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Host sexual dimorphism affects the outcome of within-host pathogen competition

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Natural infections often consist of multiple pathogens of the same or different species. When coinfections occur, pathogens compete for access to host resources and fitness is determined by how well a pathogen can reproduce compared to its competitors. Yet not all hosts provide the same resource pool. Males and females, in particular, commonly vary in both their acquisition of resources and investment in immunity, but their ability to modify any competition between different pathogens remains unknown. Using the *Daphnia magna–Pasteuria ramosa* model system, we exposed male and female hosts to either a single genotype infection or coinfections consisting of two pathogen genotypes of varying levels of virulence. We found that coinfections within females favored the transmission of the more virulent pathogen genotype, whereas coinfections within male hosts resulted in equal transmission of competing pathogen genotypes. This contrast became less pronounced when the least virulent pathogen was able to establish an infection first, suggesting that the influence of host sex is shaped by priority effects. We suggest that sex is a form of host heterogeneity that may influence the evolution of virulence within coinfection contexts and that one sex may be a reservoir for pathogen genetic diversity in nature.

KEY WORDS: Coinfection, *Daphnia magna*, *Pasteuria ramosa*, priority effects, virulence evolution, within-host interactions.

Due to the ubiquity of pathogens in natural populations, individuals are usually infected with more than one type of pathogen or multiple strains of a single pathogen (Read and Taylor 2001; Rigaud et al. 2010; Balmer and Tanner 2011). In these multiple infection contexts, pathogens compete for access to host resources and the nature of the fittest competitor is often correlated with its virulence (reviewed in Alizon et al. 2013). Host populations, however, are rarely homogeneous and pathogens will encounter hosts that vary in their age, nutritional background, immune status, or genotype; all of which leads to differences in the availability and quality of resources a given host may provide for a pathogen. Consequentially, the relative fitness of each pathogen genotype or species during coinfection (Hodgson et al. 2004; Izhar et al. 2015; Louhi et al. 2015), as well as their capacity to exclude or coexist with other competitors (de Roode et al. 2004), will depend not only on the virulence of the pathogens involved, but also on the characteristics of their hosts.

A near ubiquitous source of host heterogeneity with the potential to modify within-host pathogen competition are the differences between male and female hosts (Gipson and Hall 2016). Across many species, males and females often vary in their relative immune investment (Zuk 2009; Rolff 2002), leading to one sex experiencing higher infection rates and more intense symptoms of disease than the other (Poulin 1996; Schalk and Forbes 1997; McCurdy et al. 1998; Sheridan et al. 2000; Zuk 2009; Cousineau and Alizon 2014). The sexes may also represent hosts of varying exploitative potential due to differences in lifespan, energy acquisition, reproductive investment, or simply body or organ size (Christe et al. 2007; Duneau and Ebert 2012; Thompson et al. 2017; Gipson and Hall 2018). Together, this heterogeneity between the sexes is increasingly being linked to the performance of a single pathogen genotype within a male or female host and its subsequent transmission between susceptible hosts (e.g., Cousineau and Alizon 2014; Úbeda and Jansen 2016; Hall and Mideo 2018). Yet, it remains largely unexplored how each sex can influence the relationship between within-host pathogen competition and virulence (but see Thompson et al. 2017) and the relative transmission success of each pathogen genotype during coinfections.

Table 1. Description of the four infection types used in this study. Single infections consisted of one genotype and coinfections consisted of genotypes C19 and C24 or C19 and C1. Priority effects were manipulated via the simultaneous and sequential infection of each pathogen genotype.

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

Within-host competition can take a number of forms including, among others: (1) *Exploitation,* where pathogens compete over a limited host resource pool; (2) *Apparent,* where the immune response stimulated by one pathogen indirectly inhibits competitors; (3) *Interference,* where pathogen reproduction directly inhibits competitors through the production of toxins that kill or inhibit the growth of competitors; or (4) *Public good*s, whereby pathogens compete over resources they produce themselves (Mideo 2009; Balmer and Tanner 2011; Alizon et al. 2013; Bashey 2015). Each type of interaction is predicted to lead to specific changes in the overall virulence of any infection and the types of pathogen genotypes that are subsequently favored (see Table 1 in Alizon et al. 2013). Resource competition, for example, may cause pathogens to overexploit their host, leading to increased virulence relative to the most virulent coinfecting genotype (Chao et al. 2000; de Roode et al. 2005). Competing pathogens, however, may also antagonistically interfere with one another, leading to decreased virulence in these competition contexts (Massey et al. 2004; Choisy and de Roode 2010; Eswarappa et al. 2012).

Opportunities for the sex of a host to modulate the outcome of coinfection can thus arise in a number of ways. First, coinfection in males and females might lead to different types of competitive interactions occurring within each sex (e.g., exploitation, apparent, interference, or over public goods); each with contrasting predictions for the overall expression of virulence for each sex and how it might evolve (Alizon et al. 2013). Second, the same type of interactions may occur in males and females, but with one of the sexes instead limiting the degree to which overall virulence can be exaggerated or dampened. Differences between the sexes, for example, may cause one sex to have more resources or physical space for a pathogen to exploit (Duneau and Ebert 2012; Thompson et al. 2017; Gipson and Hall 2018), potentially intensifying the competition between pathogen genotypes over host resources and further exaggerating the expression of virulence for this sex (akin to Hall et al. 2009).

Finally, the sex of a host may modify how readily a new pathogen can establish in previously infected males or females. In a wide variety of taxa, previously established pathogens have been shown to inhibit later-arriving competitors by blocking pathogen establishment, exhausting resources, or by inducing host immune responses (de Roode et al. 2005; Lohr et al. 2010; Hoverman et al. 2013; see Table 1 in Clay et al. 2018). Priority effects are thus intimately linked to the characteristics of a host and, thereby, its sex, particularly when males and females vary in immune function or the potential resources they provide to a pathogen. It may be harder, for example, for a subsequent infection to arise in the host sex that is more difficult to infect or has less space left unoccupied by preestablished pathogens (*sensu* discussions in Bashey 2015). As of yet, however, priority effects have not been explicitly considered when studying coinfection outcomes in both sexes (e.g., Thompson et al. 2017).

In this study, we use the *Daphnia magna–Pasteuria ramosa* model system to assess the variety of ways that the sex of a host can influence the outcome of multiple infection. In general, male *Daphnia* are smaller and exhibit shorter lifespans than females. When exposed to *P. ramosa,* males are also less likely to become infected, experience lower rates of infection-induced mortality, and allow fewer pathogen spores to be produced; collectively suggesting that they are a more difficult resource to exploit than females (Duneau et al. 2012; Thompson et al. 2017; Gipson and Hall 2018; Hall and Mideo 2018). In nature, *Daphnia* may be

infected with as many as eight genotypes of *P. ramosa* (Mouton et al. 2007), with coinfections in females often favoring the most virulent competitor in isolation, except for situations in which the less virulent genotype establishes first (Ben-Ami et al. 2008; Ben-Ami and Routtu 2013). In contrast, when multiple infections occur in males, the severity of disease that results suggests that a clear winner and loser of any within-host competition may be less likely to occur (Thompson et al. 2017). Remaining to be explored is how these sex-specific patterns of coinfection impact on the types of pathogen genotypes that are subsequently favored and the implications this may have for the maintenance of genetic variation and virulence evolution.

Using pathogen genotypes of known virulence characteristics (Hall and Ebert 2012; Clerc et al. 2015; Hall and Mideo 2018), we exposed genetically identical male and female *Daphnia* to either a single pathogen genotype or to a coinfection consisting of two pathogen genotypes of varying levels of virulence. We then varied the schedule of these exposures, either exposing hosts a single time or allowing infection to establish prior to a subsequent exposure. We measured pathogen-induced reduction in lifespan (virulence), overall pathogen spore production (transmission potential), and the relative spore production of competing pathogen genotypes using microsatellite analysis. With this design, we address the following questions: (1) Do patterns of overall virulence and pathogen proliferation in males and females suggest that the same type of competitive interactions, if any, are occurring in each sex? (2) Or do females, as the more exploitable sex (larger body size, higher overall virulence, etc.), allow for more divergence in virulence between pathogen genotypes and so intensity opportunities for competition? and (3) Does the order that different pathogens encounter each sex matter for coinfection and do males, as the less exploitable host with a more limited pool of resources, limit opportunities for subsequent infections to arise? We discuss the implications of these patterns for the maintenance of pathogen genetic variation.

Methods

Daphnia magna Straus is a freshwater crustacean that produces genetically identical male and female offspring via cyclic parthenogenesis. Males are commonly produced when mothers in the population experience biotic or abiotic stressors, such as pollutants, food stress, or changes in photoperiod, and even pathogen infection (Ebert 2005). In nature, male *Daphnia* can make up a large portion of the population under these stressful environmental conditions, although females are more common in general (Duncan et al. 2006; Galimov et al. 2011). During filter feeding, *Daphnia* encounters the bacterial pathogen *Pasteuria ramosa*, which reduces the lifespan and fecundity of its host (reviewed in Ebert et al. 2016). This pathogen is an obligate killer and is transmitted horizontally after host death (see Hall and Mideo 2018 for an epidemiological model of this system).

This experiment used host genotype HU-HO-2 and novel *P. ramosa* genotypes C19, C24, and C1. Prior to the experiment, we established a parental generation by isolating juvenile female *Daphnia* from preexisting stock cultures and maintaining them in standardized conditions for three generations to minimize maternal effects. Juvenile female *Daphnia* were raised individually in 60-mL vials filled with 50 mL of artificial *Daphnia* medium (ADaM, Klüttgen et al. 1994; modified as per Ebert et al. 1998) and were transferred into fresh ADaM twice weekly. These females were maintained at 20°C, exposed to a 16-h light to 8-h dark cycle, and fed up to 5 million cells of *Scenedesmus sp.* green algae daily.

PRODUCTION OF EXPERIMENTAL ANIMALS

Once the third-generation standardized females released their first clutch, they were exposed to a short pulse of the hormone methyl farnesoate (300 µg/L, Product ID: S-0153, Echelon Biosciences, Salt Lake City, Utah) to stimulate the production of genetically identical male and female offspring. Following previously established methods (Thompson et al. 2017), the standardized females were transferred into 60-mL vials filled with 20 mL of hormone-treated ADaM and were transferred into fresh hormone-treated ADaM three times weekly. Male and female offspring were collected from the second and third clutches posthormone exposure. This treatment has previously been shown to have no detectible impact on host lifespan and fecundity nor pathogen transmission and virulence (see Table 3 within Thompson et al. 2017).

EXPERIMENTAL COINFECTION SCENARIOS

To measure the effect of host sex on the outcome of within-host pathogen competition, as well as how this competition proceeds when one genotype has already established, we randomly exposed males and females to either single infections or coinfections. These exposures were carried out in one of two exposure "schedules": (1) *simultaneous* exposure occurred once when the individual was five days old or (2) *sequential* exposure occurred at 5 and 12 days old (see Table 1 for infection design details). All exposures consisted of a 40,000 pathogen spores. In sequential coinfections, the host was exposed to 20,000 spores of one pathogen genotype a week prior to 20,000 spores of the second, thus allowing for the prior establishment of an infection. In sequential single infections, the host was simply exposed to 20,000 spores of the same genotype at each exposure period. We herein refer to the multiplicity of genotypes and schedule of exposures collectively as "coinfection treatment."

Three *P. ramosa* genotypes with previously studied disease characteristics were used in this study. When singly infecting female *Daphnia,* genotype C19 exhibits high virulence and low transmission (average infection duration: 45.29 days; average spore load: 8.56 million spores), whereas genotypes C24 and C1 cause similar infection outcomes, exhibiting lower average virulence and higher average transmission as compared to C19 (Clerc et al. 2015). Coinfections consisted of genotype C19 and either C24 or C1 as these genotype pairings represent similar virulence combinations and were thus predicted to exhibit similar patterns of competitive outcome. Additionally, genotypes C24 and C1 cannot be distinguished using our genetic analyses, whereas C19 can be distinguished from C24 or C1, allowing us to determine the relative contribution of each pathogen genotype to the total spore production of coinfected *Daphnia* (see Genetic analysis section).

Thirty-three individuals of each sex were allocated to each coinfection or uninfected control treatment. In total, this experiment consisted of 26 treatments (33 replicates $\times$ 2 sex $\times$ [3 simultaneous single infections + 3 sequential single infections + 2 simultaneous coinfections + 2 sequential coinfections treated with C19 first + 2 sequential coinfections treated with C19 second + 1 uninfected control treatment] = 858 individuals).

MEASURES OF DISEASE CHARACTERISTICS

Studies of multiple infections commonly explore how the most fit pathogen genotype is related to characteristics of virulence, such as the reduction in host fecundity or lifespan upon infection (reviewed in Alizon et al. 2013; Bashey 2015). Due to differences in the average lifespan between males and females (males: 33 days $\pm$ 1.9; females: 67 days $\pm$ 2.0, Thompson et al. 2017), we focused on two traits of common currency: the reduction in lifespan as compared to the average of the unexposed controls and the production of transmission spores at host death. To this end, survival was monitored daily, and upon host death, animals were individually frozen in 500 µL of purified water for later determination of their infection status based on the presence or absence of mature transmission spores using phase-contrast microscopy. We calculated the reduction in male and female lifespan due to infection (i.e., virulence) by subtracting individual infected male or female lifespans from the average male or female control lifespan, respectively. Thus, individuals with a positive value exhibited pathogen-induced reductions in lifespan (measured in days) as compared to the control population.

Spore loads were then quantified using an Accuri C6 flow cytometer (BD Biosciences, San Jose, California). For each infected animal, 10 µL of their sample was diluted into 190 µL of 5 mM EDTA and loaded into one well of a round-bottomed PPE 96-well plate. All wells were mixed thoroughly to prevent clumping (in addition to the dilution step) and the absence of doublets was verified via a comparison of forward scatter pulse area versus forward scatter pulse height (both a measure of cell size, clumps have larger area to height signals). Gates based on fluorescence (via the 670 LP filter) and side scatter pulse area (a measure of cell granularity) were then used to identify only mature spores based on their distinct size, morphology, and fluorescence, compared to immature spores, algae, or animal debris. Overall spore load was measured twice per individual and averaged.

GENETIC ANALYSIS AND MEASURES OF WITHIN-HOST PATHOGEN COMPETITION

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

DNA extractions were performed using the EZNA Tissue DNA kit (Omega Bio-tek, Norcross, USA) with a modified protocol based on similar studies assessing the genetic composition of *P. ramosa* infections (Ben-Ami et al. 2008; Andras and Ebert 2013; Ben-Ami and Routtu 2013). Immediately after spore counting, the crushed *Daphnia* samples were pelleted via centrifugation for 3 min at 12,205 relative centrifugal force (RCF), supernatant removed, and washed with 1 mL of double-distilled water. The samples were again centrifuged using the same settings, supernatant removed, and then suspended in 200 µL lysis buffer and 25 µL OB protease. The samples were then homogenized via bead beating with 0.25 g of 0.1 mm zirconia beads for 2 min (1 $\times$ 10 s, 1 $\times$ 20 s, and 3 $\times$ 30 s), incubated in a heat block at 55°C for 1 h, before centrifugation at 10°C for 15 min at 5005 RCF. After collecting the supernatant, the DNA extraction proceeded as directed by the manufacturer protocol, including incubating the samples for 2 min at 70°C prior to elution to increase DNA yields. Final elution volume was 100 µL.

DNA was amplified via PCR with temperature cycling methods identical to Andras and Ebert (2013). Fragment analysis and genotyping was performed on these PCR products by AGRF (Melbourne, Australia) to determine the size of microsatellite alleles and the strength of their fluorescence (represented by peak height). The peak height ratio of the microsatellite markers was interpreted as the relative proportion of spores produced by each pathogen genotype as described by Ben-Ami et al. (2008); an approach that has also been used to quantify mixed sperm stores (Hall et al. 2010). This proportion was multiplied by the absolute number of

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

Multivariate ANOVA	Pillai's trace	Approx. *F*	df	*P*-value
Production of transmission spores, reduction in host lifespan (days)				
Sex	0.907	2939.443	2, 604	<0.001 *
Pathogen coinfection treatment	0.167	5.027	22, 1210	<0.001 *
Sex by coinfection interaction	0.108	3.133	22, 1210	<0.001 *
Univariate ANOVA		***F***	**df**	***P*-value**
Production of transmission spores				
Sex		2048.260	1, 605	<0.001 *
Pathogen coinfection treatment		15.535	11, 605	<0.001 *
Sex by coinfection interaction		7.146	11, 605	<0.001 *
Reduction in host lifespan (days)				
Sex		655.365	1, 605	<0.001 *
Pathogen coinfection treatment		6.110	11, 605	<0.001 *
Sex by coinfection interaction		0.526	11, 605	0.886

spores produced within the infected host to determine the relative transmission potential of the of competing genotypes.

STATISTICAL ANALYSES

All statistical analyses were performed in R (version 3.4.1; R Development Team, available at www.r-project.org). Only 823 of the 858 individuals initially set up for this experiment were used in final analyses as some individuals either died before infection status can be adequately determined (14 days postexposure, Clerc et al. 2015) or were removed due to experimental error. These 823 individuals consisted of 65 uninfected control individuals, 129 exposed but uninfected individuals, 319 individuals infected from single infection treatments, and 310 individuals infected from coinfection treatments of which *P. ramosa* DNA was extracted from 290 individuals.

We first explored how the reduction in host lifespan and pathogen spore production changed due to host sex, pathogen coinfection treatment, and their interaction using a multivariate analysis of variance (MANOVA Type III, *car* package, Fox and Weisberg 2011). Post hoc *t*-tests and Benjamini & Hochberg adjusted *P*-values were used to characterize how each trait varied between the specific coinfection combinations (C19 and C24 or C19 and C1). We then tested whether the fitness of individual coinfecting pathogen genotypes changed due to host sex, coinfection treatment, or their interaction. To do this, we fit a linear mixed model including each coinfection treatment (*lme4* package, Bates et al. 2015) with an individual's unique ID fit as a random effect, an interaction between sex and pathogen genotype as fixed effects, and relative production of spores as the response. We also fit separate mixed models for each coinfection treatment to describe which coinfection treatment was driving patterns of significance in the full model.

Results

THE EFFECT OF HOST SEX ON PATHOGEN VIRULENCE AND PROLIFERATION

Across all single and coinfection treatments, infections in females led to higher spore loads and a greater reduction in lifespan than in males; however, the magnitude of any response to infection was not shared equally between the sexes due to the presence of the interaction term (multivariate ANOVA, Table 2). The underlying univariate ANOVAs (Table 2), and the plots of each coinfection combination (Figs. 1 and 2; see also Fig. S1), reveal that this is driven by differences in pathogen proliferation and not virulence.

The overall virulence of single and coinfection treatments was highly concordant between males and females (Fig. 1). During single infections, pathogen C19 was significantly more virulent than either C24 or C1, leading to a further reduction in lifespan of approximately 10 days compared to the less virulent genotypes, irrespective of host sex. Similar levels of overall virulence were also observed during simultaneous coinfections between C19 and its competitor (Fig. 1A and D) and sequential coinfections where C19 established first (Fig. 1B and E). Only in coinfections in which the least virulent pathogen established first (C24 and C1) did we observe lower levels of overall virulence (Fig. 1C and F versus Fig. 1B and E) that were not significantly different from the virulence of either C24 or C1 in isolation (Fig. 2C and F).

In contrast, the variation in average spore loads among all coinfection treatments was far greater in females than in males (5.9–9.6 million in females versus 1.1–1.9 million in males). In females, we found that the more virulent pathogen, C19, produced the least amount of transmission spores of all pathogen genotypes

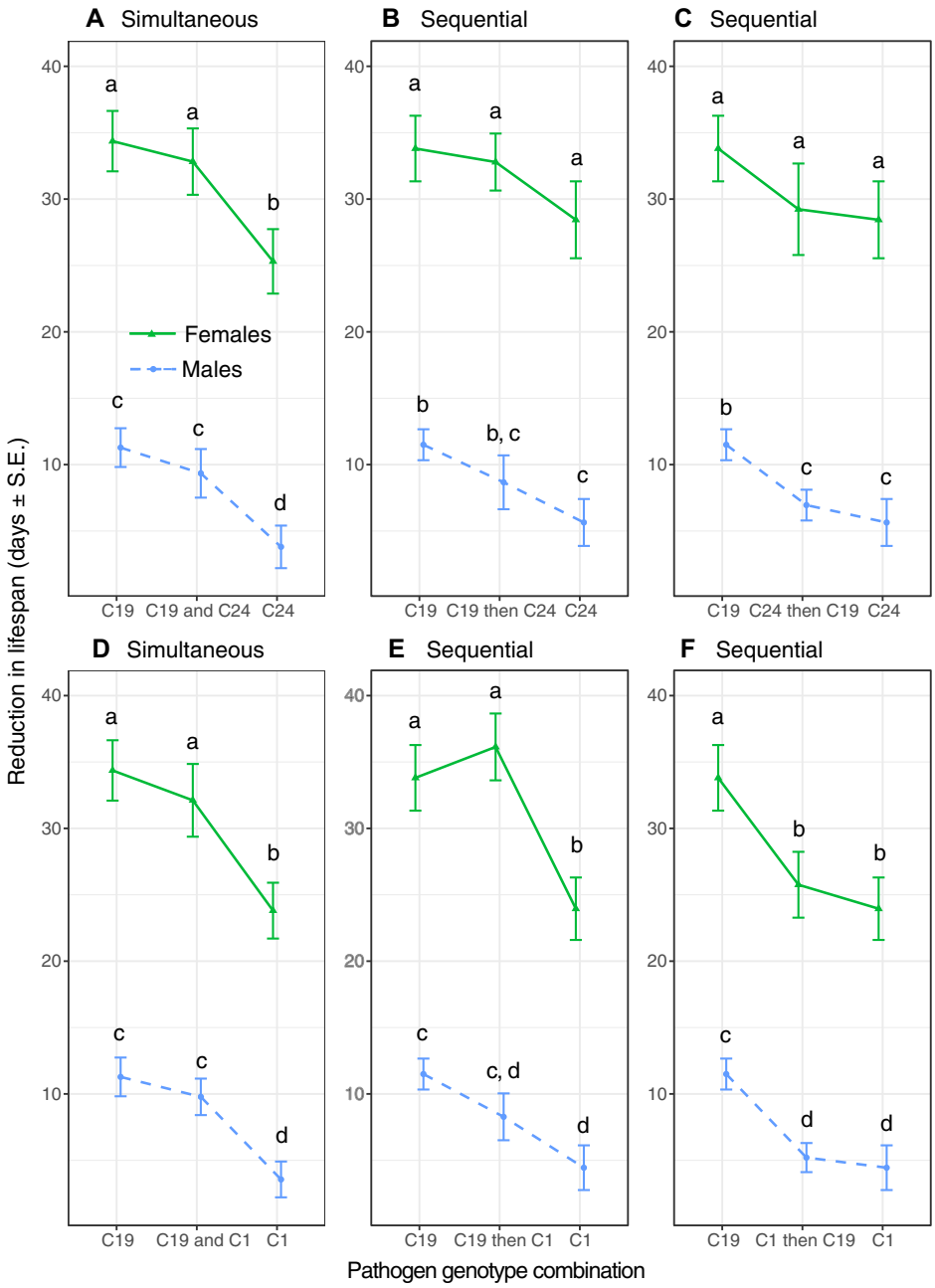

Figure 1. **The influence of host sex and coinfection treatment on overall virulence as estimated by the pathogen-induced reduction in host lifespan. Included are single infections (e.g., C19) and coinfection combinations (e.g., C19 then C1) for pathogen genotypes C19 versus C24 (A, B, and C) or C19 versus C1 (D, E, and F). Shown are treatment means and standard errors, with lowercase letters indicating significant groupings via post hoc *t*-tests and Benjamini & Hochberg adjusted *P*-values. Sequential infection labels ("then") refer to the order in which the hosts were exposed to the two pathogen genotypes, whereas simultaneous infection labels ("and") refer to the host being exposed to the two pathogen genotypes at the same time.**

during single infections. In turn, the absolute number of spores produced during simultaneous coinfections of both C19 and its competitor (Fig. 2A and D), and sequential coinfections where C19 established first (Fig. 2B and E), did not differ from the number of spores produced by C19 in isolation. Again, only when females were infected initially with the less virulent pathogen genotype (C24 or C1), were overall spore loads elevated during coinfections and not significantly different from the spore loads of C24 or C1 during single infections (Fig. 2C and F). Males exhibited comparatively similar patterns to females, but often the spore loads of each coinfection were intermediate to that of the single genotypes in isolation (Fig. 2B, D, and E), rather than clearly tracking either the most or least virulent pathogen as in females.

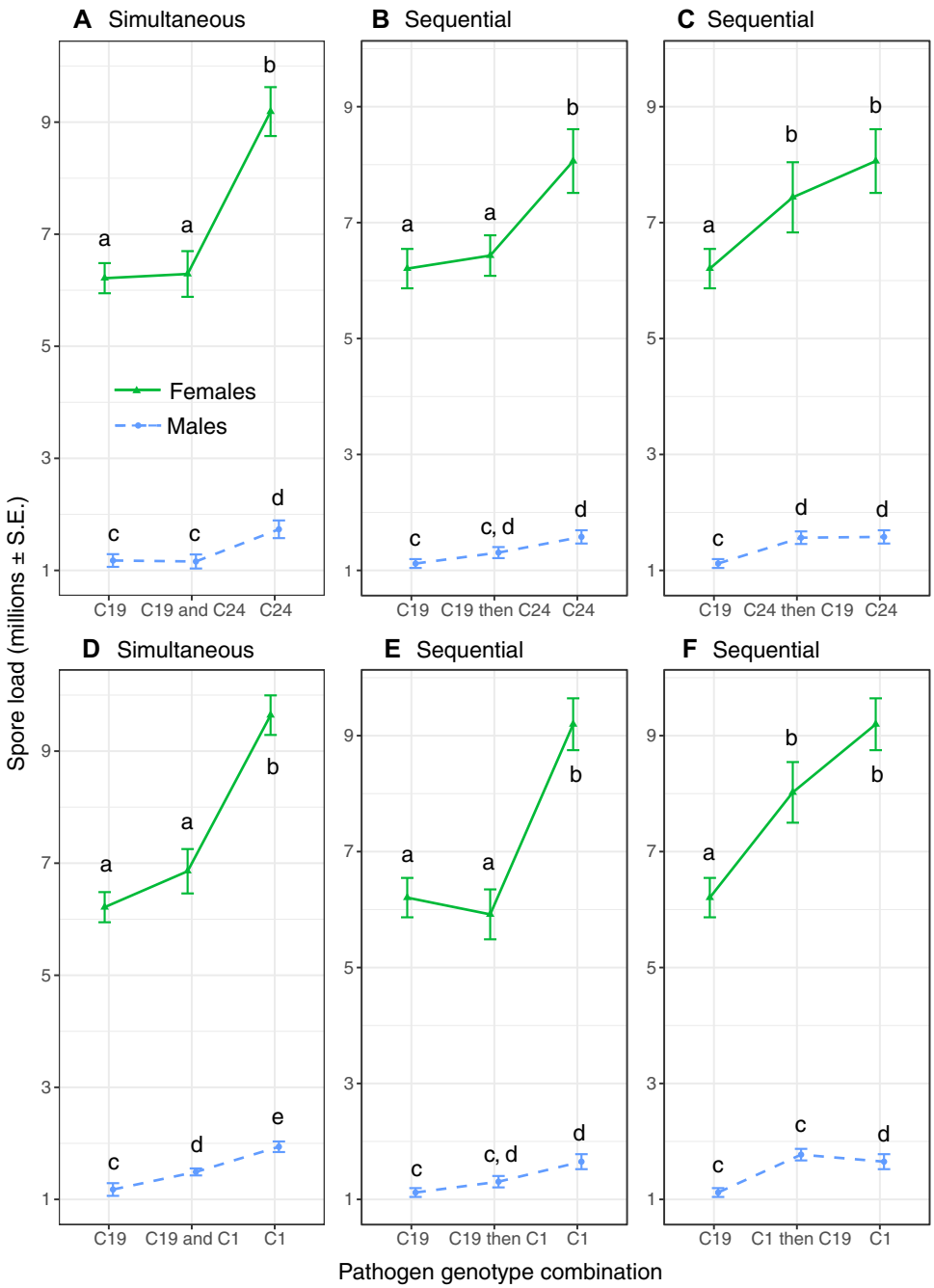

Figure 2. The influence of host sex and coinfection treatment on pathogen proliferation as estimated by the number of mature transmission spores at host death. Included are single infections (e.g., C19) and coinfection combinations (e.g., C19 then C1) for pathogen genotypes C19 versus C24 (A, B, and C) or C19 versus C1 (D, E, and F). Shown are treatment means and standard errors, with lowercase letters indicating significant groupings via post hoc *t*-tests and Benjamini & Hochberg adjusted *P*-values. Sequential infection labels ("then") refer to the order in which the hosts were exposed to the two pathogen genotypes, whereas simultaneous infection labels ("and") refer to the host being exposed to the two pathogen genotypes at the same time.

THE EFFECT OF HOST SEX ON THE PATHOGEN VIRULENCE AND PROLIFERATION TRADE-OFF

Comparing overall virulence and pathogen proliferation in both single and coinfection treatments indicates that higher overall virulence is associated with fewer total spores being produced in both sexes. However, the scale of this trade-off differs between males and females. As discussed above, this is driven by the greater variation occurring in spore loads between coinfection treatments in females (Fig. 2), whereas the relative differences in virulence among coinfection treatments remained similar for

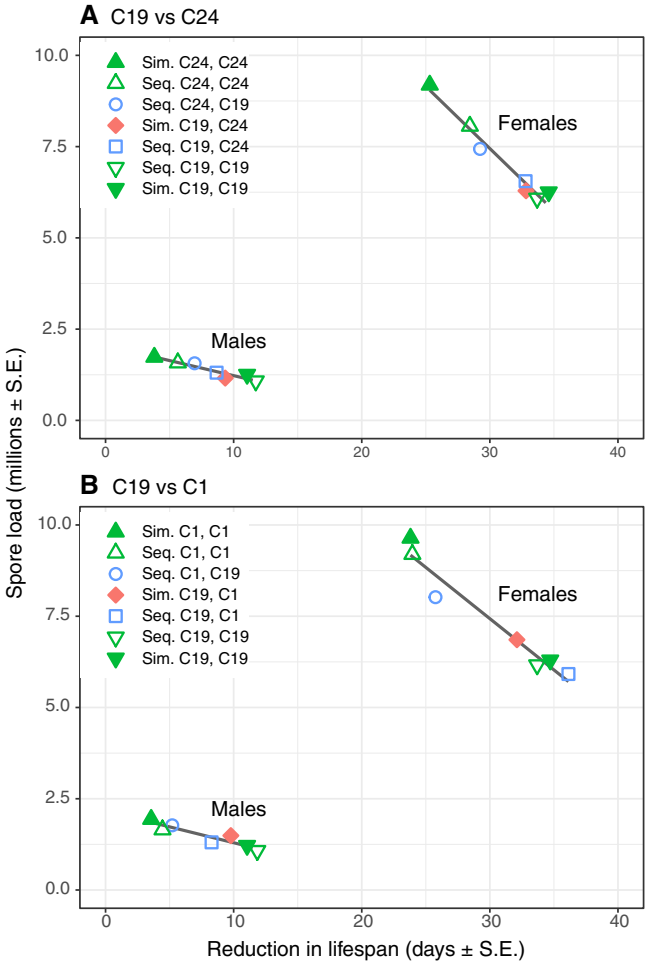

Figure 3. **The influence of host sex on the relationship between overall pathogen spore production (i.e., proliferation) and the pathogen-induced reduction in host lifespan (i.e., overall virulence) across all of the single and coinfection treatments. Shown are the treatment means for males and females exposed to either pathogen genotype C19 versus C24 (A) or pathogen genotype C19 versus C1 (B). Simultaneous (Sim.) infection labels and closed symbols refer to treatments where the host was exposed to two doses of the same pathogen (single infections, e.g., C19, C19) or to two pathogens at the same time (e.g., C19, C1). Sequential (Seq.) infection labels and open symbols also indicate the order of exposure, including treatments where the host was exposed to two doses of the same pathogen but at different times (single infections, e.g., C19, C19) or to whether the host was exposed to pathogen genotype C19 first or second, respectively (C1 then C19, e.g., Seq. C1, C19).**

both sexes (Fig. 1). The change in the steepness of this trade-off when comparing males and females is shown in Figure 3, whereby a given increase in virulence (i.e., reduction in lifespan) results in a threefold or greater decrease in spore loads for females compared to males, for both the C19 versus C1 (Slope males: $\beta = -0.087 \pm 0.016$, $P = 0.003$; Slope females: $\beta = -0.281 \pm 0.028$, $P < 0.001$) and C19 versus C24 (Slope males: $\beta = -0.083 \pm 0.010$, $P < 0.001$; Slope females: $\beta = -0.341 \pm 0.025$, $P < 0.001$) treatments. For expanded figures of each sex separately, including post hoc tests, see Figures S2 and S3.

THE EFFECT OF HOST SEX ON THE RELATIVE FITNESS AMONG COINFECTING PATHOGEN GENOTYPES

Finally, we explored how host sex influences the relative fitness of each pathogen genotype within a coinfection. We began by predicting the number of spores we might expect for each pathogen genotype in a mixed infection, based on the assumption that spore loads are determined purely by the intrinsic replication rates of each pathogen genotype (spores per day) multiplied by the duration of the infection (days until death postexposure). As previous work has shown that spore loads increase linearly over time (Hall and Mideo 2018), we used the number of spores at host death from the single infections, on average, to estimate an instantaneous replication rate for each pathogen genotype and sex combination (i.e., average spores produced per day). The average duration of infection for each pathogen genotype in a mixed infection was then calculated as the time until host death (i.e., age at host death – age of exposure) following the exposure schedules outlined by Table 1. The predicted results (Fig. 4, left column) suggest that if each pathogen were to proliferate independently within a male or female, then their share of all spores produced should be relatively equal (between 42% and 58% share) within a given sex.

Our observed results do not conform to these predictions. We found that an interaction between host sex and pathogen genotype determined relative pathogen fitness (Interaction term: $\chi^2 = 69.034$, df = 1, $P < 0.001$; see Table S1 for full model and for each coinfection treatment). In females, the more virulent genotype (C19) produced the majority of transmission spores in each coinfection context (up to 95%), except for when pathogen genotype C24 established first (Fig. 4, right column). Conversely, coinfection in males resulted in equal spore production of the competing genotypes except for when the less virulent genotypes (C24 or C1) established before C19. In both of these cases, prior establishment of the less virulent genotype resulted in C19 being competitively inferior. In general, coinfections within females were represented by the disproportionate transmission of the more virulent pathogen, but this was not observed in males. For example, in sequential infections with C24, pathogen C19 produced 90.4% of the total spores within female hosts, but only produced 49.8% of spores within males.

Discussion

In natural populations, infection is more likely to involve multiple pathogen genotypes of the same or different species (Read and Taylor 2001; Rigaud et al. 2010; Balmer and Tanner 2011). When

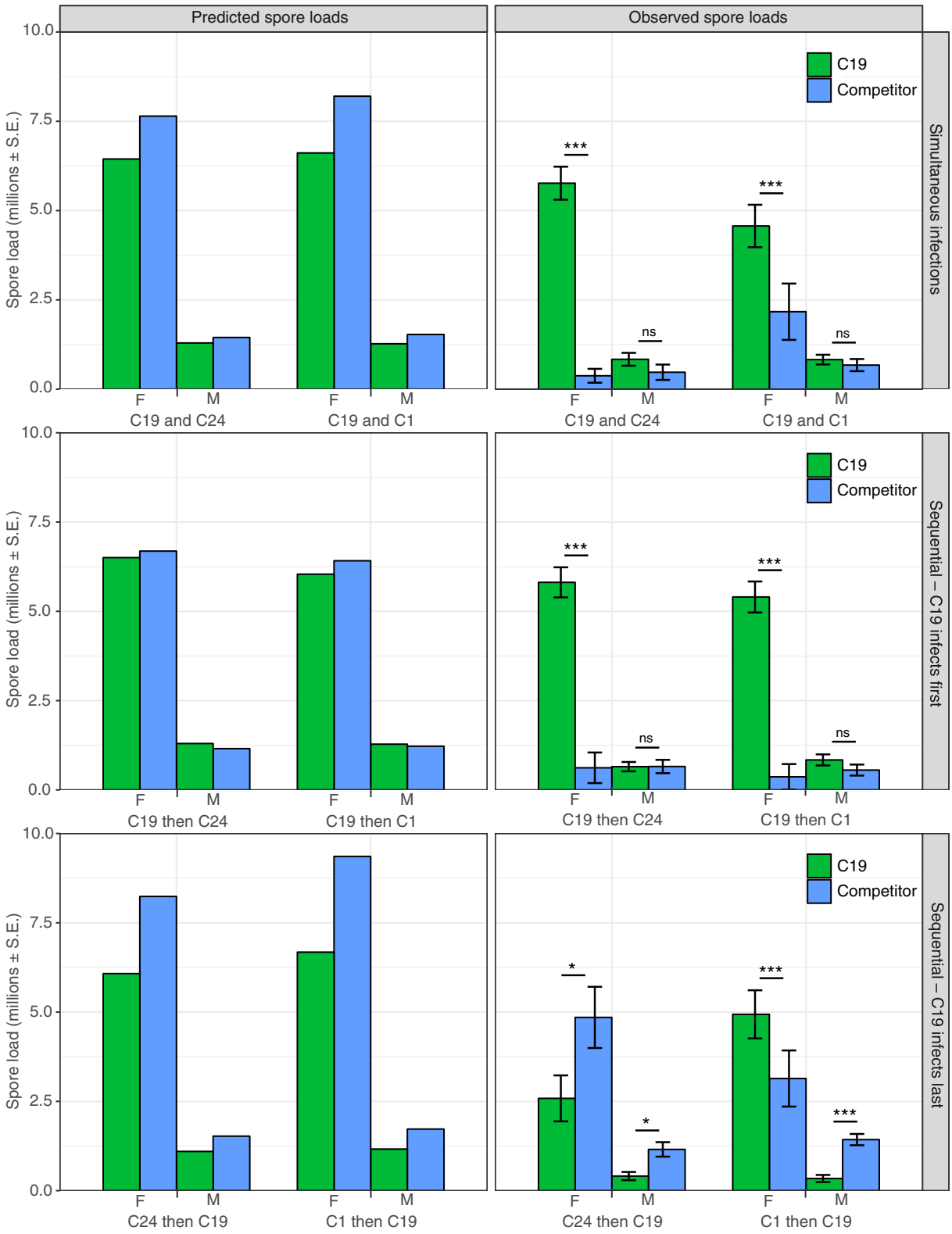

Figure 4. The predicted (based on instantaneous replication rates) and observed spore loads for each pathogen genotype competing in a coinfection (estimated using quantitative microsatellite analysis). Green bars represent spore production by pathogen genotype C19 and blue bars represent spore production by the competing pathogen genotype (either C24 or C1). Shown are individual means for each genotype and standard errors for the observed spore production. Asterisks indicate significant difference in mean spore production between competing genotypes within a single sex (two sample *t*-tests: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, ns $P > 0.05$).

multiple pathogens establish within a host, theory predicts that coinfection commonly favors more virulent pathogens through increased competition for host resources (Alizon et al. 2013). However, host heterogeneity may affect the relative fitness among competing pathogens (de Roode et al. 2004; Hodgson et al. 2004; Råberg et al. 2006; Ben-Ami et al. 2008; Ben-Ami and Routtu 2013; Izhar et al. 2015; Louhi et al. 2015) and thus the evolution of more virulent pathogens is not necessarily a universal outcome of coinfection. In this study, we considered how a common source of host heterogeneity in many species, the differences between the sexes in their capacity limit pathogen performance (see Table 1; Cousineau and Alizon 2014; Gipson and Hall 2016), can modify the expression of virulence in coinfections and the consequences this may have for the maintenance of genetic diversity in pathogen populations.

Our results indicate that the ability of males and females to modify how competition between different pathogen genotypes occurs during coinfections is multilayered. Based on the expression of overall virulence in coinfections alone, there appears, at first, to be little evidence that competition between pathogen genotypes will lead to fundamentally different virulence outcomes in males and females. In both sexes, the overall virulence and spore production of simultaneous coinfections was equal to that of the most virulent pathogen in isolation (Figs. 1 and 2). Sequential coinfections were also broadly concordant between the sexes and resembled the characteristics of the pathogen genotype that established first (Figs. 1 and 2). Instead, males appear to be limiting the degree to which overall virulence and pathogen proliferation can vary in both single and coinfections (Fig. 3). This was also observed in Thompson et al. (2017) where the scale of the trade-off between virulence and spore production varied due to host sex. Here, we formally quantified how a given change in overall virulence will lead to a reduction in spore loads that is up to three times higher in females than in males.

Despite commonalities between the sexes in their overall patterns of transmission and virulence, only females, however, appeared to allow one pathogen genotype to completely suppress the proliferation of any competitors. In all but one coinfection treatment, we found that females facilitated substantial differences in spore production between competing genotypes, with the more virulent pathogen (C19) producing up to 5.3 million spores more than its competitor and gaining a 90% or more share of the total spore loads (Simultaneous C19, C24; Fig. 4 right column). In contrast, when pathogens competed within males, each genotype produced an equal number of transmission spores in simultaneous exposures and sequential exposures when the more virulent pathogen established first (Fig. 4). This sharing of spore loads in males was much more similar to what would be expected if competitive interactions between competing genotypes were absent, or at least very weak (e.g., predicted spore loads, Fig. 4 left column). Males thus may act as a reservoir for pathogen genetic diversity, resulting in equal fitness between competing genotypes and even favoring genotypes that are frequently outcompeted within female hosts.

Our results also indicate that the arrival sequence of coinfecting pathogens will lead to different competitive outcomes depending on the host sex encountered. In infections where priority effects occur, early establishing pathogens may exhibit considerable levels of within-host replication even in competition with more virulent genotypes. Indeed, Ben-Ami et al. (2008) found in the same system that less virulent genotypes can produce substantial amounts of transmission spores when establishing first, even though they are outcompeted when establishing after more virulent genotypes. Here, females exhibited this general pattern with the more virulent C19 genotype either outcompeted by C24, or producing a similar number of spores as C1, when establishing second (Fig. 4B). Conversely, the constraints imposed by male hosts appear to keep pathogen C19 from ever outcompeting other genotypes when they establish first. Our initial prediction that the smaller pool of resources offered by males would facilitate competitive exclusion thus proves to be false. Instead, priority effects in males appear to be mediated by simple changes in the length of time in which an early- or late-arriving pathogen would be able to proliferate within the host.

One explanation for the patterns we observe is that the limited pool of resources provided by male *Daphnia* prohibits pathogens from ever diverging in their exploitation strategies. However, we cannot completely rule out that competition within a single pathogen genotype for space or resources is also contributing to the patterns of coinfection in each sex (i.e., intrastrain competition). By applying the same inoculation dose in both single and coinfection treatments (as is common in coinfection studies using genotypes of one pathogen species, for example, Klemme et al. 2015; Susi et al. 2015; Kinnula et al. 2017; but not when competing two species, for example, studies listed in Clay et al. 2018), we have also varied the initial starting density of each competing genotype. Two lines of evidence, fortunately, suggest that any density-dependent effects are unlikely to occur in our study system. First, our recent study of within-host pathogen growth (Hall and Mideo 2018) showed that spore loads in both sexes increase linearly over time (something that should be nonlinear if growth rates slowed with density). Second, using a single genotype of the same pathogen as in this study, Ben-Ami et al. (2008) directly compared a single dose of 50K spores versus single dose of 100K spores (i.e., a double dose) and found that starting densities did not influence ($df = 3, 373$; $F = 0.96$; $P = 0.41$) later spore loads (see also Ben-Ami and Routtu 2013).

Taken together, our results suggest that the evolutionary outcome of pathogen virulence in coinfection contexts will depend on how often pathogens encounter male and female hosts (i.e., van

Baalen and Sabelis 1995; Frank 1996; Mideo et al. 2008; Choisy and de Roode 2010; Alizon et al. 2013). In nature, *Daphnia* populations may consist of 0–50% male individuals at different times of the season (Duncan et al. 2006; Galimov et al. 2011). This represents a dramatic fluctuation in the availability of male and female hosts that, when considered in light of our results, may influence the frequency of pathogen transmission between each sex and thus the overall patterns of selection on virulence. For example, a higher proportion of the more exploitable sex (female *Daphnia)*, in general, should favor the transmission of more virulent pathogens for much of the year, whereas seasonal increases in the proportion of the less exploitable sex (male *Daphnia)* may instead temporarily restrain or reverse this pattern. Naturally fluctuating sex ratios may therefore provide the necessary ingredients to influence the evolution of virulence in natural populations (see also Hall and Mideo 2018).

The ubiquity of pathogens in natural populations suggests that individuals are likely to encounter and become infected by multiple pathogen genotypes. We show that the outcome of any within-host competition, as well as the trade-off between overall virulence and transmission, may depend on the sex of the host in which the multiple infections arise. This work reaffirms the role that host heterogeneity can play in affecting the outcome of within-host pathogen competition and its potential to influence the evolution of virulence (de Roode et al. 2004; Hodgson et al. 2004; Råberg et al. 2006; Ben-Ami et al. 2008; Ben-Ami and Routtu 2013; Izhar et al. 2015). Our results suggest that the often-observed relationship between virulence and pathogen competitive ability may not apply equally to each sex, providing a mechanism for the maintenance of pathogen genetic variation in sexually dimorphic host populations (see also Gipson and Hall 2016). Ultimately, how virulence should evolve within coinfection contexts will depend on the frequency that a pathogen encounters each sex, as well as variation in the exploitative potential of male and female hosts.

AUTHOR CONTRIBUTIONS

S.A.Y.G., L.J., and M.D.H designed the study. S.A.Y.G. and L.J. performed the experiments. S.A.Y.G and M.D.H analyzed the data and drafted the manuscript.

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DATA ARCHIVING

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.vb76300.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Summary of an analyses of variance describing the effect of host sex, pathogen genotype, and their interaction on relative pathogen fitness within coinfection treatments.

Figure S1. The influence of host sex and coinfection treatment on overall pathogen spore production (a) and reduction in host lifespan (b).

Figure S2. The influence of coinfection treatment on the relationship between overall pathogen spore production and reduction in host lifespan (virulence) for females exposed to pathogen genotype C19 and/or C24 (a) and females exposed to pathogen genotype C19 and/or C1 (b).

Figure S3. The influence of coinfection treatment on the relationship between overall pathogen spore production and reduction in host lifespan (virulence) for males exposed to pathogen genotype C19 and/or C24 (a) and males exposed to pathogen genotype C19 and/or C1 (b).