THE INFLUENCE OF HOST DIVERSITY,

TRANSMISSION AND MICROBIOME

ON VIRULENCE EVOLUTION

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biology

The University of Utah

August 2017

The University of Utah Graduate School

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ABSTRACT

The harm caused to a host (virulence) is an important aspect to any pathogenic infection and is influenced by many different factors. Here I seek to understand how three of these factors, host genetic diversity, transmission, and gut microbial diversity, influence the virulence of a murine specific retrovirus, Friend Virus Complex (FVC). Chapter 1 explores the effect of major histocompatibility complex (MHC) diversity on virulence. Using serial passage of FVC, through either MHC similar or MHC dissimilar mice, I show that there is a significant reduction of both fitness and virulence of FVC when a dissimilar genotype is seen than when FVC is passaged through the same genotype; suggesting that MHC diversity is an impediment to virulence evolution. Furthermore, the alternating patterns reemerged after infection with a virus adapted to resistant animals that initially swamped the alternating effect, providing evidence for negative frequency-dependent selection maintaining MHC diversity in host populations. Chapter 2 elucidates the influences natural transmission, sex, and social status have on virulence using wild-derived contact (initially uninfected) and index (initially infected) animals in seminatural enclosures. Male-male transmission is the predominant mode of transmission as minimal female transmission and no vertical transmission was observed. Moreover, natural transmission is an impediment to FVC replication as infected contact animals had lower viral titer and virulence than index animals. Finally, though dominant and nondominant males contract the virus at similar rates and experience similar

virulence, nondominant animals have higher titers. Chapter 3 seeks to understand how the microbiome influences pathogen virulence. After antibiotic treatment, animals of two different MHC congenic genotypes were reconstituted with gut microbiota from a donor of their own MHC genotype (native) or from a donor with a different MHC genotype (novel). After challenge with FVC, significantly higher titers were seen in animals receiving novel microbiota than animals receiving native microbiomes. There was only a shift down in total T-lymphocyte number in novel groups as no other cell subsets tested showed a change in abundance. The work presented here allows us to gain a better understanding of how virulence is impacted by a multitude of different forces, and that many different aspects need to be taken into account when trying to determine the evolution of virulence.

Dedicated to my wife Kylie and my family.

Thank you for always being there.

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ACKNOWLEDGMENTS

First, I would like to thank my advisor Wayne Potts for taking a chance on a less than stellar undergraduate and giving me the opportunity to learn and become the scientist that I am. Second, all of the lab members past and present that helped me through and were always available when I hit a road block in my science. I would especially like to thank James Ruff, Jason Kubinak, and Earl Middlebrook, I really don't know if I would have made it through without your help. I would also like to give a special thanks to Linda Morrison who always had an answer for me when something was going terribly wrong. I would also like to thank my committee, Drs. Frederick Adler, Sarah Bush, Kim Hasenkrug, and Jon Seger, each one of them made my science better and challenged me to think and write more like a scientist. Finally, I would like to thank all of the undergraduates, especially Elisabeth Zachary and Sophie Trujillo whom I was able to mentor, they all made my life a lot easier and I cannot express my gratitude for what they were all able to accomplish. This work was supported by the National Institutes of Health.

CHAPTER 1

INTRODUCTION

Virulence is an important component of any pathogenic infection, as it is the harm

caused to the host by the pathogen. Moreover, there are many factors that lead to

virulence, or lack of virulence, within a host; however, many of these components are

still unknown or do not have sufficient testing in the literature (Alizon *et al.*, 2009).

Genetic diversity in a host population, transmission patterns, and most recently, microbial

diversity have all been suggested to control virulence of pathogens and are the focus of

this dissertation (Ebert & Bull, 2008; Koch & Schmid-Hempel, 2012; Buffie & Pamer,

2013). Conversely, the evolutionary pressures leading to virulence have also been

thought to influence diversity in host populations through antagonistic interactions

between host and pathogen (Salathé *et al.*, 2008; Brockhurst *et al.*, 2014).

Since the discovery of the major histocompatibility complex (MHC) in the late

1940s, there has been strong interest in the unprecedented genetic diversity found at the

classic peptide-antigen presenting genes within the complex (Snell, 1948). JBS Haldane

hypothesized that the purpose of the diversity of these cell surface proteins may be to

combat the constant barrage of infectious pathogens (Haldane, 1949). Haldane's

hypothesis was later refined and dubbed the Red Queen (RQ) hypothesis (Van Valen,

1973; Bodmer *et al.*, 1986). The RQ hypothesizes that as pathogens adapt to a single

host, they become more specialized on that host genotype. It follows that those pathogens will then have a more difficult time replicating in hosts with dissimilar host genotypes. Through these interactions, and with varying amounts of allele frequencies in host populations, rare beneficial alleles (initially derived through mutations) will rise in frequency due to the decline of common alleles to which pathogens are adapting. This cyclical process is termed frequency-dependent selection and is one hypothesis for how MHC diversity is maintained in host populations (Apanius *et al.*, 1997; Sutton *et al.*, 2011; Brockhurst *et al.*, 2014).

During MHC-specific RQ coevolution, the pathogen has a selective advantage when mutations that abolish MHC binding to a particular peptide occur. However, if there are more alleles capable of recognizing a higher number of viral peptides and these alleles are distributed throughout a population, this MHC diversity can contribute to counteracting the viral fitness advantage; because escape from one allele does not mean escape from all alleles (Finlay & McFadden, 2006). Classical MHC genes have been shown to be associated with pathogen load, where an increased number of alleles in a host population correlates with a lower number of parasites and pathogens affecting that population (Jeffery & Bangham, 2000; Radwan *et al.*, 2010). Furthermore, serial passage studies have shown that the two main tenets of the RQ hold true: genotype specific adaption occurs and there is a tradeoff in pathogen virulence when new genotypes are seen (Ebert, 1998; Kubinak *et al.*, 2012, 2013). MHC diversity in a population has also been suggested to have a major role in defense against pathogen adaptation (Kubinak *et al.*, 2013). Nonetheless, it is still unknown how rapid this host genotype specific adaptation is and to what extent MHC plays a role when adaptation time is limited, as

when a pathogen is moving through a population.

The vertebrate immune system consists of two different, but interconnected responses, the innate and adaptive. Innate immune responses consist of an immediate broad response to the foreign invader, usually within a few hours of infection (Abbas *et al.*, 2012). The adaptive response, on the other hand, is a highly specific response usually driven by T-lymphocyte recognition of MHC presenting a foreign peptide (protein segments that have been degraded by cellular machinery) (Germain, 1994). After intracellular binding of MHC to a peptide, the MHC-peptide complex is presented on the outside of the cell to be interrogated by circulating T-lymphocytes (T-cells). Following recognition by a T-cell receptor (TCR), that T-cell clonally proliferates and becomes activated for cytopathic activities, allowing for recognition and destruction of other cells presenting the same MHC-peptide complex (Watts, 1997). If a viral pathogen mutates part of the peptide that the MHC molecule is binding (anchor positions), it will not be presented and will therefore escape immune recognition through that foreign peptide, usually leading to an increase in viral fitness (Finlay & McFadden, 2006).

The studies in this dissertation utilize Friend Virus Complex (FVC) as a mouse-specific pathogen to test various hypotheses concerning factors that influence virulence evolution. FVC is a murine specific gamma retrovirus that preferentially infects dividing erythroblasts, leading to enlargement of the spleen and liver (Friend, 1957). Given that one site of FVC replication is the spleen, it is ideal to use for serial passage studies as it is easily titered and passaged. FVC is referred to as a complex because it consists of two viral genomes that work together synergistically to cause disease. The first genome, Friend Murine Leukemia Virus (F-MuLV) is a replication competent retrovirus that can

complete its entire life cycle independent of other genomes or pathogens (Hasenkrug & Chesebro, 1997). Spleen Focus Forming Virus (SFFV), on the other hand, cannot complete its life cycle without the help of F-MuLV, as the envelope of the F-MuLV complements SFFV and allows SFFV to exit the cell. Though SFFV cannot produce the necessary proteins that allow it to form a capsid and exit the cell, it can stimulate the erythropoietin receptor and causes uncontrolled proliferation of the infected erythroblast (Ruscetti, 1999). In this way, SFFV creates more target cells for F-MuLV, which in turn complements SFFV, allowing for a synergistic interaction that leads to higher fitness for both viral genomes.

Inbred laboratory mice are powerful tools to understand how pathogen virulence is influenced by a multitude of factors. MHC congenic strains are identical throughout the entire genome with the exception of the three megabase gene region of the MHC. Utilizing three strains of MHC congenic mice (Balb/c^{dd}, Balb/c^{kk}, and Balb/c^{bb}) allowed interrogation of the relationship between MHC, pathogen adaptation, and the influence of gut microbiota on virulence. Though inbred congenic animals are a powerful tool in controlled laboratory settings, they do not allow for natural transmission of FVC at a level useful for experimentation, due to impaired functional behaviors (Nelson *et al.*, 2013; Chalfin *et al.*, 2014). Therefore, I also utilized wild-derived mice that engage in natural behaviors when placed in seminatural enclosures (Potts *et al.*, 1991; Meagher *et al.*, 2000; Ruff *et al.*, 2012). Wild-derived mice were useful in understanding the natural history of FVC, as well as allowing investigation of how natural transmission influences virulence of FVC.

This dissertation tests three hypotheses related to the virulence of a murine

retrovirus. First, if diversity throughout the genome is capable of impeding pathogen virulence in just one round, it follows that a single alternation of an MHC host genotype will impede FVC fitness and virulence when compared to a passage occurring through the same MHC host genotype due to its role in virulence evolution (Kubinak *et al.*, 2013, 2015a). The second set of hypotheses regard how natural transmission, host sex and social status influence virulence of FVC. FVC is thought to be transmitted through bodily fluids (Mirand *et al.*, 1966; Portis *et al.*, 1987), suggesting there may be differential transmission efficiency between the sexes with males transmitting more due to their antagonistic encounters. In addition, natural transmission barriers are expected to reduce overall rates of virulence evolution of FVC relative to investigator transmission. The third hypothesis regards microbiome diversity. It has been recently shown that MHC genotype sculpts the composition of the host gut microbiota and that transferring microbiota between two animals can shift susceptibility to enteric pathogens (Stecher *et al.*, 2005; Kubinak *et al.*, 2015b). Furthermore, transferring gut microbiome causes changes in some immune cell subsets (Cebra *et al.*, 1998; Mazmanian *et al.*, 2005; Zaph *et al.*, 2008; Hensley-McBain *et al.*, 2016). These findings suggest two possible hypotheses; (1) when gut microbiota is transferred between two different individuals with differing MHC genotypes there will be an increase in virulence of a systemic pathogen, such as FVC, due to a energetics resource tradeoff occurring within immune system because the novel transplant contains many new antigens that may be responded to hindering the ability of a systemic immune response, or (2) microbiota will interact with the host immune system as it did in the original host leading to susceptibility shifts that correspond with the resistance of the donor host. Microbial transfers have large

implications in human health as the recent literature has shown them to be successful in treatment of some enteric pathogens (Bakken *et al.*, 2011); however, it is still unknown what other physiological effects occur when there are changes in the gut microbiome.

1.1 Chapter Summaries

1.1.1 Chapter 2

The MHC is the most genetically diverse region in the vertebrate genome with over 2,500 Class I alleles known in the human population alone (Robinson *et al.*, 2015). The MHC region is vitally important in immunity due to its capability to recognize foreign peptides (Germain, 1994). It has been suggested that increased allelic diversity of MHC in host populations can have a negative influence on the presence of pathogenic infection and that there only needs to be one host change between two different genotypes to see this negative effect (Brockhurst *et al.*, 2014; Kubinak *et al.*, 2015a). In this chapter, I test the hypothesis that when FVC is passaged through hosts with alternating MHC genotypes, there will be a reduction in fitness and virulence compared to viruses passaged through a single host genotype (pure passage). These pure passages consisted of FVC being serially passaged three times through a series of genetically identical mice. The alternated passages consisted of FVC being serially passaged through three rounds of mice, but with a different MHC genotype in the second round. This protocol allowed determination of the effect of a single MHC genotype change on virulence evolution of FVC. There is a significant reduction in both fitness and virulence when the virus is passed through an alternating genotype, leading to the conclusion that increased MHC diversity is an impediment to pathogen adaptation, as exposure to just

one new MHC genotype can significantly hinder viral adaptation.

Due to the resistance of one of the MHC genotypes, I was also able to test the hypothesis that negative frequency-dependent selection is one possible mechanism by which MHC diversity is maintained in host populations. Initially, the resistant MHC genotype did not show the predicted effect of lower viral fitness and virulence when the virus was passed through alternating hosts; which may be due to the inherent resistance masking the reduction in fitness and virulent seen when MHC alternating passages are done because the resistance keeps the virus from replicating. To test this hypothesis, I modified the resistance of this MHC by using a strain of FVC that had been given the opportunity to adapt to the resistant genotype over ten rounds of passage. Using the adapted FVC virus allowed me to see a significant reduction in both fitness and virulence when the virus was passed through a dissimilar genotype, exactly what one would expect if negative frequency-dependent selection was occurring in the experimental population.

1.1.2 Chapter 3

Transmission is an important aspect of life history for any natural pathogen, as it is the process required for pathogen propagation (Antonovics *et al.*, 2017; Mccallum *et al.*, 2017). In order to determine the influence transmission, host sex and social status have on virulence, I used wild-derived animals in seminatural enclosures. The 30 m^2 enclosures allowed the mice to interact naturally, leading to competition for mates and territories between the males and selective mate choice by females (Potts *et al.*, 1991; Carroll & Potts, 2007). Since FVC is believed to transmit through bodily fluids, the natural behavior witnessed in the seminatural enclosures promotes transmission of the

virus through increasing interactions between mice. To test the hypotheses that males are the main source of transmission, that natural transmission hinders virulence and that social status can influence virulence, three different infection-treatment populations were created using both index (infected) and contact (initially uninfected) animals. The three treatments were: female only index, male only index, and both female and male index populations. This design allowed me to determine the relative transmission frequencies via each sex. Furthermore, by measuring the virulence and titer of FVC in each contact animal, I was able to determine how natural transmission affected virulence relative to the experimentally infected index animals.

I found that populations that have both male and female index animals, or male only index animals have the highest rates of transmission, whereas the populations with only females have the lowest rates of transmission. These results suggest that males are the main source of transmission events in this system. Even though there was a significant increase in viral titers in male contact animals compared to female contact animals, the higher rates of transmission did not lead to increased spleen mass in males over females. Not only are males transmitting the virus more, but they are also harboring more virus per unit of spleen after contracting the virus, providing evidence that FVC may be male adapted. Moreover, as natural transmission occurs in these populations, there is a significant reduction in overall virulence of FVC in contact animals compared to index animals. Likewise, index animals had significantly higher titers than contact animals suggesting that natural transmission is an impediment to replication as well.

1.1.3 Chapter 4

Fecal microbiome transfers (FMT) are showing promise in the treatment of

enteric pathogens (Bakken *et al.*, 2011; Vrieze *et al.*, 2013). Though there have been

many studies elucidating the effects of microbiome on enteric function, there has been

little information published about how transferring the microbiome influences systemic

immunity of the host (Sekirov *et al.*, 2010). Using established microbial transfer methods

with antibiotic treatment, followed by recolonization with new microbial constituents,

one could inquire whether fecal transplants from different host genotypes influence

systemic virulence (Kubinak *et al.*, 2015b). I propose two hypotheses; (1) that due to a

trade-off in immune function created when a host must respond to the new antigens

present when encountering a novel microbiome, a systemic viral pathogen will have

higher virulence than it would if the host had a native microbiome and (2) the resistance

to FVC of the donor microbiome will be recapitulated in the host receiving the microbial

transplant due to the cross talk between the gut microbiome and the host immune system.

To test these hypotheses, I created both treatment (novel) and control (native) groups. In

the treatment groups, novel microbiomes were transferred between two different MHC

congenic mouse strains (Balb/c^{dd} and Balb/c^{bb}), while in the control groups each animal

received native microbiome from a host with the same MHC genotype. Following

reconstitution each animal was challenged with a stock FVC.

The test resulted in virus from the novel treatment group having significantly

higher titer and virulence than the native group, partially confirming hypothesis one. This

may be due to an immune tradeoff where the host is shuttling more immune resources to

the gut and the systemic compartment is left with fewer resources capable of responding

to the pathogen (Lochmiller & Deerenberg, 2000; Bordes & Morand, 2011). However, only a reduction of overall lymphocyte number was seen in cell immune phenotyping in novel groups and there were no other significant effects. This suggests that a wider variety of immune cell subsets need to be tested in order to fully understand how microbiome transfers influence susceptibility to systemic pathogens. The finding that fecal transplants increase susceptibility to pathogens has large implications in the human health field, as using this line of treatment for enteric pathogens may make the patient more susceptible to systemic infection.

<div align="center">1.2 References</div>

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CHAPTER 2

EXPERIMENTAL MANIPULATION OF POPULATION-LEVEL

MHC DIVERSITY CONTROLS PATHOGEN VIRULENCE

EVOLUTION IN *MUS MUSCULUS*

2.1 Abstract

The virulence levels attained by serial passage of pathogens through similar host genotypes in laboratories are much higher than observed in natural systems, however, it is unknown what keeps natural virulence levels below these empirically demonstrated maximum levels. One hypothesis suggests that host diversity impedes pathogen virulence, because adaptation to one host genotype carries tradeoffs in the ability to replicate and cause disease in other host genotypes. To test this hypothesis, with the simplest level of population diversity within the loci of the major histocompatibility complex (MHC), we serially passaged Friend Virus Complex (FVC) through two rounds in hosts with either the same MHC genotypes (pure passage) or hosts with different MHC genotypes (alternated passage). Alternated passages showed a significant overall reduction of viral titer (41%) and virulence (55%) when compared to pure passages. Furthermore, a resistant host genotype initially overshadowed the effects we expected to see during alternating MHC passages, however, when FVC was allowed to adapt to the resistant host genotype, typical effects emerged in alternating lines, i.e., alternated lines

show reduced virulence. These data indicate serial exposure to diverse MHC genotypes is an impediment to pathogen adaptation, suggesting genetic variation at MHC loci is important for limiting virulence in a rapidly evolving pathogen and supports negative frequency-dependent selection as a force maintaining MHC diversity in host populations.

2.2 Introduction

Serial passage studies of pathogens have shown order-of-magnitude increases in virulence; however, many of the factors contributing to this virulence evolution are still unknown (Brown *et al.*, 2001; Kawecki *et al.*, 2012; Kubinak *et al.*, 2013). Genotype specific adaptation by the virus may be a major cause of these order-of-magnitude level virulence increases, as each experimental passage was done through similar host genotypes. The hypothesis that between host genetic diversity can influence virulence was tested in a mouse-retrovirus system and when host genotypes are alternated during serial passage, trade-offs occur as both fitness and virulence evolution are severely impeded (Kubinak *et al.*, 2015); this provides an explanation for why the virulence of pathogens in nature, seem to seldom reach the levels observed during serial passage studies in the laboratory. However, the previous study used mouse genotypes that differed across the entire genome, so that study was unable to identify any regions of particular importance for retarding virulence evolution.

Classical major histocompatibility complex (MHC) genes are the most polymorphic loci known in vertebrates and play a critical role during immune recognition (Robinson *et al.*, 2015). The high degree of allelic diversity present in host populations is thought to be due to antagonistic coevolution between hosts and their pathogens (Potts *et*

al., 1994; De Boer *et al.*, 2004). Often referred to as Red Queen (RQ) dynamics, these interactions predict reciprocal patterns of selection whereby adaptations that benefit one species will favor counter-adaptations in the interacting species (Van Valen, 1973). Furthermore, RQ dynamics may lead to maintenance of MHC diversity in populations through negative frequency-dependent selection; in turn, controlling virulence in the host population because of the trade-offs associated with genotype specific adaptation, i.e., specialization (Carius *et al.*, 2001; Meyer-Lucht & Sommer, 2005; Sutton *et al.*, 2011).

MHC class I and class II genes allow for discrimination between self and nonself peptide antigens by encoding cell surface molecules that bind and present intracellular and extracellular peptides on the cell surface for interrogation by circulating T-lymphocytes (T-cells) (Jeffery & Bangham, 2000; Wegner *et al.*, 2003; Spurgin & Richardson, 2010). Recognition by a T-cell expressing a cognate T-cell receptor to a given MHC-peptide complex, with co-stimulating factors, triggers an adaptive immune response (Watts, 1997). Consequently, circumventing antigen presentation by MHC molecules may be a primary means by which pathogens increase within-host growth rates (fitness) (Fruh *et al.*, 1999). For example, over 50% of genetic variants seen in Human Immunodeficiency Virus (HIV) occur in MHC presented epitopes (Pereyra *et al.*, 2011; Henn *et al.*, 2012); viral adaptation to escape specific MHCs could lead to trade-offs in pathogen fitness in hosts expressing different MHC molecules, due to the specialization of the viral pathogen on the first host leading to maladaptation in the second host. Multiple studies in mice have demonstrated that MHC polymorphisms influence the severity of disease associated with pathogen infection (i.e., virulence) (Meyer-Lucht & Sommer, 2005; Wedekind *et al.*, 2006; Kubinak & Potts, 2013). Furthermore, trade-offs

in pathogen fitness associated with adaptation to specific MHC genotypes between multiple hosts may explain the association between virulence and MHC polymorphism (McClelland *et al.*, 2003).

Friend Virus Complex (FVC) is a mouse specific gamma-retrovirus that has been used extensively in immunological studies and more recently in experimental evolution studies (Kubinak *et al.*, 2012b, 2013, 2015). The complex is made up of two viral genomes that work synergistically to cause an erythro-proliferative disorder in susceptible mouse strains: the replication-competent Friend Murine Leukemia Virus (F-MuLV) and the replication-incompetent Spleen Focus Forming Virus (SFFV) (Friend, 1957; Ruscetti, 1999). FVC has been shown to adapt to specific host MHC genotypes through serial passage, which results in a reduction of fitness and virulence when hosts possessing different MHC genotypes are encountered (Kubinak *et al.*, 2012b). A recent study also suggests that loci within the MHC region account for a majority of the reduction in pathogen fitness and virulence associated with exposure to a novel host genotype (Kubinak *et al.*, 2013); however, the previously mentioned studies were performed by serially exposing FVC to a single MHC genotype through ten rounds of passage, which is unlikely to happen in wild host populations because of existing genetic variation at these loci.

Here we test the simplest case of a population where we alternate virus between two different host MHC genotypes to determine whether MHC polymorphisms between two individuals is sufficient to reduce viral fitness and virulence. We hypothesize that passage of FVC through two hosts possessing unique MHC alleles will result in significantly lower viral fitness and virulence than virus serially exposed to the same

MHC genotype. The work presented here builds upon previous evidence showing benefits of genome-wide genetic diversity and suggests that between-host MHC diversity may be a major factor reducing pathogen virulence while simultaneously being maintained through selective pressures imposed by pathogens interacting with host genotypes (Kubinak *et al.*, 2012b, 2013, 2015).

2.3 Methods

2.3.1 Mouse Model

The *Mus musculus* animals used for the study were 2 to 6 month old females purchased from Jackson Laboratories and bred under specific-pathogen-free conditions. Three different MHC congenic strains of BALB/c mice were used: BALB/cByJ (Balb/c^{dd}), C.C3-H2k (Balb/c^{kk}), and C.B10-H2b (Balb/c^{bb}). Each independent experiment consisted of three viral passage lines with ten replicates per line, followed by a test phase again using ten replicates per passage line. All of the strains used were susceptible to FV infection; however, they had different susceptibility profiles with BALB/c^{bb} being most resistant, BALB/c^{dd} being least resistant, and BALB/c^{kk} being of intermediate resistance (Chesebro *et al.*, 1990; Kubinak & Potts, 2013). All animals were housed and used in accordance with the University of Utah Institutional Animal Care and Use Committee (protocol # 04-08022).

2.3.2 Pathogen Model

A previously described biological clone of an NB-tropic strain of Friend Virus Complex (FVC) was maintained in an NIH3T3 fibroblast cell line (biological clone) and

used to generate all viral stocks used in the experiments (Kubinak *et al.*, 2012b, 2013, 2015; Kubinak & Potts, 2013). Unpassaged viral stocks were generated by harvesting viral cell supernatants from an infected NIH3T3 fibroblast cell line; this bioclone stock was passaged for two rounds through BALB/c^{dd} animals in order to obtain a BALB/c^{dd} adapted stock virus; furthermore, due to the high mutation rates and large population sizes viruses are known to have, this two round passage also increases viral diversity of the stock virus allowing evolution to act (Lauring & Andino, 2010; Domingo *et al.*, 2012). The two-round adapted stock was used to start all passage lines for each individual experiment, excluding the Balb/c^{bb} adapted two round experiment. The two-round adapted stock was used to maintain sustained animal-to-animal passage in more resistant Balb/c^{bb} and Balb/c^{kk} genotypes. Spleen supernatants from the 14 animals infected in the second round were pooled in order to obtain enough volume to last the duration of the experiment. The pooled spleen supernatants were aliquoted and stored at -80°C. All viral stocks were kept on ice and each aliquot was only thawed once.

2.3.3 Virulence Estimates

FVC causes an erythro-proliferative disorder leading to gross enlargement of the spleen (splenomegaly) during acute infection. Thus, disease virulence (harm to host) was quantified by measuring spleen mass to the nearest hundredth of a gram.

2.3.4 Viral Fitness Estimates

Viral titer (retroviral genomes integrated into the host genome) was used as a proxy for viral fitness. Due to the correlated relationship between F-MuLV and SFFV (*p*

= 0.014, r^2 = 0.41) we only F-MuLV titer data was fully generated (Figure 2S.1);

however, SFFV titer data was also generated on a subset of the animals, showing a

similar effect to F-Mulv (Figure 2S.2). Viral titer was measured by using the Roche

Lightcycler 2.0 platform with primers previously published for this system (Kubinak *et al.*, 2012b). Detected F-MuLV viral genomes were normalized to glyceraldehyde-3-

phospate dehydrogenase (GAPD) copies in order to get a standardized ratio of viral

genomes per host genome copies. The viral genome by GAPD genome ratios were then

multiplied by the spleen mass in order to obtain estimates of viral genomes per spleen.

2.3.4 Serial Passage Design

The initial passage rounds were created by using either a two-round Balb/c^{dd}

passaged stock virus or a Balb/c^{bb} adapted stock virus (described below) to infect,

through intraperitoneal (ip) injection, 10 individual animals of each mouse strain. At 12

days postinfection each animal was sacrificed, infected spleens were collected for viral

load estimates, and measurements of disease were made. After organ mass was recorded

a portion of the spleen was saved in RNAlater™ and the rest combined in a 1:1 ratio of

1x Phosphate Buffered Saline (PBS) (i.e., 1g of spleen was diluted in 1ml PBS). The

spleen-PBS mixture was mechanically homogenized. Homogenates were then

centrifuged at 5000 RPM for 5 min and virus-laden supernatants were collected and

frozen at -80°C. In order to control for dose of the virus in the test phase each viral

inoculum was standardized by titering the supernatant and diluting it to the average titer

of the alternating treatment group with the lowest average titer of its replicates; any

inoculum under the average was used undiluted at a full dose (200uL) while anything

above the average was diluted before injection. Furthermore, it has been previously shown that there is no graft effect of viral proteins, from a previous animal seen, when the spleen supernatant is injected into a novel host (Kubinak *et al.*, 2015).

2.3.5 Stock Two-Round Alternating Experiments

Experiments consisted of three independent sub-experiments carried out in each BALB/c MHC congenic mouse strain (Balb/c^{dd}, Balb/c^{kk}, and Balb/c^{bb}). In each, virus was passaged twice through animals possessing the same MHC genotype or twice through animals possessing different MHC genotypes (Figure 2.1). 200 uL of the stock virus was used to inoculate 10 animals of each MHC congenic strain (Round 1). After the infection period, the animals were sacrificed and 20 uL of each individual spleen supernatant was used to infect an animal from each of the three MHC congenic host strains (Round 2). After the second infection period, the spleen supernatant was standardized and used to inoculate the original strain of passage (Test Phase). Three sub-experiments were done in total with each congenic strain of *Mus* acting as the control pure passage with their corresponding alternated passage lines through the other two MHC genotypes. In total, there were 70 infections with 30 independent passage lines in each of the three subexperiments, for a total of 210 infections.

2.3.5 Balb/c^{bb} Adapted Two-Round Alternating Experiment

Due to the unexpected result shown in the Balb/c^{bb} adapted experiment, where fitness and virulence was reduced in all treatments in the test phase, we conducted another sub-experiment with a FVC strain adapted to the Balb/c^{bb} genotype. These

additional experiments were done in order to test if the expected pattern where passages

through alternating MHC genotypes reduces titer and virulence, holds true when the virus

is adapted to the Balb/c^{bb} genotype to overcome some of the inherent host resistance. We

used a previously generated virus that was adapted to the Balb/c^{bb} host genotype through

ten rounds of passage in order to obtain an adapted virus to the resistant genotype

(Kubinak *et al.*, 2012b). The previously described Balb/c^{bb} experiment was repeated with

the new Balb/c^{bb} stock adapted through ten rounds of passage. Again, three different

passage treatments were conducted, in which the virus was passaged through either pure

passage lines (Balb/c^{bb} in each round) or alternating passage lines (different MHC

genotype in the second round of passage, Balb/c^{kk} or Balb/c^{dd}). Again, a test phase was

conducted in the original host strain of passage with the same dilution scheme, in order to

test for differences between passage treatments.

2.3.6 Statistics

All statistical analyses were completed in R (3.3.1) (R Core Team, 2016).Titer

data was multiplied by spleen mass in order to obtain total viral titer per spleen estimates

and spleen mass and titer data was log$_{10}$ transformed due to its non-normal nature. Data

pooled between the initial stock two round experiments were analyzed using linear mixed

model in the lme4 package with response variables of either viral titer or spleen mass and

a fixed effect of treatment (Bates *et al.*, 2014). A random effect of host genotype was

included in order to control for variation due to different resistance patterns in the host

strains between experiments, p-values were obtained using the R package lmerTest (2.0-

33) (Table 2S.1) (Kuznetsova *et al.*, 2016). Furthermore, each individual stock two round

experiment was analyzed by a one-way ANOVA with corresponding Tukey post-hoc

tests to determine differences between each treatment group. The Balb/c^{bb} adapted

experiment was analyzed separately from the two round alternating experiments and was

also analyzed by a one-way ANOVA with post-hoc Tukey tests to determine differences

between passage lines. All data in figures represent mean ± SEM. All data will be

uploaded to an online repository, such as DRYAD, upon acceptance.

2.4 Results

2.4.1 Stock Two-Round MHC Alternating Passages

Overall, MHC experiments show a significant reduction in viral fitness and

virulence when the virus went through an alternated passage regime versus a pure

passage regime. FVC stocks derived from alternated passage demonstrated a 41%

reduction in titers compared to pure passaged FVC (LMM; $t = -3.72$, $p < 0.001$) (Figure

2.2A). SFFV titer showed a similar overall pattern where alternating passages have lower

titers than pure passage lines (LMM; $t = -3.53$, $p = 0.001$) (Figure 2S.2). Furthermore,

virulence of FVC was reduced by 55% in alternating passages when compared to pure

passage lines (LMM; $t = -3.42$, $p < 0.001$) (Figure 2.2B).

Viral titer measures demonstrated a statistically significant overall reduction in

fitness in alternated passages when the three two-round stock experiments are pooled,

however, not all comparisons for viral titer in each individual experiment were

significantly reduced (Figure 2.3A-C). The Balb/c^{dd} experiment showed a significant

difference in viral titer between the experimental groups (Figure 2.3A) (ANOVA; $F_{(2, 31)}$

$= 4.9$, $p = 0.01$). Specifically, there was a significant reduction of FVC titer when

passaged through both alternated Balb/c^{bb} (Tukey, $p = 0.02$), and alternated Balb/c^{kk} lines (Tukey, $p = 0.05$) relative to pure passage lines. Similarly, when FVC was passaged in the Balb/c^{kk} experiment there were significant differences between viral titer of experimental groups (Figure 2.3B) (ANOVA; $F_{(2, 21)} = 11.4$, $p < 0.001$). In this case, a significant reduction in viral titer was observed only when FVC was passaged through the Balb/c^{bb} genotype when compared to pure passage lines (Tukey, Balb/c^{bb}, $p < 0.001$; Balb/c^{dd}, $p = 0.12$). The Balb/c^{bb} experiment did not show the predicted effect and there was actually an increased viral titer when FVC was passaged through the alternating Balb/c^{dd} genotype (Figure 2.3C) (Fitness, ANOVA, $F_{(2, 24)} = 10.4$, $p < 0.001$; Tukey, Balb/c^{dd}, $p = 0.02$, Balb/c^{kk}, $p = 0.27$).

Spleen mass showed similar results to the titer data; the overall data showed alternating passages had a statistically significant virulence reduction compared to pure passages, however, the trend was not consistent among sub-experiments. The Balb/c^{dd} sub-experiment showed a statistically significant relationship between groups (Figure 2.3D) (ANOVA; $F_{(2, 39)} = 4.9$, $p = 0.013$), though, there was only a statistically significant reduction in virulence when FVC was passaged through the alternated Balb/c^{bb} lines (Tukey, $p = 0.009$) and only slightly reduced in the alternated Balb/c^{kk} lines (Tukey, $p = 0.225$). The Balb/c^{kk} sub-experiment also showed a statistically significant relationship between groups (ANOVA; $F_{(2, 25)} = 8.1$, $p = 0.002$) with each of the alternated lines showing reduced virulence, but only the Balb/c^{bb} lines showing statistically significant reductions (Figure 2.3E) (Balb/c^{bb} Tukey, $p = 0.001$; Balb/c^{dd} Tukey, $p = 0.270$). Again, the Balb/c^{bb} stock experiment did not show a reduction in virulence in alternating passages and there was an increase when the virus went through an alternating Balb/c^{dd}

passage (Figure 2.3F) (ANOVA, $F_{(2,27)} = 10.9$, $p < 0.001$; Tukey; Balb/c^{dd}, $p < 0.001$).

2.4.2 Two-Round Balb/c^{bb} Adapted Experiment

In the experiment using a Balb/c^{bb} adapted virus, a reduction in both titer and virulence was observed when FVC was passaged through animals with alternated MHC genotypes, though again not statistically significant in each alternating passage (Fitness, ANOVA; $F_{(2, 23)} = 20.3$, $p < 0.0001$; Virulence, ANOVA; $F_{(2, 27)} = 19.7$, $p < 0.0001$; Figure 2.4). Individually, there was only a significant reduction in titer and virulence when passaged through the Balb/c^{kk} haplotype (Titer, Tukey $p < 0.0001$; Virulence, Tukey, $p < 0.0001$) (Figure 2.4A-B). Passaging FVC through the Balb/c^{dd} genotype did show a reduction in the means in both titer and virulence from pure passage, however, this reduction was not significant (Tukey; titer, $p = 0.93$; virulence, $p = 0.97$).

2.5 Discussion

The data presented here support the hypothesis that increased MHC diversity in host populations impedes pathogen adaptation as evidenced by reduced fitness and virulence. Though we do not see significant reduction of viral titer and virulence in each individual alternating passage, in general, there is an overall 41% reduction of viral titer and 55% reduction in virulence when FVC is passaged through alternated hosts compared to pure-passage hosts when variation between experiments is controlled in a LMM. The reductions in virulence observed here provide a mechanism for how pathogens, forced to adapt to multiple MHC genotypes encountered in natural populations, are not able to attain the virulence that has been observed in serial passages through isogenic hosts.

The initial two round stock Balb/c^{bb} experiment did not show the predicted pattern; one explaination may be that due to the high resistance of the Balb/c^{bb}, the resistance overshadows the effects that are expecting in alternating passages (Kubinak & Potts, 2013). If true, then a virus more adapted Balb/c^{bb} might allow the predicted pattern to emerge. In order to test this hypothesis, we repeated the Balb/c^{bb} experiment with a Balb/c^{bb} adapted virus serially passaged through ten rounds. When FVC was able to adapt to the Balb/c^{bb} genotype, our hypothesized pattern emerges: there is a reduction of fitness and virulence in the alternated lines when compared to the pure passage lines, though only significant in the Balb/c^{kk} alternated lines. Interestingly, the virus alternated through Balb/c^{dd} had significantly higher virulence and titer in the test phase than pure passage suggesting the susceptibility of MHC genotypes is an important interacting variable. These experimental data are consistent with other correlative studies showing a reduction in pathogen load when many different MHC alleles are present in a population (Meyer-Lucht & Sommer, 2005; Sin *et al.*, 2014). Furthermore, by allowing the virus to adapt to the most resistant MHC genotype, making these hosts more susceptible, we experimentally support the negative frequency-dependent predictions of the RQ hypothesis, demonstrating the dynamic character of such coevolutionary interactions (Borghans *et al.*, 2004; De Boer *et al.*, 2004). Though the results presented here do not completely rule out other hypotheses that may be maintaining MHC diversity in populations, it does show that negative frequency-dependent selection is one force maintaining diversity and that more work needs to be completed.

MHC diversity is thought to be maintained in vertebrate populations through three nonmutually exclusive mechanisms: non-random mate preference, heterozygote

advantage and negative frequency-dependent selection (Kubinak *et al.*, 2012a; Brockhurst *et al.*, 2014). Here we provide evidence for negative-frequency dependent selection in two ways. First, as MHC genotypes are alternated during serial passage of the virus, they supply a different selective pressure than the pure passage causing the virus to experience a fitness cost when each new MHC genotype is adapted to by the virus. These tradeoffs are essential for establishing molecular arms races and give rise to the attendant negative frequency-dependent selection. Second, the initial experiment through the resistant Balb/c^{bb} genotype did not show reduced titer or virulence during alternated passages (compared to pure passages). However, FVC is capable of adapting to the most resistant MHC genotype (Balb/c^{bb}) when passage through ten rounds, thereby making the host more susceptible and allowing the expected patterns of reduced virulence during alternated passages to emerge in subsequent experiments (Kubinak *et al.*, 2013). The adapted Balb/c^{bb} experiment models what might be expected in populations where the rare MHC alleles are initially not the targets of pathogen adaptation, but over time they become more common and consequently less resistant because they are now more frequent targets of pathogen adaptation. However, this dynamic is not seen in the initial experiment because hosts genotypes in their high resistance (rare) phase can dominate and overwhelm typical MHC-specific virulence patterns.

These data demonstrate that population-level MHC diversity is beneficial to hosts because it reduces virulence evolution. It follows that a selective advantage will arise in families where both parents are MHC dissimilar because virulence is reduced as it moves through the different MHC genotypes of a family; providing a familial level function for MHC-dissimilar mating preferences (Potts *et al.*, 1991; Eizaguirre *et al.*, 2009; Lenz *et*

al., 2009).

In many serial passage studies from *Daphnia* to mice, virulence has been shown to increase by orders of magnitude (Ebert & Bull, 2003; Kawecki *et al.*, 2012; Kubinak *et al.*, 2012b, 2013); however, the underlying mechanism of this adaptation is unknown. We provide evidence that the increases in virulence of FVC are due at least in part, to specialization of the pathogen through specific MHC genotype adaptation; as MHC plays a major role in the interactions pathogens have with hosts and as the pathogen increases in virulence by adapting to one specific MHC genotype it incurs trades-offs in other MHC genotypes (Kubinak *et al.*, 2012b). The 36% reduction in virulence observed when the pathogen is passaged through two hosts with dissimilar MHC genotypes supports the hypothesis that diversity at MHC loci is a controlling factor for virulence evolution. Increasing the amount of MHC diversity in host populations provides an advantage for each individual in that population as it reduces the probability a pathogen that infects them will have seen a similar MHC genotype during the previous infection.

Serial passage is a useful tool in coevolution studies and many have shown an increase in fitness and virulence of various pathogens using these techniques (Ebert, 1998; De Bruin *et al.*, 2008; Mackinnon *et al.*, 2008; Wei *et al.*, 2014). Many of these studies have focused on pathogen interactions with species changes or sexual vs. asexual passages (Coffey & Vignuzzi, 2011; Ciota *et al.*, 2015), but not passages of pathogens through different genotypes of the same vertebrate host species, as was done here. Furthermore, only a few of these serial passage studies have incorporated MHC allelic diversity into their passage schemes; which is thought to cause pathogen fitness tradeoffs through antagonistic relationships when the number of MHC alleles are increased in

populations (Radwan *et al.*, 2010). Only Kubinak et. al. provided experimental evidence that between host genetic diversity reduces pathogen fitness and virulence in a vertebrate *Mus* host, however this work was done with hosts that differed throughout the genome, not just at the MHC region (Kubinak *et al.*, 2015). Confirming strong effects of MHC diversity alone during experimental evolution highlights the importance MHC-specific adaptations have on viral evolution and represents the first experimental demonstration of diversity at specific loci impeding pathogen virulence evolution.

Though this study shows a strong relationship between MHC diversity and pathogen evolution, we do not take into account natural transmission or other pathogens. Removal of the cost of transmission could lead to increased virulence of the pathogen (Ebert & Bull, 2003; Alizon *et al.*, 2009), however, evidence suggests this is not causing the effect in our system. As host genotypes are alternated we see the associated costs in the new hosts with the same transmission route suggesting that viral interactions with the host MHC genotype is driving the adaptations and tradeoffs independent of removing the cost of transmission (Figure 2.2). Unfortunately, the relative importance of transmission and host genetics in virulence evolution is not known and needs to be investigated further. Moreover, FVC is a lab strain of virus and may not be found in this form in natural systems; however, other viruses such as Dengue virus have replication incompetent particles that enhance virulence much like SFFV, so this system may enable better understanding of the role of these defectives (Ke *et al.*, 2013). Furthermore, due to its similar life cycle, FVC is a good model to use to understand other ecologically relevant retroviruses, such as HIV, Simian immunodeficiency virus, Bovine leukemia virus, and Koala retrovirus (Pollari *et al.*, 1992; Tarlinton *et al.*, 2006; Myers &

Hasenkrug, 2009; Hartmann, 2012). Furthermore, we would like to point out that though we used congenic inbred animals we do not know if each strain is the same across the genome outside of the MHC region. However, if this is the case we would expect the effects to be small compared to the effects of the MHC region as there has been much less time for large changes to occur that influence pathogen defense in the rest of the genome due to the pathogen free nature of these animals. Future work should focus on the relationships between naturally transmitting pathogens and population wide MHC diversity in order to understand the relative importance each plays in their coevolutionary system.

Here we demonstrate that RQ dynamics contribute to virulence evolution by showing trade-offs in FVC fitness and virulence when it encounters a host with a novel MHC genotype, though many different factors may contribute to these trade-offs as well. These data suggest that the high virulence seen in serial passage studies may not be seen in nature due to the continual selection placed on the virus with each transmission event into a host with a novel MHC genotype. Furthermore, as the virus adapts to the most resistant genotype, we observed MHC trade-offs emerge when new (alternating) genotypes are encountered, as would be expected through negative-frequency dependent selection. These data shed light on the importance host pathogen co-evolutionary relationships play in the maintenance of MHC diversity and how this diversity is important for virulence evolution; having practical implications for endangered and agricultural species through their respective breeding programs (Frankham, 2010).

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Figure 2.1. Serial passage of FVC through MHC congenic mice. Virus was administered intraperitoneally. After 12 days of incubation it was split three ways and was passaged into a host of the similar MHC genotype (round 2 pure) as well as two different MHC genotypes (round 2 alternating). During the test phase, each round two virus was passaged into the original host of passage (round 1 genotype) in order to control for host differences in the test phase. Furthermore, each round was completed with ten replicates in each MHC genotype treatment group, denoted by x10. Each panel represents the independent experiments done in order to test each genotype in each passage scenario.

Figure 2.2. Virulence and titer of FVC in the test phase among all experimental treatments. (a) The titer of FVC was significantly reduced when passaged through alternating MHC host genotypes. (b) There was also a significant reduction of virulence (Spleen mass) when FVC was passaged through an alternating host. Symbols represent the experiment the data point came from: Balb/c^{kk} (triangle), Balb/c^{dd} (circle) and Balb/c^{bb} (square). Data represented by mean ± SE. ** Indicates a P value < 0.01, *** < 0.001

Figure 2.3. Alternating MHC genotypes reduces both titer and virulence of FV complex. (a-c) There was a significant reduction of viral titer in three out of six alternating passages across each sub-experiment; furthermore, titer was reduced below pure passages in each alternating passage, though not significantly, except the Balb/c^{bb} sub-experiment. (d-f) Virulence of FVC showed a significant reduction in two out of six possible alternating passages, again, virulence was reduced from pure passages in all cases though this reduction was not always significant. The Balb/c^{bb} experiment did not show the expected result with regard to either titer or virulence. Data represented by mean ± SE. * Indicates a P value < 0.05., ** < 0.01, *** < 0.001.

Figure 2.4. Viral titer and virulence using the Balb/c^{bb} adapted virus. (a) Alternating the MHC genotype shows a significant reduction in titer between groups, however, much of this significant effect is driven by the virus that is alternated through Balb/c^{kk} genotypes with a minor reduction in titer when passaged through Balb/c^{dd}. (b) Likewise, there was significant reductions between groups in virulence of FVC. Again, the reduction in virulence was only significant when alternated through Balb/c^{kk} MHC haplotypes, with only a slight reduction in Balb/c^{dd} passages. Data represented by mean ± SE. *** Indicates a P value < 0.001.

Figure 2S.1. Correlation between SFFV and F-MuLV titers. Only qPCR data from F-MuLV genomes was collected and reported due to the significant correlation between the two viral genomes that make up FVC: Friend Murine Leukemia Virus (F-MuLV) and Spleen Focus Forming Virus (SFFV).

Figure 2S.2. Virulence and titer of SFFV in the test phase among all experimental treatments. (a) The titer of FVC was significantly reduced when passaged through alternating MHC host genotypes. Data represented by mean ± SE. ** Indicates a P value < 0.01.

Table 2S.1. Linear mixed models comparing treatment groups (pure and alternated), controlling for host genotype.

Virulence (Spleen mass) of test phase animals by treatment

LMM (groups: Host = 3; observations 100)

Random effects	Variance	Std. Deviation		
Host	0.1059	0.3254		
Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Pure Passage)	-0.1607	0.2078	-0.773	0.50458
Treatment (Alternated Passage)	-0.3708	0.1086	-3.415	0.00094 ***

F-MuLV titers (log transformed) of test phase animals by treatment

LMM (groups: Host = 3; observations 85)

Random effects	Variance	Std. Deviation		
Host	1.4087	1.1869		
Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Pure Passage)	4.1895	0.7278	5.756	0.01952 *
Treatment (Alternated Passage)	-1.1970	0.3214	-3.724	0.00036 ***

* Indicates a P value < 0.05., *** < 0.001.

CHAPTER 3

NATURAL HORIZONTAL TRANSMISSION OF A MURINE

RETROVIRUS WITHIN SEMINATURAL

POPULATIONS OF MICE

3.1 Abstract

Transmission is a key component of a pathogen's life history and more robust

systems need to be developed to obtain reliable ecological estimates of the dominant

mode(s) of transmission and determine how host traits such as sex and social status

influence transmission dynamics. We have developed such a system, using seminatural

populations of house mice and use it to evaluate the transmission dynamics of Friend

Virus Complex (FVC). Seven seminatural populations were founded with 8 male and 16

female mice that were either "index" (initially infected) or "contact" (initially

uninfected). Three experimental population designs were implemented: female index

only, male index only, and female and male index. We found that male-male transmission

is the predominant mode of transmission with little female transmission and no evidence

of vertical transmission. Additionally, infected contact males harbor higher titers than

females while suffering a similar amount of virulence; this paired with a strong

correlation between titer and virulence in males and no correlation in females suggests

FVC may be male adapted. We also found that contact animals had lower titers than

index animals at the end of the experiment suggesting FVC replication is reduced.

Finally, social status influences infection, as though dominant and nondominant males

contract the virus at similar rates and experience similar virulence, nondominant animals

have higher titers. These results shed light on the complex life cycle of murine leukemia

viruses and their evolutionary history and may also help develop a better understanding

of the role of host sex, and social status on retroviral transmission dynamics.

3.2 Introduction

Pathogen life history is complex and consists of many barriers that when passed

coalesce in infection, and one of these consequential life history challenges is

transmission (Mccallum *et al.*, 2017). Therefore, understanding the mode(s) of

transmission and how this mode influences pathogen virulence is a large and ongoing

area of research (Antonovics *et al.*, 2017). Historically it has been difficult to obtain

reliable estimates of transmission, due to the biased nature of collection methods (i.e.,

only symptomatic individuals measured), and of the many factors controlling successful

transmission of pathogens (Antonovics, 2017; Walker *et al.*, 2017). Specifics about

pathogen transmission dynamics such as, the transmission efficiencies of various modes

and the influence of host sex, social status, and age are difficult to obtain in natural

settings due to all the competing factors involved in transmission; addressing these

shortcomings requires design of better experimental systems to obtain reliable estimates

of the movements of different pathogens under a host of ecological conditions (Arthur *et

al.*, 2017).

Transmission mode, how a pathogen moves between hosts, influences both the

likelihood of successful infection and resulting virulence experienced by the host.

Pathogens are often opportunistic and use many different modes to infect subsequent

hosts; however, some of these modes may allow for more likely transmission and higher

pathogen burden than others (Antonovics *et al.*, 2017). Experimenter transmission,

wherein researchers directly infect a host with a pathogen, does not always mimic natural

transmission modes (e.g., intraperitoneal injection with Friend Virus Complex (Portis *et

al.*, 1987; Ebert, 2013)). Also, it is thought that experimenter transmission removes the

cost of transmission, potentially artificially increasing virulence (Ewald, 1983).

Therefore, understanding the modes of transmission that a specific pathogen employs,

their relative importance, as well as shifting away from experimenter to natural

transmission is necessary to characterize the transmission ecology of host-pathogen

systems.

Through differences in physiology, hormone production, and immunity, host sex

may be a powerful force driving differences in the transmission of pathogens (Zuk &

McKean, 1996; Duneau & Ebert, 2012; Robinson & Klein, 2012). Males, for example,

produce testosterone, which has been suggested to suppress the immune system, thereby

increasing susceptibility to parasites and pathogens (Zuk, 1996). Many studies have

examined the virulence in females in regards to vertically transmitted parasites (e.g., FVC

in mice or Hepatitis C virus in humans) (Mirand *et al.*, 1966; Thaler *et al.*, 1991);

however, few have explored differences in host virulence due to horizontal transmission

or if transmission rates differ between the sexes. Furthermore, when sex biased horizontal

transmission has been tested it has been measured indirectly using measures that correlate

with transmission such as pathogen shedding or within host growth rates not successful

inoculation of another host (Perkins *et al.*, 2008; Luong *et al.*, 2009). The differences between the sexes in immunity, behavior, and other physiological processes may lead to key differences in transmission dynamics for a given parasite; unfortunately, the influence of host sex and potential sex-specific processes have not had rigorous characterization for most parasites (Klein, 2000).

Another important characteristic that can shape transmission dynamics is an individual host's social status. Individuals of a species vary in physical condition, social connectivity, dispersal ecology, or possess heterogeneity in other traits that may bear importantly on their likelihood of contracting or transmitting a parasite (Arthur *et al.*, 2017; Stephenson *et al.*, 2017); these heterogeneities and their influence on transmission dynamics may be associated with belonging to a specific social class, such as dominant or nondominant (Hausfater & Watson, 1976; Hawley *et al.*, 2007). Although specific relationships between social status and parasite transmission/virulence have been documented, general patterns across host and parasite species remain obscure (Arthur *et al.*, 2017). Therefore, in order to understand the transmission ecology of a given pathogen, studies specifically assessing the influence of host social status are required.

Friend Virus Complex (FVC) is a murine specific retrovirus that has been widely used in laboratories to understand various aspects of the retroviral life cycle and immunity (reviewed in Hasenkrug & Chesebro, 1997; Myers & Hasenkrug, 2009). FVC has been shown to be transmitted both vertically (through milk) and horizontally (sexually from males to females), at least, within susceptible strains of laboratory mice in artificial caged environments (Mirand *et al.*, 1966; Portis *et al.*, 1987). Additionally, a related wild murine ecotropic virus has been shown to transmit between males in cage

trials, though, neither of these studies have focused on disentangling the various modes of natural transmission, even in caged environments, to determine their relative importance (Portis *et al.*, 1987). Moreover, simply demonstrating that transmission can occur via a specific mode does not provide information about which mode of transmission is the most common in nature, or the route to which a pathogen is primarily adapted.

The commensal nature of house mice (*Mus musculus*) provides a unique opportunity to manipulate aspects of transmission ecology (e.g., density, sex-ratio of infected individuals) to determine how their pathogens move through a population in a controlled experimental setting. Experimental infections in house mouse populations inhabiting seminatural enclosures, when paired with appropriate titering, allow for quantifying contraction and transmission rates of pathogens and resulting virulence measures in an ecologically-relevant setting. The use of such enclosures has been advocated by the evolutionary and functional genomics community (Carroll & Potts, 2006, 2007); however, until now their novel attributes have yet to be harnessed for disease ecology and epidemiology.

Here we use wild-derived house mouse populations living under seminatural conditions to evaluate the virulence and transmission dynamics of a murine retrovirus (FVC). Specifically, we assess: whether horizontal or vertical is the dominant mode of transmission; which sex, if either, drives horizontal transmission; if there is a corresponding sex-specific virulence and pathogen titer pattern accompanying differences in transmission; if virulence and titer measures resulting from experimenter and natural transmission differ; and if male social status influences the observed virulence, titer, or transmission patterns. Regarding the dominance of horizontal vs. vertical transmission,

specific *a priori* predictions are difficult to formulate due to conflicting cage studies (Mirand *et al.*, 1966; Portis *et al.*, 1987). However, if sexual transmission is the main route of horizontal transmission then female mice should contract the virus at a higher rate than males (while males transmit at higher rates than females (e.g., Padian *et al.*, 1991; Looker *et al.*, 2008), but if aggressive interactions are the main transmission mechanism, then males should have higher rates of both contraction and transmission as they more readily engage in fighting (Singleton & Krebs, 2007). Correspondingly, we predict that if one sex is primarily responsible for transmission then it will experience lower virulence due to infection than the other, according to theory (Anderson & May, 1982). Also, if natural transmission involves more barriers to transmission, such as less opportunity, dose or different routes, than experimenter transmission, we predict that animals naturally contracting the virus will have lower titers and virulence than animals infected intraperitoneally (Ewald, 1983). Finally, if dominant males are in better condition than nondominant males they will have lower titers and virulence; however, if they expend resources defending territories and this results in a tradeoff with immune function they will develop more severe infection (Arthur *et al.*, 2017).

3.3 Methods and Materials

3.3.1 General Methods

3.3.1.1 Animals

To assess transmission dynamics, mice with functional, wild type, behaviors are needed and it has been documented that standard laboratory strains have lost many of these behaviors (Nelson *et al.*, 2013a; Chalfin *et al.*, 2014). Therefore, we employed

wild-derived genetically outbred house mice (*Mus musculus*) from the 14th and 16th generation of a colony initially described in reference (Meagher *et al.*, 2000). For cage-based experiments and before release into seminatural enclosures, individuals were provided access to food and water *ad libitum* and maintained on a 12:12 h light:dark cycle. All procedures were approved by the IACUC of the University of Utah (protocol #14-08022).

3.3.1.2 Pathogen

FVC is an NB-Tropic gamma retrovirus that primarily infects erythroblasts causing gross splenomegaly in its murine host. FVC is composed of two separate genomes that work synergistically to cause disease. First, Friend murine Leukemia Virus (F-MuLV) is a prototypic gamma retrovirus which has all the proper machinery to infect and exit erythroblasts (for review see (Hasenkrug & Chesebro, 1997)). The second genome, Spleen Focus Forming Virus (SFFV), is capable of inserting into the genome, but cannot exit a cell without being pseudo-typed (packaged) in an F-MuLV capsid. However, SFFV's truncated envelope gene can stimulate the Erythropoietin receptor (EpoR) causing uncontrolled proliferation of erythroblasts allowing for more target cells for F-MuLV and increasing the probability of the SFFV genome being pseudo-typed and exiting the host cell (Ruscetti, 1999; Kosmider & Moreau-Gachelin, 2006).

Due to the erythropoietic nature of FVC the main sight of replication is the spleen (Metcalf *et al.*, 1959). The dramatic increase in the size of the spleen allows for an estimate of the amount of harm (i.e., virulence) the host is experiencing during infection. After sacrifice, the spleen is harvested and the mass measured.

F-MuLV viral titers were measured by qPCR amplification of retroviral integrations from the host genome (provirus) using primer sets and protocols described previously (Kubinak *et al.*, 2012). Provirus quantification, as opposed to Ribonucleic Acid (RNA) measurements, was used because it is a measure of productive viral genomes that have inserted into the genome. Quantitative PCR was used to obtain estimates of viral fitness from spleen homogenates using the Lightcycler 2.0 platform. Crossing points above cycle 36 were requantified and if still over that threshold the sample was treated as uninfected per the manufacturer's recommendation due to the unreliability of quantifiable estimates at that number of cycles. Each proviral estimate was normalized to a Glyceraldehyde Phosphate Dehydrogenase (GAPD) reference to obtain relative per cell genome estimates. Furthermore, each proviral GAPD ratio was multiplied by spleen mass in order to get proviral load per spleen estimates; this estimate was used in all analysis except the correlations of F-MuLV proviral load by spleen mass, due to its potential to inflate the significance of the correlation.

Only SFFV infection status (positive/negative) was measured due to the high level of endogenous DNA and expressed RNA similar to SFFV found in wild-derived mice. SFFV was amplified from RNA with the following primer sets, Fwd – 5'GTTCGCGCGCTTCTGCTTCC3', Rev – 5'ACAGCGAGACCACGAGTCGG3', using the Verso RT-PCR kit by Thermo Scientific on a Biorad c1000 touch machine used per manufacturer recommendations. PCR products were run on a 1% agarose gel in order to determine the presence or absence of the ~170 bp fragment of SFFV (Figure 3S.1). RNA extractions were completed using the Zymo Quick-RNA MiniPrep kit in order to obtain pure RNA template for PCR.

A Bioclone FVC stock was cultured from a transfected a NIH3T3 cell line, culture supernatants were collected, aliquoted and frozen at -70°C until needed. The resulting Bioclone stock was not virulent enough to cause disease in wild-derived mice; therefore, a *Mus*-adapted stock (as opposed to cell-culture adapted) capable of infecting animals in the study was generated. To create this stock, Bioclone was serially passaged through two rounds of BALB/c female mice. After two rounds of passage, each of the 14 animal's spleens were homogenized and pooled. Supernatants were collected from the pooled spleens and stored at -70°C until needed. The resulting virulent FVC stock did produce larger spleens in wild-derived mice than Bioclone (virulent stock: 0.27 ± 0.07 g, Bioclone: 0.05 ± 0.008 g; WRS; $n = 48$, $W = 168.5$, $p = 0.019$). Furthermore, within cages female mice experienced higher virulence from FVC infections with our virulent FVC stock than did males, but only a marginal increase of F-MuLV titers was observed in females over males. Twelve days postinfection females had spleens averaging 0.45 ± 0.13 g (M ± SEM), which were 3.46 times the mass of male spleens which were 0.11 ± 0.04 g (WSR; $n = 28$, $W = 158.5$, $p = 0.005$; Figure 3S.2A). However, only a marginally statistically significant difference in F-MuLV titers per spleen (F:M; 2.07 ± 0.33: 1.17 ± 0.32) was observed between the sexes (t-test; $t = 1.97$, $p = 0.060$ [Figure 3S.2B]). Interestingly, a correlation between spleen mass and titer was not identifiable in this data set (LM; $t = -0.55$, $p = 0.209$; Table 3S.1).

3.3.1.3 Seminatural Enclosures

Enclosures are 30 m^2 and subdivided into six subsections by wire mesh to promote territorial formation (Figure 3.1). Subsections contain *ad libitum* food and water

dispensers associated with a set of nest boxes in one of the four "optimal" territories, enclosed dark nesting sites, or two "suboptimal" territories, with nest boxes exposed to the light. Descriptions of enclosure, including additional photographs, and establishment/maintenance methods have been provided elsewhere in more detail (Ruff *et al.*, 2013, 2015a; b, Gaukler *et al.*, 2014, 2016). Populations are founded by 24 mice (16 females; 10 males); densities and sex ratio were selected to be similar to those reported in the wild (Singleton & Krebs, 2007). Populations in this study were maintained for 7 weeks, at which point founders and any offspring born within enclosures were sacrificed and organs were harvested to assess virulence measures and viral titers.

Prior to release in seminatural enclosures both contact and index female founders were genotyped and categorized by a common mitochondrial allelic variant. Offspring collected at the termination of the populations were then typed so that the treatment of their mother could be determined. Mitochondrial types from all 108 offspring were gathered from populations containing female index animals and offspring were classified as having come from either contact (83) or index females (25). Information on primer design and PCR and gel electrophorese protocols have been described previously (Meagher *et al.*, 2000).

Prior to release into enclosures, founders of both sexes were implanted with unique passive integrated transponder (PIT) tags (TX1400ST, BioMark, Boise ID). Location data were gathered via a set of PIT antennae and readers (FS2001F-ISO, BioMark, Boise ID) placed at each of the feeders and data are recorded with data-logging software (Minimon, Culver City, CA). Dominance was assigned when a male had more than 80% of all male readings at a single feeder over the majority of the study as in

previous studies (Morris *et al.*, n.d.; Nelson *et al.*, 2013b).

3.3.2 Study Design and Statistical Analyses

3.3.2.1 Initial Evaluation of FVC Natural Transmission

To determine if natural transmission of FVC is possible within seminatural enclosure populations a single pilot population of 24 mice (16 female and 8 male) was established with both female and male index mice (initially infected via IP injection) as well as contact mice (initially uninfected) of both sexes, resulting in half of the animals being contact and half being index. These mice were from the 14th-generation of the aforementioned colony and were 76.9 ± 12.1 (M $\pm$ SD) weeks of age.

3.3.2.2 Relative Importance of Horizontal Versus Vertical Transmission

Infection status was assessed in the offspring of index females as described above and the rate of infected offspring was compared to the observed rates of horizontal transmission in adults. As no offspring tested positive for F-MuLV titers a one-sample t-test was used to compare rates of horizontal transmission across populations to the hypothetical value of 0.

3.3.2.3 Influence of Host Sex on Horizontal Transmission Dynamics

Once natural transmission of FVC was confirmed six additional populations were established to determine the influence of host sex on FVC transmission dynamics. Two

populations were designed as described above with both female and male index founders, two populations had only female index founders, and the last two had only male index founders (Figure 3.1B). All populations contained 30 mice (20 female and 10 male) that were 13.6 ± 2.7 weeks old and study duration was again 7 weeks. To assess the effect of contact (female and male) and index host sex (female, male, and both sexes) on contact FVC infection status, we used a generalized linear mixed model (GLMM) to predict the probability of infection status; this and all other GLMMs were fit in R using the *glmer* function of the lme4 library (Bates *et al.*, 2015; R Core Team, 2016). As infection status was defined as either infected or uninfected, we used a model assuming a binomial distribution with a logit link. Infection status was assessed in contact animals that survived the duration of the trial ($n = 112$) and these mice were grouped into seven populations, (the initial pilot population was included) from which individual observations for female and male contact mice were made (observations = 14). Contact and index sex were treated as fixed effects and population was modeled as a random effect. The model intercept was set for female contact animals from populations with only female index animals.

3.3.2.4 Influence of Host Sex and Transmission on Virulence and Pathogen Fitness

Comparisons of spleen mass and viral titers were obtained between index and contact animals in order to determine the effect natural transmission has on FVC's ability to replicate and cause disease. All surviving animals were assessed for infection status by spleen mass and viral titer then each measure was pooled in order to obtain overall

virulence and titer estimates; in total, 75 observations were assessed out of the seven

populations. Comparisons between contact and index virulence and titer were performed

using linear mixed models (LMM) from R packages lmer4 and degrees of freedom and

resulting p-values were estimated using lmerTest (Kuznetsova *et al.*, 2016). Furthermore,

in order to determine the effect host sex has on titer and virulence models were

performed on each viral measure of contact animals; host sex and infection status were

used as fixed effects and population as a random effect, as was done previously. To

ensure that population design (sex of index mice) did not influence virulence and viral

titer, and impair the ability to compare virulence and F-MuLV titers of contact and index

mice, we performed a LMM. No significant effect of population design was observed

(Figure 3S.3, Table 3S.2).

3.3.2.5 Influence of Male Social Status on Transmission,

Virulence and Titer of Naturally Transmitted FVC

To determine the effect social dominance has on transmission, virulence, and titer,

comparisons were made using both GLMMs and LMMs. The influence of social

dominance on transmission was determined by comparisons made between the

transmission efficiency between surviving dominant and nondominant contact animals (*n*

= 40). Due to the binomial nature of transmission data GLMMs were utilized again

assuming a binomial distribution and a logit link. Social status was used as the fixed

effect with population again used as a random effect. In order to determine the influence

of social status on virulence and titer, LMMs were utilized due to the continuous nature

of the data analyzed. Each LMM was conducted on 22 observations throughout the seven

populations and each model consisted of social status as the single fixed effect followed by population as the lone random effect.

3.4 Results

Overall rates of horizontal transmission to contact founders in seminatural populations, regardless of the sex of index and contact mice, were 47.6 ± 7.6% (M ± SEM) ($n = 7$). Contrastingly, no offspring (0/25) born to index females had detectable F-MuLV titers showing that vertical transmission did not occur. The observed difference in rates between horizontal and vertical transmission were statistically significant (t-test; $t = 6.29$, $p = 0.001$).

Male mice within seminatural enclosures were the major drivers of viral transmission dynamics as they were both more likely to contract and transmit F-MuLV than females. Across all populations contact males had contraction rates of 61.2 ± 10.3% and were 1.63 times more likely to contract F-MuLV than contact females who were infected with F-MuLV at a rate of 37.5 ± 10.3%; this difference was statistically significant (GLMM; Z = -2.37, P = 0.018; Figure 3.2A; Table 3S.3). Similarly, populations founded with only index males had transmission frequencies of 50.0 ± 7.1% ($n = 2$) (across both sexes of contacts), which were 2.51 times higher than rates in populations containing only index females (19.9 ± 2.3%; $n = 2$); again, this difference was found to be statistically significant (GLMM; $Z = -3.135$, $p = 0.002$; Figure 3.2B). Further support for male-driven transmission was observed as populations containing both female and male index mice did not have higher rates of transmission (63.5 ± 4.5%; $n = 2$) than those containing only index males (GLMM; $Z = 1.04$, $p = 0.299$; Figure

3.2B), despite containing more index individuals in total.

Furthermore, SFFV transmission was also male biased, though males were not significantly more likely to contract SFFV than females they are more likely to transmit SFFV. Across all populations contact males had marginally significantly higher rates of SFFV contraction (GLMM; $Z = 1.79$, $p = 0.073$; Figure 3.2C; Table 3S.2). Furthermore, there is 2.31 times more transmission in populations founded by only male index mice than those founded by only female index mice (GLMM; $Z = 4.14$, $p < 0.0001$; Figure 3.2D). Interestingly, populations founded with both male and female index animals did not have significantly higher SFFV transmission rates than female index populations (GLMM; $Z = 1.74$, $p = .082$; Figure 3.2D, 3S.3).

Contact males showed a significantly increased F-MuLV titer over contact females (LMM; $t = 2.61$, $p = 0.011$; Figure 3.3B; Table 3S.4), though there was no difference between the sexes in index animals. A strong correlation exists between virulence and titer in contact males (LM; $r^2 = 0.398$, $F_{1,20} = 13.23$, $n = 22$, Table 3S.5), however, there is no relationship seen between virulence and titer in contact females (LM; $r^2 = 0.048$, $F_{1,27} = 1.326$, $n = 29$; Figure 3.3C). Furthermore, both male and female index mice had 2.39 times larger spleen mass than contact mice (LMM; $t = 3.02$, $p = 0.004$; Figure 3.3A; Table 3S.4). However, there is no significant difference in virulence between the sexes in either contact or index mice (LMM; $t = 0.25$, $p = 0.807$; Figure 3.3A). F-MuLV titer was significantly higher in index males and females when compared to contact animals (LMM; $t = 4.35$, $p < 0.0001$; Figure 3.3B).

No effect of male social status was seen in regards to susceptibility as both socially dominant and nondominant male contact mice contracted F-MuLV at similar

frequencies (GLMM; Z = -0.08, p = 0.938; Figure 3.4A; Table 3S.6). Similar patterns

were seen for rates of SFFV contraction (GLMM; Z = -0.03, p = 0.973; Figure 3.4B). Of

males that contracted F-MuLV, social status did not influence spleen mass (LMM; t = -

1.19, p = 0.250; Figure 3.4C; Table 3S.6); However, F-MuLV titers were higher in

nondominant relative to dominant males (LMM; t = -2.59, p = 0.018: Figure 3.4D).

3.5 Discussion

Within seminatural enclosures we demonstrated that horizontal transmission (and

not vertical) is the primary mode of transmission, characterized asymmetrical rates of

contraction and transmission between the sexes, that natural transmission results in less

virulent infection than direct injection, that males which contract F-MuLV have higher

titers than do females despite experiencing similar levels of virulence, and that male

social status influences infection severity. Across all contact mice, rates of horizontal

transmission of F-MuLV were 47.6%, while no offspring born to index females within

enclosures tested positive. Male contact mice were 1.63 times more likely to contract F-

MuLV than contact females. Though there was no difference in spleen mass between

male and female contact animals there was a significant increase (~ 1 order of magnitude)

in titer in males over females. Index mice had 6.92 times the spleen mass as did contact

mice. Finally, it was observed that social status did not influence contraction rates of

FVC, nor the resulting virulence, but nondominant males were found to harbor F-MuLV

titers that were ~ 2 orders of magnitude higher than dominant males.

FVC horizontal transmission through sex and vertical transmission has been

explicitly tested using inbred mouse strains in cages (Mirand *et al.*, 1966; Portis *et al.*,

1987); however, these studies did not take into account all modes of transmission simultaneously to determine their relative importance. Horizontal transmission is dominant to vertical transmission in this FVC system, as no evidence of vertical transmission was observed, even though previous studies have shown vertical transmission to occur at ~16% (Mirand *et al.*, 1966). Because of the high rates of male contraction and transmission male-male agonistic encounters emerge as the likely main mode of FVC transmission. If sexual transmission were the main mode, we would predict patterns of high rates of female contraction and high rates of male transmission, which were not observed.

The observations that males primarily contract and transmit FVC within seminatural enclosures, while possessing higher titers, but similar levels of virulence to females, is indicative that FVC, and potentially other MuLVs, may have an evolutionary history transmitting through males. Sex-specific transmission and virulence is a key life history characteristic of many parasites and pathogens (Zuk & McKean, 1996). Host sex can be an important selective pressure on pathogens, which are more likely to be transmitted or contracted by one sex than the other due to sex-based differences in behavior and physiology (Duneau & Ebert, 2012; Robinson & Klein, 2012). Sex-specific pathogen adaptation has been documented in invertebrate host-pathogen systems (Høeg, 1995; Werren *et al.*, 2008; Rosenkranz *et al.*, 2010), and in the vertebrate systems of *Myxozoa* in fish (Whipps & Kent, 2006), and the mite *Spinturnix andegavinus* in bats (Christe *et al.*, 2007). Experimental demonstration of pathogen adaptation based on host sex is currently limited to invertebrates (e.g., *Daphnia* (Duneau *et al.*, 2012)). Mammalian models, such as the FVC/*Mus* system, are needed to explore the generality of

these patterns in species with adaptive immunity. Mammalian models will also be better models for human disease, where recent evidence is indicating that sex-specific adaption may be an important phenomenon (Úbeda & Jansen, 2016).

The finding that male social status influences F-MuLV titers, but not spleen size or prevalence, highlights the complicated interactions between social status, individual immune function, and virulence (reviewed in Fairbanks & Hawley, 2012). Two main hypothetical frameworks exist to explain the relationship between social status and cost of infection in vertebrates: 1) that dominant individuals expend more resources acquiring a territory and matings, leaving them less capable of investing in immune defenses to ward off infections (Folstad & Karter, 1992); and 2) that dominant individuals are in better condition, and therefore more resistant to pathogens as subordinates undergo stress associated with poor habitat and constant antagonism by dominant individuals (Sapolsky, 2004) (for recent review of both paradigms see Habig *et al.*, 2015). Unfortunately, the unusual pattern between viral titers and spleen size (virulence) in this study can be viewed to support either paradigm. Specifically, dominant males may be getting sicker per unit virus as they have lower titers but similar sized spleens as nondominate males; conversely dominant males may be in better condition than subordinates, and thus able to better regulate their viral load.

In regard to prevalence in social vertebrates, typically dominant individuals are more parasitized than subordinates due to more frequent social interactions (Habig *et al.*, 2015); however, in this study both classes of males contracted F-MuLV at similar frequencies possibility due to male-male interactions as the primary driver of transmission (i.e., dominant males have more interaction with females but they do not

readily transmit the virus). To further dissect the interplay between social status, immune parameters, and pathogen virulence seminatural systems, like this one, are needed to help merge laboratory measures of immune function with truly natural systems.

Although this study advances our understanding of how a model retrovirus transmits in a naturalistic setting, there are a few limitations in interpreting the results. First, variation exists in the infection duration of contact animals (i.e., some contact mice contract FVC earlier in the study than others) likely leading to inflated variation in titers and spleen mass of contact mice; this may cause us to miss patterns that would become apparent with more temporal control. Likewise, comparisons between contact and index mice are temporally disjointed in the sense that index mice are all infected prior to release into enclosures and contacts are infected throughout the study; additionally, index mice are likely infected with higher doses of virus via IP injection than contact mice. Moreover, vertical transmission may not have been seen in this study due to two reasons: first, wild-derived mice are much more resistant to FVC than the inbred mice used previously; second, vertical transmission was measured at 1-4 weeks postbirth in this study and anywhere from 5 to 8 months postbirth in previous studies (Mirand *et al.*, 1966). Finally, the inability to use qPCR-based techniques to quantify SFFV due to expression of endogenous retroviral RNA limits our ability to integrate SFFV fully into our understanding of FVC transmission dynamics.

Understanding how MuLVs naturally move through populations is important for more than just *Mus* populations, as related retroviruses have been found across taxa of terrestrial vertebrates and are thought to be important for emerging infectious diseases (Martin *et al.*, 1999). Furthermore, the transmission of leukemia viruses such as Feline

Leukemia Virus in the endangered Florida Panther (*Puma concolor coryi)* are of great interest to conservationists trying to restore natural populations of these animals not to mention the effects of these viruses on domestic felines (Cunningham *et al.*, 2008; O'Brien *et al.*, 2012). The power of seminatural enclosures allows for the elucidation of the factors contributing to the natural history of murine leukemia like viruses. Furthermore, characterizations of the transmission dynamics of various pathogens under natural or seminatural conditions, such as the one done here, may provide insight into better combating emerging infectious disease, and established wildlife and human pathogens (Antonovics *et al.*, 2017).

Using seminatural enclosures and wild-derived mice we have characterized the transmission dynamics of a classic model retrovirus. In doing so, we have demonstrated that the prevailing understanding—that FVC is primarily vertically transmitted through mothers milk, or transmitted horizontally through sex (Mirand *et al.*, 1966; Portis *et al.*, 1987)—is not supported and that male-male transmission during aggressive interactions is likely the predominant mode of transmission. Furthermore, we illustrate that within seminatural enclosures males harbor higher titers than females while suffering similar virulence; collectively, with the observation that there is a strong correlation between nonspleen corrected titer and virulence in males and none in females suggests that FVC is male adapted. Additionally, we demonstrated that male social status influences aspects of infection in an unexpected way: though dominant and nondominant males contract the virus at similar rates, and experience similar levels of virulence, dominant males have lower titers than do nondominant individuals. Understating the natural transmission dynamics of model pathogens will lead to better interpretations of the results of

laboratory experiments by framing them in an ecological and evolutionary context, while

studies with seminatural host-pathogen systems promise to bridge the gap between

laboratory and field studies and potentially shed light on the complex interactions of

sociality and disease ecology.

3.6 References

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Figure 3.1. Seminatural enclosure and population design. (A) Seminatural enclosure set up with six territories with four optimal territories (bins) and two suboptimal territories (small bins). (B) Study design showing three different population design groups: female and male index (left), male only index (center) and female only index (right).

Figure 3.2. Transmission dynamics of F-MuLV and SFFV in seminatural enclosures by contact (A-C) and index (B-D) mouse sex. (A) Male contact mice (squares) contracted F-MuLV at rates 1.63 times higher than contact females (circles). (B) Populations founded with only index males (filled triangles) had transmission rates 2.51 times higher across all contacts than those founded with only index females (open triangles), while rates did not differ in populations with only male index relative to those containing index animals of both sexes (diamonds). (C) No significant difference was seen between contraction in males and females in SFFV. However, male populations had 2.31 times higher transmission than females. Interestingly, populations founded with both male and female index animals have reduced transmission efficiency than those founded by only index males and are only marginally significantly different from those founded solely by female index mice. Data points represent populations ($n = 7$), lines represent population means, and error bars standard error. * Indicates a P value < 0.05., **< 0.01, ***< 0.001.

Figure 3.3. Spleen mass (A), F-MuLV titers (B), and their correlation (C) in female and male mice from seminatural enclosures. (A) Index mice (open symbols) of both sexes had spleens 6.92 times the mass of contact (closed symbols) animals, a log scale is used to enhance visualization. **(B)** Males (squares) had higher F-MuLV titers, by ~ 1 order of magnitude, than females (circles), but posthoc tests only confirmed this sexually dimorphic pattern between contact mice. Additionally, index mice of both sexes had titers ~ 2 orders of magnitude higher than contacts. **(C)** Though spleen mass and F-MuLV titers were positively correlated in contact males, no such relationship was observed in females and the relationship between spleen mass and FMuLV titers significantly differed between the sexes. Data points represent individual mice, in A and B lines indicate means and error is standard error, while in panel C lines are regressions. from post-hoc Tukey multiple comparison tests. Dashed lines indicates that origin is below zero. * Indicates a P value < 0.05, ** < 0.01, *** < 0.001

Figure 3.4. Influence of male social dominance on F-MuLV (A) and SFFV (B) contraction, virulence (C), and titer (D) in seminatural enclosures. (A) The dominance status of contact males did not influence their likelihood of contracting F-MuLV; (B) this was also the case for SFFV. (C) Likewise, virulence as measured by spleen mass was not influenced by social status. (D) However, F-MuLV titers were higher in nondominant relative to dominant males Data points represent populations ($n = 7$) in A, and individuals ($n = 22$) in B and C. lines represent means, and error bars standard error. Dashed line indicates that origin is below zero.

3.7 Supplementary Material

Figure 3S.1. Gel showing the ~170bp SFFV fragment. Positive control (lane 2) shows the positive SFFV RNA fragment at ~170bp whereas the negative RNA controls (lanes 3-4) do not. The ladder is represented in lane 1 and has the 500bp, 200bp and 100bp bands labelled.

Figure 3S.2. Spleen mass (A) and F-MuLV titer (B) in female and male caged mice.
(A) Females had spleens 3.46 times the mass of those of males twelve days post-infection; a log scale was used to enhance visualization. (B) A trend was detected in regards to females having higher F-MuLV titers than males. **Indicates a P value < 0.01).

Figure 3S.3. Influence of population design on virulence and titer(A) and F-MuLV titer (B). No significant influence of population design was seen on either spleen mass or F-MuLV titer of contact mice. For statistics see (Table 3S.5); no comparisons between groups were significantly different. Dashed line indicates that origin is below zero.

Table 3S.1. Summary of LM results concerning viral titers and spleen mass of caged animals.

F-MuLV titers (log transformed) by spleen mass (log transformed) of both sexes				
Fixed effects	*Estimate*	*Std. Error*	*t value*	*Pr(>|t|)*
Intercept (Female)	2.49	0.43	5.75	<0.0001***
Sex (Male)	-0.30	0.88	1.07	0.296
Spleen mass (log transformed)	0.94	0.54	-0.55	0.209
Sex (Male) X Spleen mass (log transformed)	1.14	0.80	1.43	0.165

SFFV RNA titers (log transformed) by spleen mass (log transformed) of both sexes				
Fixed effects	*Estimate*	*Std. Error*	*t value*	*Pr(>|t|)*
Intercept (Female)	-0.35	0.40	-0.88	0.39
Spleen mass (log transformed)	1.13	0.46	2.45	0.02*
Sex (Male)	-0.005	0.73	-0.006	0.995
Sex (Male) X Spleen mass (log transformed)	0.19	0.67	0.29	0.77

SFFV RNA titers (log transformed) by F-MuLV titers (log transformed) of both sexes				
Fixed effects	*Estimate*	*Std. Error*	*t value*	*Pr(>|t|)*
Intercept (Female)	4.91	2.10	2.34	0.03*
SFFV titer (log transformed)	-0.37	0.42	-0.88	0.39
Sex (male)	-2.50	2.34	-1.07	0.30
SFFV Titer (log transformed) x Sex (Male) (log transformed)	0.35	0.48	0.72	0.48

* Indicates a P value < 0.05., ***< 0.001.

Table 3S.2. Summary of LMMs concerning the virulence and titer of F-MuLV of contact mice by population design.

Virulence (Spleen mass) of contact mice by index sex

LMM (groups: 7; observations = 51)

Random effects	Variance	Std. Deviation		
Population	0.020	0.142		
Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	0.717	0.121	5.92	0.001**
Sex (Female)	-0.123	0.103	-1.19	0.241
Index sex (Female)	-0.060	0.199	-0.30	0.771
Index sex (Male)	0.152	0.164	0.93	0.405

F-MuLV titers (log transformed) of contact mice by index sex

LMM (groups: 7; observations = 51)

Random effects	Variance	Std. Deviation		
Population	0.061	0.247		
Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-0.561	0.360	-1.56	0.162
Sex (Female)	-1.409	0.387	-3.65	0.001 **
Index sex (Female)	0.084	0.602	0.14	0.892
Index sex (Male)	0.626	0.431	1.45	0.265

** Indicates a P value < 0.01, *** < 0.001.

Table 3S.3. Summary of GLMM results for FVC transmission dynamics in seminatural enclosures.

F-MuLV Transmission by contact and index sex

GLMM with binomial distribution and logit link (groups: populations = 7; observations = 14)

Random effects	Variance	Std. Deviation		
Population	0.00	0.00		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept (Contact Female, Index Female)	-2.12	0.56	-3.81	<0.0001***
Sex of contact mouse (Male)	1.16	0.49	2.37	0.018*
Sex of index mice (Male only)	1.85	0.59	3.14	0.002**
Sex of index mice (Both Female and Male)	2.35	0.61	3.88	<0.0001***

SFFV Transmission by contact and index sex

GLMM with binomial distribution and logit link (groups: populations = 7; observations = 14)

Random effects	Variance	Std. Deviation		
Population	0.00	0.00		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept (Contact Female, Index Female)	-1.94	0.55	-3.54	0.0004***
Sex of contact mouse (Male)	0.91	0.50	1.79	0.0727
Sex of index mice (Male only)	2.55	0.62	4.14	<0.0001***
Sex of index mice (Both Female and Male)	1.02	0.59	1.74	0.0823

F-MuLV Transmission to contact males by social status

GLMM with binomial distribution and logit link (groups: populations = 7; observations = 14)

Random effects	Variance	Std. Deviation		
Population	0.00	0.00		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept (Nondominant contact males)	0.17	0.41	0.41	0.683
Social status (Dominant)	-0.05	0.64	-0.08	0.938

SFFV Transmission to contact males by social status

GLMM with binomial distribution and logit link (groups: populations = 7; observations = 14)

Random effects	Variance	Std. Deviation		
Population	0.00	0.00		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept (Nondominant contact males)	-0.20	0.45	-0.45	0.655
Social status (Dominant)	-0.02	0.65	-0.03	0.973

* Indicates a P value < 0.05., ** < 0.01, *** < 0.001.

Table 3S.4. Summary of LMM results concerning titer and virulence measures between index and contact animals.

Virulence (Spleen mass) of contact and index mice by sex

LMM (groups: populations = 7; observations = 75)

Random effects	Variance	Std. Deviation		
Population	0.0137	0.117		
Fixed effects	*Estimate*	*Std. Error*	*t value*	*Pr(>|t|)*
Intercept	0.704	0.098	7.19	<0.0001***
Sex (Male)	0.032	0.130	0.25	0.807
Type (Index)	0.489	0.162	3.02	0.004 **
Interaction (Male:Index)	0.327	0.238	1.37	0.176

FMuLV titers (log transformed) of contact and index mice by sex

LMM (groups: populations = 7; observations = 75)

Random effects	Variance	Std. Deviation		
Population	0.353	0.594		
Fixed effects	*Estimate*	*Std. Error*	*t value*	*Pr(>|t|)*
Intercept	-1.475	0.364	-4.06	0.001 **
Sex (Male)	1.095	0.419	2.61	0.011 *
Type (Index)	2.310	0.531	4.35	<0.0001***
Interaction (Male:Index)	-0.181	0.789	-0.23	0.819

* Indicates a P value < 0.05., ** < 0.01, *** < 0.001.

Table 3S.5. Summary table of correlations of titer and virulence in contact animals

FMuLV titers (log transformed) by Virulence (Spleen mass) of contact females

LM; r^2 = 0.048 , $F_{1,27}$ = 1.326, n = 29

Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	1.077	0.385	2.80	0.009**
Spleen mass (log transformed)	-0.583	0.500	-1.17	0.253

FMuLV titers (log transformed) by Virulence (Spleen mass) of contact males

LM; r^2 = 0.398 , $F_{1,20}$ = 13.23, n = 22

Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	0.053	0.544	0.10	0.924
Spleen mass (log transformed)	2.472	0.680	3.63	0.002**

FMuLV titers (log transformed) by Virulence (Spleen mass) of both contact sexes

LM; r^2 =0.421 , $F_{3,47}$ =11.4, n = 51

Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Female)	1.077	0.397	2.71	0.009**
Sex (Male)	-0.583	0.657	-1.13	0.126
Spleen mass (log transformed)	-1.024	0.516	-1.56	0.264
Sex (Female) X Spleen mass (log transformed)	3.055	0.832	3.67	<0.0001***

** Indicates a P value < 0.01, *** < 0.001.

Table 3S.6. Summary table of GLMMs of male social status on F-MuLV titer and virulence

F-MuLV Transmission to contact males by social status

GLMM with binomial distribution and logit link (groups: populations = 7; observations = 14)

Random effects	Variance	Std. Deviation		
Population	0.00	0.00		
Fixed effects	Estimate	Std. Error	Z value	Pr(>|z|)
Intercept (Nondominant contact males)	0.17	0.41	0.41	0.683
Social status (Dominant)	-0.05	0.64	-0.08	0.938

Virulence (Spleen mass) of contact males by social status

LMM (groups: populations = 7; observations 22)

Random effects	Variance	Std. Deviation		
Population	0.00	0.01		
Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Nondominant contact males)	0.80	0.09	8.94	<0.0001***
Social status (Dominant)	-0.17	0.14	-1.19	
				0.250

FMuLV titers (log transformed) of contact males by social status

LMM (groups: populations = 7; observations 22)

Random effects	Variance	Std. Deviation		
Population	0.08	0.29		
Fixed effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Nondominant contact males)	1.22	0.38	3.21	0.007**
Social status (Dominant)	-1.48	0.57	-2.59	0.018*

CHAPTER 4

MICROBIAL TRANSPLANT INCREASES

SUSCEPTIBILITY TO A SYSTEMIC

VIRAL INFECTION

4.1 Abstract

Many physiological processes in the host are influenced by the microbial

communities living within the gut. These influences range from digestion of fibrous

foods to immunity influencing susceptibility to enteric pathogens. Though much work

has shown a strong relationship with enteric pathogen susceptibility and the microbiome,

little has been done on how changes in the microbiome influence systemic infection. We

seek to differentiate between two hypotheses that may lead to changes in FVC virulence:

(1) that because of trade-offs between resources available for systemic and enteric

immunity of the host, microbial transfers of the gut microbiota will cause an increase in

susceptibility to the systemic pathogen Friend Virus Complex (FVC); (2) there will be a

shift in FVC susceptibility that corresponds with host resistance of the donor

microbiome. Using established microbial transfer techniques, we reconstituted two

different treatment groups with different microbiotas: native treatments consisted of hosts

receiving gut microbiome stock from animals with their same MHC genotype, and novel

treatments consisted of hosts receiving gut microbiome stock from animals of a different

MHC genotype. Novel treatment groups had higher titer and virulence than the native groups, suggesting that the animals in the novel treatment are more susceptible to FVC infection. Furthermore, there was a marginally significant reduction in overall lymphocyte percentage in the novel treatment group, suggesting animals receiving novel microbiomes are potentially immunocompromised; However, there was a significant increase in proportion of CD4+ T-cells These results suggest that caution should be taken when fecal transplants are done to combat enteric infections as the treatment may cause a patient to become more susceptible to other systemic pathogens and that more work needs to be done in order to fully understand the immune factors leading to this virulence increase.

4.2 Introduction

Gut microbial communities are known to play a role in the maintenance of homeostasis and health in the host where specific microbial constituents have been shown to aid in digestion of fibrous foods, immunity, and in the ability to detoxify toxins (reviewed in Dethlefsen *et al.*, 2007; Sekirov *et al.*, 2010). Colonization immunity, or the ability of the microbiome associated with a host to confer resistance to enteric pathogens, has implications in human health; leading to a potential therapeutic capacity to combat enteric disease (Stecher *et al.*, 2007; Sekirov *et al.*, 2010; Buffie & Pamer, 2013). However, it is still unclear to what extent physiological processes can be influenced, positively or negatively, by microbiome transfer.

Microbiomes residing within the host gut have a large influence on host immune function and changes in microbial composition of communities alters overall immune

function (Maynard *et al.*, 2012). These dynamics are clearly seen in germ-free mouse systems where the absence of microbiota leads to severely immunocompromised hosts (Cebra, 1999; Khosravi *et al.*, 2014). Microbiome transfers have shown shifts in immune function; even the ability to shift susceptibility to enteric pathogens (Willing *et al.*, 2011; Kubinak *et al.*, 2015). These immunity shifts have been found to be driven by specific immune cell subtypes and differential cytokine signaling that change when interacting with novel microbial constituents (Chen *et al.*, 1999; Hensley-McBain *et al.*, 2016). Relationships with particular constituents associated with microbial colonization (i.e., liposaccharide [LPS]) and increased inflammation have also be found, which may shift immunity away from antiviral immunity, allowing viruses to replicate more freely (Pullen *et al.*, 1995; Jude *et al.*, 2003). However, the microbiota may be interacting with systemic components of the immune system, which may, in turn be influencing interactions with systemic pathogens.

The major histocompatibility complex (MHC) is the key interface involved in the differentiation of self from nonself and mediates much of the adaptive immune response. It is the most diverse region in the vertebrate genome with over 2,400 alleles for one locus in the human population alone (Bodmer *et al.*, 1986; Wegner *et al.*, 2003; Robinson *et al.*, 2015). MHC genotypes are highly associated with resistance and susceptibility profiles to numerous pathogens (Jeffery & Bangham, 2000). Furthermore, the host MHC genotype has a strong influence on shaping the microbial communities in the gut, which has been demonstrated using MHC congenic mice (*Mus musculus*) (Kubinak *et al.*, 2015). Collectively, the centrality of the MHC in adaptive immunity, its high degree of polymorphism, and its proven ability to sculpt microbial communities make MHC

congenic mouse lines an ideal model in which to characterize the influence of microbiota on systemic pathogen virulence.

Friend Virus Complex (FVC) is a systemic murine retrovirus that is made up of two independent genomes that work synergistically to cause an erythro-proliferative disease: Friend Murine Leukemia Virus (F-MuLV) and Spleen Focus Forming (SFFV) (Friend, 1957; Ruscetti, 1999). Due to FVC being well characterized and its ability to be used as an acute acting virus, it is ideal to use in the short infection study completed here (Hasenkrug & Chesebro, 1997; Kubinak *et al.*, 2012, 2013). Previous work with FVC has shown increases in virulence in the absence of host microbiota; however, it has not been assessed if different microbial communities influence FVC virulence (Mirand & Grace, 1963).

Here we test the influence fecal microbial transplants (FMT) have on virulence of a systemic pathogen—FVC. We accomplish the FMT by transferring the gut microbial constituents of two different strains of MHC congenic mice. Two independent groups were created: the novel group received microbiota from the dissimilar MHC genotype whereas the native group received microbiota from the same MHC genotype. We seek to differentiate between two hypotheses: (1) that transferring gut microbiota between two different genotypes of hosts will increase the susceptibility to viral infection by FVC due to the tradeoff in immunity, or (2) there will be a shift in susceptibly that corresponds to the resistance of the host microbiome transferred.

4.3 Methods

Mice *(Mus musculus)* were purchased from Jackson laboratories and bred under specific pathogen free conditions. Two different strains of MHC congenic Balb/c mice were used: BALB/cByJ (Balb/c^{dd}), and C.B10-H2b (Balb/c^{bb}). All animals used in the experiment were females between two and six months of age. Twenty-eight animals were initially placed on antibiotic treatment before infection and were grouped into four treatments (Figure 4.1), two control groups and two treatment groups. Due to severe weight loss during antibiotic treatment, seven animals were removed from the study before infection leading to 21 total animals being infected.

All viral stocks were generated from a previously described biological clone of the NB-tropic strain of Friend Virus Complex (Kubinak *et al.*, 2012). The viral stock used to infect the experimental animals was generated by passaging the biological clone through one round of highly susceptible DBA mice in order to produce a virus that was virulent enough to cause disease in both host genotypes used in the study. After infection, all spleens were collected, mixed in a 1:1 ratio with 1x PBS, homogenized, and centrifuged at 5,000 RPM for 5 min. Supernatants were collected, mixed with each other, then aliquoted and stored at -80°C. All viral stocks were kept frozen until use and only thawed once.

All infections were intraperitoneal (IP) and were injected at a 20 µL volume. After infection, the virus was incubated *in vivo* for 12 days at which time animals were sacrificed. At sacrifice, spleens were harvested and portions were removed and weighed for use in flow cytometry. The remainder of each spleen was weighed then homogenized in a 1:1 ratio with 1x PBS.

To obtain estimates of the number of viral insertions into the host genome, quantitative PCR (qPCR) was conducted on F-MuLV using a previously described protocol (Kubinak *et al.*, 2012). Due to a strong correlation and previous work showing similarities between F-MuLV and SFFV titers only F-MuLV was quantified here (Kubinak *et al.*, 2012). Viral titers were standardized to total GAPDH estimates to obtain a viral genome by GAPDH ratio. Furthermore, the F-MuLV/GAPD ratios calculated previously were multiplied by spleen mass in order to obtain total viral genomes by spleen estimates. All qPCR reactions were conducted on a Biorad CFX96 system.

All microbiota stocks were generated by sacrificing four animals of each MHC genotype and collecting microbiota from their intestines (duodenum to anus). The microbiome from each genotype was then combined in a 1:1 ratio with 1x PBS in order to create the stocks used for oral gavage. All microbiota stocks were spun at 5,000 RPM for 5 min., supernatants were aliquoted, flash frozen in liquid nitrogen and then kept at -80°C until use.

Animals were treated daily for two weeks with 0.5mg/mL of each of the following antibiotics: neomycin sulfate, ampicillin, gentamicin, and erythromycin. Following antibiotic treatment mice were put into their respective native or novel groups. Native groups consisted of animals receiving a daily oral gavage (100 µL) of microbiota from a donor of their own genotype (i.e., Balb/c^{bb} genotype animals received Balb/c^{bb} microbiota). Novel groups consisted of animals that received a daily oral gavage (100 µL) of microbiota from the different host genotype (i.e., Balb/c^{bb} genotype animals received Balb/c^{dd} microbiota). Oral gavage occurred daily for one week to allow for sufficient reconstitution to occur. One day after the last oral gavage procedure, each

animal was infected with a standardized volume of stock FVC.

Flow Cytometry was conducted at the flow cytometry core at the University of Utah using a FACs Canto cytometer running FACsDiva software. All samples were isolated from the spleen and run through a 0.22μ filter in order to obtain a single cell suspension. Cells were stained per BioLegend cell staining protocol using the following conjugated antibody stains: antimouse CD4-FITC, antimouse CD8-PercyPcy5.5, anti-mouse CD3-Pacific Blue, antimouse CD11a-PE/Cy7, antimouse CD19-PE. These conjugated antibodies were chosen to obtain estimates of standing and activated T-cell abundance along with standing and activated B-cell abundance. All conjugated antibody stains were ordered from BioLegend and used per manufacturer recommendations.

Linear models were run with host genotype and microbiome treatment groups as fixed effects and either spleen mass, proviral load, or spleen corrected proviral load as the response variable. These models accounted for variation due to both host genotype and microbiome treatment group and their interactions. Post-hoc t-tests were completed when a significant interaction term was seen between genotype and treatment group. When interaction terms in the linear models were not significant those terms were removed from the model as in the titer and spleen corrected titer models. Models were conducted with 21 observations each and an intercept set at zero. All statistical analyses were conducted on $\log_{10}$ transformed data to improve normality and completed in R statistical modeling software (3.3.1) (R Core Team, 2016).

4.4 Results

Novel microbiome caused a significant increase in virulence at the model

intercept (0.2218 ± 0.0681) when compared to spleen size from animals with native

microbiomes (LM; $t = 3.255$, $p = 0.005$, Table 4.1). There was also a large effect of host

genotype with the more susceptible genotype (Balb/c^{dd}) having significantly higher

virulence than the resistant genotype (Balb/c^{bb}) (LM; $t = 14.44$, $p < 0.0001$). Furthermore,

the interaction between host genotype and treatment was also significant (LM; $t = -2.177$,

$p = 0.045$); though post hoc tests indicate the effect is only significant in the Balb/c^{bb}

genotype (Post hoc Welch T test; Balb/c^{dd}, $t = -0.031$, $p = 0.976$; Balb/c^{bb}, $t = -3.085$, $p =$

0.0150) (Figure 4.2A–B).

Mice with novel microbiomes had marginally significantly increased ($0.4922 \pm$

0.2339) F-MuLV titers relative to those with native microbiomes (LM; $t = 1.75$, $p =$

0.098). Furthermore, the Balb/c^{dd} genotype showed a significant increase of F-MuLV

viral titer over the Balb/c^{bb} genotype (LM; $t = 4.44$, $p < 0.001$). There was not a

significant interaction between host genotype and microbiome treatment as both

genotypes showed an increase in titer in novel treatment, so the interaction term was

removed from the final model (LM; $t = -1.190$, $p = 0.254$) (Figure 4.2C–D).

The total number of viral genomes per spleen also showed a marginally

significant increase (0.6646 ± 0.2515) in hosts receiving novel microbiomes over native

microbiomes (LM; $t = 1.92$, $p = 0.071$) (Figure 4.2E–F). Again, host genotype had a

significant effect on spleen corrected titer with Balb/c^{dd} genotypes having increased

levels over Balb/^{bb} genotypes (LM; $t = 6.84$, $p < 0.0001$). There was no significant

interaction showing that both genotypes are acting similarly when novel microbiomes are

present, so the interaction term was not included in the final model (LM; $t = -1.527$, $p = 0.1464$).

In novel mice, there was a marginally significant reduction on overall lymphocyte proportion (LM: $t = -1.90$; $p = 0.074$; Figure 4.3; Table 4.2); however, no other significant relationships between tested cell subsets were seen. However, activated CD3+ and CD4+ T-cell subsets showed marginally significant and significant (respectively) increases in proportion when novel microbiomes were seen (CD3+, LM: $t = 1.982$, $p = 0.0649$; CD4+, LM: $t = 2.36$, $p = 0.031$; Figure 4.3; Table 4.2).

4.5 Discussion

The results presented here support the hypothesis that there may be a tradeoff in immunity between systemic and enteric immunity of the host, where the enteric immune response is pulling resources away from the systemic response, as we did not see a shift in susceptibility corresponding with resistance to FVC as would be expected in hypothesis 2. Comparisons between mice receiving either native or novel microbiome show an increase in susceptibility, though not significant with each measure, to FVC when a novel microbiome is seen; however, the virulence is driven by the significant increase in the Balb/c^{bb} genotype. Furthermore, a marginally significant reduction in overall lymphocyte proportion was seen in hosts receiving novel microbiomes, potentially leaving them less able to fight a systemic pathogen; this result is likely driven by a sharp reduction in lymphocytes when Balb/c^{bb} hosts receive Balb/c^{dd} microbiota. Although lymphocyte proportion was reduced by a marginal amount, there were increases in CD3+ and CD4+ T-cell subsets, again in the mice receiving a novel

microbiome. The patterns seen in the lymphocyte, but not the downstream T-cells, suggests that there are other important subsets playing a role and need to be determined in future work.

It is thought that cross talk between host and microbiome is important in the microbiomes ability to influence disease outcomes of pathogens (Buffie & Pamer, 2013). Multiple studies have demonstrated the interconnectivity of the host and microbiome; for example, how the Major histocompatibility complex (MHC) shapes the microbial community in the gut and how the microbial constituents of the gut influence cytokine signaling and immune response outside of the gut (Kubinak *et al.*, 2015). However, little work has been completed that has shown an influence of differential communities of microbiota on virulence of systemic pathogens.

Mirand and colleagues in the 1960s used germ free animals to understand the effect the microbiome has on FVC virulence (Mirand & Grace, 1963); though they showed an increase in virulence in germ-free animals, this may be due to the severely immunocompromised nature of germ-free animals as their immune systems never have the opportunity to mature (Marietta *et al.*, 2015). Using antibiotic treatment allows us to determine the effect of gut microbiota with fully immunologically competent animals. Furthermore, other immune responses such as T helper, IL-23, IL-17 and development of lymphoid tissue have been correlated with microbiota shifts (Cebra *et al.*, 1998; Mazmanian *et al.*, 2005; Zaph *et al.*, 2008). More specifically, T-cell effector cells, such as Th17+, Th22 and CD4+ cell, fluctuate in number when rhesus macaques are infected with simian immunodeficiency virus (SIV) after the microbiota is perturbed (Hensley-McBain *et al.*, 2016). Here we do not see a strong influence on the basic systemic

adaptive immune response (i.e. CD4+, CD8+ and B-cells). However, the drastic decrease in total lymphocyte proportion suggests there is another cell type that is influencing the effect. These data suggest more detailed immune cell phenotyping is warranted and perhaps from more tissues than just spleen.

Due to human health related pathogens, such as causative agents for diarrhea, the majority of studies involving microbiota have been done in the context of enteric pathogens (Kolling *et al.*, 2012). As a result, specific microbial constituents have been found that confer resistance to enteric pathogens, such as *Salmonella enterica* or *Clostridium* sp. (reviewed in Kamada *et al.*, 2013). However, these same relationships are not known for systemic pathogens. Moreover, the work presented here indicates that microbiota transferred between individuals influences the virulence of a systemic pathogenic infection. Furthermore, the finding that there is an increase in susceptibility when microbiota is transferred between MHC congenic animals, provides further evidence that MHC may be influential in shaping the protective nature of the microbial community in the gut. The increase in fitness and virulence of FVC may be due to multiple physiological responses such as immune resources being taken away from the systemic compartment to fight the novel microbiome in the gut or it may also be a change in the target cell number present when reconstitution occurs. The latter hypothesis seems less likely as data suggest that animals treated with antibiotics have reduced myeloid precursor cells suggesting a reduction in target cells for FVC to infect (Hill *et al.*, 2012; Khosravi *et al.*, 2014).

It has become clear in the last decade that the resident microbiome is acting like another organ of the host and that perturbations to that community have rippling effects

throughout host physiology, especially on immunity (Rohmer *et al.*, 2011). By treating animals with antibiotics then transferring intact microbiomes, we simulate a more ecologically relevant situation, as these conditions occur naturally in humans or domestic animals when they are prescribed antibiotics for pathogenic bacterial infections or during microbial transplant treatment routines (Sullivan *et al.*, 2001; Noverr & Huffnagle, 2004). Furthermore, fecal transplants are becoming more accepted and commonplace, especially due to the success medical professionals have had in treating *Clostridium difficile* infections with fecal transplants from healthy donors (Bakken *et al.*, 2011; Vrieze *et al.*, 2013). Though more work is needed to determine the generality of this pattern across systemic pathogens; the findings presented here suggest that caution is warranted during fecal transfers as treating the enteric disease could make the host more susceptible to a systemic viral infection.

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Figure 4.1. Microbiota transfer experimental design. Microbiota stocks were harvested from either Balb/c^{bb} or Balb/c^{dd} hosts. The microbiota stocks were transferred into hosts with either the same host genotype (Native) or into host of a different host genotype (Novel).

Figure 4.2. Virulence and fitness of FVC in hosts receiving either native or novel microbiomes. (A) A significant increase in virulence was seen in novel (open symbols) over the native (filled symbols) treatment, however, there is also a significant interaction suggesting the two genotypes are responding differently. Due to the significant interaction in virulence post hoc t-tests were performed which showed the effect of the LM is driven by the Balb/c^{bb} genotype (square symbols). (B) Proviral load showed a marginally significant increase in hosts receiving novel microbiomes. (C) Furthermore, spleen corrected titer also showed a marginally significant increase in the novel group with both groups showing a similar pattern. Round symbols represent Balb/c^{dd} genotypes.

Figure 4.3. Immune cell subset proportions of total cells or total lymphocytes. (A) Total lymphocyte proportion of total cells counted, showing a reduction in proportion in novel treatment groups when compared to native treatment groups. (B) CD3+ (T-lymphocytes) proportion of total lymphocyte population. There was a marginally significant increase in CD3+ T-cells in novel treatment groups over native treatment groups. However, this increase is driven by (C) CD4+ T-cells having a significant increase in novel treatment groups. Symbols denote genotype with filled symbols indicating native treatemtn group and open symbols indicating novel treatments. Data are represented by mean ± SEM.

Table 4.1. Linear models comparing treatment groups (native and novel), and host genotype.

Virulence (Spleen mass) of treatment groups

LM ($F_{3,16}$ = 125.9; R^2 = 0.9594; p-value = 2.44e-11)

Fixed Effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Native Microbiota)	-0.6956	0.0503	-13.823	2.59e-10 ***
Host (Balb/c^{dd})	1.0899	0.0755	14.438	1.35e-10 ***
Treatment (Novel Microbiota)	0.2218	0.0681	3.255	0.00497 *
Interaction (Balb/c^{dd}:Novel)	-0.2214	0.1017	-2.177	0.04480 *

F-MuLV titers (log transformed) of treatment groups.

LM ($F_{3,16}$ = 8.551; R^2 = 0.6159; p-value = 0.00128)

Fixed Effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Native Microbiota)	-2.6409	0.2593	-10.186	1.18e-08 ***
Host (Balb/c^{dd})	1.3328	0.2999	4.444	0.00037 **
Treatment (Novel Microbiota)	0.5247	0.2999	1.750	0.0982 .

Spleen Corrected F-MuLV titers (log transformed) of treatment groups.

LM ($F_{3,17}$ = 25.35; R^2 = 0.7193; p-value < 0.0001)

Fixed Effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Native Microbiota)	-3.2825	0.2910	-11.280	2.58e-09 ***
Host (Balb/c^{dd})	2.3009	0.3366	6.836	2.90e-05 ***
Treatment (Novel Microbiota)	0.6473	0.3366	1.923	0.0714 .

* Indicates a P value < 0.05., ** < 0.01, *** < 0.001.

Table 4.2. Linear models comparing treatment groups (native and novel), and host genotype of Total immune cell subsets.

Total Lymphocyte proportion by treatment groups

LM ($F_{2,17}$ = 4.127; R^2 = 0.2476; p-value = 0.035)

Fixed Effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Native Microbiota)	0.3694	0.0476	7.754	5.57e-07 ***
Host (Balb/c^{dd})	-0.1176	0.0551	-2.135	0.047 *
Treatment (Novel Microbiota)	-0.1047	0.0551	-2.901	0.074 .

Total Activated CD3+ proportion by treatment group

LM ($F_{2,17}$ = 1.93; R^2 = 0.089; p-value = 0.176)

Fixed Effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Native Microbiota)	0.128	0.0240	5.341	5.4e-05 ***
Host (Balb/c^{dd})	-0.005	0.0278	-0.163	0.872
Treatment (Novel Microbiota)	0.054	0.0278	1.960	0.067 .

Total Activated CD4+ proportion by treatment group

LM ($F_{2,17}$ = 10.59; R^2 = 0.5023; p-value = 0.001)

Fixed Effects	Estimate	Std. Error	t value	Pr(>|t|)
Intercept (Native Microbiota)	0.086	0.0259	3.312	0.0041 **
Host (Balb/c^{dd})	0.118	0.0301	3.928	0.0011 **
Treatment (Novel Microbiota)	0.071	0.0301	2.356	0.0307 *

* Indicates a P value < 0.05., ** < 0.01, *** < 0.001.

APPENDIX A

SERIAL INFECTION OF DIVERSE HOST (*MUS*)

GENOTYPES RAPIDLY IMPEDES

FITNESS AND VIRULENCE

PROCEEDINGS B

Research

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Serial infection of diverse host (*Mus*) genotypes rapidly impedes pathogen fitness and virulence

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Reduced genetic variation among hosts may favour the emergence of virulent infectious diseases by enhancing pathogen replication and its associated virulence due to adaptation to a limited set of host genotypes. Here, we test this hypothesis using experimental evolution of a mouse-specific retroviral pathogen, Friend virus (FV) complex. We demonstrate rapid fitness (i.e. viral titre) and virulence increases when FV complex serially infects a series of inbred mice representing the same genotype, but not when infecting a diverse array of inbred mouse strains modelling the diversity in natural host populations. Additionally, a single infection of a different host genotype was sufficient to constrain the emergence of a high fitness/high virulence FV complex phenotype in these experiments. The potent inhibition of viral fitness and virulence was associated with an observed loss of the defective retroviral genome (spleen focus-forming virus), whose presence exacerbates infection and drives disease in susceptible mice. Results from our experiments provide an important first step in understanding how genetic variation among vertebrate hosts influences pathogen evolution and suggests that serial exposure to different genotypes within a single host species may act as a constraint on pathogen adaptation that prohibits the emergence of more virulent infections. From a practical perspective, these results have implications for low-diversity host populations such as endangered species and domestic animals.

1. Introduction

Genetic variation in host populations can prevent the emergence of infectious diseases in two non-mutually exclusive ways: by directly reducing pathogen transmission rates (e.g. resistant individuals in a contact network block potential routes of transmission), and by creating variable host environments that result in fitness trade-offs to rapidly adapting pathogens [1]. In the latter case, fitness trade-offs arise as a consequence of pathogen adaptive responses that enhance fitness in one host genotype at the cost of fitness in another host genotype. This phenomenon is often referred to as antagonistic pleiotropy.

Low genetic diversity in host populations may reduce fitness trade-offs associated with specialization and promote the expansion of highly virulent pathogens [1], a phenomenon long appreciated by plant pathologists and termed the monoculture effect [2–7]. Given the practical implications of this phenomenon, it is surprising that only a handful of empirical studies have quantified the role of genetic variability among animal hosts in limiting infectious disease. Numerous serial passage experiments have shown that fitness trade-offs arise as a consequence of pathogen adaptation to a novel host [8,9]. However, most of these studies focus on adaptation to new host species rather than genotypes within a single host. Empirical evidence that genetic

diversity in an animal species limits pathogen adaptation and virulence evolution comes primarily from invertebrate studies [10–12]. There is a need to empirically address this hypothesis in vertebrate host–pathogen systems, particularly because their unique adaptive immune system will heavily influence the outcome of host–pathogen interactions.

The mouse-specific pathogen Friend virus (FV) complex is a well-studied host–pathogen model. The term 'complex' refers to the presence of two viruses: the replication-competent Friend murine leukaemia virus (F-MuLV) and the replication incompetent spleen focus-forming virus (SFFV). Synergistic interactions between these viruses promote the erythroproliferative disease observed in susceptible mouse genotypes (electronic supplementary material, figure S1) [13–15], and multiple host polymorphisms have been identified that influence the susceptibility of mice to infection [16–19]. Previous work in our laboratory has shown that serial infection of FV complex through a single mouse genotype can promote adaptive responses that enhance fitness in that host genotype at the cost of reduced fitness in other host genotypes [20,21]. Moreover, resistance variability among host genotypes can significantly influence both the magnitude of an adaptive response and the degree of host specialization (i.e. magnitude of fitness trade-offs) exhibited by this pathogen [22]. These data suggest that genetic diversity among hosts may constrain the evolution of more virulent infectious diseases.

Expanding upon these previous results, the experiments described here sought to empirically address the hypothesis that genetic variation among individuals of the same mammalian species, the house mouse (*Mus musculus*), acts as a constraint on pathogen adaptation and virulence evolution. To that end, we serially passaged FV complex through a series of individuals from the same mouse genotype or through a series of individuals representing different mouse genotypes and compared patterns of viral fitness (i.e. viral titres) and disease virulence that emerged in response to these differing selection regimes. We predicted that serial passage of FV complex in a single host genotype would result in adaptive responses that increased pathogen fitness and virulence and that FV complex serially passaged through a series of genetically distinct hosts would result in significantly lower fitness and virulence. Experimental results demonstrate that serial infection of a diverse array of host genotypes, and even a single round of host alternation, places a powerful constraint on the capacity of FV complex to evolve higher fitness and virulence.

2. Material and methods

(a) Host model
Several genotypes of inbred mice were purchased from Jackson laboratories for use in the experiments described here, namely BALB/cJ, C.C3 (a.k.a. BALB.KK), DBA/2J, A.SW, A/J, C3H/HeJ and 129X1/svj (see the electronic supplementary material, table S1, for more details on mouse strains). Animals were either bred under specific-pathogen-free conditions at the University of Utah or purchased as needed. All experimental animals were females between 8 and 16 weeks of age. The BALB/c and DBA/2J genotypes were chosen as test hosts for two primary reasons. First, we wanted to standardize the host environment across virus stocks to focus on the effect of selection regime on patterns of pathogen fitness and virulence, and using these two genotypes provided us with independent experimental

replicates. Second, because of their high sensitivity to FV complex infection, we reasoned that these genotypes would be useful models for readout of low fitness/low virulence viral phenotypes, whereas other more resistant genotypes might clear or be refractory to such infections. Owing to constraints on availability of mouse strains used in five-round experiments (A.SW, BALB.KK), and the requirement of susceptibility to FV complex infection and FV-induced disease, we chose to use the 129X1/svj, A/J and C3H/HeJ strains as alternate host genotypes in two-round serial passage experiments.

(b) Pathogen model
An NIH3T3 fibroblast cell line (3–6a) containing a biological clone of an NB-tropic strain of FV complex (i.e. an NB-tropic strain of F-MuLV complexed with the polycythemia strain of SFFV) was kindly provided by Dr Sandra Ruscetti (NIAID). This biological clone was grown in tissue culture and supernatants were collected to produce stocks of 'unpassaged' FV complex, which were subsequently used to initiate all serial passage experiments.

(c) Serial passage of FV complex
All passage lines were initiated by infecting animals via intraperitoneal (i.p.) injection with equivalent volumes (200 µl) of the unpassaged FV complex stock described above. Mouse-to-mouse passage of FV complex consisted of serially passaging FV complex from the spleens of infected animals to a new host via i.p. injection of virus-laden spleen supernatants. Infected spleens were homogenized in 15 ml conical tubes containing an equivalent weight/volume of sterile $1\times$ phosphate-buffered saline (PBS) (0.1 g = 100 µl 1X PBS), centrifuged at 5000 r.p.m. for 5 min at 4°C, and their supernatants were stored at −80°C in 500 µl aliquots until use. Virus stocks were thawed once immediately prior to use. Infected materials were kept on ice during all steps of virus stock preparation. Variability in infectious dose between passage rounds was controlled by infecting animals with equivalent volumes of different virus stocks whose concentrations were adjusted during the w/v dilution of spleen homogenates. Two independent serial passage designs were employed in this study and are described in detail below.

(i) Five-round serial passage experiments
Two independent experiments were conducted (designated BALB/c or DBA/2J after the host genotypes that served as test-phase genotypes). For each experiment, FV complex was serially passaged five times through a series of female animals from the same *Mus* inbred strain (BALB/c or DBA/2J) or through females from five different *Mus* strains (figure 1a). Thus, one 'same host genotype' and one 'different host genotypes' virus stock were created for each of the independent BALB/c and DBA/2J experiments. A total of six different mouse strains were used for these five-round serial-passage experiments and all have previously been shown (BALB/c, DBA/2J, 129X1/svj, A.SW, BALB.KK) [20–22], or are shown here (electronic supplementary material figure S2), to be susceptible to FV complex infection. Infected animals were monitored daily, with each round of infection lasting 10–12 days depending on the health status of the animal. To increase the likelihood of successful passage as well as increase volumes of passage stocks, two animals from the same strain were infected per round of infection and their spleen supernatants were pooled. To expand our stock of passaged virus for test-phase infections, 5–10 animals were infected during the fifth passage round. At the test phase, independent cohorts of female DBA/2J and BALB/c animals ($n = 8$–10) were infected with equivalent volumes of virus stocks derived by serial passage through the same or different host genotypes. Cohorts of animals from each genotype (DBA/2J = 20 animals, BALB/c = 40 animals) were

Figure 1. The design of serial passage experiments. Letter designations in each mouse represent different mouse genotypes. (*a*) To create the 'same host genotype' and 'different host genotypes' passage lines in the five-round serial passage experiments the following host genotypes were used: (BALB/c experiment: A, BALB/c; B, DBA/2J; C, BALB.KK; D, A.SW; E, 129X1/svj; F, A/J)(DBA/2J experiment: A and B, DBA/2J; C, BALB.KK; D, A.SW; E, 129X1/svj; F, BALB/c). (*b*) To create the 'same host genotype' and 'different host genotypes' passage lines in the two-round serial passage experiments the following genotypes were used: (BALB/c experiment: A, BALB/c; B, A/J; C, 129X1/svj; D, C3H/HeJ) (DBA/2J experiment: A, DBA/2J; B, A/J; C, 129X1/svj; D, C3H/HeJ). During the test phase all virus stocks were tested in the same host genotype. (PR, 'passage round').

also infected with unpassaged virus to obtain baseline viral fitness and virulence estimates. To further control infectious dose in the test phase, viral stocks were titred using an infectious particle assay and animals received roughly 1500 focus-forming units (FFU) (i.e. infectious particles) per 200 µl i.p. injection. Test-phase infections lasted 10 days.

(ii) Two-round serial passage experiments

Two independent experiments were conducted (again denoted as 'BALB/c' or DBA/2J' experiments). Experiments consisted of serially passaging FV complex for two sequential rounds of infection through animals from the same host genotype or through animals representing two different host genotypes (figure 1*b*). A/J, C3H/HeJ and 129X1/svj inbred *Mus* strains were chosen for use as alternate host genotypes because they are susceptible to FV complex infection (electronic supplementary material, figure S2). To increase statistical power, a total of 10 independent 'same host genotype' and 30 independent 'differnt host genotypes' passage lines (10 independent lines for each of our three alternate host genotypes) were created for comparison in the test phase for both the DBA/2J and BALB/c experiments. Spleen supernatants were prepared as described above. Briefly, after the first round of infection, spleen supernatant from one BALB/c or DBA/2J animal was split into aliquots of equivalent volumes and used to infect either another animal of that respective genotype or an animal representing one of the three 'alternate' host genotypes. Spleen supernatants were diluted as described above, and all animals during second round infections received between 75 and 150 µl i.p. injections.

At the test phase, independent cohorts of female DBA/2J and BALB/c animals were infected with equivalent doses of virus derived by passage through the same or different host genotypes. To do this, volumes of supernatants used to infect test-phase animals were adjusted based on viral titres of infecting stocks. Specifically, the lowest mean proviral load estimate from spleen supernatants derived from one of the four cohorts of second round animals was designated the 'target' dose and the fold difference of all stocks above this value was used as the dilution factor. Viral stock dilutions were made in sterile 1X PBS immediately prior to i.p. injection of test animals. The entirety (approx. 250 µl) of spleen supernatants that had proviral load estimates lower than the target dose was used to infect test-phase animals.

(d) FV complex fitness estimate

An important parameter of pathogen fitness is within-host growth rate, which we estimated by measuring viral titres in infected animals. A quantitative PCR assay was developed using the Lightcycler 2.0 platform (Roche) to measure the number of integrated retroviral genomes (proviruses) in host DNA and has been detailed previously [20,21]. Briefly, after weighing spleens from euthanized infected animals to estimate virulence, organs were suspended in an equivalent weight/volume of sterile $1\times$ PBS and then mechanically homogenized. All work with infected animal tissues was carried out in a certified BSL2 laminar flow hood. Homogenates were kept on ice until being centrifuged at 5000 r.p.m. for 10 min. High-quality genomic DNA was extracted from 100 µl of spleen supernatant using a DNeasy DNA extraction kit (Qiagen). Concentration (ng µl^{-1}) and quality (260/280 ratio of approx. 1.8–2.0) of DNA samples were checked using a Nanodrop spectrophotometer (Thermo Scientific). Stock DNA samples were used to create 20 ng µl^{-1} sample dilutions for use in quantitative

PCR assays (50 ng total gDNA per reaction). Because spleens were homogenized in an equal weight/volume ratio of $1\times$ PBS during tissue processing and because we standardized qPCR reactions to contain 50 ng of genomic DNA, qPCR estimates only reflect proportional differences in viral titres (i.e. viral genomes per copies of GAPD). To account for these dilution effects, we analyse viral fitness data standardized (i.e. multiplied) by spleen weight to approximate absolute loads of virus (i.e. viral genomes per spleen). Both standardized values (main document) and unstandardized values (electronic supplementary material) are reported, but we limit our description of results to standardized data for brevity. Viral load estimates are represented as proviral copies per 1 000 000 host cells (i.e. 10^6 GAPD copies).

(e) Virulence estimates

Infection by FV complex of susceptible animals results in an erythroproliferative disorder (electronic supplementary material, figure S1). Major symptoms associated with acute disease caused by FV complex infection are the development of gross organ enlargement (-megaly) due to clonal expansion of virally infected erythroblasts in the spleen and liver (primary and secondary sites of terminal haematopoeisis, respectively), as well as elevated haematocrit values due to the overabundance of RBCs in the blood (i.e. polycythemia) [23] (electronic supplementary material, figure S1). Thus, to estimate disease associated with acute FV complex infection, animals were euthanized and their spleens and livers were removed and weighed to the nearest 100th of a gram. Haematocrit was measured as the percentage of red blood cells (RBCs) per volume of whole blood. Splenomegaly is used as our primary estimate of virulence for two reasons. First, the spleen is the primary site of FV complex replication [23]. Second, hepatomegaly is only weakly correlated with F-MuLV titres in our experiments and haematocrit is not correlated with F-MuLV titres at all (electronic supplementary material, figure S3). A total of 75 μl of whole blood was drawn from the femoral artery of euthanized animals into a heparinized haematocrit tube and spun at 2500 r.p.m. for 5 min in a centrifuge. The length of the RBC pack was divided by the total length of volume in the haematocrit tube (measured to the nearest millimetre) to obtain the haematocrit value (%RBCs) for each animal. Splenomegaly and hepatomegaly are correlated estimates of virulence, whereas polycythemia is not correlated with either splenomegaly or hepatomegaly.

(f) Statistical analysis

All statistical analyses were carried out using PRISM v. 5 (Graphpad) or JMP v. 9.0 (SAS). Owing to a lack of normality and/or unequal variance in many datasets, a non-parametric Mann–Whitney U-test was used for all pair-wise statistical comparisons. Linear correlation analysis was performed on virulence and proviral load estimates (genomes per 10^6 GAPD copies).

3. Results

(a) Five-round serial passage experiments

Thirty-seven experimental infections were conducted during the test phase of five-round serial passage experiments. Serial infection of animals from the same host genotype resulted in significant increases in viral fitness compared with unpassaged control virus in both the BALB/c (40-fold increase) and DBA/2J experiments (20-fold increase; figure 2a; electronic supplementary material, figure S4). Serial infection of different host genotypes led to a significant increase in fitness over unpassaged virus in the BALB/c experiment (Mann–Whitney

Figure 2. Results from five-round serial passage experiments. In two independent experiments (BALB/c and DBA/2J) FV complex was serially passaged through the same host genotype or through five rounds of alternating host genotypes and tested in cohorts of BALB/c ($n = 9-10$ per group) or DBA/2J animals ($n = 8-10$ per group), respectively. (a) FV titres and (b) splenomegaly are compared. Mean value from cohorts of animals infected with unpassaged virus is depicted as a dashed line for reference. p-values shown above are the results of pair-wise Mann–Whitney U-tests. Bars represent group means.

U-test, $p = 0.0002$), but the magnitude of fitness increase (4-fold) was significantly lower than that observed after serial infection of the same host genotype (figure 2a; electronic supplementary material, figure S4). Serial infection of different hosts in the DBA/2J experiment did not result in any change in fitness compared to unpassaged virus (Mann–Whitney U-test, $p = 0.95$; figure 2a; electronic supplementary material, figure S4).

We next contrasted patterns of disease severity resulting from infection with FV complex stocks derived from our two selection regimes. Across all infected animals, there is a significant positive correlation between F-MuLV fitness and splenomegaly up to about 1 g of spleen, after which the relationship plateaus ($R^2 = 0.34$, ANOVA, $F_{1,175} = 87.9$, $p < 0.0001$; electronic supplementary material, figure S5). In comparison

Figure 3. Results from two-round serial passage experiments. In two independent experiments (BALB/c and DBA/2J), FV complex was serially passaged under two different selection regimes: for two rounds through the same host genotype or for two rounds through two different host genotypes. Two-round serial passage through alternating host genotypes was carried out in three different host genotypes for each experiment. Genotype designations in parentheses represent the 'different' host genotype used to produce a given viral stock. (a) Viral fitness and (b) its' associated virulence are compared between cohorts of BALB/c (n = 10 per group) or DBA/2J animals (n = 10 per group) infected with their respective viral stocks. The mean value from cohorts of animals infected with unpassaged virus is depicted as a dashed line for reference. p-values are the results of pair-wise Mann–Whitney U-tests. Bars represent group means.

with unpassaged virus, splenomegaly was significantly increased during infection with virus stocks derived by serial passage through individuals from the same host genotype, but was not different (BALB/c experiment) or was significantly reduced (DBA/2J experiment) in animals infected with virus stocks derived by passage through different host genotypes (figure 2b; electronic supplementary material, figure S6).

During five-round serial passages, we observed that exposure to the 129X1/svj genotype in passage round 4 was associated with a significant decline in FV-induced virulence (splenomegaly) in both passage lines (electronic supplementary material, figure S7). This effect persisted in subsequent infections even when the virus was exposed to the highly susceptible BALB/c genotype in the DBA/2J alternating passage line (electronic supplementary material, figure S7a). Thus, the reduction in F-MuLV fitness and FV complex-induced disease observed after infection with alternately passaged FV complex could have been due to the 129X1/svj genotype. To address this possibility, we followed up five-round serial passage experiments with a series of two-round experiments where FV complex was alternated through a single host genotype.

(b) Two-round serial passage experiments

There were a total of 80 experimental infections during the test phase of two-round serial passage experiments. In the second passage round, F-MuLV fitness and virulence was significantly reduced during infection of alternate A/J, 129X1/svj and C3H/HeJ host genotypes compared with a second round of infection in the same BALB/c or DBA/2J host genotypes (electronic supplementary material, figure S8). These

dramatic reductions in viral fitness and virulence were also observed in test-phase infections. Serial infection of the same host genotype resulted in significant increases in viral fitness in both experiments (BALB/c = 16-fold increase; DBA/2J = 22-fold increase) compared with unpassaged control virus (figure 3a; electronic supplementary material, figure S9). By contrast, serial infection of different host genotypes resulted in a general decrease in FV complex fitness compared with unpassaged virus (BALB/c experiment-average approximately 4-fold decrease; DBA/2J experiment-average approximately 25-fold decrease; figure 3a; electronic supplementary material, figure S9). These results were correlated with patterns of virulence. Compared to unpassaged virus, serial infection of the same host genotype led to a significant 6-fold (BALB/c experiment) and 4-fold (DBA/2J experiment) increase in splenomegaly, while serial infection of different host genotypes resulted in an average 1.3-fold (BALB/c experiment) and 2.6-fold (DBA/2J experiment) reduction in splenomegaly (figure 3b; electronic supplementary material, figure S10). Because similar effects emerged in response to alternation through 129X1/svj, A/J and C3H/HeJ genotypes, the five-round result is unlikely to be explained by some unique attribute of the 129X1/svj genotype.

(c) Host diversity impedes the pathogenic determinant of FV complex

Given the importance of the replication-defective SFFV virus in promoting acute infection (electronic supplementary material, figure S1), we reasoned that the failure of FV complex to promote splenomegaly after serial passage through different

host genotypes might be due to defects in SFFV replication. This is further suggested by the fact that despite substantial overlap in the range of F-MuLV fitness among the three selection regimes (electronic supplementary material, figure S5, inset), resulting patterns of virulence were substantially different (figures 2*b* and 3*b*; electronic supplementary material, figures S5, S6 and S10). Analysis of SFFV titres in test-phase samples from our two-round serial passage experiments revealed that serial passage through different host genotypes resulted in undetectable SFFV titres (electronic supplementary material, figure S11). This was unexpected given that all three host genotypes used for alternation in two-round serial passage experiments (A/J, 129svj, and C3H/HeJ) were confirmed to be susceptible to viral replication and susceptible to SFFV-induced splenomegaly (electronic supplementary material, figure S2). It is possible that differences in the production or quality of antiviral factors derived from the previous host that are co-transferred along with virus into new hosts could confound our results. Experiments to address this possibility did not support this explanation (electronic supplementary material, figure S12*a*). We also tested whether co-transfer of syngeneic (self) versus allogeneic (foreign) spleen tissues impacted viral fitness or virulence (electronic supplementary material, figure S12*b*), the availability of host target cells in the spleen (electronic supplementary material, figure S13*a*) or relevant immune system parameters (electronic supplementary material, figure S13*b*). Tissue transplant did not affect any of these parameters.

4. Discussion

Previous serial passage experiments have shown that pathogen adaptation leads to increased fitness and virulence in the host-of-passage, and attenuation of these traits in former host species [8]. These results are consistent with the argument that pathogen fitness constraints arise as a consequence of adaptation to specific host species, but importantly they also imply that variable host genotypes within a species might impede pathogen adaptation and virulence evolution. Collectively, results from our experiments demonstrate that serial exposure of a viral pathogen to different host genotypes within a single host species impedes the emergence of high fitness/high virulence viral phenotypes compared with the same number of infections among genetically identical hosts. Even a single round of host alternation was sufficient to produce this constraint (figure 3; electronic supplementary material, figures S9 and S10). To the best of our knowledge, this is the first time this has been demonstrated in a vertebrate host species.

The best empirical evidence in animals that genetic diversity within a host species negatively impacts pathogen adaptation and virulence evolution comes from studies using invertebrate host–pathogen systems. Serial passage of the fungal pathogen *Metarhizium anisopliae* through individuals of the leafcutter ant species *Acromyrmex echinatior* showed that increasing diversity among ant hosts was associated with reduced virulence and increased likelihood of extinction of the pathogen [10]. Recently, using an elegant series of experiments, Morran *et al.* [12] demonstrated that outcrossing lines of the nematode *Caenorhabditis elegans* produced less virulent strains of the coevolving bacterial pathogen *Serratia marcescens* than obligately selfing *C. elegans* lines [12]. These experiments

support the hypothesis that diversity-promoting mechanisms like sexual reproduction may have evolved as mechanisms to counter the negative effects of rapid pathogen adaptation and virulence evolution [24,25]. This has become known as the sex-against-virulence hypothesis, and our data provide additional support for this hypothesis in vertebrates.

Results from our experiments are consistent with theoretical and empirical work demonstrating that genetic diversity in host populations can limit the spread and severity of infectious diseases [11,26–35]. Theoretical models have argued that when virulence is correlated with pathogen replication, within-host growth rates are predicted to be optimal at just below the point where host immunity can no longer control infection [32], or at some intermediate level determined by the average resistance among hosts in a population [33]. This maximizes transmission rates and ultimately pathogen fitness. These models argue that host resistance against infection is a major constraint on virulence evolution. Studies of virulence evolution of rodent malaria (*Plasmodium chabaudi*) in response to infection of immunocompromised or immunocompetent hosts have shown that the relative abundance of virulent clones is determined by how host immunity influences competition with avirulent competitors [36–38]. Using the same pathogen model, Grech *et al.* [39] demonstrated that pathogen growth rates are strongly influenced by host genotype (i.e. they are host genotype-dependent), and an earlier study using an ancestral and C57Bl/6 mouse-adapted line of *P. chabaudi* provided similar conclusions [40]. Host genotype-dependent pathogen fitness and virulence patterns have also been demonstrated previously with our *Mus*-FV model system [20–22]. Experiments from our laboratory [20–22] also provide evidence to support the argument that pathogen fitness trade-offs that emerge as a consequence of host-specific adaptation can influence virulence evolution. This contrasts with previous work in *P. chabaudi* where serial passage in C57Bl/6 mice did not result in virulence trade-offs in other mouse genotypes [40]. Many variables could account for this discrepancy (differing passage methodologies, different pathogens, etc.), and more studies in different host–pathogen systems are needed to establish the generality of this phenomenon. However, collectively these results indicate that host resistance can influence virulence evolution by affecting within-host pathogen growth rates through direct elimination of pathogens, by modulating competitive interactions among pathogen variants or by creating fitness trade-offs that will impact the dynamics of subsequent infections.

Multiple host loci are known to influence susceptibility to FV complex infection and disease, and numerous polymorphisms have been characterized among mouse strains (electronic supplementary material, table S2) [17,18,41]. Broadly, these 'resistance elements' can be characterized as restriction factors (retrovirally derived host-encoded genetic elements that interfere with the virus life cycle), or host genes whose products directly impact immunity against viral infection or that control haematopoiesis [17,19]. Results from our experiments indicate that serial infection of different host genotypes disrupted the erythroproliferative potential of FV complex by severely restricting SFFV replication. Consequently, infection by alternately passaged virus stocks was rendered avirulent even in highly susceptible DBA/2J and BALB/c host genotypes. The severity of this phenotype suggests that perhaps the genotypes used for alternation

possessed resistance elements that abolished the ability of SFFV to replicate and be passaged to susceptible BALB/c or DBA/2J hosts in the test phase of infection. Two lines of evidence argue against this. First, mouse strains were chosen for these experiments based on the presence of susceptibility alleles at loci (e.g. FV2, FV1, FV4) that would render them permissive to FV complex infection and SFFV-induced erythroblastosis (electronic supplementary material, table S2). Second, in this (electronic supplementary material, figure S2) and previous experiments from our laboratory [20–22], we have shown that FV complex is able to both replicate and cause disease in all of the host genotypes used in this study.

The production of FV-specific neutralizing antibodies is important for anti-FV immunity and could explain our result if A/J, 129X1/svj and C3H/HeJ mice have potent neutralizing antibody responses, whereas BALB/c or DBA/2J animals do not. While addressing this possibility is beyond the scope of this study, we do know that at least the BALB/c, A/J and 129X1/svj mouse strains carry the susceptibility allele at the Rfv3 locus, which is associated with weaker neutralizing antibody responses (electronic supplementary material, table S2). Additionally, immune responses generated against non-self proteins that are co-transferred in spleen homogenates with virus might contribute to lower viral titres. Results from experiments to address this possibility did not support this argument (electronic supplementary material, figures S12 and S13) and are consistent with previous work demonstrating that allograft responses do not prime immune responses against FV complex infection [42]. Thus, we are currently unable to explain loss of SFFV below levels of detection for our qPCR assay. Perhaps fitness trade-offs that emerge during host alternation (or another currently unidentified resistance element) drop F-MuLV or SFFV titres below a critical multiplicity of infection threshold that blocks disease progression. The striking nature of this phenotype warrants further investigation.

Serial passage experiments are valuable in addressing the role of fitness trade-offs on virulence evolution [8,43], but they have important limitations that should be acknowledged [44]. First, relaxed costs of transmission inherent in serial passage experiments may result in levels of adaptation and virulence unachievable under natural settings [45]. However, this is unlikely to explain differences in the trajectory of pathogen adaptation and virulence evolution in our experiments because control and alternating passages were transmitted in the same manner. Second, while murine leukaemia viruses have been shown to circulate in wild mouse populations [46], the rapid adaptation and virulence increases in FV complex (a spontaneously occurring laboratory isolate [47]) shown here and in previous studies from our laboratory [20–22] might not represent a natural pathogen response. Third, serial passage of FV complex promotes expansion of the defective SFFV component of FV complex that drives virulence. Severe mortality associated with infection by highly virulent oncogenic retroviruses in nature would probably preclude their persistence and expansion in host populations. However, notable examples of ecologically relevant retroviruses include simian and human immunodeficiency viruses, feline immunodeficiency virus and feline leukaemia virus [48], as well as the emerging koala retrovirus [49]. Thus, while serial passage experiments are a useful first step for exploring the role of host genetic variation as an impediment to virulence evolution, results must be interpreted in light of the limitations to these models.

Inbred mice are powerful research tools because their inbred status removes the confounding effect of genetic variation for studies on the physiologic response to a stimulus, and because comparisons of different strains (and their relevant crosses) have been instrumental in our understanding of the functional significance of host polymorphisms. In this study, different mouse strains were used to model different genotypes present in a population of a single host species. However, two caveats must be made. First, it is difficult to know at what level variability among different mouse strains approximates genetic variability among individuals in a wild *Mus* population. Given that the pedigrees of most classic inbred mouse strains share a common and very recent ancestry [50,51], one could argue that our genetic diversity might be on the low end of natural populations, making our results conservative. Second, inbred mice are genetic mosaics of multiple *Mus* subspecies (*M. m. domesticus, M. m. musculus, M. m. castaneus*) [52,53], and most of these strains were selected based on the presence of specific traits. Thus, the suite of polymorphisms relevant to FV complex infection and the penetrance of these traits among strains may not represent the natural condition. Ultimately, we believe that experimental evolution studies using wild mice housed under semi-natural conditions will help establish the ecological relevance of the findings in this study.

Results from our experiments are of practical significance because they highlight the potential risks associated with low genetic diversity in host populations. Endangered species and livestock may be particularly susceptible. In the case of endangered species, enhanced disease virulence associated with low diversity among founding individuals might contribute to the high failure rate of reintroductions. Intensive livestock farming practices house related individuals under high density [54,55]. Results from serial passage studies suggest that low genetic diversity among individuals and reduced barriers to transmission may rapidly promote the emergence of more virulent infectious diseases. Intensive farming practices are of particular concern for two reasons. First, virulence evolution in this setting could jeopardize global food security as exemplified by the huge costs associated with periodic epidemics of foot-and-mouth disease virus in livestock [56]. Second, more severe/frequent disease outbreaks might lead to increased use of antimicrobial drugs that could exacerbate the evolution of microbial resistance [57]. Genetic management of endangered species and livestock herds (i.e. manipulating genetic diversity to augment resistance to infection) may be a useful strategy for limiting these risks.

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rspb.royalsocietypublishing.org *Proc. R. Soc. B* **282**: 20141568

APPENDIX B

SEXUAL SELECTION CONSTRAINS BODY MASS

OF FEMALE BUT NOT MALE MICE

ORIGINAL RESEARCH

WILEY Ecology and Evolution
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Sexual selection constrains the body mass of male but not female mice

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Funding information
National Institute of General Medical Sciences, Grant/Award Number: R01-GM109500

Abstract

Sexual size dimorphism results when female and male body size is influenced differently by natural and sexual selection. Typically, in polygynous species larger male body size is thought to be favored in competition for mates and constraints on maximal body size are due to countervailing natural selection on either sex; however, it has been postulated that sexual selection itself may result in stabilizing selection at an optimal mass. Here we test this hypothesis by retrospectively assessing the influence of body mass, one metric of body size, on the fitness of 113 wild-derived house mice (*Mus musculus*) residing within ten replicate semi-natural enclosures from previous studies conducted by our laboratory. Enclosures possess similar levels of sexual selection, but relaxed natural selection, relative to natural systems. Heavier females produced more offspring, while males of intermediate mass had the highest fitness. Female results suggest that some aspect of natural selection, absent from enclosures, acts to decrease their body mass, while the upper and lower boundaries of male mass are constrained by sexual selection.

KEYWORDS

fecundity, intrasexual selection, mammals, sexual selection, sexual size dimorphism, stabilizing selection

1 | INTRODUCTION

Body size is influenced by natural and sexual selection with both female- and male-biased sexual size dimorphism (SSD), as well as monomorphism, common across vertebrates (Andersson, 1994). Selective forces for increased female size include a positive relationship with fecundity, enhanced resources for parental care, and dominance over resources, while those for decreased female size include increased maturation rate and decreased energy demands; conversely, male-biased SSD is primarily driven by physical competition for mates with the largest individuals having the highest fitness (Andersson, 1994; Clutton-Brock, 2009; Cluttonbrock & Parker, 1992; Schulte-Hostedde,

2007). Taken together, fecundity selection in females and sexual selection in males are largely thought to be the primary selective forces driving larger body size across organisms; however, it has proven more difficult to understand the counteracting selection which constrains body size.

Blanckenhorn (2000) suggested four costs due to larger body size: (1) viability costs in juveniles due to longer development (or faster growth); (2) viability costs in adults due to predation, parasitism, or starvation; (3) decreased mating success of large males due to lack of agility or high energy costs; and (4) decreased fitness in both sexes due to late reproduction associated with longer development. These four hypotheses include pressures due to both natural (1 and 2) and

sexual (3 and 4) selection; however, supporting evidence in vertebrates has been difficult to obtain for the two sexual selection hypotheses. Specifically, within vertebrates, costs associated with relatively large body size, in the context of male sexual selection, have only been demonstrated in the pied flycatcher (*Ficedula hypoleuca*) and serrate-legged small treefrogs (*Philautus odontarsus*) (Alato & Lundberg, 1986; Zhu et al., 2016).

House mouse (*Mus musculus*) populations inhabiting semi-natural enclosures are well suited for quantifying selective forces operating on a variety of phenotypes and provide a unique opportunity to assess the natural and sexual selective forces that constrain body size (Carroll & Potts, 2007). Within these enclosures some, but not all, pressures of natural selection (e.g., predation) are absent, and most sexual selection pressures are present (including male–male competition and female choice ((Meagher, Penn, & Potts, 2000; Nelson, Colson, Harmon, & Potts, 2013))). Therefore, by assessing the reproductive success of mice in semi-natural enclosures, one can evaluate a trait's influence on fitness in the context of moderate levels of natural selection and high levels of sexual selection.

Here we assess the relationship between body mass (a measure of body size) and fitness in both sexes of house mice. Due to the nature of our study we control for three of the four hypothesized selective pressures on body size (1, 2, and 4 above) allowing us to assess whether male sexual section might act to constrain body size with counteracting pressures on males that are too small as well as those who are too large. We do this by retrospectively analyzing parentage and body mass data from three previous studies using our mouse semi-natural enclosure system. Each of these studies directly tested outbred control mice in direct competition with experimentally manipulated mice; only control mice are analyzed here. The first study (S1) assessed the fitness consequences of inbreeding; parentage was conducted subsequently to evaluate the deleterious nature of the *t*-complex (Carroll, Meagher, Morrison, Penn, & Potts, 2004; Meagher et al., 2000). The second (S2) and third (S3) studies assessed fitness consequences of pharmaceuticals (Gaukler et al., 2015, 2016). Collectively, the fitness and body mass data from these studies provide a unique opportunity to test the selective pressures that may constrain body mass in vertebrates.

2 | MATERIALS AND METHODS

2.1 | Animals

From 55 litters, 113 (75 female and 38 male) outbred wild-derived mice were assessed. Mice from S1 ($n = 77$) were from the second generation of a colony initially described by Meagher et al. (2000), while those in S2 ($n = 24$) and S3 ($n = 12$) were from the twelfth. Mice entered enclosures as sexually mature adults (S1: 23.0 ± 9.5 weeks old, S2: 26.2 ± 7.1, S3: 27.1 ± 2.3, mean ± SD) and were weighed prior to release. Ten populations (S1: $n = 7$, S2: $n = 2$, S3: $n = 1$) were established with 16 females and eight males, half of whom were controls, and seven mice were not weighed. Collectively, these populations represent all published accounts from our laboratory with complete body mass and parentage data. The assessed studies were approved by the Institutional Care and Use Committee at the University of Utah (protocol #s 97-11011, 07-8002, and 10-08002).

2.2 | Semi-natural enclosures

Indoor enclosures are 30–50 m^2 and are subdivided to promote territory formation. Subsections have food and water provided ad libitum associated with nest-boxes in either "optimal" territories (with enclosed nest-boxes) or "suboptimal" territories (with exposed boxes). Photographs and diagrams of enclosures may be found in the initial studies (Gaukler et al., 2015, 2016; Meagher et al., 2000). Offspring born within S1 populations were removed at ~6.4 weeks of age, while in S2 and S3 all offspring were collected at eight weeks into the study and then again at five-week intervals; after removal, offspring were euthanized and tissues were harvested. Populations were maintained for 30.0 ± 4.3 weeks.

2.3 | Parentage

Four–17 autosomal microsatellite loci were amplified per offspring. Primers were tagged with CY-5 or CY-3 fluorescent dye. DNA samples were PCR-amplified and run on 6.25% denaturing acrylamide gel at 40 W for 3–7 hours. Gels were imaged on a FluorImager. Additional details on parentage analysis, including loci used, can be found in original reports (Carroll et al., 2004; Gaukler et al., 2015, 2016).

2.4 | Data analysis

For an initial approach, offspring counts of both sexes were first modeled together using a generalized linear mixed model (GLMM) with a Poisson distribution and logarithmic link. We predicted offspring counts of mice across populations by modeling the fixed effects of body mass (at the time of entrance into enclosures), sex, and a sex-by-mass interaction, while study, population (nested within study), and litter were included as random effects. This initial model resulted in an unexpected negative correlation between body mass and fitness in males [contrary to published findings (Franks & Lenington, 1986; Krackow, 1993)]. Therefore, we next assessed the presence of a reproductive optimum for the male data alone by performing a GLMM with the same structure (excluding sex and its interaction) above and a generalized nonlinear mixed model (GNLMM) with a second-order polynomial term for mass and the aforementioned random effects; the GLMM and GNLMM were then compared by Akaike information criterion.

As nonlinear models can be sensitive to extreme values we also evaluated the presence of linear versus negative-quadratic relationships between mass and fitness using a bootstrapping approach. Specifically, separate Poisson generalized linear models (GLMs) (1,000 iterations) were used to assess the influence of body mass (second-order polynomial), and to calculate an optimum if applicable, for each sex. Assessment between linear and quadratic relationships was

performed by evaluating the consistency of positive and negative values (95% CIs) of first- and second-order polynomial terms for mass. The influence of extreme values is mitigated as bootstrapping utilizes random sampling with replacement, which ensures that overall patterns are not driven by individual data points. Importantly, both analysis approaches reached almost identical conclusions. All models were run in R (3.3.1) using lme4 and boot (Bates, Maechler, Bolker, & Walker, 2015; Canty & Ripley, 2016; R Core Team, 2016). Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.v3p2g.

3 | RESULTS

Mice weighed 15.2 ± 3.4 g (mean ± SD) range from 7.7 to 26.6 g and were sexually dimorphic with females weighing 14.3 ± 3.0 g and males weighing 17.1 ± 3.4 g (t test; t_{67} = −4.22, p < .0001). Offspring counts per mouse ranged from 0 to 109 with males producing more (36.1 ± 29.0) pups than females (12.0 ± 11.0; Wilcoxon; W = 661, p < .0001) as expected based on the 2:1 sex ratio.

Female fitness increased with increasing body mass (GLMM; Z = 2.44, p = .015; Figure 1a; Table 1A), while this relationship differed in males (GLMM; Z = −6.60, p < .0001). Male body mass had a negative-quadratic relationship with high fitness possessing an optimal mass, as indicated by the GLMM having essentially no support (ΔAIC = 24.9) relative to the GNLMM (Figure 1b; Table 1B). Moreover, bootstrap models indicate a positive (95% CI: 1.80, 5.09) first-order polynomial term for mass in females and negative (95% CI: −5.25, −0.21) second-order mass terms in males (Figure 1c); these

bootstrapping results are indicative that overall patterns are not driven by extreme points (e.g., the heaviest mice).

4 | DISCUSSION

We demonstrate a positive relationship between body mass and female fitness and a negative-quadratic relationship in males. The positive relationship in females likely indicates larger females have higher fecundity, a pattern also seen within other rodents such as deer-mice (*Peromyscus* sp.*)* and voles (Arvicolinae) (García-Navas, Bonnet, Bonal, & Postma, 2016; Myers & Master, 1983), although alternative hypotheses such as differential resource control cannot be eliminated. As sexual selective forces are largely present within enclosures it is likely these forces which influence the observed optimum in male mass; for example, it is possible there is an optimal mass for winning agonistic contests, perhaps balancing strength/agility, or that females prefer to mate with males of intermediate size. These observations suggest that house mouse body size is, at least partially, constrained by male sexual competition and that the simple paradigm of "bigger is better" in regard to sexual selection is not applicable to this species.

We are able to assess the possibility of male sexual selection constraining body size because our study design and species selection control for three of the four costs of large body size suggested by Blanckenhorn (2000). By releasing all mice as adults we control for juvenile viability selection and by excluding predators and most parasites, while providing ample access to food/water we greatly reduce the pressure of adult viability selection. Likewise, the proposed cost of

FIGURE 1 Influence of body mass on fitness. (a) For female mice, body mass is positively correlated with fitness. (b) For males, there is a negative-quadratic relationship with an optimal mass (arrow) for fitness. For (a,b) points represent individuals, grouped by population (shapes) from three studies (colors; S1: white/open. S2: black, S3: gray), solid lines indicate best fits, while vertical lines represent medians and quartiles. (c) Different patterns between females and males are demonstrated by first- and second-order polynomial coefficients of mass from bootstrap GLMs. For females, first-order terms are consistently positive, while second-order straddle zero, suggesting a positive relationship between fitness and mass. For males, first-order terms span zero, while second-order terms are negative, suggesting a negative-quadratic relationship. Gray centers demark mean values, and ellipses indicate 95% CIs

(A) Influence of body mass on fitness by sex. GLMM with Poisson distribution and logarithmic link (intercept at 15.23 g; 113 mice born in 60 cages, founded 10 populations nested in three studies)

Random effects	Variance			Std. deviation
Study	0.2789			0.5281
Population	0.0384			0.1960
Litter	0.5273			0.7261

Fixed effects	Estimate	Std. error	Z value	Pr(>|z|)
Intercept	2.6354	0.3453	7.63	<0.0001
Mass (g)	0.0622	0.0255	2.44	0.0148
Sex (male)	1.0206	0.0899	11.35	<0.0001
Mass × sex (male)	−0.1558	0.0235	−6.60	<0.0001

(B) Influence of body mass on male fitness. GNLMM with Poisson distribution and logarithmic link (38 mice born in 33 cages, founded 10 populations nested in three studies)

Random effects	Variance			Std. deviation
Study	0.3045			0.5518
Population	0.4262			0.6528
Litter	1.0437			1.0216

Fixed effects	Estimate	Std. error	Z value	Pr(>|z|)
Intercept	3.4038	0.5830	5.84	<0.0001
First-order term (mass)	−5.2139	1.3775	−3.79	0.0002
Second-order term (mass)	−3.6361	0.8205	−4.43	<0.0001

TABLE 1 Body mass and fitness model results

"late reproduction" is thought to be of primary importance in species with low encounter rates or with constrained mating periods, neither of which apply to house mice (Singleton & Krebs, 2007). The elimination of three of the four characterized costs of large body size allows us to conclude that stabilizing selection on body size, due to male–male competition, female mate choice, or a combination of the two is sufficient to constrain house mouse body size—an intriguing finding in a polygynous mammal.

Previous studies investigating relationships between fitness and body mass of house mice in semi-natural enclosures have relied on dominance status as a proxy for fitness. A study of 32 mice found a marginally significant trend that "fitness-rank," based on social dominance, was positively correlated with male mass, but not female mass (Krackow, 1993), while another larger study found positive relationships for "dominance-rank" and body mass in both sexes (Franks & Lenington, 1986). Importantly, neither study considered an optimal mass nor directly assessed fitness. One caveat concerning our study is that the analyses were limited to un-manipulated control mice, who were cohoused with treatment individuals. Although this asymmetry in individual quality could influence the observed relationships, we find this unlikely as control mice were primarily in competition with each other, and gradients of individual quality are the norm in nature. In light of this caveat, it should be acknowledged that a study designed to directly test the influence of body mass on mouse fitness within semi-natural enclosures considering nonlinear selection would be definitive; however, the results provided here are unique and illuminating on the selective forces shaping the evolution of body size.

Although the notion that a causal relationship between male body size and success in acquiring mates leads to increased body size in both sexes is well supported in vertebrates (Fairbairn, 1997), it may not explain patterns of SSD wherein maximal male size is not optimal. Typically it is assumed that sexual selection for increased size in males is counteracted by natural selection (e.g., predation, interspecific competition); however, the presence of an optimal body mass in semi-natural enclosures with high levels of sexual selection (and reduced levels of natural selection) indicates house mice may be an exception to this rule. Broadly, our results support that fecundity selection in females may be a primary selective agent for large body size, but question the extent to which larger body size in males is universally beneficial in the context of sexual selection. Moreover, based on the observation herein, that larger females have more offspring when natural selection is relaxed, perhaps instead of asking "why are males relatively large?" we should ask "why are females small?."

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

J.S.R., J.W.C., A.C.N, and W.K.P., designed the study. S.M.G., S.M., conducted enclosure studies. S.M.G. and L.S.C., assessed parentage. L.C.M. curated data. J.S.R and D.H.C. analyzed data. J.S.R. and W.K.P. wrote the manuscript.

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APPENDIX C

MHC HETEROZYGOTE DISADVANTAGE DURING

INFECTION OF FRIEND VIRUS COMPLEX

<u>C.1 Introduction</u>

Animals heterozygous at the major histocompatibility complex (MHC) are thought to be more protected against pathogens as they have the potential to recognize twice the pathogenic peptide as homozygotes (Kubinak *et al.*, 2012a). Furthermore, it has been shown that vertebrates increase the MHC heterozygosity of their offspring through disassortative mating preferences, suggesting heterozygosity is beneficial in natural populations (Penn & Potts, 1999; Mays & Hill, 2004). However, heterozygosity is not universally protective against pathogens, as there are MHC alleles that have dominate susceptibility profiles; this has been shown in salmonella and Theiler's virus where some heterozygote combinations are more susceptible to the pathogens than either of the homozygotes, though when both pathogens are coinfected together the heterozygote genotypes seem to be protective relative to the homozygotes (McClelland *et al.*, 2003).

Here we use Friend Virus Complex (FVC) to test whether MHC heterozygote animals are more resistant to the pathogen than MHC homozygote animals through serial passage, we would expect that if there was an advantage to being an MHC heterozygote, heterozygote animals lower virulence and viral titer in the MHC homozygote animals. Furthermore, if MHC heterozygotes are more difficult for FVC to adapt to, then we would hypothesize that homozygote passage would increase in fitness and virulence more rapidly, as has been shown previously, whereas heterozygote passages will take a higher number of passage rounds to increase to the same level of fitness and virulence as the homozygotes.

C.2 Methods

Ten animals of each MHC genotype were infected in order to understand how virulent the virus is during the initial infection: Balb/c^{dd}, Balb/c^{bb}, Balb/c^{kk}, Balb/c^{dk}, and Balb/c^{bd}. The second and third rounds consisted of five animals of each genotype due to constraints in animal number. FVC was serial passaged through each genotype three times to further test whether the virus has a more difficult time adapting to heterozygote animals than homozygote animals. Animals were sacrificed 12 days postinfection at which time spleen mass (virulence) was measured. After measurement, the spleens were mixed in a 1:1 ratio with 1x Phosphate Buffered Saline (PBS), homogenized and spun at 5000 RPM. Supernatants were collect and frozen at -80°C until thawed to infect the next round of animals. DNA was extracted from spleen homogenates and qPCR was done in order to determine the number of proviral insertions occurred which is a measure of viral titer (fitness).

C.3 Results and Discussion

After the first 12 day infection there was no heterozygote advantage seen as each of the homozygote genotypes tested had significantly reduced virulence than their heterozygote counterparts; however, heterozygotes did not show a significant decrease in viral fitness (LMM; Virulence, $t = -2.42$, $p = 0.019$; Fitness, $t = -3.59$, $p = 0.19$; Fig C1A-B). Furthermore, this disadvantage continued into the second round of serial passage, with virulence still showing an increase in heterozygotes over homozygotes (LMM; Round 2, $t = -2.34$, $p = 0.029$; Fig C2A); though the effect was lost in the third round where there was no significant difference in virulence between the two treatment groups

(LMM; t = -1.11, p = 0.382; Fig C2B).

Here we show that there is an overall disadvantage to being an MHC heterozygote when infected with FVC, though this disadvantage disappears through passage of the virus. The lack of increase through serial passage in homozygote animals has not been seen before as previously each time FVC has been serially passaged through these MHC homozygote genotypes there has been a dramatic increase in both fitness and virulence, which we do not see here (Kubinak *et al.*, 2012b, 2013, 2015). Regardless of the lack of increase through passage there was still an initial disadvantage in heterozygote animals; however, more work needs to be completed to determine if the heterozygote disadvantage seen in the first two rounds of infection is accurate.

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Figure C.1. Spleen mass and proviral estimates by treatment group in round 1 passages. (A) Heterozygotes had significantly higher spleen masses than homozygotes in the initial round of infection; (B) whereas there was no difference detected in viral titer. Data represented by mean ± SEM.

Figure C.2. Virulence estimates in the second and third round of infections. (A) The heterozygote disadvantage persisted into the second round as heterozygotes had significantly larger spleens than homozygotes. (B) In the third round there was no detectable difference in spleen mass. Data represented by mean ± SE.