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

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Abstract

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

Introduction

Resulting from the contrasting strategies by which males and females maintain fitness, the sexes are expected to vary in their relative immune investment, condition and morphology (Zuk and Stoehr 2002; Zuk 2009), all of which can lead to sex biases in the outcome of infection (Poulin 1996; Schalk and Forbes 1997; McCurdy et al. 1998; Sheridan et al. 2000). In humans, for example, males are generally more susceptible to infectious diseases such as tuberculosis and hepatitis B (Giefing-Kröll et al. 2015; vom Steeg and Klein 2016) and harbour a larger viral load than females in the case of HIV (Napravnik et al. 2002; Donnelly et al. 2005). However, the 'sicker sex' can vary from species to species (see table 1 in Cousineau and Alizon 2014) and many studies have considered the consequences this has for host susceptibility and mortality (Giefing-Kröll et al. 2015; Klein and Flanagan 2016) as well as mate choice (Hamilton and Zuk 1982). Recently, attention has turned to how sexual dimorphism relates to the evolution of pathogen fitness itself, leading to predictions, many yet untested, for how male–female differences might impact on the evolutionary dynamics of host–pathogen interactions (Duneau and Ebert 2012; Cousineau and Alizon 2014; Gipson and Hall 2016).

A component of pathogen fitness that may be particularly susceptible to disruption by sexual dimorphism is the fundamental trade-off between transmission and virulence. In natural populations, pathogens are inevitably transmitted through hosts that differ in their capacity to fight infection (Altizer et al. 2006; Wolinska

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and King 2009), spurring many theoretical predictions on how a heterogeneous host population will affect the optimal level of virulence (Regoes et al. 2000; Gandon 2004; Osnas and Dobson 2012; Williams 2012). Gandon (2004), for example, highlighted how a specialist virulence strategy is predicted if the frequency of encountering only one host type is high. In this context, by adapting to the most common host, a pathogen can become maladapted to the less common one as a consequence of either suboptimal exploitation or overexploitation. This cost becomes progressively smaller as the chance of encountering the less common host type is reduced. In contrast, when the likelihood of transmission between different host types is higher, more generalist strategies are now favoured that can select for intermediate values of virulence (assuming all things being equal in other demographic parameters such as background mortality, Gandon (2004)).

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

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

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

Via a series of experimental infection trials, we exposed genetically identical male and female *Daphnia* to one of two compatible *P. ramosa* genotypes at either 10, 20, 30 or 40 days old. We then measured the resulting changes in the costs of infection via age-specific mortality rates and linked them to aspects of pathogen fitness such as infection rate, pathogen-induced reduction in lifespan (virulence) and the production of spores (transmission). In the light of previous studies which have investigated the effects of host sex or age on disease in isolation (Duneau et al. 2012; Clerc et al. 2015; Izhar and Ben-Ami 2015; Clark et al. 2017; Thompson et al. 2017), we predicted that mortality rates and virulence should increase with the age at which a host encounters a pathogen as a consequence of the ageing process (Adamo et al. 2001; Doums et al. 2002; Zerofsky et al. 2005), and that these increasing costs would be felt most strongly by the less resistant sex, which in *Daphnia* is females (Duneau et al. 2012; Thompson et al. 2017). We discuss how our results may impact on the evolution of optimal infectivity and virulence for a pathogen and contribute to the maintenance of variability in infectious disease in natural populations.

Materials and methods

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

Production of experimental animals

Prior to the experiment, juvenile female *Daphnia* were collected from stock cultures and individually maintained in 60-mL vials filled with 50 mL of artificial *Daphnia* medium (ADaM, Klüttgen et al. 1994; modified by Ebert et al. 1998). *Daphnia* were transferred into fresh media twice weekly, maintained under standard conditions (20 °C, 16L: 8D) for three generations and fed up to five million *Chlorella vulgaris* algal cells daily, steadily increasing from birth to accommodate the growing needs of the animal. Experimental males and females were produced using methods from Thompson et al. (2017). In short, females were exposed to 300 μg L^{-1} of the hormone methyl farnesoate (Product ID: S-0153, Echelon Biosciences, Salt Lake City, UT) after producing their first clutch and then transferred into fresh hormone-treated media every 2 days. Subsequent clutches were collected and the sex of all offspring determined. This method can be used to reliably produce male and female *Daphnia* while having no detectable impact on lifespan, fecundity, infection rates or spore loads (Thompson et al. 2017).

Infection experiment design and estimates of pathogen fitness

Male and female *Daphnia* were randomly exposed to one of the two pathogen genotypes at ages 10, 20, 30 or 40 days. For each treatment combination, we individually placed between 60 and 120 males and females in jars, with the larger sample sizes allocated to the older ages to compensate for natural deaths occurring before pathogen exposure, with an additional 50 individuals of each sex as unexposed controls (2 sex × 2 pathogen genotypes × 4 ages × 60 to 120 replicates + [100 female and 110 male control replicates]). Infections took place in 60-mL vials filled with 20 mL of media. The infection process occurred over 2 days wherein 20 000 pathogen spores were added daily (40 000 spores total). This process was applied to a new group of animals at each infection date. Animals were maintained under standard conditions as above.

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

Immortal time bias and survival analysis

All statistical analyses were performed using R (version 3.3.1; R Development Core Team, available at www.r-project.org). One of the challenges of analysing cohort based survival data is a phenomenon known as 'immortal time bias' (Ho et al. 2012). Immortal time bias occurs when the survival rate of an experimental treatment is inflated simply because those individuals must live long enough to receive treatment whereas control individuals experience mortality from the beginning of an experiment. To control for this bias, we paired exposed and infected animals with a matched control cohort (as per Lévesque et al. 2010). Age-matched control cohorts were created by filtering control survival data to animals which lived at least 14 days after each exposure age as this is the earliest point in which infection status can be accurately diagnosed.

Using the age-matched cohorts, we modelled the time to death of each individual using a Weibull hazard function as estimated via the *survival* package, and visualized these trends via Kaplan–Meier survival curves using the *ggfortify* package. Variation in mortality was estimated as a function of the main effects of exposure outcomes (exposed, infected or control), host sex (male or female) and age of exposure (days 10, 20, 30 or 40), as well as their interactions, using a three-factor analysis of variance (Type III). To help explain any observed

treatment differences, we estimated hazard ratios for infected or exposed individuals (relative to the age-matched control cohort baseline) for each combination of sex and age of exposure using the *SurvRegCensCov* package. Here, hazard rates were parameterized as odds of death at any given time due to exposure to or infection by a given pathogen.

Characterizing age-specific trends

Complementing the analysis of mortality, we also analysed traits directly related to pathogen fitness and the virulence–transmission trade-off. Due to differences in the average lifespan between males and females (males: 33 days ± 1.9, females: 67 days ± 2.0; Thompson et al. 2017), we focused on three traits of common currency: the proportion of exposed animals that became infected; the reduction in lifespan as compared to the average of the age-matched control cohorts; and the production of transmission spores at host death (analyses included only infected individuals, see Table S1). Comparisons between the sexes and pathogen genotypes were analysed using a generalized linear model (binomial error distribution, logit link function) for infection rates and a least-squares linear model for both the reduction in lifespan and the production of spores. Before analysis, we square root transformed spore loads to meet the requirement of normality, although data are presented on the original scale to aid interpretation.

For each sex, we used a sequential model fitting approach (e.g. Hall et al. 2008) to describe the relationship between pathogen fitness traits and age of infection. This approach begins with a model containing only pathogen genotype (C20 or C24) as a factor, with each of the following terms added sequentially: the linear terms for age of infection; the corresponding quadratic term; the interaction between the linear term and the pathogen factor; and finally the interaction between the quadratic term and the pathogen factor. A partial *F*-test was used to assess whether the relationship between age of infection and each trait was linear or nonlinear (based on the significance of the linear and quadratic terms, respectively), and if these trends differed between the pathogen genotypes (based on the interaction of this factor with either the linear or quadratic regression coefficient). We then visualized the most appropriate model and compared them between the two sexes using the most complex model possible and the 'drop1' model simplification function in R.

Results

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

Our results point to a complex interaction between exposure outcomes (control vs. exposed and uninfected vs. exposed and infected), host sex and the age of exposure on patterns of host mortality. Comparison of the survival curves for control, exposed but not infected (herein exposed), and infected animals (Fig. 1), showed that, as expected, the median lifespan of control males was shorter than control females for all ages of exposure (e.g. day 10 controls, male: 36.32 ± 0.99; female: 65.74 ± 1.48), and not surprisingly, that the duration of life remaining decreased as animals got older, irrespective of whether the host was exposed or not (e.g. day 40 controls, male: 11.66 ± 0.87; female: 35.74 ± 1.48). In addition to these obvious sex ($P < 0.001$) and age effects ($P < 0.001$), we found a significant interaction between host sex and age on host mortality, with marginal contributions from interactions between age and exposure outcome ($P = 0.068$) and a three-way interaction between all factors ($P = 0.075$, see Table S2 for ANOVA specifics).

Further exploration of the hazard rates for each treatment combination highlighted the driving forces behind these patterns of mortality (Table 1). Relative to the age-matched control cohort, we found that infections in females resulted in higher mortality rates, and that the odds of death at any given age were between 2.5-fold and five-fold across all ages of exposure. Conversely, mortality was only increased in males infected at 10 days old, with a two-fold increase in the likelihood of death (Fig. 1b). Infected male hazard rates at all other age classes were indistinguishable from those of the control cohorts. For both sexes, individuals that were exposed to the pathogen but did not become infected showed no increase in hazard ratio (no hazards were greater than 1), except for one marginal case for females at age 40 (Fig. 1g).

Host sex changes the relationship between virulence and pathogen fitness

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

The probability of infection differed between pathogen genotypes in female hosts, but not in males (Table 2). For females, the interaction between

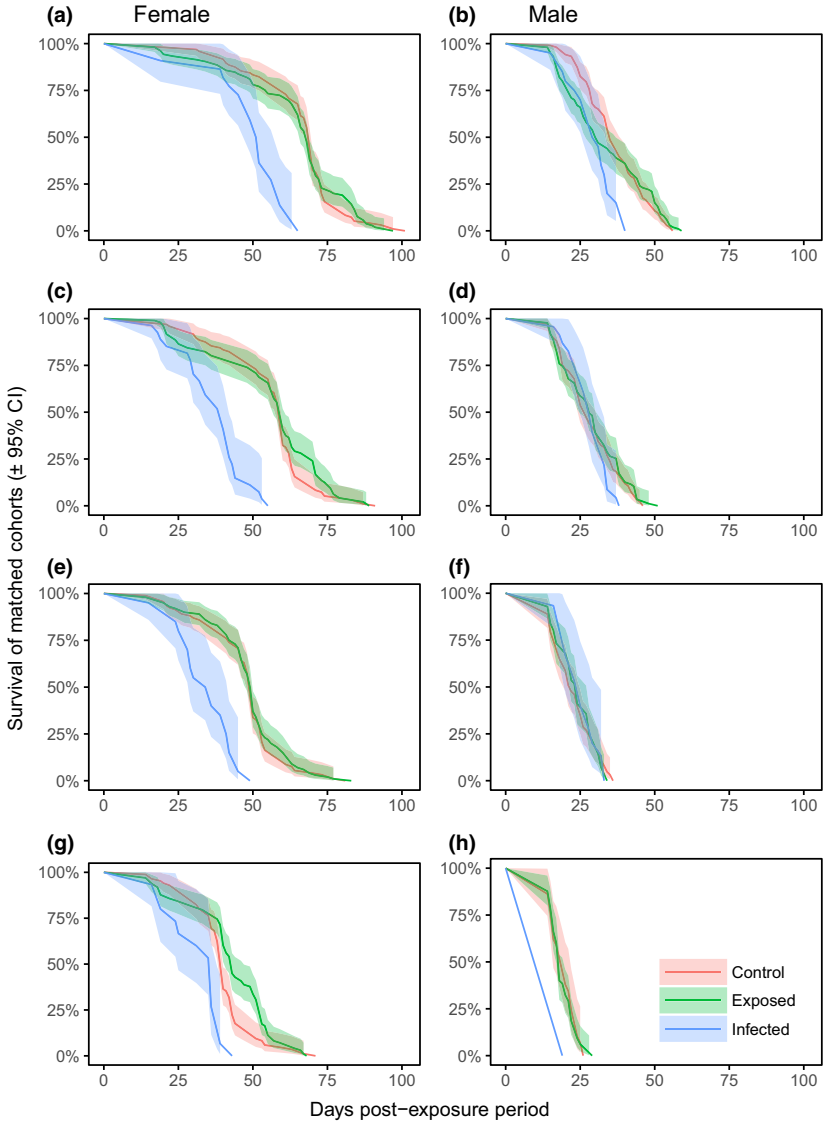

Fig. 1 Kaplan–Meier survival curves describing the influence of sex and outcome of pathogen exposure on survival in female (left column) and male (right column) individuals following exposure at 10 (a, b), 20 (c, d), 30 (e, f) or 40 (g, h) days old. Red lines denote control individuals, green lines denote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

pathogen genotype and the quadratic term arose because intermediate ages led to the highest success for pathogen C20 (negative quadratic: Age^2: -0.004 ± 0.002, $P = 0.015$), whereas C24 showed the opposite pattern, albeit weaker and nonsignificant (positive quadratic: Age^2: 0.002 ± 0.002, $P = 0.399$, Fig. 2a). In contrast, for males, the lack of any significant interactions indicates that the relationship between age of infection and infection success was the same for both pathogens (Table 2), with infection success declining nonlinearly with age (age: 0.186 ± 0.078, $P = 0.017$; age^2: -0.005 ± 0.002, $P = 0.002$, Fig. 2b). Although this would suggest that the influence of host sex depends on the pathogen genotype and age of infection in combination, the three-way interaction between sex, pathogen and age of exposure was dropped from a model including both male and female data (see Table S3), suggesting the contrast between males and females may be marginal.

Similar patterns were observed for both virulence and spore loads, with the pathogen effect only detected for females (pathogen by age or age^2 interactions, Table 2). In females, the virulence of pathogen C20 declined in an accelerating manner (age: 0.884 ± 0.540, $P = 0.108$; age^2: -0.028 ± 0.01, $P = 0.013$), whereas for pathogen C24, it peaked at intermediate ages (age: 2.161 ± 1.019, $P = 0.043$; age^2: -0.041 ± 0.020, $P = 0.055$). This trend was reversed for spore loads (Fig. 2e), with C20 displaying no significant change with increasing age (age: 0.013 ± 0.033, $P = 0.687$), compared to the rapid decline in spores for C24 (age: -0.250 ± 0.062, $P = <0.001$). In males, no difference between the genotypes was detected (Table 2). Both virulence (age: -1.367 ± 0.503,

Table 1 Summary of statistical analyses describing the differences in survival between control, exposed but not infected, and infected groups for each combination of sex and age of exposure.

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

Asterisks denote significant effects ($\alpha = 0.05$). Hazard ratios indicate the increased odds of death at any given age compared to the control group, with values equal to 1 indicating equal mortality rates between groups and values greater than 1 indicate an increase in mortality rates. Bold indicates values significantly different from 1.

Table 2 Results of the sequential model fitting approach describing the effects of pathogen genotype and age of exposure on infection rate, virulence and spore load.

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

Each term was added sequentially beginning with a model containing the pathogen genotype (C20 or C24) as a factor, followed by the linear and quadratic terms for age of infection and finally the interaction between these terms and the pathogen factor. Asterisks denote significant effects ($\alpha = 0.05$).

$P = 0.008$; age^2: 0.024 ± 0.012, $P = 0.046$, Fig. 2d) and spore loads (age: -0.033 ± 0.013, $P = 0.012$, Fig. 2f) peaked in males exposed at day ten and then declined. Models including both male and female trends always retained a three-way interaction with either the linear or quadratic term, supporting the conclusion that the influence of host sex on pathogen fitness is specific to both the pathogen genotype and the age of infection (see Table S3).

Discussion

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

When two hosts differ in the amount of resources they provide to a pathogen, theory predicts that

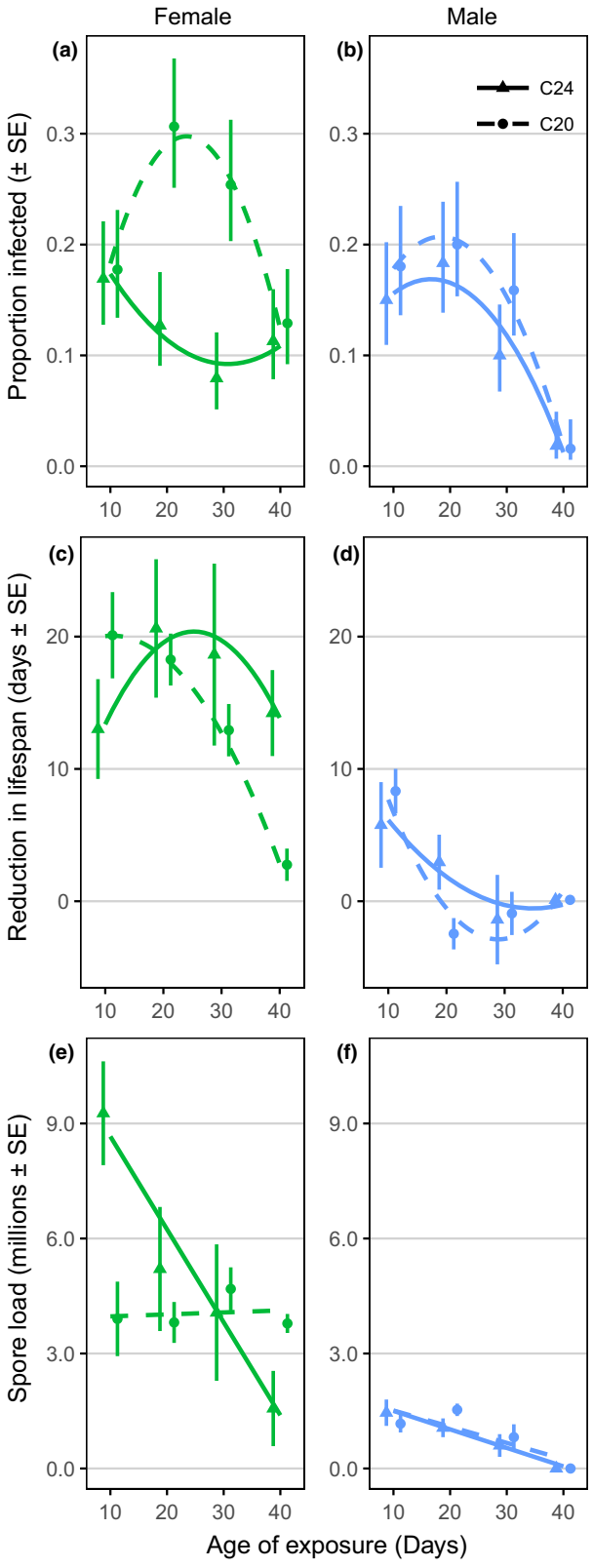

Fig. 2 The influence of host sex and age of pathogen exposure on infection rate (a, b), pathogen-induced reduction in lifespan (c, d) and spore production (e, f). Shown are the treatment means, standard errors and the trends over time as suggested by the best fitting linear models (see Table 2). Female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

pathogen reproduction will accelerate in the more exploitable host at the expense of decreasing host fitness (Hall et al. 2009). Although previously applied only in the context of host differences in the acquisition of nutrients, the contrast between male and female hosts in our study system presents an analogous scenario. Female *Daphnia* are larger and longer lived than males and have a significant pool of resources available to invest in producing clutches of offspring every 3 days (e.g. Clerc et al. 2015). In contrast, males represent a more difficult host to exploit, providing less physical space and fewer resources for pathogen growth (e.g. Thompson et al. 2017). Our results suggest that old age may further impact on the resources that each sex cedes to a pathogen. As the remaining lifespan of females is greater than males at any given age (Fig. 1), the higher overall infection rate, virulence and spore load observed in females (Fig. 2) may be a product of the increased time allowed for exploitation by the pathogen. Fewer resources to exploit and the short lifespan of males may simply not provide enough time and energy for the pathogen to either establish a successful infection or reach the intensity of infection that would facilitate a substantial increase in the rate of mortality (*c.f.* females).

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

which a host encounters a pathogen can prevent a single pathogen strategy from maximizing fitness.

Ultimately it will be the frequency of encountering different sexes or host ages that will determine how much diversity in different strategies is maintained (see Gipson and Hall 2016). In the wild, we expect *Daphnia* populations to be predominately female biased, but males can still constitute up to half of the population for two to three months of the season (e.g. Galimov et al. 2011), and the increase in male production has been shown, at least in one case study, to occur prior to a disease outbreak (Duncan et al. 2006). Age structure is less well known, but evidence suggests that the age classes, on the scale explored here, are likely relevant: *Daphnia* must survive approximately 2 weeks to produce their first clutch (Ebert 2005); have been observed to overwinter in nature (Gliwicz et al. 2001; Slusarczyk 2009); and can live as long as 150 days in laboratory settings (Ebert et al. 2016). Under these conditions, female-biased populations of mixed age classes will occur for much of the year and will be particularly labile for pathogen evolution, with changes in the rank order of pathogen genotypes occurring for transmission, virulence and infection rates whenever a pathogen encounters animals of different ages. In contrast, males will present a simpler scenario with infection rates and spore loads highest at early ages for all pathogen genotypes. Thus, deviations from an even sex ratio and young cohort can lead to situations where one pathogen genotype is more consistently favoured (i.e. females, Fig. 2e) or mask the variation between pathogen genotypes (i.e. males, Fig. 2f).

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

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

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Table S1 Sample sizes for analysis of infection rate, virulence (parasite induced reduction of lifespan), and transmission (mature spore load).
Table S2 Summary of analysis of variance (Type III) describing the effects of host sex, host age at exposure to a pathogen, and exposure outcome on survival. Asterisks denote significant effects ($\alpha = 0.05$), Hashes denote marginally significant effects.
Table S3 Candidate regression models describing the effects of host sex (sex), pathogen genotype (par), and age of exposure (age) on infection rate, virulence, and spore load.

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

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