# Sexual dimorphism and its effect on the evolutionary potential of infectious disease

Stephen Andrew Yandle Gipson

BSc Biological Sciences

A thesis submitted for the degree of Doctor of Philosophy at

Monash University in 2018

School of Biological Sciences

## Copyright notice

## Abstract

Within natural populations, males and females can be strikingly different. Often exhibiting differences in body size, reproductive investment, longevity, ornamentation, or behaviour, the sexes represent some of the greatest variation within a species. These differences reflect the diverging life-history strategies employed by each sex, that is, how males and females allocate their finite resources into attracting mates and producing offspring. An often observed consequence of sexually dimorphic life-histories is a difference between the sexes in susceptibility to disease or severity of infection, which are reported in many taxa from mammals to invertebrates. While well studied in the context of host fitness, how sexual dimorphism in disease outcome impacts on pathogen fitness has received comparatively little attention.

The aim of my thesis is to explore how sexual dimorphism in its many forms can impact on pathogen fitness and shape the evolution of disease. I first address this aim with a literature review, highlighting the unrecognized implications that sexual dimorphism may have for the genetic basis of host-pathogen coevolution. I subsequently perform a series of experiments using the planktonic crustacean, *Daphnia magna*, and its obligate bacterial endoparasite, *Pasteuria ramosa*. Using quantitative and molecular approaches, I demonstrate how host sex, as well as its interaction with age or dietary condition, can determine pathogen fitness, the outcome of competition between co-infecting pathogen genotypes, the evolution of virulence, and the maintenance of pathogen genetic variation.

My results demonstrate the ability of male-female differences to affect infectious disease evolution through their interaction with naturally varying population characteristics such as age structure and nutrient availability. I found that changes in optimal virulence due to the interaction of host sex and age may favour the maintenance of different pathogen virulence strategies. Next, I found that sex-specific resource investment fundamentally changes how pathogens exploit hosts of each sex and contributes to variation in disease outcome. Finally, I found that differences in the exploitation potential of each sex can change the outcome of within-host pathogen competition and maintain pathogen genetic diversity. Collectively, my results suggest that the pervasive source of host heterogeneity provided by sex will affect many aspects of pathogen fitness and should encourage further research into the role sex plays in the evolution and spread of disease.

## Publications during enrolment

Gipson, S.A.Y. and M.D. Hall. 2016. The evolution of sexual dimorphism and its potential impact on host-pathogen coevolution. Evolution 70 (5), 959-968.

Thompson, O., S.A.Y. Gipson, and M.D. Hall. 2017. The impact of host sex on the outcome of co-infection. Scientific Reports 7, 910.

Gipson, S.A.Y. and M.D. Hall. 2018. Interactions between host sex and age of exposure modify the virulence-transmission trade-off. Journal of Evolutionary Biology 31 (3), 428-437.

## Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer reviewed journals and two unpublished papers intended for submission in peer reviewed journals. The core theme of the thesis is evolutionary ecology. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Dr Matt Hall.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of Chapters 2, 3, 4, and 5 my contribution to the work involved the following:

| Thesis Chapter | Publication Title | Status | Nature and % of student contribution | Co-author name(s) Nature and % of Co-author's contribution* | Co-author(s), Monash student Y/N* |
|---|---|---|---|---|---|
| 2 | The evolution of sexual dimorphism and its potential impact on host-pathogen coevolution | Published | 70%. Concept and writing of manuscript. | 30%. M.D. Hall: concept, edits to manuscript. | No |
| 3 | Interactions between host sex and age of exposure modify the virulence-transmission trade-off | Published | 75%. Design, data collection, data analysis, and writing of manuscript. | 25%. M.D. Hall: concept, design, analysis, edits to manuscript. | No |
| 4 | Host sex modulates the condition-dependence of pathogen fitness | Not submitted | 75%. Design, data collection, data analysis, and writing of manuscript. | 20%. M.D. Hall: concept, design, analysis, edits to manuscript.<br>5%. A.K. Pettersen: design. | No<br>Yes * |
| 5 | Sexual dimorphism in disease affects the outcome of within-host pathogen competition | Not submitted | 85%. Design, data collection, data analysis, and writing of manuscript. | 15%. M.D. Hall: concept, design, and edits to manuscript. | No |

* A.K. Pettersen submitted her PhD thesis prior to the drafting of Chapter 3.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

**Student signature:**  **Date:** 28 March, 2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

**Main Supervisor signature:**  **Date:** 28 March, 2018

## Acknowledgements

First, a big thanks to my supervisor Matt. Coming to Australia to study in your lab has been an incredible experience for me and I only had the opportunity due to a great deal of your effort and generosity. Throughout these three and a half years you always made time for me, helping me through any statistics or *Daphnia* related crises I managed to kick up and investing much of your busy schedule into my education. Ultimately, I think we learned a lot from our research and I am grateful to have done this work in your lab. Thanks for your patience and guidance while I clumsily figured out what research is about during these last few years. I would also like to thank my co-supervisor Anne Peters and the members of my review panel: Tim Connallon, Beth McGraw, and Carla Sgrò. I am truly thankful to each of you for the meetings, pep talks, and general advice in navigating the PhD. I would also like to thank the Academic and Professional staff within the School of Biological Sciences including Damian Dowling, Barbara Hardie, Fiona Hibbert, Susie Ho, Keyne Monro, Craig White, and Bob Wong.

The PhD could be challenging at times, but I would happily do it all over again knowing that I would meet some wonderful friends in the Hall lab. Louise and Toby, it has been great getting to know you both as we worked on our PhDs together. Louise, thank you for all of the nice dinners at your home, teaching me the best parts of the Danish language (including "frog structure"), and for being an example of how to work hard and stay positive. Toby, thanks for having my back whenever I somehow got stuck manning the BBQ (I'll always remember *"rotation, rotation, rotation"*), for making water changes more enjoyable (even if we had to listen to your Country Pop playlists), and for letting me bother you with stats questions. Lindsey, thanks for making our lab the efficient and positive place that it is. You do so much to keep everything running smoothly, but somehow made time to help me with my experiments, share your great taste in music (Coffee Cola is an all-time jam), and just generally make the lab a fantastic place to work in. Luis, the only thing that kept me sane during our DNA extractions were the utterly nonsense conversations we had. I don't regret trying to make a pun out of every PCR plate position even though it led to a few small mistakes. Love your work. Isobel, thanks for being the only other person who respected the scientific process of our weekly taste testing. Thanks also for your advice on millions of drafts and statistical analysis. Vignesh, the early days of the lab were filled with a great deal of construction, trial and error, and much more hitting things with hammers than either of us were expecting.

Thanks for helping me get my PhD started and for teaching me many of the skills I still use today. Finally, thanks to the many other members of the Hall lab and volunteers- Clare Bracey, Eleonora Kay, Kyle Kelly, Tess Laidlaw, Jessica Lever, Jared Lush, Liam McNamara, Olivia Thompson, and Jasmine di Valentine- for your friendship and support.

I have met many friends within the School of Biological Sciences who played a large part in making my PhD experience an enjoyable one. First, thanks to my officemates: Julie "Jukie" Groce, Fariba Pakdel, Kelsey Roberts, Stefanie Rog, Emily De Stigter, Melissa Wartman, and Stephanie Watts-Williams. Thanks also to my fellow members of the Postgrad Committee: Lotte van Boheemen, Evatt Chirgwin, Clémentine Lasne, Qamariya Nasrullah. Finally, thanks to the many others I met in journal clubs, Postgrad camping trips, or over beers in the tea room: Shane Baylis, Candice Bywater, Cathy Cavallo, Helena Chen, Ashraful Islam, Manoj Kamalanathan, Emily Kerton, Cassandra Koh, Tom Lines, Marcus Michelangeli, Paulina Mikulic, Cat Mills, Katie Moon, Rowan Mott, Nancy Van Nieuwenhove, Mario Novelo Canto, Yussi Palacios, Ruzeen Patwa, Amanda Pettersen, Emma Razeng, Michael Roast, Sonia Sànchez, Aysha Sezmis, Will Sowersby, Gerard Terradas, Niki Teunissen, Beccaa Vaught, Thomas Wilkins, Sean Williamson, and Felix Zajitschek. I cannot wait to celebrate with you all once I submit this thesis.

Despite the countless hours of water changes and jar washing, I somehow made time to get out of the lab. In those precious few hours you would probably find me at The Curtin with Alex Bennetts, Vee Campbell, Tom Eccles, Jake Lawrence, Alexandra Neill, Steven Throup, and Penny Wilson. I am going to miss you guys a *gondolot* (*Get it?*). Otherwise you would find me at The Alma House with Matthew Giuffre, Michael Harris, Ryan Kort, Aurora McCaig, Brendan McFarlane, and Yukari Sakai. I have made so many friends, both within and outside of Monash, which I will dearly miss when it is finally time to head back home. Thank you for making Savanna and I feel welcome here in Melbourne. Make sure to come visit me in New Orleans, otherwise I hope to visit Melbourne again soon. To my friends back home, my only hesitation about moving to Melbourne was at the thought of us growing apart. It was tough at first, but we somehow got used to hanging out over Skype like nothing had changed. To Michael Arruebarrena, Tierney Brinkman, Ian Christie, Adam Gnuse, Wayne del Corral Jr., Annie Cespedes, Jessica Edwards, Michael Moises, Jake Nesbit, Louis Parsons, Justin Triay, Andrew Tyler, and many others: thanks for cheering me on this whole time and for being as excited to have me back as I am to spend time with you again.

To my family, thank you for being (mostly) ok with my decision to move to Melbourne for a while. Thanks especially to my mom and dad for bringing me camping, fuelling my interest in science, and helping to foster the appreciation of nature that brought me out here. Thanks to my grandmaw, grandpaw and my brothers Sean, Robert, and Kevin for all of the letters, packages, airplane tickets and everything else that reminded me how proud and supportive you were even though you would really rather have me back home. To my niece, Mei Mei, thank you for the beautiful watercolours that you sent me for Christmas! I cannot wait to finally meet you in person. Thanks also to Ray and Barbara for all of the support over the years and making sure Savanna and I never went hungry in Melbourne. I'm looking forward to catching up and spending some time on the beach with you while I recover from my studies. And, above all, thank you Savanna for your love, support, and putting up with me in general. I only worked up the nerve to leave home and get my PhD knowing you would be with me the whole way. Thanks for illustrating my talks, listening to my presentations, and being the only one that could cheer me up during the hardest parts of this experience. It is going to be sad for us to finally leave Melbourne, but I know we will make the most out of whatever is next together.

## Table of Contents

Copyright notice ..... 2
Abstract ..... 3
Publications during enrolment ..... 4
Thesis including published works declaration ..... 5
Acknowledgements ..... 7
Table of Contents ..... 10
Chapter 1 – General introduction ..... 12
The many forms of sexual dimorphism and their impact on host-pathogen coevolution ..... 13
Host sex, age of exposure, and pathogen fitness ..... 14
Pathogen exploitation is sex-specific and underlies dimorphism in disease outcome ..... 15
Host sex changes the competitive outcome of co-infection ..... 16
Thesis organization ..... 17
Chapter 2 – The evolution of sexual dimorphism and its potential impact on host-pathogen coevolution ..... 18
Abstract ..... 19
The Interplay between Sexual Dimorphism and Infection ..... 19
The Sex-Specific Evolutionary Potential of an Invading Pathogen ..... 20
Sex-Specific Selection and the Constraint of Host-Pathogen Dynamics ..... 23
The Implications of Male-Biased Mutation for Genetic Variability in Resistance ..... 25
Future Directions ..... 26
Concluding Remarks ..... 27
Acknowledgments ..... 28
Chapter 2 boxes and figures ..... 29
Chapter 3 – Interactions between host sex and age of exposure modify the virulence-transmission trade-off ..... 34
Abstract: ..... 35
Introduction ..... 35
Materials and methods ..... 38
Results ..... 41
Discussion ..... 43
Acknowledgments ..... 46
Chapter 3 tables and figures ..... 47
Chapter 3 supplementary material ..... 51
Chapter 4 – Host sex modulates the condition-dependence of pathogen fitness ..... 53

Abstract .....54
Introduction .....54
Methods .....57
Results .....61
Discussion .....62
Acknowledgements .....66
Chapter 4 tables and figures .....67
Chapter 5 – Sexual dimorphism in disease affects the outcome of within-host pathogen competition .....72
Abstract .....73
Introduction .....73
Methods .....77
Results .....81
Discussion .....83
Acknowledgements .....85
Chapter 5 tables and figures .....86
Chapter 6 – The consequences of sexual dimorphism for host-pathogen coevolution .....93
Host sex and the genetic basis of host-pathogen coevolution .....93
Host sex and age of exposure shape the virulence-transmission trade-off .....94
Optimal pathogen exploitative strategies underlie sexual dimorphism in disease outcome .....95
Host sex changes the competitive outcome of coinfection, favouring different pathogen virulence strategies .....96
Future directions .....97
References .....99
Appendix – The impact of host sex on the outcome of co-infection ..... 117

# Chapter 1 – General introduction

Stemming from anisogamy and the unequal costs of producing male and female gametes, the sexes exhibit fundamentally different strategies for producing offspring (Andersson 1994). These divergent strategies are often typified by males that compete for access to multiple mates and females that must balance the cost of producing expensive gametes by choosing to mate with only high-quality males. An early observation of this pattern was described by Bateman (1948), where the fitness of male *Drosophila melanogaster* increased with the number of mates whereas female fitness was limited by egg production. These mating strategies ultimately result in selection favouring different phenotypic or behavioural characteristics which increase fitness for each sex (Schärer et al. 2012). Yet, organisms must invest into fitness-related traits from a finite pool of resources (Rowe and Houle 1996; Hunt et al. 2004) and life-history decisions must balance the fitness gain of investment into one trait with the consequences of limiting resources to another. Such life-history trade-offs can result in a variety of evolutionary consequences, such as how males and females balance their investment in reproduction with varying levels of investment in defence against the constant threat of parasitism (Zuk and Stoehr 2002; Zuk 2009).

The differential costs of reproduction for males and females play a central role in life-history trade-offs related to immunity. In general, females are expected to invest more resources into immunity due to its benefits to longevity and thus the production of eggs or parental investment (Rolff 2002). Conversely, males achieve higher fitness by investing a greater proportion of their resources into traits that increase mating success at the expense of lower investment into immunity. These common life-history differences may underlie the observation that males are often the “sicker sex”, becoming infected more frequently and more severely than females (Poulin 1996; Schalk and Forbes 1997; Zuk 2009; Cousineau and Alizon 2014). However, host populations experience a range of sex-specific effects on life-history that are relevant to immunity, including strong sex differences in how infection affects fitness, variation in the degree of sexual dimorphism, or sex biases in exposure to pathogens. Whether one sex is in fact “sicker”, and which is the sicker sex, will therefore depend on the host-pathogen system of interest (McCurdy et al. 1998; Sheridan et al. 2000; Stoehr and Kokko 2006).

From the perspective of pathogen evolution, however, the more interesting problem is that such heterogeneity in disease outcome exists, rather than which particular sex is more susceptible. In nature, host populations often vary in sex ratio (Clutton-Brock and Iason 1986; Pusey 1987; Duneau and Ebert 2012) which, in turn, will impact on the likelihood of a pathogen encountering either sex. Pathogen fitness may thus be affected not only by sex-specific patterns of disease, but also the likelihood of encountering the more or less susceptible sex, yet both theory and empirical studies have only recently begun to address the fitness consequences for pathogen populations infecting sexually dimorphic host populations (Duneau and Ebert 2012; Cousineau and Alizon 2014).

The aim of my thesis is to explore the variety of ways by which host sex can influence pathogen fitness as well as their evolutionary implications. I first address this aim with a literature review, exploring how host sex influences the nature and pace of host-pathogen coevolution. I then use the *Daphnia magna* - *Pasteuria ramosa* host-pathogen model system to experimentally test how common occurrences in natural populations, such as variation in age-structure or nutritional condition, might interact with host sex to influence pathogen fitness in single and co-infections. Recognizing that host sex provides a major source of variation in the exploitative environment experienced by an invading pathogen, I investigated how male and female differences affect the relationship between pathogen virulence and transmission, and play an integral role in the maintenance of genetic diversity in pathogen populations.

## The many forms of sexual dimorphism and their impact on host-pathogen coevolution

In Chapter 2, I explore how sexual dimorphism affects the maintenance of genetic variation in host and pathogen fitness as well as the rate of host-pathogen coevolution. This review builds upon studies that discuss how sexual dimorphism in immunity, and importantly other sources of sexual dimorphism such as behaviour, physiology, or varying rates of pathogen exposure to male or female hosts, impact on pathogen evolution. Duneau and Ebert (2012) were the first to frame cases of sex differences in disease outcome in the context of pathogen evolution. Drawing on examples like the specialisation of *Kudoa ovivora* parasites to the ovaries of female *Thalassoma bifasciatum* (Swearer and Robertson 1999), the authors described how sex differences can skew the establishment of pathogens to a single host sex and how this may ultimately lead to patterns of sex-specific pathogen adaptation analogous to pathogen local adaptation (Lively 1989). Subsequently, Cousineau and Alizon (2014) reviewed sex differences in immune defence

strategies, finding that while males are often more susceptible to disease, they can better tolerate the harmful effects of infection. Cousineau and Alizon (2014) then modelled the differences in selection that resistance and tolerance exert on pathogen virulence, and showed that such sex-specific immune strategies can select for varying levels of virulence depending on patterns of contact between the sexes. Collectively, these studies present some of the first formal arguments that host sex can affect pathogen evolution.

While each study explored the evolutionary optima of pathogens which encounter sexually dimorphic host populations, they did not consider how pathogen evolution may be affected by the genetic architecture underlying sexually dimorphic host characteristics. In addressing this issue, I discuss how varying patterns of pathogen fitness between the sexes or the shape of cross-sex genetic correlations affect the speed of coevolution or maintenance of pathogen genetic variation. I first consider these concepts in the context of genotype-by-environment interactions (GxE, Hunt et al. 2004; Ingleby et al. 2010), where the relative difference or rank order of pathogen fitness may vary within each host sex. Then I consider how genetic correlations stemming from a shared genetic architecture between males and females (Lande 1980; Bonduriansky and Chenoweth 2009) affect a host population's adaptive response to exploitation by a pathogen population. Finally, I consider how a male-biased mutation rate (Haldane 1935), coupled with more severe fitness consequences of deleterious mutations for infected males than females (Sharp and Vincent 2015), can cause variation in the selection imposed on the pathogen population by male and female hosts.

## Host sex, age of exposure, and pathogen fitness

Natural populations consist of individuals that vary in their capacity to fight infection (Altizer et al. 2006; Wolinska and King 2009), causing heterogeneity in the level of host harm upon infection and in pathogen fitness. The life-history strategies employed by each sex will change not only the patterns of disease between males and females, but also how disease outcome varies over the course of male or female development. For example, the less choosy sex is often described by an early reproductive peak followed by a decline in survival with increasing age (Vinogradov 1998; Sgrò and Partridge 1999; Bonduriansky et al. 2008). Partially underlying these sex differences in survival are the sex-specific patterns of susceptibility to disease or severity of pathogen-induced harm with increasing age (Giefing-Kröll et al. 2015; Klein and Flanagan 2016). As both sex ratio (Clutton-Brock and Iason 1986; Duneau and Ebert 2012 and table 2 therein) and age structure

(Charlesworth 1994) vary within natural populations, so too may a pathogen's exploitation strategy when encountering different hosts, having unexplored consequences for the evolution of disease.

A variety of theoretical predictions have been made for how pathogens should adapt to contrasting patterns of selection imposed by different hosts (Regoes et al. 2000; Gandon 2004; Osnas and Dobson 2012; Williams 2012). If pathogen fitness varies across host types, selection may favour exploitation strategies that lead to greater average fitness across host types rather than pathogens adapted to a single host type. Yet a successful generalist strategy depends on a high probability of encountering each host type, and pathogens may be better off specializing on the most common host when fitness differences between hosts are large. In Chapter 3, I consider how an individual's sex and age collectively influence pathogen fitness to better understand how these natural sources of host heterogeneity may influence the evolution of disease. To do so, I infected male and female *Daphnia* at one of four age groups, spanning from a young age, to well after the onset of reproductive maturity. I then measured the age-specific patterns of mortality within each sex, relating these patterns to the relationship between pathogen virulence and transmission. In this study, I provide some of the first insight into how host sex and age can interact to influence pathogen fitness and the maintenance of diverse exploitation strategies among pathogens.

### Pathogen exploitation is sex-specific and underlies dimorphism in disease outcome

Infection is characterized by a conflict between a pathogen and host over a finite pool of resources. This principle drives the virulence-transmission trade-off whereby pathogen reproduction is inherently harmful to the host and fitness is maximized by efficiently exploiting resources before host death (Jensen et al. 2006; Alizon et al. 2009). Upon pathogen exposure, hosts are expected to mount an immune response, sequestering resources from the pathogen (Lee 2006; Hall et al. 2017). Meanwhile, pathogen reproduction directly appropriates host resources (*e.g.* host castration, Ebert et al. 2004; Clerc et al. 2015). An intimate link thus exists between the outcome of infection and the host's pool of resources, or condition (Rowe and Houle 1996; Hunt et al. 2004), leading to several predictions and observations on how host condition can change the outcome of infection (Laine 2007; Seppälä et al. 2008; Wolinska and King 2009; Vale et al. 2011). In addition, because the different life-history strategies of males and females lead to sex-specific patterns of resource acquisition or allocation to fitness-related traits (Rowe and Houle

1996; Stoehr and Kokko 2006; Boggs 2009; Schärer et al. 2012), from a pathogen's perspective the net amount of exploitable resources as well as how pathogens can best exploit those resources may differ in male and female hosts. Consideration of the role that host sex plays in creating a variable resource environment for an invading pathogen may thus be important in understanding the basis for sexual dimorphism in the outcome of infection.

One way to explore how resources underlie sex differences in disease is to compare patterns of disease outcome across male and female hosts to those predicted when pathogens encounter hosts of varying condition. Hall *et al.* (2009c) provide an invaluable model that describes how the relationship between pathogen virulence and transmission is expected to change across hosts of varying condition. In the model, infection progresses rapidly within hosts of high condition and results in high pathogen fitness as well as high levels of pathogen virulence in these hosts. Conversely, low condition hosts lead to lower levels of pathogen exploitation, characterised by low virulence and pathogen reproduction. Yet as the sexes often vary in resource allocation as well as acquisition, they may not only vary in the size of their exploitable resource pool but also in which traits those resources are invested, potentially altering the relationship between host condition and pathogen exploitation. In Chapter 4, I infected male and female *Daphnia* raised on either a high-quality or low-quality diet and measured the effect of host sex on the condition-dependence of pathogen fitness and the form of pathogen exploitation. In doing so, I explored how resource availability differentially affects pathogen fitness within male or female hosts, and how sex-specific resource allocation influences the pathogen exploitation strategy.

### Host sex changes the competitive outcome of co-infection

Within natural populations, individuals are often infected with multiple types or strains of pathogens (Read and Taylor 2001; Rigaud et al. 2010; Balmer and Tanner 2011). Such multiple infections are particularly interesting in the context of pathogen evolution due to their potential to select for more competitive pathogen strains; in contrast to single infections which select on the absolute performance of a pathogen in isolation (Alizon et al. 2013; Seppälä and Jokela 2016). Fuelling this fitness variation is how virulence (often explored through reduction in lifespan or host fitness within multiple infection studies, see Alizon et al. 2013) affects pathogen fitness across these different infection contexts. While low virulence may be favoured in single infections so that pathogens have more time to reproduce before killing their hosts (Bull 1994; Frank 1996; Alizon et al. 2009), more virulent pathogens often outcompete less virulent pathogens in multiple infection

contexts because virulence relates to a pathogen's exploitative potential (Alizon et al. 2013; Cressler et al. 2016). Interestingly, pathogens may express different levels of virulence within male or female hosts (see Table 1, Cousineau and Alizon 2014) and thus the relative competitive ability of pathogen genotypes may also vary between the sexes.

Chapter 5 explores how host sex may impact on the relative fitness between co-infecting pathogen genotypes. In this study I co-infected male and female *Daphnia* with two pathogen genotypes that exhibit strong variation in virulence when singly infecting female hosts (Clerc et al. 2015). I also exposed hosts to single infections to compare the disease outcome for each pathogen genotype within single and co-infection contexts. As a measure of individual pathogen fitness, I used molecular analyses to measure the relative contribution of each co-infecting pathogen to the overall production of spores. The outcome of multiple infection can have a variety of evolutionary implications, from selecting on increased levels of virulence (Alizon et al. 2013; Sofonea et al. 2018) to maintaining pathogen genetic variation by increasing variation in fitness (Seppälä and Jokela 2016). As pathogen virulence and fitness can vary when infecting male or female hosts in single infection contexts, host sex affects the traits which may determine pathogen competitive ability and impact on pathogen evolution.

## Thesis organization

This thesis is presented as a "thesis including published works" consisting of two peer-reviewed and published papers and two manuscripts intended for publication in peer-reviewed journals, as well as a general introduction (Chapter 1) and a conclusion (Chapter 6). Chapter 2 is published in *Evolution* and Chapter 3 is published in *The Journal of Evolutionary Biology.* The Appendix includes a paper I co-authored during my thesis and which was published in *Scientific Reports*.

With the exception of the fragment analysis and genotyping performed by the Australian Genome Research Facility and described in Chapter 5, I was responsible for the collection of the data presented in this thesis. Additionally, I was responsible for the planning, research, laboratory work, data analysis, and manuscript preparation for each presented chapter. However, the first-person plural is used in subsequent chapters to reflect the collaborative nature of my research.

## Chapter 2 – The evolution of sexual dimorphism and its potential impact on host-pathogen coevolution

Stephen A.Y. Gipson[1, 2] and Matthew D. Hall[1, 3]

1. School of Biological Sciences, Monash University, Melbourne, Victoria 3800, Australia

2. [redacted]

3. [redacted]

Keywords: sexual dimorphism; genotype-by-environment interactions; genetic correlations; mutation; host-pathogen interactions; coevolution

Manuscript published in May 2016, *Evolution* 70 (5)

## Abstract

Sex and infection are intimately linked. Many diseases are spread by sexual contact, males are thought to evolve exaggerated sexual signals to demonstrate their immune robustness, and pathogens have been shown to direct the evolution of recombination. In all of these examples, infection is influencing the evolution of male and female fitness, but less is known about how sex differences influence pathogen fitness. A defining characteristic of sexual dimorphism is not only divergent phenotypes, but also a complex genetic architecture involving changes in genetic correlations amongst shared fitness traits, and differences in the accumulation of mutations – all of which may affect selection on an invading pathogen. Here, we outline the implications that the genetics of sexual dimorphism can have for host-pathogen coevolution and argue that male-female differences influence more than just the environment that a pathogen experiences.

## The Interplay between Sexual Dimorphism and Infection

Within any population there is often no bigger difference between individuals than the dimorphism that can occur between males and females. Driven by the fundamental differences in reproductive investment between the sexes, males and females commonly vary in body size and shape, reproductive investment, longevity, choosiness (investment in selecting a mate) and the degree of sexual ornamentation (Schärer et al. 2012). Sex-specific differences also extend to a host's investment in immune defence (Zuk and Stoehr 2002). Males, for example, are typically thought of as the "sicker sex" (Zuk 2009), investing fewer resources in maintaining an effective immune response in favour of their costly sexual displays (but see Box 1 for discussion). Each sex, therefore, differs in important properties that help to define pathogen fitness, such as encounter rates, degree of resistance, exploitative potential (body or organ size) and resource availability (*e.g.* Christe et al. 2007).

Despite the well-documented differences between males and females in behaviour, physiology, and immunity, only recently has the interplay between sexual dimorphism and infection been considered in light of infectious disease evolution (Box 1). Duneau and Ebert (2012) were first to develop explicit hypotheses on how a pathogen should adapt to selection imposed by each sex, noting that this process might eventually lead to pathogens that are either optimally adapted to one sex, or are able to plastically respond to the challenges of sexual dimorphism. Building on this insight, Cousineau and Alizon (2014) modelled how the evolution of pathogen virulence depends on whether sexual dimorphism occurs in the probability of becoming infected (resistance) or in

mitigating the damage caused by a pathogen once infected (tolerance). Together, these two studies highlight how sex differences can be an underappreciated driver of pathogen evolution; yet they only formalise one component of sex-specific pathogen evolution – the evolutionary optima of a pathogen when faced with sexual dimorphism in a host's internal environment.

In this article, we outline the unrecognised implications that sexual dimorphism can have for the genetic basis of host-pathogen coevolution. The struggle between pathogens and hosts has long fascinated biologists, and has been fundamental to our understanding of the very basis of male and female differences – the origin of sexual recombination (see Box 2). Driven by differences in the type of gametes they produce, however, each sex is often pushed towards different evolutionary optima, a process that often requires some important genetic architecture to proceed. The decline in positive genetic correlations amongst shared fitness traits, the cyclical coevolution of sex-specific behaviours, and the differential accumulation of mutations are all aspects of genetic architecture that underlie the evolution of sexual dimorphism. Drawing on the type of insights common to studies of mate choice evolution, we discuss three key implications arising from the complex genetics of male-female differences: i) the importance of genotype-by-environment interactions for shaping the evolutionary potential of a pathogen; ii), the role of cross-sex genetic correlations in enhancing or constraining host-pathogen coevolutionary cycles; and iii) the potential for sex-specific mutation patterns to influence the rate at which a host can respond to the threat of parasitism.

## The Sex-Specific Evolutionary Potential of an Invading Pathogen

Host populations are rarely homogeneous, and pathogens will encounter individuals that differ in age, nutritional condition, body size, immune status, and physiology (Schmid-Hempel 2013). By modelling the epidemiological characteristics of disease when there are two or more host classes, theory has established how this heterogeneity can affect the evolution of pathogen fitness (Regoes et al. 2000; Gandon 2004; Williams 2012). These approaches have recently been extended to include variability in the internal host environment as caused by male-female differences (Box 1). For simplicity, however, the emerging study of sex-specific pathogen evolution has yet to consider explicitly how the impact of host sex may depend on the genotype of the invading pathogen. Such genotype-specific responses are nonetheless a common feature of both a host and pathogen's response to environmental change (reviewed in Wolinska and King 2009). Here we consider the adaptive implications of male-female differences in light of the extensive body of

research on the evolutionary importance of such genotype-by-environment interactions (GxEs, see Hunt et al. 2004; Ingleby et al. 2010). We focus on two important aspects of GxEs – the maintenance of genetic variation, and the change in evolutionary potential.

In general, GxEs arise when the relative fitness of different genotypes, or sometimes strains in the case of pathogens, is dependent on the environment in which that they are expressed (Fig. 1). In the absence of a GxE (Fig. 1A), the relative differences among pathogen genotypes remain unaltered in each sex, and so it is only the overall degree of sexual dimorphism in resistance that influences pathogen evolution. This scenario is most analogous to the theoretical considerations of host sex heterogeneity so far, whereby one sex, typically females, is more able to supress pathogen fitness. The evolution of disease thus progresses depending on how often the invading pathogens encounter the two environmental classes and how well invading pathogens are able to exploit host resources and transmit to new uninfected animals (*e.g.* Box 1). However, a more complicated scenario for host-pathogen coevolution, and one that is likely to be biologically widespread, occurs when the response of a pathogen to the sex of a host is genotype specific – either involving a change in genotype rank order, or a change in the scale of genetic variation.

In situations where the rank order of fitness among genotypes changes (Fig. 1B), GxEs have the potential to influence the maintenance of genetic polymorphisms in natural populations. As long as there is gene flow between environments, no one genotype will be consistently favoured, allowing fluctuating selection to maintain genetic variation in fitness (Gillespie and Turelli 1989). This process helps to explain why male sexual traits typically show significant heritability (Pomiankowski and Møller 1995), despite the prediction that female choice should rapidly erode genetic variation in sexual signals (Kirkpatrick and Ryan 1991). Similar arguments help explain why we observe considerable variation in infectious disease characteristics, despite the expectation that selection should favour completely resistant hosts or infective pathogens (*e.g.* Blanford et al. 2003; Vale et al. 2008; Hall and Ebert 2012). Indeed, environment specific responses of pathogens may be almost universal, with GxEs occurring for a wide range of environmental conditions such as temperature, food quality, and the maternal environment (Wolinska and King 2009). Given the widespread potential for heterogeneity to arise due to male and female differences, we therefore suggest that sex driven GxEs may be another factor helping to maintain genetic variation in pathogen populations.

A more subtle version of a GxE can also occur when the relative fitness of a pathogen genotype changes across environments, but rank order of the genotypes remains the same (Fig. 1C). Such changes in the scale of variation do not directly help to maintain genetic variation, as one genotype is always superior. Instead genotypes differ in their sensitivity to the alternative environments, altering the intensity of any response to selection as the expression of genetic variance has now changed in each environment (Hoffmann and Merilä 1999). Work on infectious disease has already shown that pathogen genetic variability can increase or decrease with environmental change, such as with the age of a host (Mideo et al. 2011; Clerc et al. 2015). More broadly across a landscape of environmental variability, it has also been suggested that changes in genetic variation can substantially influence the intensity of host-pathogen coevolution, creating evolutionary "cold spots" or "hot spots", depending on the degree of difference amongst genotypes in each environment (Thompson 1994; Gomulkiewicz et al. 2000). Extrapolating the results would suggest that host sex is another possible environmental variable that could enhance or constrain pathogen evolution, contributing to local hot spots or cold spots in the environmental landscape that a pathogen experiences.

Genotype-by-environment interactions clearly have potential to shape the evolutionary dynamics experienced by an invading pathogen; but their precise outcome will depend on the type of trait experiencing such interactions. Broadly speaking, the fitness of a pathogen depends on a variety of traits related to the force of infection (the number of infections acquired over time), namely, infection rates, transmission potential, and the length of the infection period (Anderson and May 1991). Evolutionary theory, however, makes distinctions between the outcomes that arise from each of these different parameter types (*e.g.* Anderson and May 1982; van Baalen 1998; Gandon et al. 2002; Råberg et al. 2009). As discussed in Box 1, for example, variation in investment between males and females in resistance (limiting pathogen burden) or tolerance (limiting severity of pathogen burden), will lead to different evolutionary outcomes for a pathogen. Similar arguments could also be made for changes in pathogen genetic variation that arise for traits related to either partial (quantitative) or complete (qualitative) resistance against a pathogen (Gandon and Michalakis 2000). Thus, while the general form of a GxE (rank order or scale changes) defines the broad evolutionary relevance of an interaction between males, females, and pathogens – the precise outcome will depend on the trait and host-pathogen system of study.

## Sex-Specific Selection and the Constraint of Host-Pathogen Dynamics

Central to the understanding of Red Queen dynamics are the repeated coevolutionary cycles between hosts and pathogens (Box 2). At the heart of this process are the genetic associations that build up and break down between the antagonistic combatants, whereby selection on one party results in a correlated response in the other (Fig. 2A). But what if there is an additional party in this process that is also undergoing coevolution with one of the combatants? While the Red Queen concept has been applied to a wide range of evolutionary problems, such scenarios have traditionally been limited to simple two party systems in isolation – either host and pathogen, or predator and prey (Brockhurst et al. 2014). A rare example of a study examining coevolutionary patterns within a three-party system is that of Dercole *et al.* (2010) which modelled predator-prey coevolution in a three species food chain (Fig. 2B). The inclusion of a coevolving super-predator forced the normally stable evolutionary cycles between predator and prey to become chaotic, with both the direction and strength of selection becoming intrinsically unpredictable beyond a short evolutionary time. In stark contrast to the predictable cycles of typical Red Queen dynamics, therefore, the addition of another external source of genetic variation vastly complicates the process of coevolution.

Here we consider what happens to Red Queen dynamics when the genetic architecture of sexual dimorphism is introduced into the Red Queen process (Fig. 2c). A defining characteristic of sexual populations is that males and females can undergo their own coevolutionary struggle, as each sex experiences contrasting patterns of natural and sexual selection (Parker 2006; Cox and Calsbeek 2009). At the heart of this tension are the cross-sex genetic correlations amongst homologous traits ($r_{MF}$) that arise as a consequence of a shared gene pool (Lande 1980, 1987; Bonduriansky and Chenoweth 2009). Tight genetic correlations between shared traits act to prevent phenotypic divergence between males and females. With different evolutionary optima for each sex, this inevitably constrains adaptation, as selection on one sex will now produce a correlated response in the other. To overcome these limitations, $r_{MF}$ values should diminish or even become negative. When selection is concordant but varies in strength, a decline in $r_{MF}$ allows shared traits to evolve independently and more readily reach their separate optima (Lande 1980). However, if selection is sexually antagonistic and differs in its direction, a negative genetic correlation between traits can build up as alleles are favoured that benefit one sex, but are detrimental to the other (intralocus sexual conflict, reviewed in Bonduriansky and Chenoweth 2009). Evolution in sexual populations, therefore, is characterised by a spectrum of $r_{MF}$ values, from the strong and positive values for

many shared traits, through to those which diminish from unity as traits become more closely related to fitness (Poissant et al. 2010; Griffin et al. 2013).

An initial glimpse into the consequences of sexual dimorphism for disease is offered by models which have previously explored the impact of separate sexes on the rate of adaptation for a species in general (Lande 1980; Connallon and Clark 2014). Strong and positive correlations between traits are predicted to enhance the rate of adaptation only when selection on males and females is tightly aligned (Lande 1980). This is essentially the null model for host-pathogen dynamics, reflecting that male and female defence components are assumed to share the exact same genetic architecture, and undergo stable coevolutionary cycles as part of a normal two-party system (*sensu* Dercole et al. 2010). As the fitness correlation between the sexes diminishes ($r_{MF} < 1$), however, so to can the rate of adaptation if selection remains sexually concordant (*e.g.*) (Connallon and Clark 2014). With the uncoupling of male and female resistance ($r_{MF} \approx 0$), adaptive changes in one sex would no longer be reflected in the other due to a lack of any correlated response. Chaotic cycling is now a possibility, as by uncoupling male and female traits, Red Queen dynamics will most closely reflect the three-party dynamic of Dercole *et al.* (2010). Finally, as cross-sex genetic correlations change sign ($r_{MF} < 0$), the change in resistance of both sexes can become suboptimal, with selection on one sex directly impeding the adaptive evolution of the other (Connallon and Clark 2014). Thus, under the constant threat of parasitism, the genetic architecture of sexual dimorphism could potentially reduce the rate of host adaptation, thereby modifying the process of host-pathogen coevolution.

Despite the well-developed predictions for the evolution of infectious disease (Box 2) and sexual dimorphism (Lande 1980; Bonduriansky and Chenoweth 2009; Connallon and Clark 2014), evolutionary theory has yet to explore the implications of sex-specific adaptation for host-pathogen coevolution. Yet the evolutionary decoupling of male and female resistance is likely, at least in part, given the presence of considerable sexual dimorphism in traits related to host resistance (Vincent and Sharp 2014). What remains unclear, however, is the ecological and evolutionary conditions that permit cross-sex genetic correlations to constrain or enhance the rate at which hosts can adapt to pathogens. While the work by Dercole *et al.* (2010) has shown that genetic variation introduced by another evolving party can fundamentally change the direction and strength of selection during Red Queen dynamics, it is important to note that genetic constraints can eventually be overcome if selection is strong enough. Studies of sexual dimorphism, in general, have shown that stressful conditions can cause $r_{MF}$ for fitness to change

sign and become more positive as increased sexually concordant selection favours overall genetic quality (Long et al. 2012; Berger et al. 2014). Identifying how strong and frequent Red Queen cycles need to be before sexual dimorphism ceases to matter will be essential for unravelling the potential importance of cross-sex genetic correlations for host-pathogen coevolution.

## The Implications of Male-Biased Mutation for Genetic Variability in Resistance

A key requirement for host-pathogen coevolution via selective sweeps is that novel pathogen infectivity and host resistance alleles must constantly arise for coevolution to occur (Box 2). In this case, mutations are commonly the source of novel genetic variation and provide the raw fuel for evolutionary change. However, while the consequences of mutation on the rate of evolution are well understood (Maynard Smith 1976), an important consideration is how differences in mutation rates amongst hosts may impact on pathogen evolution. It is well known that genes involved in defence against a pathogen, such as those within the major histocompatibility complex or plant Resistance (R) pathways, evolve at high rates (Bergelson et al. 2001; Borghans et al. 2004). Additionally, theoretical studies have shown populations with higher mutation rates are better able to track the constantly moving optima driven by their antagonistic species (Gandon and Michalakis 2002; Salathé et al. 2005; Tellier et al. 2014). Less attention, however, has been directed towards the implications for infectious disease if some individuals within a population are more likely to accumulate mutations. We discuss how biases in mutation rate between males and females, together with the observation that the fitness consequences of mutations are often more severe in males, may fundamentally alter the outcome of host-pathogen coevolution.

Although the occurrence of novel mutations has traditionally been considered to be random, it has long been proposed that the rate at which mutations arise may be higher in males than females (Haldane 1935). The mutation bias stems from a fundamental difference in the production of male and female gametes, with spermatogenesis requiring more cell divisions than oogenesis, and thus more opportunity for replication errors (Hurst and Ellegren 1998). Consequentially, if adaptation depends on the supply of beneficial new mutations, then sex biases in mutation will substantially alter the rates of evolutionary change. Indeed, in the context of sex linkage, theory has shown how male-biased mutation can accelerate the accumulation of beneficial changes on specific chromosomes, with male sexual displays predicted to evolve most rapidly when Z-linked (Kirkpatrick and Hall 2004). However, reducing the waiting-time for an adaptive mutation to arise is even more important in the context of Red Queen dynamics and selective sweeps, where

pathogens typically have effective population sizes that far outstrip that of their hosts (*i.e.* Lanfear et al. 2014). While the potential for male-biased mutation to accelerate host-pathogen coevolution has not yet been considered, a study of baculovirus resistance in the codling moth linked the rapid emergence of host resistance to the rise of Z-linked resistance alleles (Asser-Kaiser et al. 2007), suggesting a role, at least in principle, for male-biased mutation.

In general, however, most mutations are predicted to be deleterious (Eyre-Walker and Keightley 2007). Theory has previously explored the link between pathogens, mutations, and sex, in terms of the maintenance of sexual recombination. By magnifying the negative effects of mutation accumulation, pathogens strengthen the opportunity for sex to overcome the advantages of remaining asexual (Park et al. 2010). Yet an interesting finding from studies of spontaneous mutation is that the severity of the fitness loss is felt much stronger by males, even though mutations are generally sexually concordant (Sharp and Agrawal 2013). Driving this pattern is the elevated intensity of selection acting on male mating success, whereas females are typically able to find a mate even if under mutational duress (Agrawal 2001; Whitlock and Agrawal 2009). But what about the consequences of mutation load for the ability of a male to fight or tolerate infection? To date, only Sharp and Vincent (2015) have considered how pathogens and mutation interact with male-female differences. Their results show how pathogen exposure further exacerbates the fitness loss experienced by males, again contributing to the effective purging of deleterious alleles. Nonetheless, the evolutionary implications of this process for pathogen transmission or virulence remain unexplored. On one hand males are more susceptible to infection and less likely impose strong selection on pathogen infectivity; but on the other, total selection on males is likely to be stronger and males are predicted to evolve more rapidly. Understanding the tension between these two contrasting patterns will shed light on whether males or females are the optimal sex for a pathogen to exploit.

## Future Directions

While recent attention has been given to the role that sex differences play in pathogen evolution, we believe that this review highlights unrecognized ways in which sexual dimorphism can impact on the genetic basis of host-pathogen coevolution. A logical next step will be to formalize theory which explores the interplay between sex-specific adaptation and the rapid coevolutionary cycles that result from host-pathogen coevolution. Key to this process will be understanding the impact of male-female genetic correlations on rates of adaptation when patterns of selection change

rapidly in both direction and magnitude. In general, theory predicts that when selection is sexually concordant, positive genetic correlations will accelerate adaptation to natural selection, whereas negative correlations will constrain how quickly a population can adapt to change (or vice versa with sexually antagonistic selection, Lande 1980, 1987; Bonduriansky and Chenoweth 2009). However, the constraints that arise from cross-sex genetic correlations have yet to be studied in the context of a rapidly changing environment, such as the once provided by a coevolving pathogen (but for some related contexts, see Chevin 2013; Kopp and Matuszewski 2014). We may expect that if resistance is strongly positively correlated between the sexes, then host adaptation to a pathogen will proceed as expected by current theory. For other genetic correlation values, however, the rate of coevolution may decline or become unpredictable, particularly in the sex that less sensitive to the threat of infection (*sensu* Connallon and Clark 2014).

Complementing theory will be empirical work which explicitly examines the impact of host sexual dimorphism on pathogen adaptation. While it is both empirically and theoretically well-established that males and females can differ in their susceptibility to infectious disease (Sheridan et al. 2000; Cousineau and Alizon 2014), progress now depends on estimating and interpreting parameters directly relevant to the impact of host sex on pathogen populations (*e.g.* infection rates, transmission potential and infection period, Anderson and May 1991). Study systems well suited to this work will utilize hosts which are sexually dimorphic in immunity, plus have experimentally manipulable sex ratios or sex-role reversal – thus presenting the pathogen with two selectively distinct environments, as well as variability in the frequency of encountering each environment (*e.g.* Rolff 2002; Duneau et al. 2012; Masri et al. 2013). Additionally, comparisons between population that have differed for many generations in the rate at which a pathogen encounters each sex offer a powerful way to find evidence for sex-driven pathogen evolution, whether via the classic cross-infection experiments (discussed in Schmid-Hempel 2013), or more modern developments in unravelling molecular signals of local adaptation (Balenger and Zuk 2014).

## Concluding Remarks

We believe that an emerging challenge for disease research is to consider how the evolution of male-female differences permeates through the process of host-pathogen coevolution. The view that the sexes differ in a range of characteristics is well established and has recently been implicated in host resistance and pathogen adaptation (Duneau and Ebert 2012; Cousineau and

Alizon 2014). Recognising the importance of the genetic architecture of sexual dimorphism in these contexts, however, will offer new opportunities to understand the drivers and constraints placed on host-pathogen coevolution. Encouraged by the universal nature of sexual dimorphism and the recent results discussed in this review, we anticipate a greater exploration of male-female differences will offer new insight into how infectious disease evolves and the opportunities available to predict or control the spread of pathogens.

## Acknowledgments

We thank T. Connallon, A. Peters and L. van Boheemen for comments and valuable discussion. This work was supported by an Australian Research Council (ARC) Discovery Early Career Researcher Award to MDH.

## Chapter 2 boxes and figures

*Box 1: Insight into the Impact of Male-Female Differences on Pathogen Adaptation*

Evidence for the interplay between host sex and pathogen evolution is rare, but awareness that heterogeneity between sexes can influence disease evolution is growing. In birds and mammals, surveys of the literature have found that males are often more susceptible to infection and consequently experience higher infection rates and intensities than females (Poulin 1996; Schalk and Forbes 1997; Zuk 2009; Cousineau and Alizon 2014). This pattern, however, is not necessarily universal, and predictions for which sex should invest more in immunity will likely vary with the study species or environmental context, particularly in invertebrates (McCurdy et al. 1998; Sheridan et al. 2000; Stoehr and Kokko 2006). Indeed, what matters most is not whether males or females are generally the "sicker sex", but that sexual dimorphism in host resistance exists and can drive pathogen adaptation to the differences between male and female hosts (Christe et al. 2007; *e.g.,* Duneau et al. 2012). With the widespread occurrence of sex-ratio biases, social hierarchies, and differences in male and female explorative behaviours (Duneau and Ebert 2012 and Table 2 therein) there is ample opportunity for pathogens to encounter males and females at different rates, and experience differences in the patterns of selection that a sexually dimorphic host can generate.

In developing the arguments for the link between host sex heterogeneity and infectious disease, Duneau and Ebert (Duneau and Ebert 2012) established the evolutionary requirements for how host sex could drive pathogen evolution. Based on the expectation that a pathogen will adapt to the characteristics of its most common host (Lively 1989), they outlined how the degree of host sexual dimorphism, and the probability of encountering the opposite sex, will define how a pathogen evolves. A generalist pathogen is predicted to evolve if the frequency of encountering multiple sexes is high and fitness trade-offs between environments are small. Conversely, pathogens are expected to become specialised to one sex when the sexes strongly differ, but the pathogen is unlikely to encounter both sexes. Finally, a pathogen may evolve a plastic response to host sex if both the frequency of experiencing male and female hosts, together with the fitness differences between environments, is high.

Complementing this work, Cousineau and Alizon (2014) focused on the evolution of pathogen virulence in response to how males and females defend against infection. In surveying a diverse range of mammalian pathogens, from viruses like influenza to macroparasites such as *Schistosoma*

(Cousineau and Alizon 2014 and Table 1 therein), they found that males are typically more susceptible to initial infection than females, but are better able to cope with higher pathogen loads once infected. With these differences in mind, Cousineau and Alizon (2014) modelled how sex differences in either resistance or tolerance shape pathogen evolution. Sexual dimorphism in resistance was found to yield an increase in pathogen virulence only when pathogen transmission occurs only between members of opposite sexes, as this is when the most resistant sex will always be encountered. Conversely, dimorphism in tolerance accelerated the evolution of virulence under any pattern of contact between the sexes. Their results demonstrate how pathogen virulence will depend on a complex interaction between the degree of sexual dimorphism, the form of defence (resistance or tolerance), and the pattern of contact between the sexes.

*Box 2: Pathogens, Red Queen Dynamics, and Sex*

Infections are a powerful evolutionary force, driving "arms races" between hosts and pathogens, as host are forced to repeatedly evolve counter-adaptations to the constant threat of parasitism (May and Anderson 1983). This process of cyclical adaptation and counter-adaptation is commonly known as a "Red Queen" dynamic (van Valen 1973; Bell 1982). Originally attributed to the probability of taxon extinction, the Red Queen process has come to define an important concept in biology – the role of biotic interactions for rapidly shaping evolutionary outcomes (reviewed in Brockhurst et al. 2014). Of particular interest for infectious disease are the ways in which antagonistic interactions can lead to perpetual evolutionary change (Woolhouse et al. 2002; Lively 2010). Models of coevolution by negative frequency dependent selection, for example, describe a process whereby pathogens adapt to the most common host, providing a selective advantage to rare host genotypes (Hamilton 1980). Selection thus occurs in a time-lagged and negative frequency-dependent manner and genetic diversity within a population is maintained (*e.g.* Dybdahl and Lively 1998). Antagonistic coevolution can also occur via recurrent selective sweeps. Here coevolution proceeds via the repeated occurrence, spread and fixation of beneficial mutations, which inevitably reduces genetic diversity within populations (Levin 1981; Buckling and Rainey 2002).

Not only has the pervasive nature of the host-pathogen antagonism been fundamental to our understanding of infectious disease evolution, it has also provided an explanation for the very basis of male and female differences – the origin and maintenance of sexual recombination (Jaenike 1978; Hamilton et al. 1990; Lively 2010). In this context, sex is most strongly associated

with negative frequency-dependent oscillations between host and pathogen genotypes. By breaking up statistical associations amongst loci, and increasing the frequency of rare alleles under the threat of parasitism (Peters and Lively 1999), recombination allows sexual organisms to overcome the “two-fold” reproductive advantage of asexual competitors (Maynard Smith 1978). Although widely accepted as a fundamental component of infectious disease evolution, it is difficult to demonstrate Red Queen patterns in the field (but see Dybdahl and Lively 1998; Decaestecker et al. 2007; Koskella and Lively 2007; Thrall et al. 2012), and correlative evidence from genomic studies is not without its controversy (Hall and Ebert 2013). Nonetheless, the antagonistic coevolution between hosts and pathogens is integral to the evolution of sex, and the genesis of reproductive and behavioural differences between males and females.

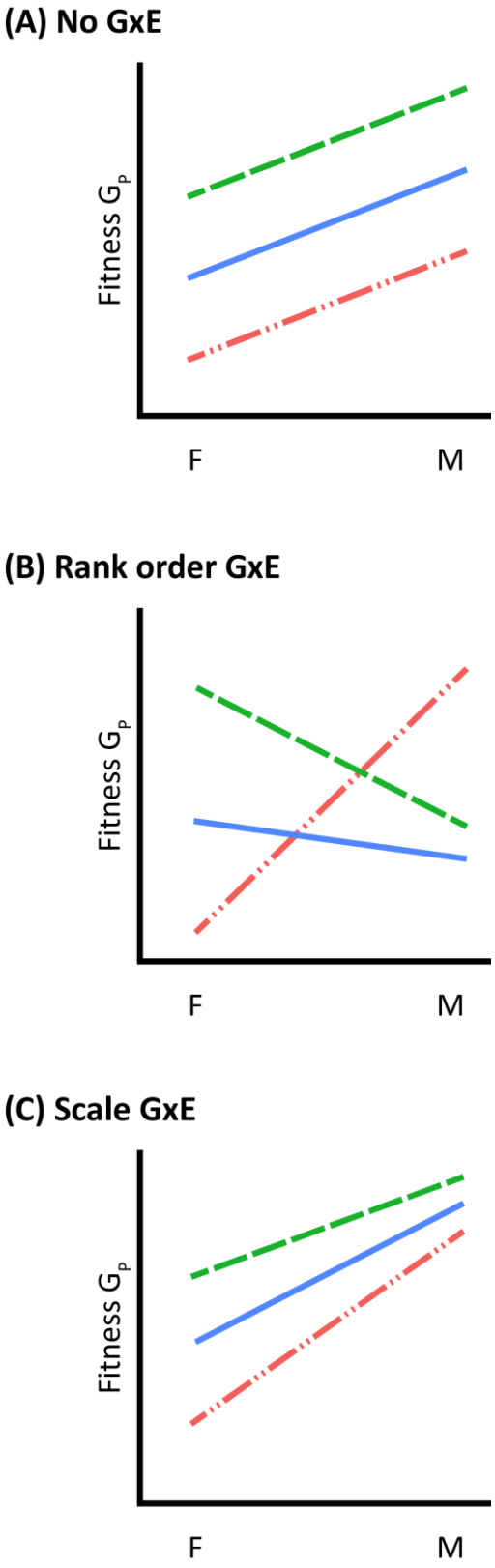


**Figure 1.** Conceptual reaction norms for the relative fitness of three pathogen genotypes ($G_P$) and two host sexes (male or female). (A) In the absence of a $G_P$ x E, pathogen genotypes (represented by different colours) may differ in their fitness, and each sex may be more or less resistant, but the effects are the same for each genotype. In the presence of a $G_P$ x E, (B) the rank order of the pathogen genotypes may change between the sexes, or (C) the scale of genetic variation changes between the sexes, but the rank order of pathogen genotypes remains the same. Each scenario has different consequences for the intensity of a selection response and the maintenance of genetic variation.


**Figure 2.** Illustrative examples of different Red Queen interactions. (A) The basic Red Queen process describes hosts and pathogens as existing in an indefinite and predictable cycle of adaptation followed by a proportional counter adaptation (Brockhurst et al. 2014). (B) Red Queen "Chaotic" hypotheses (Dercole et al. 2010) describe the unpredictability of both strength and direction of selection of classic Red Queen models with the addition of a third coevolving species. (C) Similar to Red Queen Chaotic, patterns of host-pathogen coevolution with the added complexity of males and females may be difficult to predict using classic Red Queen models. In this scenario, pathogens will adapt to the different selective pressures presented by each sex, while the sexes will independently develop reciprocal adaptations as modulated by cross-sex correlations ($r_{MF}$).

## Chapter 3 – Interactions between host sex and age of exposure modify the virulence-transmission trade-off

Stephen A.Y. Gipson[1, 2] and Matthew D. Hall[1]

1. School of Biological Sciences and Centre for Geometric Biology, Monash University, Melbourne, Victoria 3800, Australia

2. [redacted]

Keywords: age-structured interactions; genotype-by-environment interactions; host–pathogen interactions; optimal virulence; sexual dimorphism; virulence–transmission trade-off

Manuscript published in March 2018, *The Journal of Evolutionary Biology* 31 (3)

**Abstract:**

The patterns of immunity conferred by host sex or age represent two sources of host heterogeneity that can potentially shape the evolutionary trajectory of disease. With each host sex or age encountered, a pathogen's optimal exploitative strategy may change, leading to considerable variation in expression of pathogen transmission and virulence. To date, these host characteristics have been studied in the context of host fitness alone, overlooking the effects of host sex and age on the fundamental virulence-transmission trade-off faced by pathogens. Here we explicitly address the interaction of these characteristics and find that host sex and age at exposure to a pathogen affect age-specific patterns of mortality and the balance between pathogen transmission and virulence. When infecting age-structured male and female *Daphnia magna* with different genotypes of *Pasteuria ramosa*, we found that infection increased mortality rates across all age classes for females, whereas mortality only increased in the earliest age class for males. Female hosts allowed a variety of trade-offs between transmission and virulence to arise with each age and pathogen genotype. In contrast, this variation was dampened in males, with pathogens exhibiting declines in both virulence and transmission with increasing host age. Our results suggest that differences in exploitation potential of males and females to a pathogen can interact with host age to allow different virulence strategies to coexist, and illustrate the potential for these widespread sources of host heterogeneity to direct the evolution of disease in natural populations.

## Introduction

Resulting from the contrasting strategies by which males and females maintain fitness, the sexes are expected to vary in their relative immune investment, condition, and morphology (Zuk and Stoehr 2002; Zuk 2009), all of which can lead to sex biases in the outcome of infection (Poulin 1996; Schalk and Forbes 1997; McCurdy et al. 1998; Sheridan et al. 2000). In humans, for example, males are generally more susceptible to infectious diseases such as tuberculosis and hepatitis B (Giefing-Kröll et al. 2015; vom Steeg and Klein 2016) and harbor a larger viral load than females in the case of HIV (Napravnik et al. 2002; Donnelly et al. 2005). However, the "sicker sex" can vary from species to species (see Table 1, Cousineau and Alizon 2014) and many studies have considered the consequences this has for host susceptibility and mortality (Giefing-Kröll et al. 2015; Klein and Flanagan 2016) as well as mate choice (Hamilton and Zuk 1982). Recently, attention has turned to how sexual dimorphism relates to the evolution of pathogen fitness itself,

leading to predictions, many yet untested, for how male-female differences might impact on the evolutionary dynamics of host-pathogen interactions (Duneau and Ebert 2012; Cousineau and Alizon 2014; Gipson and Hall 2016).

A component of pathogen fitness that may be particularly susceptible to disruption by sexual dimorphism is the fundamental trade-off between transmission and virulence. In natural populations, pathogens are inevitably transmitted through hosts that differ in their capacity to fight infection (Altizer et al. 2006; Wolinska and King 2009), spurring many theoretical predictions on how a heterogeneous host population will affect the optimal level of virulence (Regoes et al. 2000; Gandon 2004; Osnas and Dobson 2012; Williams 2012). Gandon (2004), for example, highlighted how a specialist virulence strategy is predicted if the frequency of encountering only one host type is high. In this context, by adapting to the most common host, a pathogen can become maladapted to the less common one as a consequence of either suboptimal exploitation or overexploitation. This cost becomes progressively smaller as the chance of encountering the less common host type is reduced. In contrast, when the likelihood of transmission between different host types is higher, more generalist strategies are now favoured that can select for intermediate values of virulence (assuming all things being equal in other demographic parameters such as background mortality, Gandon 2004).

In the context of host heterogeneity, sex is simply another factor, much like species, genotype, or immune status that leads to pathogens potentially encountering different hosts at each transmission event (see Osnas and Dobson 2012). What matters for pathogen evolution will be the degree to which a pathogen differentially exploits males and females, and the proportions of each sex that remain uninfected in a population (Gandon 2004). To date, very few empirical studies (Duneau et al. 2012; Thompson et al. 2017; Willink and Svensson 2017), and only one theoretical model (Cousineau and Alizon 2014), have explicitly considered male-female differences in the context of virulence evolution. Notably, Cousineau and Alizon (2014) modelled how a pathogen might evolve depending on whether sex differences occur in preventing infection (resistance) or in minimizing the damage caused by given a pathogen load (tolerance). Their approach highlighted the need to consider the interaction between host sex and multiple components of pathogen fitness and revealed how variation in host sex ratios, either at birth or due to differential mortality, can modify the effect of host heterogeneity on pathogen evolution.

Male-female heterogeneity will arise not only from average differences in immunity, reproduction, or energy acquisition between the sexes (Stoehr and Kokko 2006), but also their timing of investment in these components of fitness (Rolff 2002). Heterogeneity due to sex, therefore, has an inherently dynamic aspect. The "live fast, die young" reproductive strategy that often typifies the less choosy sex, for example, describes a shift in reproductive peak towards an earlier age, followed by a continual decline in general performance and survival (Vinogradov 1998; Sgrò and Partridge 1999; Bonduriansky et al. 2008). Age-specific sex differences extend to patterns of immunity, represented as changes in susceptibility with increasing age (Giefing-Kröll et al. 2015; Klein and Flanagan 2016). Given the propensity for natural populations to vary both in their sex ratio (Clutton-Brock and Iason 1986; Duneau and Ebert 2012 and Table 2 therein) and age structure (Charlesworth 1994), it is likely that the interaction between the age and sex of a host, rather than solely their independent effects, will be a pervasive source of heterogeneity governing the evolution of infectious disease.

In this study, we consider how interactions between host sex and age at pathogen exposure influence pathogen fitness. We use the crustacean *Daphnia magna* and its bacterial pathogen *Pasteuria ramosa* to measure the impact of sex on age-specific patterns of mortality and how these modulate the relationship between pathogen virulence and transmission. In this system, infection is associated with castration, gigantism and reduced survival (Ebert et al. 2016), but these patterns are modified by a range of host genetic, pathogen genetic, and environmental factors (*e.g.* Carius et al. 2001; Vale et al. 2008; Hall and Ebert 2012). Both sex- and age-specific differences have been previously explored in isolation. In terms of age structure, for example, both host resistance (Garbutt et al. 2014), and the relationship between pathogen virulence and transmission (Clerc et al. 2015; Izhar and Ben-Ami 2015), depend on the age of the host; indicating that the optimal infectivity and virulence strategy for a pathogen may be age-specific. In turn, research has shown that males are more resistant to infection, that mean pathogen fitness is greater within the female host (Duneau et al. 2012; Thompson et al. 2017), and that the relative fitness advantage of any given pathogen genotype is greater within females (Thompson et al. 2017).

Via a series of experimental infection trials, we exposed genetically identical male and female *Daphnia* to one of two compatible *P. ramosa* genotypes at either 10, 20, 30 or 40 days old. We then measured the resulting changes in the costs of infection via age-specific mortality rates and linked them to aspects of pathogen fitness such as infection rate, pathogen-induced reduction in

lifespan (virulence), and the production of spores (transmission). In light of previous studies which have investigated the effects of host sex or age on disease in isolation (Duneau et al. 2012; Clerc et al. 2015; Izhar and Ben-Ami 2015; Clark et al. 2017; Thompson et al. 2017), we predicted that mortality rates and virulence should increase with the age at which a host encounters a pathogen as a consequence of the ageing process (Adamo et al. 2001; Doums et al. 2002; Zerofsky et al. 2005), and that these increasing costs would be felt most strongly by the less resistant sex, which in *Daphnia* is females (Duneau et al. 2012; Thompson et al. 2017). We discuss how our results may impact on the evolution of optimal infectivity and virulence for a pathogen and contribute to the maintenance of variability in infectious disease in natural populations.

## Materials and methods

*Daphnia magna* Straus is a freshwater crustacean that reproduces via cyclic parthenogenesis and can produce genetically identical male and female clones (Ebert 2005). *Pasteuria ramosa* Metchnikoff (Green 1974; Ebert et al. 2016) is a common pathogen of *D. magna* that invades the host via attachment to the oesophagus and subsequently reproduces within the hemolymph of the infected *Daphnia,* filling the body with transmission spores. *P. ramosa* transmission is exclusively horizontal, occurring after the release of spores from a dead host. This experiment utilized male and female *Daphnia* of genotype HU-HO-2 and novel *P. ramosa* genotypes C20 or C24 that have previously been shown to strongly vary in their expression of fitness characteristics and capture a range of possible transmission-virulence combinations (Clerc et al. 2015; Thompson et al. 2017). On average, C20 produced less spores and reduced the lifespan of the host more than C24 (Clerc et al. 2015 and Table 1 therein).

### *Production of experimental animals*

Prior to the experiment, juvenile female *Daphnia* were collected from stock cultures and individually maintained in 60-mL vials filled with 50 mL of artificial *Daphnia* medium (ADaM, Klüttgen et al. 1994; modified by Ebert et al. 1998). *Daphnia* were transferred into fresh media twice weekly, maintained under standard conditions (20°C, 16L: 8D) for three generations, and fed up to 5 million *Chlorella vulgaris* algal cells daily, steadily increasing from birth to accommodate the growing needs of the animal. Experimental males and females were produced using methods from Thompson et al. (2017). In short, females were exposed to 300 µg/L of the hormone methyl farnesoate (Product ID: S-0153, Echelon Biosciences, Salt Lake City, Utah) after producing their

first clutch and then transferred into fresh hormone-treated media every two days. Subsequent clutches were collected and the sex of all offspring determined. This method can be used to reliably produce male and female *Daphnia* while having no detectable impact on lifespan, fecundity, infection rates, or spore loads (Thompson et al. 2017).

### *Infection experiment design and estimates of pathogen fitness*

Male and female *Daphnia* were randomly exposed to one of the two pathogen genotypes at ages 10, 20, 30, or 40 days. For each treatment combination, we individually placed between 60 and 120 males and females in jars, with the larger sample sizes allocated to the older ages to compensate for natural deaths occurring before pathogen exposure; with an additional 50 individuals of each sex as unexposed controls (2 sex × 2 pathogen genotypes × 4 ages × 60 to 120 replicates + [2 sex × 50 control replicates]). Infections took place in 60-mL vials filled with 20 mL of media. The infection process occurred over two days wherein 20,000 pathogen spores were added daily (40,000 spores total). This process was applied to a new group of animals at each infection date. Animals were maintained under standard conditions as above.

Survival was checked for daily and upon death, *Daphnia* were frozen in 500 µL of water for subsequent assessment of infection success and production of mature transmission spores. Before spore counting, *Daphnia* were thawed, crushed, and checked for infection using phase-contrast microscopy to assess the presence or absence of spores at any stage of development. If infection was detected, the sample was counted using an Accuri C6 flow cytometer (BD Biosciences, San Jose, California). Custom gates based on fluorescence (FL3) and side scatter (SSA) were used to quantify mature transmission spores only, with fluorescence used to omit algae from the final counts, and side scatter used to isolate only mature spores based on their differences in morphology and size relative to animal debris and immature spores (*i.e.* Ebert et al. 2016). In one counting round 32 wells of a round-bottomed PPE 96-well plate were filled with 190 µL of 5mM EDTA and mixed with 10 µL of crushed *Daphnia* sample. Each sample was counted twice and averaged.

### *Immortal time bias and survival analysis*

All statistical analyses were performed using R (version 3.3.1; R Development Core Team, available at www.r-project.org). One of the challenges of analysing cohort based survival data is a phenomenon known as “immortal time bias” (Ho et al. 2012). Immortal time bias occurs when the

survival rate of an experimental treatment is inflated simply because those individuals must live long enough to receive treatment whereas control individuals experience mortality from the beginning of an experiment. To control for this bias, we paired exposed and infected animals with a matched control cohort (as per Lévesque et al. 2010). Age-matched control cohorts were created by filtering control survival data to animals which lived at least 14 days after each exposure age as this is the earliest point in which infection status can be accurately diagnosed.

Using the age-matched cohorts, we modelled the time-to-death of each individual using a Weibull hazard function as estimated via the *survival* package, and visualized these trends via Kaplan-Meier survival curves using the *ggfortify* package. Variation in mortality was estimated as a function of the main effects of exposure outcomes (exposed, infected or, control), host sex (male or female), and age of exposure (days 10, 20, 30 or 40), as well as their interactions, using a three-factor analysis of variance (Type III). To help explain any observed treatment differences, we estimated hazard ratios for infected or exposed individuals (relative to the age-matched control cohort baseline) for each combination of sex and age of exposure using the *SurvRegCensCov* package. Here, hazard rates were parameterized as odds of death at any given time due to exposure to or infection by a given pathogen.

### *Characterizing age-specific trends*

Complementing the analysis of mortality, we also analysed traits directly related to pathogen fitness and the virulence-transmission trade-off. Due to differences in the average lifespan between males and females (males: 33 days ± 1.9, females: 67 days ± 2.0; Thompson et al. 2017), we focused on three traits of common currency: the proportion of exposed animals that became infected; the reduction in lifespan as compared to the average of the age-matched control cohorts; and the production of transmission spores at host death (analyses included only infected individuals, see supplementary Table 1). Comparisons between the sexes and pathogen genotypes were analysed using a generalized linear model (binomial error distribution, logit link function) for infection rates and a least-squares linear model for both the reduction in lifespan and the production of spores. Before analysis, we square root transformed spore loads to meet the requirement of normality, although data are presented on the original scale to aid interpretation.

For each sex, we used a sequential model fitting approach (*e.g.* Hall et al. 2008) to describe the relationship between pathogen fitness traits and age of infection. This approach begins with a

model containing only pathogen genotype (C20 or C24) as a factor, with each of the following terms added sequentially: the linear terms for age of infection; the corresponding quadratic term; the interaction between the linear term and the pathogen factor; and, finally the interaction between the quadratic term and the pathogen factor. A partial F-test was used to assess whether the relationship between age of infection and each trait was linear or nonlinear (based on the significance of the linear and quadratic terms respectively), and if these trends differed between the pathogen genotypes (based on the interaction of this factor with either the linear or quadratic regression coefficient). We then visualized the most appropriate model and compared them between the two sexes using the most complex model possible and the "drop1" model simplification function in R.

## Results

### *Host mortality depends on both the sex and age of a host*

Our results point to a complex interaction between exposure outcomes (control versus exposed-and-uninfected versus exposed-and-infected), host sex, and the age of exposure on patterns of host mortality. Comparison of the survival curves for control, exposed but not infected (herein exposed), and infected animals (Fig. 1), showed that, as expected, the median lifespan of control males was shorter than control females for all ages of exposure (*e.g.* day 10 controls, male: 36.32 ± 0.99; female: 65.74 ± 1.48); and not surprisingly, that the duration of life remaining decreased as animals got older, irrespective of whether the host was exposed or not (*e.g.* day 40 controls, male: 11.66 ± 0.87; female: 35.74 ± 1.48). In addition to these obvious sex ($P < 0.001$) and age effects ($P < 0.001$), we found a significant interaction between host sex and age on host mortality, with marginal contributions from interactions between age and exposure outcome ($P = 0.068$) and a three-way interaction between all factors ($P = 0.075$, see supplementary Table 2 for ANOVA specifics).

Further exploration of the hazard rates for each treatment combination highlighted the driving forces behind these patterns of mortality (Table 1). Relative to the age-matched control cohort, we found that infections in females resulted in higher mortality rates, and that the odds of death at any given age were between 2.5- and 5-fold across all ages of exposure. Conversely, mortality was only increased in males infected at 10 days old, with a 2-fold increase in the likelihood of death (Fig. 1b). Infected male hazard rates at all other age classes were indistinguishable from

those of the control cohorts. For both sexes, individuals that were exposed to the pathogen but did not become infected showed no increase in hazard ratio (no hazards were greater than 1), except for one marginal case for females at age 40 (Fig. 1g).

*Host sex changes the relationship between virulence and pathogen fitness*

While hazard rates are informative in describing the odds of death at any given time due to exposure to or infection by a given pathogen, what is important from the pathogen perspective is the margin by which pathogen exposure reduces survival at each age class, and the relationships that this has with other aspects of pathogen fitness, namely infection success and spore loads. Irrespective of the sex of the host, we found that the relationships between age of infection and components of pathogen fitness were non-linear in the case of infection rates and virulence (reduction in host lifespan), but linear for the production of transmission spores (Table 2, Fig. 2). Sex did, however, affect the strength and sign of the above relationships, and whether or not they depended on the genotype of the infecting pathogen.

The probability of infection differed between pathogen genotypes in female hosts, but not in males (Table 2). For females, the interaction between pathogen genotype and the quadratic term arose because intermediate ages led to the highest success for pathogen C20 (negative quadratic: $Age^2$: -0.004 ± 0.002, P = 0.015), while C24 showed the opposite pattern, albeit weaker and non-significant (positive quadratic: $Age^2$: 0.002 ± 0.002, P = 0.399, Fig 2a). In contrast, for males, the lack of any significant interactions indicates that the relationship between age-of-infection and infection success was the same for both pathogens (Table 2), with infection success declining non-linearly with age (age: 0.186 ± 0.078, P = 0.017; $age^2$: -0.005 ± 0.002, P = 0.002, Fig 2b). Although this would suggest that the influence of host sex depends on the pathogen genotype and age of infection in combination, the three-way interaction between sex, pathogen and age-of-exposure was dropped from a model including both male and female data (see supplementary Table 3), suggesting the contrast between males and females may be marginal.

Similar patterns were observed for both virulence and spore loads, with the pathogen effect only detected for females (pathogen by age or $age^2$ interactions, Table 2). In females, the virulence of pathogen C20 declined in an accelerating manner (age: 0.884 ± 0.540, P = 0.108; $age^2$: -0.028 ± 0.01, P = 0.013), while for pathogen C24 it peaked at intermediate ages (age: 2.161 ± 1.019, P = 0.043; $age^2$: -0.041 ± 0.020, P = 0.055). This trend was reversed for spore loads (Fig. 2e), with C20

displaying no significant change with increasing age (age: 0.013± 0.033, P = 0.687), compared to the rapid decline in spores for C24 (age: -0.250± 0.062, P = <0.001). In males, no difference between the genotypes was detected (Table 2). Both virulence (age: -1.367 ± 0.503, P = 0.008; $age^2$: 0.024 ± 0.012, P = 0.046, Fig. 2d) and spore loads (age: -0.033 ± 0.013, P = 0.012, Fig. 2f) peaked in males exposed at day ten and then declined. Models including both male and female trends always retained a three-way interaction with either the linear or quadratic term, supporting the conclusion that the influence of host sex on pathogen fitness is specific to both the pathogen genotype and the age of infection (see supplementary Table 3).

## Discussion

The process of aging is expected to place considerable stress on the capacity of a host to fight infection (Adamo et al. 2001; Doums et al. 2002; Zerofsky et al. 2005). Arising from either the increasing costs of mounting an immune response (High 2004), or a decline in immune function with age (Katz et al. 2004; Plowden et al. 2004), we predicted that the costs of infection, as estimated by an increase in mortality rates, should increase with the age of infection and that these increasing costs would be felt most strongly by the less resistant sex, in this case females. In the end, though, our results were more complex. While hazard rates were indeed higher in females, consistent with the idea that they suffer the greatest fitness loss due to infection (Thompson et al. 2017), the trend with age was sex-specific; mortality rates were always higher in infected females across the four age classes, whereas mortality only increased in the earliest age class for males. Based on these findings, we suggest that the observed age-specific patterns may have more to do with the differences in the exploitation potential of males and females to a pathogen, than simply an ageing immune system.

When two hosts differ in the amount of resources they provide to a pathogen, theory predicts that pathogen reproduction will accelerate in the more exploitable host at the expense of decreasing host fitness (Hall et al. 2009c). Although previously applied only in the context of host differences in the acquisition of nutrients, the contrast between male and female hosts in our study system presents an analogous scenario. Female *Daphnia* are larger and longer lived than males, and have a significant pool of resources available to invest in producing clutches of offspring every three days (*e.g.* Clerc et al. 2015). In contrast, males represent a more difficult host to exploit, providing less physical space and fewer resources for pathogen growth (*e.g.* Thompson et al. 2017). Our results suggest that old age may further impact on the resources that each sex cedes to a

pathogen. As the remaining lifespan of females is greater than males at any given age (Fig. 1), the higher overall infection rate, virulence, and spore load observed in females (Fig. 2) may be a product of the increased time allowed for exploitation by the pathogen. Fewer resources to exploit and the short lifespan of males may simply not provide enough time and energy for the pathogen to either establish a successful infection or reach the intensity of infection that would facilitate a substantial increase in the rate of mortality (*c.f.* females).

In general, evolution favours pathogens which strike a balance between transmission and virulence (Alizon et al. 2009). However, when the difference between host environments is large, as we have shown between young and old individuals, or between males and females, then a range of possible virulence strategies may be maintained (Regoes et al. 2000; Gandon 2004; Osnas and Dobson 2012). Indeed, when infecting females, the two pathogen genotypes displayed a range of relationships between transmission (spore loads) and virulence (relative reduction in lifespan) at each age class (Fig. 2c), Pathogen C24 matched the results of a previous study whereby time to host death remained constant across exposure ages, while transmission declined with age (see Izhar and Ben-Ami 2015); C20 showed the reverse pattern with constant transmission across each age class at the expense of virulence. In contrast, both virulence and pathogen transmission were highest at earlier ages in males, irrespective of pathogen genotype. These findings highlight how complex interactions between host sex and the age at which a host encounters a pathogen can prevent a single pathogen strategy from maximizing fitness.

Ultimately it will be the frequency of encountering different sexes or host ages that will determine how much diversity in different strategies is maintained (see Gipson and Hall 2016). In the wild, we expect *Daphnia* populations to be predominately female biased, but males can still constitute up to half of the population for two to three months of the season (*e.g.* Galimov et al. 2011), and the increase in male production has been shown, at least in one case study, to occur prior to a disease outbreak (Duncan et al. 2006). Age-structure is less well known, but evidence suggests that the age classes, on the scale explored here, are likely relevant: *Daphnia* must survive to approximately two weeks to produce their first clutch (Ebert 2005); have been observed to overwinter in nature (Gliwicz et al. 2001; Slusarczyk 2009); and can live as long as 150 days in laboratory settings (Ebert et al. 2016). Under these conditions, female biased populations of mixed age classes will occur for much of the year and will be particularly labile for pathogen evolution, with changes in the rank order of pathogen genotypes occurring for transmission, virulence, and infection rates whenever a pathogen encounters animals of different ages. In contrast, males will present a simpler scenario

with infection rates and spore loads highest at early ages for all pathogen genotypes. Thus, deviations from an even sex-ratio and young cohort can lead to situations where one pathogen genotype is more consistently favoured (*i.e.* females, Fig. 2e) or mask the variation between pathogen genotypes (*i.e.* males, Fig. 2f).

Given the likelihood that a pathogen will encounter males and females of varying ages at some stage during a season, our results can also be interpreted in light of theory on the evolution of optimal virulence (Regoes et al. 2000; Gandon 2004; Osnas and Dobson 2012; Williams 2012; Cousineau and Alizon 2014). In considering variation in the level of resistance or the frequency of encountering each sex, Cousineau and Alizon (2014) found that the optimal level of virulence decreased when transmission frequently occurred within only one sex. This is because pathogen reproduction occurs mainly through the least resistant sex, where selection for increased virulence is weaker. As with broader theory on host heterogeneity, if between-type transmissions rates increase, then virulence levels are predicted to shift (Gandon 2004). Pathogens are now forced to overcome any maladaptation associated with a previously uncommon host, and if this leads to increased contact with a more resistance host type, then elevated levels of virulence will also ensue. In the *Daphnia–Pasteuria* system, therefore, we might predict that the more frequently the pathogen encounters male *Daphnia* the less likely it is to underexploit this host relative to females, and the greater chance that the more resistant males will facilitate the evolution of increased virulence in general.

In summary, we have shown how basic characteristics of natural populations, such as sex and age heterogeneity, can impact on patterns of host mortality and pathogen fitness. In females, age-specific infection gives rise to phenomena that fundamentally change the pace of infectious disease evolution, whereas in males disease outcomes are more dampened (see also Thompson et al. 2017). What happens outside the host, in terms of the within and between host-type transmission rates, will be key to understanding the evolution of virulence in this system. Quantifying natural variation in sex-ratios and age-structure over time will help to define how much *P. ramosa* may have experienced prior adaptation to younger female hosts. Yet, if host sex and age impact on pathogen fitness in other systems as they have here, regardless of whether males or females are the more resistant sex, we propose that an understanding of a population's sex ratio and age structure are crucial in predicting the severity and spread of disease.

## Acknowledgments

We thank V. Sundaramoorthi for assistance with laboratory work and F. Zajitschek and F. Camus for the helpful discussions on mortality analysis.

Data deposited at Dryad: https://doi.org/10.5061/dryad.bs387

## Chapter 3 tables and figures

Table 1: Summary of statistical analyses describing the differences in survival between control, exposed but not infected, and infected groups for each combination of sex and age of exposure. Asterisks denote significant effects (α = 0.05). Hazard ratios indicate the increased odds of death at any given age compared to the control group, with values equal to one indicating equal mortality rates between groups and values greater than one indicate an increase in mortality rates. Bold indicates values significantly different from one.

| Sex | Age of exposure | Deviance ($\chi^2$) | P-value | Hazard ratio | |
|---|---|---|---|---|---|
| | | | | Exposed [95% C.I.] | Infected [95% C.I.] |
| Male | Day 10 | 8.602 | 0.014 * | 1.027 [0.779, 1.354] | **2.191 [1.346, 3.566]** |
| | Day 20 | 1.970 | 0.374 | 0.894 [0.667, 1.198] | 1.246 [0.787, 1.972] |
| | Day 30 | 0.190 | 0.910 | 0.969 [0.676, 1.389] | 0.884 [0.503, 1.553] |
| | Day 40 | 0.315 | 0.854 | 0.992 [0.640, 1.537] | 1.527 [0.362, 6.435] |
| Female | Day 10 | 25.675 | <0.001 * | 0.981 [0.744, 1.294] | **4.032 [2.472, 6.577]** |
| | Day 20 | 42.328 | <0.001 * | 0.865 [0.651, 1.149] | **5.074 [3.204, 8.034]** |
| | Day 30 | 25.374 | <0.001 * | 0.925 [0.697, 1.228] | **4.121 [2.482, 6.843]** |
| | Day 40 | 18.794 | <0.001 * | **0.691 [0.516, 0.926]** | **2.570 [1.465, 4.507]** |

Table 2: Results of the sequential model fitting approach describing the effects of pathogen genotype and age of exposure on infection rate, virulence, and spore load. Each term was added sequentially beginning with a model containing the pathogen genotype (C20 or C24) as a factor, followed by the linear and quadratic terms for age of infection, and finally the interaction between these terms and the pathogen factor. Asterisks denote significant effects (α = 0.05).

| | Females only | | | Males only | | |
|---|---|---|---|---|---|---|
| | F or $\chi^2$ | df | P-value | F or $\chi^2$ | df | P-value |
| **Probability of infection** | | | | | | |
| Pathogen factor | 8.02 | 1 | 0.005* | 0.54 | 1 | 0.463 |
| Age | 1.97 | 1 | 0.160 | 14.07 | 1 | <0.001* |
| Age$^2$ | 1.93 | 1 | 0.164 | 10.25 | 1 | 0.001* |
| Pathogen: Age | 0.21 | 1 | 0.650 | 0.04 | 1 | 0.839 |
| Pathogen: Age$^2$ | 4.89 | 1 | 0.027* | 0.01 | 1 | 0.917 |
| **Virulence and the relative reduction in lifespan** | | | | | | |
| Pathogen factor | 0.24 | 1, 77 | 0.629 | 0.55 | 1, 55 | 0.460 |
| Age | 4.42 | 1, 77 | 0.039* | 13.32 | 1, 55 | <0.001* |
| Age$^2$ | 10.43 | 1, 77 | 0.002* | 4.21 | 1, 55 | 0.045* |
| Pathogen: Age | 11.28 | 1, 77 | 0.001* | 0.23 | 1, 55 | 0.636 |
| Pathogen: Age$^2$ | 0.40 | 1, 77 | 0.530 | 2.45 | 1, 55 | 0.123 |
| **Production of transmission spores** | | | | | | |
| Pathogen factor | 5.06 | 1, 79 | 0.027* | 0.25 | 1, 55 | 0.616 |
| Age | 10.98 | 1, 79 | 0.001* | 6.81 | 1, 55 | 0.012* |
| Age$^2$ | 0.04 | 1, 79 | 0.844 | 2.38 | 1, 55 | 0.129 |
| Pathogen: Age | 16.91 | 1, 79 | <0.001* | 0.63 | 1, 55 | 0.430 |
| Pathogen: Age$^2$ | 0.83 | 1, 79 | 0.364 | 1.34 | 1, 55 | 0.252 |

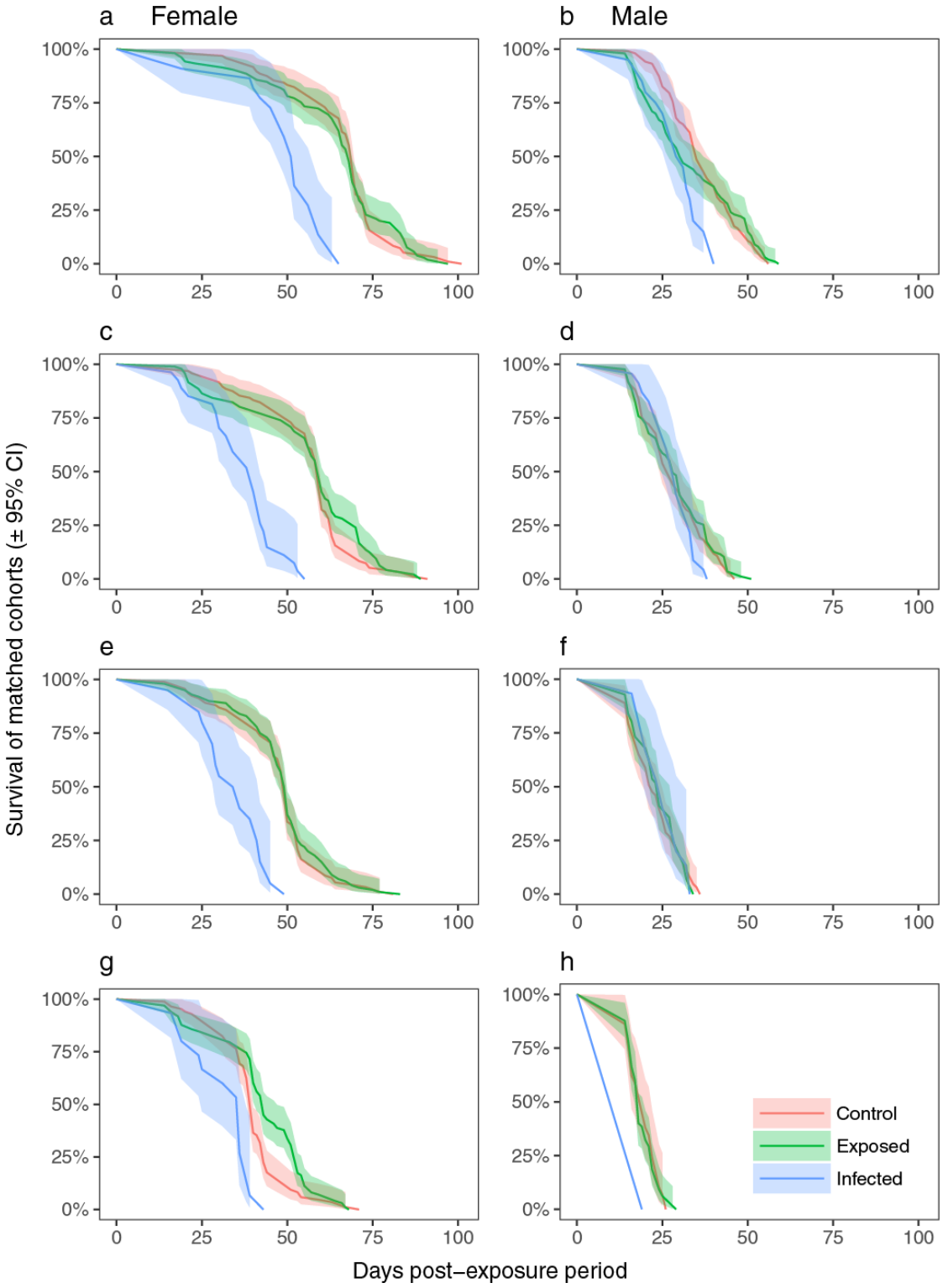


Figure 1: Kaplan-Meier survival curves describing the influence of sex and outcome of pathogen exposure on survival in female (left column) and male (right column) individuals following exposure at 10 (a, b), 20 (c, d), 30 (e, f), or 40 (g, h) days old. Red lines denote control individuals, green lines denote exposed individuals which did not become infected, and blue lines indicate infected individuals. Shading indicates 95% confidence intervals.

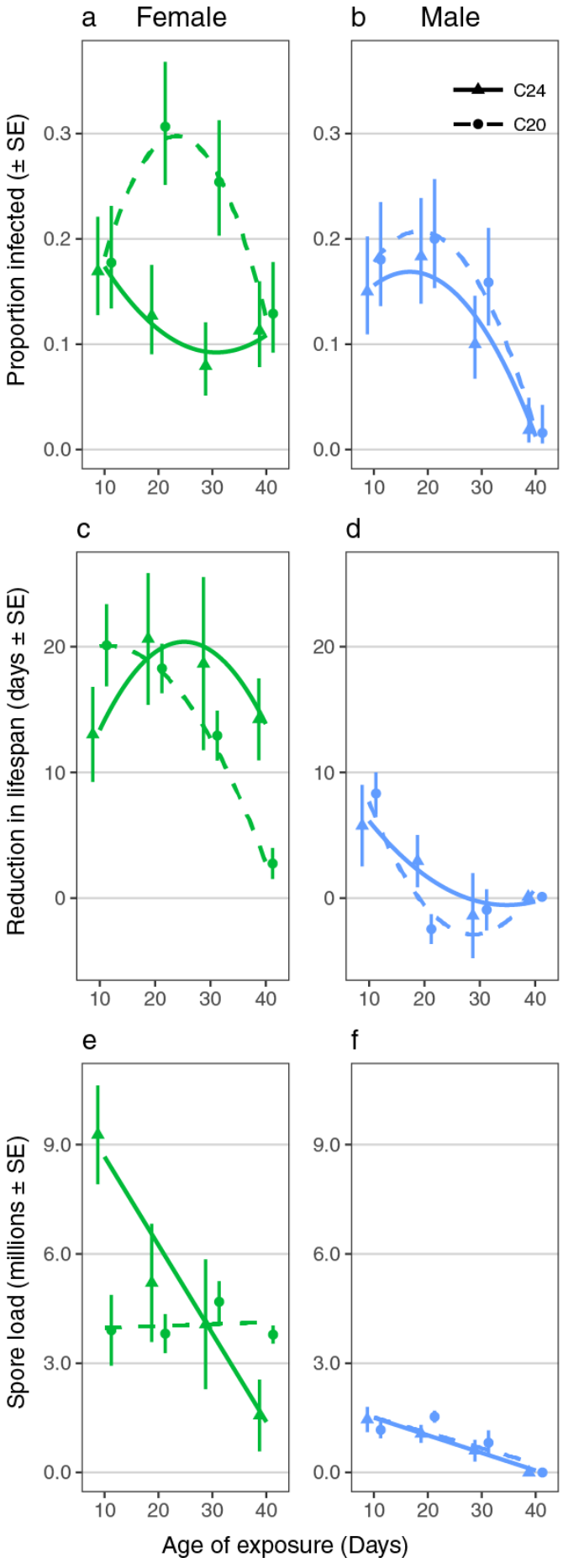


Figure 2: The Influence of host sex and age of pathogen exposure on infection rate (a, b), pathogen induced reduction in lifespan (c, d), and spore production (e, f). Shown are the treatment means, standard errors, and the trends over time as suggested by the best fitting linear models (see Table 2). Female results and male results are visualized in the left and right columns respectively. A solid line with triangles or a dashed line with circles represents the C24 or C20 pathogen genotype respectively.

## Chapter 3 supplementary material

Table S1: Sample sizes for analysis of infection rate, virulence (pathogen induced reduction of lifespan), and transmission (mature spore load).

| | Age of exposure (days) | Pathogen genotype | Initial *n* | Infected (*n*) | Virulence (*n*) | Transmission (*n*) |
|---|---|---|---|---|---|---|
| Male | 10 | C20 | 61 | 11 | 11 | 11 |
| | 20 | C20 | 60 | 12 | 12 | 12 |
| | 30 | C20 | 63 | 10 | 10 | 10 |
| | 40 | C20 | 63 | 1 | 1 | 1 |
| | 10 | C24 | 60 | 9 | 9 | 9 |
| | 20 | C24 | 60 | 11 | 11 | 11 |
| | 30 | C24 | 60 | 6 | 6 | 6 |
| | 40 | C24 | 54 | 1 | 1 | 1 |
| Female | 10 | C20 | 62 | 11 | 11 | 11 |
| | 20 | C20 | 62 | 19 | 19 | 19 |
| | 30 | C20 | 63 | 16 | 16 | 16 |
| | 40 | C20 | 62 | 8 | 8 | 8 |
| | 10 | C24 | 65 | 11 | 11 | 11 |
| | 20 | C24 | 63 | 8 | 8 | 8 |
| | 30 | C24 | 63 | 5 | 5 | 5 |
| | 40 | C24 | 62 | 7 | 7 | 7 |

Table S2: Summary of analysis of variance (Type III) describing the effects of host sex, host age at exposure to a pathogen, and exposure outcome on survival. Asterisks denote significant effects (α = 0.05), hashes denote marginally significant effects.

| | Deviance ($\chi^2$) | df | P-value |
|---|---|---|---|
| Sex of animal | 191.267 | 1 | < 0.001 * |
| Age of exposure | 107.674 | 2 | < 0.001 * |
| Exposure outcomes | 12.251 | 2 | 0.002 * |
| Sex x Age | 7.362 | 2 | 0.025 * |
| Sex x Exposure | 0.377 | 2 | 0.828 |
| Age x Exposure | 8.736 | 4 | 0.068 # |
| Sex x Age x Exposure | 8.501 | 4 | 0.075 # |

Table S3: Candidate regression models describing the effects of host sex (sex), pathogen genotype (par), and age of exposure (age) on infection rate, virulence, and spore load. The least complex model includes a linear relationship for each combination of sex and pathogen genotype and the most complex model estimates separate relationships.

| Trait | Model type | Model terms added | AIC | AIC Weight |
|---|---|---|---|---|
| Infection rate | Nonlinear | ~ sex + par + sex : par | 821.915 | 0.000 |
| | | ~ sex + par + sex : par + age + age$^2$ | 805.212 | 0.080 |
| | | ~ sex + par + sex : par + age + age$^2$ + sex : age + sex : age$^2$ | 801.689 | 0.450 |
| | | ~ sex + par + sex : par + age + age$^2$ + par : age + par : age$^2$ | 806.030 | 0.051 |
| | | ~ sex + par + sex : par + par : (age + age$^2$) + sex : (age + age$^2$) | 802.299 | 0.319 |
| | | ~ sex + par + sex : par + sex : par (age + age$^2$) | 804.544 | 0.099 |
| Virulence | Nonlinear | ~ sex + par + sex : par | 1047.187 | 0.000 |
| | | ~ sex + par + sex : par + age + age$^2$ | 1037.891 | 0.000 |
| | | ~ sex + par + sex : par + age + age$^2$ + sex : age + sex : age$^2$ | 1028.485 | 0.016 |
| | | ~ sex + par + sex : par + age + age$^2$+ par : age + par : age$^2$ | 1031.534 | 0.003 |
| | | ~ sex + par + sex : par + par : (age + age$^2$) + sex : (age + age$^2$) | 1021.242 | 0.420 |
| | | ~ sex + par + sex : par + sex : par (age + age$^2$) | 1019.862 | 0.561 |
| Spore load | Linear | ~ sex + par + sex : par | 713.969 | 0.000 |
| | | ~ sex + par + sex : par + age | 702.688 | 0.000 |
| | | ~ sex + par + sex : par + age + sex : age | 702.267 | 0.000 |
| | | ~ sex + par + sex : par + age + par : age | 685.670 | 0.052 |
| | | ~ sex + par + sex : par + age + par : age + sex : age | 685.191 | 0.059 |
| | | ~ sex + par + sex : par + age + par : age + sex : age + sex : par : age | 679.480 | 0.889 |

## Chapter 4 – Host sex modulates the condition-dependence of pathogen fitness

Stephen A.Y. Gipson[1, 2], Amanda K. Pettersen[1], and Matthew D. Hall[1]

1. School of Biological Sciences and Centre for Geometric Biology, Monash University, Melbourne, Victoria 3800, Australia

2. [redacted]

Keywords: sexual dimorphism; host-pathogen interactions; virulence; metabolic rate; *Daphnia magna*; *Pasteuria ramosa*

Manuscript under review in *Functional Ecology*

## Abstract

Sexual selection theory predicts that males and females will differentially invest resources into fitness-related traits such as immunity. These immune differences may underlie the frequently observed sexual dimorphism in disease outcomes. Yet, the sexes will not only vary in how resources are invested, but also in how they are acquired and processed. As hosts and pathogens are at conflict over a shared pool of resources, such sex-specific variation in acquisition or allocation may impact both the net amount of resources a pathogen can access and the exploitation strategy they use to convert these resources to transmission stages. In this study, we explore the role that resource dynamics play in varying infection outcomes between the sexes. To do this we raised male and female *Daphnia magna* on a high or low diet treatment and exposed them to the bacterial pathogen *Pasteuria ramosa.* We found that females are a greater resource pool for *P. ramosa* as represented by increased virulence, pathogen reproduction, and pathogen-induced change in metabolic rate as compared to males. We additionally found these traits to vary due to dietary treatment in females, but remain largely unchanged across diet in males. Finally we found that host sex and dietary treatment interact to influence optimal pathogen exploitation strategy. Collectively, these results provide evidence that variation in exploitable resources underlie considerable variation in disease outcome, but that sex-specific resource acquisition and use fundamentally changes the way in which pathogens extract resources from their hosts.

## Introduction

Sex differences in the prevalence, course, and severity of infection are universal. Across the animal kingdom, one sex is often described as the “sicker sex” with females typically more susceptible to infection in invertebrates, versus males in mammals and birds (Poulin 1996; Schalk and Forbes 1997; McCurdy et al. 1998; Sheridan et al. 2000; Zuk 2009; Cousineau and Alizon 2014). In explaining these common differences, life-history theory has focused on the ways that males and females differentially invest in immunity versus other components of fitness such as reproduction or growth (Rolff 2002; Zuk and Stoehr 2002; Stoehr and Kokko 2006; Zuk 2009). This host-centric perspective, however, captures only part of the complexity of how sex differences influence disease outcomes (Duneau and Ebert 2012; Gipson and Hall 2016). From the perspective of a pathogen, the sexes of a host may represent different resources that can be exploited to differing extents, an effect that potentially contributes to the frequently observed sexual dimorphism in the outcome of disease.

Competition over a shared pool of resources underlies much of the conflict that arises whenever hosts and pathogens interact. Upon pathogen exposure, hosts are expected to mount an immune response, which will sequester resources from the pathogen and reduce its growth (reviewed in Ong et al. 2006; Hall et al. 2017). In turn, essential functions like host growth or maintenance may be affected by pathogen appropriation of host resources (Hall et al. 2009c). Any differences in the way males or females acquire, store, or allocate nutrients (Stoehr and Kokko 2006; Boggs 2009; Schärer et al. 2012) will thus impact on the way conflict over a shared pool of resources is resolved. As a result, the next availability of resources available for exploitation by a pathogen (herein host condition, *sensu* Rowe and Houle 1996), and the way in which pathogen exploitation relates to host damage (De Roode et al. 2008; Seppälä et al. 2008; Hall et al. 2009c), will both depend on the sex of a host. Yet so far pathogen exploitation strategies have not been considered in light of fundamental differences in the "quality" of each sex as a potential host (but see Thompson et al. 2017; Gipson and Hall 2018).

In exploring how host condition affects the interplay between host harm and pathogen reproduction, Hall *et al.* (Hall et al. 2009c) modelled the effects of a host's nutritional environment on multiple aspects of the infection process. They found that with increasing host condition, in terms of the net availability of resources for the pathogen to exploit, disease progressed more rapidly and allowed for greater pathogen reproduction at the expense of increased virulence. In contrast, hosts of lower condition were constrained in both growth and food intake, limiting the resources of which a pathogen can exploit and thus pathogen reproduction. Applying the insight of this model to sex differences and pathogen exploitation would suggest that the sex that provides a greater pool of resources to the pathogen, or is more able to translate nutrients in the environment into accessible resources, will most likely suffer an accelerated progression of infectious disease. Our goal for this study was to uncover if the relationships between host harm and pathogen exploitation progress similarly between males and females, or if these relationships are differentially modulated by changes in the availability of resources a given sex has access to in their environment.

One way to explore how host sex and condition interact to influence disease outcome is to manipulate resource availability and characterize how changes in the availability and use of energy translates to variation in disease symptoms. Indeed, much work has shown that when resource availability is modulated, many components of host and pathogen fitness vary, presumably due to changes in either immune investment (Brown et al. 2000; Seppälä et al. 2008) or the pool of

resources which a pathogen can exploit (Ebert et al. 2000, 2004; Bedhomme et al. 2004; De Roode et al. 2008). Yet, sex-specific patterns of resource acquisition or allocation may further complicate the relationships between a host's use of resources and infectious disease. Obtaining measures of how infected hosts use, transform, and expend energy is key to linking resource availability to pathogen exploitation strategies. Metabolic rates provide such a bridge (Kearney and White 2012; Arnold et al. 2013). Indeed, previous work has found that males and females vary in the way metabolic rates respond to infection or immune activation (Evans et al. 2009) and that these differences are mediated through sex-specific physiology or energy use (Bashir-Tanoli and Tinsley 2014). Infection thus represents a repartitioning of host resources (Ots et al. 2001; Gray and Bradley 2006; Arnold et al. 2013), which may be particularly susceptible to variation due to sex-specific patterns of resource allocation and acquisition.

In this study, we utilize the *Daphnia magna* - *Pasteuria ramosa* model system to explore how differences in the way each sex acquires and expends energy can help explain the diversity of disease outcomes seen in sexually dimorphic species (Sheridan et al. 2000; see also Table 1, Cousineau and Alizon 2014). Previous studies have shown that female *Daphnia* are more susceptible to infection, allow for greater pathogen reproduction, and live longer post-infection than males, all of which suggest that females are the more exploitable sex for a pathogen (Duneau et al. 2012; Thompson et al. 2017; Gipson and Hall 2018). Additionally, studies focusing on female *Daphnia* have found that energy availability and use is likely to play an important role in these differences. For example, dietary manipulations have shown that host condition influences the relationship between pathogen reproduction and host fitness or mortality as well as pathogen infection rate (Hall et al. 2009c; Vale et al. 2011; Hall and Ebert 2012). Furthermore, *P. ramosa* has been shown to increase *Daphnia* metabolic rate upon infection, affirming a potential energetic cost of infection in this system (Hall et al., in prep). *Daphnia magna* is thus an excellent model to explore how sexual dimorphism and its relationship to pathogen exploitation of resources may underlie some of the diversity in disease outcome we observe between males and females in general.

Here, we investigate how host sex and condition interact to influence pathogen exploitative potential, defined here as the ability of pathogens to utilise host resources for their own reproduction. To do this we designed a cross-infection experiment using genetically identical male and female *Daphnia* raised on different levels of food availability. We infected male and female individuals from each diet treatment with one of two pathogen genotypes, and measured the

effect of each treatment on characteristics which collectively influence pathogen fitness: spore production, changes in host lifespan, and changes in host body size. We then related patterns of pathogen fitness within each sex to changes in host mass-corrected metabolic rate upon infection. With this design, we explored how food acquisition differentially affects the outcome of disease within male and female hosts, and how differences in the exploitative potential of males and females underlie sexual dimorphism in disease outcome.

## Methods

*Daphnia magna* Straus is a globally distributed freshwater crustacean which produces genetically identical male and female offspring via cyclic parthenogenesis (Ebert 2005). *Daphnia* filter feed on planktonic algae which brings them in contact with their natural pathogen *Pasteuria ramosa* Metchnikoff (Smirnov 2014; Ebert et al. 2016). *P. ramosa* is a bacterial, spore forming pathogen which decreases the lifespan and fecundity of infected hosts (Ebert et al. 2016). *P. ramosa* transmission occurs exclusively horizontally via the production of spores that, once taken up by *Daphnia* hosts, reproduce and fill the body cavity before being released upon host death (Ebert et al. 2016). This experiment utilized male and female *Daphnia* from genotype HU-HO-2 and novel *P. ramosa* genotypes C20 or C24 which have been previously shown to vary in pathogen induced reduction of lifespan and the production of transmission spores (Clerc et al. 2015; Thompson et al. 2017).

Prior to the experiment, all animals were maintained under standardized conditions for three generations in order to minimize any potential maternal effects. Juvenile female *Daphnia* were collected from stock cultures and raised individually in 60-mL vials filled with 50 mL of artificial *Daphnia* medium (ADaM) (Klüttgen et al. 1994; modified by Ebert et al. 1998). Thereafter, *Daphnia* were transferred into fresh media twice weekly and maintained at 20°C and exposed to a 16-hour light to 8-hour dark cycle. Animals were fed daily with green algae (*Scenedesmus* sp.), beginning with 0.5 million cells per animal per day at birth and increasing gradually to 5 million cells per animal per day to meet the growing energy demands of the animals.

### *Production of experimental animals*

Genetically identical male and female *Daphnia* were produced by exposing the standardized females to a short pulse of the hormone methyl farnesoate (300 µg/L, Product ID: S-0153, Echelon Biosciences, Salt Lake City, Utah). Following previously established protocols (Thompson et al.

2017), animals were moved into artificial media treatment upon the release of their first clutch. The third clutch produced post-exposure to the hormone was collected and returned to normal media. The sex of each *Daphnia* was determined by presence or absence of a modified appendage which is unique to male *Daphnia* (Ebert 2005). This method of producing male *Daphnia* has been shown to have no detectable impact on either the lifespan and fecundity of control animals, nor the virulence or spore production exhibited by infected individuals (see Thompson et al. 2017).

### *Infection experiment design and measures of pathogen fitness*

Male and female *Daphnia* were randomly exposed to either pathogen C20 or C24 and assigned to either a high or low diet treatment of 4 million or 1 million algal cells daily as part of a factorial design. Similar dietary treatments have been previously used in this system, whereby females raised on a diet of 1 million spores exhibit reduced size and delayed production of their first clutch as well as lower overall host fecundity and pathogen spore production (Ben-Ami et al. 2010; Hall and Ebert 2012). Each treatment combination consisted of 160 individuals with an additional 50 males and 50 females assigned as uninfected controls (2 sex x 2 pathogen genotypes x 2 diets x 160 replicates + [2 sex x 50 control replicates] = 1380 individuals). *Daphnia* were infected over the course of two days at four and five days old. Infections took place in 60-mL vials filled with 20 mL of ADaM and 20,000 spores were added daily (40,000 spores total). On day six, animals were transferred to fresh jars filled with 50 mL of ADaM and maintained under standardized conditions as outlined above.

Survival of *Daphnia* was checked daily and upon death, individuals were frozen in 500 µL of purified water for later determination of infection status and measurement of transmission spores. All *Daphnia* were thawed, crushed with a pestle, and sampled individually for infection status and spore count using an Accuri C6 flow cytometer (BD Biosciences, San Jose, California). Each counting round consisted of 32 wells of a round-bottomed PPE 96-well plate filled with 190 µL of 5mM EDTA and 10 µL of a single crushed *Daphnia.* Custom gates based on fluorescence and side scatter were used to measure the number of mature spores produced. Each *Daphnia* was measured twice and spore counts were recorded as the average of the two measurements. Infection status was additionally assessed, based on the presence or absence of mature spores using phase-contrast microscopy.

*Measurements of host metabolic rate*

Oxygen consumption rates (V̇O2) were used as a proxy for metabolic rate. Measurements were conducted using a fluorescence-based respirometry system on *Daphnia* at three weeks post exposure to *P. ramosa*. This age was selected as it captures a phase of intense within-host competition between host and pathogen for resources (Clerc et al. 2015; Ebert et al. 2016). *Daphnia* were first rinsed in ADaM and loaded individually into 4-mL vials with non-consumptive oxygen sensor spots adhered to the bottom. Prior to measurement, oxygen sensor spots were calibrated using air-saturated ADaM (100% air saturation) and ADaM containing 2% sodium sulfite (0% air saturation). *Daphnia* media was autoclaved and returned to 20$^{o}$C prior to use to remove any bacteria which may impact on oxygen consumption measurements. For each air-tight vial, oxygen consumption rates were measured using multiple 24-channel readers (SDR SensorDish Reader, PreSens Precision Sensing GmbH, Germany), run in parallel (8 x 24-channel readers at a time) (White et al. 2011). Each run took place in a dark, temperature-controlled room at 20$^{o}$C for 1.5 hours with oxygen consumption measurements taken once every minute. The experimental period began at 40 minutes post initiation of the measurement software as measurements before this time can be unreliable as conditions have yet equilibrate after the loading process.

For each 24-channel reader, we randomly allocated 20 animals and 4 blank controls containing only sterile artificial media. *Daphnia* from each sex, diet, and pathogen genotype combination were evenly distributed among each oxygen reader. For each run, $\dot{V}O_2$ was calculated from the change in oxygen saturation over time (% $h^{-1}$) as per White et al. (2011) as $\dot{V}O_2 = -[(m_a - m_c)/100] \times V \times \beta O_2$; where $m_a$ is the rate of change of oxygen saturation for an experimental animal, $m_c$ is the per run average rate of change for the controls, $\beta O_2$ is the oxygen capacitance of air-saturated water at 21$^{o}$C (6.40, Cameron 1986), and V is the water volume of the vials (0.004 L). The parameters $m_a$ and $m_c$ were estimated using the *LoLinR* package in R (Olito et al. 2017), which uses local linear regressions to objectively estimate the monotonic rates from oxygen consumption data. $\dot{V}O_2$ estimates (in mL $O_2$ $h^{-1}$) were then converted to metabolic rate (milliJoules $h^{-1}$) using the calorific conversion factor of 20.08 J $mL^{-1}$ $O_2$ (Lighton 2008). Immediately following oxygen consumption measurements, the body size of each individual was measured using a dissecting microscope and converted to dry weight (mg) as per Yashchenko et al. (2016).

*Statistical analyses*

Of the 1380 animals which initiated the study, not all were available for the metabolic assay at three weeks post exposure to *P. ramosa* or were removed from the study due to sampling errors. In total, we were able to measure 574 out of the 928 initially measured animals at three-week post exposure (87 male and 79 female controls, 118 males and 123 females exposed to C20, and finally 67 males and 100 females exposed to C24) split across the low and high diet treatment (292 and 282 individuals respectively).

All statistical analyses were performed in R (version 3.4.1; R Development Team, available at www.r-project.org). We focused on four traits that are directly comparable between infected males and females: the change in (i) lifespan, (ii) body size, and (ii) mass-corrected metabolic rate following infection; and, (iv) the production of mature transmission spores at host death. Following Hall *et al.* (Hall et al., in prep), mass-corrected metabolic rates (hereafter "metabolic rate") were derived from the residuals of a second order polynomial regression of raw metabolic rates on $\log_{10}$ mass. Next, the relative changes in lifespan, body size, and metabolic rate were calculated as the difference between an infected individual's trait values and the average of the sex-specific control individuals. Traits were analysed using a full-factorial analysis of variance (white corrected ANOVA Type III, *car* package, Fox and Weisberg 2011) with host sex (male or female), pathogen genotype (C20 or C24), diet (low or high), and their interactions as fixed effects. We also fit separate ANOVAs for each sex for the purpose of determining which sex was driving patterns of significance in the full models.

Finally, based on the predictions of Hall *et al.* (2009c), we explored how changes in how an infected animal uses, transforms, and expends energy (*i.e.* its metabolic rate) combined with the virulence of disease (*i.e.* the reduction in lifespan) to predict the fitness of a pathogen. To do so we used response surface analysis, as implemented using general additive models (GAMs, *mgcv*, Wood 2017), to test whether these relationships varied by sex or diet. Response surfaces are a common approach in evolutionary studies, whereby a second-order polynomial regression or GAM is used to characterize the relationship between multiple traits and an estimate of fitness (Hall et al. 2008, 2009a; Polak et al. 2017). With this approach, we first scaled our fitness measure, spore production, to a relative fitness measure by dividing by the mean within each treatment. We then

standardized all other traits to a mean of zero and standard deviation of one. For all GAMs, we analysed the standardized change in virulence and relative metabolic rate, as well as their interaction as nonlinear smoothing splines, with pathogen genotype a fixed factor (to control mean difference between pathogens) and relative spore numbers as the predictor variable. Differences in the response surfaces between each sex and diet was assessed using likelihood ratio tests.

## Results

### *The influence of food availability and host sex on disease characteristics*

In all measured traits we found that interactions between diet treatment and sex or pathogen genotype defined variation in disease outcome. Both spore load (pathogen fitness) and mass-corrected metabolic rates were influenced by an interaction between diet and the sex of the host, whereas pathogen-induced reduction in lifespan (virulence) and relative body size was determined by a three-way interaction between host sex, pathogen genotype, and diet (Table 1). Overall the results were characterized by the greater response of females to the high diet treatment. High diet in females resulted in a threefold reduction in lifespan, a doubling in both metabolic rates and spore loads, and a decrease in body size. The responses were concordant for both pathogen genotype, except for body size where the magnitude of change was pathogen-specific. In contrast, for males the pathogen C24 produced more spores and C20 exhibited higher levels of virulence, but the effect of diet was limited to a reduction in lifespan, with high diets leading to lower virulence. There was no effect of diet nor pathogen genotype in the cases of body size and metabolic rates in males.

### *The sex-specific relationship between pathogen exploitation and pathogen fitness*

Having established that interactions between diet and sex impact on patterns of infection, we then explored how these characteristics influence how virulence and changes in metabolic rate interact to predict pathogen transmission. Using generalized additive models, we found that the best fitting response surface was one that allowed for an interaction between host sex and diet treatment (Table 2). We then tested whether the response surfaces varied: 1) between diets within a given sex; and, 2) between sexes within a given diet. Within a given sex, the surfaces were not significantly different between the high and low diet treatment for males (d.f. = 3.509, $\chi^2$ = 0.697, $P$ = 0.167), but were significantly different for females (d.f. = 6.260, $\chi^2$ = 2.565, $P$ = 0.034).

Within a given diet treatment, the exploitation surfaces were not significantly different between the sexes within the high diet (d.f. = 3.693, $\chi^2$ = 0.188, $P$ = 0.804), but were significantly different within the low diet (d.f. = 2.807, $\chi^2$ = 3.153, $P$ < 0.001). Collectively, these results suggest that pathogens in males and females on a high diet alone share a common exploitation strategy, but females on a low diet represent a fundamental shift in how energy use and virulence are translated into pathogen fitness.

To visualize these exploitation surfaces we estimated a single response surface for males from both diets with pathogen genotype and diet as additional fixed effects, and separate response surfaces for females raised on the low or high diet with pathogen genotype as a fixed effect. The parameter estimates for these models are shown in Table 3. For both males and females on the high diet, we found that both the standardized reduction in lifespan and the standardized increase in mass-corrected metabolic rates were important for predicting pathogen fitness either independently or in combination. Relative pathogen fitness in each context was lowest when the reduction in lifespan was the most extreme and mass corrected metabolic rates following infection increased by the least amount (Fig. 2a and 2b). In contrast, for females on the low diet the standardized virulence and the standardized increase in mass-corrected metabolic rate acted independently to influence the production of pathogen transmission spores. The response surface was described by a ridge of high spore production with relative pathogen fitness peaking at mid to low standardized values for the reduction in lifespan and increased with increasing standardized relative mass-corrected metabolic rates from there (Fig. 2c).

## Discussion

A host's nutritional status can have far reaching effects on the outcome of disease (Wolinska and King 2009), as well as the relationship between host and pathogen fitness characteristics (Vale et al. 2011). When hosts vary in condition, and thus the amount of exploitable resources, the optimal level of pathogen exploitation will change, impacting on both the level of host harm and pathogen reproduction (Hall et al. 2009c). The sexes are also predicted to vary in their energy use due to differences in physiology, behaviour or immune investment (Rolff 2002; Zuk and Stoehr 2002; Zuk 2009; Duneau and Ebert 2012), yet how energy intake and expenditure influence the net amount of exploitable resources and the outcome of disease for each sex has yet to be fully addressed. In this study, we explored how an individual's sex can modify the relationship between condition, the flow of energy from environment to pathogen, and the capacity of pathogens to exploit their host.

We found that both sex and diet interact to influence the severity of disease in infected animals as well as how the relationship between virulence and energy flux translate into pathogen performance for each sex. We discuss how sex differences in the way energy is acquired, used, and allocated may help to explain the diversity of disease outcomes that are observed in species with two-separate sexes. We further discuss the relevance of these results in natural contexts where simple variation in the energetic demands of males and females, and thus variation in the impact of relative resource availability, may drive the observed patterns.

*Resource availability on sex-specific patterns of pathogen fitness and evolution*

Variation in environmental conditions such as resource availability can affect multiple aspects of host-pathogen interactions (Wolinska and King 2009; Vale et al. 2011). By influencing an individual's acquisition of resources, such environmental variation can impact on immune investment or resources within a host, changing the patterns of exploitation by pathogens (Brown et al. 2000; Ebert et al. 2000, 2004; Bedhomme et al. 2004; De Roode et al. 2008; Seppälä et al. 2008). Here, we considered how such condition-dependent disease outcomes may be influenced the diverging patterns of resource acquisition or allocation between the sexes (Stoehr and Kokko 2006; Boggs 2009; Schärer et al. 2012). We found that females represent a much larger exploitative environment for pathogens than males as indicated by increased levels of spore production, virulence and metabolic rate (Table 1, Fig. 1). Females also more readily allowed for resources in the environment to be translated to pathogen exploitation as represented by increased pathogen spore production and virulence when raised on a high diet. These patterns were qualitatively consistent with the model predictions of Hall *et al.* (Hall et al. 2009c), where increasing resources of the host should relate to greater pathogen reproduction and virulence. Conversely, pathogens infecting males expressed constant levels of spore production and more subtle changes in virulence across diets.

These sex-specific patterns of diet treatment on disease outcome may have a variety of evolutionary implications. By increasing the costs or likelihood of becoming infected, changes in the environment may accelerate cycles of coevolution between host and pathogen (Lively 1999; Blanford et al. 2003; Vale et al. 2011). In this study, shifts from the low to high diet are associated subtle changes in virulence within males, yet strong differences in virulence within females (Fig. 1b). Resource fluctuations in natural environments may then result in evolutionary "hot" or "cold spots" (Thompson 1994; Gomulkiewicz et al. 2000) where the speed or intensity of host-pathogen

coevolution will depend on an interaction between resource acquisition and host sex. Furthermore, these patterns of virulence are associated with strong variation in pathogen fitness; with two- to four-fold differences in spore production between males and females in low or high diet respectively (Fig. 1a). Consequently, diet influences the difference in pathogen fitness between male and female hosts, which may increase the likelihood of sex-specific pathogen adaptation within high resource conditions (Duneau and Ebert 2012).

*Sex-specific patterns resource use favour different pathogen exploitation strategies*

Sex differences in immunity may underlie much of the observed variation in disease outcome between the sexes, either by changing patterns of susceptibility (Zuk 2009) or varying the amount of resources sequestered from pathogens as part of immune defence (reviewed in Ong et al. 2006; Hall et al. 2017). Yet the sexes will also vary in their relative resource allocation into fitness related traits (Rowe and Houle 1996; Stoehr and Kokko 2006; Boggs 2009; Schärer et al. 2012), their allocation strategies based on patterns of resource acquisition (Houslay et al. 2015), and ultimately the proportion of resources invested at different levels of condition (Zajitschek and Connallon 2017). Collectively this suggests that changes in nutritional environment may fundamentally affect how resources are used between males and females and, as a consequence, the optimal pathogen strategy to exploit host resources. Patterns of resource use may thus underlie variation in disease between males and females in tandem with sex-specific patterns of immunity.

Our results show that the sexes vary in their sensitivity to the availability of resources in the environment. This is represented, as discussed above, by sex-specific effects of diet treatment on pathogen induced changes metabolic rate or virulence (Fig. 1). Irrespective of these shifts in mean trait values, however, are differences between the sexes in the relationships between the properties of an infection that determine pathogen fitness. In males from either dietary treatment or females raised specifically on a high diet, we found that increases in virulence and decreases in metabolic rate lead to low pathogen reproduction (Fig. 2a, b). Yet, this pattern changed within females raised on a low diet where these traits acted independently, favouring an increase in pathogen reproduction with increasing metabolic rate, but only at intermediate values for the reduction in host lifespan (Fig. 2c). Collectively, these results suggest that what best predicts pathogen fitness will change depending on patterns of resource acquisition within females, yet remain robust to this variation within males. In this way, a host's resource environment can affect

the optimal pathogen exploitation strategy and may thus be an additional factor driving sex-specific outcomes of infection.

### *The consequences of sex differences in exploitation for disease spread in the wild*

In nature, organisms may experience variation in their ability to acquire resources from the environment, either due to seasonal resource fluctuations (Sommer et al. 1986; Reynolds et al. 2017) or environmental feedbacks between resource consumption and availability (McCauley et al. 1999; Schenk et al. 2002). In this study, we find that changes in resource acquisition can have far-reaching impacts on disease due to sex differences in how resources translate into pathogen fitness. Specifically, we found that the flow of resources from the environment to the pathogen population occurs disproportionately through female hosts. Fluctuating food availability coupled with naturally varying sex ratios (Clutton-Brock and Iason 1986; Duneau and Ebert 2012 and Table 2 therein) will likely drive patterns of disease where varying resource acquisition may have little impact on disease prevalence in male-biased populations, but strongly influence both pathogen reproduction and virulence in female-biased populations.

These dynamics may be important for the spread of disease in natural populations. For example, Hall *et al.* (Hall et al. 2009b) found that how host resources are made available for exploitation by pathogens may also be important in the timing of disease epidemics: hosts providing few exploitable resources will delay the start of epidemics, whereas increased host resources may decrease patterns of host susceptibility and thus inhibit epidemics. If difference between the sexes hold true in other species, then one sex may have strong impacts on the timing of epidemics in natural populations whereas the other may be less influential in these disease dynamics even as environmental conditions fluctuate. A logical next step would thus be empirical studies which directly examine the effect of host food availability on pathogen resources (*e.g.* iron, reviewed in Ong et al. 2006) within each sex or long-term mesocosm studies measuring pathogen transmission under a variety of resource availabilities and sex ratios.

Partially underlying the sex differences observed in this study may be variation in the resource requirements of male and female *Daphnia.* Female *Daphnia* are the larger sex (Duneau et al. 2012; Thompson et al. 2017) and exhibit substantial resource investment into producing a clutch of offspring every three days post reproductive maturity (Ebert 2005). As a result, our diet treatment may simply not have stressed male *Daphnia* to the extent that it stressed females. Nevertheless,

we have shown that identical changes in resource availability can have strong differences on pathogen exploitative potential when encountering male or female hosts. In this way, host sex can influence patterns of exploitation by pathogens simply if one sex is more impacted by fluctuations in resource availability. Yet males and females frequently exhibit variation in physiology, behaviour, and resource investment (Schärer et al. 2012); suggesting that resource acquisition may often differentially affect the sexes as well as the optimal strategy for exploitation by pathogens.

*Conclusion*

In this study, we found that male *Daphnia* are a poorer environment for pathogen exploitation but conclude that intrinsic differences between the sexes alone are insufficient to explain why disease outcome so often varies between males and females (Sheridan et al. 2000; see also Table 1, Cousineau and Alizon 2014). Instead, we find that sex-specific differences in the use of resources fundamentally change the way pathogens exploit their hosts. In females, dietary treatment impacts on the optimal pathogen exploitation strategy whereas males are robust to these changes, favouring a single pathogen exploitation strategy. Yet, males and females may vary in their resource requirements due patterns of sexual dimorphism. Further, the relative abundance of resources may influence an individual's metabolic rate (Bohrer and Lampert 1988). Nevertheless, we have shown how fluctuations in resource availability that may be expected in natural populations can dramatically influence sex-specific patterns of pathogen fitness. If the specific sex and environmental context influence pathogen exploitation strategy as described here, the pervasive differences in acquisition and allocation strategy between the sexes (Rowe and Houle 1996; Stoehr and Kokko 2006; Boggs 2009) may underlie much of the sex-specific patterns of disease outcome observed in nature.

**Acknowledgements**

We would like to thank Lindsey Heffernan for extensive help in experiment planning, animal care, and metabolic rate measurements. We would additionally like to thank Isobel Booksmythe, Tobias Hector, Jared Lush, Martino Malerba, and Louise Solveig Nørgaard for additional help and advice.

## Chapter 4 tables and figures

Table 1: Summary of analyses of variance (white corrected, Type III) describing the effects of host sex, pathogen genotype, and diet treatment on pathogen production of transmission spores, change in host lifespan, change in host body size, and change in host mass-corrected metabolic rate as compared to control. Asterisks denote significant effects ($\alpha = 0.05$).

| | *F* | d.f. | *p*-value |
|---|---|---|---|
| **Production of transmission spores** | | | |
| Sex | 333.630 | 1,400 | <0.001 * |
| Pathogen genotype | 4.396 | 1,400 | 0.037 * |
| Diet | 63.105 | 1,400 | <0.001 * |
| Sex : Pathogen | 0.113 | 1,400 | 0.737 |
| Sex : Diet | 50.113 | 1,400 | <0.001 * |
| Pathogen : Diet | 0.001 | 1,400 | 0.969 |
| Sex : Pathogen : Diet | 0.607 | 1,400 | 0.436 |
| **Change in host lifespan (days)** | | | |
| Sex | 56.218 | 1,393 | <0.001 * |
| Pathogen genotype | 42.187 | 1,393 | <0.001 * |
| Diet | 107.606 | 1,393 | <0.001 * |
| Sex : Pathogen | 7.186 | 1,393 | 0.008 * |
| Sex : Diet | 263.828 | 1,393 | <0.001 * |
| Pathogen : Diet | 0.694 | 1,393 | 0.405 |
| Sex : Pathogen : Diet | 5.420 | 1,393 | 0.020 * |
| **Change in host body size (mm)** | | | |
| Sex | 201.990 | 1,400 | <0.001 * |
| Pathogen genotype | 30.195 | 1,400 | <0.001 * |
| Diet | 47.429 | 1,400 | <0.001 * |
| Sex : Pathogen | 13.763 | 1,400 | <0.001 * |
| Sex : Diet | 30.096 | 1,400 | <0.001 * |
| Pathogen : Diet | 10.676 | 1,400 | 0.001 * |
| Sex : Pathogen : Diet | 9.141 | 1,400 | 0.003 * |
| **Change in host metabolic rate (mJ/h)** | | | |
| Sex | 66.405 | 1,400 | <0.001 * |
| Pathogen genotype | 0.612 | 1,400 | 0.434 |
| Diet | 20.244 | 1,400 | <0.001 * |
| Sex : Pathogen | 0.185 | 1,400 | 0.668 |
| Sex : Diet | 37.582 | 1,400 | <0.001 * |
| Pathogen : Diet | 0.052 | 1,400 | 0.819 |
| Sex : Pathogen : Diet | 0.440 | 1,400 | 0.508 |

Table 2: Candidate generalized additive models (GAMs) describing the effects of host sex and diet treatment on the response surface of pathogen fitness represented by relative changes in host mass-corrected metabolic rate and the reduction in lifespan. The least complex model estimates no sex- or diet-specific response surface and the most complex model estimates separate response surfaces for each treatment combination. Models were ranked by AIC and the best-fitting model selected by AIC weight.

| **Candidate response surfaces** | **Number of model terms** | **AIC** | **ΔAIC** | **AIC weight** |
|---|---|---|---|---|
| 1. No response surface | 0 | 515.703 | 100.700 | 0.000 |
| 2. Single response surface for all treatments | 3 | 425.665 | 10.662 | 0.005 |
| 3. Response surface varies by sex only | 6 | 420.634 | 5.631 | 0.056 |
| 4. Response surface varies by diet only | 6 | 424.932 | 9.929 | 0.007 |
| 5. Response surface varies by sex and diet | 12 | 415.003 | - | **0.933** |

Table 3: Summary of generalized additive models (GAMs) describing the effect of virulence, mass-corrected host metabolic rate (millijoules), and their interaction on pathogen spore production. Asterisks denote significant effects and hashes denote marginally significant effects (α = 0.05).

| | | | | | |
|---|---|---|---|---|---|
| Male, Combined diet | **Parametric** | **Estimate** | **S.E.** | **T value** | **P value** |
| | Pathogen | 0.122 | 0.048 | 2.565 | **0.011 *** |
| | Diet | 0.024 | 0.038 | 0.625 | 0.533 |
| | **Smooth** | **e.d.f** | **Ref. d.f.** | **F value** | **P value** |
| | Virulence | 1.000 | 1.000 | 5.249 | **0.023 *** |
| | Millijoules | 1.000 | 1.000 | 2.863 | 0.092 # |
| | Vir. X mJ | 3.237 | 27.000 | 0.171 | 0.166 |
| Female, High diet | **Parametric** | **Estimate** | **S.E.** | **T value** | **P value** |
| | Pathogen | -0.016 | 0.053 | -0.297 | 0.767 |
| | **Smooth** | **e.d.f** | **Ref. d.f.** | **F value** | **P value** |
| | Virulence | 1.000 | 1.000 | 1.418 | 0.237 |
| | Millijoules | 1.000 | 1.000 | 4.811 | **0.031 *** |
| | Vir. X mJ | 3.201 | 27.000 | 0.218 | 0.067 # |
| Female, Low diet | **Parametric** | **Estimate** | **S.E.** | **T value** | **P value** |
| | Pathogen | 0.072 | 0.065 | 1.102 | 0.273 |
| | **Smooth** | **e.d.f** | **Ref. d.f.** | **F value** | **P value** |
| | Virulence | 4.324 | 5.340 | 10.645 | **< 0.001 *** |
| | Millijoules | 1.000 | 1.000 | 7.648 | **0.007 *** |
| | Vir. X mJ | <0.001 | 27.000 | 0.000 | 0.999 |

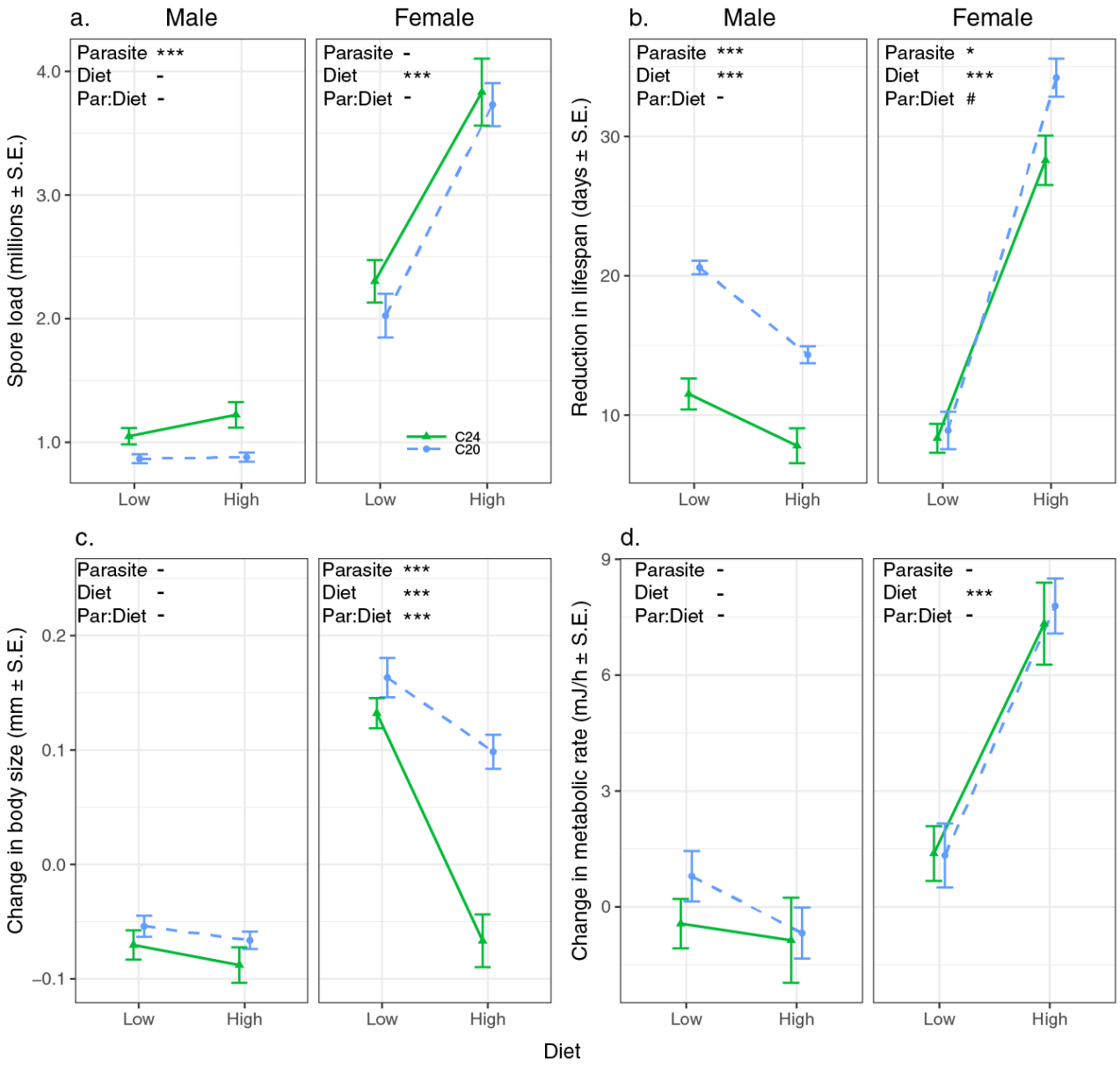


Figure 1: The influence of host sex and diet treatment on pathogen spore production (a), change in host lifespan (b), change in host body size (c), and change in host metabolic rate. Shown are treatment means and standard errors for each diet treatment. Male and female results are presented in the left and right columns respectively. A solid line with triangles or a dashed line with circles represents pathogen C24 or C20 respectively. Asterisks indicate significant effects (α = 0.05, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; # $p < 0.10$).

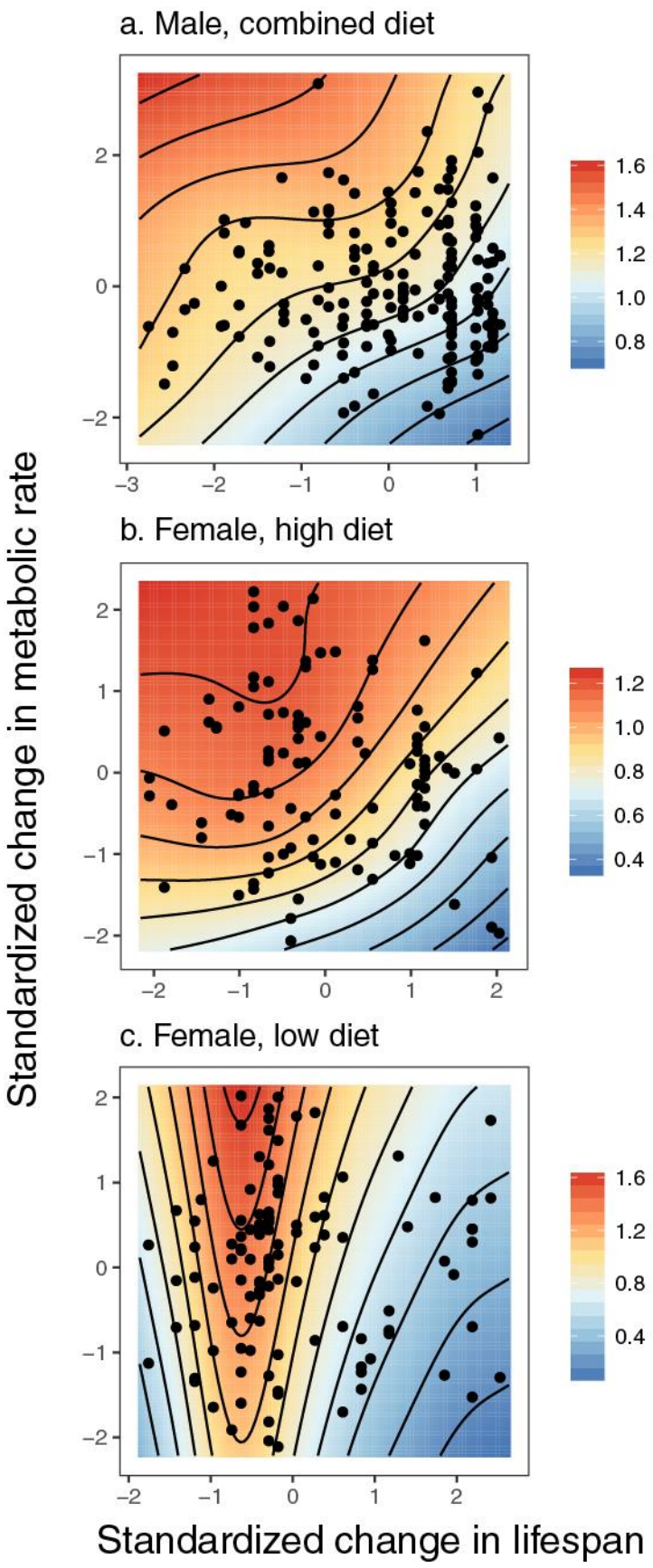


Figure 2: Response surfaces represented by relative changes in host mass-corrected metabolic rate and the reduction in lifespan which predict pathogen spore production for infected males (a), females raised on high diet (b), and females raised on low diet (c). Spore production is mean centred whereas metabolic rate and change in lifespan are in units of standard deviation from mean values of their respective axes. Infected individuals exhibited lifespans ranging from those similar to controls, to those much shorter than controls. Red or blue coloration represents high or low spore production respectively.

## Chapter 5 – Sexual dimorphism in disease affects the outcome of within-host pathogen competition

Stephen A.Y. Gipson[1, 2] and Matthew D. Hall[1]

1. School of Biological Sciences and Centre for Geometric Biology, Monash University, Melbourne, Victoria 3800, Australia

2. [redacted]

Keywords: sexual dimorphism; co-infection; pathogen genetic variation; virulence evolution; *Daphnia magna*; *Pasteuria ramosa*

Manuscript under review in *Evolution*

## Abstract

Natural infections often consist of multiple pathogens of the same or different species. In multiple infections pathogens compete for access to host resources and fitness is determined by how well a pathogen can reproduce compared to its competitors. Here, we consider how the relative fitness between competing pathogens is affected by sexual dimorphism in the outcome of disease, such as the varying patterns of host harm or reproduction within male and female hosts. Using the *Daphnia magna* - *Pasteuria ramosa* model system, we exposed male and female hosts to either a single genotype infection or co-infections consisting of two pathogen genotypes of varying levels of virulence, measured as pathogen-induced reduction in host lifespan. We found that the relationship between pathogen fitness and virulence was similar between males and females, yet the scale of this relationship was much greater in females. This resulted in strong variation in fitness between co-infecting pathogen genotypes in females where the most virulent genotype frequently outcompeted the less virulent genotype. Conversely, co-infecting pathogens expressed similar levels of fitness within males or favoured the transmission of less virulent pathogens in some sequential co-infection contexts. These results suggest that infection in males may maintain pathogen genetic variation as no one genotype is always most fit, whereas infection in females may favour the transmission of more virulent pathogen genotypes, potentially impacting on pathogen evolution. Collectively, these results suggest that host sex may be a powerful selective force on pathogens in natural populations.

## Introduction

Due to the ubiquity of pathogens in natural populations, individuals are usually infected with more than one type of pathogen or multiple strains of a single pathogen (Read and Taylor 2001; Rigaud et al. 2010; Balmer and Tanner 2011). Such multiple infections have been observed in humans which may carry as many as five strains of the malaria causing parasite *Plasmodium falciparum* (Babiker et al. 1999; Lord et al. 1999). In multiple infections, pathogen types or strains compete for access to host resources where the most fit competitor is determined by the relative difference in host exploitation between competitors, often explored through impacts on host fecundity or lifespan (Alizon et al. 2013; Bashey 2015). It is in this relationship between host exploitation and pathogen fitness that the predictions for multiple infections vary from those of single infections. While prudent pathogen exploitation leads to the highest pathogen fitness in single infection contexts represented by a trade-off between pathogen reproduction and host harm (Bull 1994;

Frank 1996; Alizon et al. 2009), a pathogen's ability to acquire more resources than competing strains largely dictates relative pathogen fitness in multiple infections. Yet, host populations are rarely homogeneous and host heterogeneity in characteristics such as age at exposure, nutritional background, or genotype have been previously shown to influence patterns of infection-induced mortality, and ultimately the relative fitness of co-infecting pathogens (Hodgson et al. 2004; Izhar et al. 2015; Louhi et al. 2015). As such, it is important to explore how additional sources of variation between hosts may influence patterns of within-host pathogen competition as well as their evolutionary consequences.

In this study, we consider the role of host sex in affecting the competitive ability of pathogens and thus outcome of multiple infection. Due to differences in life-history strategies, physiology, or behaviour (Parker 2006; Schärer et al. 2012) the sexes represent varying exploitative environments from the perspective of a pathogen (Duneau et al. 2012; Gipson and Hall 2018), yet how these sex differences impact on the outcome of multiple infections has received limited consideration (but see Thompson et al. 2017). In general, female fitness is related to longevity, resulting in the prediction that females should invest relatively more resources into immunity than should males (Rolff 2002; Zuk 2009). Yet the sicker sex can vary from species to species (Poulin 1996) and males may invest more in immunity relative to females in certain environmental contexts or patterns of sexual selection (Sheridan et al. 2000; Stoehr and Kokko 2006). Consequently, the sexes exhibit variation in pathogen-induced decreases in fecundity or lifespan (see Table 1, Cousineau and Alizon 2014; Giefing-Kröll et al. 2015; Klein and Flanagan 2016), the same virulence characteristics which determine pathogen fitness within multiple infection contexts (Alizon et al. 2013; Bashey 2015). If males and females vary in the effects of exploitation by pathogens then the sex encountered by pathogens may either accentuate or mask pathogen variation in exploitative potential, influencing the competitive outcome of within-host pathogen competition.

Competition between multiple pathogen genotypes can take a number of forms including, among others: 1) *Exploitation*- where pathogens compete for a limited pool of resources; 2) *Apparent competition*- where stimulation of the immune response by one pathogen indirectly impacts on competitors; or 3) *Interference competition*- where pathogen reproduction directly limits exploitation by other strains either through prior establishment or competition over physical space (Mideo 2009; Balmer and Tanner 2011; Alizon et al. 2013; Bashey 2015; Cressler et al. 2016). These different forms of competition may have a variety of effects on pathogen virulence.

Competition for resources, for example, may increase the level of virulence due to the "tragedy of the commons" where pathogen genotypes over exploit their hosts (Chao et al. 2000; de Roode et al. 2005); yet competing pathogens may interfere with one another, leading to lower levels of exploitation and thus virulence in these competition contexts (Massey et al. 2004; Eswarappa et al. 2012). Inhibition may also extend to infections where one genotype establishes prior to the other; often favouring the earlier establishing pathogen regardless of their virulence characteristics (de Roode et al. 2005; Eswarappa et al. 2012). Yet predictions for how co-infections should impact on virulence are often complicated or conflicting, due either to the variety of strategies by which pathogens can extract resources from their hosts (reviewed in Alizon et al. 2013) or how host genotypes interact with co-infecting pathogen genotypes to produce a variety of co-infection outcomes (GxG interactions, see Louhi et al. 2015).

Within the varying forms of within-host pathogen competition exist opportunities, many unexplored, for host sex to modulate the way pathogens exploit their host and thus influence the outcome of co-infection. The previously discussed effects of host sex on fecundity and lifespan, for example, may affect the relative competitive ability of pathogens by impacting on patterns of pathogen virulence. Additionally, physiological differences between the sexes may cause one sex to have fewer resources or less physical space for a pathogen to exploit (Duneau and Ebert 2012; Thompson et al. 2017; Gipson and Hall 2018), potentially intensifying the competition between pathogens over host resources, or limiting the potential for pathogens to establish in previously infected hosts. The effect of prior pathogen establishment may further be influenced by sex differences in patterns of immunity where sex differences in the immune response to infection may impact on the likelihood of subsequent pathogen establishment. Given the propensity for males and females to vary in the characteristics which may affect within-host pathogen competition, the sex encountered by a pathogen may create substantial variation in the outcome of multiple infections, factoring into the variety of coinfection outcomes which ultimately determine pathogen fitness (Alizon et al. 2013).

In this study, we use the *Daphnia magna- Pasteuria ramosa* model system to assess the impact that host sex has on the outcome of multiple infection. In this system, male *Daphnia* are smaller, exhibit shorter lifespans, are more resistant to infection, experience lower rates of infection induced mortality, and allow for lower production of pathogen spores; collectively suggesting that they are a more difficult resource to exploit than females (Duneau et al. 2012; Thompson et al. 2017; Gipson and Hall 2018). In nature, *Daphnia* may be infected with as many as eight genotypes

of *P. ramosa* (Mouton et al. 2007) and have consequently been used to study the effects of multiple infection on both the outcome of competition between pathogens as well as the evolution of virulence (Ben-Ami et al. 2008; Andras and Ebert 2013; Ben-Ami and Routtu 2013; Izhar et al. 2015). Co-infections in this system often favour the reproduction of more virulent genotypes, are as virulent as the most virulent genotype in isolation, and will only favour the reproduction of less virulent genotypes when they establish first (Ben-Ami et al. 2008; Ben-Ami and Routtu 2013). Yet co-infections play out differently between males and females, where females exhibit pathogen virulence and reproduction intermediate to that of the competing pathogens in isolation, whereas the outcome within males is more variable (Thompson et al. 2017). Remaining to be explored though is how these sex-specific patterns of co-infection may impact on the relative fitness between pathogen genotypes and the implications this may have for the maintenance of genetic variation.

Using pathogen genotypes of known virulence characteristics (Clerc et al. 2015), we exposed genetically identical male and female *Daphnia* to either a single pathogen genotype or to a co-infection consisting of two pathogen genotypes of varying levels of virulence. We then varied the schedule of these exposures, either exposing the host a single time, or allowing infection to establish prior to a subsequent exposure. Upon host death, we measured pathogen-induced reduction in lifespan (virulence), overall pathogen spore production (transmission), as well as the relative spore production of competing pathogen genotypes using microsatellite analysis. Due to the relationship between virulence and pathogen competitive ability, as well as the often observed sex-specific patterns of disease outcome, we make the following predictions: 1) the less exploitable sex will suppress variation of pathogen genotypes in virulence, resulting in similar reproductive output between competing pathogen genotypes; 2) the more exploitable sex will allow for pathogens to exhibit variation in virulence, causing the more virulent pathogen to reproductively outcompete the less virulent genotype; and consequently 3) the patterns of sequential co-infections in the more exploitable sex will closely match those of theory and empirical studies (de Roode et al. 2005; Ben-Ami et al. 2008; Eswarappa et al. 2012), whereas the less exploitable sex will allow for more equal reproduction of each genotype regardless of the relative virulence of the previously established genotype. We discuss the implications of these patterns for the maintenance of genetic variation in pathogens and the evolution of virulence.

## Methods

*Daphnia magna* Straus is a freshwater crustacean found throughout the globe which produces genetically identical male and female offspring via cyclic parthenogenesis (Ebert 2005). During filter feeding, *Daphnia* encounter the bacterial pathogen *Pasteuria ramosa* which reduces the lifespan and fecundity of its host (reviewed in Ebert et al. 2016). *P. ramosa* is an obligate killing pathogen, transmitting exclusively horizontally after inducing host death and spores being released from the decaying cadaver. This experiment utilized host genotype HU-HO-2 and novel *P. ramosa* genotypes C19, C24, and C1. Prior to the experiment, we established a parental generation by isolating juvenile female *Daphnia* from pre-existing stock cultures and maintaining them in standardized conditions for three generations to minimize maternal effects. Juvenile female *Daphnia* were raised individually in 60-mL vials filled with 50 mL of artificial *Daphnia* medium (ADaM, Klüttgen et al. 1994; Ebert et al. 1998) and were transferred into fresh ADaM twice weekly. These females were maintained at 20°C, exposed to a 16-hour light to 8-hour dark cycle, and fed up to 5 million cells of algae daily.

### *Production of experimental animals*

Once the third-generation standardized females released their first clutch, they were exposed to a short pulse of the hormone methyl farnesoate (300 µg/L, Product ID: S-0153, Echelon Biosciences, Salt Lake City, Utah) to stimulate the production of genetically identical male and female offspring. Following previously established methods (Thompson et al. 2017), the standardized females were transferred into 60-mL vials filled with 20 mL of hormone treated ADaM and were transferred into fresh hormone treated ADaM three times weekly. Male and female offspring were collected from the second and third clutches post hormone exposure. This treatment has previously been shown to have no detectible impact on host lifespan and fecundity, nor pathogen transmission and virulence (Thompson et al. 2017).

### *Infection design*

To measure the effect of host sex on the outcome of within-host pathogen competition, as well as how this competition proceeds when one genotype has already established, we randomly exposed males and females to either single infections or co-infections (infections consisting of two pathogen genotypes). These exposures were carried out in one of two exposure "schedules": 1) *simultaneous*- exposure occurred once when the individual was five days old or 2) *sequential*-

exposure occurred at 5 and 12 days old (see Table 1 for infection design details). All exposures consisted of a 40,000 pathogen spores of either a single pathogen genotype (in the case of single infections) or evenly split among two pathogen genotypes (in the case of co-infections). Consistent spore doses among exposed individuals were ensured by using a Neubauer haemocytometer to count the total number of spores in a homogenized sample for each pathogen genotype before preparing dilutions. In sequential co-infections, the host was exposed to 20,000 spores of one pathogen genotype a week prior to 20,000 spores of the second genotype to allow for prior establishment of infection. Replicate treatments were created so that each possible order of pathogen genotype exposures were represented. In sequential single infections, the host was simply exposed to 20,000 spores at each of the exposure periods by the same genotype. We herein refer to the multiplicity of genotypes and schedule of exposures collectively as "co-infection treatment."

Three *P. ramosa* genotypes with previously studied disease characteristics were used in this study: C19, C24, and C1. When singly infecting female *Daphnia,* genotype C19 exhibits high virulence and low transmission (average infection duration: 45.29 days; average spore load: 8.56 million spores) whereas genotypes C24 and C1 cause similar infection outcomes, exhibiting lower average virulence (higher durations of infection) and higher average transmission as compared to C19 (Clerc et al. 2015). Co-infections consisted of genotype C19 and either C24 or C1 as these genotype pairings represent similar virulence combinations and were thus predicted to exhibit similar patterns of competitive outcome. Additionally, genotypes C24 and C1 cannot be distinguished using our genetic analyses whereas C19 can be distinguished from C24 or C1, allowing us to determine the relative contribution of each pathogen genotype to the total spore production of co-infected *Daphnia* (see Genetic analysis section). 33 individuals from each sex were allocated to each co-infection treatment with an additional 33 individuals of each sex allocated as uninfected controls. In total this experiment consisted of 26 treatments (33 replicates * 2 sex * [3 simultaneous single infections + 3 sequential single infections + 2 simultaneous co-infections (C19 with C24 or C1) + 2 sequential co-infections treated with C19 first + 2 sequential co-infections treated with C19 second + 1 uninfected control treatment] = 858 individuals).

### *Measures of disease characteristics*

Survival was checked daily for the assessment of lifespan of control and infected individuals. We calculated the pathogen-induced reduction in lifespan by subtracting the average lifespans of

control male or female treatments from the lifespan measurement of infected male or female individuals respectively. Upon host death, *Daphnia* were individually frozen in 500 µL of purified water for later determination of infection status and pathogen fitness as measured by overall production of transmission spores. Infection status was assessed by thawing a *Daphnia* sample, crushing it with a pestle, and noting the presence or absence of mature transmission spores using phase-contrast microscopy. Individuals identified as infected were immediately assessed for overall spore production using an Accuri C6 flow cytometer (BD Biosciences, San Jose, California). The spore load of each infected *Daphnia* was counted twice by diluting 10 µL of *Daphnia* sample into 190 µL of 5mM EDTA and loading this dilution into one well of a round-bottomed PPE 96-well plate. Custom gates based on fluorescence (FL3) were used to omit algae cells from the final count whereas custom gates based on side scatter (SSA) were used to identify only mature spores based on their distinct size and morphology compared to immature spores and animal debris (Ebert et al. 2016). The final overall spore load was calculated from the average of the two flow cytometer counts.

### *Genetic analysis and measures of within-host pathogen competition*

To assess the fitness of co-infecting pathogen genotypes, we performed DNA extractions on infected *Daphnia* from co-infection treatments and determined the relative contribution of each pathogen genotype using variable number tandem repeats (VNTRs, (Mouton et al. 2007)). Specifically, pathogen genotypes were distinguished using primer sequences Pr1, Pr2, and Pr3 (Table 2, Mouton et al. 2007) and have been previously used to distinguish *P. ramosa* isolate P1 from isolates P4 and P5 (Ben-Ami and Routtu 2013) from which *P. ramosa* clones C19, or C24 and C1 are respectively derived (Luijckx 2012). As clones from isolates P4 and P5 cannot be distinguished using this method, co-infections always consisted of pathogen genotype C19 and C24 or C1.

DNA extractions were performed using the EZNA Tissue DNA kit (Omega Bio-tek, Norcross, Georgia) with a modified protocol based on similar studies assessing the genetic composition of *P. ramosa* infections (Ben-Ami et al. 2008; Andras and Ebert 2013; Ben-Ami and Routtu 2013; Izhar et al. 2015). Immediately after spore counting, the crushed *Daphnia* samples were pelleted via centrifugation for 3 minutes at 12,205 RCF, supernatant removed, and washed with 1 mL of double-distilled water. The samples were again centrifuged using the same settings, supernatant removed, and suspended in 200 µL lysis buffer and 25 µL OB protease. The samples were then

homogenized via bead beating with approximately 0.25 g of 0.1 mm zirconia beads for 2 minutes (1 x 10s, 1 x 20s, and 3 x 30s). Subsequently, samples were incubated in a heat block at 55°C for 1 hour before centrifugation at 10°C for 15 minutes at 5005 RCF. After collecting the supernatant, the DNA extraction proceeded as directed by the manufacturer protocol. An optional step of incubating samples for 2 minutes at 70 °C prior to elution greatly increased DNA yields. Final elution volume was 100 µL. DNA was amplified via PCR with temperature cycling methods identical to Andras and Ebert (2013). Fragment analysis and genotyping was performed on these PRC products by AGRF (Melbourne, Australia) to determine the size of microsatellite alleles and the strength of their fluorescence (represented by peak height). The peak height ratio of the microsatellite markers were interpreted as the relative proportion of spores produced by each pathogen genotype as described by Ben-Ami et al. (2008). This proportion was multiplied by the absolute number of spores produced within the infected host to determine the relative transmission of competing genotypes.

*Statistical analyses*

Only 823 of the 858 individuals initially set up for this experiment were used in final analyses as some individuals either died before infection status can be adequately determined (14 days post exposure, Clerc et al. 2015) or were removed due to experimental error. These 823 individuals consisted of 65 uninfected control individuals, 129 exposed but uninfected individuals, 319 individuals infected from single infection treatments, and 310 individuals infected from co-infection treatments of which *P. ramosa* DNA was extracted from 290 individuals. All statistical analyses were performed in R (version 3.4.1; R Development Team, available at www.r-project.org). Studies of multiple infection commonly explore how the most fit pathogen genotype is related to reduction in host fecundity or lifespan upon infection (reviewed in Alizon et al. 2013; Bashey 2015). Here, we focused on pathogen induced reduction in lifespan as a comparable measure of pathogen virulence between males and females. We then related patterns of virulence to overall pathogen transmission (total production of pathogen spores) as well as the transmission of individual co-infecting pathogen genotypes.

We first analysed the effects of sex, pathogen co-infection treatment, and their interaction on pathogen spore production and virulence using full-factorial analyses of variance (white corrected ANOVA Type III, *car* package, Fox and Weisberg 2011). Then we explored how the relationship between pathogen virulence and overall pathogen fitness changes due to host sex, pathogen co-

infection treatment and their interaction using a multivariate analysis of variance (MANOVA Type III, *car* package). Using the *eemeans* package (https://github.com/rvlenth/emmeans) to perform post-hoc comparisons of the multivariate means for equivalence, we then explored how the relationship between virulence and overall spore production varied due to host sex or either of the two pathogen genotype combinations (C19 and C24 or C19 and C1). Finally, we tested whether the fitness of individual co-infecting pathogen genotypes changed due to host sex, co-infection treatment, or their interaction. To do this we fit linear mixed models for each co-infection treatment (*lme4* package, Bates et al. 2015) with an individual's unique ID fit as a random intercept, an interaction between sex and pathogen genotype as fixed effects, and relative production of spores as the response. We also fit separate ANOVAs for each co-infection treatment in order to describe which co-infection treatment was driving patterns of significance in the full model.

## Results

### *The effects of host sex on pathogen virulence, transmission, and their interaction*

Our results indicate interactive effects of host sex and patterns of co-infection on the combined influence of pathogen fitness (the total spore production of co-infections or single genotype infections) and pathogen induced reduction in host lifespan (virulence). Across all single and co-infection treatments, the multivariate ANOVA reveals how infections in females lead to higher spore loads and a greater reduction in lifespan than in males (Fig. 1); but that the magnitude of any response to infection is not shared equally between the sexes due to the presence of the interaction term (Table 2). Figure 1 and Table 2 (univariate ANOVA) suggest that this interaction is largely driven by greater variation in spore loads between co-infection treatments occurring in females (Fig 1a), with pathogens producing between 9.6 million and 5.9 million average spores within females and between 1.9 million and 1.1 million average spores within males. In contrast, females experienced higher levels of virulence than males, but the relative differences in virulence among co-infection treatments was similar between the sexes (Fig. 1b). Collectively this suggests that while pathogens which delay host death produce the greatest amount of spores in females, this trade-off is more dampened in males where variation in virulence relates to a much smaller range in pathogen reproduction (Fig. 1).

Despite the difference in the scale of the trade-off between males and females, within each sex the relationship between pathogen transmission and virulence across each co-infection treatment remained qualitatively similar. As expected, in females we found pathogen genotype C19 was more virulent and produced less transmission spores than C24 (Fig. 2a) or C1 (Fig. 2b) in single infection contexts. Similarly, sequential single genotype infections were indistinguishable from simultaneous single infections. Simultaneous co-infections always behaved like the more virulent pathogen in single infection contexts (C19), yet the order of sequential co-infections influenced the relationship between virulence and transmission differently based on the genotype of the competitor. In competitions with between C19 and C1, sequential co-infections always behaved like the genotype that established first (*i.e.* sequential C19, C1 behaved like single infections with C19, whereas sequential C1, C19 behaved like single infections with C1; Fig. 2b). In contrast, the relationship between virulence and transmission of sequential co-infections were indistinguishable in competitions between C19 and C24 (Fig. 2a). Males exhibited comparatively similar patterns to females, with C19 more virulent and producing fewer spores than C24 (Fig. 3a) or C1 (Fig. 3b) in single infections; sequential single infections exhibiting similar patterns to simultaneous single infections, sequential co-infections with C24 exhibiting similar patterns regardless of infection order, and sequential co-infections with C1 behaving like the genotype which established first.

### *The effects of host sex on the relative fitness among co-infecting pathogen genotypes*

Finally, we explored how host sex and pathogen genotype collectively influence the relative pathogen fitness within co-infection contexts. We found that an interaction between host sex and pathogen genotype determined relative pathogen fitness (Table 3). Upon examining each pathogen co-infection treatment individually, we found that an interaction between sex and pathogen genotype influenced relative pathogen fitness in every co-infection treatment except for sequential C24, C19 (Table 3). In females, the more virulent genotype (C19) produced the majority of transmission spores in each co-infection context except for when pathogen genotype C24 established first (Fig. 4). Conversely, co-infection in males resulted in equal spore production of the competing genotypes except for when the less virulent genotypes (C24 or C1) established before C19. In both of these cases, prior establishment of the less virulent genotype resulted in C19 being competitively inferior.

## Discussion

Much of our understanding on how pathogens should exploit hosts and how exploitation strategies should evolve are influenced by studies focusing on single infections. Yet in nature, infection is more likely to co-occur between multiple pathogen genotypes of the same or different species (Read and Taylor 2001; Rigaud et al. 2010; Balmer and Tanner 2011). When multiple pathogens establish within a host, theory predicts that co-infection commonly favours more virulent pathogens through increased competition for host resources (Alizon et al. 2013). However, many hosts within a given population will vary in their ability to acquire and store energy (*i.e.* condition, Rowe and Houle 1996; Hunt et al. 2004), thus the evolution of more virulent pathogens is not necessarily a universal outcome of co-infection (Alizon et al. 2013; Cressler et al. 2016). In this study we considered how the expression of virulence and the maintenance of pathogen genetic variation within co-infections are influenced by a common source of host heterogeneity: the difference between the sexes in their capacity limit pathogen performance (see Table 1, Cousineau and Alizon 2014).

Our results indicate that the ability of co-infections to modify the relationship between pathogen growth (*i.e.* spore production) and pathogen induced reduction in host lifespan (*i.e.* virulence) will depend on the sex of the host. While infection in females is marked by a negative relationship between spore production and virulence, similar virulence patterns in male hosts correspond with more limited variation in spore production (Fig. 1). This was also observed in Thompson *et al.* (2017) where the scale of the trade-off between virulence and spore production varied due to host sex, driven largely by a reduction in overall variation in pathogen fitness across co-infection treatments in males. Despite this lower variation in spore production within male hosts, we found that the relationship between pathogen virulence and spore production was qualitatively similar between the sexes. Regardless of sex, simultaneous co-infections often exhibited relationships between pathogen virulence and spore production equal to that of the most virulent pathogen in isolation (Fig. 2, 3). Likewise, the relationship between virulence and spore production in sequential co-infections depended on pathogen genotype and order of establishment, but was unaffected by sex. These patterns culminate with females representing an environment within which pathogens can attain the highest levels of reproduction; yet co-infections within females also represent a substantial decrease in pathogen spore production as compared to males.

Despite commonalities between the sexes in the overall patterns of transmission and virulence, the relative fitness between co-infecting pathogen genotypes varied strongly between males and females. We found that females exhibited significant differences in spore production between competing genotypes in all but one co-infection treatment, with the more virulent pathogen (C19) producing up to 5.3 million spores more than its competitor (Simultaneous C19, C24; Fig. 4). In contrast, when pathogens competed within males, each genotype produced an equal number of transmission spores in simultaneous exposures and sequential exposures when the more virulent pathogen established first (Fig. 4). Explaining these results may be that the weaker exploitative environment of male *Daphnia* (*i.e.,* the amount of host resources that pathogens may utilize for their own reproduction, Thompson et al. 2017; Gipson and Hall 2018) prohibits pathogens from exhibiting variation in exploitation strategies. Males then may be a reservoir for pathogen genetic diversity, resulting in equal fitness between competing genotypes and even favouring genotypes which are frequently outcompeted within female hosts.

Our results also indicate that the arrival sequence of co-infecting pathogens will lead to different competitive outcomes depending on the host sex encountered. Previously established pathogens may inhibit later arriving competitors by blocking pathogen establishment, exhausting resources, or by inducing host immune responses (de Roode et al. 2005; Lohr et al. 2010; Hoverman et al. 2013). In these situations, early establishing pathogens may exhibit considerable levels of reproduction even in competition with more virulent genotypes. Indeed, Ben-Ami *et al.* (2008) found that less virulent genotypes can exhibit substantial levels of reproduction when establishing first even though they are outcompeted when establishing after more virulent genotypes. Here, females exhibited this general pattern with the more virulent C19 genotype outcompeted by C24 and producing a similar number of spores as C1 when establishing second (Fig. 4). Conversely, the constraints imposed by male hosts appear to keep pathogen C19 from ever outcompeting other genotypes when they establish first. Consequently, male hosts may thus maintain pathogen genetic variation that would otherwise be eroded by infection in females where the more virulent genotype is more frequently transmitted.

Taken together, our results suggest that the evolutionary outcome of pathogen virulence in co-infection contexts will depend on how often pathogens encounter male and female hosts. In co-infections, more virulent pathogens often transmit more than their less virulent competitors, potentially favouring the evolution of higher virulence (Bell et al. 2006; Råberg et al. 2006; Ben-Ami et al. 2008; Balmer et al. 2009; Ben-Ami and Routtu 2013). Here, we find that co-infections in

female hosts favour the transmission of the more virulent pathogen, potentially selecting for higher levels of virulence. In contrast, co-infection in males is characterized by either equal fitness of competing pathogens or higher fitness of less virulent pathogen genotypes. Co-infection in males may then either slow the evolution of virulence or completely reverse selection on virulence depending on the exposure context. Yet, the evolutionary outcome of virulence will depend not only on the within-host outcomes of infection, but also in how disease is transmitted between hosts (van Baalen and Sabelis 1995; Frank 1996; Mideo et al. 2008; Alizon et al. 2013). Indeed, sex ratio can vary within a variety of species (Clutton-Brock and Iason 1986; Pusey 1987; Duneau and Ebert 2012 and Table 2 therein), including *Daphnia* (Galimov et al. 2011), which may influence the frequency of pathogen transmission between each sex and thus the overall patterns of selection on virulence. A higher proportion of the more exploitable sex, for example, may favour the transmission of more virulent pathogens, whereas population shifts to the less exploitable sex may restrain or reverse this pattern.

The ubiquity of pathogens in natural populations suggests that individuals are likely to encounter and become infected by multiple pathogen genotypes. We show that host sex may further impact on the relative fitness among pathogen genotypes due to within-host competition, finding that differences in the scale of the trade-off between pathogen virulence and transmission between the sexes affect the relative fitness among competing pathogens. Ultimately, how virulence should evolve within co-infection contexts will then depend on frequency of encountering each sex as well as variation in the exploitative potential of male and female hosts. Collectively, our results suggest that the often observed relationship between virulence and pathogen competitive ability may not apply equally to each sex, providing a mechanism for the maintenance of pathogen genetic variation in sexually dimorphic host populations.

## Acknowledgements

We would like to thank Luis Jimenez for invaluable assistance in planning and performing our genetic analyses. Thanks also to Isobel Booksmythe, Toby Hector, Louise Nørgaard, and Kyle Kelly for helpful conversations, laboratory assistance, and advice on statistical analyses.

## Chapter 5 tables and figures

Table 1: Description of the four infection types used in this study. Single infections consisted of one genotype and co-infections consisted of genotypes C19 and C24 or C19 and C1.

| Infection type | Description | Dose at 5 days old | | Dose at 12 days old | |
|---|---|---|---|---|---|
| | | Geno. A | Geno. B | Geno. A | Geno. B |
| Simultaneous single infection | Individual was exposed once to a single pathogen genotype | 40,000 spores | - | - | - |
| Simultaneous co-infection | Individual was exposed once to two pathogen genotypes | 20,000 spores | 20,000 spores | - | - |
| Sequential single infection | Individual was exposed to a pathogen at day 5 and then to the same pathogen at day 12. | 20,000 spores | - | 20, 000 spores | - |
| Sequential co-infection | Individual was exposed to one pathogen at day 5 and then to a different pathogen at day 12. | 20,000 spores | - | - | 20,000 spores |

Table 2: Summary of univariate and multivariate analyses of variance (white corrected, Type III) describing the effects of host sex, pathogen co-infection treatment, and their interaction on pathogen production of transmission spores, pathogen induced reduction in host lifespan and their multivariate interaction. Asterisks denote significant effects ($\alpha = 0.05$).

| Univariate ANOVA | ***F*** | **d.f.** | ***p*-val** |
|---|---|---|---|
| **Production of transmission spores** | | | |
| Sex | 2048.260 | 1,605 | <0.001 * |
| Pathogen co-infection treatment | 15.535 | 11,605 | <0.001 * |
| Sex : Pathogen | 7.146 | 11,605 | <0.001 * |
| **Reduction in host lifespan (days)** | | | |
| Sex | 655.365 | 1,605 | <0.001 * |
| Pathogen co-infection treatment | 6.110 | 11,605 | <0.001 * |
| Sex : Pathogen | 0.526 | 11,605 | 0.886 |

| Multivariate ANOVA | **Pillai's trace** | **Approx. *F*** | **d.f.** | ***p*-val** |
|---|---|---|---|---|
| **Production of transmission spores, reduction in host lifespan (days)** | | | | |
| Sex | 0.90683 | 2939.4 | 2,604 | <0.001 * |
| Pathogen co-infection treatment | 0.16749 | 5.0 | 22,1210 | <0.001 * |
| Sex : Pathogen | 0.10780 | 3.1 | 22,1210 | <0.001 * |

Table 3: Summary of an analyses of variance (white corrected, Type III) describing the effect of host sex, pathogen genotype, and their interaction on relative pathogen fitness within co-infection treatments. Asterisks denote significant effects (α = 0.05).

| **Relative pathogen fitness (relative spore production)** | ***F*** | **d.f.** | ***p*-val** |
|---|---|---|---|
| **Full model including all co-infection treatments** | | | |
| Sex | 170.335 | 1,574 | <0.001 * |
| Pathogen genotype | 49.093 | 1,574 | <0.001 * |
| Sex : Pathogen genotype | 69.034 | 1,574 | <0.001 * |
| **Simultaneous C19, C24** | | | |
| Sex | 40.511 | 1,72 | <0.001 * |
| Pathogen genotype | 57.442 | 1,72 | <0.001 * |
| Sex : Pathogen genotype | 43.890 | 1,72 | <0.001 * |
| **Sequential C19, C24** | | | |
| Sex | 47.780 | 1,98 | <0.001 * |
| Pathogen genotype | 48.988 | 1,98 | <0.001 * |
| Sex : Pathogen genotype | 49.133 | 1,98 | <0.001 * |
| **Sequential C24, C19** | | | |
| Sex | 26.541 | 1,88 | <0.001 * |
| Pathogen genotype | 6.991 | 1,88 | 0.008 * |
| Sex : Pathogen genotype | 1.776 | 1,88 | 0.183 |
| **Simultaneous C19, C1** | | | |
| Sex | 29.257 | 1,84 | <0.001 * |
| Pathogen genotype | 6.960 | 1,84 | 0.008 * |
| Sex : Pathogen genotype | 5.386 | 1,84 | 0.020 * |
| **Sequential C19, C1** | | | |
| Sex | 49.962 | 1,96 | <0.001 * |
| Pathogen genotype | 73.905 | 1,96 | <0.001 * |
| Sex : Pathogen genotype | 58.960 | 1,96 | <0.001 * |
| **Sequential C1, C19** | | | |
| Sex | 42.688 | 1,106 | <0.001 * |
| Pathogen genotype | 0.538 | 1,106 | 0.463 |
| Sex : Pathogen genotype | 8.934 | 1,106 | 0.003 * |

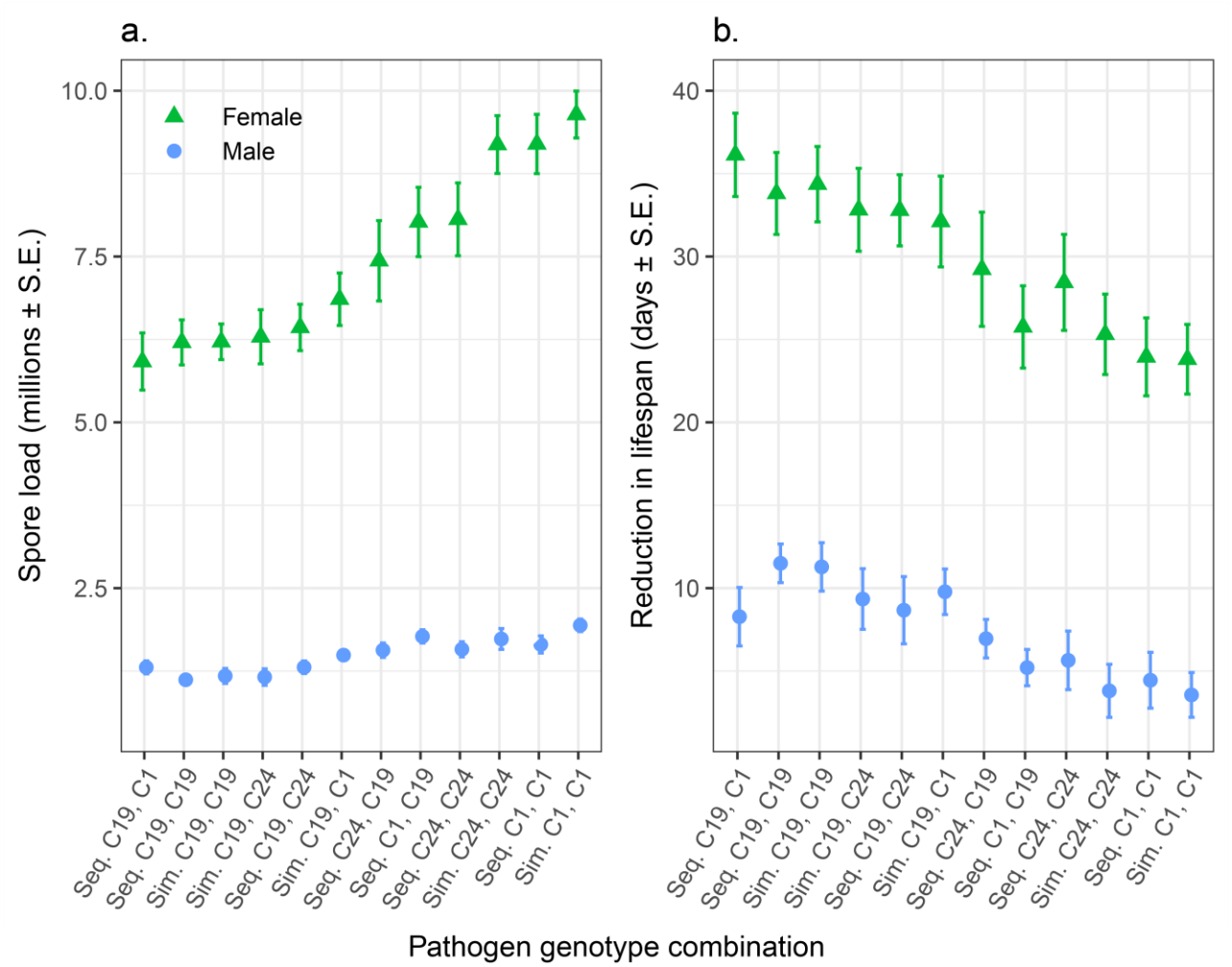


Figure 1: The influence of host sex and co-infection treatment on overall pathogen spore production (a) and reduction in host lifespan (b). Shown are treatment means and standard errors. Female results are represented by a solid green line with triangles and male results are represented by a dashed blue line with circles. Pathogen co-infection treatments are ordered by ascending average spore production in females. Sequential (Seq.) infection labels refer to the order in which the two pathogen genotypes established in their hosts whereas simultaneous (Sim.) infection labels refer to the two pathogen genotypes which established in their host at the same time.

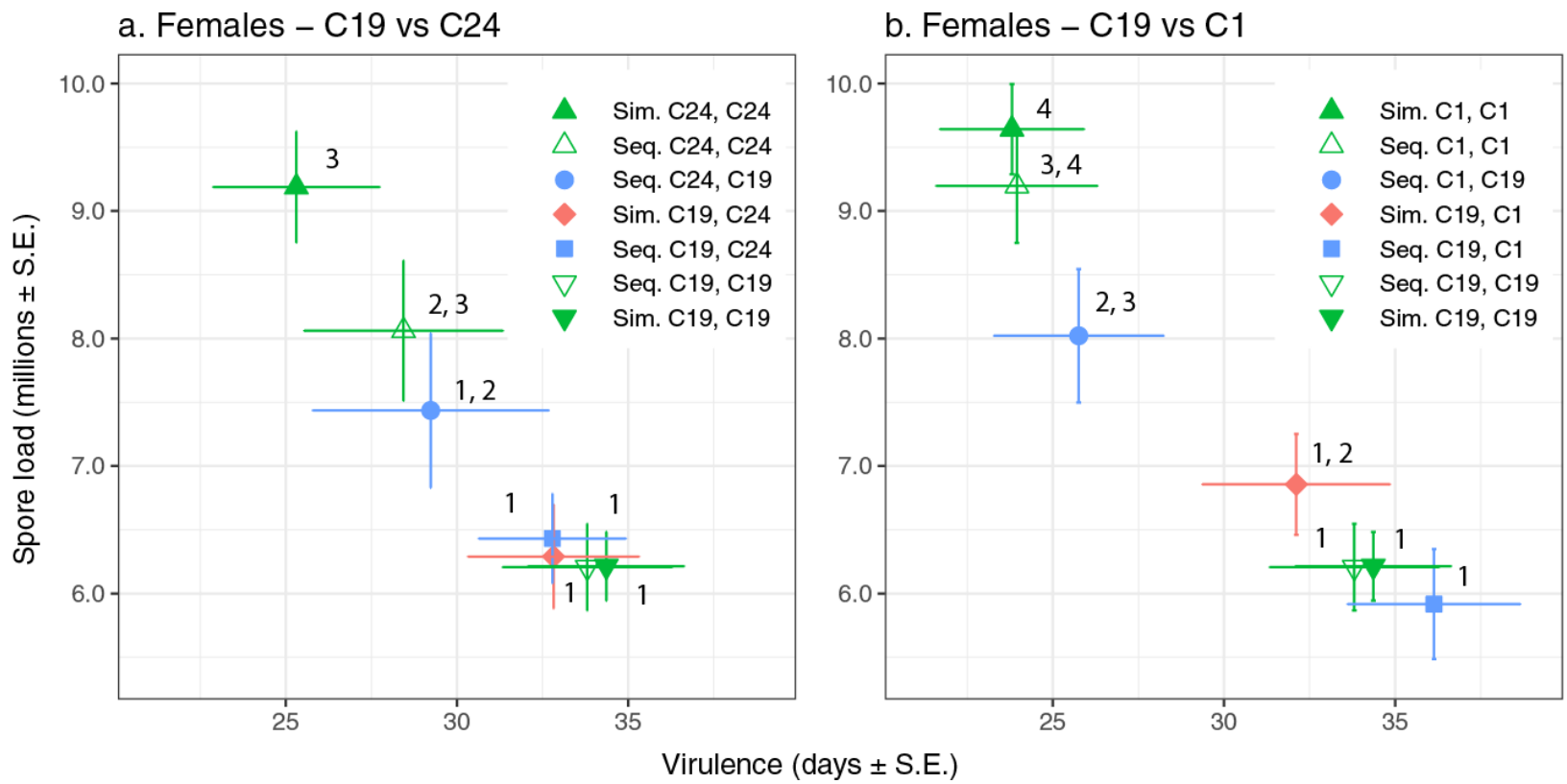


Figure 2: The influence of co-infection treatment on the relationship between overall pathogen spore production and reduction in host lifespan (virulence) for females exposed to pathogen genotype C19 and/or C24 (a) and females exposed to pathogen genotype C19 and/or C1 (b). Solid green triangles indicate single simultaneous infections, open green triangles indicate single sequential infections, blue squares or circles refer to sequential co-infections where the host was exposed to pathogen genotype C19 first or second respectively, and red diamonds refer to simultaneous co-infections. Shown are multivariate treatment means and standard errors with different associated numbers signifying a significant difference in multivariate mean between treatments. Sequential (Seq.) infection labels refer to the order in which the two pathogen genotypes established in their hosts whereas simultaneous (Sim.) infection labels refer to the two pathogen genotypes which established in their host at the same time.

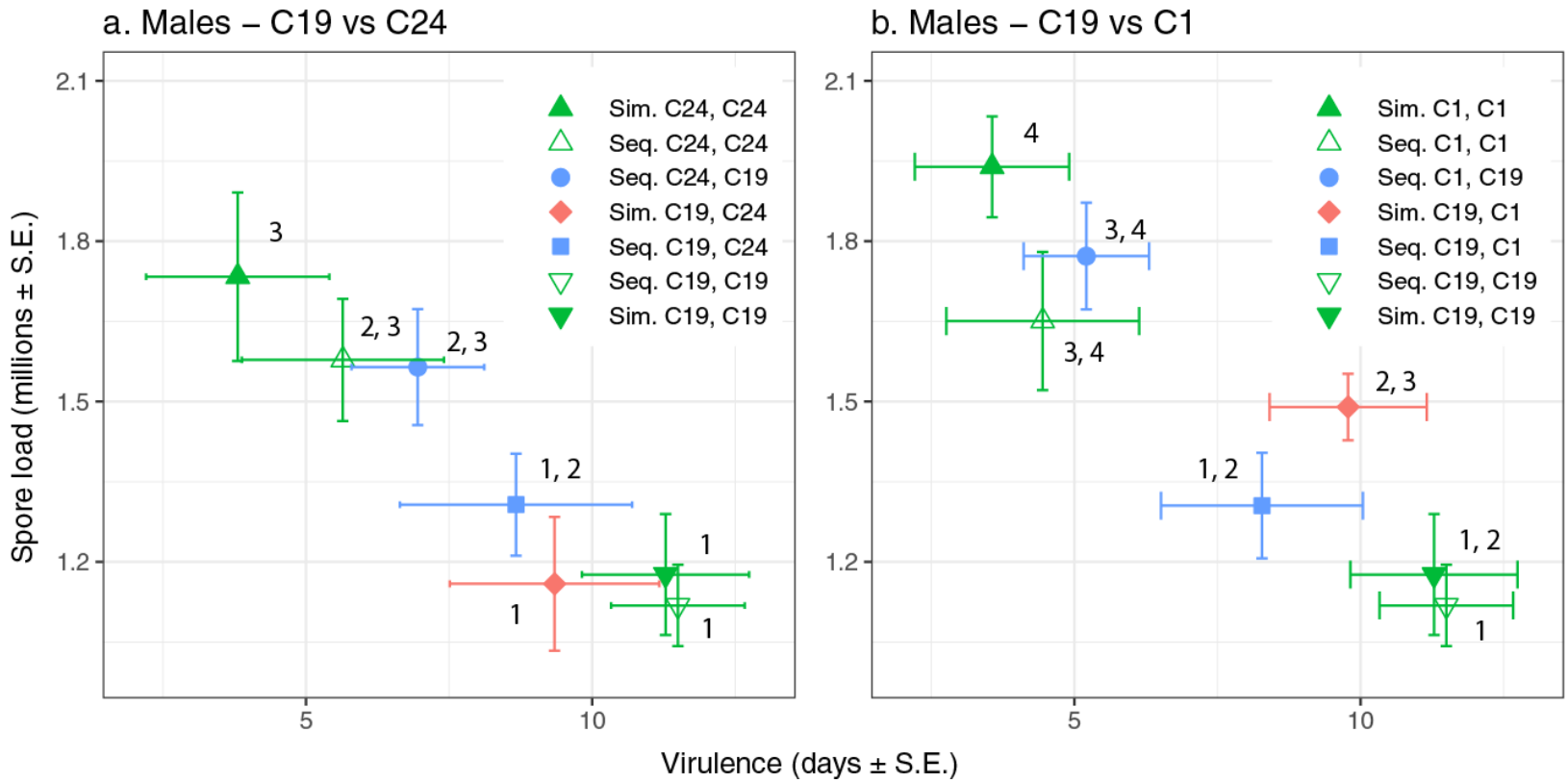


Figure 3: The influence of co-infection treatment on the relationship between overall pathogen spore production and reduction in host lifespan (virulence) for males exposed to pathogen genotype C19 and/or C24 (a) and males exposed to pathogen genotype C19 and/or C1 (b). Solid green triangles indicate single simultaneous infections, open green triangles indicate single sequential infections, blue squares or circles refer to sequential co-infections where the host was exposed to pathogen genotype C19 first or second respectively, and red diamonds refer to simultaneous co-infections. Shown are multivariate treatment means and standard errors with different associated numbers signifying a significant difference in multivariate mean between treatments. Sequential (Seq.) infection labels refer to the order in which the two pathogen genotypes established in their hosts whereas simultaneous (Sim.) infection labels refer to the two pathogen genotypes which established in their host at the same time.

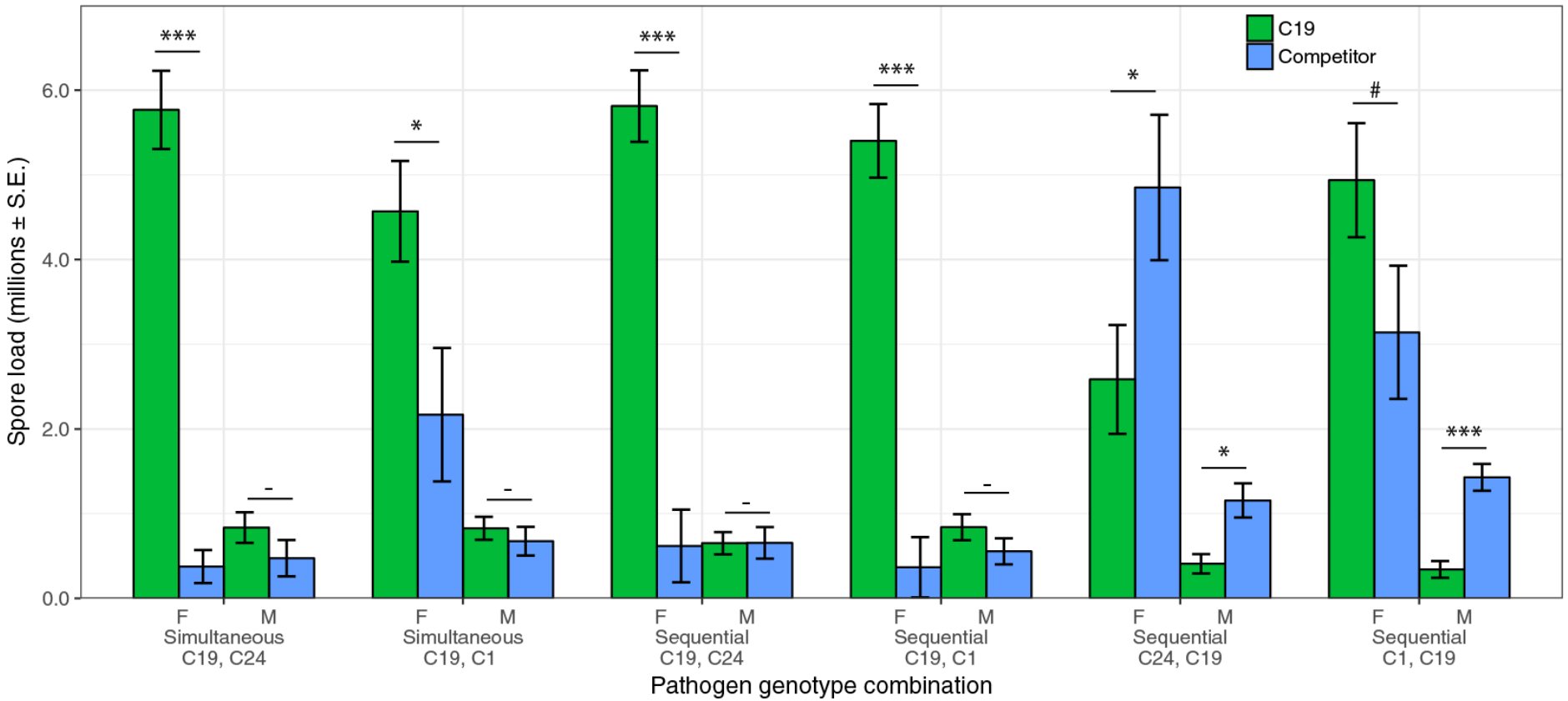


Figure 4: The influence of host sex and co-infection treatment on within-host pathogen competition as measured by relative spore production of individual genotypes. Green bars represent spore production by pathogen genotype C19 and blue bars represent spore production by the competing pathogen genotype (either C24 or C1). Shown are individual means for each genotype and standard errors. Asterisks indicate significant difference in mean spore production between competing genotypes within a single sex (two sample t-tests: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # $p < 0.10$, – > 0.10).

# Chapter 6 – The consequences of sexual dimorphism for host-pathogen coevolution

In this thesis, I explored the evolutionary consequences of sexual-dimorphism for host-pathogen coevolution. First, I reviewed theory on the response of pathogen fitness to environmental changes, on how cross-sex genetic correlations can impact on the population level response to selection, and how sex biases in mutation rates will influence evolutionary change. I considered these works in light of pathogen adaptation to host sex. I then performed a series of empirical studies using the waterflea host *Daphnia magna* and its obligate bacterial pathogen *Pasteuria ramosa.* With this model system I used infection designs, dietary manipulation, within-host pathogen competition experiments, and genetic analysis to explore the mechanisms underlying variation in disease outcome and pathogen fitness. My results collectively show how host sex can be a powerful selective force on pathogens by exhibiting varying impacts on relative pathogen fitness throughout age, interacting with diet to determine the optimal pathogen exploitation strategy, and by exhibiting potential to favour different levels of pathogen virulence in co-infection contexts.

## Host sex and the genetic basis of host-pathogen coevolution

In a literature review, I explored some previously overlooked consequences of sexual dimorphism for the nature and pace of host-pathogen coevolution (Chapter 2). I first drew from literature on genotype by environment interactions (GxEs), considering host sex as a variable environment for pathogens and the evolutionary consequences of sex-specific pathogen fitness. Previous work has theorized that when pathogen fitness varies between each sex, adaptation will progress depending on the extent of the fitness trade-off between the two sexes as well as the frequency of encountering each sex (Duneau and Ebert 2012; Cousineau and Alizon 2014). Here, I considered two more complex, but biologically relevant scenarios for host-pathogen coevolution. First, if the rank order of pathogen fitness changes across each host sex, pathogens may experience a fluctuating selective environment which will maintain pathogen genetic variation as no single genotype is consistently most fit (Gillespie and Turelli 1989). Second, host sex may instead impact on the variance in fitness among pathogen genotypes, yet the rank order remains the same. Here, even though one genotype is always most fit, the intensity of selection on fitness differences will

vary across environments (Hoffmann and Merilä 1999) potentially impacting on the speed of coevolutionary cycles (Gomulkiewicz et al. 2000).

I next considered the role of cross-sex genetic correlations in accelerating or constraining patterns of host-pathogen coevolution. When the genetic basis of a trait is positively correlated and the sexes experience similar patterns of selection, theory predicts rapid adaptive responses to selection (Lande 1980). In the context of host pathogen co-evolution, a positive correlation for traits underlying immunity may allow for hosts to exhibit a rapid adaptive response to selection by pathogens. If there is no cross-sex genetic correlation for immunity, the adaptive response of one sex will not be reflected in the other potentially allowing for sex-specific adaptive responses to selection. In this situation, the male, female, and pathogen population are best viewed as three different coevolving entities which may result in "chaotic" cycles of coevolution (Dercole et al. 2010). Finally, if immunity is negatively correlated an adaptive response of one sex may disturb the phenotypic optima of the other. In this scenario, selection on one sex will impede on the adaptive evolution on the other (Connallon and Clark 2014), potentially reducing the rate of host adaptation.

Finally, I explored how male biases in mutation rate (Haldane 1935) stemming from replication errors during the production of gametes (Ellegren and Fridolfsson 2003) may impact on host-pathogen coevolution. Previous work has shown that males experience stronger selection on deleterious mutations and that the resulting purging of mutations through males can improve mean fitness in females (Sharp and Vincent 2015). Yet, it remains unclear how these patterns will impact on pathogen evolution and should be explicitly explored in the future. For example, if males are the more susceptible host they may also impose weak selection on the pathogen and thus lower rates of pathogen adaptation. Conversely, stronger selection on male fitness may result in the rapid fixation of beneficial immune-related mutations, potentially accelerating the adaptive response of the host population.

### Host sex and age of exposure shape the virulence-transmission trade-off

Using male and female *Daphnia* of four different age groups, I measured how sex and age of exposure to pathogens collectively affect the outcome of disease. In females, I found that infection with *P. ramosa* increased mortality rate as compared to uninfected females at each age group and that the odds of death were 2.5 - 5 fold greater than controls. Conversely, mortality rate only increased in males infected at the earliest age class as represented by two-fold increase

in odds of death. The observation that mortality rate only increased due to infection in the earliest age class for males, but was consistently higher across age in females suggests that males are a less exploitable sex. The relatively lower post-infection lifespan of males may simply not allow for disease to progress to the point where pathogen reproduction impacts on host mortality.

The increased mortality rate of infected females was mirrored by greater pathogen infection rates, virulence (reduction in lifespan), and fitness (spore production) within female hosts. Furthermore, pathogen genotypes exhibited variation in these traits within females, whereas the two pathogen genotypes used in this study behaved identically in males. Interestingly, we observed that the most fit pathogen genotype changes with age within females. Again, the longer lifespan of female *Daphnia* (Duneau et al. 2012; Thompson et al. 2017) may be driving these differences, allowing *P. ramosa* more time to exploit its host and thus express variation in disease characteristics (Clerc et al. 2015). This study suggests that naturally varying population characteristics like age distribution or sex ratio (Clutton-Brock and Iason 1986; Charlesworth 1994; Duneau and Ebert 2012), by affecting the relative fitness between pathogen genotypes, may represent a mechanism to maintain genetic variation within pathogen populations.

### Optimal pathogen exploitative strategies underlie sexual dimorphism in disease outcome

I then explored how sex-specific patterns of resource acquisition or allocation (Stoehr and Kokko 2006; Boggs 2009; Schärer et al. 2012) affect pathogen fitness and optimal exploitation strategy (Chapter 4). Using dietary manipulations, I infected male and female *Daphnia* hosts with *P. ramosa* and measured the resulting impacts of host condition on pathogen fitness within each sex. I found that the sexes vary in how acquired resources are transferred to the pathogen. Specifically, I found that shifts from the low to the high diet in females were represented by increased pathogen spore production and virulence, whereas these changes were more subtle within males. Mirroring these results was the effect of host condition on pathogen-induced changes in metabolic rate; measured to assess the effects of infection on the use, transformation, and expenditure of energy (Kearney and White 2012; Arnold et al. 2013). While metabolic rate remained unchanged across diet within males, we observed a strong increase in metabolic rate in females raised on the high diet, suggesting that the overall level of pathogen exploitation varied due to diet in female hosts.

The dietary treatment, however, did not only affect the relationship between host condition and pathogen fitness, but also the relationship between the characteristics of pathogen exploitation which best predict pathogen fitness. In considering how pathogen-induced reduction in lifespan or

increases in metabolic rate interact to determine spore production, we found that diet impacted on the relationship among these traits only within female hosts. Indeed, in males from either dietary treatment or females raised specifically on a high diet, low pathogen fitness was predicted by an interaction of low increases in metabolic rate or high virulence. Conversely, shifts to the low diet only changed the optimal exploitation strategy in females where pathogen fitness increased only along a narrow ridge of increased metabolic rate and small reductions in virulence. These findings provide evidence that inherent differences in patterns of resource allocation between the sexes may favour different strategies of pathogen exploitation.

## Host sex changes the competitive outcome of coinfection, favouring different pathogen virulence strategies

Finally, I explored how variation in the expression of virulence due to host sex (see Table 1 Cousineau and Alizon 2014; Giefing-Kröll et al. 2015; Klein and Flanagan 2016) may influence the relative fitness among co-infecting pathogen genotypes. To do so, I infected male and female *Daphnia* with combinations of *P. ramosa* genotypes which exhibit varying impact on host lifespan within single infection contexts (Clerc et al. 2015). I then measured how co-infections in each sex impact on measures of pathogen fitness and used microsatellite analysis to measure the relative fitness of competing pathogen genotypes. I first found that the virulence-transmission trade-off within co-infection contexts was qualitatively similar among male and female hosts. In general, when pathogens were exposed to their hosts simultaneously, virulence and transmission resembled that of the more virulent pathogen in single infection contexts. When one pathogen established first, however, virulence and transmission resembled that of the earlier arriving genotype regardless of its virulence characteristics in single infections.

Despite these similar patterns between the sexes, we found that an interaction between sex and the order of pathogen establishment influenced the relative fitness among competing pathogen genotypes. In females, co-infections generally favoured the transmission of the more virulent genotype regardless of co-infection treatment. In contrast, co-infection in males resulted in either equal fitness among competing pathogens or higher fitness of less virulent pathogen genotypes. While co-infections are often predicted to favour the transmission of more virulent pathogen genotypes (Alizon et al. 2013), we provide evidence that host sex may constrain or even reverse this pattern and may thus be a powerful force in the maintenance of pathogen genetic diversity.

## Future directions

My studies emphasise the critical role that host sex plays in influencing multiple aspects of the infection process. There remains a distinct shortage of disease research that either incorporates both male and female individuals or formally analyses sex-specific effects on the progression and outcomes of infection. Unfortunately, this routine omission of host sex in the primary literature has led to a variety of real world problems, ranging from poor representation of females in clinical trials to medicinal dosages tailored to only half of the population (Johnson et al. 2014). Furthermore, while there has been a growing appreciation for how host heterogeneity may influence disease spread or management in natural populations (Johnson et al. 2015), the lack of studies incorporating both sexes may be ignoring an important, inherent source of natural host variation. Indeed, in my thesis I have presented several lines of evidence that the sexes can vary in the rate of becoming infected, the amount of within-host pathogen reproduction, and how these traits are influenced by age or nutritional condition - all sources of heterogeneity which may underlie disease dynamics in natural populations (Lloyd-Smith et al. 2005; Hall et al. 2009b; Beldomenico and Begon 2010). A practical implication of my thesis work is therefore the importance of incorporating host sex differences into studies of disease at all stages, from primary research to the development of disease treatments.

My work demonstrates that a greater focus on the effects of host sex – both sex-specific responses to infection in hosts, and host sex-specific effects on pathogen virulence and transmission – will lead to new insights into disease evolution and the dynamics of epidemics. The approach taken in my thesis thus has implications for theory on host-pathogen coevolution, and for modelling the spread and severity of disease through host populations. These are both vital to our ability to manage disease in natural populations, yet more data on more host-pathogen systems is needed to inform models and generate theory.

In conclusion, my thesis has highlighted just a few of the many ways in which sexual dimorphism can impact on the evolution of infectious disease. I have shown that male and female hosts can represent substantial variation in the selective environment for pathogens, and that host sex interacts with other sources of heterogeneity such as host age or acquisition of resources to influence the relative fitness of pathogen genotypes, and thus, the evolutionary potential of disease. Yet, studies of disease evolution are frequently performed on a single sex, overlooking the ubiquity of sexual dimorphism across most complex organisms. There remain numerous open

questions about the role of sex in the outcome of disease. It is my hope that this research, and the recent work of other researchers on this topic, will inspire a greater appreciation for how host sex affects the spread and evolution of disease in nature.

## References


Adamo, S. A., M. Jensen, and M. Younger. 2001. Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. integer*): trade-offs between immunity and reproduction. Anim. Behav. 62:417–425.

Agrawal, A. F. 2001. Sexual selection and the maintenance of sexual reproduction. Nature 411:692–695.

Alizon, S., J. C. de Roode, and Y. Michalakis. 2013. Multiple infections and the evolution of virulence. Ecol. Lett. 16:556–567.

Alizon, S., A. Hurford, N. Mideo, and M. Van Baalen. 2009. Virulence evolution and the trade-off hypothesis: History, current state of affairs and the future. J. Evol. Biol. 22:245–259.

Altizer, S., A. Dobson, P. Hosseini, P. Hudson, M. Pascual, and P. Rohani. 2006. Seasonality and the dynamics of infectious diseases. Ecol. Lett. 9:467–484.

Anderson, R. M., and R. M. May. 1982. Coevolution of hosts and parasites. Parasitology 85:229–239.

Anderson, R. M., and R. M. May. 1991. Infectious diseases of humans: dynamics and control. Oxford University Press, Oxford, UK.

Andersson, M. B. 1994. Sexual Selection. Princeton University Press, Princeton.

Andras, J. P., and D. Ebert. 2013. A novel approach to parasite population genetics: Experimental infection reveals geographic differentiation, recombination and host-mediated population structure in Pasteuria ramosa, a bacterial parasite of *Daphnia.* Mol. Ecol. 22:972–86.

Arnold, P. a, K. N. Johnson, and C. R. White. 2013. Physiological and metabolic consequences of viral infection in *Drosophila melanogaster.* J. Exp. Biol. 216:3350–7.

Asser-Kaiser, S., E. Fritsch, K. Undorf-Spahn, J. Kienzle, K. E. Eberle, N. A. Gund, A. Reineke, C. P. W. Zebitz, D. G. Heckel, J. Huber, and J. A. Jehle. 2007. Rapid emergence of baculovirus resistance in codling moth due to dominant, sex-linked inheritance. Science 317:1916–1918.

Babiker, H. A., L. C. Ranford-Cartwright, and D. Walliker. 1999. Genetic structure and dynamics of *Plasmodium falciparum* infections in the Kilombero region of Tanzania. Trans. R. Soc. Trop. Med. Hyg. 93:S11–S14.

Balenger, S. L., and M. Zuk. 2014. Testing the Hamilton-Zuk hypothesis: past, present, and future. Integr. Comp. Biol. 54:601–613.

Balmer, O., S. C. Stearns, A. Schötzau, and R. Brun. 2009. Intraspecific competition between co-infecting parasite strains enhances host survival in African trypanosomes. Ecology 90:3367–3378.

Balmer, O., and M. Tanner. 2011. Prevalence and implications of multiple-strain infections. Lancet Infect. Dis. 11:868–878.

Bashey, F. 2015. Within-host competitive interactions as a mechanism for the maintenance of parasite diversity. Philos. Trans. R. Soc. B 370:20140301.

Bashir-Tanoli, S., and M. C. Tinsley. 2014. Immune response costs are associated with changes in resource acquisition and not resource reallocation. Funct. Ecol. 28:1011–1019.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila*. Heredity 2:349–368.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1–48.

Bedhomme, S., P. Agnew, C. Sidobre, and Y. Michalakis. 2004. Virulence reaction norms across a food gradient. Proc. R Soc. B 271:739–744.

Beldomenico, P. M., and M. Begon. 2010. Disease spread, susceptibility and infection intensity: vicious circles? Trends Ecol. Evol. 25:21–27.

Bell, A. S., J. C. de Roode, D. Sim, and A. F. Read. 2006. Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. Evolution 60:1358–1371.

Bell, G. 1982. The masterpiece of nature: the evolution and genetics of sexuality. University of California Press, Berkeley.

Ben-Ami, F., L. Mouton, and D. Ebert. 2008. The effects of multiple infections on the expression and evolution of virulence in a *Daphnia*-endoparasite system. Evolution 62:1700–1711.

Ben-Ami, F., and J. Routtu. 2013. The expression and evolution of virulence in multiple infections: the role of specificity, relative virulence and relative dose. BMC Evol. Biol. 13:1.

Ben-Ami, F., D. Ebert, and R. R. Regoes. 2010. Pathogen dose infectivity curves as a method to analyze the distribution of host susceptibility: a quantitative assessment of maternal effects after food stress and pathogen exposure. Am. Nat. 175:106–115.

Bergelson, J., M. Kreitman, E. a Stahl, and D. Tian. 2001. Evolutionary dynamics of plant r-genes. Adv. Sci. 292:2281–2285.

Berger, D., K. Grieshop, M. I. Lind, J. Goenaga, A. A. Maklakov, and G. Arnqvist. 2014. Intralocus sexual conflict and environmental stress. Evolution 68:2184–2196.

Blanford, S., M. B. Thomas, C. Pugh, and J. K. Pell. 2003. Temperature checks the Red Queen? Resistance and virulence in a uctuating environment. Ecol. Lett. 6:2–5.

Boggs, C. L. 2009. Understanding insect life histories and senescence through a resource allocation lens. Funct. Ecol. 23:27–37.

Bohrer, R. N., and W. Lampert. 1988. Simultaneous measurement of the effect of food concentration on assimilation and respiration in *Daphnia magna* Straus. Funct. Ecol. 2:463–471.

Bonduriansky, R., and S. F. Chenoweth. 2009. Intralocus sexual conflict. Trends Ecol. Evol. 24:280–288.

Bonduriansky, R., A. Maklakov, F. Zajitschek, and R. Brooks. 2008. Sexual selection, sexual conflict and the evolution of ageing and life span. Funct. Ecol. 22:443–453.

Borghans, J. A. M., J. B. Beltman, and R. J. De Boer. 2004. MHC polymorphism under host-pathogen coevolution. Immunogenetics 55:732–739.

Brockhurst, M. A., T. Chapman, K. C. King, J. E. Mank, S. Paterson, and G. D. D. Hurst. 2014. Running with the Red Queen: the role of biotic conflicts in evolution. Proc. R. Soc. B 281: 20141382.

Brown, M. J. F., R. Loosli, and P. Schmid-Hempel. 2000. Condition-dependent expression of virulence in a trypanosome infecting bumblebees. Oikos 91:421–427.

Buckling, A., and P. B. Rainey. 2002. Antagonistic coevolution between a bacterium and a bacteriophage. Proc. R Soc. B 269:931–936.

Bull, J. J. 1994. Virulence. Evolution 48:1423–1437.

Cameron, J. N. 1986. Appendix 2 - Solubility of O2 and CO2 at different temperatures and salinities. P. 255 *in* Principles of Physiological Measurement. Academic Press, London.

Carius, H. J., T. J. Little, and D. Ebert. 2001. Genetic variation in a host-parasite association: potential for coevolution and frequency-dependent selection. Evolution 55:1136–1145.

Chao, L., K. A. Hanley, C. L. Burch, C. Dahlberg, and P. E. Turner. 2000. Kin selection and parasite evolution: higher and lower virulence with hard and soft selection. Q. Rev. Biol. 75:261–275.

Charlesworth, B. 1994. Evolution in age-structured populations. 2nd ed. Cambridge University Press, Cambridge.

Chevin, L. M. 2013. Genetic constraints on adaptation to a changing environment. Evolution 67:708–721.

Christe, P., O. Glaizot, G. Evanno, N. Bruyndonckx, G. Devevey, G. Yannic, P. Patthey, A. Maeder, P. Vogel, and R. Arlettaz. 2007. Host sex and ectoparasites choice: Preference for, and higher survival on female hosts. J. Anim. Ecol. 76:703–710.

Clark, J., J. S. Garbutt, L. McNally, and T. J. Little. 2017. Disease spread in age structured populations with maternal age effects. Ecol. Lett. 20: 445-451.

Clerc, M., D. Ebert, and M. D. Hall. 2015. Expression of parasite genetic variation changes over the course of infection: implications of within-host dynamics for the evolution of virulence. Proc. R. Soc. B 282:20142820.

Clutton-Brock, T. H., and G. R. Iason. 1986. Sex ratio variation in mammals. Q. Rev. Biol. 61:339–374.

Connallon, T., and A. G. Clark. 2014. Evolutionary inevitability of sexual antagonism. Proc. R. Soc. B

281: 20132013.

Cousineau, S. V., and S. Alizon. 2014. Parasite evolution in response to sex-based host heterogeneity in resistance and tolerance. J. Evol. Biol. 27:2753–2766.

Cox, R. M., and R. Calsbeek. 2009. Sexually antagonistic selection, sexual dimorphism, and the resolution of intralocus sexual conflict. Am. Nat. 173:176–187.

Cressler, C. E., D. V. McLeod, C. Rozins, J. Van Den Hoogen, and T. Day. 2016. The adaptive evolution of virulence: a review of theoretical predictions and empirical tests. Parasitology 143:915–930.

de Roode, J. C., M. E. H. Helinski, M. A. Anwar, and A. F. Read. 2005. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. Am. Nat. 166:531–542.

De Roode, J. C., A. B. Pedersen, M. D. Hunter, and S. Altizer. 2008. Host plant species affects virulence in monarch butterfly parasites. J. Anim. Ecol. 77:120–126.

Decaestecker, E., S. Gaba, J. A. M. Raeymaekers, R. Stoks, L. Van Kerckhoven, D. Ebert, and L. De Meester. 2007. Host–parasite "Red Queen" dynamics archived in pond sediment. Nature 450:870–874.

Dercole, F., R. Ferriere, and S. Rinaldi. 2010. Chaotic Red Queen coevolution in three-species food chains. Proc. R. Soc. B 277:2321–2330.

Donnelly, C., L. Bartley, A. Ghani, A. Le Fevre, G. Kwong, B. Cowling, A. van Sighem, F. de Wolf, R. Rode, and R. Anderson. 2005. Gender difference in HIV-1 RNA viral loads. HIV Med. 6:170–178.

Doums, C., Y. Moret, E. Benelli, and P. Schmid-Hempel. 2002. Senescence of immune defence in *Bombus* workers. Ecol. Entomol. 27:138–144.

Duncan, A. B., S. E. Mitchell, and T. J. Little. 2006. Parasite-mediated selection and the role of sex and diapause in *Daphnia*. J. Evol. Biol. 19:1183–1189.

Duneau, D., and D. Ebert. 2012. Host sexual dimorphism and parasite adaptation. PLoS Biol. 10:e1001271.

Duneau, D., P. Luijckx, L. F. Ruder, and D. Ebert. 2012. Sex-specific effects of a parasite evolving in a female-biased host population. BMC Biol. 10:104.

Dybdahl, M. F., and C. M. Lively. 1998. Host-parasite coevolution: evidence for rare advantage and time-lagged selection in a natural population. Evolution 52:1057–1066.

Ebert, D. 2005. Ecology, epidemiology, and evolution of parasitism in *Daphnia*. 1st ed. National Library of Medicine (US), National Center for Biotechnology Information, Bethesda, MD.

Ebert, D., H. J. Carius, T. Little, and E. Decaestecker. 2004. The evolution of virulence when parasites cause host castration and gigantism. Am. Nat. 164:S19–S32.

Ebert, D., D. Duneau, M. D. Hall, P. Luijckx, J. P. Andras, L. Du Pasquier, and F. Ben-Ami. 2016. A population biology perspective on the stepwise infection process of the bacterial pathogen *Pasteuria ramosa* in *Daphnia*. Adv. Parasitol. 91:265–310.

Ebert, D., C. D. Zschokke-Rohringer, and H. J. Carius. 2000. Dose effects and density-dependent regulation of two microparasites of *Daphnia magna*. Oecologia 122:200–209.

Ebert, D., C. D. Zschokke-Rohringer, and H. J. Carius. 1998. Within–and between–population variation for resistance of Daphnia magna to the bacterial endoparasite *Pasteuria ramosa*. Proc. R. Soc. B 265:2127–2134.

Ellegren, H., and A. K. Fridolfsson. 2003. Sex-specific mutation rates in salmonid fish. J. Mol. Evol. 56:458–463.

Eswarappa, S. M., S. Estrela, and S. P. Brown. 2012. Within-host dynamics of multi-species infections: Facilitation, competition and virulence. PLoS One 7.

Evans, O., E. P. Caragata, C. J. McMeniman, M. Woolfit, D. C. Green, C. R. Williams, C. E. Franklin, S. L. O'Neill, and E. A. McGraw. 2009. Increased locomotor activity and metabolism of *Aedes aegypti* infected with a life-shortening strain of Wolbachia pipientis. J. Exp. Biol. 212:1436–1441.

Eyre-Walker, A., and P. D. Keightley. 2007. The distribution of fitness effects of new mutations. Nat. Rev. Genet. 8:610–618.

Fox, J., and S. Weisberg. 2011. An {R} Companion to Applied Regression. Second ed. Sage,

Thousand Oaks, CA.

Frank, S. A. 1996. Models of parasite virulence. Q. Rev. Biol. 71:37–78.

Galimov, Y., B. Walser, and C. R. Haag. 2011. Frequency and inheritance of non-male producing clones in *Daphnia magna*: evolution towards sex specialization in a cyclical parthenogen? J. Evol. Biol. 24:1572–1583.

Gandon, S. 2004. Evolution of multihost parasites. Evolution 58:455–469.

Gandon, S., and Y. Michalakis. 2000. Evolution of parasite virulence against qualitative or quantitative host resistance. Proc. R. Soc. B 267:985–990.

Gandon, S., and Y. Michalakis. 2002. Local adaptation, evolutionary potential and host-parasite coevolution: Interactions between migration, mutation, population size and generation time. J. Evol. Biol. 15:451–462.

Gandon, S., M. van Baalen, and V. A. A. Jansen. 2002. The evolution of parasite virulence, superinfection, and host resistance. Am. Nat. 159:658–669.

Garbutt, J. S., A. J. P. O'Donoghue, S. J. McTaggart, P. J. Wilson, and T. J. Little. 2014. The development of pathogen resistance in *Daphnia magna*: implications for disease spread in age-structured populations. J. Exp. Biol. 217:3929–3934.

Giefing-Kröll, C., P. Berger, G. Lepperdinger, and B. Grubeck-Loebenstein. 2015. How sex and age affect immune responses, susceptibility to infections, and response to vaccination. Aging Cell 309–321.

Gillespie, J. H., and M. Turelli. 1989. Genotype-environment interactions and the maintenance of polygenic variation. Genetics 121:129–138.

Gipson, S. A. Y., and M. D. Hall. 2018. Interactions between host sex and age of exposure modify the virulence-transmission trade-off. J. Evol. Biol. 31:428-437.

Gipson, S. A. Y., and M. D. Hall. 2016. The evolution of sexual dimorphism and its potential impact on host-pathogen coevolution. Evolution 70:959–968.

Gliwicz, M. Z., A. Slusarczyk, and M. Slusarczyk. 2001. Life history synchronization in a long-lifespan

single-cohort *Daphnia* population in a fishless alpine lake. Oecologia 128:368–378.

Gomulkiewicz, R., J. N. Thompson, R. D. Holt, S. L. Nuismer, and M. E. Hochberg. 2000. Hot spots, cold spots, and the geographic mosaic theory of coevolution. Am. Nat. 156:156–174.

Gray, E. M., and T. J. Bradley. 2006. Malarial infection in Aedes aegypti: effects on feeding, fecundity and metabolic rate. Parasitology 132:169–176.

Green, J. 1974. Parasites and epibionts of Cladocera. Trans. Zool. Soc. Lond. 32(6):417–516.

Griffin, R. M., R. Dean, J. L. Grace, P. Rydén, and U. Friberg. 2013. The shared genome is a pervasive constraint on the evolution of sex-biased gene expression. Mol. Biol. Evol. 30:2168–2176.

Haldane, J. B. S. 1935. The rate of spontaneous mutation of a human gene. J. Genet. 31:317–326.

Hall, M. D., G. Bento, and D. Ebert. 2017. The evolutionary consequences of stepwise infection processes. Trends Ecol. Evol. 32:612–623.

Hall, M. D., L. F. Bussière, and R. Brooks. 2009a. Diet-dependent female evolution influences male lifespan in a nuptial feeding insect. J. Evol. Biol. 22:873–81.

Hall, M. D., L. F. Bussière, J. Hunt, and R. Brooks. 2008. Experimental evidence that sexual conflict tnfluences the opportunity, form and intensity of sexual selection. Evolution 62:2305–2315.

Hall, M. D., and D. Ebert. 2012. Disentangling the influence of parasite genotype, host genotype and maternal environment on different stages of bacterial infection in *Daphnia magna*. Proc. R. Soc. B 279:3176–3183.

Hall, M. D., and D. Ebert. 2013. The genetics of infectious disease susceptibility: has the evidence for epistasis been overestimated? BMC Biol. 11:79.

Hall, M. D., B. L. Phillips, D. J. Marshall, and C. R. White. n.d. Host-pathogen interactions and the influence of pathogen exposure on metabolic rates. In prep.

Hall, S. R., C. J. Knight, C. R. Becker, M. A. Duffy, A. J. Tessier, and C. E. Cáceres. 2009b. Quality matters: Resource quality for hosts and the timing of epidemics. Ecol. Lett. 12:118–128.

Hall, S. R., J. L. Simonis, R. M. Nisbet, A. J. Tessier, and C. E. Cáceres. 2009c. Resource ecology of

virulence in a planktonic host-parasite system: an explanation using dynamic energy budgets. Am. Nat. 174:149–162.

Hamilton, W. D. 1980. Sex versus non-sex versus parasite. Oikos 35:282–290.

Hamilton, W. D., R. Axelrod, and R. Tanese. 1990. Sexual reproduction as an adaptation to resist parasites (a review). Proc. Natl. Acad. Sci. U. S. A. 87:3566–3573.

Hamilton, W. D., and M. Zuk. 1982. Heritable true fitness and bright birds: a role for parasites? Science 218:384–387.

High, K. P. 2004. Infection as a cause of age-related morbidity and mortality. Ageing Res. Rev. 3:1–14.

Ho, A. M.-H., P. W. Dion, C. S. H. Ng, and M. K. Karmakar. 2012. Understanding immortal time bias in observational cohort studies. Anaesthesia 68:126–130.

Hodgson, D. J., R. B. Hitchman, A. J. Vanbergen, R. S. Hails, R. D. Possee, and J. S. Cory. 2004. Host ecology determines the relative fitness of virus genotypes in mixed-genotype nucleopolyhedrovirus infections. J. Evol. Biol. 17:1018–1025.

Hoffmann, A. a., and J. Merilä. 1999. Heritable variation and evolution under favourable and unfavourable conditions. Trends Ecol. Evol. 14:96–101.

Houslay, T. M., J. Hunt, M. C. Tinsley, and L. F. Bussière. 2015. Sex differences in the effects of juvenile and adult diet on age-dependent reproductive effort. J. Evol. Biol. 28:1067–1079.

Hoverman, J. T., B. J. Hoye, and P. T. J. Johnson. 2013. Does timing matter? How priority effects influence the outcome of parasite interactions within hosts. Oecologia 173:1471–1480.

Hunt, J., L. F. Bussière, M. D. Jennions, and R. Brooks. 2004. What is genetic quality? Trends Ecol. Evol. 19:329–333.

Hurst, L. D., and H. Ellegren. 1998. Sex biases in the mutation rate. Trends Genet. 14:446–452.

Ingleby, F. C., J. Hunt, and D. J. Hosken. 2010. The role of genotype-by-environment interactions in sexual selection. J. Evol. Biol. 23:2031–2045.

Izhar, R., and F. Ben-Ami. 2015. Host age modulates parasite infectivity, virulence and

reproduction. J. Anim. Ecol. 84:1018–1028.

Izhar, R., J. Routtu, and F. Ben-ami. 2015. Host age modulates within-host parasite competition. Biol. Lett. 11.

Jaenike, J. 1978. An hypothesis to account for the maintenance of sex within populations. Evol. Theory 3:191–194.

Jensen, K. H., T. Little, A. Skorping, and D. Ebert. 2006. Empirical support for optimal virulence in a castrating parasite. PLoS Biol. 4:1265–1269.

Johnson, P. A., T. Fitzgerald, A. Salganicoff, S. F. Wood, and J. Goldstein. 2014. Sex-Specific Medical Research: why women's health can't wait. A report of the Mary Horrigan Connors Center for Women's Health & Gender Biology at Brigham and Women's Hospital. Brigham and Women's Hospital.

Johnson, P. T. J., J. C. De Roode, and A. Fenton. 2015. Why infectious disease research needs community ecology. Science 349:1259504.

Katz, J. M., J. Plowden, M. Renshaw-Hoelscher, X. Lu, T. M. Tumpey, and S. Sambhara. 2004. Immunity to influenza: the challenges of protecting an aging population. Immunol. Res. 29:113–124.

Kearney, M. R., and C. R. White. 2012. Testing Metabolic Theories. Am. Nat. 180:546–565.

Kirkpatrick, M., and D. W. Hall. 2004. Male-biased mutation, sex linkage, and the rate of adaptive evolution. Evolution 58:437–440.

Kirkpatrick, M., and M. J. Ryan. 1991. The evolution of mating preferences and the paradox of the lek. Nature 350:33–38.

Klein, S. L., and K. L. Flanagan. 2016. Sex differences in immune responses. Nat. Rev. Immunol. 16:626.

Klüttgen, B., U. Dülmer, M. Engels, and H. T. Ratte. 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Res. 28:743–746.

Kopp, M., and S. Matuszewski. 2014. Rapid evolution of quantitative traits: Theoretical

perspectives. Evol. Appl. 7:169–191.

Koskella, B., and C. M. Lively. 2007. Advice of the rose: experimental coevolution of a trematode parasite and its snail host. Evolution 61:152–159.

Laine, A. L. 2007. Pathogen fitness components and genotypes differ in their sensitivity to nutrient and temperature variation in a wild plant-pathogen association. J. Evol. Biol. 20:2371–2378.

Lande, R. 1987. Genetic correlations between the sexes in the evolution of sexual dimorphism and mating preferences. P. *in* J. W. Bradbury and M. B. Andersson, eds. Sexual selection: Testing the alternatives. John Wiley & Sons, Chichester, N.Y.

Lande, R. 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. Evolution 34:292–305.

Lanfear, R., H. Kokko, and A. Eyre-Walker. 2014. Population size and the rate of evolution. Trends Ecol. Evol. 29:33–41.

Lee, K. A. 2006. Linking immune defenses and life history at the levels of the individual and the species. Integr. Comp. Biol. 46:1000–1015.

Lévesque, L. E., J. A. Hanley, A. Kezouh, and S. Suissa. 2010. Problem of immortal time bias in cohort studies: example using statins for preventing progression of diabetes. BMJ 340:907–911.

Levin, B. R. 1981. Periodic selection, infectious gene exchange and the genetic structure of E. coli populations. Genetics 99:1–23.

Lighton, J. R. B. 2008. Measuring metabolic rates: a manual for scientists. Oxford University Press, Oxford.

Lively, C. M. 2010. A review of red queen models for the persistence of obligate sexual reproduction. J. Hered. 101:S13–S20.

Lively, C. M. 1989. Adaptation by a parasitic trematode to local populations of its snail host. Evolution 43:1663–1671.

Lively, C. M. 1999. Migration, Virulence, and the Geographic Mosaic of Adaptation by Parasites.

Am. Nat. 153:S34–S47.

Lloyd-Smith, J. O., S. J. Schreiber, P. E. Kopp, and W. M. Getz. 2005. Superspreading and the effect of individual variation on disease emergence. Nature 438:355–359.

Lohr, J. N., M. Yin, and J. Wolinska. 2010. Prior residency does not always pay off - co-infections in *Daphnia*. Parasitology 137:1493–1500.

Long, T. A. F., A. F. Agrawal, and L. Rowe. 2012. The effect of sexual selection on offspring fitness depends on the nature of genetic variation. Curr. Biol. 22:204–208.

Lord, C. C., B. Barnard, K. Day, J. W. Hargrove, J. J. McNamara, R. E. Paul, K. Trenholme, and M. E. Woolhouse. 1999. Aggregation and distribution of strains in microparasites. Trans. R. Soc. B 354:799–807.

Louhi, K.-R., L.-R. Sundberg, J. Jokela, and A. Karvonen. 2015. Interactions among bacterial strains and fluke genotypes shape virulence of co-infection. Proc. R. Soc. B 282:20152097.

Luijckx, P. 2012. Host specificity and genetics of host resistance in the *Daphnia-Pasteuria* host-parasite system. Doctoral Thesis, University of Basel.

Masri, L., R. D. Schulte, N. Timmermeyer, S. Thanisch, L. L. Crummenerl, G. Jansen, N. K. Michiels, and H. Schulenburg. 2013. Sex differences in host defence interfere with parasite-mediated selection for outcrossing during host-parasite coevolution. Ecol. Lett. 16:461–468.

Massey, R. C., A. Buckling, and R. ffrench-Constant. 2004. Interference competition and parasite virulence. Proc. R. Soc. B 271:785–788.

May, R. M., and R. M. Anderson. 1983. Epidemiology and genetics in the coevolution of parasites and hosts. Proc. R. Soc. B 219:281–313.

Maynard Smith, J. 1978. The evolution of sex. Cambridge University Press, Cambridge.

Maynard Smith, J. 1976. What determines the rate of evolution? Am. Nat. 110:331–338.

McCauley, E., R. M. Nisbet, W. W. Murdoch, A. M. de Roos, and W. S. C. Gurney. 1999. Large-amplitude cycles of *Daphnia* and its algal prey in enriched environments. Nature 402:653–656.

McCurdy, D. G., D. Shutler, A. Mullie, and M. R. Forbes. 1998. Sex-biased parasitism of avian hosts: relations to blood parasite taxon and mating system. Oikos 82:303–312.

Mideo, N. 2009. Parasite adaptations to within-host competition. Trends Parasitol. 25:261–268.

Mideo, N., S. Alizon, and T. Day. 2008. Linking within- and between-host dynamics in the evolutionary epidemiology of infectious diseases. Trends Ecol. Evol. 23:511–517.

Mideo, N., W. A. Nelson, S. E. Reece, A. S. Bell, A. F. Read, and T. Day. 2011. Bridging scales in the evolution of infectious disease life histories: application. Evolution 65:3298–3310.

Mouton, L., G. Nong, J. F. Preston, and D. Ebert. 2007. Variable-number tandem repeats as molecular markers for biotypes of *Pasteuria ramosa* in *Daphnia* spp. Appl. Environ. Microbiol. 73:3715–3718.

Napravnik, S., C. Poole, J. C. Thomas, and J. J. Eron. 2002. Gender difference in HIV RNA levels: a meta-analysis of published studies. J. Acquir. Immune Defic. Syndr. 31:11–9.

Olito, C., C. R. White, D. J. Marshall, and D. R. Barneche. 2017. Estimating monotonic rates from biological data using local linear regression. J. Exp. Biol. jeb.148775.

Ong, S. T., J. Z. Shan Ho, B. Ho, and J. L. Ding. 2006. Iron-withholding strategy in innate immunity. Immunobiology 211:295–314.

Osnas, E. E., and A. P. Dobson. 2012. Evolution of virulence in heterogeneous host communities under multiple trade-offs. Evolution 66:391–401.

Ots, I., A. B. Kerimov, E. V. Ivankina, T. A. Ilyina, and P. Horak. 2001. Immune challenge affects basal metabolic activity in wintering great tits. Proc. R. Soc. B 268:1175–1181.

Park, A. W., J. Jokela, and Y. Michalakis. 2010. Parasites and deleterious mutations: interactions influencing the evolutionary maintenance of sex. J. Evol. Biol. 23:1013–1023.

Parker, G. A. 2006. Sexual conflict over mating and fertilization: an overview. Trans. R. Soc. B 361:235–259.

Peters, A. D., and C. M. Lively. 1999. The red queen and fluctuating epistasis: a population genetic analysis of antagonistic coevolution. Am. Nat. 154:393–405.

Plowden, J., M. Renshaw-Hoelscher, C. Engleman, J. Katz, and S. Sambhara. 2004. Innate immunity in aging: impact on macrophage function. Aging Cell 3:161–167.

Poissant, J., a. J. Wilson, D. W. Coltman, and J. Alastair. 2010. Sex-specific genetic variance and the evolution of sexual dimorphism: A systematic review of cross-sex genetic correlations. Soc. Study Evol. 64:97–107.

Polak, M., L. W. Simmons, J. B. Benoit, K. Ruohonen, S. J. Simpson, S. M. Solon-biet. 2017. Nutritional geometry of paternal effects on embryo mortality. Proc. R. Soc. B 284:20171492.

Pomiankowski, A., and A. P. Møller. 1995. A resolution of the lek paradox. Proc. R. Soc. B. 260:21–29.

Poulin, R. 1996. Sexual inequalities in helminth infections: a cost of being a male. Am. Nat. 147:287–295.

Pusey, A. 1987. Sex-biased dispersal and inbreeding avoidance in birds and mammals. Trends Ecol. Evol. 2:295–299.

Råberg, L., J. C. de Roode, A. S. Bell, P. Stamou, D. Gray, and A. F. Read. 2006. The role of immune-mediated apparent competition in genetically diverse malaria infections. Am. Nat. 168:41–53.

Råberg, L., A. L. Graham, and A. F. Read. 2009. Decomposing health: tolerance and resistance to parasites in animals. Trans. R. Soc. B 364:37–49.

Read, A. F., and L. H. Taylor. 2001. The ecology of genetically diverse infections. Science 292:1099–1102.

Regoes, R. R., M. a Nowak, and S. Bonhoeffer. 2000. Evolution of virulence in a heterogeneous host population. Evolution 54:64–71.

Reynolds, J. J., E. Vander Wal, B. K. Adams, R. M. Curran, and C. M. Doucet. 2017. Does prey density predict characteristics of primiparity in a solitary and specialized predator, the Canada lynx (*Lynx canadensis*)? Can. J. Zool. 95:779–785.

Rigaud, T., M.-J. Perrot-Minnot, and M. J. F. Brown. 2010. Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. Proc. R. Soc. B 277:3693–3702.

Rolff, J. 2002. Bateman's principle and immunity. Proc. Biol. Sci. 269:867–72.

Rowe, L., and D. Houle. 1996. The lek paradox and the capture of genetic variance by condition dependent traits. Proc. R. Soc. B 263:1415–1421.

Salathé, M., A. Scherer, and S. Bonhoeffer. 2005. Neutral drift and polymorphism in gene-for-gene systems. Ecol. Lett. 8:925–932.

Schalk, G., and M. R. Forbes. 1997. Male biases in parasitism of mammals: effects of study type, host age, and parasite taxon. Oikos 78:67–74.

Schärer, L., L. Rowe, and G. Arnqvist. 2012. Anisogamy, chance and the evolution of sex roles. Trends Ecol. Evol. 27:260–264.

Schenk, D., D. Schenk, S. Bacher, and S. Bacher. 2002. Functional response of a generalist insect predator to one of its prey species in the eld. J. Anim. Ecol. 524–531.

Schmid-Hempel, P. 2013. Evolutionary Parasitology. Oxford University Press, Oxford, UK.

Seppälä, O., and J. Jokela. 2016. Do coinfections maintain genetic variation in parasites? Trends Parasitol. 32:930–938.

Seppälä, O., K. Liljeroos, A. Karvonen, and J. Jokela. 2008. Host condition as a constraint for parasite reproduction. Oikos 117:749–753.

Sgrò, C. M., and L. Partridge. 1999. A delayed wave of death from reproduction in *Drosophila*. Science 286:2521–2524.

Sharp, N. P., and A. F. Agrawal. 2013. Male-biased fitness effects of spontaneous mutations in *Drosophila melanogaster*. Evolution 67:1189–1195.

Sharp, N. P., and C. M. Vincent. 2015. The effect of parasites on sex differences in selection. Heredity 114:367–372.

Sheridan, L. a D., R. Poulin, D. F. Ward, and M. Zuk. 2000. Sex differences in parasitic infections among arthropod hosts: is there a male bias? Oikos 88:327–334.

Slusarczyk, M. 2009. Extended lifespan traded for diapause in *Daphnia*. Freshw. Biol. 54:2252–2262.

Smirnov, N. N. 2014. Physiology of the Cladocera. Academic Press.

Sofonea, M. T., S. Alizon, and Y. Michalakis. 2018. Why multiple infections favour virulent parasites. bioRxiv 258004.

Sommer, U., Z. M. Gliwicz, W. Lampert, and A. Duncan. 1986. The PEG-model of seasonal succession of planktonic events in fresh waters. Arch. Hydroviol. 106:433–471.

Stoehr, A. M., and H. Kokko. 2006. Sexual dimorphism in immunocompetence: what does life-history theory predict? Behav. Ecol. 17:751–756.

Swearer, S. E., and D. R. Robertson. 1999. Life history, pathology, and description of *Kudoa ovivora* n. sp. (Myxozoa, Myxosporea): an ovarian parasite of Caribbean labroid fishes. J. Parasitol. 85:337–353.

Tellier, A., S. Moreno-Gámez, and W. Stephan. 2014. Speed of adaptation and genomic footprints of host-parasite coevolution under arms race and trench warfare dynamics. Evolution 68:2211–2224.

Thompson, J. N. 1994. The coevolutionary process. Chicago University Press, Chicago.

Thompson, O., S. A. Y. Gipson, and M. D. Hall. 2017. The impact of host sex on the outcome of co-infection. Sci. Rep. 7:1–7.

Thrall, P. H., A.-L. Laine, M. Ravensdale, A. Nemri, P. N. Dodds, L. G. Barrett, and J. J. Burdon. 2012. Rapid genetic change underpins antagonistic coevolution in a natural host-pathogen metapopulation. Ecol. Lett. 15:425–435.

Vale, P. F., M. Stjernman, and T. J. Little. 2008. Temperature-dependent costs of parasitism and maintenance of polymorphism under genotype-by-environment interactions. J. Evol. Biol. 21:1418–1427.

Vale, P. F., A. J. Wilson, A. Best, M. Boots, and T. J. Little. 2011. Epidemiological, evolutionary, and coevolutionary implications of context-dependent parasitism. Am. Nat. 177:510–21.

van Baalen, M. 1998. Coevolution of recovery ability and virulence. Proc. R. Soc. B 265:317–325.

van Baalen, M., and M. W. Sabelis. 1995. The Dynamics of Multiple Infection and the Evolution of

Virulence. Am. Nat. 146:881–910.

van Valen, L. 1973. A new evolutionary law.

Vincent, C. M., and N. P. Sharp. 2014. Sexual antagonism for resistance and tolerance to infection in *Drosophila melanogaster*. Proc. R. Soc. B 281:20140987.

Vinogradov, A. E. 1998. Male reproductive strategy and decreased longevity. Acta Biotheor. 46:157–160.

vom Steeg, L. G., and S. L. Klein. 2016. SeXX Matters in Infectious Disease Pathogenesis. PLoS Pathog. 12:1–6.

White, C. R., M. R. Kearney, P. G. D. Matthews, S. A. L. M. Kooijman, and D. J. Marshall. 2011. A manipulative test of competing theories for metabolic scaling. Am. Nat. 178:746–754.

Whitlock, M. C., and A. F. Agrawal. 2009. Purging the genome with sexual selection: reducing mutation load through selection on males. Evolution 63:569–582.

Williams, P. D. 2012. New insights into virulence evolution in multigroup hosts. Am. Nat. 179:228–239.

Willink, B., and E. I. Svensson. 2017. Intra- and intersexual differences in parasite resistance and female fitness tolerance in a polymorphic insect. Proc. R. Soc. B 284:20162407.

Wolinska, J., and K. C. King. 2009. Environment can alter selection in host-parasite interactions. Trends Parasitol. 25:236–244.

Wood, S. N. 2017. Generalized additive models: an introduction with R. Second Edi. Chapman and Hall/CRC Press, Boca Raton, FL.

Woolhouse, M. E. J., J. P. Webster, E. Domingo, B. Charlesworth, and B. R. Levin. 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nat. Genet. 32:569–577.

Yashchenko, V., E. I. Fossen, Ø. N. Kielland, and S. Einum. 2016. Negative relationships between population density and metabolic rates are not general. J. Anim. Ecol. 85.

Zajitschek, F., and T. Connallon. 2017. Partitioning of resources: the evolutionary genetics of sexual

conflict over resource acquisition and allocation. J. Evol. Biol. 30:826–838.

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

Zuk, M. 2009. The sicker sex. PLoS Pathog. 5:e1000267.

Zuk, M., and A. M. Stoehr. 2002. Immune defense and host life history. Am. Nat. 160:S9–S22.

## Appendix – The impact of host sex on the outcome of co-infection

Olivia Thompson, Stephen A.Y. Gipson, and Matthew D. Hall*

School of Biological Sciences, Monash University, Melbourne, Victoria 3800, Australia

*Corresponding author. Email: [redacted]

Keywords: host-pathogen interactions, multiple infection, sexual dimorphism, infectious disease

Manuscript published in April 2017, *Scientific Reports* 7, 910

## Abstract

Males and females vary in many characteristics that typically underlie how well a host is able to fight infection, such as body-size, immune capacity, or energy availability. Although well studied in the context of sexual signalling, there is now growing recognition that these differences can influence aspects of pathogen evolution as well. Here we consider how co-infection between multiple pathogen strains is shaped by male-female differences. In natural populations, infections by more than one pathogen strain or species are believed to be a widespread occurrence. Using the water flea, *Daphnia magna*, we exposed genetically identical males and females to replicated bacterial co-infections. We found that pathogen transmission and virulence were much higher in females. However, males did not simply lower average pathogen fitness, but rather the influence of co-infection was more varied and less defined than in females. We discuss how pathogens may have more fitness benefits to gain, and consequently to lose, when infecting one sex over the other.

## Introduction

Males and females of the same species can be strikingly different from each other. They may differ in many overt traits, such as size, morphology or colouration [1]; but these differences can also extend to the immune response, with one sex often being more severely affected by infections [2,3]. In birds and mammals, for example, males are often more susceptible to infection and develop higher parasite loads than females [4,5], whereas the reverse pattern is often observed in invertebrates [6-8]. When combined with the diversity of sex ratios, sex biases in dispersal, and general behavioural difference between males and females [1,9,10], there is ample opportunity for dimorphism between the sexes to influence many aspects of disease epidemiology and evolution [3,8,11].

Here we consider how differences between males and females can influence a widespread feature of host-pathogen interactions – the influence of co-infections on the outcome of infection. Multiple infections are believed to be more common in nature than infections composed of only a single parasite species or genotype [12,13]. Both theory and empirical studies have shown that the overall virulence of a co-infection can increase, decrease, or remain intermediate to the intrinsic properties of each infection pathogen [14]. Rarely, however, have these concepts been explored within the context of sexual dimorphism in host resistance and exploitative potential. Co-infection

studies typically either explore infection outcomes in only one sex [15,16], or have not explicitly accounted for males-female differences [17].

We explored the interplay between host sex and co-infection using the well-studied crustacean *Daphnia magna* and its obligate bacterial pathogen *Pasteuria ramosa* [15,18,19]. In *Daphnia* up to 50 percent of all individuals in a population can be infected with two or more pathogen species [20], or eight or more genotypes of the one pathogen species [21]. Male *Daphnia*, however, are significantly smaller, more resistant, and die younger than females [22]; potentially offering a parasite a more difficult pool of resources to exploit than females. To study how the contrasting environments provided by males and females impacted on host and pathogen fitness, we manipulated pathogen competition in genetically identical male and female *Daphnia* (genotype HU-HO-2), using three *P. ramosa* genotypes (C1, C14, and C19) with known infection characteristics. In line with theory [14], time to host death and the total production of pathogen transmission spores were used as estimates of co-infection outcomes. We hypothesised that any differences between single and multiple infections seen in females would be dampened in males as a result of the sexual dimorphism in physiological characteristics.

## Results

Our results reveal that the production of transmission spores under single and co-infections depended on the sex of the host (Fig. 1). While all pathogen genotypes performed significantly better in females (overall sex effect $P < 0.01$, Table 1), the influence of the pathogen combinations was sex-specific (overall infection term, $P < 0.05$, Table 1). This significant interaction was driven by the co-infection combinations where the two pathogens differ in their capacity to produce spores (Figures 1C and 1D, see Table 1; but not Figure 1B). Corresponding to the pattern of pathogen growth, we found that the relative changes in virulence were concordant for each sex (Fig. 2). Overall the average reduction in host lifespan was strongly and negatively correlated with the average production of mature transmission spores (correlation [95% CI]: females -0.93 [-0.99, -0.45]; males -0.98 [-0.99, -0.78]). Although females suffered a much higher reduction in lifespan than males (overall sex effect: $P < 0.01$, Table 1), there was no evidence for differences between the sexes in how well different pathogen combinations induced virulence (overall interaction term, $P > 0.05$, Table 1).

We compared virulence under coinfection with that expected based on interference competition (intermediate values), competition for resources (higher values than most virulent) or spite (lower values than the least virulent). Post-hoc comparisons, as indicted in Figures 1 and 2, revealed that co-infection scenarios similar to resource competition or spite are least unlikely, as co-infection never significantly exceeded the most virulent pathogen, nor was lower than the least virulent. Instead, co-infection largely matched the average of the innate characteristics of each infecting genotype in isolation; albeit with some sex-specific differences. When the two pathogens differed in their virulence or spore production, co-infection in females was indistinguishable from the average of the two single infections, and different to that predicted by either the least or most virulent strain (see Table 2). In contrast, coinfection in males was more varied, with one co-infection (C1 + C19) not significantly different to the predicted intermediate values, and another (C14 + C19) more similar to the most virulent and least transmissive strain (C19).

## Discussion

Males and females vary in many characteristics that combine to offer pathogens two environments that differ in their exploitative potential [3]. Here, males of *Daphnia magna* are smaller and more resistant to infection than their genetically identical female counterparts [22], a pattern common to many other arthropods [6,7]. Our results show that these differences change how well a pathogen can exploited a host, with pathogen transmission (mature spore loads) and virulence (reduction in expected lifespan) being much higher in females. However, the presence of the significant interaction term for pathogen transmission indicates that males do not simply lower average pathogen fitness, but rather the transition from the worst performing pathogen, to the mixed co-infection, to the best performing pathogen was different in each sex.

Emerging from our results is that the best performing pathogens have the most to gain from single infections in females, but also the most to lose from co-infections in the same sex. High-growth pathogen strains (C1 or C14) outperformed their lesser performing competitor (C19) in single infections by 2.1 million spores on average in females, versus only 0.55 million spores in males. As the evolutionary advantage of a pathogen depends its success relative to other competitors, not its absolute transmission, this represents a nearly four-fold improvement in relative fitness. Conversely, the same pathogens also experienced the greatest reduction in transmission success when co-infections occurred in females. Total spore loads for a co-infection were always less than that of the high-growth pathogen strain in isolation; a substantial loss of potential fitness even if

the other pathogen was outcompeted completely. We also cannot rule out that the lesser performing pathogen in isolation (C19) is a superior competitor in a co-infection, a fact that would further decrease the fitness of the pathogen ranked best in isolation. Overall, this loss of fitness for high growth pathogens (and potential gain for low growth pathogens) was three-fold higher in females (1.2 million spores) than males (0.41 million spores).

In the context of the co-infection virulence the distinction between males and females was more subtle. For females, virulence in co-infections closely matched those expected by interference competition or immune-mediated competition between competing strains [14]. Overall virulence of a co-infection was firmly intermediate to that of the two competing pathogens, ruling out alternate explanations that predict either an increase (competition for a finite pool of resources, or facilitation via immune depletion) or a decrease (generation of spite) in virulence [14]. Evidence for interference competition-like mechanisms in males was less well-supported, as certain co-infection combinations tracked the overall characteristics of the least virulent pathogen. Given the lack of an overall significant interaction term for virulence, however, it appears that co-infection in males does not fundamentally change the driver of overall virulence, but rather that the patterns of co-infection in males may be less defined or extreme than in females.

As with any form of sexual dimorphism, underlying the observed differences in pathogen virulence and transmission may be a range of morphological, physiological, or immune based processes. In this system, males are more resistant (in terms of infection success) and so presumably also better equipped to limit the growth of a pathogen. For male and female *Daphnia*, however, an obvious point of difference is the divergence in body size [22], whereby males are approximately 25 percent smaller than females. This could also explain the results we observe; irrespective of any immune response. With the larger body size and greater capacity for gigantism (*i.e.* pathogen induced growth) in females, there is naturally more resources available for a pathogen to exploit (energy or space for replication, for example). Infections in males may just be more constrained by the host, limiting both the potential fitness gains, and even loses, that a pathogen can derive from infection in this sex.

Our findings divert somewhat from previous studies in this system which have typically assumed that virulence tracks the most virulent co-infecting strain [15], but increasingly it is becoming clear that co-infections depend as much on the specific genotype of the pathogens involved, as well as that of the host, and their interaction [17,24]. We suggest that heterogeneity between the sexes is an

additional factor that is likely to influence co-infection outcomes by reducing the intensity of co-infection scenarios when males are infected. Extending this work to include genetic difference in the degree of sexual dimorphism, which we expect given the strong clonal differences observed in *Daphnia*, would further integrate the study of host-pathogen co-evolution with the evolution of male-female differences (see [3]).

## Methods

The host and pathogen genotypes used in this study were chosen as completely compatible and displayed strong genetic interactions for the severity of infectious disease in earlier studies [19,23]. The host genotype (HU-HO-2) originates from Hungary, while the three pathogen genotypes were C1 originating from Moscow, Russia; C19, derived from North Germany; and, C14 from Finland, Tvärminne. Various combinations of these clones, in some form, have been used previously in studies of co-infection [24,25].

### *Production of male and female Daphnia*

To reliably induce the production of males and females from a single *Daphnia* genotype, we used a modified protocol based on the work of Olmstead and Keblanc [26]. Briefly, we exposed the parental generation to a juvenoid hormone, methyl farnesoate (Echelon Biosciences, product number S-0153), after their first and second clutches, and then collected the third and fourth clutches for use in this experiment. Individuals were placed in 20-mL of the standard *Daphnia* media (ADaM; [27], modified after [28]), but supplemented with methyl farnesoate at a concentration of 300 μg/L. This media was changed either every two days, or when an animal produced a clutch. Importantly, we found that the hormone had no detectable effect on the subsequent fitness of the offspring.

A short pulse of hormone has been used in previous studies to study male-female differences in genetically identical *Daphnia* [29]. To confirm that the hormone had negligible impact on the life-history of exposed animals, we conducted a series of experimental trials (see Table 3) using female *Daphnia* raised from mothers that were either exposed to the hormone or raised normally in standard *Daphnia* media. We found that there was no significantly difference between the animals collected from treated and untreated mothers in the trait means and variances of both general life-history traits (lifespan and clutch production), and the characteristics of infection (infection rates; spore loads, lifespan, and clutch production of infected animals). Using this approach

removes the need to stress mothers (low-light, high-density, or starvation) to induce male production; a process which can also influence infection characteristics [19,30].

### *Experimental infection trials*

Animals were first raised individually in 60-mL jars containing 50 mL of artificial *Daphnia* medium following standard protocols [18, 19], and fed daily with algae (*Chlorella vulgaris*, up to 5 million cells animal$^{-1}$ day$^{-1}$). At four days old, individual males and females were exposed to 20,000 spores from a single *P. ramosa* genotype (C1, C14, and C19), or 20,000 spores containing an equal mix of two genotypes (C1+C14, C1+C19, or C14+C19, 10,000 per genotype). As per standard approaches, this process was repeated the following day when the animals were five days old (40,000 spores in total) to ensure that an internal immune or physiological response, rather than moulting, contributed to differences in infection (see [31]). In total, there were 12 treatment groups (two host sex groups × [3 single infections + 3 multiple infections]), with 36 animals initially assigned to each, plus 20 unexposed controls per sex (472 initial animals).

Survival was monitored daily and animals frozen on the day of death for the assessment of infection status and spore production using a Neubauer haemocytometer. As expected, overall infection rates were lower in males (mean ± SE: 0.58 ± 0.06; range: 0.35 to 0.70) than females (mean ± SE: 0.83 ± 0.04; range: 0.69 to 0.92). Analysis of variance revealed that infection rates indeed varied significantly with host sex ($\chi^2$ = 33.18, df = 1, P < 0.01) and the co-infection treatments ($\chi^2$ = 18.91, df = 5, P < 0.01), but were otherwise largely consistent (interaction term: $\chi^2$ = 6.13, df = 5, P = 0.29). Due to difference in the average lifespan between males and females (males: 33 days ± 1.9; females: 67 days ± 2.0), we subsequently focused on two traits of common currency: the production of transmission spores at host death; and, the reduction in lifespan as compared to the average of the unexposed controls.

### *Statistical analysis*

All statistical analyses were performed in R (ver. 3.2.4; R Development Core Team, available at www.r-project.org). Initially there were 472 animals exposed to the co-infection treatments, arising from approximately 36 animals each assigned to 12 treatment groups (two host sex groups × [3 single infections + 3 multiple infections]) plus 20 male and 20 female unexposed controls. However, this sample size was reduced by the end of the experiment as: i) not all animals became infected; ii) they died before day 15 post-infection and so could not reliably have their infection

status assessed; or iii) were removed from the study due to sampling errors (see supplementary Information for more details). Before analyses, we checked for the assumptions of linear models (normality, homogeneity of variances). In the case of the spore loads at host death and the reduction in average lifespan, we used a white-adjusted analysis of variance to correct for unequal variances as implement using the *car* package of R [32].

Traits were first analysed using a full-factorial analysis of variance (Type III) with the infection treatment (various single or multiple infections), host sex (male or female), and their interaction as fixed effects. For each trait of interest, we began first with an overall test, using the entire dataset (both single and multiple infection together). If a significant effect was detected (host sex, Infection treatment, or their interaction) we then explored each subset of pathogen combinations (*e.g.* C1 versus C14 versus C1+C14) to see if specific single versus multiple infections combinations were contributing to the overall pattern. In this way, we avoid issues of multiple testing that would arise if we explored every related combination of single and multiple infections in isolation. Finally, one sample t-tests were used to compare the values for co-infection treatment groups against expected value based on interference competition (intermediate values), competition for resources (higher values than most virulent) or spite (lower values than the least virulent).

## Acknowledgements

We thank V. Sundaramoorthi for assistance. This work was supported by an ARC Discovery Early Career Researcher Award to MDH.

## Author contributions

OT, SG, and MH conceived and designed the study. OT and SG carried out the lab work. OT and MH drafted the manuscript. All authors gave final approval for publication.

## Availability of materials and data

Datasets supporting the conclusions of this article are available via a figshare repository at https://dx.doi.org/10.4225/03/585732493b9ad

## Competing interests

The authors declare no competing financial interests.

## References


1. Andersson, M. B. Sexual Selection. (Princeton University Press, 1994).
2. Zuk, M. The sicker sex. PLoS Path 5, e1000267 (2009).
3. Gipson, S. A. Y. & Hall, M. D. The evolution of sexual dimorphism and its potential impact on host-pathogen coevolution. Evolution 70, 959–968 (2016).
4. Poulin, R. Sexual inequalities in helminth infections: A cost of being a male? Am Nat 147, 287–295 (1996).
5. Schalk, G., Forbcs, M. R., Schalk, G. & Forbes, M. R. Male biases in parasitism of mammals: Effects of study type, host age, and parasite taxon. Oikos 78, 67–74 (1997).
6. Stoehr, A. M. & Kokko, H. Sexual dimorphism in immunocompetence: what does life-history theory predict? Sexual dimorphism in immunocompetence: what does life-history theory predict? Behav Ecol 17, 751–756 (2006).
7. Sheridan, L. A. D., Poulin, R., Ward, D. F. & Zuk, M. Sex differences in parasitic infections among arthropod hosts: Is there a male bias? Oikos 88, 327–334 (2000).
8. Duneau, D. & Ebert, D. Host sexual dimorphism and parasite adaptation. Plos Biol 10, e1001271 (2012).
9. Cluttonbrock, T. H. & Iason, G. R. Sex-ratio variation in mammals. Q Rev Biol 61, 339–374 (1986).
10. Pusey, A. E. Sex-biased dispersal and inbreeding avoidance in birds and mammals. Trends Ecol Evol 2, 295–299 (1987).
11. Cousineau, S. V. & Alizon, S. Parasite evolution in response to sex-based host heterogeneity in resistance and tolerance. J Evol Biol 27, 2753–2766 (2014).
12. Read, A. F. The ecology of genetically diverse infections. Science 292, 1099–1102 (2001).
13. Petney, T. N. & Andrews, R. H. Multiparasite communities in animals and humans: frequency, structure and pathogenic significance. Int J Parasitol 28, 377–393 (1998).
14. Alizon, S., de Roode, J. C. & Michalakis, Y. Multiple infections and the evolution of virulence. Ecol Lett 16, 556–567 (2013).
15. Ben Ami, F., Mouton, L. & Ebert, D. The effects of multiple infections on the expression and evolution of virulence in a Daphnia-endoparasite system. Evolution 62, 1700–1711 (2008).
16. Taylor, L. H., Mackinnon, M. J. & Read, A. F. Virulence of mixed-clone and single-clone infections of the rodent malaria Plasmodium chabaudi. Evolution 52, 583–591 (1998).
17. Louhi, K.-R., Sundberg, L.-R., Jokela, J. & Karvonen, A. Interactions among bacterial strains

and fluke genotypes shape virulence of co-infection. Proc R Soc B 282, 20152097–8 (2015).

18. Clerc, M., Ebert, D. & Hall, M. D. Expression of parasite genetic variation changes over the course of infection: implications of within-host dynamics for the evolution of virulence. Proc R Soc B 282, 20142820 (2015).
19. Hall, M. D. & Ebert, D. Disentangling the influence of parasite genotype, host genotype and maternal environment on different stages of bacterial infection in Daphnia magna. Proc R Soc B 279, 3176–3183 (2012).
20. Decaestecker, E., Declerck, S., De Meester, L. & Ebert, D. Ecological implications of parasites in natural Daphnia populations. Oecologia 144, 382–390 (2005).
21. Mouton, L., Nong, G., Preston, J. F. & Ebert, D. Variable-number tandem repeats as molecular markers for biotypes of Pasteuria ramosa in Daphnia spp. Appl Environ Microbiol 73, 3715–3718 (2007).
22. Duneau, D., Luijckx, P., Ruder, L. F. & Ebert, D. Sex-specific effects of a parasite evolving in a female-biased host population. BMC Biol 10, 104 (2012).
23. Luijckx, P., Ben Ami, F., Mouton, L., Pasquier, Du, L. & Ebert, D. Cloning of the unculturable parasite Pasteuria ramosa and its Daphnia host reveals extreme genotype-genotype interactions. Ecol Lett 14, 125–131 (2010).
24. Ben Ami, F. & Routtu, J. The expression and evolution of virulence in multiple infections: the role of specificity, relative virulence and relative dose. BMC Evol Biol 13, 97–1 (2013).
25. Izhar, R., Routtu, J. & Ben Ami, F. Host age modulates within-host parasite competition. Biol Lett 11, 20150131–20150131 (2015).
26. Olmstead, A. W. & Leblanc, G. A. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean Daphnia magna. J Exp Zool 293, 736–739 (2002).
27. Klüttgen, B., Dülmer, U., Engels, M. & Ratte, H. T. ADaM, an artificial freshwater for the culture of zooplankton. Water Research 28, 743–746 (1994).
28. Ebert, D., Zschokke-Rohringer, C. D. & Carius, H. J. Within-and between-population variation for resistance of Daphnia magna to the bacterial endoparasite Pasteuria ramosa. Proc R Soc B 265, 2127–2134 (1998).
29. Lohr, J. N., David, P. & Haag, C. R. Reduced lifespan and increased ageing driven by genetic drift in small populations. Evolution 68, 2494–2508 (2014).
30. Hall, M. D., Vettiger, A. & Ebert, D. Interactions between environmental stressors: the influence of salinity on host-parasite interactions between Daphnia magna and Pasteuria ramosa. Oecologia 171, 789–796 (2013).

31. Duneau, D. & Ebert, D. The role of moulting in parasite defence. Proc R Soc B 279, 3049–3054 (2012).
32. Fox, J. & Weisberg, S. An R Companion to Applied Regression. (SAGE Publications, 2011).

**Table 1:** Influence of host sex and co-infection on the production of transmission spores, and the relative reduction in host lifespan. In bold are the overall tests using the entire dataset for the influence of host sex on the outcome of all infection treatments (all single and co-infections together). Below these are the individual analyses for each co-infection combination.

| | | **Infection treatment** | | | **Host sex** | | | **Interaction** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | F-ratio | df | P-value | F-ratio | df | P-value | F-ratio | df | P-value |
| **Spore loads at death** | | **8.80** | **5, 259** | **<0.001** | **170.41** | **1, 259** | **<0.001** | **3.02** | **5, 259** | **0.011** |
| *Subset by:* | *C1 + C14* | 0.33 | 2, 133 | 0.723 | 105.19 | 1, 133 | <0.001 | 1.632 | 2, 133 | 0.199 |
| | *C1 + C19* | 10. | 2, 125 | <0.001 | 75.30 | 1, 125 | <0.001 | 4.61 | 2, 125 | 0.012 |
| | *C14 + C19* | 10.01 | 2, 116 | <0.001 | 67.17 | 1, 116 | <0.001 | 6.55 | 2, 116 | 0.002 |
| **Reduction in lifespan** | | **12.18** | **5, 263** | **<0.001** | **288.26** | **1, 263** | **<0.001** | **1.60** | **5, 263** | **0.159** |
| *Subset by:* | *C1 + C14* | 0.20 | 2, 136 | 0.819 | 119.69 | 1, 136 | <0.001 | 0.90 | 2, 136 | 0.407 |
| | *C1 + C19* | 10.96 | 2, 127 | <0.001 | 182.94 | 1, 127 | <0.001 | 1.00 | 2, 127 | 0.368 |
| | *C14 + C19* | 11.36 | 2, 117 | <0.001 | 119.02 | 1, 117 | <0.001 | 3.44 | 2, 117 | 0.036 |

**Table 2:** Tests of equality between the production of spores and the relative reduction in lifespan in the co-infection treatments. Presented are one sample t-tests comparing the co-infection treatment groups against the predicted means of various single infection outcomes. We compared virulence under coinfection with that expected based on interference competition (intermediate values), competition for resources (higher values than most virulent) or spite (lower values than the least virulent). The sign of the t-value indicates whether the co-infection outcome was lower or higher than expected.

| | | **Female co-infections** | | | **Male co-infections** | | |
|---|---|---|---|---|---|---|---|
| | | t-value | df | P-value | t-value | df | P-value |
| **True mean is equal to the average of the two single infections** | | | | | | | |
| *Spore loads:* | *C14 + C1* | -2.243 | 29 | 0.033 | 0.669 | 20 | 0.511 |
| | *C19 + C1* | 0.830 | 31 | 0.413 | -0.866 | 21 | 0.396 |
| | *C19 + C14* | -0.718 | 26 | 0.479 | -2.329 | 17 | 0.032 |
| *Lifespan reduction:* | *C14 + C1* | 0.113 | 31 | 0.912 | -0.957 | 20 | 0.350 |
| | *C19 + C1* | -0.558 | 31 | 0.581 | -0.781 | 21 | 0.444 |
| | *C19 + C14* | 0.804 | 26 | 0.429 | 4.510 | 17 | <0.001 |
| **True mean is equal to the average of the most virulent strain and least transmissive strain** | | | | | | | |
| *Spore loads:* | *C14 + C1* | -2.182 | 29 | 0.037 | 1.483 | 20 | 0.154 |
| | *C19 + C1* | 5.846 | 31 | <0.001 | 1.536 | 21 | 0.140 |
| | *C19 + C14* | 2.082 | 26 | 0.048 | -0.756 | 17 | 0.460 |
| *Lifespan reduction:* | *C14 + C1* | -0.893 | 31 | 0.389 | -1.300 | 20 | 0.209 |
| | *C19 + C1* | -3.086 | 31 | 0.004 | -3.275 | 21 | 0.003 |
| | *C19 + C14* | -1.981 | 26 | 0.053 | 2.139 | 17 | 0.047 |
| **True mean is equal to the average of the least virulent and most transmissive strain** | | | | | | | |
| *Spore loads:* | *C14 + C1* | -2.305 | 29 | 0.029 | -0.145 | 20 | 0.886 |
| | *C19 + C1* | -4.187 | 31 | 0.002 | -3.268 | 21 | 0.004 |
| | *C19 + C14* | -3.517 | 26 | 0.002 | -3.902 | 17 | 0.001 |
| *Lifespan reduction:* | *C14 + C1* | 1.112 | 31 | 0.273 | -0.615 | 20 | 0.546 |
| | *C19 + C1* | 1.970 | 31 | 0.058 | 1.713 | 21 | 0.104 |
| | *C19 + C14* | 3.589 | 26 | 0.001 | 6.877 | 17 | <0.001 |

**Table 3:** The influence of the hormone treatment on the fitness characteristics of the following offspring generation. Equality of means was assessed using a least-squares linear model in the case of lifespan and spore loads, and a generalised linear model for the number of clutches produced (Poisson distribution, log link function) and infection rates (Binomial distribution, logit link function). Where appropriate, Levene's test was used to assess homogeneity of variances between the offspring of untreated and treated mothers. All measures were estimated using a single genotype of both *D. manga* (HU-HO-2) and *P. ramosa* (C14), following the same basic infection process outlined in this study.

| | **Untreated mothers** | | **Treated mothers** | | **Equality of means** | | | **Equality of variances** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | F or $\chi^2$ | df | P-value | F or $\chi^2$ | df | P-value |
| **Trial 1: Characteristics of uninfected animals or controls** | | | | | | | | | | |
| Lifespan (days) | 66.86 | 5.04 | 67.36 | 4.71 | 0.705 | 1, 268 | 0.402 | 0.570 | 1, 268 | 0.451 |
| Clutches produced | 11.73 | 1.68 | 11.49 | 1.62 | 0.334 | 1, 268 | 0.563 | 0.201 | 1, 268 | 0.654 |
| **Trial 2: Characteristics of infected animals** | | | | | | | | | | |
| Infection rates | 0.89 | 0.05 | 0.86 | 0.054 | 0.002 | 1, 69 | 0.966 | – | – | – |
| Spore loads (millions) | 3.97 | 1.79 | 3.89 | 1.94 | 0.029 | 1, 61 | 0.866 | 0.076 | 1, 61 | 0.784 |
| Lifespan (days) | 51.88 | 11.82 | 50.55 | 13.38 | 0.174 | 1, 61 | 0.678 | 0.137 | 1, 61 | 0.713 |
| Clutches produced | 3.03 | 1.28 | 3.10 | 0.83 | 0.022 | 1, 61 | 0.882 | 1.362 | 1, 61 | 0.248 |

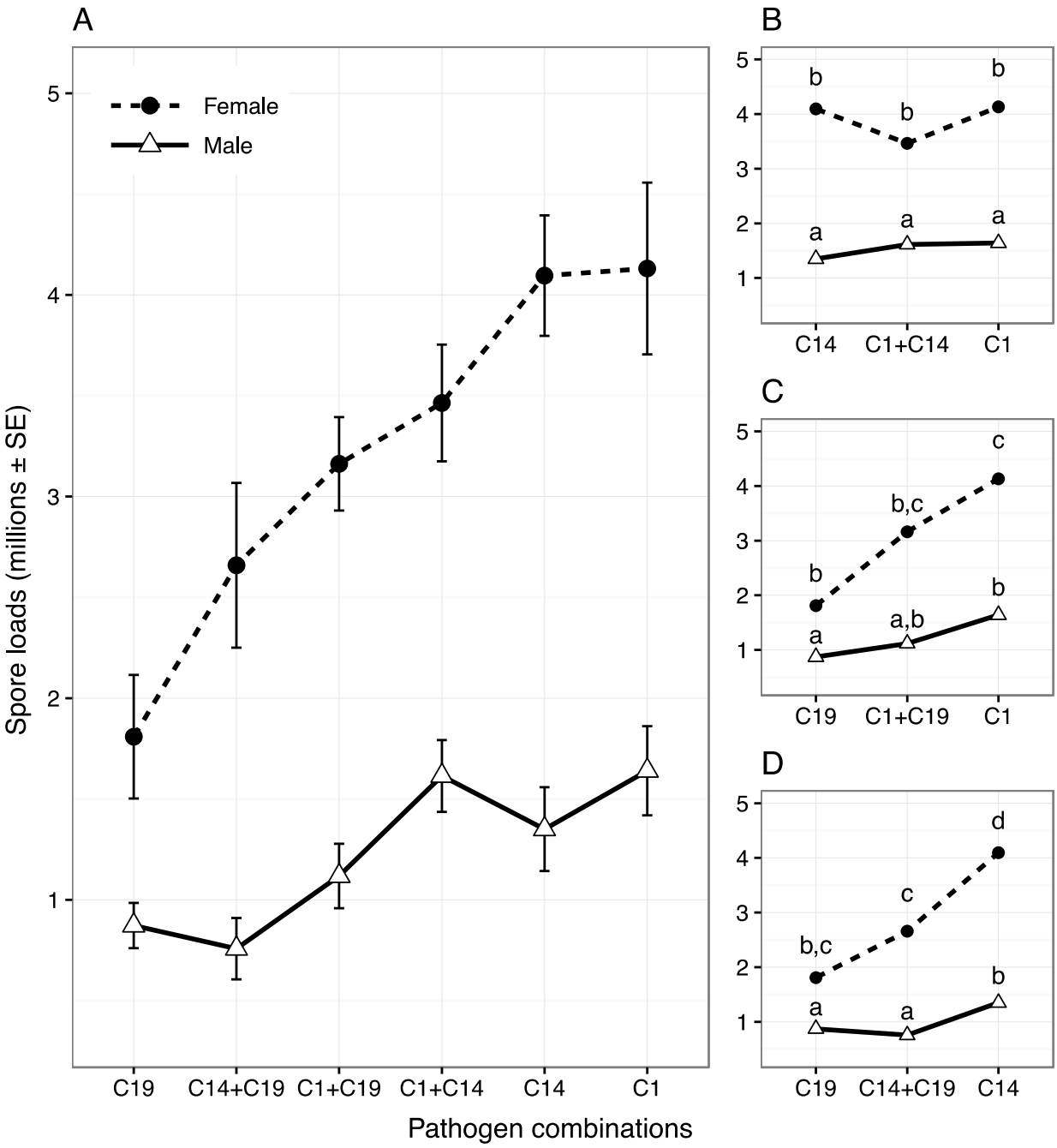


**Figure 1:** Transmission spores at death for all co-infection treatments. Pathogen combinations in panel A are ordered by spore loads in infected females (left to right, lowest to highest). Panels B to D show the same results subset by the co-infection combinations, with lower-case letters indicating significant groupings via post-hoc t-tests and Benjamini & Hochberg adjusted p-values.

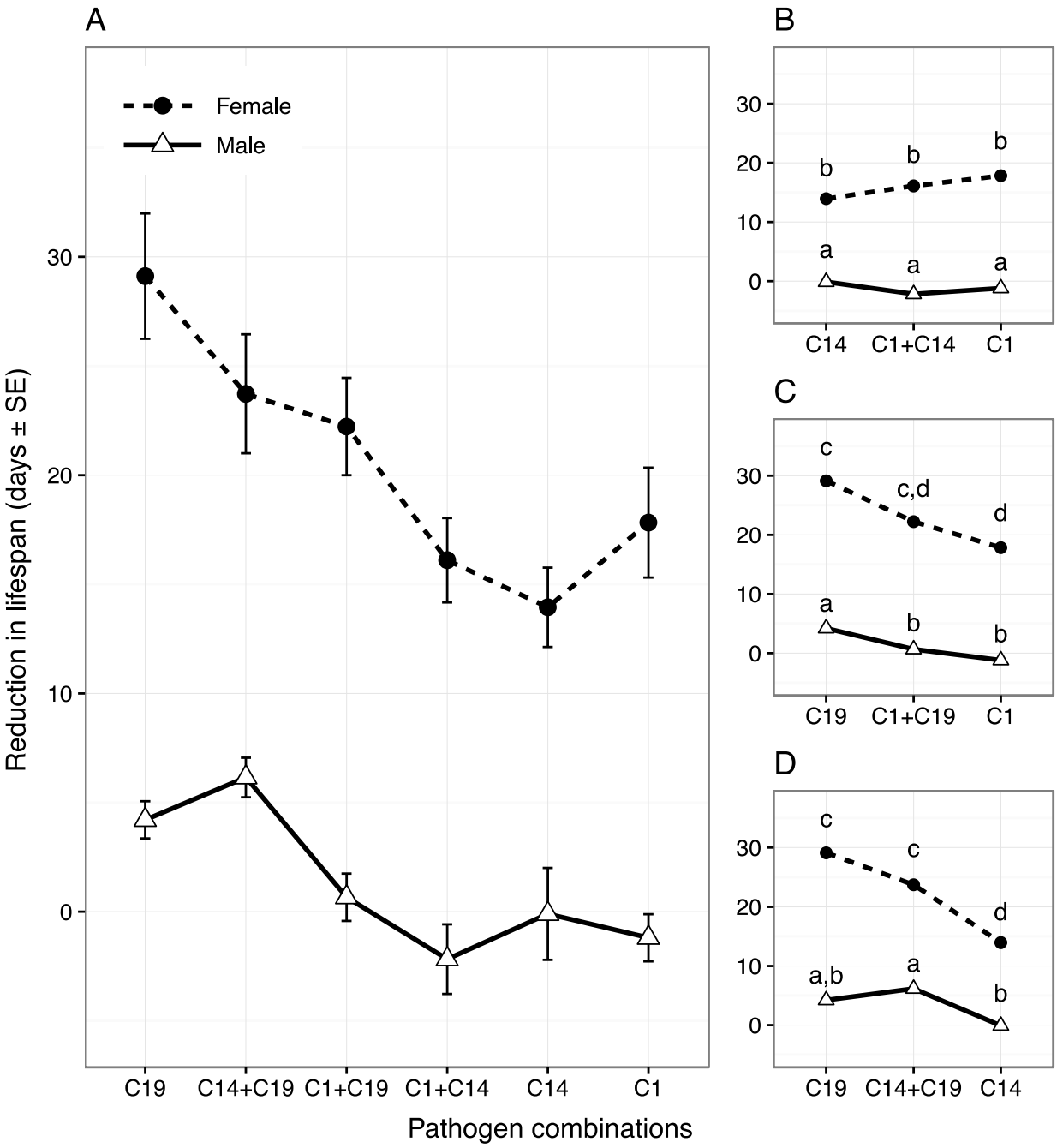


**Figure 2:** Relative reduction in host lifespan for all co-infection treatments. Pathogen combinations in panel A are ordered by spore loads in infected females (left to right, lowest to highest). Panels B to D show the same results subset by the specific co-infection combinations, with lower-case letters indicating significant groupings via post-hoc t-tests and Benjamini & Hochberg adjusted p-values.