspring survival or other non-sexual aspects of offspring fitness. A fitness-related trait often of interest both in the context of natural selection and sexual selection is pathogen resistance, the ability of an individual to fight against or tolerate the negative impacts of pathogens. Existing empirical work provides equivocal evidence for the correlation between sexual success and pathogen resistance (Folstad and Karter, 1992, Wu et al., 2018, Hall et al., 2004, Kruuk et al., 2002, Parrett et al., 2022), indicating a multifaceted relationship between these evolutionary processes.

Two mechanisms have been proposed on how a link between pathogen resistance and traits that affect sexual success can be generated and they differ in the assumptions on the genetic architecture of resistance and the importance of environment context (summarized in (Westneat and Birkhead, 1998)). The first proposed mechanism posits that sexual selection favors general immunocompetence which is effective against a broad range of pathogens (hereafter, "general immunocompetence" scenario). Within this theoretical framework, the level of both pathogen resistance and sexual trait expression are directly linked with the individual's condition. Condition refers to the individual's capacity to cope with environmental challenges, capturing a large part of the individual's genetic variance (Rowe and Houle, 1996, Hill, 2011). The common condition-dependency generates a positive genetic correlation between pathogen resistance and sexual selection as they share a similar set of genes distributed across the whole genome (Tomkins et al., 2004). Further, it is assumed that many of these genetic variants are additive and thus inherited by the offspring. In this scenario, the direction of the relationship between sire sexual success and offspring pathogen resistance is not affected by the epidemiological context, meaning that sexually

successful male sires more resistant offspring regardless of the occurrence of a specific pathogen. On the other hand, the Hamilton-Zuk hypothesis considers a scenario where the genetic variants confer specific pathogen resistance independent of the general condition (Hamilton and Zuk, 1982). When a specific pathogen is around, males having this specific resistance will have better condition, therefore a better sexual performance compared to the ones without, and eventually a higher sexual success (hereafter, "specific resistance" scenario). This pathogen resistance should also be heritable so that when the pathogen in the parent environment is the same as the one in the offspring environment, the offspring can benefit from the outcome of sexual selection. However, if these two pathogenic environments do not align (simply missing this specific pathogen in the offspring environment), such positive genetic correlation will be violated as the selected specific resistance is not only a futile but also an expensive trait to express in the absence of the corresponding pathogen (Adamo and Spiteri, 2005). The sign of sire sexual success-offspring pathogen resistance correlation in this case is mediated by the occurrence of pathogens.

Only a few studies were designed to experimentally distinguish between these two scenarios. A study by Joye and Kawecki (2019) using *Drosophila melanogaster* and the bacterial pathogen *Pseudomonas entomophila* shows that males winning the mating contest sire more pathogen-resistant sons than loser males when they are exposed to pathogens prior to mating and that this sire-son correlation turns negative when the pathogen is absent. This finding provides valuable empirical evidence supporting the "specific resistance" scenario, yet it has its limitation: the winner-loser metric only captures the one-off sexual success by scoring who mates first with a single virgin female, which may not represent the overall results of sexual selection. The relationship between sexual success and non-sexual success is not necessarily unidirectional across different episodes of sexual selection (Rowe and Rundle, 2021). The selection for good genes can be rather stronger if other episodes of sexual selection also act on promoting good genes (Vuarin et al., 2019, House et al., 2016) or of little effect if the cost of sex-specific selection or sexual conflict outweighs the benefits (Baur et al., 2023, Okada et al., 2014, Pizzari and Birkhead, 2000). A large array of genes shows sex-specific/biased expression (Ellegren and Parsch, 2007, Ingleby et al., 2014), and many fitness-related traits even those essential for both sexes like pathogen resistance, have sexual dimorphic values (Belmonte et al., 2020, Duneau et al., 2017). Therefore, this potential offspring-sex-specific effect should also be included in experimental design testing the "good genes".

In this study, we are employing a more inclusive assay of competitive male sexual success that potentially reflects the net effect of the diverse facets of sexual selection. We also use a different pathogen that progresses in an extended timeframe, leaving more time for sexual selection to act. We use *D. melanogaster* and the fungal pathogen *Metarhizium brunneum* as our experimental system to investigate whether sire sexual success (defined by sire paternity share after all episodes of sexual selection) and offspring pathogen resistance (offspring survival post infection) depends on the occurrence of pathogens (i.e., the epidemiological context of where sexual selection happens). We hypothesize that (1) sire's sexual success is positively correlated with offspring's pathogen resistance when sire is exposed to the pathogen prior to the mating trials (i.e., sexual selection) and the link will be negative or no correlation when there is no pathogen exposure before sexual selection takes place, which supports the abovementioned "specific resistance" scenario and (2) the sire-offspring relationship is sex-specific due to the wide-spread sex-biased gene expression in *Drosophila* (Ayroles et al., 2009, Innocenti and Morrow, 2010).

## Materials and Methods

### Fly origin and maintenance

Wild-type flies used in the experiment originated from a lab-adapted, outbred population collected in 2007 in the Canton of Valais, Switzerland (Valais 07) and maintained in the lab since at a population size of more than 1000 adults with overlapping generations. To generate a fluorescently labeled competitor strain, we backcrossed GFP-ProtB (courtesy of Stefan Lüpold, University of Zürich, Zürich, Switzerland (Manier et al., 2010)) into this background (Valais 07) for five generations. GFP-tagged flies used in the experiment were more than 95% genetically identical to the wild-type population. The ubiquitous GFP marker is a very effective dominant phenotypic marker for paternity assignment as it is already visible at the embryo stage.

Adult flies used in this experiment were raised on standard cornmeal-yeast-agar media with Nipagin at 25°C, 55% relative humidity, and 12L:12D photoperiod. The larval density was controlled by transferring about 200 eggs to each bottle containing 40ml food. Virgin wild-type flies of both sexes were collected within 6-8h post emergence and then maintained in same-sex groups of 10 in the food vials with 10ml food until used in the experiment. GFP-tagged non-virgin males were collected three days after emergence and also maintained in groups of 10. All fly transfers were done under light $CO_2$ anesthesia.

### Fungal culture and infection protocol

The pathogen used in this experiment is *Metarhizium brunneum* KVL 03-143. Pathogen origin and infection protocol were previously described in Chapter 1. Sires assigned to the infection treatment were individually dipped into 2ml spore suspension (spores stored in 0.05% Triton X-100) with an adjusted concentration of $10^7$ spores/ml for 10 seconds. Sires assigned to sham treatment were individually dipped into spore-free 0.05% Triton X-100 for 10 seconds. To study the offspring resistance, offspring of the same family were submersed in the spore suspensions ($10^7$ spores/ml) for 30 seconds in groups of 20. Infection treatment was applied to daughters and sons separately.

## Experiment design

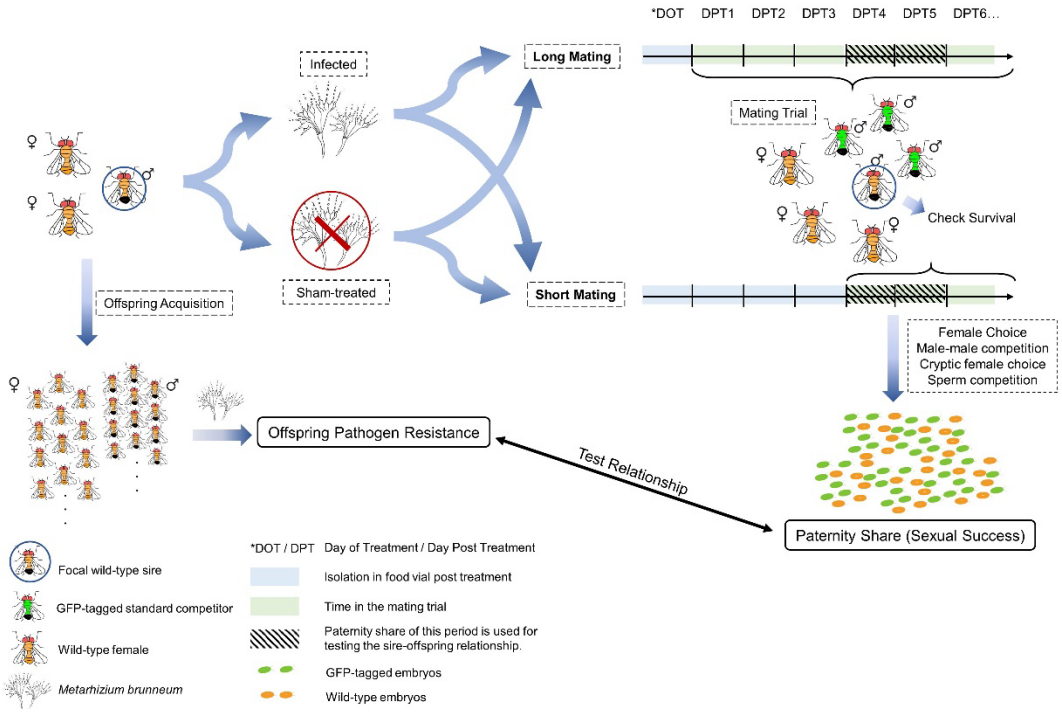


*Figure 3-1 Experimental design to study the relationship between sire sexual success and offspring resistance to M.brunneum with/without exposure to the pathogen before sire sexual success is measured. Each sire was coupled with two random virgin females and offspring from these matings were allowed to develop [Offspring Acquisition]. After mating, sires were subjected to either sham treatment or infection. Next, sires were kept in isolation and placed into mating trials together with three virgin females and three GFP-tagged standard competitors on day 1 or day 4 post-treatment depending on the assigned mating scenario [Long Mating vs. Short Mating]. Sire's sexual success was determined by the combined paternity share on day 4 and day 5 post-treatment. When offspring from the offspring acquisition vials reached adulthood, their resistance to M.brunneum was assessed in terms of survival post-infection (20 offspring per offspring sex per sire).*

The design of our main experiment is summarized in Figure 3-1. To test our hypothesis, we acquired a measure of sexual success for each sire and a measure of pathogen resistance of each sire's offspring. To rule out any potential effect of sire treatment on the measurement of offspring's resistance, we obtained offspring from each sire prior to any treatment. Each two-day-old wild-type virgin male (sire) was coupled with two random wild-type virgin females in a vial with standard food and given 24 hours to mate before being removed for the next step of the experiment. Females were given another 48 hours to lay eggs in the same vial before being removed. All vials at this step (offspring acquisition vials) were kept for offspring collection.

Subsequently, we quantified the sires' sexual competitiveness following infection or sham treatment. Six hours after being removed from the offspring acquisition vials, each sire was subjected at random to either infection or sham treatment and then kept individually in a food vial until the mating trial. In each mating trial, a single sire was placed in a bottle with grape juice agar and yeast (i.e., oviposition medium) together with three virgin females and three GFP-tagged competitor males. GFP-tagged males outnumbered focal males in the mating trial because they are less competitive in sexual competition (Sharda et al., 2023). Upon the onset of the mating trial, virgin females were 4 days old and uninfected. The competitor males were of the same age as the sire, were not infected, and for workload reasons were not isolated as virgins. Flies in the mating trial were transferred to a new oviposition medium following a 10h (daytime):14h (nighttime) interval. All embryos laid during the nighttime hours were counted (by a single experimenter blind to sample identity) under fluorescence stereomicroscope to acquire the number of GFP-tagged and wild-type embryos (the percentage of non-GFP embryos used as the paternity share of the wild-type sire).

To understand if the interactions within the mating trials and the sire's infection status upon the onset of the mating trial affect the relationship between the sire's sexual success and offspring pathogen resistance, we have designed two mating scenarios: Long Mating and Short Mating. Sires assigned to "Long Mating" had a longer time (3 days) to interact with females and standard competitors in the mating trial before scoring the sexual success for testing the sire-offspring relationship (day 4-5 post-treatment). Sires assigned to "Short Mating" had less time to interact (less than 1 day). On day 7 post-treatment, all flies in the mating trials were transferred to standard food bottles, and mortality of the flies was recorded daily until day 10 post treatment. No mortality of the sham-treated sire nor fungal infection of the females in contact with infected males was seen during the experiment.

To assess the offspring pathogen resistance, from each sire, we randomly collected 20 4-6-day-old non-virgin offspring of each sex and infected them with *M. brunneum* spore suspension ($10^7$ spores/ml). After infection, offspring were kept in same-sex groups of 10 in the food vials. Dead flies within 2 hours post infection were considered as mortality from handling rather than infection and were removed from the vials and the analysis (727 flies were removed; 7551 offspring for the final analysis). Mortality due to infection was then recorded daily until day 9 post infection. The

entire experiment was conducted in three blocks over two months (see Table S 3-1 for detailed experiment setup).

Paternity share of day 4-5 post treatment was chosen as a proxy for sire sexual success as at this time the infection is established, the immune response is fully activated within the infected host, and mortality of the infected sire starts on day 6 post infection (see Chapter 1). It is calculated as the sum of the wild-type egg count from day 4 and day 5 divided by the total number of eggs on day 4-5.

To measure a male's ability to mate in a competing environment and to be able to attribute any observed relationships to sexual selection, within the main experiment work, we also recorded the paternity share of sires in the "Long Mating" scenario from day 1-6 post treatment and that of sires in "Short Mating" scenario from day 4-6 post treatment. Sires which found dead during the mating trials were removed and we have stopped tracking the paternity share of the corresponding sire since the day of mortality.

**Statistical analysis**

All statistical analyses were performed with R (v. 4.1.2) (R Core Team, 2020). Visualization of the results was conducted with package *ggplot2* (v.3.4.1) (Wickham, 2016) and *ggpubr* (v.0.4.0) (Kassambara, 2020). All generalized linear mixed models (GLMM) were done with package *lme4* (v.1.1-27.1) (Bates et al., 2015). All continuous covariates in the model were mean-centered (i.e., subtracting the overall mean value across all experiment blocks). We used the *DHARMa* package (v.0.4.5) (Hartig, 2022) to check for the distribution of the residuals and overdispersion. Effects of the fixed factors in the GLMMs were subject to the likelihood ratio test (LRT) with the *mixed()* function of the *afex* package (v.1.0-1) (Singman et al., 2021). Major axis regression was performed with the *lmodel2* package (v.1.7-3) (Legendre, 2018).

*Effects of the pathogen on sire sexual success in a competing environment*

We compared the paternity share in the mating trial of the infected sires to that of the sham-treated sires. We fitted the data to a GLMM (binomial distribution, logit link) with day post treatment (a continuous variable), sire infection treatment (infected vs. sham-treated), and their interaction as fixed factors, and experiment block with sire identity nested in it as the random factors. The

analysis was done for data from days 4-6 post treatment and for the "Long Mating" and "Short Mating" scenarios separately.

*Offspring survival post infection*

To test if there is any difference between the post infection survival of sons and daughters which indicates a sex-specific immune response, we performed a GLMM (binomial distribution, logit link) with the number of alive offspring versus the number of dead offspring as the response variable, day post treatment (a continuous variable), offspring sex and their interactions as fixed factors and sire identity nested in experiment block as the random factors. In addition, we fitted linear models to investigate the relationship between the daughter's and son's survival on each day post infection, and the correlation was tested with Pearson's correlation method. To include the block effects in the survival data, the offspring survival was centered on zero by experiment block before feeding into the analysis.

The analysis of the sire-offspring relationship required a value of post-infection survival of offspring from a given family collapsed to a single measure of resistance per family per sex. We used the offspring survival data from the day when the average proportion of alive offspring dropped down to around 50% as the proxy for offspring pathogen resistance and the chosen day could be different for daughters and sons. To examine the relationship between daughter's pathogen resistance and son's pathogen resistance, we performed a major axis regression (MA) based on the assumption that the strength of prediction is the same when using daughter pathogen resistance to predict son pathogen resistance and when vice versa. The regression line was not forced through the origin to capture the sexual differences in the susceptibility to the pathogen. In addition, we tested the correlation with Pearson's correlation method.

*Sire sexual success, sire pathogen resistance, and offspring pathogen resistance*

We then examined the relationship between sire's sexual success and sire's pathogen resistance and only infected sires were included in the analysis. Sires that died prematurely (i.e., before DPI6) were excluded from the analysis. For this analysis, sires were divided into two classes, relatively resistant (i.e., those alive after the day when 50% mortality was observed) and relatively susceptible (i.e., those that died before and on the day when 50% mortality was observed). We did

not use the actual day of death because ~25% of sires survived until day 10 (the last day of the experiment) and would thus have been censored. The splitting time points for the two mating scenarios are day 9 post infection for the "Long Mating" scenario and day 7 post infection for "Short Mating" scenario (Figure S 3-1). We fitted the data with a GLMM (binomial distribution, logit link), including sire paternity share as the response variable, sire pathogen resistance level as a fixed factor, and experiment block and observation identity as the random factors.

To investigate the heritability of pathogen resistance, we also investigated the link between the sire's pathogen resistance level and the offspring's pathogen resistance. We fitted the data to a GLMM (binomial distribution, logit link), using offspring pathogen resistance (number of alive offspring versus the number of dead offspring on the chosen day) as the response variable, sire pathogen resistance level (relatively resistant versus relatively susceptible), and mating scenario as fixed factors and experiment block and observation identity as the random factors. The analysis was done for daughters and sons separately.

We applied the same set of analyses to the dataset where sires were divided into six levels based on their mortality time (see Appendix 1), and similar conclusions were reached.

*Relationship between sire sexual success and offspring pathogen resistance*

To examine the relationship between sire sexual success and offspring pathogen resistance, we fitted the data to a GLMM with binomial distribution and a logit link function. The full model included offspring pathogen resistance as the response variable, sire paternity share, sire infection treatment, mating scenario, offspring sex and their interactions as fixed factors, experiment block with sire identity nested in it as the random effects, and an observational level random factor to correct for overdispersion.

Based on the interactions observed in the full model, we split the analysis by offspring sex, resulting in GLMMs with sire paternity share, sire infection treatment, mating scenario, and their interactions as fixed factors and experiment block and observation identity as random factors. We then did a model selection and chose the model with the lowest AIC as the best model describing the data. Estimates of the relationship (i.e., slope) between offspring's pathogen resistance and

sire's sexual success and the associated standard errors were from GLMMs with only sire paternity share as the fixed factor, experiment block, and observation identity as the random factors.

## Results

### Effects of the pathogen on sire's sexual success in a competing environment

Paternity share serves as a proxy for the focal sire's competitiveness. In the mating trial, each focal sire was outnumbered by the GFP-tagged males (1 vs. 3), but on average half of the embryos were sired by the focal males, which further confirms GFP-tagged males being the inferiors in sexual selection (Sharda et al., 2023). Paternity share scored on a given day does not necessarily reflect the mating on the day as females can store sperms and would get less receptive once mated. In the Long Mating scenario, the dynamics of paternity share on days 1-3 in the mating trial were different from that on days 4-6 (Figure 3-2). On days 4-6 both paternity share of infected and sham-treated sires decreased, with infected sires experiencing a greater decrease (LRT, sire infection treatment × day post treatment, $\chi^2_1 = 43.5$, $p < 0.001$; estimated slopes (log-odds scale)±SE: infected, -0.266±0.016, $p < 0.001$; sham-treated, -0.122±0.015, $p < 0.001$). In the Short Mating scenario, the mating trial started on day 4 post treatment, and infected sires entered the mating trial when infection was well established (Chapter 1). Since the start of the mating trial, infected sires had a lower paternity share than the sham-treated sires. As in the Long Mating scenario, paternity share decreased over time, particularly for infected sires (Figure 3-2; sire infection treatment × day post treatment, $\chi^2_1 = 12.2$, $p < 0.001$; estimated slopes (log-odds scale)±SE: infected, -0.333±0.023, $p < 0.001$; sham-treated, -0.225±0.021, $p < 0.001$). These findings show that infected males could still court and mate but infection negatively affected the sire's competitiveness. In the subsequent analyses, we used paternity share on day 4-5 post treatment as the measure of the sire's sexual success given that it captured the treatment effects in both mating scenarios (Figure S 3-2) and that no major sire mortality was observed during this timeframe.

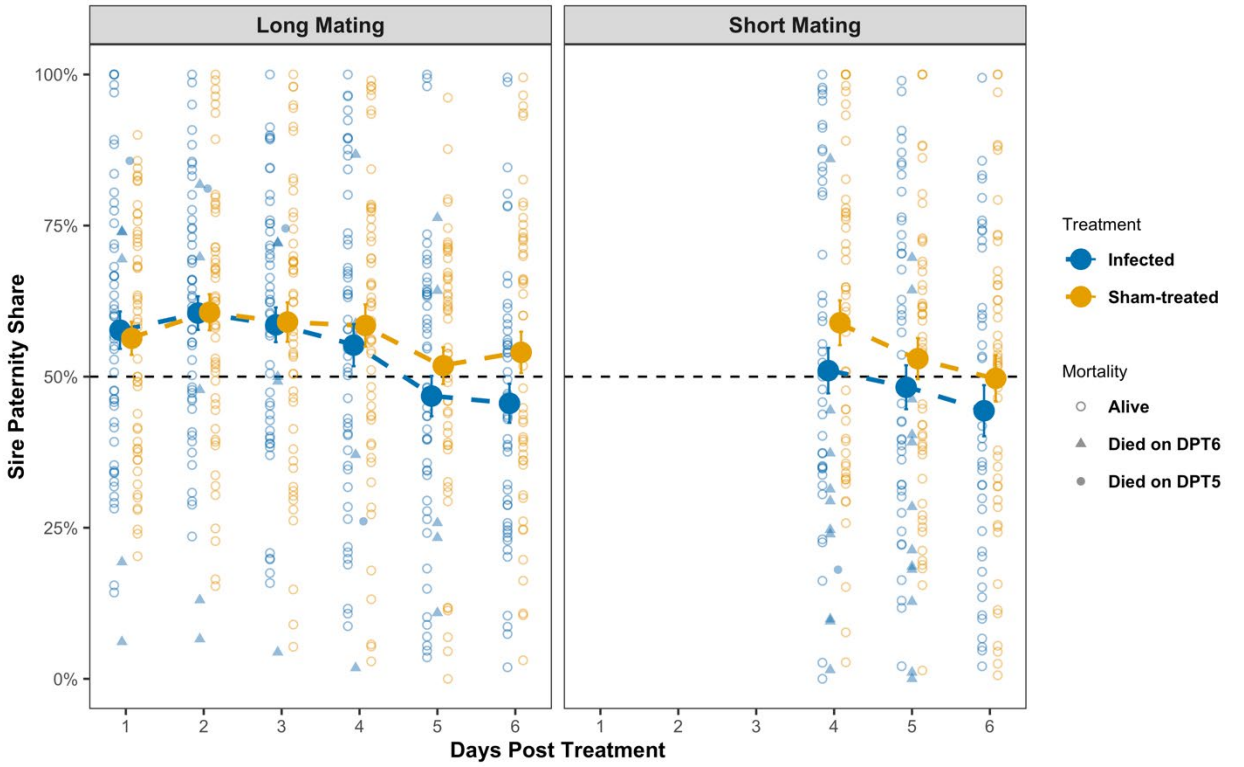


*Figure 3-2 Paternity share of the infected sires and sham-treated sires in the mating trials (means ± SE). Each dot represents a measure of each sire on each day post treatment. Mortality indicates on which day post treatment the sire was found dead and the paternity share of the corresponding sire was only plotted until the day before the mortality day. "Alive" means that the sire remained alive until the end of day 6 post treatment. No mortality was observed in the sham-treated sires. Only 1 infected sire died before day 5 post treatment and it was excluded from the analysis.*

## Offspring survival post infection

For examining the sire-offspring relationship, we need to acquire a measure of offspring pathogen resistance and we looked into the offspring survival post infection. Following infection, sons died slower compared to daughters (Figure 3-3A; estimated coefficient (log-odds scale, reference level = daughter), son, 0.88, $p < 0.001$; LRT, offspring sex, $\chi^2_1 = 594.6$, $p < 0.001$; Figure S 3-3). For further analysis, we used survival data from the day where mortality was close to 50%: day 7 post infection (DPI7) for daughters and day 9 post infection (DPI9) for sons as the measure of their pathogen resistance level. The survival of sons on DPI9 and daughters on DPI7 was positively correlated (Figure 3-3B; major axis slope = 1.416; Pearson's correlation coefficient = 0. 242, $p <$ 0.001; see Figure S 3-4 for daughter-son survival relationship on each DPI).

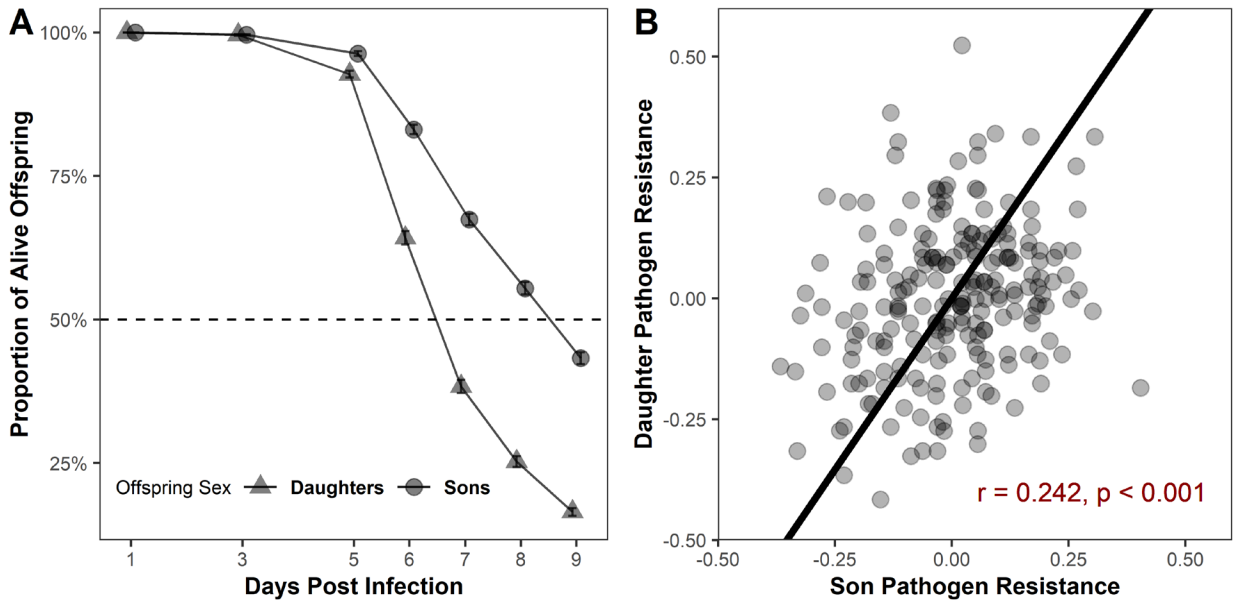


*Figure 3-3* ***A****: Survival post infection for daughters and sons (means ± SE);* ***B****: Correlation between the pathogen resistance of sons and daughters of the same sire. Survival data for sons is from day 9 post infection (DPI9) and for daughters is from DPI7. x-axis and y-axis indicate the difference between the observed survival to the mean offspring survival of the corresponding experiment block. Each dot represents the offspring coming from the same sire. The solid line displays the fitted line from major axis regression. Pearson's correlation coefficient (r) and the corresponding significance level are indicated in the figure.*

## Resistant sires have higher sexual success but not more resistant offspring.

Before examining the relationship between sire sexual success and offspring pathogen resistance, we first looked into whether there is a link between sire sexual success and sire pathogen resistance and investigated the heritable potential of pathogen resistance. Sires of higher pathogen resistance (i.e., relatively resistant group which lived longer than 50% of the infected sires) had higher sexual success in the mating trials than the relatively susceptible sires (Figure 3-4A; Figure S 3-5A; sire pathogen resistance level: $\chi^2_1 = 5.9$, $p = 0.015$; estimated coefficient (log-odds scale) for relatively susceptible group versus the relatively resistant group = -0.595, z = -2.3, $p = 0.0221$). There was no significant correlation between sire pathogen resistance and offspring pathogen resistance (pairwise comparison, relatively resistant sire vs. relatively susceptible sire, son, $p=0.951$, daughter, $p=0.282$ ; Figure 3-4B, C; Figure S 3-5B, C).

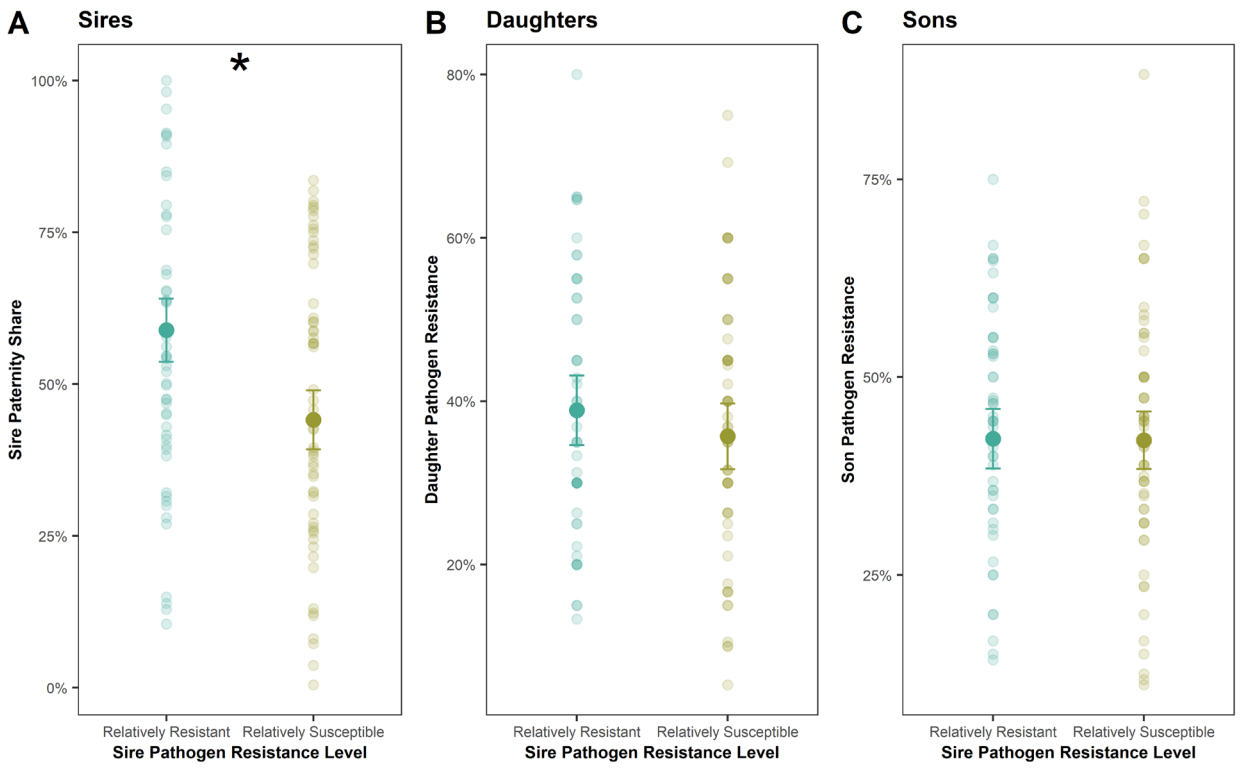


*Figure 3-4 Relationship between sire's pathogen resistance level with **A**: sire's paternity share; **B**: daughter's pathogen resistance and **C**: son's pathogen resistance. Each dot in A represents measures from each sire and in B-C represents the survival of ~20 offspring from each sire on the chosen day. Symbols show the estimated marginal means ± SE. Results of pairwise comparison are indicated with asterisk (*, $p \leq 0.05$).*

## Sire-offspring relationship is offspring-sex-specific and depends on the pathogenic environment.

The relationship between sire sexual success and offspring pathogen resistance varied depending on the sire's treatment prior to the mating trial and offspring sex as indicated by the results of the generalized linear mixed model (GLMM) on the whole dataset (Full model, sire infection treatment × paternity share × offspring sex, $\chi^2_1 = 5.4$, $p = 0.021$; See Table S 3-2 for the complete model statistics). Therefore, further analyses were split by offspring sex to test the relationship between sire sexual success and offspring pathogen resistance. For sons, the best GLMM describing the data includes sire paternity share, sire infection treatment, and their interaction as fixed factors. For daughters, the best GLMM includes sire paternity share, mating scenario, and their interaction as fixed factors. Both GLMMs include experiment block as the random factor and observation identity was also included as a random factor to correct for overdispersion.

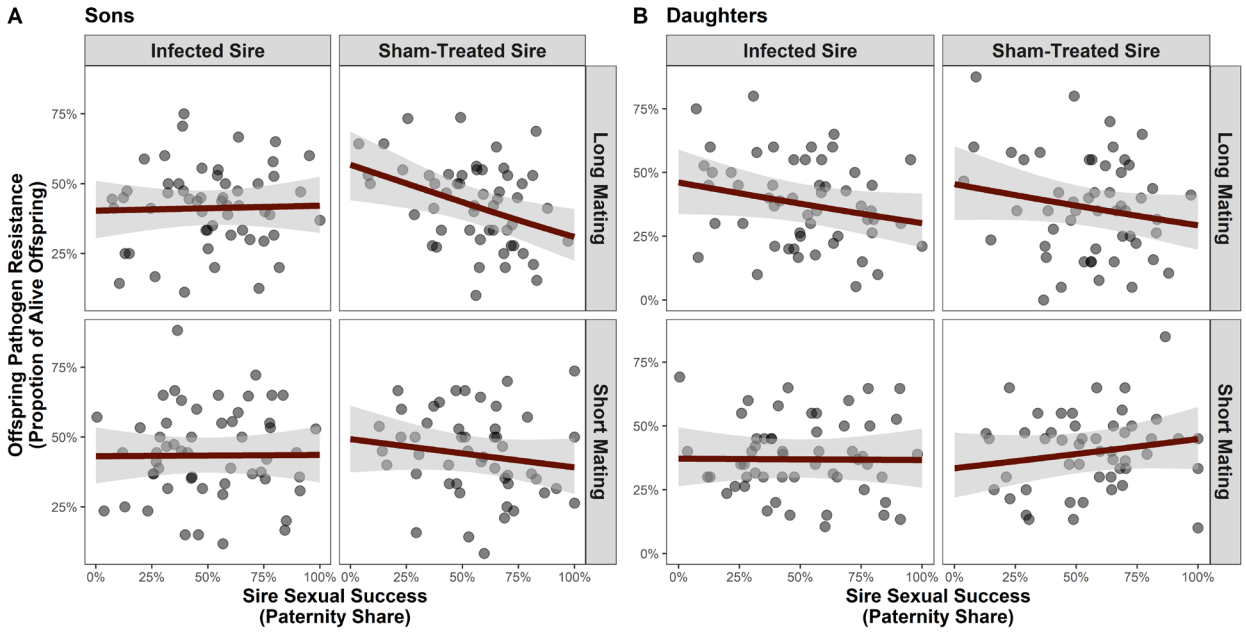


*Figure 3-5 Relationship between sire sexual success and pathogen resistance of **A**: Sons; **B**: Daughters. Each dot represents ~20 offspring from the sire. Solid lines represent the predicted values of a generalized linear mixed model (sire's infection treatment, mating scenarios, and paternity share as fixed factors; experiment block and observation identity as the random factors). Grey shadows around lines indicate the predicted 95% confidence intervals.*

For sons, the mating scenario did not significantly affect the relationship between their pathogen resistance and sire sexual success (the best model fitting the data is a GLMM without mating scenario), which is also confirmed by the similar sire-son relationship seen in the Long Mating scenario and the Short Mating scenario (Figure 3-5A). A significant effect of the interaction between sire's paternity share (i.e., sexual success) and sire treatment was found (Figure 3-5A; sire infection treatment × paternity share, $\chi^2_1 = 4.3$, $p = 0.038$; Table S 3-3). For sires subject to sham treatment prior to mating trial, sires of higher paternity share had sons of lower survival post infection (paternity share, $\chi^2_1 = 7.0$, $p = 0.008$; estimated slope (log-odds scale)±SE = -0.73±0.27, $p = 0.008$), but when sires were infected prior to the mating trial, such negative correlation was no longer detectable (paternity share, $\chi^2_1 = 0.004$, $p = 0.95$; estimated slope±SE = 0.02±0.25, $p = 0.95$).

For daughters, sire infection treatment did not significantly affect the relationship between their survival and the sire's paternity share (the best model fitting the data is the GLMM without sire infection treatment), but the sire-daughter relationships observed in the two mating scenarios were different (Figure 3-5B; paternity share × mating scenario, $\chi^2_1 = 4.8$, $p = 0.029$; Table S 3-4). When measured in the Long Mating scenario, sire's paternity share was negatively linked with the

daughters' pathogen resistance regardless of the sire's infection treatment (paternity share, $\chi^2_1$ = 4.5, $p$ =0.035; estimated slope±SE = -0.77±0.36, $p$ =0.034). In the Short Mating scenario, we found no correlation between daughters' pathogen resistance and sire sexual success regardless of whether sires were subjected to infection prior to the mating trial.

## Discussion

In this study, we set out to test the hypothesis that the relationship between sire sexual success and offspring pathogen resistance would depend on the epidemiological context under which sexual selection takes place. Specifically, we predicted that this correlation would be positive if the sires were exposed to the pathogen prior to the action of sexual selection, but none or negative otherwise. We found that this focal relationship indeed depends on the sire pathogen exposure, but the pattern turned out to be more complicated than our relatively simple prediction. The first and the most significant pattern we found is that the sire-offspring relationship is different for daughters and for sons. The best models describing the sire-offspring relationship differ for each sex, indicating that the consequences of sexual selection on daughters and sons depend on different circumstances.

For sons, the sign of the sire-son relationship was mediated by the occurrence of the pathogen. When sires were sham-treated prior to mating trials, more sexually successful sires had more susceptible sons against the fungal infection, but when sires were exposed to pathogens before entering the mating trial, the negative correlation between sires and sons was no longer detectable. This finding, in line with Joye and Kawecki (2019), shows that the link between sire sexual success and offspring pathogen resistance is context-dependent, partially supporting the "specific resistance" hypothesis (Westneat and Birkhead, 1998). Infected sires had a lower paternity share than control sires in a competing environment, revealing the negative impacts of infection and demonstrating the potential of sexual selection to promote resistant males (Jacobs et al., 2015). Sires with high resistance levels will be able to minimize the negative effects of infection on condition, and have better sexual signals, leading to higher sexual success. The positive genetic covariance component in the "good genes" hypothesis posits that the offspring of the more sexually successful sires should also have higher pathogen resistance (Hamilton and Zuk, 1982). Therefore, we should expect to find a positive sire-offspring relationship when pathogens are present. However, in both mating scenarios, we did not find any significantly positive sire-son relationship, suggesting no "good genes" for sons with respect to pathogen resistance.

For daughters, the relationship between pathogen resistance and sire sexual success depends on the mating scenarios (i.e., length of interactions in the mating trial). In the Long Mating scenario, regardless of sire infection treatment, daughters of more sexually successful sires were more susceptible. The negative sire-daughter relationship observed when the sires were not exposed to the pathogen can also be explained by the fact that in an environment without a specific pathogen, carrying resistance for a specific pathogen is costly, leading to reduced capacity for sexual performance compared to the sire with lower resistance level (Folstad and Karter, 1992, Sheldon and Verhulst, 1996). Specific pathogen resistance is a "bad gene" in this scenario and thus would not be favored by sexual selection, which explains the negative sire-offspring relationship.

As for the negative sire-daughter relationship observed when sires were exposed to the pathogen, we argue that the reason behind is different from the one detected when sires were not exposed to the pathogen, and it is likely due to sexually antagonistic selection. Although sons were more resistant than daughters, we have demonstrated that the pathogen resistance of sons and daughters was positively correlated. Such positive covariance indicates that some of the genes encoding pathogen resistance are shared by both sexes. Nonetheless, a negative sire-daughter correlation was detected instead of the no correlation seen for sons. It could be that some of the genes favored by sexual selection on sires have antagonistic effects on daughters or at best, sexually selected genes in sons may not be as beneficial to daughters (sex-specific effects), as has been reported in Guncay et al. (2017) and Joye and Kawecki (2019), respectively.

The fact that the mating scenario has a stronger impact on the sire-daughter relationship than on the sire-son relationship further consolidates our speculation on the sexually antagonistic selection. In the Short Mating scenario, the negative sire-daughter relationship became no correlation between daughter pathogen resistance and sire sexual success. While in the "Short Mating" scenario. most of the females were mated but not yet ready to remate, females in the "Long Mating" scenario had already recovered from previous mating and became receptive again by the time sexual success was measured. Therefore, in the Long Mating scenario, the captured sire sexual success likely reflects the sire's competitiveness for (re)mated females, whereas, in the Short Mating scenario, the scored sexual success mostly reflects the outcome of the competition for virgin females. Mated females often have a stronger selection for certain male traits (Kohlmeier et al., 2021, Jennions and Petrie, 2000, Kokko and Mappes, 2005), thus resulting in a potentially

stronger preference for male-benefit traits in the Long Mating scenario and potentially a stronger antagonistic gene effects on daughters.

Sire of a higher pathogen resistance level did not have offspring of significantly higher resistance. The correlation between sire pathogen resistance and offspring pathogen resistance is not as high as for the son-daughter pathogen resistance correlation. This could be because some of the sons and daughters were full sibs, so there is also dominant genetic variance contributing to the son-daughter correlation but not the sire-offspring correlation. Another possible explanation is that sire pathogen resistance was measured within a sexual selection context which may strengthen the immunity-reproduction trade-off (Kawecki, 2020), while the measured offspring pathogen resistance mainly captured the immune responses post infection without the confounding reproductive efforts.

The "good genes" version of sexual selection is often thought to reinforce the alignment of sexual selection and natural selection because males of high condition will be favored by females, and by doing so, population mean fitness is expected to increase in the long run. In nature, males get infected and continue to interact with the other males and females during the course of infection, which is similar to what has been shown in our Long Mating scenario. We found no evidence for "good genes" sexual selection, which is in line with findings from Sharda et al. (2022) that sexual selection does not promote resistant genes in the population. The context- and sex-dependent sire-offspring relationship we detected here demonstrates that the cost-benefit balance of carrying pathogen resistance was mediated by the context where sexual selection happens and how sex-specific or sexually antagonistic selection may break the alignment of the two forms of selections on promoting resistant genes (Foerster et al., 2007, Hollis and Houle, 2011, Rice and Chippindale, 2001, Pischedda and Chippindale, 2006). However, pathogen resistance is only one measure of fitness. To fully understand the relationship between sire's sexual success and offspring's non-sexual fitness and its implications on the population fitness, future experiments should cover more fitness-related traits to understand how the synergy between natural selection and sexual selection acts on a larger temporal and spatial scale.

## Data accessibility

All data and R scripts will be made available on Zenodo (DOI:xxxx) upon acceptance. For peer review, these materials are included as supplementary materials.

## Author contributions

A.L. and T.J.K. conceptualized the study; A.L. collected the data; A.L. and T.J.K. wrote the manuscript.

## Competing interests

We declare no competing interests.


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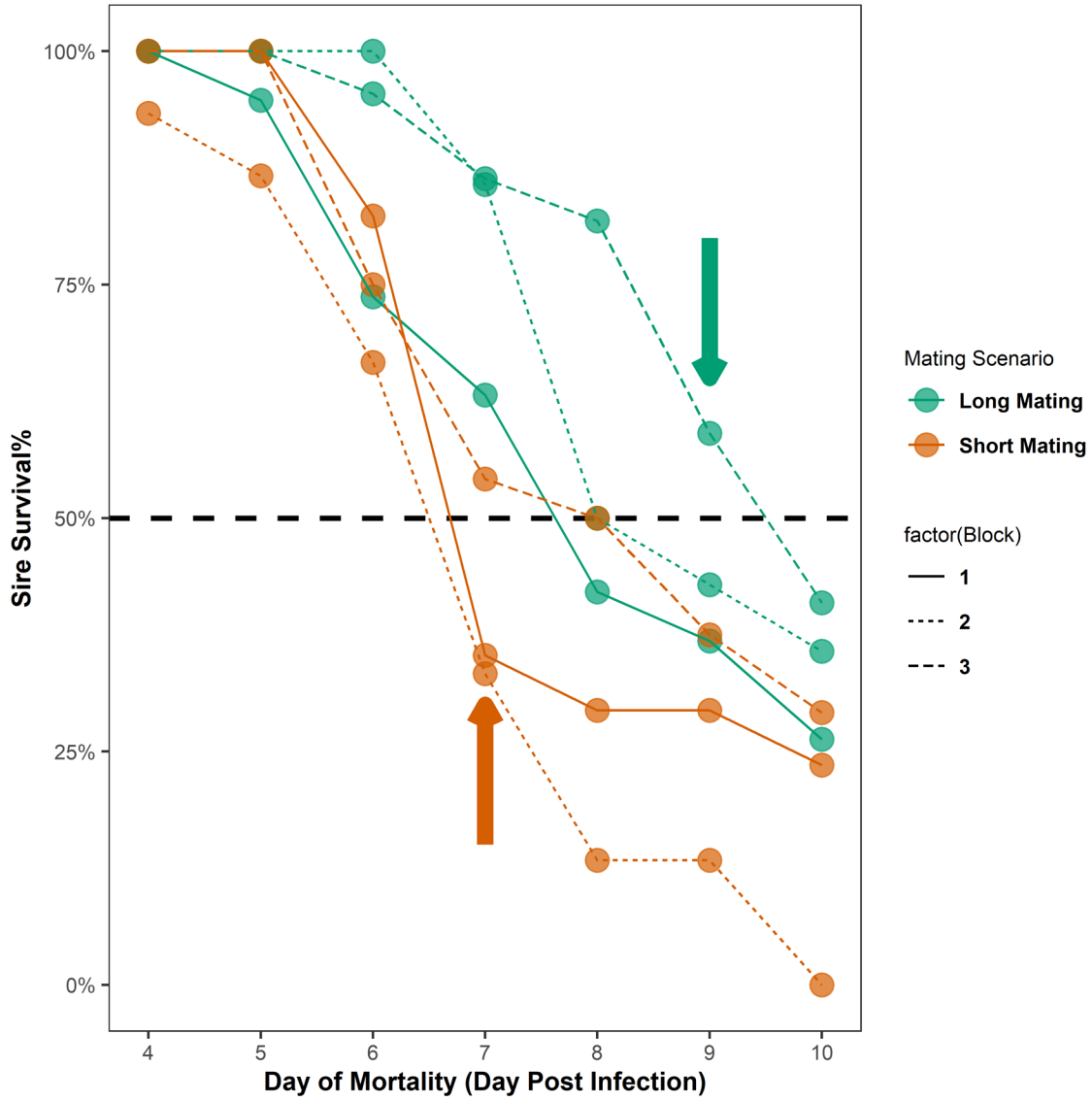


*Figure S 3-1 Survival curves of infected sires following infection in the two mating scenarios. Arrows indicate splitting time points for sire pathogen resistance levels: relatively susceptible (sires dead before or on the splitting time point) and relatively resistant (sires alive after the splitting time point). The splitting time point is different for the "Long Mating" scenario and "Short Mating" scenario.*

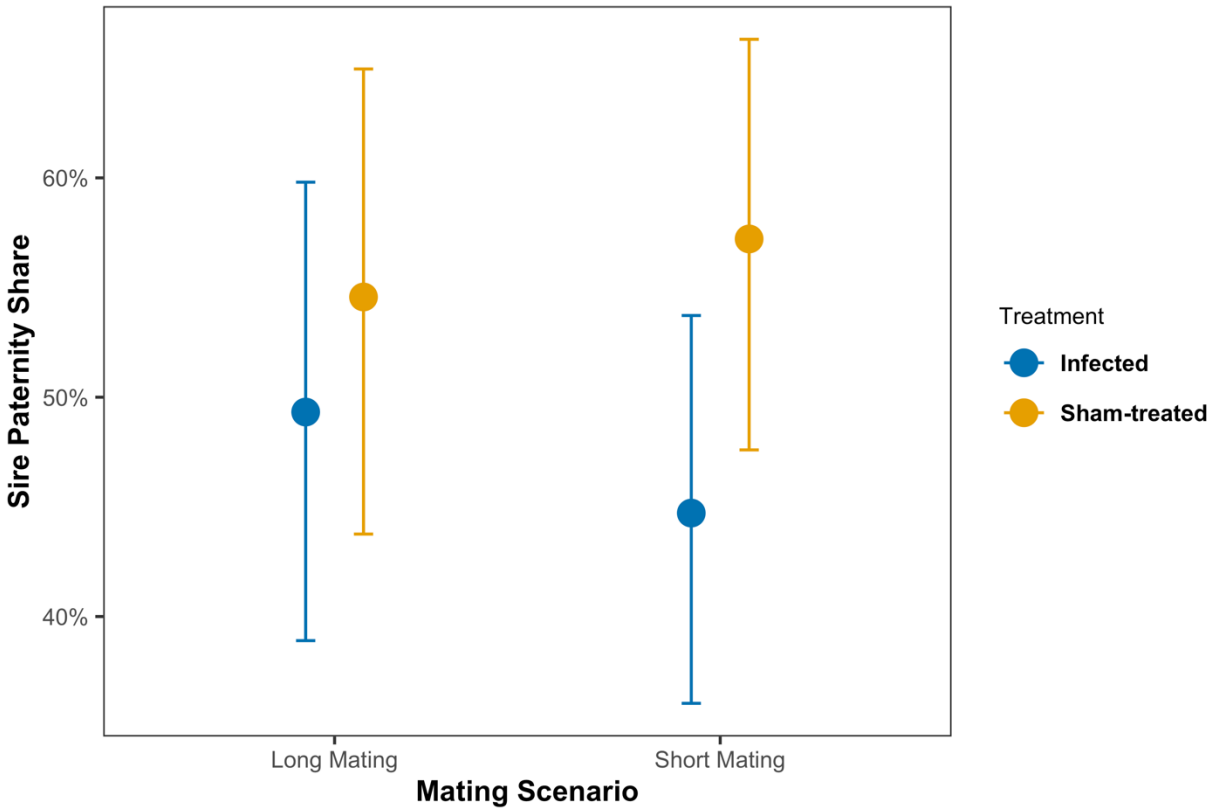


*Figure S 3-2 Estimated sire paternity share (Estimated marginal means and the 95% confidence intervals) Dataset used for both mating scenarios was **paternity share from day 4-6 post treatment**. Pairwise comparison showed that the paternity share of the infected sire is not statistically different from that of the sham-treated sire (p = 0.326, 0.063 respectively)*

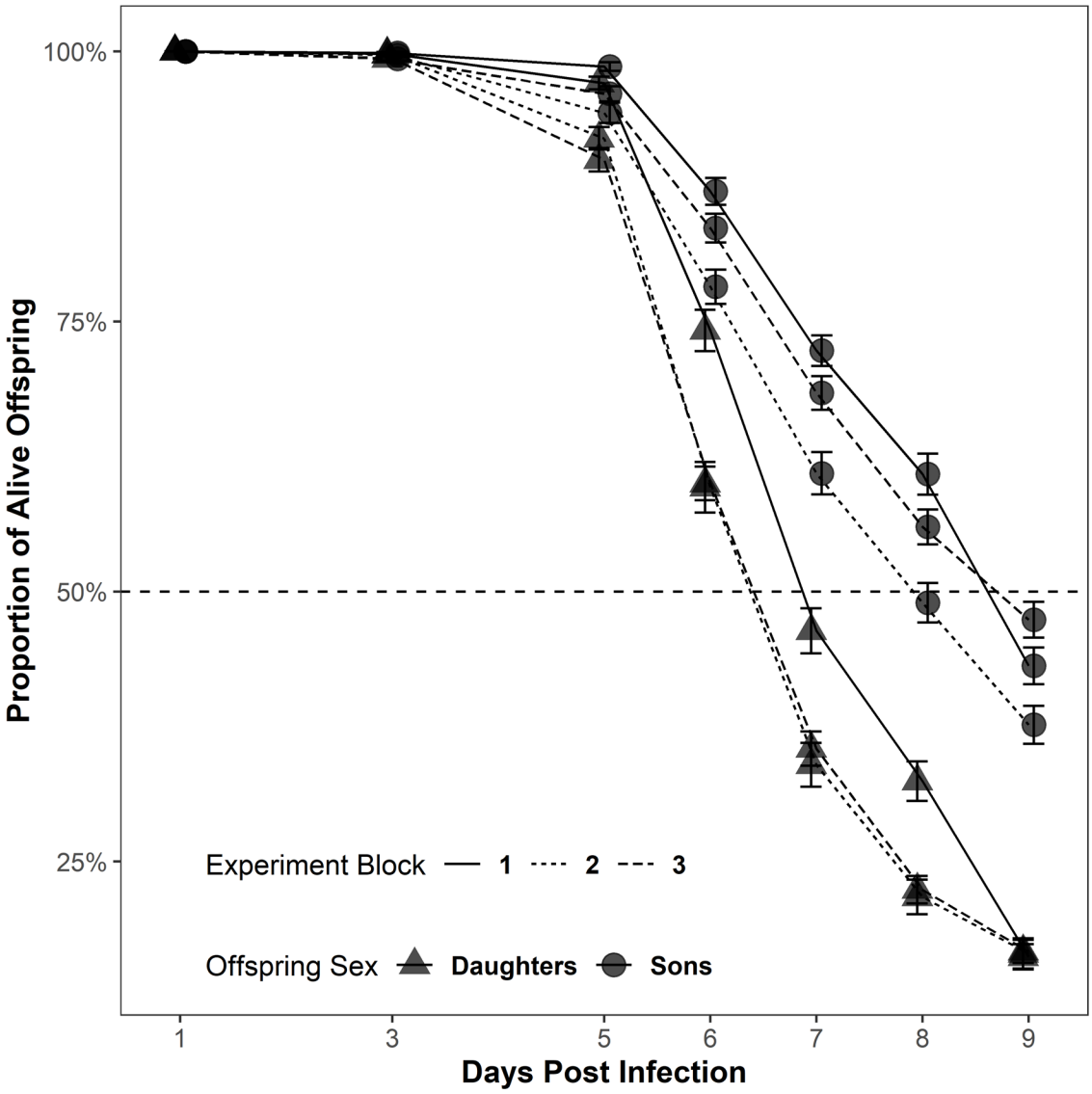


*Figure S 3-3 Survival of daughters and sons post infection (means ± SE). Different experiment blocks are indicated by different line types.*

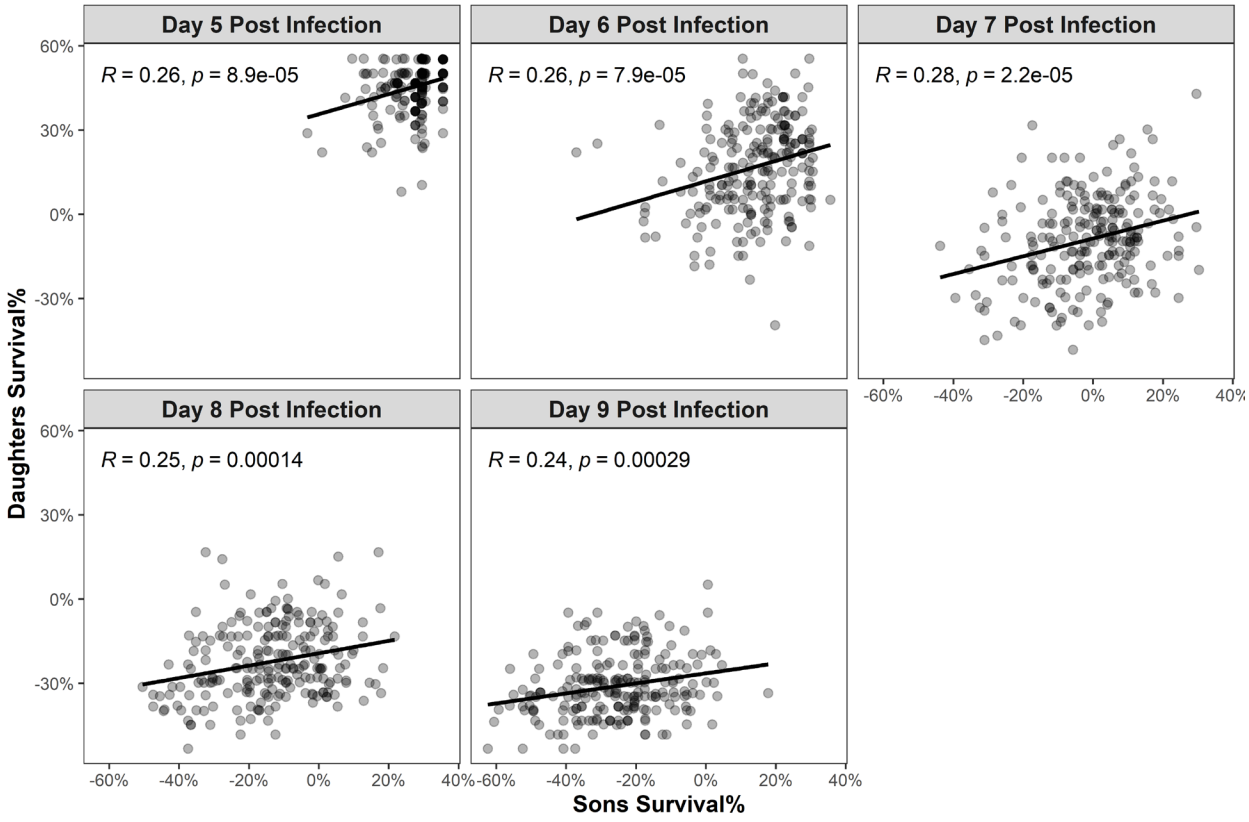


*Figure S 3-4 Relationship between son survival% and daughter survival% on each day post infection. Each dot represents the offspring from the same sire. x axis and y axis indicate the difference between the observed survival% to the mean offspring survival% of the corresponding experiment block. Solid lines are the fitted lines from linear regression. Pearson's correlation coefficient (R) and the corresponding significance level are also shown in the figure.*

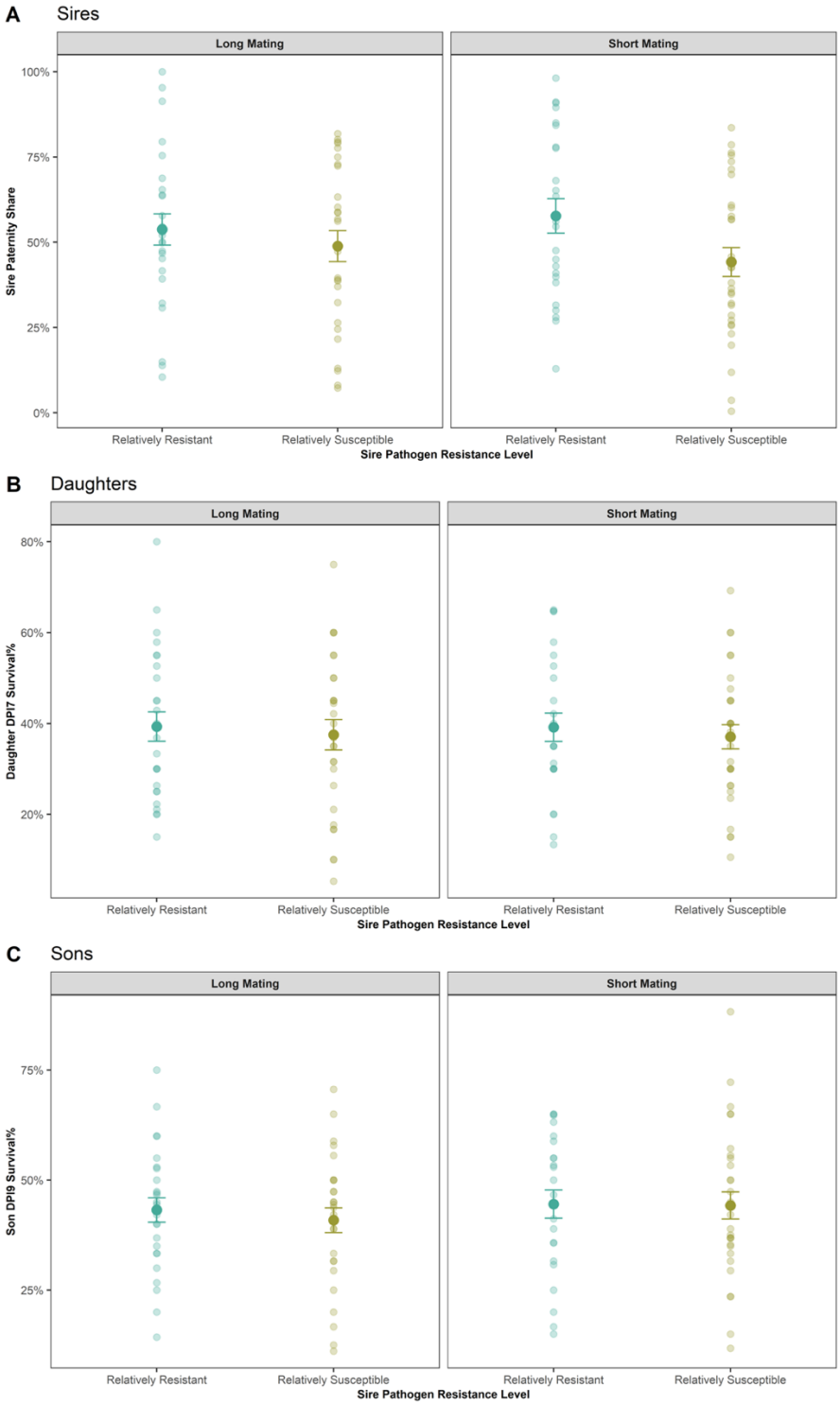


*Figure S 3-5 Relationship between sire's pathogen resistance level with **A**: sire's paternity share; **B**: daughter's pathogen resistance and **C**: son's pathogen resistance in each mating scenario. Each dot in **A** represents measures from each sire and in **B-C** represents the survival of ~20 offspring from each sire. Error bars show the means ± SE.*

*Table S 3-1 Sample size (number of sires) of the main experiment*

| Experiment Block | Block 1 | | Block 2 | | Block 3 | |
|---|---|---|---|---|---|---|
| Mating Scenario | Long Mating | Short Mating | Long Mating | Short Mating | Long Mating | Short Mating |
| Infected | 18 | 18 | 15 | 14 | 22 | 24 |
| Sham-treated | 15 | 16 | 13 | 14 | 24 | 19 |

*Table S 3-2 Effects of the fixed factors on offspring survival based on the Likelihood Ratio Test (**Whole dataset**). The generalized linear mixed model includes offspring pathogen resistance as the response variable, sire infection treatment, paternity share, mating scenario, offspring sex and their interactions as fixed factors, and experiment block with focal sire identity nested in it and observation identity as the random factors. Significance terms ($p \leq 0.05$) are indicated in bold.*

**FULL MODEL (Whole dataset)**

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| Sire Infection Treatment | 0.2 | 1 | 0.625 |
| Paternity Share | 3.3 | 1 | 0.067 |
| **Offspring Sex** | **15.4** | **1** | **<0.001** |
| Mating Scenario | 0.7 | 1 | 0.394 |
| Sire Infection Treatment × Paternity Share | 0.8 | 1 | 0.385 |
| Sire Infection Treatment × Offspring Sex | 0.0 | 1 | 0.969 |
| Paternity Share × Offspring Sex | 0.1 | 1 | 0.810 |
| Sire Infection Treatment × Mating Scenario | 0.1 | 1 | 0.766 |
| Paternity Share × Mating Scenario | 3.7 | 1 | 0.053 |
| Offspring Sex × Mating Scenario | 0.0 | 1 | 0.923 |
| **Sire Infection Treatment × Paternity Share × Offspring Sex** | **5.4** | **1** | **0.021** |
| Sire Infection Treatment × Paternity Share × Mating Scenario | 0.9 | 1 | 0.353 |
| Sire Infection Treatment × Offspring Sex × Mating Scenario | 0.8 | 1 | 0.366 |
| Paternity Share × Offspring Sex × Mating Scenario | 2.0 | 1 | 0.153 |
| Sire Infection Treatment × Paternity Share × Offspring Sex × Mating Scenario | 0.2 | 1 | 0.685 |

*Table S 3-3 Effects of the fixed factors based on the likelihood ratio tests (**Sons only dataset**). The best GLMM fitting to the data (with the lowest AIC) includes offspring pathogen resistance as the response variable, sire infection treatment, paternity share and their interaction as fixed factors, and experiment block and observation identity as the random factors. Significance terms (p < 0.05) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| Sire Infection Treatment | 0.096 | 1 | 0.756 |
| Paternity Share | 2.645 | 1 | 0.104 |
| **Sire Infection Treatment × Paternity Share** | **5.198** | **1** | **0.023** |

*Table S 3-4 Effects of the fixed factors based on the likelihood ratio tests (**Daughters only dataset**). The best GLMM fitting to the data (with the lowest AIC) includes offspring survival as the response variable, mating scenario, paternity share and their interaction as fixed factors, and experiment block and observation identity as the random factors. Significance terms (p < 0.05) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| Paternity Share | 3.090 | 1 | 0.079 |
| Mating Scenario | 0.384 | 1 | 0.535 |
| **Paternity Share × Mating Scenario** | **5.439** | **1** | **0.020** |

## Appendix 1

As we only checked the survival of the infected sires until day 10 post infection, sires that were alive at the end of the experiment were noted as right-censored data. In order to properly include these censored data in the data analysis, we have created a new variable called "sire pathogen resistance level". In addition to what has been reported in the main text of this chapter, we have repeated the data analysis testing the relationship between sire sexual success and sire pathogen resistance level as well as between sire pathogen resistance level and offspring pathogen resistance using a 6-level sire pathogen resistance (Figure A 3-1; sire died on day 6 post infection as the lowest level and sire alive on day 10 post infection as the highest level). Conclusions drawn from these additional analyses are the same as reported in the chapter: sire pathogen resistance level was positively correlated with sire sexual success, but no significant correlation was observed between sire pathogen resistance and offspring pathogen resistance.

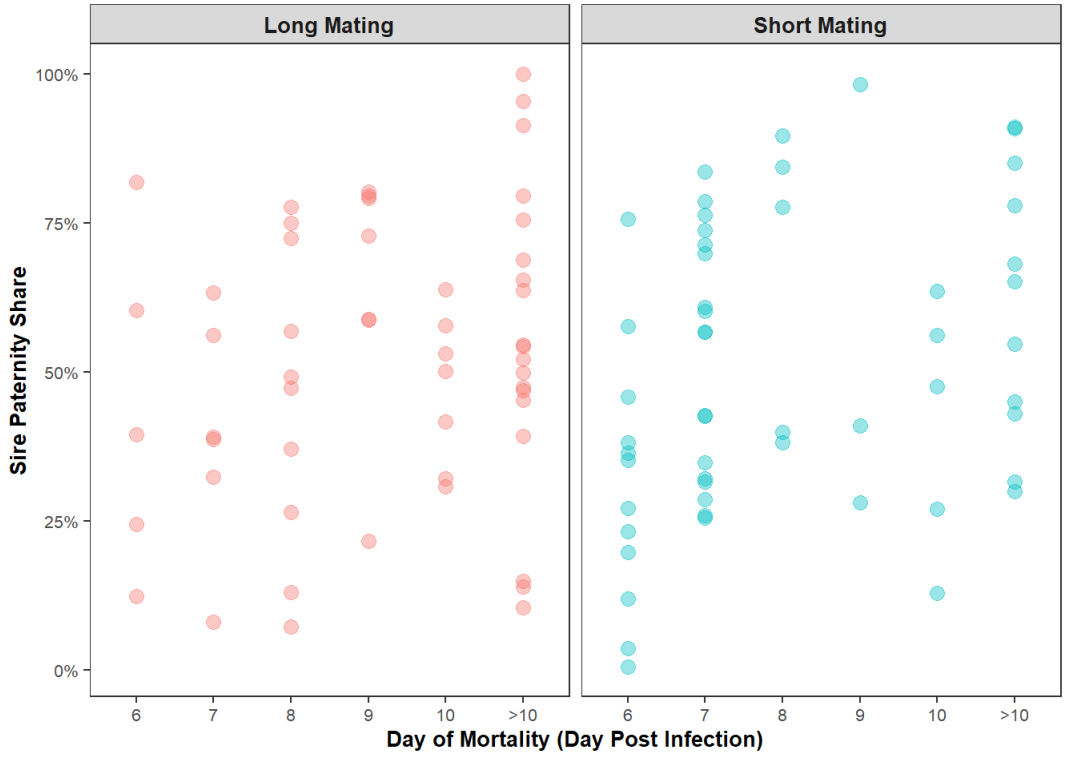


*Figure A 3-1 Relationship between sire paternity share (i.e. sire sexual success) and day of mortality (i.e. the day post infection when the sire was found dead). Sires that were alive at the end of the observation were grouped into ">10". Each transparent dot represents a sire.*

***Treating "sire pathogen resistance level" as a continuous variable (with censored data)***

In this dataset, the value for each sire's pathogen resistance level is the same as the number of day post infection upon death, and sires that did not die at the end of the observation are given a value of 15 (a value chosen randomly). The best GLMM (binomial distribution, logit link) describing any of the three response variables is with sire pathogen resistance level (centered on zero) as the fixed factor, experiment block as the random factor, and an observation level random factor to correct for overdispersion. The full statistic output (Table A 3-1), estimated slopes (Table A 3-2), and figures (Figure A 3-2, Figure A 3-3) are listed below.

*Table A 3-1 Effects of fixed factors using likelihood ratio test (**With Censored Data**). Significance terms ($p \leq 0.05$) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| Response Variable: Sire Sexual Success | | | |
| **Sire Pathogen Resistance Level** | **5.8** | **1** | **0.016** |
| Response Variable: Son Pathogen resistance | | | |
| Sire Pathogen Resistance Level | 0.3 | 1 | 0.575 |
| Response Variable: Daughter Pathogen Resistance | | | |
| Sire Pathogen Resistance Level | 0.5 | 1 | 0.482 |

*Table A 3-2 Estimated slopes of the relationship between sire pathogen resistance level and each of the response variables **(With Censored Data)**. Significance terms except for intercepts ($p \leq 0.05$) are indicated in bold.*

| Response Variable | Term | Estimate | Std.Error | z value | Pr(>\|z\|) |
|---|---|---|---|---|---|
| Sire Sexual Success | Intercept | 0.029 | 0.186 | 0.2 | 0.878 |
| | **Sire Pathogen Resistance Level** | **0.094** | **0.038** | **2.4** | **0.015** |
| Son Pathogen Resistance | Intercept | -0.318 | 0.140 | -2.3 | 0.022 |
| | Sire Pathogen Resistance Level | 0.010 | 0.018 | 0.6 | 0.574 |
| Daughter Pathogen Resistance | Intercept | -0.526 | 0.163 | -3.2 | 0.001 |
| | Sire Pathogen Resistance Level | 0.013 | 0.019 | 0.7 | 0.481 |

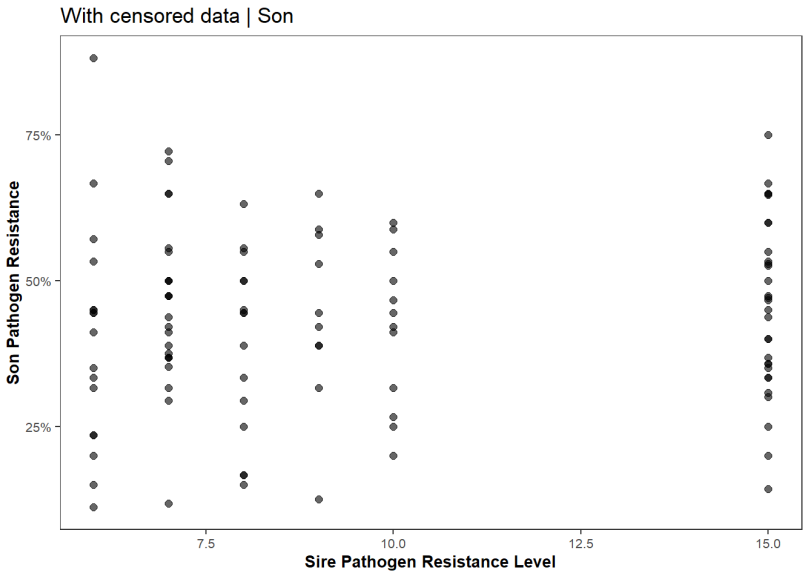


*Figure A 3-2 Relationship between sire pathogen resistance level and son's pathogen resistance* ***(With Censored Data)****. Each transparent dot represents ~20 sons of the sire.*

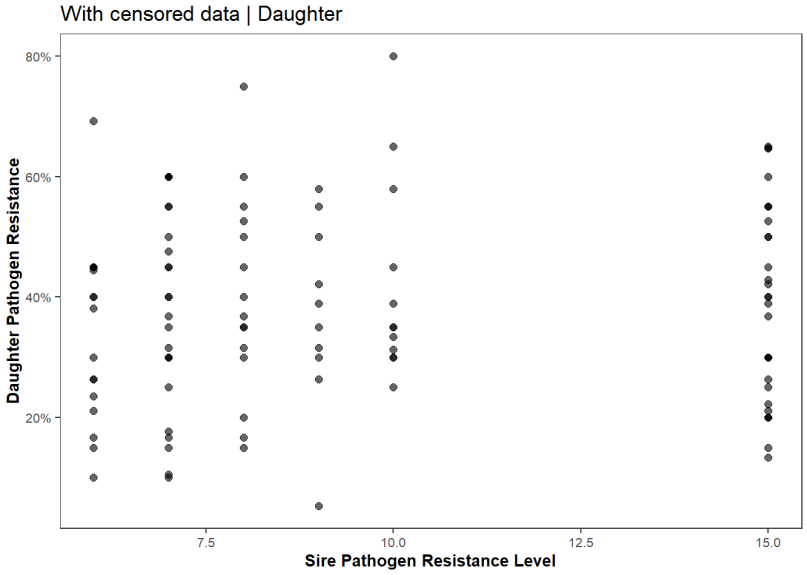


*Figure A 3-3 Relationship between sire pathogen resistance level and daughter's pathogen resistance* ***(With Censored Data)****. Each transparent dot represents ~20 daughters of the sire.*

***Treating "sire pathogen resistance level" as a continuous variable (withOUT censored data)***

Next, we removed the censored data from the dataset and checked if the correlation observed above has changed. The best GLMM (binomial distribution, logit link) describing sire sexual success and son pathogen resistance only includes sire pathogen resistance level as a fixed factor, whereas the best model describing daughter pathogen resistance includes sire pathogen resistance level, mating scenario, and their interactions as fixed factors. All models are with experiment block as the random factor and also include an observation level random factor to correct for overdispersion. The full statistical output (Table A 3-3) and the figures (Figure A 3-4, Figure A 3-5)are listed below.

*Table A 3-3 Effects of fixed factors **(WithOUT Censored Data)**. Significance terms ($p \leq 0.05$) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| Response Variable: Sire Sexual Success | | | |
| Sire Pathogen Resistance Level | 3.7 | 1 | 0.054 |
| Response Variable: Son Pathogen Resistance | | | |
| Sire Pathogen Resistance Level | 0.0 | 1 | 0.892 |
| Response Variable: Daughter Pathogen Resistance | | | |
| **Sire Pathogen Resistance Level** | **7.3** | **1** | **0.007** |
| Mating Scenario | 0.1 | 1 | 0.767 |
| **Mating Scenario × Sire Pathogen Resistance Level** | **4.0** | **1** | **0.045** |

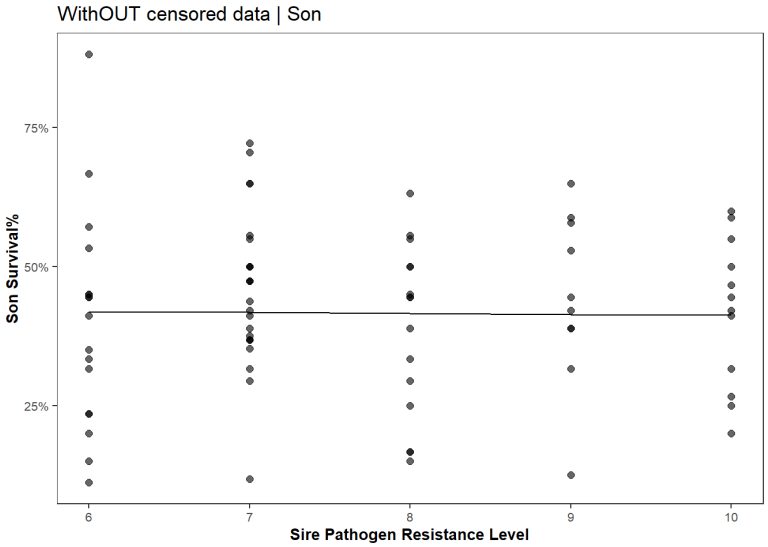


*Figure A 3-4 Relationship between sire pathogen resistance level and son survival% post infection (i.e. son pathogen resistance)* ***(WithOUT Censored Data)****. Each transparent dot represents ~20 sons of the sire. The solid line demonstrates the predicted values from the linear regression.*

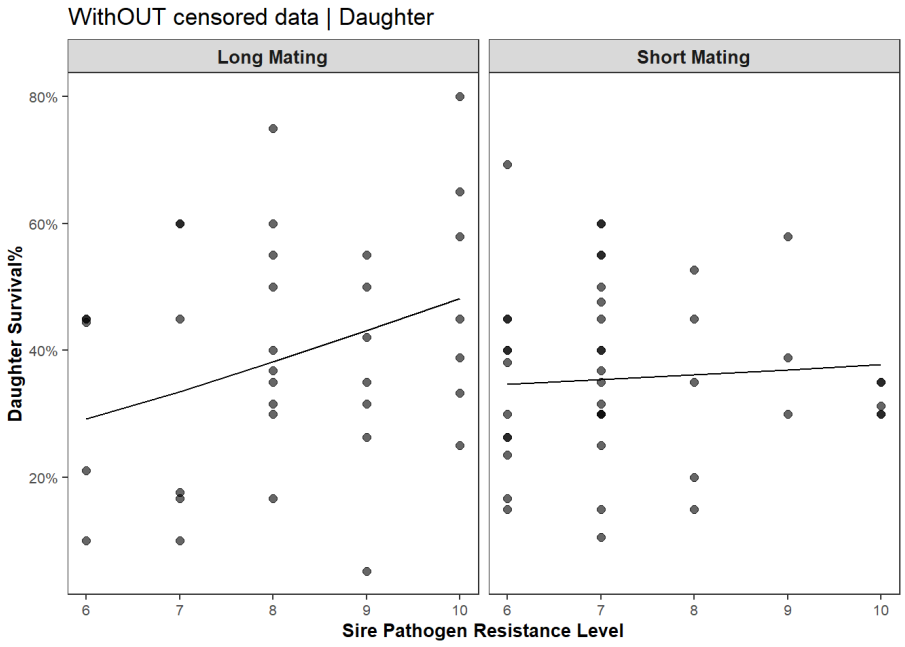


*Figure A 3-5 Relationship between sire pathogen resistance level and daughter survival% post infection (i.e. daughter pathogen resistance) in different mating scenarios* ***(WithOUT Censored Data)****. Each transparent dot represents ~20 daughters of the sire. The solid line demonstrates the predicted values from the linear regression.*

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# Chapter 4 From sire to offspring: tracing the link between sexual success and non-sexual fitness in different pathogenic environments

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Abstract

Few experiments have demonstrated the genetic correlation between sire sexual success and offspring reproductive value with consideration of trade-offs between life history traits and with consideration of varying the sexual selection context and the offspring environments. However, it holds the key to understanding why the results of research on "good genes" are often ambiguous. Here, we explored the potential effect of good genes sexual selection in different sire-offspring pathogenic environments and tested the link between sexual success, offspring pathogen resistance, and offspring reproductive fitness. Our experimental system was a trio system involving the fruit fly *Drosophila melanogaster*, the fungal pathogen *Metarhizium brunneum*, and the bacterial pathogen *Pseudomonas entomophila*. Sons experienced stronger selection on their reproductive fitness than daughters. A positive correlation between pathogen resistance and reproductive fitness was seen, but only in daughters and only when both traits were measured in an environment with pathogens. Although we found no support for "good genes" in any specific sire-offspring pathogenic environment combination, we observed a subtle trend. The relationship between sire sexual success and offspring reproductive fitness seemed to be different when the epidemiological environment of the sire and offspring aligns (both facing the same pathogen or both in the pathogen-free environment), compared to when the two pathogenic environments mismatch. Moreover, in some specific environments, an opposite correlation of sire-daughter and sire-son was found, highlighting the prevalence of sexually antagonistic gene expression even for important life history traits. Our results illustrate that the relationship between sire sexual success and offspring fitness is sex-specific and context-dependent, which at its core aligns with the "genotype by environment" interactions and has important implications on how sexual selection operates in changing environments.

## Introduction

The "good genes" sexual selection, asserting the positive additive genetic correlation between sexual success and non-sexual fitness (Rosenthal, 2017, Kokko et al., 2003), is recognized as an important evolutionary force. It, aligning with natural selection, promotes genes that facilitate individuals' adaptation to the environment they inhabit (Rowe and Rundle, 2021). Within the "good genes" framework, males of high genetic quality (i.e., the breeding value (Hunt et al., 2004)) have higher sexual success. However, evidence from empirical experiments is mixed (Baur and Berger, 2020, Garcia-Gonzalez and Simmons, 2011, Byers and Waits, 2006). How male genetic quality is expressed and perceived by females varies in different environmental settings (Robinson et al., 2008, Gosden and Svensson, 2008), making also the association between male sexual success and overall offspring fitness potentially variable (Robinson et al., 2012). Yet, this aspect is often overlooked.

The occurrence of pathogens is one source of fluctuations in the organism's living environment. Pathogen resistance is considered an important fitness-related trait in the presence of pathogens. How does the link between sexual success and pathogen resistance look like across varying epidemiological environments? Some researchers argued that the consequences of sexual selection are pathogen-mediated (Hamilton and Zuk, 1982): sexual selection favors specific resistance (effective against one type of pathogens) and a positive link can be detected when the sexual selection happens in the presence of pathogens and when both sire and offspring are facing the same (type of) pathogens. Conversely, there is a viewpoint that sexual selection favors general immunocompetence against a diverse array of pathogens. It suggests that the same genetic variants conferring pathogen resistance are favored in any pathogenic environment and that a consistently positive link between sexual success and pathogen resistance exists across all pathogenic environments due to the common condition dependency (Westneat and Birkhead, 1998). Studies testing the relationship between sexual success (mainly sexual ornaments) and pathogen resistance (e.g., parasite load, immune response) are mostly motivated by the "general immunocompetence" model. Sexual success is often measured in the absence of pathogens and mixed results have been yielded (Roberts et al., 2004, Kilpimaa et al., 2004, Drayton et al., 2012). It has also been shown in Kawecki (2020) and Chapter 3 that carrying pathogen resistance is costly to sexual selection in the absence of pathogens. Moreover, Joye and Kawecki (2019) have provided evidence that sexual

selection favors “specific pathogen resistance”, but the “general immunocompetence” scenario is not directly rejected as the experiment only includes the presence/absence of a pathogen and does not directly show that the increased pathogen resistance is not effective against another pathogen.

The “specific pathogen resistance” scenario and the “general immunocompetence” scenario are not mutually exclusive. How an organism detects “intruders” and the activation of immune responses are manifested in multiple ways. Some of these aspects are shared among defense against multiple pathogens, such as the physical barriers, the humoral response, melanization and encapsulation, and the cellular defenses (Hultmark, 2003), which makes it possible that improved resistance to one (type of) pathogen can also lead to improved resistance against another. For instance, Wang et al. (2017) find that certain genetic variants in *Drosophila melanogaster* confer resistance against both *Metarhizium anisophliae* and *Pseudomonas aeruginosa*. Similarly, Aparajita et al. (2021) show that *D.melanogaster* evolved to resist *P.entomophila* or *Enterococcus faecalis* also gain higher resistance against multiple novel pathogens. However, immune responses can be fairly specific and may not have the capacity for defense against other pathogens. For instance, in *D. melanogaster*, antimicrobial peptides (AMPs) work in a high specificity, with only a subset of AMPs involved in effectively combating a specific pathogen rather than the entire repertoire of AMPs (Hanson et al., 2019). Given the complex host-pathogen interactions, determining which type of pathogen resistance is favored by sexual selection will require testing the pathogen resistance across a range of different pathogens.

An aspect of the genetic basis for the link between sexual success and pathogen resistance is that genes encoding these traits have pleiotropic effects. Genetic variants conferring high pathogen resistance can also be responsible for traits contributing to sexual success. However, genetic trade-offs also exist. Trade-offs at the physiological level between fitness-related traits are also common due to the limited resources and energy of the individuals (Stearns, 1989, Flatt and Heyland, 2011). If sire sexual success confers high pathogen resistance but low fitness in other aspects, the “good genes” version of sexual selection would not facilitate natural selection as the theory predicts. However, one may also expect that individuals of higher pathogen resistance in the presence of pathogens are more likely to survive to a reproductive age and thus may have higher chances to pass on their genes. Such a positive correlation between pathogen resistance and reproductive fitness may be more evident in females as females usually invest more in somatic maintenance

than males (Zuk and Stoehr, 2002). If sexual selection favors specific resistance, what would be the fitness of offspring when they are exposed to another type of pathogens or when they are exposed to no pathogens in terms of survival and in terms of overall reproductive success? One should also keep in mind that sexual success for males may also be the result of the Fisherian runaway process, where certain traits, though not necessarily advantageous for survival, are favored by females, providing benefits predominantly to sons (Prokop et al., 2012, Fisher, 1930). Trade-offs can also be extended to the dichotomy between females' and males' interest in shared traits. The divergence between female fitness and male fitness can also be mediated by pathogens. The alignment of natural selection and sexual selection through "good genes" requires that the net effect of sexual selection should favor improved overall offspring fitness despite potential sex-specific/biased sexual selection. Together, all these complexities listed above highlight the need to not only look at potential trade-offs between life history traits, but also to consider sexual dimorphism in life histories when testing the "good genes".

In light of the yet-to-be-resolved matters stated previously, we aimed to explore the "good genes" hypothesis by testing the link between male sexual success and components of offspring fitness under various epidemiological contexts, while also dissecting the potential different impacts for daughters and sons. We used the fruit fly *Drosophila melanogaster* and two contrasting pathogens, a fungal pathogen *Metarhizium brunneum,* and a gram-negative bacterial pathogen, *Pseudomonas entomophila* as our experiment system to test the relationship between sexual success, pathogen resistance, and reproductive fitness in the same experiment framework.

First, to directly investigate whether sexual selection favors pathogen resistance and if so, what kind of pathogen resistance is favored, we looked into the relationship between sire sexual success (sire paternity share in competing mating trials) and offspring pathogen resistance (survival post infection) when sexual selection happened with or without exposure to *M.brunneum* and when offspring were exposed to *M.brunneum* or *P.entomophila*. Fluctuations caused by pathogens are not limited to the occurrence of pathogens but are also linked to the dose of pathogens. The abundance of pathogens is in most cases directly associated with the virulence of pathogens. A more natural scenario would be that individuals encounter low doses of pathogens and are still able to reproduce during the course of infection. This is more relevant to bacterial infection than fungal infection because infection by a high dose of *P.entomophila* is acute and severe and

infection with a lower dose gives the host some buffering time similar to the incubation period post fungal infection. Therefore, to examine the potential trade-offs between offspring resistance and offspring reproductive fitness, we varied the levels of pathogen exposure when measuring reproductive fitness and tested the link between these two traits in three scenarios: a high dose of *M.brunnuem*, a low dose of *P.entomophila*, and no pathogen exposure for reproductive fitness. Lastly, to investigate whether sexual selection improves offspring net fitness (i.e., reproductive value) and whether such a link is affected by the alignment of the pathogenic environment of the sire and offspring, we looked into the relationship between sire sexual success and offspring reproductive fitness (relative contribution to the grand-offspring generation) across different epidemiological contexts (i.e., different sire-offspring pathogenic environment combinations). To investigate the potential differences between the two sexes, we looked into all the relationships mentioned above separately for daughters and sons. Although we did not reach a definite conclusion on whether sexual selection promotes pathogen resistance or offspring reproductive fitness, we found weak evidence[4] showing that the sire-offspring relationship was sex-specific and context-dependent, indicating a complex interplay between sexual selection, pathogen resistance, and reproductive fitness and the multifaceted nature of evolutionary strategies in pathogen defense and reproduction.

[4] In order to highlight that testing "good genes" hypothesis is not a simple binary null hypothesis testing (either accept or reject), this chapter is written in an evidence-based style as suggested by Muff et al (2022).
MUFF, S., NILSEN, E. B., O'HARA, R. B. & NATER, C. R. 2022. Rewriting results sections in the language of evidence. *Trends in Ecology & Evolution,* 37**,** 203-210.

## Materials and Methods

### Fly origin, pathogen origins, infection

We used flies from a lab-adapted outbred population and a GFP population of similar genetic backgrounds in this experiment (previously described in Chapter 3). All flies were raised at a controlled density (~200 eggs per 40ml food) and maintained at 25°C, 55% relative humidity, and 12L:12D photoperiod on standard cornmeal-yeast-agar media. When virgin flies were required for the experiment, they were collected 6-8h post emergence and maintained in food vials until used in the experiment. Female virginity was further confirmed by the absence of larvae in the vials. All fly transfers were done under light $CO_2$ anesthesia.

Pathogens used in this experiment are *Metarhizium brunneum* (a fungal pathogen) and *Pseudomonas entomophila* (a gram-negative bacterium). Pathogen origins and infection protocols were previously described in Chapter 1 and in Joye and Kawecki (2019), respectively.

### Experiment procedure

The full experiment design is summarized in Figure 4-1. This experiment was designed to investigate the relationship between sire sexual success, offspring pathogen resistance, and offspring reproductive fitness.

To obtain offspring from each sire prior to any treatment, we began by pairing each sire with three virgin females and let them mate for 20h on orange juice agar with yeast (oviposition bottle). Females were fed with extra yeast the day before mating to increase egg yield. Following the mating, each sire was then transferred to an individual food vial until the next step of the experiment. Females were also transferred to new food vials for another round of egg-laying. To avoid larval over-crowding, after 24 hours, females were then transferred to new food vials to lay eggs, and we kept the old vials for collecting offspring (offspring acquisition vials batch#1). After another 24 hours, females were removed, and food vials were also kept for offspring collection (offspring acquisition vials batch#2). From each oviposition bottle (i.e., sire), we collected 200 eggs into food bottles (fitness assay bottles).

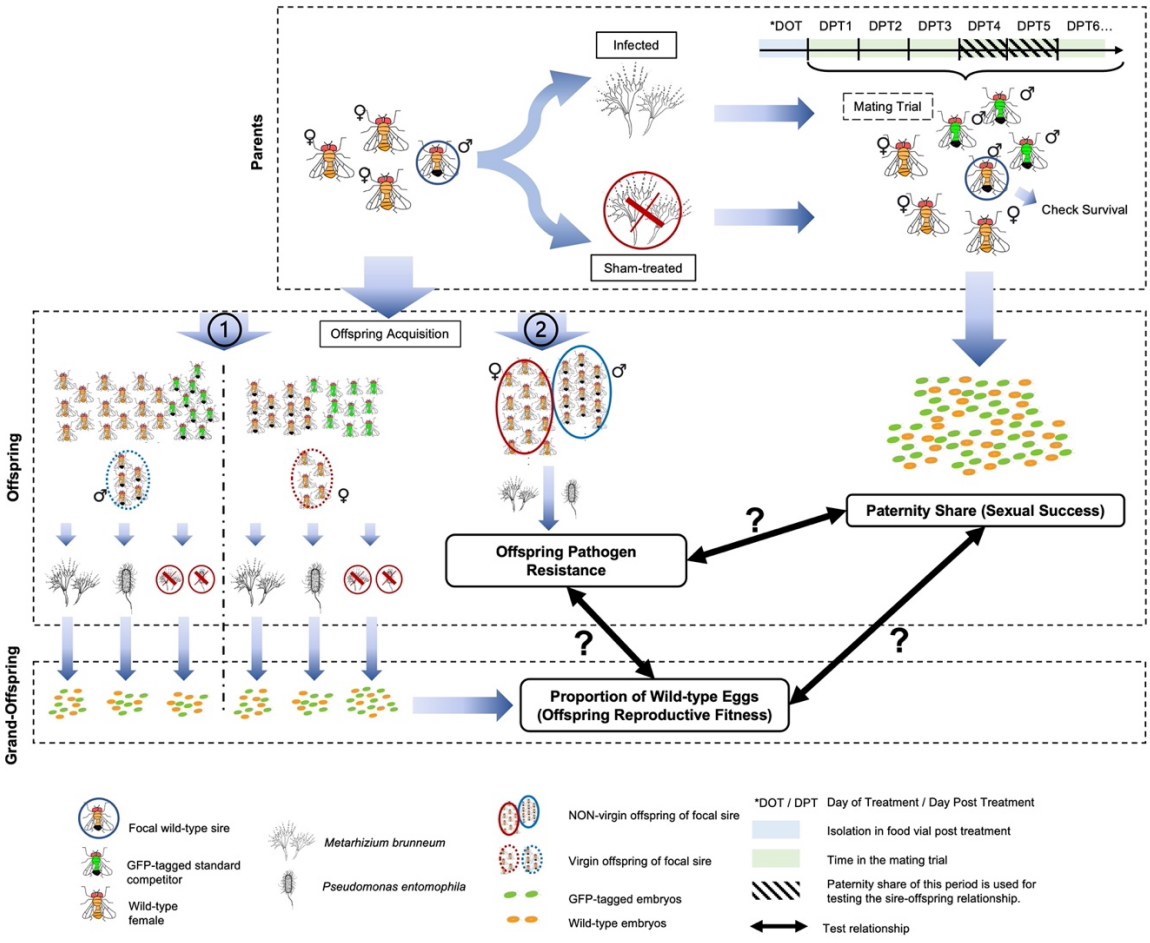


*Figure 4-1 Experimental design to study the relationship between sire sexual success, offspring pathogen resistance, and offspring reproductive success across various epidemiological contexts. First, each sire was coupled with three random virgin females and offspring from this mating were allowed to develop [Offspring Acquisition]. Then, after mating, sires were subjected to either sham treatment or Metarhizium infection. Next, sires were kept in isolation and placed into mating trial together with three virgin females and three GFP-tagged standard competitors on day 1 post treatment. Sire's sexual success was determined by the combined paternity share on day 4 and day 5 post treatment. When offspring ② from offspring acquisition reached adulthood, their resistance to Metarhizium or Pseudomonas was assessed in terms of survival post infection (20 offspring per offspring sex per sire). Offspring ① from the offspring acquisition were raised at controlled density (~200 eggs per 40ml food) and collected and sexed as virgins. Every 5 virgin offspring were paired with 10 standard GFP-tagged competitors and 15 standard wild-type mates [Mating Groups]. These mating groups were subjected to either Metarhizium infection, Pseudomonas infection, or no treatment (sham-treated). A subsample of eggs laid by females in the mating groups was counted on day 3 and 5 post treatment and the proportion of wild-type eggs was used as the proxy of offspring reproductive success.*

Six hours after being removed from the oviposition bottle, each sire was randomly subjected to infection (*M.brunneum* spore suspensions $10^7$ spores/ml) or sham treatment [spore-free 0.05% Triton X-100 (Sigma-Aldrich)] and kept individually until the mating trial. Each mating trial started on day 1 post treatment. At the start of the mating trial, we paired each sire with three non-infected GFP-tagged standard competitors and three virgin non-infected wild-type females and put

them on standard cornmeal-yeast-agar media. At the end of day 2 post treatment, we transferred all flies into grape juice agar with yeast. All flies were transferred to fresh grape juice agar with yeast every 12 hours. We counted all eggs laid by females during day 4 and 5 post treatment. The number of wild-type eggs over total number of eggs laid during these two days (i.e., paternity share) was then used as a proxy for sire sexual success. We used paternity share from day 4-5 post treatment because at this time the infection is established, the immune response is fully activated within the infected host, and there is no mortality due to infection but negative effects on sire competitiveness are always shown (See Chapter 1 and Chapter 3).

To measure the offspring pathogen resistance post infection, we pooled all emerged flies from both offspring acquisition batch#1 and batch#2 and randomly selected 20 flies for each sex from each sire for each infection treatment. Upon infection, all offspring were 4-6-day-old. They were subjected to either *M.brunneum* ($10^7$ spores/ml) or *P.entomophila* ($OD_{600}$ = 40) infection and then kept in same-sex groups of 10 in food vials. Mortality was recorded daily until day 10 post infection for *M.brunneum* and day 5 post infection for *P.entomophila*.

To measure the offspring's reproductive fitness, we randomly selected 15 virgin focal offspring per sex per sire from those that emerged from the fitness assay bottles. Then we transferred 5 virgin focal offspring together with 10 virgin GFP-tagged standard competitors and 15 virgin wild-type standard mates (sex ratio = 1:1) onto grape juice agar with yeast. Hereafter, we call this setup mating groups. Prior to the mating groups, virgin focal offspring were subjected to either *M.brunneum* ($10^7$ spores/ml), *P.entomophila* ($OD_{600}$ = 10), or no infection, and were 3-day-old upon treatment. Standard mates and standard competitors were also raised at a controlled density (~200 eggs per 40ml food) and were of the same age as the focal offspring upon the onset of the mating groups. Mating groups were transferred to fresh grape juice agar with yeast every 12 hours. On day 3 and 5 post treatment, we randomly and non-hazardously selected and counted a subsample of eggs (100 ~ 200 eggs per subsample) from the pool of eggs in each mating group. The number of wild-type eggs over the total number of eggs counted was taken as the proxy for the focal offspring's reproductive fitness.

**Statistical Analysis**

All analyses were conducted using R v.4.1.2 (R Core Team, 2020) and visualizations were done using '*ggplot2*' v. 3.4.1 (Wickham, 2016). Generalized linear mixed models (GLMMs) were run using the *glmer*() function of the package '*lme4'* v 1.1-27.1 (Bates et al., 2015). Model assumptions were verified using the '*DHARMa*' package v.0.4.5 (Hartig, 2022) and if any over-dispersion was detected, GLMMs would be updated to include an observational level random factor (i.e., observation identity). Effects of fixed factors were tested using the likelihood ratio test (LRT) with the *mixed*() function of the '*afex*' package v.1.0-1 (Singman et al., 2021).

*The relationship between antifungal pathogen resistance and antibacterial pathogen resistance*

To investigate the link between sire sexual success and offspring pathogen resistance, we needed a single offspring survival measure as the proxy for offspring pathogen resistance. First, we tested whether daughters and sons have different dynamics of survival post infection, using a GLMM (binomial distribution, logit link) with offspring sex, day post infection (centered on zero), and their interactions as fixed factors, and experiment block with sire identity nested in it as the random factors. We used the offspring survival data from the day when the average proportion of alive offspring dropped down to around 50% as the measure of offspring pathogen resistance. We then tested the correlation between daughter pathogen resistance to son pathogen resistance to examine the heritability potential of pathogen resistance, and the correlation between pathogen resistance to *P.entomophila* and to *M.brunneum* to investigate whether and how these two types of pathogen resistance are correlated.

*The relationship between offspring pathogen resistance and offspring reproductive fitness*

To investigate whether there is a trade-off between offspring pathogen resistance and offspring reproductive fitness and whether it applies to all pathogenic environments, we fitted a GLMM (binomial distribution, logit link) with offspring reproductive fitness as the response variable, offspring combined pathogen resistance, offspring treatment (*M.brunneum*, *P.entomophila*, or no infection) and their interactions as the fixed factors and experiment block as the random factor. An observational level random factor was also included to correct for overdispersion. Offspring combined pathogen resistance was calculated as number of survivors post *P.entomophila* infection

plus number of survivors post *M.brunneum* infection divided by total number of flies infected by *P.entomophila* plus total number of flies infected by *M.brunneum*. This analysis was done for daughters and sons separately. The combined pathogen resistance is centered on zero by experiment block.

The same analysis was repeated by replacing the offspring combined pathogen resistance with resistance to *P.entomophila* or resistance to *M.brunneum* and similar conclusions were drawn (see Appendix 2). If there is a significant correlation between daughter's pathogen resistance and son's pathogen resistance, we also looked into the link between daughter's reproductive success and son's pathogen resistance and vice versa (see Appendix 2).

*The relationship between sire sexual success and offspring pathogen resistance*

To test whether sexual selection favors pathogen resistance and if so, what kind of pathogen resistance is favored, we need to look at the relationship between sire sexual success and offspring pathogen resistance when the pathogenic environment where sexual selection happens aligns with the pathogenic environment of the offspring (both exposed to the same pathogens or no pathogen) and when they do not (e.g., absent of pathogens or a different set of pathogens in one of the generation).

To test the sire-offspring relationship when the two environments align and when the pathogen is absent in the sire generation, we first conducted the analysis separately for offspring of *M.brunneum*-infected sires and of sham-treated sires. We fitted a GLMM (binomial distribution, logit link) with offspring resistance to *M.brunneum* infection (alive versus dead) as the response variable, paternity share (center-scaled by subtracting the overall mean), offspring sex and their interactions as fixed factors, and experiment block with sire identity nested in it and observation identity as the random factors. Then, we applied the same GLMMs but with offspring resistance to *P.entomophila* infection as a response variable to examine the link between sire sexual success and pathogen resistance when the offspring are exposed to a different pathogen than the sire. Variation in survival post *P.entomophila* between the two experiment blocks should be accounted for with the experiment block included in the model as a random factor. Yet, to fully put our mind at ease, we repeated the analysis for *P.entomophila* for each experiment block and for two new datasets where survival from different day post infection was chosen (in one, DPI5 for sons and

DPI2 for daughters and in the other one, DPI5 for sons and DPI3 for daughters). Similar conclusions were reached (results not shown).

*The relationship between sire sexual success and offspring reproductive fitness*

To investigate whether sire sexual success can be used to predict offspring reproductive success and whether such a link depends on the alignment of the sire-offspring epidemiological environments, we created a new variable called "socombo" and it has two categories: match and mismatch. We then fitted the data to a GLMM (binomial distribution, logit link) with offspring reproductive success (number of wild-type eggs vs. number of GFP-tagged eggs) as the response variable, sire paternity share, socombo and their interactions as fixed factors, and experiment block and observation identity as random factors. Then to further investigate the sire-offspring relationship in any specific pathogenic environment combination, we fitted a GLMM (binomial distribution, logit link) with offspring reproductive success as the response variable, sire treatment (*M.brunneum*-infected vs. sham-treated), sire paternity share (center-scaled), offspring treatment (*M.brunneum*-infected vs. *P.entomophila*-infected vs. pathogen-free control), offspring sex and their interactions as fixed factors, and experiment block with sire identity nested in it and observation identity as random factors. The analysis was then split by sex to simplify the model and the interpretations.

## Results

### Offspring pathogen resistance

Sons had higher resistance against both the *P.entomophila* and *M.brunneum* than daughters in terms of post infection survival (Figure S 4-1; Table S 4-1). The time until 50% mortality post infection ($LT_{50}$) for females and males after fungal infection was day 7 post infection (DPI7) and DPI9 respectively, and after bacterial infection, it was DPI5 for both sexes. We used the survival data from these days as the measure for offspring pathogen resistance. Daughter's pathogen resistance against *P.entomophila* was significantly correlated with son's resistance against *P.entomophila*, though the correlation was modest in size (Pearson's correlation $r$=0.23, $p$=0.049; Figure 4-2A). We did not find any daughter-son correlation in terms of resistance against *M.brunneum* (Figure 4-2B).

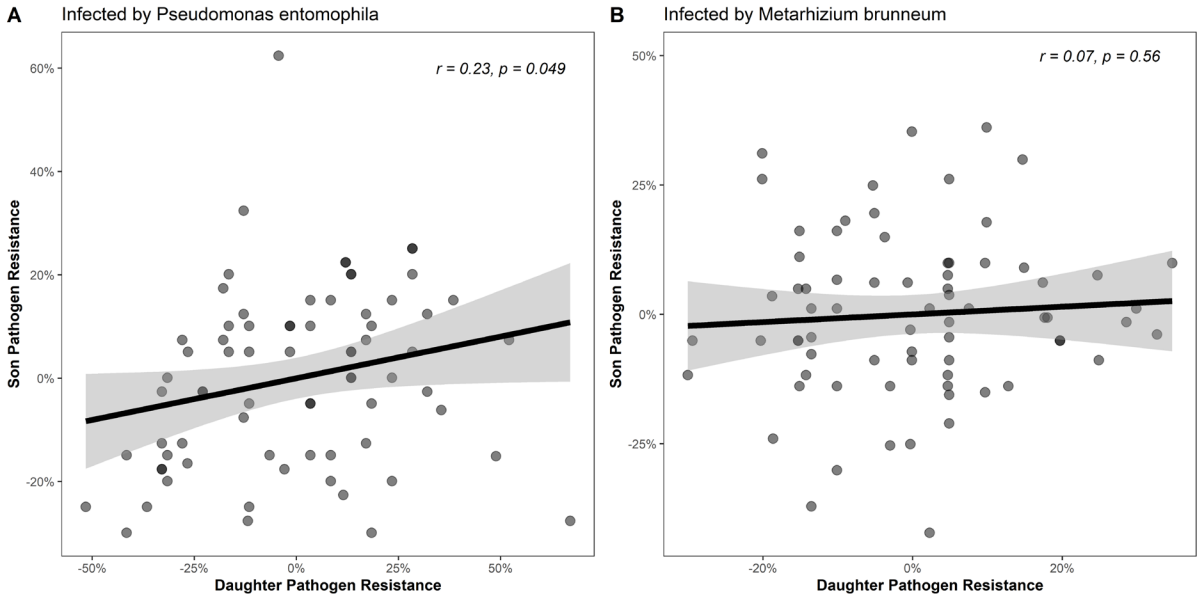


*Figure 4-2 Relationship between daughters' pathogen resistance and sons' pathogen resistance. **A**: When offspring were infected by P.entomophila; **B**: when infected by M.brunneum. Each dot represents the offspring of the same sire. The solid line and grey shade indicate the predicted values from linear models and the 95% confidence interval. Pearson's correlation r and the corresponding p values are also indicated in the figure.*

No significant correlation was found between the resistance to *P.entomophila* and *M.brunneum* regardless of the offspring sexes (Figure 4-3; overall Pearson's correlation $r$ = 0.136, $p$= *0.117*).

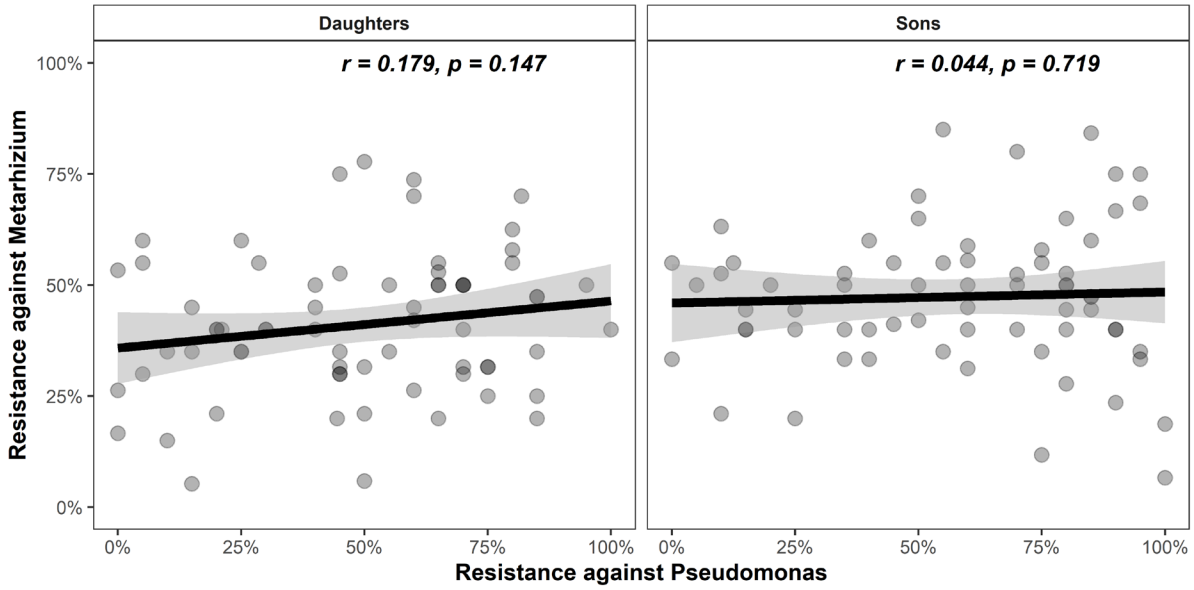


*Figure 4-3 Relationship between offspring resistance against Pseudomonas and resistance against Metarhizium.* ***A****: for daughters;* ***B****: for sons. Each dot represents 20 offspring of the same family. Solid lines and grey shades indicate the predicted values from linear models and the 95% confidence interval. Pearson's correlation r and the corresponding p values are also indicated in the figure.*

## Offspring reproductive fitness

For daughters, infection treatment significantly reduced daughters' reproductive fitness (Figure 4-4A; LRT, offspring treatment, $\chi^2_2$ = 14.4, $p$ <0.001). The lowest values were seen when daughters were infected by *M.brunneum* (estimated marginal mean ± SE = 33.5±1.8%; pairwise comparison, $p$ <0.001 and $p$ <0.001 in comparison to those infected by *P.entomophila* and those subjected to no treatment, respectively), while daughters exposed to *P.entomophila* had similar reproductive success to those in pathogen-free environments (37.8±1.9%, 38.5±1.9% respectively). For sons, in all environments, the variations in reproductive success were higher than in daughters (indicated by the larger predicted 95% confidence intervals). As for daughters, offspring treatment significantly affected sons' reproductive fitness (Figure 4-4B; offspring treatment, $\chi^2_2$ = 344.9, $p$<0.001). Infection by *M.brunneum* reduced the son's reproductive success the most (63.8±4.2%; $p$ =0.026 and $p$ <0.001 in comparison to those infected by *P.entomophila* and those subjected to no treatment, respectively). Sons infected by *P.entomophila* also had reduced reproductive success compared to sons in pathogen-free environments (70.1±3.8% vs. 77.5±3.2%, $p$ =0.007).

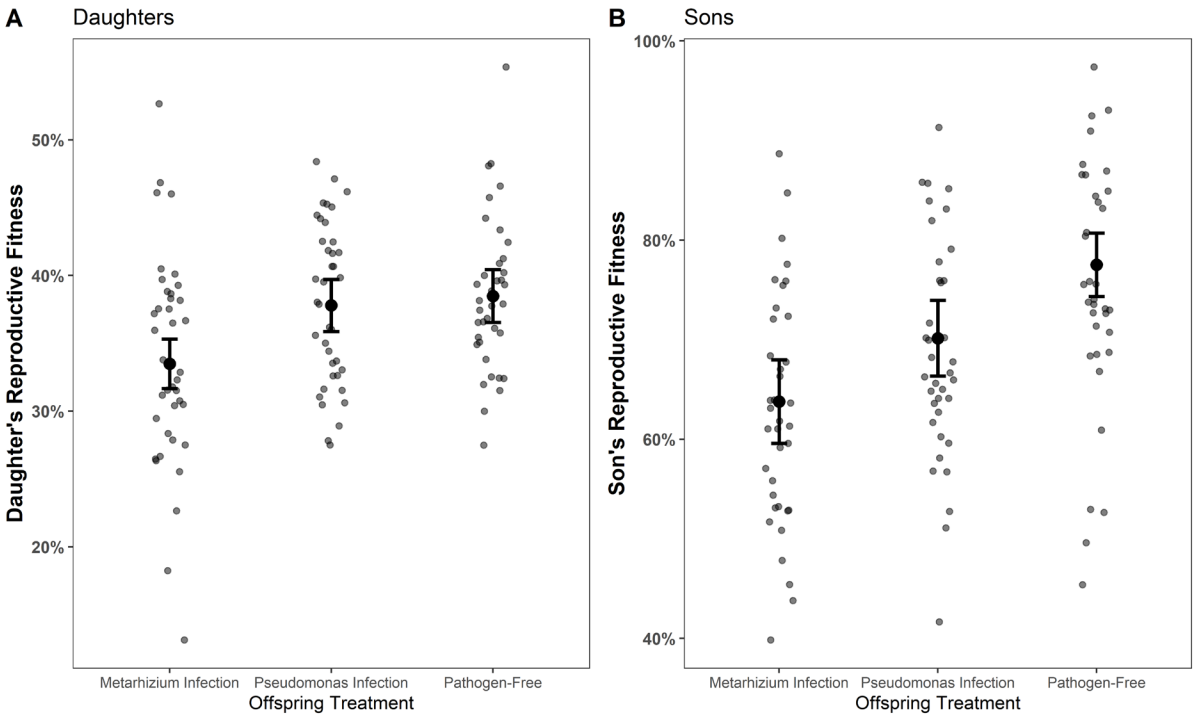


*Figure 4-4 Reproductive fitness of **A**: daughters and **B**: sons in the mating groups after treatment. Each transparent dot represents 5 offspring from each sire. Dark dots and error bars indicate the estimated marginal means and the 95% confidence interval.*

## No trade-offs between offspring pathogen resistance and offspring reproductive fitness

To investigate whether there is a trade-off between pathogen resistance and reproductive fitness, we looked into the relationship between combined pathogen resistance and reproductive fitness and we did the analysis separately for daughters and sons. For daughters, the higher the pathogen resistance they had, the higher reproductive fitness they had in the presence of pathogens, while in a non-pathogenic environment, there was no such correlation (Figure 4-5). For sons, there was no correlation between pathogen resistance and reproductive fitness (Figure 4-6). Altogether, we did not find any trade-offs between offspring pathogen resistance and offspring reproductive fitness.

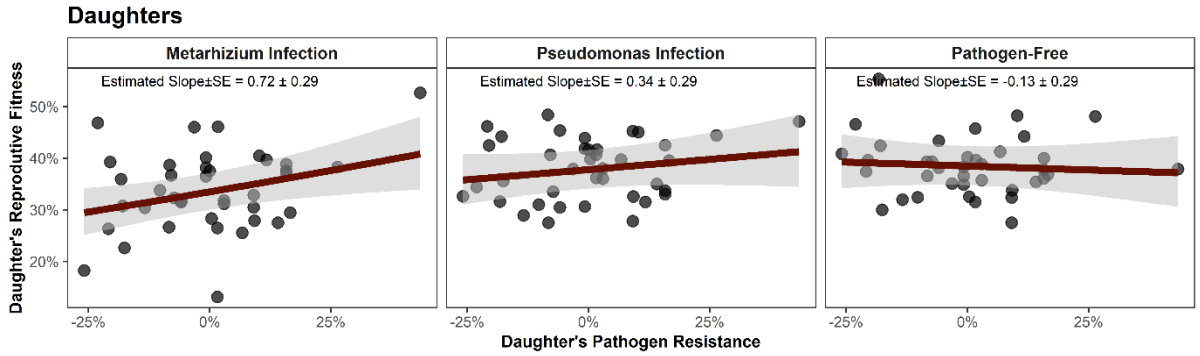


*Figure 4-5 Relationship between daughter's combined pathogen resistance and reproductive fitness. x axis indicates the difference between the observed survival% (i.e., pathogen resistance) to the mean offspring survival% of the corresponding experiment block. Each dot represents the daughters of each sire. Solid lines represent the predicted values of a generalized linear mixed model (combined pathogen resistance, offspring treatment, and their interactions as fixed factors; experiment block with focal sire identity nested in it as the random factors and an observation level random factor to correct for overdispersion). Grey shadows around lines indicate the predicted 95% confidence intervals. Estimated slopes (log-odds scale) ± SE predicted by the model are also provided in the figure.*

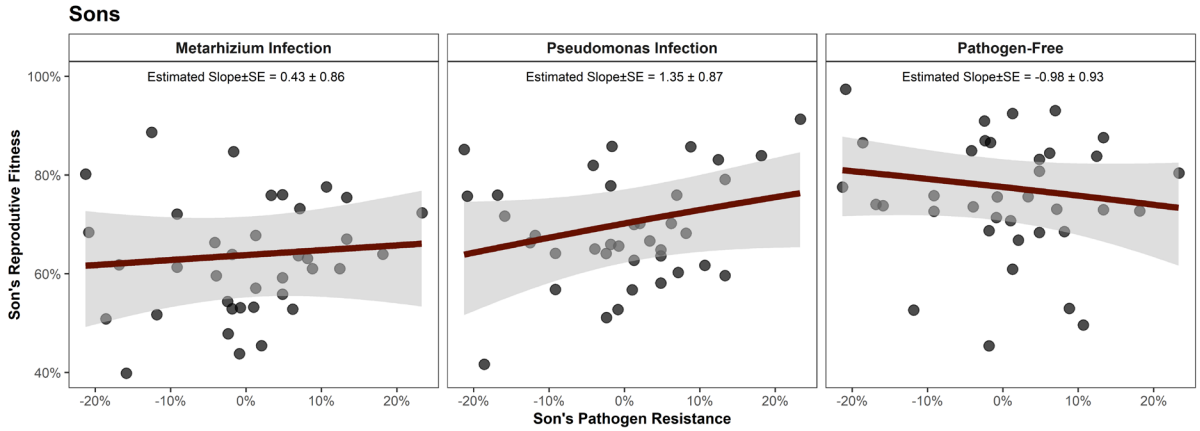


*Figure 4-6 Relationship between son's combined pathogen resistance and reproductive fitness. x axis indicates the difference between the observed survival% (i.e., pathogen resistance) to the mean offspring survival% of the corresponding experiment block. Each dot represents the sons of each sire. Solid lines represent the predicted values of a generalized linear mixed model (combined pathogen resistance, offspring treatment, and their interactions as fixed factors; experiment block with focal sire identity nested in it as the random factors and also an observation level random factor to correct for overdispersion). Grey shadows around lines indicate the predicted 95% confidence intervals. Estimated slopes (log-odds scale) ± SE predicted by the model are also provided in the figure.*

## The relationship between sire sexual success and offspring pathogen resistance

To find out which type of pathogen resistance is favored by sexual selection, we examined the sire-offspring relationship across multiple sire-offspring pathogenic environment combinations (See Table S 4-2 for full model output of the corresponding GLMMs and Table S 4-3 for estimated slopes). First, when the pathogenic environment where sexual selection happened and the offspring

pathogenic environment aligned, that is, when both sire and offspring were exposed to *M.brunneum*, we found no evident correlation between sire sexual success and offspring resistance to *M.brunneum* (Figure 4-7A; Table S 4-2). However, there was a trend of sex-specific sire-offspring relationships (offspring sex × sire paternity share, $\chi^2_1 = 3.0$, $p = 0.083$). Then when the two environments did not align because the sire was not exposed to pathogens prior to the mating trial, we observed no link between sire sexual success and offspring resistance. This was true for both the antifungal resistance (Figure 4-7A; Table S 4-2) and the anti-bacterial resistance (Figure 4-7B; Table S 4-2). Lastly, when offspring were exposed to a different pathogen than the sire, sires of higher sexual success when exposed to *M.brunneum* seemed to have offspring of higher resistance to *P.entomophila* (sire paternity share, $\chi^2_1 = 3.1$, $p = 0.081$; estimated slope (log-odds scale) ± SE = 1.38±0.77, $p = 0.073$; Figure 4-7B; Table S 4-2, Table S 4-3).

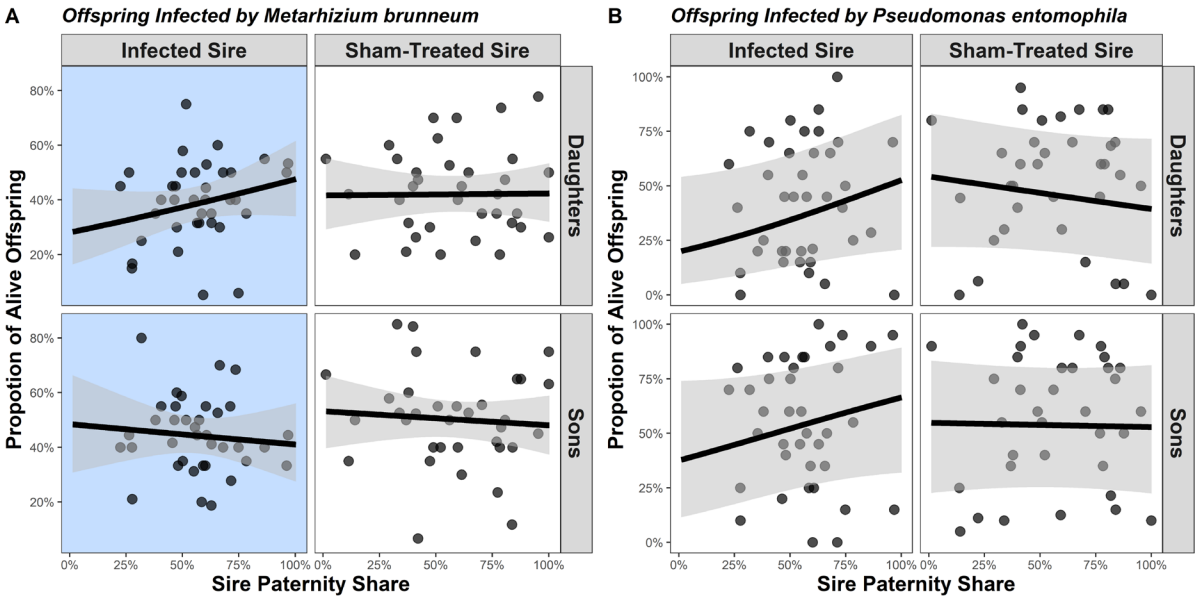


*Figure 4-7 Relationship between sire sexual success and offspring pathogen resistance when offspring were infected by **A**: Metarhizium brunneum; **B**: Pseudomonas entomophila. Each dot represents ~20 offspring from the sire. Solid lines represent the predicted values of a generalized linear mixed model (sire infection treatment, sire paternity share, offspring sex, and their interactions as fixed factors; experiment block with focal sire identity nested in it as the random factors). Grey shadows around lines indicate the predicted 95% confidence intervals. The blue panel background signifies that the sire's pathogenic environment matches with the offspring's pathogenic environment.*

## The relationship between sire sexual success and offspring reproductive fitness

When only considering whether the sire pathogenic environment matched with the offspring pathogenic environment in the sire-offspring analysis, we found a weak trend showing that the

relationship between sire sexual success and offspring reproductive fitness was different when both sires and offspring were in the same pathogenic environment (both facing the same pathogen or both facing no pathogen) and when they were in different ones (sire paternity share × socombo, $\chi^2_1 = 2.54$, $p = 0.111$; Figure S 4-2; Table S 4-4). However, when taking a closer look at the sire-offspring relationship at a specific pathogenic environment combination, the association between sire sexual success and offspring reproductive success is rather weak and there was no evident correlation between the two traits (Figure 4-8, Figure 4-9, Table S 4-5, Table S 4-6).

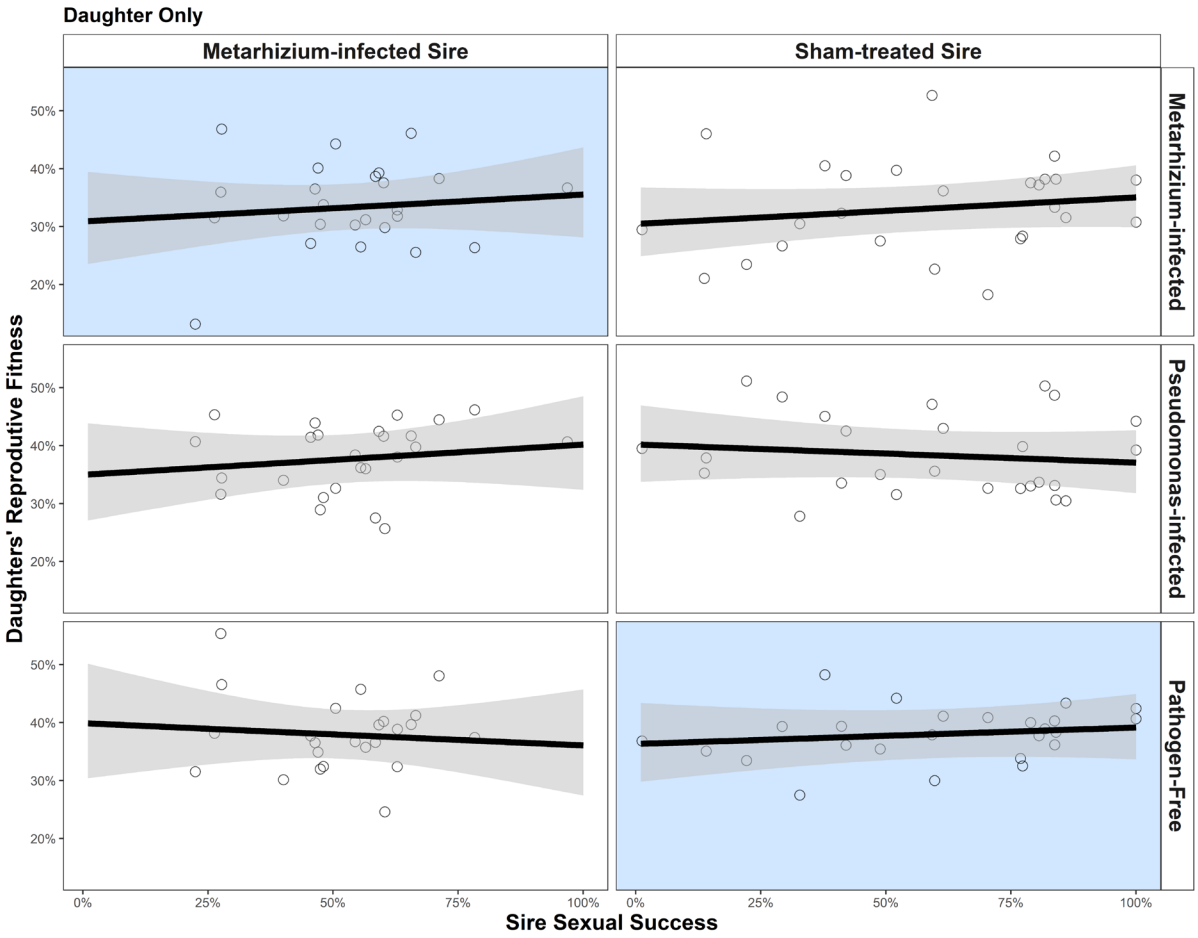


*Figure 4-8 Relationship between sire sexual success and **daughters**' reproductive fitness. Each dot represents 5 daughters from each sire. Solid lines represent the predicted values of a generalized linear mixed model (sire infection treatment, sire paternity share, offspring treatment and their interactions as fixed factors; experiment block and observation identity as random factors). Grey shadows around lines indicate the predicted 95% confidence intervals. The blue panel background signifies that the sire's pathogenic environment matches with the offspring pathogenic environment.*

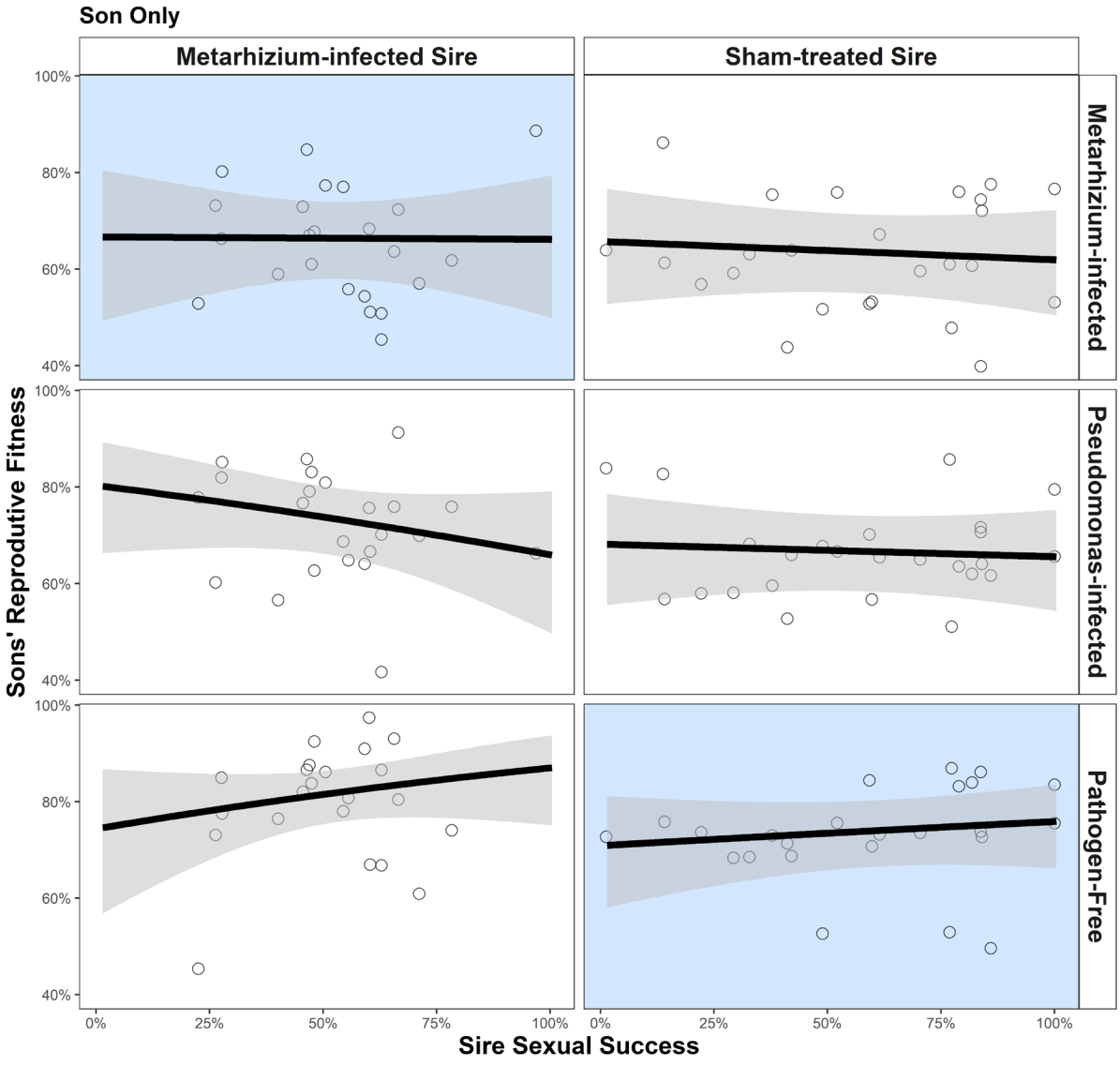


*Figure 4-9 Relationship between sire sexual success and **sons**' reproductive fitness. Each dot represents 5 sons from each sire. Solid lines represent the predicted values of a generalized linear mixed model (sire infection treatment, sire paternity share, offspring treatment and their interactions as fixed factors; experiment block and observation identity as random factors). Grey shadows around lines indicate the predicted 95% confidence intervals. The blue panel background signifies that the sire's pathogenic environment matches with the offspring pathogenic environment.*

## Discussions

In this study, we aimed to experimentally test whether sexual selection favors specific resistance or general immunocompetence and to test whether sire sexual success can be used to predict offspring reproductive fitness in different epidemiological environments. We found no evidence supporting that pathogen resistance was favored by sexual selection. Moreover, we were unable to conclusively determine how sire sexual success affects offspring fitness in specific pathogenic settings because no evident sire-offspring relationship was seen in most cases. Nonetheless, we did find weak evidence of a context-dependent and sex-specific sire-offspring relationship.

Findings from Chapter 3 have shown that the relationship between sire sexual success and offspring pathogen resistance is sex-specific, even pointing towards a potential sexually antagonistic selection on pathogen resistance. Using the same experiment system, a trend of opposite correlation for sire-son and sire-daughter was also observed when both sires and offspring were exposed to *M.brunneum*. This result, together with previous findings, highlights the presence of sex-specific gene expression in both sexual traits and pathogen resistance and the potential of balancing selection in maintaining genetic variation (Mank, 2017). However interestingly, this trend of contrasting sire-offspring correlation was not seen when offspring were exposed to a different pathogen than the sires: sire sexual success measured in the environment with *M.brunneum* tended to be positively linked to improved resistance to *P.entomophila* of both daughters and sons. Improved resistance to *P.entomophila* can only be selected through sexual selection in this case as there was no correlation between resistance against *M.brunneum* and resistance against *P.entomophila*. Using *D.melanogaster* and *P. aeruginosa*, Guncay et al. (2017) report that sexually successful males compared to sexually unsuccessful males sire sons that carry less bacterial loads when facing an immune challenge. Similarly, female house mice mated with preferred males produce offspring with higher resistance to *Salmonella* (Raveh et al., 2014). Our result, in line with these studies, partially supported the "general immunocompetence" hypothesis but to our knowledge, we are the first study reporting that sexual success measured in one pathogenic environment correlated with pathogen resistance measured in another pathogenic environment. We found no correlation between sire sexual success measured without pathogen exposure and offspring pathogen resistance to *P.entomophila*. Our result is different from what has been reported in Joye and Kawecki (2019) where male sexual success in the mating contest in

the absence of pathogens is negatively correlated with son's pathogen resistance to *P.entomophila*. Altogether, we found no support indicating that sexual selection promotes pathogen resistance across different epidemiological contexts.

As evident in many other polygamous species (Janicke et al., 2016, Davies et al., 2023), females experienced weaker selection compared to males with a much higher variation seen in males' reproductive fitness. No trade-off was found between offspring pathogen resistance and offspring reproductive fitness. Although such a finding was contrary to our expectations, it was in line with many previous studies that do not detect trade-offs between immunity and reproduction (Faria et al., 2015, Hangartner et al., 2013). Yet, here we only measured a snapshot of offspring reproductive success. It does not preclude the possibility that trade-offs may happen between early-life reproduction and late-life reproduction (Travers et al., 2015), especially when terminal investment is involved (Foo et al., 2023), which may then change the correlation between pathogen resistance and reproductive fitness. While we did not detect any cost of carrying pathogen resistance, we found that females of higher pathogen resistance had higher reproductive fitness when pathogens were around, a pattern not seen in males. This result demonstrates that the relationship between life history traits is also sex-specific and context-dependent.

A tendency of context-dependent correlation was also seen between sire sexual success and offspring reproductive fitness: a positive trend when the epidemiological environment of the two generations aligned (either facing the same pathogen or both in a pathogen-free environment), but no correlation when otherwise. Such a trend of positive correlation became undetectable when tested under a specific offspring pathogenic environment. Yet, combining the positive link seen between daughters' pathogen resistance and their reproductive fitness only when both measured in environments with pathogens, it is reasonable to speculate that one would have a higher chance of detecting positive links between traits when the epidemiological context where sexual selection happens aligns with the context where the consequences of sexual selection are measured, compared to scenarios when the two contexts do not align. Such finding at its core is in line with what has been termed as "genotype by environment interaction" (Ingleby et al., 2010, Hunt et al., 2004). However, in sexual selection studies, the environment is no longer restricted to the environment where individuals are in but also includes offspring sex as a unique internal environment for gene expression. Although we did not find a significant link between sire sexual

success and offspring reproductive fitness in most of the environment combinations, it was also noticeable that when there was a trend towards a positive or negative sire-daughter correlation, the sire-son correlation tended to be in the opposite direction, further demonstrating the prevalence of sex-biased gene expression.

The absence of support for "good genes" is not a surprise. First of all, it is not easy to evolve resistance to pathogens even under an artificial selection regime (Kraaijeveld and Godfray, 2008, Sharda et al., 2022). Although the mechanism is not clear, it still implies that sexual selection operating at a single generation may not have enough power to select for higher pathogen resistance. Second, how well individuals cope with a pathogen or infection is decided by multiple factors: active pathogen avoidance, resistance (direct combat), and tolerance (cost mitigation). Female choice is also often a multi-signal process (Kraak et al., 1999, Candolin, 2003). Even though females in generally choose males of higher pathogen resistance, each female may adopt a different strategy and hence different elements of the increased pathogen resistance would be favored. The same logic applies to other episodes of sexual selection and the outcome would become more unpredictable. Moreover, the stability of the environment where sexual selection happens may also affect the outcome of sexual selection (Martinossi-Allibert et al., 2019). In this experiment, before sexual success was measured, all mating trials were subjected to less frequent food transfers than in Chapter 3 as they were on standard fly food for two days before getting to the grape juice agar for scoring the paternity share. A much lower sire mortality was observed during this experiment compared to the mortality reported in Chapter 3. The virulence of the fungus is not in question here, as we used the same set of spore suspensions for infecting both the sires and the offspring, and based on Figure S 4-1, the killing of the fungal infection was effective and consistent with other published data. Infected sires may have a different strategy for coping with an infection under more stable environments. Lastly, on the genetic level, the overall effect of sexual selection is equal to the net effect of all loci under selection, which will be even more difficult to predict when factors like G×Es and sex-specific/biased selection are involved. In our results, sexual dimorphism was shown at multiple levels from immune responses to reproductive fitness, indicating the high prevalence of sex-specific/biased gene expression. In summary, this high level of stochasticity in both molecular and biological processes results in a situation where the overall effects of "good genes" are, in most cases, undetectable on the phenotypic level.

Overall, our results showed no support for “good genes” sexual selection. Sire sexual success was in most of the environment contexts not correlated with offspring pathogen resistance nor offspring reproductive fitness. Yet, the intricacies of the sire-offspring correlation uncovered in this study underscore the significance of incorporating the context of sexual selection and the context of the realization of “good genes” in studies of “good genes”.

**Acknowledgment**

We would like to thank all the student assistants in the Kawecki’s lab for preparing all the food used in this experiment.

## Supplementary Materials

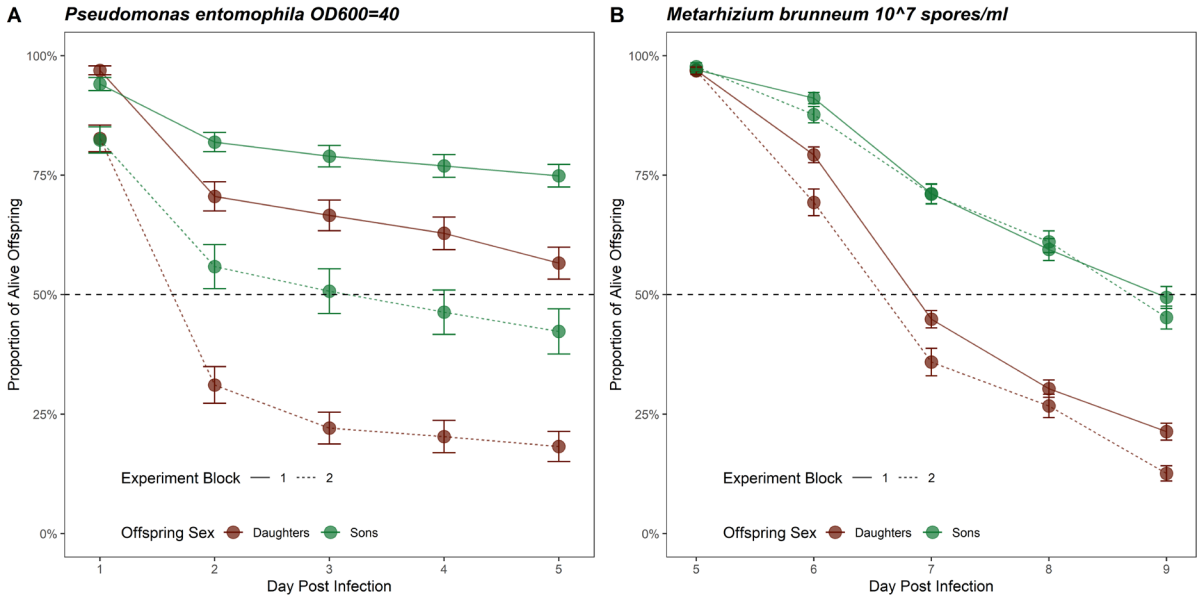


*Figure S 4-1 Post infection survival of daughters and sons. **A**: When treated with P.entomophila ($OD_{600}$=40); **B**: when treated with M.brunneum ($10^7$ spores/ml)*

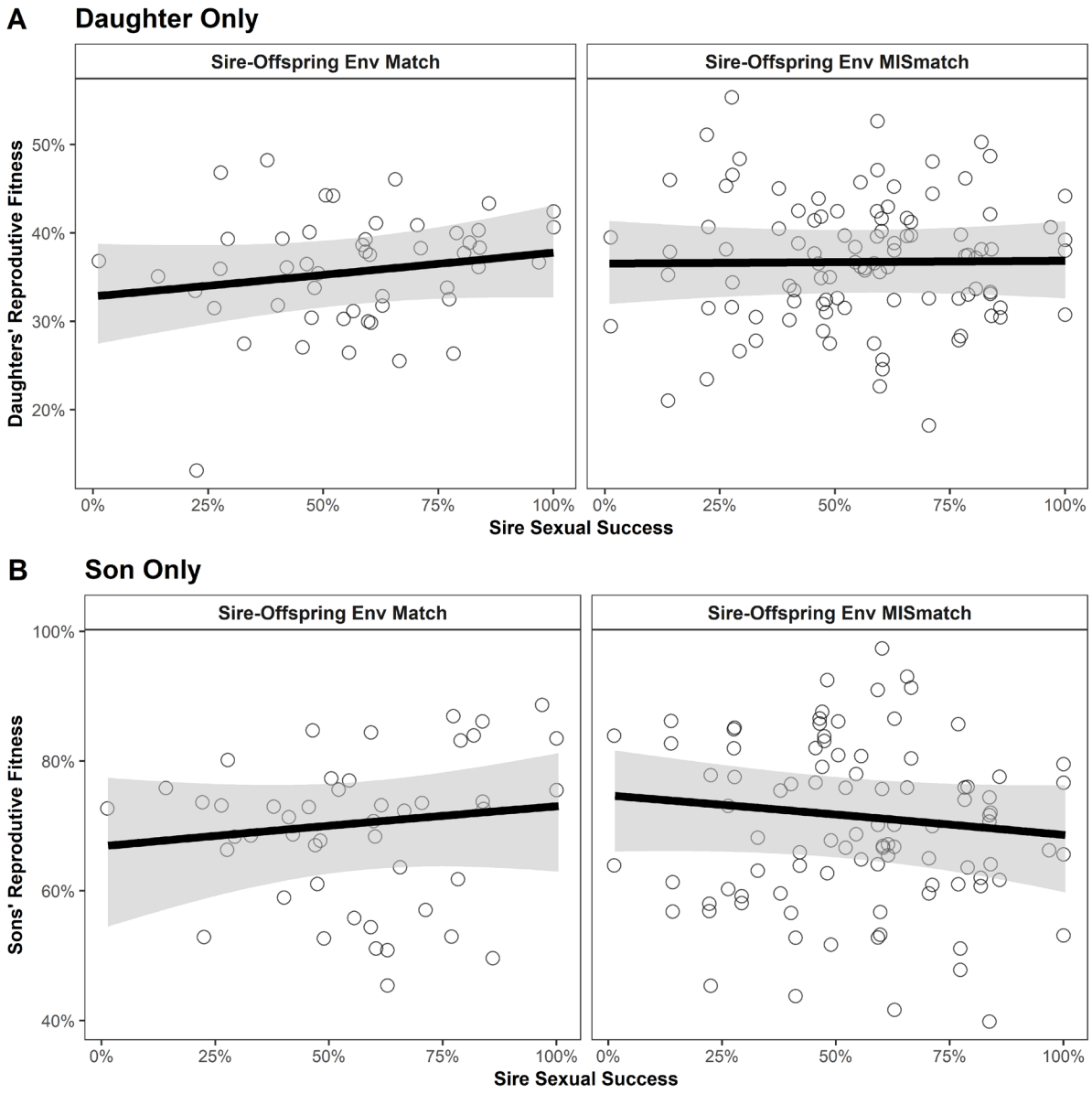


*Figure S 4-2 Relationship between sire sexual success and offspring reproductive fitness when the pathogenic environment of the sire aligned with that of the offspring (either both with the same pathogen or both pathogen-free; "Sire-Offspring Env Match") and when the two environments did not align ("Sire-Offspring Env MISmatch). **A**: for daughters and **B**: for sons. Each open circle represents 5 offspring in the mating group from each sire. Solid lines represent the predicted values of a generalized linear mixed model (socombo, sire sexual success, offspring treatment and their interactions as fixed factors; experiment block with focal sire identity nested in it as random factors; an observational level random factor was added to correct for overdispersion). Grey shadows around lines indicate the predicted 95% confidence intervals. Check Table S 4-4 for the statistical summary.*

*Table S 4-1 Effects of fixed factors on **offspring survival post infection** based on the Likelihood Ratio Test. The generalized linear mixed model includes offspring survival as the response variable, day post treatment, offspring sex and their interactions as fixed factors, and experiment block with focal sire identity nested in it and observation identity as the random factors. Significance terms ($p \leq 0.05$) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| Infection of *P.entomophila* | | | |
| **Day Post Infection** | **394.0** | **4** | **< 0.001** |
| **Offspring Sex** | **67.6** | **1** | **< 0.001** |
| **Day Post Infection × Offspring Sex** | **31.0** | **4** | **<0.001** |
| Infection of *M.brunneum* | | | |
| **Day Post Infection** | **1735.3** | **4** | **< 0.001** |
| **Offspring Sex** | **251.7** | **1** | **< 0.001** |
| **Day Post Infection × Offspring Sex** | **27.7** | **4** | **<0.001** |

*Table S 4-2 Effects of fixed factors on **offspring pathogen resistance** based on the Likelihood Ratio Test. The generalized linear mixed model testing the relationship between sire sexual success and offspring pathogen resistance includes offspring survival post infection as the response variable, offspring sex, sire paternity share and their interactions as fixed factors, and experiment block with focal sire identity nested in it and observation identity as the random factors. Significance terms ($p \leq 0.05$) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| ***M.brunneum*-infected Sire & *P.entomophila*-infected Offspring** | | | |
| **Offspring Sex** | **36.2** | **1** | **<0.001** |
| Sire Paternity Share | 3.1 | 1 | 0.081 |
| Offspring Sex × Sire Paternity Share | 0.2 | 1 | 0.664 |
| **Sham-treated Sire & *P.entomophila*-infected Offspring** | | | |
| Offspring Sex | 1.9 | 1 | 0.174 |
| Sire Paternity Share | 0.3 | 1 | 0.558 |
| Offspring Sex × Sire Paternity Share | 0.4 | 1 | 0.529 |
| ***M.brunneum*-infected Sire & *M.brunneum*-infected Offspring** | | | |
| **Offspring Sex** | **4.3** | **1** | **0.038** |
| Sire Paternity Share | 0.3 | 1 | 0.556 |
| Offspring Sex × Sire Paternity Share | 3.0 | 1 | 0.083 |
| **Sham-treated Sire & *M.brunneum*-infected Offspring** | | | |
| **Offspring Sex** | **9.2** | **1** | **0.002** |
| Sire Paternity Share | 0.2 | 1 | 0.675 |
| Offspring Sex × Sire Paternity Share | 0.2 | 1 | 0.646 |

*Table S 4-3 Estimated slopes(log-odds scale) for the relationship between sire paternity share and offspring pathogen resistance using lstrends() from 'emmeans' package based on GLMMs including sire treatment, offspring sex, sire paternity share, and their interactions as fixed factors, and experiment block with focal sire identity nested in it as the random factors.*

| | Estimated Slope | SE | asymp.LCL | asymp.UCL |
|---|---|---|---|---|
| ***M.brunneum*-infected Sire & *P.entomophila*-infected Offspring** | | | | |
| Daughter | 1.504 | 0.877 | -0.214 | 3.223 |
| Son | 1.192 | 0.865 | -0.504 | 2.888 |
| **Sham-treated Sire & *P.entomophila*-infected Offspring** | | | | |
| Daughter | -0.603 | 0.661 | -1.899 | 0.693 |
| Son | -0.079 | 0.648 | -1.348 | 1.190 |
| ***M.brunneum*-infected & *M.brunneum*-infected Offspring** | | | | |
| Daughter | 0.842 | 0.594 | -0.322 | 2.006 |
| Son | -0.302 | 0.640 | -1.556 | 0.952 |
| **Sham-treated Sire & *M.brunneum*-infected Offspring** | | | | |
| Daughter | 0.028 | 0.427 | -0.809 | 0.865 |
| Son | -0.206 | 0.424 | -1.038 | 0.626 |

*Table S 4-4 Effects of fixed factors on **offspring reproductive fitness (model simple)** based on the Likelihood Ratio Test. The generalized linear mixed model includes sire paternity share, socombo (sire-offspring environment combination), and their interactions as fixed factors, and experiment block and observation identity as the random factors. Significance terms ($p \leq 0.05$) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| **Full Model** | | | |
| Sire Paternity Share | 0.2 | 1 | 0.649 |
| Sire-Offspring Env Combo (match vs. mismatch) | 0.7 | 1 | 0.394 |
| **Offspring Sex** | **344.0** | **1** | **<0.001** |
| Sire Paternity Share × Sire-Offspring Env Combo | 2.5 | 1 | 0.111 |
| Sire Paternity Share × Offspring Sex | 0.1 | 1 | 0.741 |
| Sire-Offspring Env Combo × Offspring Sex | 0.0 | 1 | 0.960 |
| Sire Paternity Share × Sire-Offspring Env Combo × Offspring Sex | 0.6 | 1 | 0.435 |
| **Daughter Only** | | | |
| Sire Paternity Share | 1.1 | 1 | 0.293 |
| Sire-Offspring Env Combo (match vs. mismatch) | 1.0 | 1 | 0.309 |
| Sire Paternity Share × Sire-Offspring Env Combo | 0.9 | 1 | 0.352 |
| **Son Only** | | | |
| Sire Paternity Share | 0.0 | 1 | 0.990 |
| Sire-Offspring Env Combo (match vs. mismatch) | 0.2 | 1 | 0.628 |
| Sire Paternity Share × Sire-Offspring Env Combo | 1.6 | 1 | 0.201 |

*Table S 4-5 Effects of fixed factors on **offspring reproductive fitness (model full)** based on the Likelihood Ratio Test. The generalized linear mixed model includes sire treatment, sire paternity share, offspring treatment, offspring sex, and all interaction terms between them as fixed factors and experiment block with focal sire identity nested in it and observation identity as the random factors. Significance terms ($p \leq 0.05$) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| **Sire Treatment** | **7.7** | **1** | **0.006** |
| Sire Paternity Share | 0.1 | 1 | 0.742 |
| **Offspring Treatment** | **50.1** | **2** | **<0.001** |
| **Offspring Sex** | **426.5** | **1** | **<0.001** |
| Sire Treatment ×Sire Paternity Share | 0.0 | 1 | 0.928 |
| Sire Treatment ×Offspring Treatment | 2.1 | 2 | 0.346 |
| Sire Paternity Share ×Offspring Treatment | 2.3 | 2 | 0.317 |
| **Sire Treatment ×Offspring Sex** | **10.4** | **1** | **0.001** |
| Sire Paternity Share ×Offspring Sex | 0.0 | 1 | 0.886 |
| **Offspring Treatment ×Offspring Sex** | **18.2** | **2** | **<0.001** |
| Sire Treatment ×Sire Paternity Share ×Offspring Treatment | 0.3 | 2 | 0.875 |
| Sire Treatment ×Sire Paternity Share ×Offspring Sex | 0.0 | 1 | 0.976 |
| Sire Treatment ×Offspring Treatment ×Offspring Sex | 2.8 | 2 | 0.248 |
| Sire Paternity Share ×Offspring Treatment ×Offspring Sex | 3.1 | 2 | 0.213 |
| Sire Treatment ×Sire Paternity Share ×Offspring Treatment ×Offspring Sex | 2.2 | 2 | 0.326 |

*Table S 4-6 Effects of fixed factors **on daughter's reproductive fitness or son's reproductive fitness** based on the Likelihood Ratio Test. The generalized linear mixed model includes sire treatment, sire paternity share, offspring treatment and their interactions as fixed factors, and experiment block with focal sire identity nested in it and observation identity as the random factors. Significance terms ($p \leq 0.05$) are indicated in bold.*

| Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|
| Daughter Only | | | |
| Sire Treatment | 0.0 | 1 | 0.954 |
| Sire Paternity Share | 0.4 | 1 | 0.504 |
| **Offspring Treatment** | **19.8** | **2** | **<0.001** |
| Sire Treatment × Sire Paternity Share | 0.0 | 1 | 0.943 |
| Sire Treatment × Offspring Treatment | 0.2 | 2 | 0.903 |
| Sire Paternity Share × Offspring Treatment | 0.8 | 2 | 0.684 |
| Sire Treatment × Sire Paternity Share × Offspring Treatment | 1.5 | 2 | 0.478 |
| Son Only | | | |
| **Sire Treatment** | **10.0** | **1** | **0.002** |
| Sire Paternity Share | 0.0 | 1 | 0.965 |
| **Offspring Treatment** | **34.7** | **2** | **<0.001** |
| Sire Treatment × Sire Paternity Share | 0.0 | 1 | 0.910 |
| Sire Treatment × Offspring Treatment | 3.2 | 2 | 0.207 |
| Sire Paternity Share × Offspring Treatment | 3.4 | 2 | 0.185 |
| Sire Treatment × Sire Paternity Share × Offspring Treatment | 1.4 | 2 | 0.487 |

# Appendix 2

## Using specific pathogen resistance to predict reproductive fitness

The use of combined pathogen resistance, encompassing both resistance to *M.brunneum* and *P.entomophila*, decreased data variability, and to some extent, it represented the individual's overall pathogen resistance, given that these are two distinct pathogens. Yet, some may challenge this decision. Therefore, here we provide the figure and statistics when using either resistance to *M.brunneum* or resistance to *P.entomophila* to predict offspring reproductive fitness in different epidemiological contexts. Our results show that the basic correlation predicted using combined pathogen resistance is similar to those predicted with resistance to a specific pathogen (Figure A 4-1, Figure A 4-2, Figure 4-5, Figure 4-6). However, using combined pathogen resistance enhances the statistical power (Table A 4-1).

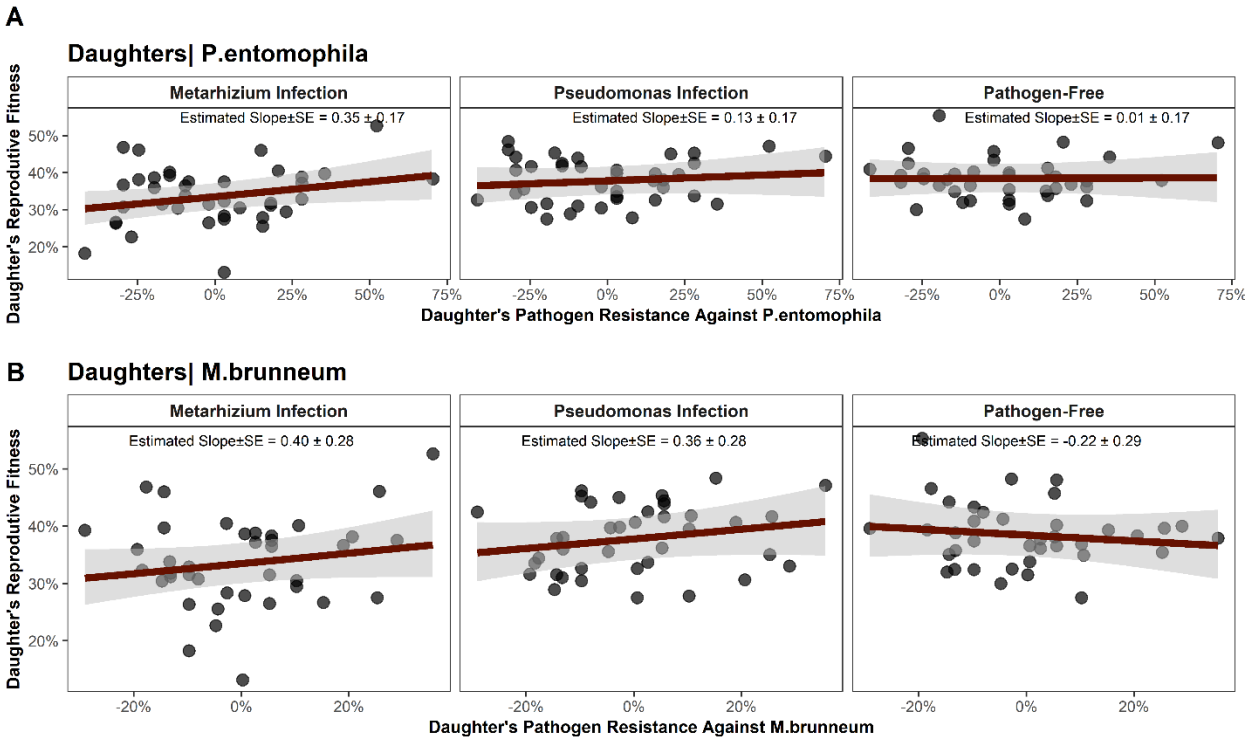


*Figure A 4-1 Relationship **A**: between **daughter's** resistance to **P.entomophila** and its reproductive fitness; **B**: between **daughter's** resistance to **M.brunnuem** and its reproductive fitness. x axis indicates the difference between the observed survival% (i.e., pathogen resistance) to the mean offspring survival% of the corresponding experiment block. Each dot represents the daughters of each sire. Solid lines represent the predicted values of a generalized linear mixed model (specific pathogen resistance, offspring treatment, and their interactions as fixed factors; experiment block with focal sire identity nested in it as the random factors and also an observation level random factor to correct for overdispersion). Grey shadows around lines indicate the predicted 95% confidence intervals. Estimated slopes (log-odds scale) ± SE predicted by the model are also provided in the figure.*

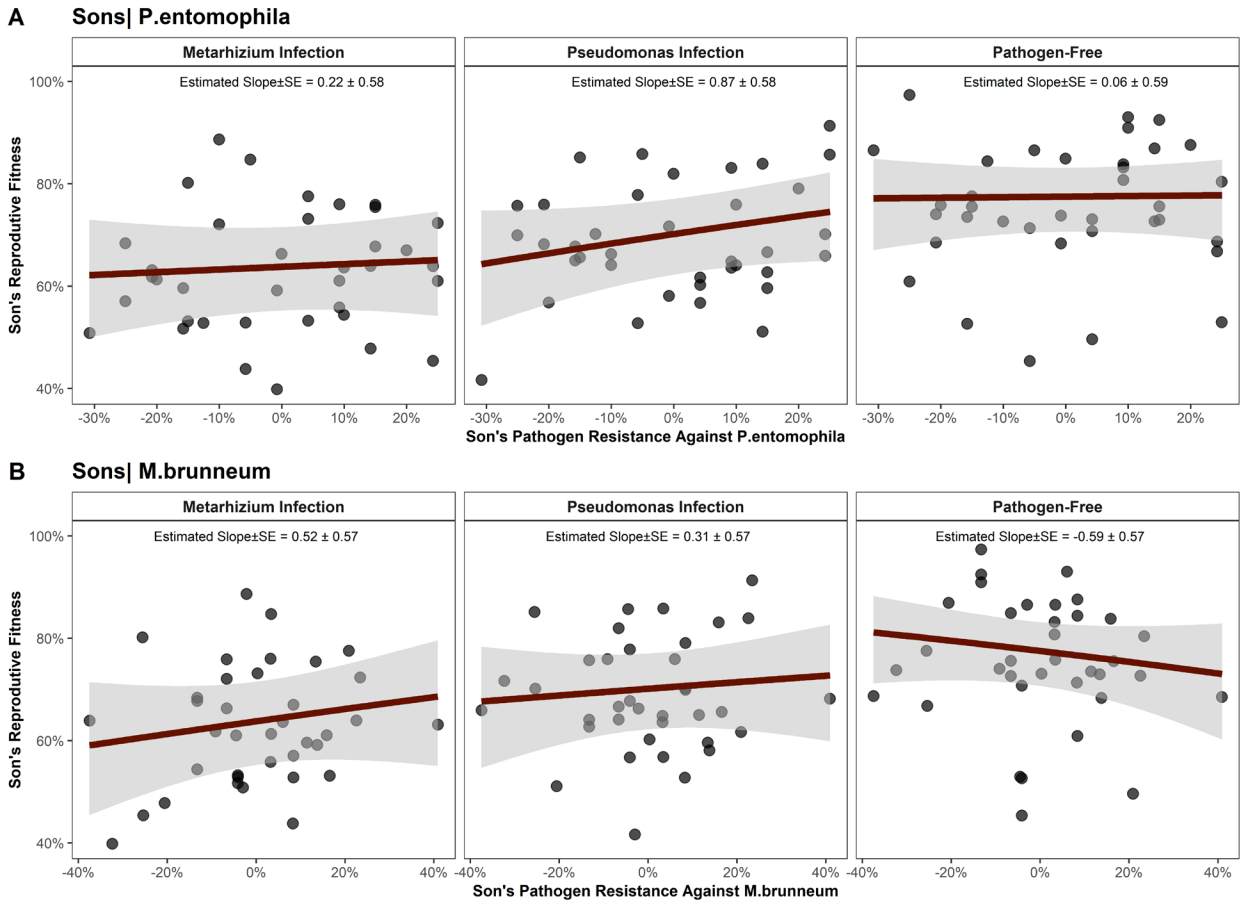


*Figure A 4-2 Relationship **A**: between **son's** resistance to **P.entomophila** and its reproductive fitness; **B**: between **son's** resistance to **M.brunneum** and its reproductive fitness. x axis indicates the difference between the observed survival% (i.e., pathogen resistance) to the mean offspring survival% of the corresponding experiment block. Each dot represents the sons of each sire. Solid lines represent the predicted values of a generalized linear mixed model (specific pathogen resistance, offspring treatment, and their interactions as fixed factors; experiment block with focal sire identity nested in it as the random factors and also an observation level random factor to correct for overdispersion). Grey shadows indicate the predicted 95% confidence intervals. Estimated slopes ± SE predicted by the model are also provided in the figure.*

*Table A 4-1 Effects of fixed factors **on daughter's reproductive fitness or son's reproductive fitness** based on Likelihood Ratio Test. The generalized linear mixed model includes pathogen resistance, offspring treatment and their interactions as fixed factors, and experiment block with focal sire identity nested in it and observation identity as the random factors. This analysis was done for daughters and for sons separately. Significance terms ($p \leq 0.05$) are indicated in bold.*

| Predicting Variable | Offspring Sex | Pathogen Resistance | | Offspring Treatment | | Pathogen Resistance × Offspring Treatment | |
|---|---|---|---|---|---|---|---|
| | | $\chi^2_1$ | Pr(>Chisq) | $\chi^2_2$ | Pr(>Chisq) | $\chi^2_2$ | Pr(>Chisq) |
| Combined pathogen resistance | daughter | 3.1 | 0.079 | **15.2** | **0.001** | 4.3 | 0.114 |
| | son | 0.3 | 0.610 | **24.2** | **<0.001** | 3.6 | 0.168 |
| Resistance against *P.entomophila* | daughter | 2.5 | 0.111 | **14.7** | **0.001** | 2.1 | 0.345 |
| | son | 1.3 | 0.260 | **23.7** | **<0.001** | 1.1 | 0.584 |
| Resistance against *M.brunneum* | daughter | 1.1 | 0.304 | **15.0** | **0.001** | 2.9 | 0.229 |
| | son | 0.1 | 0.808 | **23.8** | **<0.001** | 2.2 | 0.335 |

## Using pathogen resistance of the opposite sex to predict reproductive fitness

Given the significant positive correlation (though modest in size) between daughter's and son's resistance to *P.entomophila*, one could in theory use the resistance of one sex to predict the reproductive fitness of the other. Such an approach may not be so straightforward and is rather challenging because many traits linked to pathogen resistance and reproductive fitness are sex-specific, which is evident not only in the genetic makeups and general trait expressions but also in their interactions with the environments (Tarka et al., 2018, Lange et al., 2021). Moreover, one cannot decide how much the non-additive genetic variance, e.g. dominance, contributed to the observed correlation. Yet, adopting such an approach will still help us to understand in what way pathogen resistance and reproductive fitness are genetically linked and in what way such correlation will affect population fitness.

We found no correlation between son's pathogen resistance and daughter's reproductive success in any of the epidemiological contexts (Figure A 4-3A, Table A 4-2). However, daughter's pathogen resistance was negatively correlated with son's reproductive success when measured after *M.brunneum* infection or in a pathogen-free environment, and no correlation was found in the environment with *P.entomophila* (Figure A 4-3B, Table A 4-2). These findings should be interpreted with caution due to the complexity mentioned above. However, we could still speculate

that the benefits of certain traits have to be weighed against their costs in different aspects of fitness, which again may lead to a balancing selection.

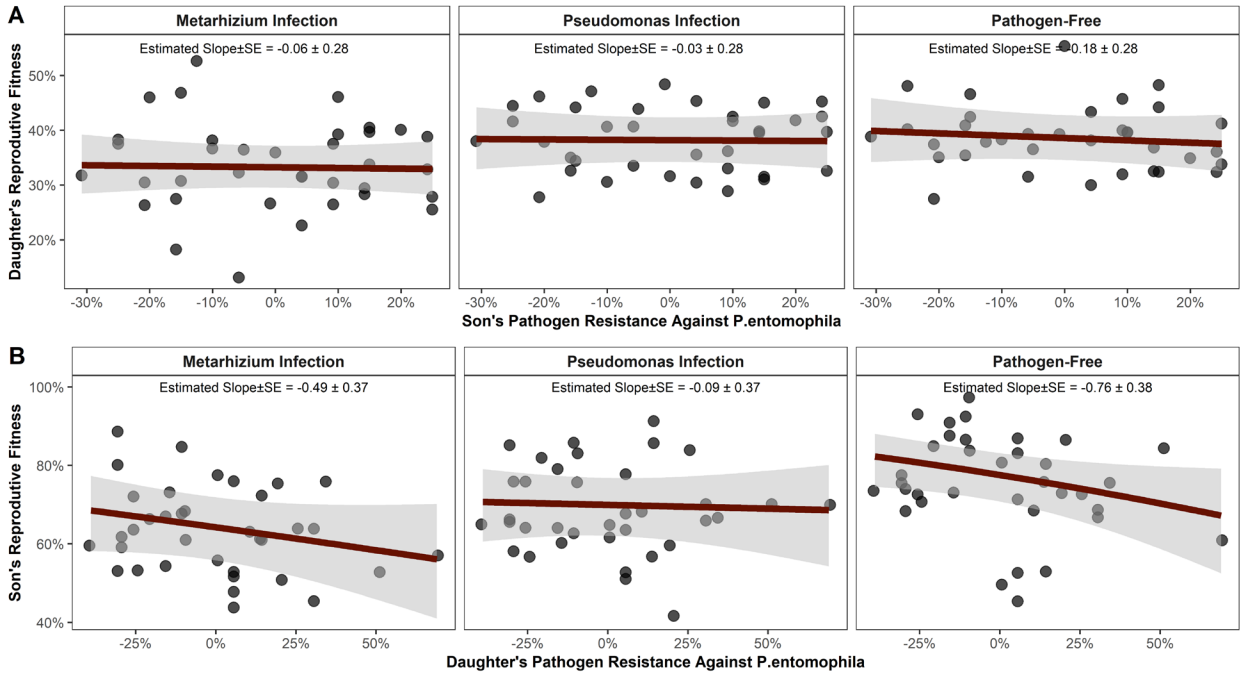


*Figure A 4-3 Relationship **A**: between **son's** resistance to P.entomophila and **daughter's** reproductive fitness; **B**: between **daughter's** resistance to P.entomophila and **son's** reproductive success. x axis indicates the difference between the observed survival% (i.e., pathogen resistance) to the mean offspring survival% of the corresponding experiment block. Each dot represents 5 offspring of each focal sire. Solid lines represent the predicted values of a generalized linear mixed model (specific pathogen resistance, offspring treatment, and their interactions as fixed factors; experiment block with focal sire identity nested in it as the random factors and also an observation level random factor to correct for overdispersion). Grey shadows indicate the predicted 95% confidence intervals. Estimated slopes ± SE predicted by the model are also provided in the figure.*

*Table A 4-2 Effects of fixed factors on offspring reproductive fitness based on Likelihood Ratio Test. The generalized linear mixed model includes **pathogen resistance to P.entomophila of the opposite sex**, offspring treatment, and their interactions as fixed factors, and experiment block with focal sire identity nested in it and observation identity as the random factors. Significance terms ($p \leq 0.05$) are indicated in bold.*

| | Term | Chisq | Chi Df | Pr(>Chisq) |
|---|---|---|---|---|
| Using son's pathogen resistance to predict daughter's reproductive success | Pathogen Resistance | 0.3 | 1 | 0.608 |
| | **Offspring Treatment** | **15.7** | **2** | **<0.001** |
| | Pathogen Resistance × Offspring Treatment | 0.2 | 2 | 0.917 |
| Using daughter's pathogen resistance to predict son's reproductive success | **Pathogen Resistance** | **4.2** | **1** | **0.042** |
| | **Offspring Treatment** | **22.7** | **2** | **<0.001** |
| | Pathogen Resistance × Offspring Treatment | 1.6 | 2 | 0.445 |

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# General Discussion and Perspectives

## Major Findings and Discussions

It's not a novel idea that pathogen plays a role in sexual selection but decades after Hamilton and Zuk (1982) published their famous paper on the matter, in what way and to what extent, pathogens affect the process and consequence of sexual selection remain ambiguous. Few experiments have considered the complex environments that individuals inhabit and the divergence in females' and males' fitness. To help address this, this thesis explores the role of pathogens in sexual selection in two aspects:1) direct consequences of host-pathogen interactions on reproduction and 2) indirect consequences on the link between sexual success and offspring fitness as a mediator.

The direct consequences of host-pathogen interactions decide how much scope is left for sexual selection to operate on for promoting beneficial genes. We began in Chapter 1 by investigating how infection by *M.brunneum* affects males' sexual success and fecundity in a non-competing setting and with an excess number of available mates. Although strong immune responses were activated and high mortality occurred immediately after the incubation period, we found little evidence showing that infection negatively affected male attractiveness, reproductive efforts, and overall reproductive outcome. Our results imply that selection for resistant males is mainly through mortality and little scope is added by sexual selection in a setting with no rival males and more than enough mates. This finding also brings into question the role of female behaviors in the power of sexual selection. As a simple attempt to investigate the topic, in Chapter 2, we tested whether females actively avoid oviposition sites of infection risk and the fitness consequences of choosing the infectious site. We found that females actively avoided infectious oviposition sites, but contrary to our prediction, no reduced egg viability was detected when eggs were laid on infectious sites. Fitness consequences may be revealed at the later life stages or in the sexual selection context (Kawecki, 2020).

Chapter 1 and 2 explores the potential of sexual selection in selecting for improved fitness in rather simplified scenarios with the aim to provide baseline results for the subsequent chapters. Sexual selection often involves more complex environments and the host-pathogen interaction is not

limited to direct consequences. Chapter 3 and 4 demonstrate that selection for pathogen resistance and offspring fitness in a pathogenic environment is indeed a multifaceted process.

The first question we explored in Chapter 3 was how sexual success is affected by infection in a competing setting. Different from the results of Chapter 2, sire sexual success was significantly reduced by infection, and sire of higher pathogen resistance had higher sexual success, indicating the potential for sexual selection to promote pathogen resistance. The second question we asked was how sexual success is linked with pathogen resistance when the pathogen is present /absent in sexual selection (i.e., the context of sexual selection). We showed that the link between these two traits was context-dependent which reveals the cost of pathogen resistance in the absence of pathogens and partially supports the "specific resistance" hypothesis. We also showed that the link was different for daughters and sons, displaying signs of sexually antagonistic selection on pathogen resistance. The opposite sire-daughter and sire-son correlation was less obvious when sexual selection was relaxed. Chapter 4 consolidates findings from Chapter 3 that the correlation between sexual success and non-sexual fitness is context-dependent and sex-specific. It is interesting to see that sire of higher sexual success measured in the environment with *M.brunneum* had offspring (both daughters and sons) of higher resistance against *P.entomophila*. As *M.brunneum* and *P.entomophila* are two distinct types of pathogens and there was no correlation between the resistance against these two, this finding partially supports the "general immunocompetence" hypothesis. In more complex sire-offspring environment combinations, there was a higher likelihood to find potential positive links between sire sexual success and offspring reproductive fitness, and between offspring pathogen resistance and offspring reproductive fitness when the epidemiological context of sexual selection aligns with the pathogenic environment where offspring are in, compared to when they do not align. Both findings from Chapter 3 and Chapter 4 confirm the role of pathogens as the mediator in sexual selection and that offspring sex is an important factor to consider in experiment design given the prevalence of sex-specific sire-offspring relationships.

In summary, despite extensive analysis, we found no evidence indicating that sexual selection directly contributes to or enhances pathogen resistance. This lack of positive correlation challenges the core premise of the "good genes" hypothesis. Further investigation on "good genes" should emphasize the environmental contexts to better understand the multifaceted dynamics.

## PhD Q&A (Perspectives)

Here I would like to address some of the often-asked questions throughout my PhD journey and share my thoughts on future directions on the topic.

### To what extent is it important or not that these pathogens are "natural" enemies of Drosophila? How much do we expect the questions to depend on whether genetic variation has been shaped by a history of exposure to the pathogen in question or close relatives? Or in other words, do you expect a stronger correlation if you use a natural pathogen?

It is not necessary to use natural enemies of *D.melanogaster* in experiments testing the link between sexual success and offspring fitness. The selection for "good genes" acts through the pleiotropic effects of the loci under selection and traits of interest often have a genome-wide genetic signature. The requirement of the experiment is more on the population under sexual selection: is there enough genetic variation for sexual selection to act on? In my thesis, I used a lab-adapted outbred population and there is ample genetic variation (personal communication, Dr. Patrick Joye from University of Lausanne).

Basic requirements on the pathogen characteristics are that (1) infection by the pathogen should affect the signaling of the male's condition so that females can distinguish males of different genetic quality and (2) resistance to the pathogen should be heritable. In "good genes" research, pathogen resistance is more referred to immune-based resistance to infection, although other processes like pathogen avoidance and starvation responses also contribute to infection success.

Both *P.entomophila* and *M.brunneum* fulfill these basic requirements mentioned above and are natural generalist pathogens, unlikely to be involved in strict coevolutionary arms races with *D.melanogaster*. In all measures of sexual success, males were exposed to *M.brunneum* infection. The major advantage of using *M.brunneum* is that the buildup of infection to death takes time (with a $10^7$ spores/ml concentration, ~5 days), leaving more time margins for sexual selection to act on before mortality occurs compared to other acute pathogen infections.

Most of the existing studies use pathogens that the host will naturally encounter. When using natural enemies, meaning that the genetic variation of the population has likely been shaped by a history of exposure to the pathogen in question or close relatives, the host-pathogen interactions

are better studied, and the selection pressure is relevant to natural selection and realistic. When individuals are introduced to new pathogens, most individuals could be susceptible. Under this situation, individuals of high general immunocompetence are likely the ones who survive the infection and pass on the genes to the next generation. If the standing genetic variation of the population is high, there could be existing genetic materials for resistance against such novel pathogens. There is no easy answer to whether a stronger correlation could be seen or not when using a natural pathogen or not. It depends on the nature of the pathogen, the host, and the host-pathogen interactions. With that being said, I cannot help but wonder: what about using a pathogen against which the pathogen resistance has a relatively simple genetic architecture, e.g., the *Drosophila C virus* (Cogni et al., 2016)? What about using a fly-specific behavior-manipulating fungus, e.g., *Entomophthora muscae* (Elya et al., 2018, Wang et al., 2020)? What if instead of using a "wild population", we use populations that are genetically resistant to certain pathogens? I came to realize that we know so little about pathogens as a source of environmental fluctuations in sexual selection although we have been researching host-pathogen interactions for decades.

You argued that there is a sign of sexually antagonistic selection on pathogen resistance. Are you suggesting there is a balancing selection?

**Yes**. Balancing selection maintains genetic variation in a population by favoring different alleles or trait variations in different environmental or social contexts. In the case of sexually antagonistic selection, an allele confers benefits in one sex but disadvantages in the other. Balancing selection may act to preserve genetic variation related to the pathogen resistance in the population but decrease the efficacy of "good genes" sexual selection on promoting resistant genes.

**But also no**. Genetic trade-offs do not equal physiological trade-offs. What I found may not be the direct selection on pathogen resistance and it could be the result of trade-offs between fitness components. In both experiments from Chapter 3 and 4, only when both sire and offspring were subjected to *M.brunneum* infection was the sign of sexually antagonistic selection on pathogen resistance detected. The potential sexual antagonism seemed to be corrected by other life history processes as the opposite correlation was no longer seen when testing the link between sire sexual success and offspring reproductive fitness. Interestingly, sexual antagonism was not seen in pathogen resistance to *P.entomophila* but was revealed in reproductive fitness. These findings

demonstrate that not only the genetic architecture of the resistance but also the relationship between resistance and other life history traits are pathogen-specific.

### Are you rejecting the "good genes" hypothesis according to your experiment results?

No, the fact that no positive correlation between sexual success and offspring fitness was found challenges the "good genes" hypothesis. I am rather raising attention to include different contexts in "good genes" studies. Findings from Chapter 3 and Chapter 4 have real-world implications for "good genes" sexual selection in populations exposed to a changing environment. I investigated the net effects of sexual selection on pathogen resistance and reproductive value. No "good genes" were found. That's likely due to the genotype by environment interactions in the traits of interest and if sire and offspring are exposed to the same environment, the odds of finding "good genes" would be higher than when the two are not the same, as indicated in Chapter 4.

Chapters 1 and 2, besides showing the direct interactions between *M.brunneum* and *D.melanogaster*, also indirectly demonstrate how experiment design would affect our understanding on the system. For instance, infection had little impact on male reproductive potential in a non-competing setting but significantly reduced male paternity share in a competing setting. How much of the little support for "good genes" and the prevalence of sexual-specific correlation/ sexual antagonism reported in this thesis can be attributed to the experiment setting? Long et al. (2012) have shown that while in an off-peak population sire of higher sexual success have offspring of higher fitness, supporting the "good genes" sexual selection, attractive fathers have unfit daughters, indicating sexual antagonism, in a well-adapted population. Similarly, Flintham et al. (2023) using a theoretical model demonstrate that male harm inflicted by males of high condition (genetically encoded) on females offsets the benefits of "good genes". Using a well-adapted and well-fed population for the experiment might have skewed our findings toward an increased level of sexual conflict. However, sexual dimorphism is a universal phenomenon. Moreover, it has also been shown that selection for high-condition males can also indirectly select for high fecundity in females through genes with positive effects on both sexes (Buzatto and Clark, 2020). Experiments done in the wild also have other problems (Hamon and Foote, 2005, Singh and Punzalan, 2018, Gómez-Llano et al., 2020). Mixed results are a norm in sexual selection studies. The complexities of experimentally testing sexual selection theories stem from the theory

itself (Kuijper et al., 2012). It will be interesting to do a review/meta-analysis on how terminological definitions affect our research into the relationship between sexual success and non-sexual fitness. Different researchers define key terms like “sexual success”, and “condition” in different ways. Some definitions are agnostic and some are rather specific/narrow. Such variations can significantly influence the design, outcomes, and conclusions of the study (Achorn and Rosenthal, 2019, Anthes et al., 2017, Alonzo and Servedio, 2019).

One should also acknowledge that “good gene” sexual selection is only one of the processes where indirect benefits can be gained. While “good genes” studies center on the additive genetic variation, there is another stream of research that focuses on the non-additive genetic variation, the “compatible genes” (Neff and Pitcher, 2005). The magnitude of genetic benefits gained through “good genes” and “compatible genes” is comparable across animal populations, and for some species, “compatible genes” is more important than “good genes” (Mikael et al., 2009). Moreover, life history traits might have low heritability ($h^2$=$V_A$/$V_P$) simply because they are encoded by multiple loci, which inflates both $V_A$ and $V_P$, and $V_P$ will be further inflated when the expression of traits changes with the changes in the environment. Therefore, even though $V_A$ is large, it is still difficult to detect high $h^2$ in practice because $V_P$ could be so much larger, which increases the difficulties in finding “good genes” on the phenotypic level.

Studying sexual success as a whole is a big pie to swallow. To make things more feasible and easier to decipher, one can revisit the basic principles of “good genes” and focus on one episode of sexual selection at a time. For example, a long-standing puzzle in “good genes” is how females distinguish males of high genetic quality. Within the context of pathogens, one can ask: which traits capture the information of condition/ pathogen resistance in the presence/absence of pathogens? In *D.melanogaster*, courtship behavior is a plastic trait. It has been shown that males adjust their courtship efforts in different social environments (Marie-Orleach et al., 2019) and this was further confirmed by model simulations (Hollon et al., 2023). It is likely that courtship behavior may also vary when facing different pathogens but may also vary in a temporal manner, which leads to the next questions: how does male signaling vary in different epidemiological contexts and how does it change during the course of infection? The same set of questions can be also applied when studying pheromones (CHC, juvenile hormone, etc.).

Our understanding on the process of sexual selection will be more complete if together with phenotyping, one also looks at what is happening on the genetic level. To do so, one could adopt an evolve & re-sequence approach to directly test the genetic correlations and to point out the genetic components of traits of interest (Parrett et al., 2022, Schlotterer et al., 2015, Shahrestani et al., 2021). Several attempts have been made with experimental evolution but yielded mixed results (Hollis et al., 2009, Sharda et al., 2022, Hollis and Houle, 2011). Building on these previous findings, further study can tailor the manipulations more effectively to the specific contexts, pathogens, and populations involved.

### Why is there so much "noise" in the data?

It is clear that the high level of individual variation, especially in sexual success measure (evident in Chapters 1, 3, 4), contributes to finding no support for the "good genes" hypothesis. Such noise can be generated for multiple reasons. Globally, all the measured traits are complex traits encoded by numerous loci, and taking into account stochastic molecular processes and G×Es, noise is inevitable in the trait value. However, one can also consider a scenario where less noise is expected: if sexual success is mainly conferred by body size. Even in optimal conditions, the size range is generally constrained within the species' genetic limits, and these variations are typically within a relatively narrow range. Paternity share or reproductive success, on the other hand, is a combined outcome of multiple processes, such as female perception, mate choice, male-male competition, sexual conflict or harassment, terminal investment, etc. Each of these elements adds a layer of complexity and uncertainty to the final sexual or reproductive success. To reach the same fitness, there could be multiple combinations of strategies depending on the individuals' life history trade-offs at the genetic level. However, because individuals are living in a fluctuating environment, certain levels of noise can still be seen even if there is one optimal strategy for each environment. For example, all males were raised in a pathogen-free environment in this thesis. If some of these males happen to develop attractive signals despite not being resistant in the presence of pathogens, such males would be favored, which does not contribute to "good genes" but adds another layer of blurring to the data.

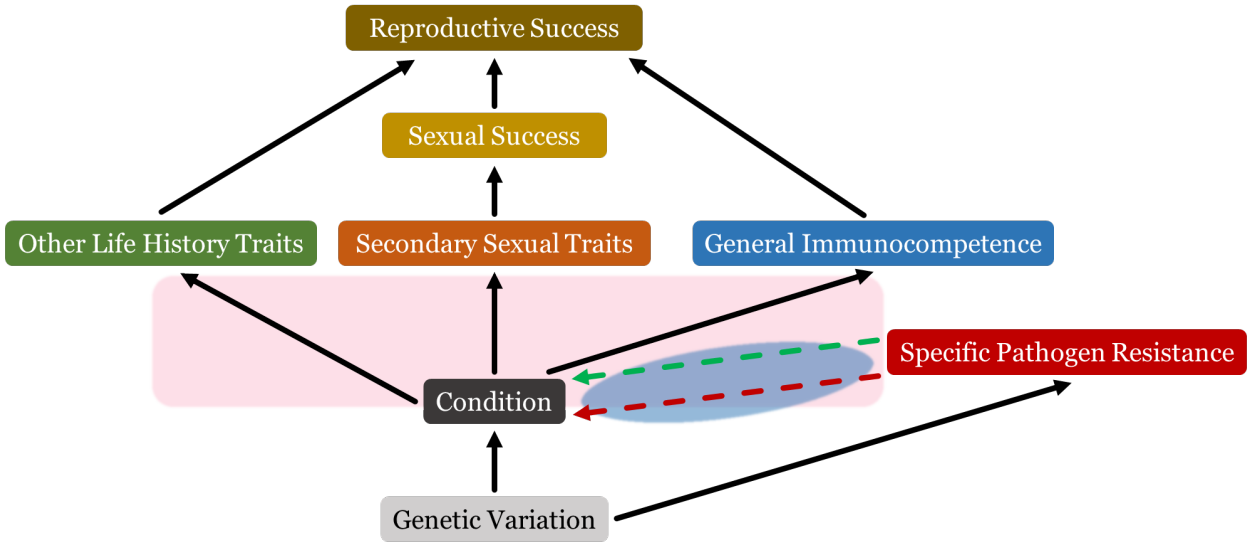
But is it possible that some of the noise in individual behavioral variation is meaningful and is the product of natural selection? Perhaps trying to find the genetic link between behaviors and life-

history traits will shed some light on the topic. But one thing is for sure: *Drosophila melanogaster* will be a good tool for such behavior studies as it has both "personality" (Buchanan et al., 2015) and "sexiness" (Spieth, 1974).

How would you improve the models from Westneat and Birkhead (1998) based on your current knowledge on the topic?

*All models are wrong but some are useful – George Box (1919-2013)*

Findings from this thesis highlight the role of genotype by environment interactions in shaping sexual traits (sexual success) and the link between sexual traits and offspring fitness components. The environment here is not restricted to the occurrence of pathogens but also includes offspring sex. Thus, the new model should also include sex-specific/biased sexual selection. The current models listed in Westneat and Birkhead (1998) lay a solid foundation for this thesis but questions are often raised about whether condition has the same definition in the "specific resistance" and "general immunocompetence" model. Therefore, the new model should also address such confusion and clarify the genetic underpinnings of the condition. Based on my current understanding on the topic, I propose the following model:




This model is individual-based and emphasizes the "trade-off" between the traits rather than the actual cost of carrying the traits (Getty, 2006, Számadó et al., 2023).

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