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Jae Hoon Cho April 11, 2017

Parasite virulence and the maintenance of outcrossing in the *Caenorhabditis elegans* and *Serratia marcescens* system

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Abstract

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The Red Queen hypothesis (RQH) predicts that coevolving parasites can impose persistent selection against common host genotypes, and ultimately select against uniparental reproduction. Thus, coevolving parasites are predicted to constrain self-fertilizing lineages from invading obligately outcrossing host populations, despite the inherent costs of biparental reproduction. However, this prediction is contingent on parasite virulence. Only highly virulent parasites are expected to maintain obligate outcrossing, which may limit the scope of the RQH as a broad explanation for the maintenance of outcrossing. Therefore, we tested whether the invasion of self-fertilization within obligately outcrossing host populations was dependent on the virulence of coevolving parasites. We introduced wild-type *Caenorhabditis elegans* hermaphrodites, capable of both self-fertilization and outcrossing, into *C. elegans* populations fixed for a mutant allele conferring obligate outcrossing. Replicate *C. elegans* populations were exposed to one of four strains of *Serratia marcescens* parasites (Sm2170, SmD1, Sm933, or Db11), varying in virulence, for 24 generations under three treatments: a control (avirulent) parasite treatment, a fixed (non-evolving) parasite treatment, and a copassaged (potentially coevolving) parasite treatment. As predicted, self-fertilization invaded *C. elegans* host populations in the control and fixed-parasite treatments for all bacterial strains. In the copassaged treatment, self-fertilization invaded host populations in the two least virulent strains (Sm933 and Db11), but remained rare in the two most virulent bacterial strains. Indeed, the obligate outcrossing allele was maintained at high frequencies in multiple populations copassaged with the two most virulent parasite strains, but decreased in frequency in all other populations. Consistent with the RQH, highly virulent coevolving parasites are capable of impeding the invasion of self-fertilization in outcrossing host populations. However, selection imposed by more moderately virulent coevolving parasites was not sufficient to maintain obligate outcrossing. Therefore, the RQH may not be sufficient as the sole explanation for the maintenance of biparental sex.

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Introduction

Biparental outcrossing is a dominant mode of reproduction among plants and animals. Less than 1% of animal species are obligately asexual, and few plant and animal species reproduce by obligate self-fertilization (Bell 1982; Judson and Normark 1996). This finding is surprising, however, because substantial inherent costs are associated with biparental mating systems, and not uniparental mating systems (Williams 1975; Smith 1978; Bell 1982). For example, unlike biparental populations, a uniparental population (i.e. one that reproduces via self-fertilization or parthenogenesis) does not bear the "two-fold cost of males," whereby the number of offspring-bearing progeny is halved due to the production of males who are incapable of bearing offspring themselves (Smith 1978). In an obligately outcrossing population, this cost reduces the numerical growth of lineages every generation and makes the population susceptible to invasion and replacement by individuals with mutant alleles conferring uniparental reproduction (Fisher 1941; Smith 1978; Charlesworth 1980; Agrawal and Lively 2001). If obligate outcrossing is to be maintained, biparental reproduction must confer substantial advantages over uniparental reproduction that outweigh its inherent costs.

The Red Queen Hypothesis (RQH) predicts that coevolution with parasites can allow for the maintenance of host obligate outcrossing. It proposes that coevolving parasites select against common host genotypes, and therefore favor host outcrossing over self-fertilization ("selfing") since outcrossing has the capacity to generate genetically variable offspring (Jaenike 1978; Hamilton 1980). Empirical studies of both natural and laboratory host populations have supported this prediction. Studies on natural snail populations, for example, have shown that the frequencies of parasites were positively correlated with rates of host sexual reproduction across multiple lakes in New Zealand and across multiple sites within lakes (Lively 1987; King et al.

2011; Vergara et al. 2014). Furthermore, laboratory experiments exposed mixed-mating populations of the nematode host *Caenorhabditis elegans* to coevolving bacterial parasites and found that higher rates of outcrossing evolved and were maintained over time (Morran et al. 2011). Further experiments have shown that host survival against coevolving parasites was positively correlated with host outcrossing rates (Morran et al. 2011). While obligately selfing populations of *C. elegans* went extinct within 20 generations of coevolution with *S. marcescens,* host populations that were capable of outcrossing survived for the full duration of the experiment (30 generations).

Although these studies support the prediction that coevolving parasites can impose selection favoring host outcrossing over selfing, Slowinski et al. (2016) directly tested the ability of coevolving parasites to impede the invasion of self-fertilization into obligately outcrossing host populations. Approximately 50 wild-type *C. elegans* hermaphrodites, capable of self-fertilization and outcrossing, were combined into a larger population of approximately 950 mutant individuals, fixed for a mutant allele conferring obligate outcrossing. Replicate populations of the combined wild type and mutant *C. elegans* were then exposed to one of three bacterial treatments: (1) a control (avirulent) parasite treatment, (2) a fixed (non-evolving) parasite treatment, and (3) a copassaged (potentially coevolving) parasite treatment of *Serratia marcescens* strain Sm2170. Host selfing rates and the genotypic frequencies of the mixed-mating allele conclusively showed that self-fertilization invaded into the obligately outcrossing host population in both the control and fixed treatments, but not in the copassaged treatment. As predicted, parasite-mediated selection alone was capable of impeding the invasion of self-fertilization into obligately outcrossing populations.

Yet, while those results strongly supported the RQH, the population genetics models produced by Otto and Nuismer (2004) offered that, generally, species interactions were not predicted to select for sex. Given the limitations that biologically relevant parameters impose, they argued that the RQH alone could not explain the prevalence of outcrossing in nature. Instead, the RQH would only hold true in certain parameter space when species interactions induce strong selection. Because the strain of *S. marcescens* (Sm2170) used in the study by Slowinski et. al (2016) is highly virulent (~90% host mortality) and capable of imposing strong selection against host nematodes, the results of Slowinski et al. (2016) may be explained by the models of Otto and Nuismer (2016). However, it has not been empirically tested whether the RQH is applicable in systems where coevolving parasites are low-to-moderately virulent.

If only highly virulent parasites are expected to maintain host outcrossing because strong selection is required for outcrossing to overcome its substantial costs relative to self-fertilization, then the RQH is limited in its account of the ubiquity of outcrossing, especially since not all parasites and pathogens are highly virulent (Lenski and May 1994; Bonhoeffer et al. 1996; Carval and Ferriere 2010). Thus, in the present study, we tested the prediction that the invasion of self-fertilization into an outcrossing population was contingent on the virulence of the coevolving parasite. We closely replicated the experiment described in Slowinski et al. (2016), but expanded the bacterial treatments to four total *S. marcescens* strains that vary in virulence (Db11, Sm933, SmD1, and Sm2170). We predicted that in the control treatments, self-fertilization would rapidly invade host populations for all bacterial strains. Self-fertilization was also expected to invade host populations exposed to fixed-parasites for all bacterial strains, though we expected the invasion to lag—especially in populations exposed to the more virulent strains—since host populations would need to adapt to novel parasite environments. For the

copassaged treatment, we predicted that the negative frequency-dependent selection induced by highly virulent coevolving parasites would constrain the invasion of self-fertilization into host populations, but not those induced by low-to-moderately virulent bacteria.

Methods

Establishment of obligately outcrossing and mixed mating *C. elegans* host populations

Obligately outcrossing C. elegans

The starting population of obligately outcrossing *Caenorhabditis elegans* (CF3 INV) was established by and is further detailed in Slowinski et al. (2016) and Morran et al. (2011). In short, the obligately outcrossing *C. elegans* strain (PX386) was first generated by backcrossing the obligately outcrossing allele, fog-2 (q71), into an inbred strain of CB4856 (PX382). During three consecutive generations, four near-isogenic replicate populations of the generated PX386 were independently mutagenized at 40mM of EMS for 4 hours. Afterwards, each of the four populations was divided into either a control or a fixed-parasite treatment and underwent 30 generations of experimental evolution. In the control treatment, *C. elegans* were exposed to heat-killed *Serratia marcescens* (Sm2170) every generation, while in the fixed-parasite treatment, to the same stock population of *S. marcescens* (Sm2170). At the end of the 30 generations, one of the populations exposed to the control treatment (CF3) was chosen to be used in the current study as the obligately outcrossing host (CF3 INV).

Mixed-mating C. elegans

To generate a population of mixed-mating *C. elegans* (WT CF3 INV) that retained the genetic background of the obligately outcrossing population, the obligately outcrossing worms (CF3 INV) were mated with wild-type CB4856 worms. Offspring capable of self-fertilization were backcrossed with obligately outcrossing nematodes for five generations. The resulting population was inbred for ten generations, and then used to establish the mixed-mating population (WT CF3 INV) in the current study.

We obtained separate frozen populations of these obligately outcrossing and mixed-mating *C. elegans* from Slowinski et al. (2016), maintaining them at -80°C until their use in the current study, at which time they were grown to required numbers on plates seeded with *E. coli*.

Serratia marcescens parasite strains

We chose the parasite *S. marcescens* because it is naturally occurring and capable of killing *C. elegans* after the establishment of intestinal infection via consumption, (Kurz et al. 2003; Pradel et al. 2006). The following *S. marcescens* strains were selected because they vary in virulence: Db11, Sm933, SmD1, and Sm2170. The *S. marcescens* strain Db11 was originally isolated by H. Boman (Schulenburg and Ewbank 2004), while both Sm933 and SmD1 were obtained from Carolina Biological Supply Company (Burlington, NC)**.** The strain Sm2170 was obtained from T. Watanabe (Schulenburg and Ewbank 2004).

Serratia Selection Plates (SSPs)

The protocol for preparing *Serratia* Selection Plates (SSPs) has been described in Morran et al. (2011) and Slowinski et al. (2016), and has been modified slightly for this study. SSPs were

prepared by pouring 30 mL of autoclaved NGM Lite (US Biological, Swampscott, MA) into 10 cm Petri dishes. Petri dishes were then marked and divided laterally into thirds. The middle third of the dish remained unseeded, separating a third of the plate containing 35 µl of *E. coli* (a common, avirulent laboratory food source) from the remaining third, containing 35 µl of a *S. marcescens* parasite treatment. The area of the plate designated for the *S. marcescens* parasite treatments contained experimental bacterial strains (Db11, Sm933, SmD1, or Sm2170). Inocula were spread using sterile spreaders after bacterial solutions were first incubated for 24 hours at 28°C in test tubes of 5 mL Lysogeny broth (LB). The plate was then incubated for an additional 24 hours at 28°C to produce a thick lawn of *S. marcescens* and *E. coli* prior to nematode exposure. After incubation, 40 µl of ampicillin (400 mg/mL) was streaked down the middle section of the plate to reduce the spread of *S. marcescens* into the *E. coli* lawns.

Treatment groups

Fixed-parasite and control parasite treatments

Inocula for fixed parasite treatment plates were produced using bacterial colonies taken from ancestral stock populations of each *S. marcescens*. The inocula for the control parasite treatment plates were also generated using these ancestral stock populations, but were first heat-killed at 80°C for 6 hours following their incubation in LB.

Copassaged parasite treatment.

The ancestral stock populations of the strains of *S. marcescens* were used to inoculate the first generation of *Serratia* Selection Plates (SSPs) for copassaged parasite treatments. Thereafter, inocula were generated by first harvesting the bodies of 10-20 dead or morbid nematodes in the

current SSPs. Once harvested and picked into 1 mL of M9 buffer, nematodes were centrifuged in solution at 3000 RPM for 3 minutes. After the supernatant was discarded, nematodes were rinsed five times with 1 mL of M9 and then crushed. Bacterial cells were then streaked on NGM lite agar (100 µl) and incubated for 24 hours at 28°C. A random sample of 10-20 bacterial colonies was selected to inoculate 5 mL of LB. SSPs were then seeded and incubated, as described in "*Serratia* Selection Plates (SSPs)." Thus, the copassaged treatments pit host resistance against coevolving parasite infectivity and virulence.

Passage of host populations for experimental evolution

Approximately 900 obligately outcrossing nematodes and 100 mixed-mating nematodes were transferred in M9 buffer onto the parasite treatment side of all plates at generation 0. In order to reach their *E. coli* food source, nematodes were required to crawl through *S. marcescens*. After four days, all nematodes on the *E. coli* side of the SSPs plates were transferred off the plates. They were then washed 3 times with M9 buffer and a random sample of approximately 1000 nematodes, most of which were L4 larvae, was then transferred to the next generation of SSPs. This protocol was repeated for 24 total host generations. The virulence of coevolved parasites was measured against contemporary hosts at generations 12 and 22 using survival assays; the male frequency of each population was counted every 8 generations to determine selfing rates.

We noticed some bacterial contamination in control Sm2170 plates at generation 15, but the contaminant did not appear to have any effects on the host population. Still, preventative measures were taken to reduce the likelihood of its transfer into the following SSPs. Initially, areas of the *E. coli* side of the SSPs that did not appear to be contaminated were chunked onto the next generation of SSPs. Later, we modified the original protocol to include two additional

washes in affected populations. Because the *E. coli* appeared to outcompete the unknown contaminant, the amount of *E. coli* seeded onto the SSPs was also increased from 35 µl to 50 µl, for all control Sm2170 replicates.

Survival assays and parasite virulence

Bacterial virulence was measured using survival assays (SAs) on *Serratia* selection plates (SSP), as described in Morran et al. (2011). SAs were conducted by transferring a predetermined number of *C. elegans* in SSPs seeded with OP50 and the tested parasite. Nematode survival in OP50 and in the unseeded region was scored after 48 hours. Nematodes on the *S. marcescens* side of the plate were not counted. Mortality rates were determined by subtracting host survival rates from 1. The initial virulence of *S. marcescens* strains (Db11, Sm933, SmD1, and Sm2170) was determined separately for CF3 INV (obligately outcrossing) and WT CF3 INV (mixed-mating) *C. elegans.* SAs were also used to determine the virulence of copassaged parasites at generations 12 and 22 against contemporary hosts.

Calculating selfing rates

The protocol for determining male frequencies, outcrossing rates, and selfing rates are described in Stewart and Phillips (2002) and Slowinski et al. (2016). Briefly, the sex of a sample of 200 adult nematodes from a cross section of the *E. coli* side of the SSPs were scored at generations 8, 16, and 24 to determine male frequency. Outcrossing rates were determined by multiplying the male frequency by 2 and adjusting for additional males produced by nondisjunction of the X chromosome during meiosis; selfing rates were subsequently calculated by subtracting the outcrossing rate from 1 (Stewart and Phillips 2002). The initial selfing rates of all populations

were determined to be 0.10 due to the starting frequencies of the transferred mixed-mating and obligately outcrossing worms.

Measuring mixed-mating allele frequencies

Allele frequency was measured after passage 24 of three replicate populations from every experimental treatment group, as well as an additional replicate population for fixed SmD1 and fixed Sm2170. From each population, twenty-seven nematodes were randomly selected. PCR was performed and fog-2 genotypes were scored to determine the frequency of the mixed-mating allele. The protocol for measuring *fog-2* genotype frequencies is further described in Slowinski et al. (2011).

Resampling of the Db11 populations yielded inconsistent genotypic frequencies. Thus, the Db11 populations were removed from our analyses of the frequency of the obligate-outcrossing allele.

Statistical methods

The initial virulence of the parasite strains, as determined by average host mortality rates, was analyzed using Wilcoxon / Kruskal-Wallis tests because of the violation of the assumption of normality. To compare the mortality rates of obligate outcrossing and mixed-mating hosts, Wilcoxon / Kruskal-Wallis tests were again used. We also used an ANOVA to test whether the virulence of the bacterial parasites (as measured by host mortality) differed significantly at generation 22; we used Tukey post hoc tests for all pairwise treatment comparisons. Wilcoxon / Kruskal-Wallis tests were again used to compare the initial virulence of copassaged *S. marcescens* strains to their virulence at generation 22.

An ANOVA was used to test whether the generation 8 and generation 24 mean selfing rates of replicate populations differed by treatment, parasite, and treatment by parasite. The mean selfing rates violated the assumption of homogeneity of variance, so the data was transformed using the arcsine square root transformation. We used Tukey post hoc tests on all pairwise treatment comparisons, except in the analysis of treatment by parasite effects of the fixed parasite treatment at generation 8, in which a least squared mean contrast test was performed. A least squared mean contrast test was also performed to compare fixed SmD1 and fixed Sm2170 selfing rates to those of control SmD1 and control Sm2170 treatments.

We used a binomial logistic regression to test whether the frequency of individuals with the mixed-mating genotypes at generation 24 was associated with treatment, parasite, and/or treatment by parasite effects.

Results

Parasite virulence over time

The *S. marcescens* strains Db11, Sm933, SmD1, and Sm2170 exhibit varying degrees of virulence for both obligately outcrossing and mixed mating host populations (Fig. 1; Wilcoxon / Kruskal-Wallis tests; obligately outcrossing: $\chi^2_3 = 25.56$, $P < 0.0001$; mixed-mating: $\chi^2_3 = 26.13$, $P < 0.0001$) and are listed in order of increasing virulence, as determined by average host mortality rates at generation 0. The parasites Db11 and Sm933 exhibited low to moderate levels of virulence, relative to SmD1 and Sm2170 (Fig. 1). Because our experiment was set up as an

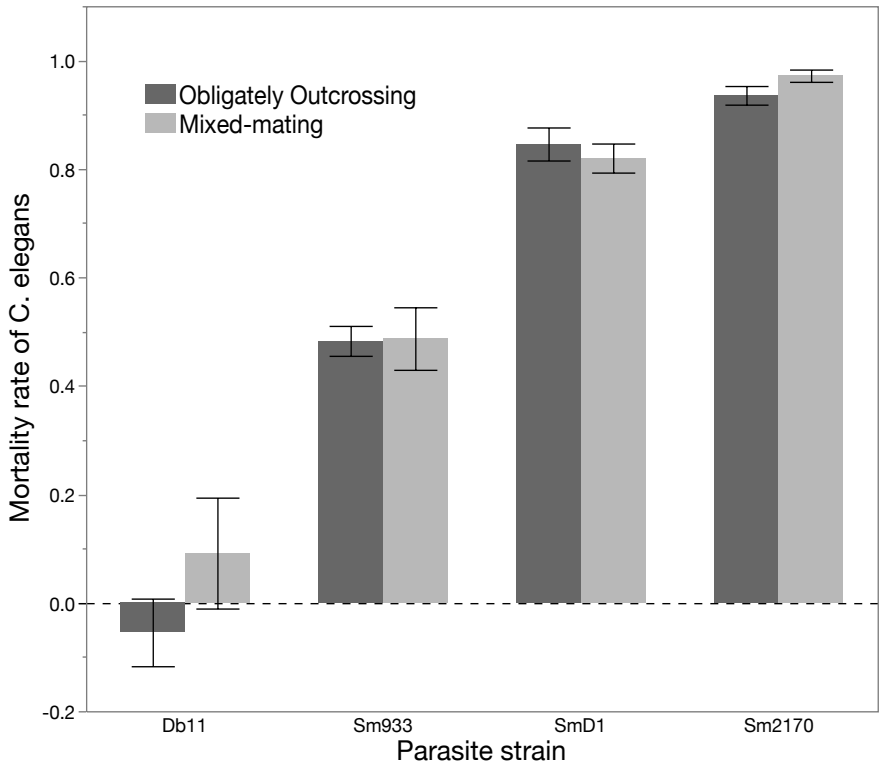

Figure 1. Average mortality rates of *C. elegans* exposed to *S. marcescens* strains at generation 0 (± one standard error). The average mortality rates of obligately outcrossing *C. elegans* are signified by dark grey bars, and those of mixed-mating *C. elegans* are signified by light grey bars. Three to ten replicate populations of approximately 175 nematodes were assayed against the various bacterial parasites. There was no difference between the average mortality rates of obligately outcrossing and mixed-mating *C. elegans* when exposed to the same parasite strain (Wilcoxon, $p < 0.05$ for each pairwise comparison).

invasion of mixed-mating individuals within an obligately outcrossing population, we then determined whether these parasites had disproportionate effects on the mixed-mating individuals relative to obligately outcrossing individuals. We found that there was no significant difference between the average host mortality rates of obligately outcrossing and mixed-mating nematodes against each parasite strain (Db11: $\chi^2_1 = 1.09$, $P = 0.30$; Sm933: $\chi^2_1 = 0.01$, $P = 0.94$; SmD1: $\chi^2_1 = 0.74$, $P = 0.39$; Sm2170: $\chi^2_1 = 1.87$, $P = 0.17$).

The virulence that parasites from the copassaged treatment imposed on their

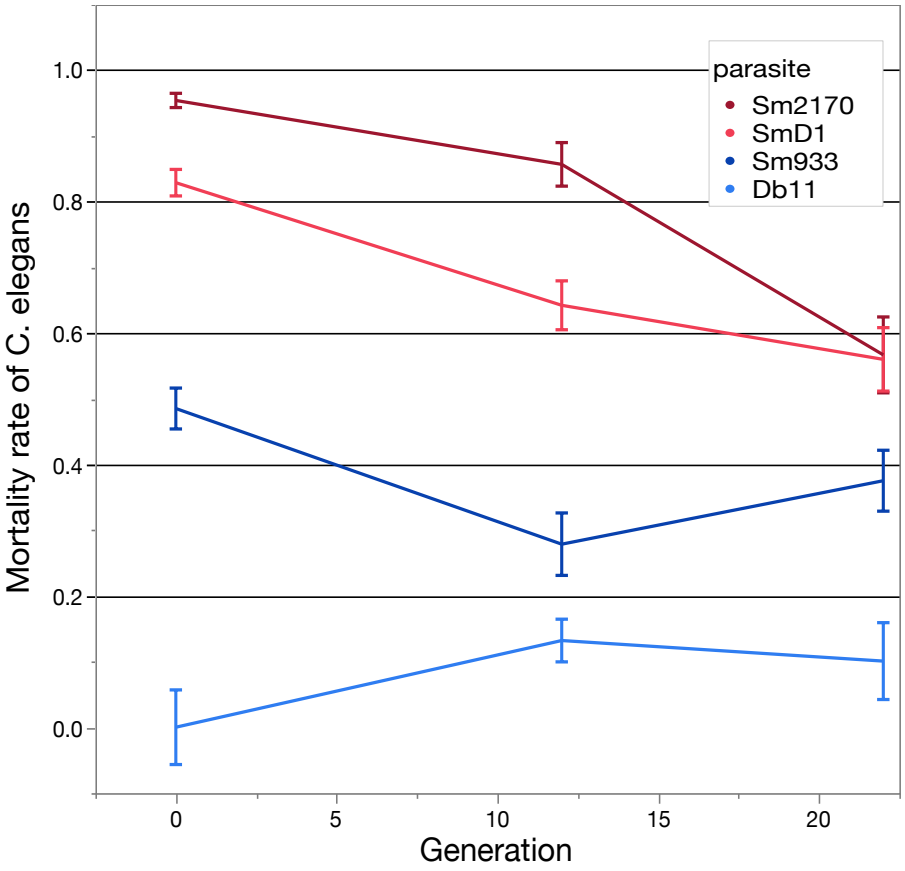

Figure 2. The virulence of copassaged parasites over 22 generations, as measured by the average mortality rates of contemporary *C. elegans* populations (± one standard error). Three replicate samples of 100-250 nematodes were assayed for each replicate copassaged population. The bacterial strains Sm2170, SmD1, Sm933, and Db11 are signified by dark red, light red, dark blue, and light blue lines, respectively.

contemporary host populations was monitored at generation 12 and 22 as a means to observe changes in overall virulence and relative levels of virulence between strains (Fig. 2). Parasite virulence deviated from their initial measurements for all strains, but the rank order of parasite mean virulence was consistent throughout the experiment (Fig. 2). For the two highly virulent strains, SmD1 and Sm2170, virulence decreased over 22 generations (Fig. 2; Wilcoxon / Kruskal-Wallis tests; SmD1: $\chi^2_2 = 18.16$, $P < 0.001$; Sm2170: $\chi^2_2 = 27.12$, $P < 0.001$); host mortality rates were more than 30% lower at generation 22 compared to generation 0 for both

strains and were not significantly different from that of Sm933, the relatively moderately virulent strain (Table 1; Tukey's HSD; $p < 0.05$). For the two low to moderately virulent strains, Db11 and Sm933, virulence did not significantly change over 22 (Fig. 2; Wilcoxon / Kruskal-Wallis tests, Db11: $Z = -0.97$, $P = 0.33$; Sm933: $Z = 1.74$, $P = 0.08$).

Table 1. ANOVA table for parasite virulence at generation 22

Source	Sum of Squares	df	Mean Square	F	P
Model	0.96	3	0.32	12.46	0.0001
Error	0.46	18	0.03		
Total	1.42	21			
Parasite	0.96	3		12.46	0.0001

Selfing rate

To measure the invasion of self-fertilization into obligately outcrossing host populations, selfing rates were calculated based on male frequencies over time. Self-fertilization was considered to have invaded into a population if there were measurable increases in the selfing rates of populations, relative to generation 0. The effects of treatment, parasite, and treatment by parasite on selfing rates at generation 8 and generation 24 were all significant (Table 2; Table 3). Self-fertilization rapidly invaded into control treatment host populations for all tested parasites; it also rapidly invaded into fixed treatment host populations exposed to Db11 and Sm933. However, the invasion of self-fertilization into fixed treatment host populations exposed to SmD1 and Sm2170 was delayed (Fig. 3). At generation 8, the selfing rates of fixed SmD1 and Sm2170 host

populations were lower than those of fixed Db11 and fixed Sm933 host populations (Table 2; LS-means contrast test, $F_{1, 42} = 21.39$; $p < 0.0001$). The selfing rates of fixed SmD1 and Sm2170 host populations were also lower than those of control SmD1 and Sm2170 host population at generation 8 (Table 2; $F_{1, 42} = 16.22$; $p = 0.0002$). Despite the fact that fixed SmD1 and fixed Sm2170 parasites were not evolving against their hosts, host outcrossing was maintained at high rates, comparable to those of host populations exposed to copassaged SmD1 and copassaged Sm2170 parasites, respectively (Table 2; Tukey's HSD; $p < 0.05$). Finally, we found that at generation 24, the selfing rates of all control and fixed parasite treatments were similar (Table 3; Tukey's HSD; $p < 0.05$). Therefore, self-fertilization invaded all control and fixed parasite treatments, but the exposure to highly virulent parasites was capable of constraining the invasion of self-fertilization into obligately outcrossing host populations.

Compared to the selfing rates of hosts of the control and fixed parasite treatment, those of the copassaged parasite treatment were significantly lower (Table 2; Tukey's HSD; $p < 0.05$). Also, while the selfing rates were low in the copassaged of the most virulent parasites, those of copassaged Sm933 host populations were significantly greater (Table 2; Tukey's HSD; $p < 0.05$). The selfing rates of copassaged SmD1 host populations were similar to those of copassaged Sm933 populations, but different from those of copassaged Db11 populations (Table. 3; Tukey's HSD; $p < 0.05$). Finally, copassaged Sm933 and copassaged Db11 host populations had similar selfing rates at generation 8 (Table 2; Tukey's HSD; $p < 0.05$).

We also found that at generation 24, host populations exposed to copassaged SmD1 and copassaged Sm2170 had significantly lower selfing rates than did those of all other parasite treatments (Table 3; Tukey's HSD; $p < 0.05$). Three of the four replicate populations of copassaged Sm2170 exhibited mean selfing rates equal to or less than the initial selfing rate of

0.10, and four of the six replicate populations of copassaged SmD1 four had selfing rates equal to or less than the initial selfing rate of 0.10. All other treatment populations had selfing rates greater than the initial selfing rate. Therefore, the invasion of self-fertilization within copassaged treatment was dependent on parasite virulence.

Table 2. ANOVA table for selfing rates at generation 8.

Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**P**
Model	1.33	11	0.12	8.67	<0.0001
Error	0.59	42	0.01		
Total	1.92	53			
Treatment	0.53	2		19.17	<0.0001
Parasite	0.48	3		11.57	< 0.0001
Treatment x Parasite	0.35	6		4.17	0.0022

Table 3. ANOVA table for selfing rates at generation 24.

Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**P**
Model	2.06	11	0.19	14.10	<0.0001
Error	0.56	42	0.01		
Total	2.61	53			
Treatment	0.96	2		36.26	<0.0001
Parasite	0.20	3		5.14	0.0041
Treatment x Parasite	0.97	6		12.16	<0.0001

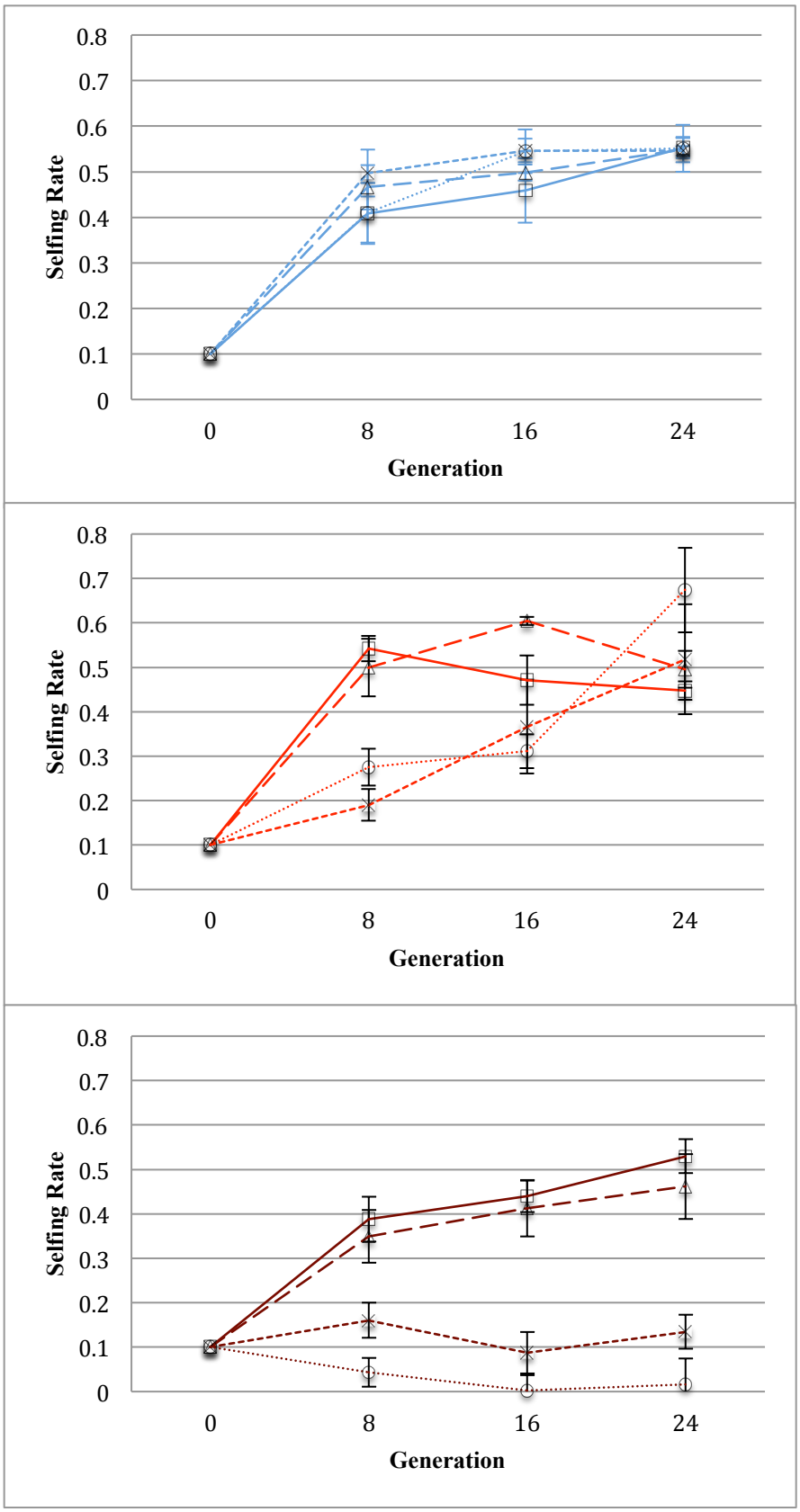

Figure 3. Mean selfing rates (± one standard error) of host populations exposed to control (top, light blue), fixed (middle, light red), and copassaged (bottom, dark red) parasite treatments, over the 24 generations. Approximately 200 worms from each replicate population were sexed to determine male frequency, which was then used to calculate selfing rates. Host populations exposed to Db11, Sm933, SmD1, or Sm2170 are indicated by solid lines with square markers, long dashed lines with triangle markers, short dashed lines with 'x' markers, or dotted lines with circle markers, respectively.

Obligate outcrossing and mixed-mating genotypic frequencies

We found that the frequency of the obligate outcrossing genotype at generation 24 was significantly associated with treatment ($\chi^2_2 = 73.40$, $p < 0.0001$) and the interaction between treatment and parasite strain ($\chi^2_4 = 96.23$, $p < 0.0001$). Compared to those in the control treatment populations, the frequencies of the obligate outcrossing genotype in the copassaged treatment populations were not significantly different ($\chi^2_1 = 3.48$, $p = 0.062$), while those of fixed treatment populations were significantly lower ($\chi^2_1 = 60.24$, $p < 0.0001$). The frequency of the obligately outcrossing genotype in the fixed treatment was also significantly lower than both the copassaged and control treatments ($\chi^2_1 = 33.54$, $p < 0.0001$). We found that the frequencies of the obligate outcrossing genotype were significantly greater in copassaged Sm2170 ($\chi^2_1 = 58.00$, $p < 0.0001$) and copassaged SmD1 ($\chi^2_1 = 4.43$, $p = 0.035$) populations, relative to control Sm2170 and control SmD1, respectively (Fig. 4). The frequencies of the obligate outcrossing genotype were significantly lower in the copassaged Sm933 populations ($\chi^2_1 = 58.00$, $p < 0.0001$) compared to control Sm933 host populations (Fig. 3). Finally, we found two of three host populations exposed to copassaged Sm2170 and one of three host population exposed to copassaged SmD1, in which the obligate outcrossing genotype was the only genotype detected in the 20+ individuals sampled each. This suggests that the mixed-mating allele was lost in these populations or existed at very low frequencies

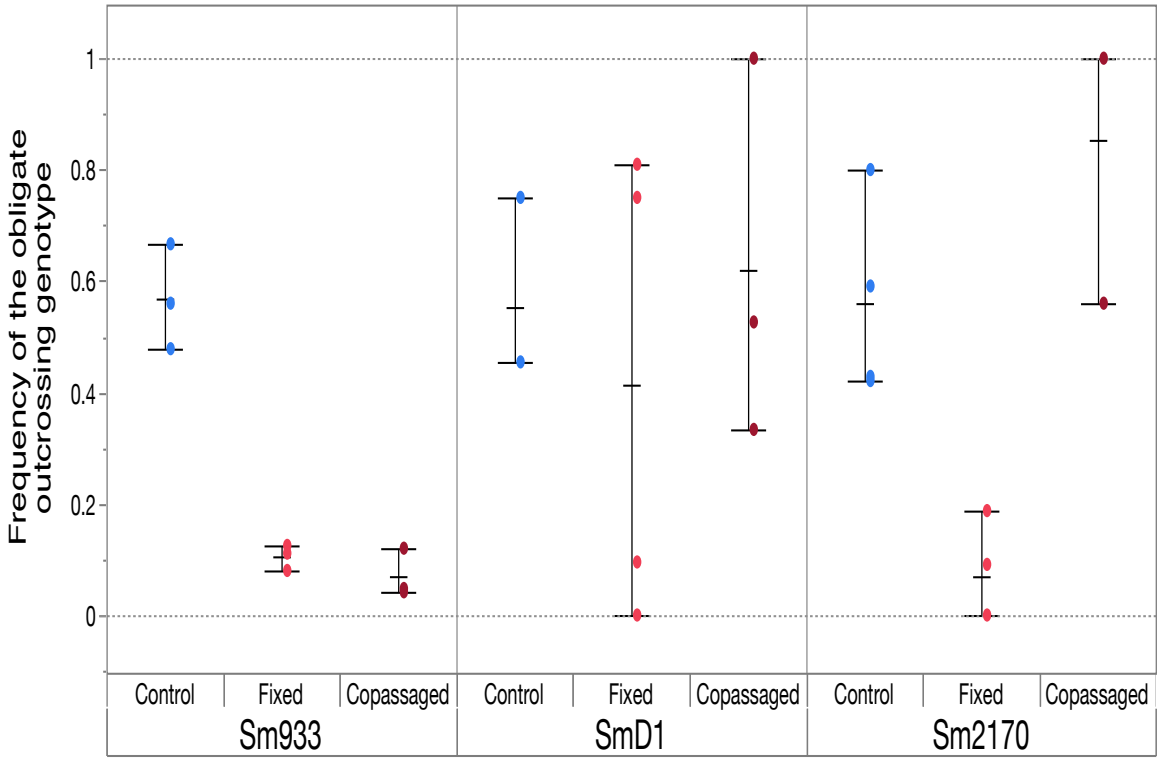

Figure 4. The genotypic frequencies of the obligate outcrossing allele of host populations at generation 24. Twenty-seven individuals from three replicate populations from every experimental treatment group, as well as an additional replicate population for fixed SmD1 and fixed Sm2170, were sampled. The average frequencies of the obligate outcrossing genotype of treatment populations are presented. PCR was performed to score *fog-2* genotypes to determine the frequency of the obligate-outcrossing allele. Db11 populations were removed from our analyses of the frequency of the obligate-outcrossing allele because resampling of the Db11 populations yielded inconsistent genotypic frequencies.

Discussion

The Red Queen hypothesis suggests that strong selection is required for coevolving parasites to maintain host outcrossing. Therefore, we tested the prediction that only highly virulent coevolving parasites could constrain the spread of self-fertilization (i.e. a measurable increase in the selfing rates of populations, relative to generation 0) within outcrossing host populations. As predicted, self-fertilization was able to invade *C. elegans* host populations in all control and fixed

parasite treatments, but not in all copassaged parasite treatments, wherein coevolution was possible (Fig. 3). The invasion of self-fertilization was only stopped in host populations exposed to the copassaged parasite treatment in such cases where the copassaged parasite was highly virulent. At generation 24, three of four copassaged Sm2170 and four of six replicate populations of copassaged SmD1 had selfing rates that were equal to or less than the initial selfing rate of 0.10. The selfing rates of all other populations at generation 24, however, were greater than the initial selfing rate. Indeed, the selfing rates of populations exposed to the highly virulent copassaged SmD1 and copassaged Sm2170 were significantly lower than those of all other treatment groups. This suggests that highly virulent, coevolving parasites, such as copassaged SmD1 and copassaged Sm2170, may be required to impede the invasion of self-fertilization and maintain obligate outcrossing. Yet, we found that over the course of experimental evolution, the virulence of both copassaged SmD1 and Sm2170 decreased, while those of the low to moderately virulent copassaged parasites stayed relatively constant (Fig. 2).

Overall, our results were consistent with the findings of Slowinski et al (2016). That is, coevolution with parasites was necessary to stop the invasion of self-fertilization. We also found that the invasion of self-fertilization was linked to an increase in the frequency of the mixed-mating allele over time (Fig. 4). Populations in which the invasion of self-fertilization was stopped, such as those exposed to the highly virulent copassaged strains SmD1 and Sm2170, had higher frequencies of the obligate outcrossing genotype than did the less virulent strain Sm933. We also measured very low frequencies of the obligate outcrossing genotype in the fixed parasite treatments. As Slowinski et al. (2016) had suggested, we believe that the initial selection for host outcrossing after exposure to a novel parasite environment was reversed following host adaptation to the non-evolving parasite. Once resistant host genotypes became common, selfing

could have, instead, become favored over outcrossing since outcrossing has the potential to break up of resistant gene combinations (Charlesworth and Charlesworth 1987).

However, our results were also able to add to the findings of Slowinski et. al (2016). Because we sampled replicate populations, we could examine the genotypic frequencies of individual populations. We found that even in the highly virulent copassaged parasite treatments, only two of three copassaged Sm2170 and one of three copassaged SmD1 host populations contained just the obligately outcrossing allele, suggesting that the mixed-mating allele was lost in these populations or existed at very low frequencies. In other words, the invasion of the mixed mating allele into obligately outcrossing host populations was permitted to some degree in replicate populations containing highly virulent copassaged parasites. This could be, perhaps, because the selection for outcrossing occurred after an early invasion by the mixed-mating allele. Since mixed-mating individuals can still reproduce via outcrossing, the mixed-mating allele could have easily propagated within the host populations before parasites adapted to common host genotypes. This is evidenced, in part, by the fact that high rates of outcrossing were maintained even in replicate populations with high frequencies of the mixed-mating genotype.

The finding that high rates of outcrossing were maintained in copassaged SmD1 and copassaged Sm2170 host populations is also interesting in that it suggests an explanation for the decrease in the virulence of copassaged SmD1 and copassaged Sm2170 against contemporary host populations. Since the selfing rates of the replicate populations were maintained at very low levels (Fig. 3), it is unlikely that an early loss of the mixed mating allele is a sufficient explanation. Rather, the loss in virulence may be attributed to parasite-mediated negative frequency-dependent selection.

If common genotypes were over-infected and rarer host genotypes were under-infected,

then individuals with these rare genotypes would have incurred lower fitness costs relative to the total population. As the number of individuals with genotypes under-infected by parasites increased, the frequencies of these formerly rare genotypes would have also increased. Until parasites capable of infecting the new common host genotype, arose or increased in numbers, mean mortality rate would have been observed as decreasing over time. In other words, host specificity, or differential rates of infection based on the host genotype, is likely the cause of the decrease in parasite virulence.

We, therefore, suspect that mean virulence may be a poor measure of determining a parasite population's propensity to maintain outcrossing. Instead, the key measure of virulence may be parasite virulence against susceptible hosts. While copassaged SmD1 and Sm2170 parasite populations exhibited declines in mean virulence over time, they may have still exhibited very high levels of virulence against the originally common genotypes. Under this scenario, even seemingly moderately virulent parasites could favor the maintenance of outcrossing. Although these findings are consistent with our current data as well as the predictions of the RQH, we cannot definitively show over only 24 host generations that negative-frequency dependent selection is the underlying cause for the observed decrease in parasite virulence.

The rapid invasion and spread of self-fertilization in control populations behaved according to theoretical models of the costs of biparental sex (Fisher 1941, Smith 1978, Charlesworth 1980; Agrawal and Lively 2001). Although obligately outcrossing *C. elegans* did not incur the full two-fold cost of males, relative to mixed-mating individuals, who could also reproduce via outcrossing, the obligately outcrossing population was invaded by mixed-mating individuals in control and fixed parasite conditions. Additionally, the delay in the invasion of

self-fertilization within obligately outcrossing host populations in the fixed parasite treatment was consistent with previous work with mixed-mating *C. elegans* demonstrating the temporary increase in host outcrossing rates following exposure to non-evolving *S. marcescens* (Slowinski 2016). Taken altogether, these results support the RQH and the capacity for coevolutionary interactions between hosts and parasites to maintain host outcrossing.

Despite the fact that our results generally support the Red Queen hypothesis, our results also support the prediction that the maintenance of obligate outcrossing via Red Queen dynamics alone is potentially limited to species interactions characterized by strong selection. We found that obligately outcrossing populations exposed to low-moderately virulent coevolving parasites were susceptible to invasion by self-fertilization. Therefore the Red Queen hypothesis may only be applicable in limited portions of parameter space, wherein virulent parasites impose strong selection on host populations. This is potentially limiting for the Red Queen hypothesis as a general explanation the widespread maintenance of outcrossing because highly virulent parasites appear to be relatively rare in nature. Additionally, selection imposed by highly virulent parasites may not always be sufficient to stop invasion by self-fertilization. Even in host populations exposed to highly virulent parasites, the invasion of the mixed mating allele was permitted in some cases (Fig. 4). While highly virulent parasites may be necessary to halt the invasion of mixed mating into obligately outcrossing host populations, they are not guaranteed to stop invasion completely.

The Red Queen hypothesis alone may not be a broad explanation for the maintenance of outcrossing. However, that does not mean that parasite-mediated selection and Red Queen dynamics are not a critical part of the broad explanation. Indeed, other factors like inbreeding depression or Hill-Robertson interference may impose additional selection favoring the

maintenance of outcrossing (Hartfield and Keighley 2012) and act in synergy with parasite-mediated selection and negative frequency-dependent selection (Hodgson and Otto 2012). As predicted by the pluralistic hypothesis, which invokes selection imposed by deleterious mutations acting in concert with Red Queen dynamics as a broad explanation for outcrossing, parasite-mediated selection may frequently act synergistically with other selective forces to maintain outcrossing (West et al. 1999).

References

Agrawal, A.F., and Lively, C.M. (2001). Parasites and the evolution of self-fertilization. Evolution 55, 869-879.

Becks, L., and Agrawal, A.F. (2012). The Evolution of Sex Is Favoured During Adaptation to New Environments. Plos Biology 10, 11.

Bell, G. (1982). The Masterpiece of Nature: The Evolution and Genetics of Sexuality. University of California Press.

Bonhoeffer, S., Lenski, R.E., and Ebert, D. (1996). The curse of the pharaoh: The evolution of virulence in pathogens with long living propagules. Proc R Soc Lond [Biol] 263, 715-721.

Carval, D., and Ferriere, R. (2010). A unified model for the coevolution of resistance, tolerance, and virulence. Evolution 64, 2988-3009.

Charlesworth, D., Charlesworth, B. (1987). Inbreeding Depression and its Evolutionary Consequences. Annu Rev Ecol Syst. 18, 237

Charlesworth, B. (1980). The cost of sex in relation to mating system. J Theo Biol. 84, 655-671.

Fisher, R.A. (1941). Average excess and average effect of a gene substitution. Annal Eugenics 11, 53–63.

Glater, E.E., Rockman, M.V., and Bargmann, C.I. (2014). Multigenic Natural Variation Underlies Caenorhabditis elegans Olfactory Preference for the Bacterial Pathogen Serratia marcescens. G3-Genes Genomes Genetics 4, 265-276.

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

Hartfield, M., and Keightley, P.D. (2012). Current Hypotheses For The Evolution Of Sex And Recombination. Integrative Zoology. 7.2, 192-209

Hodgson, E.E., and Otto S.P. (2012). The red queen coupled with directional selection favours the evolution of sex. Journal of Evolutionary Biology. 4, 797-802

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

Judson, O.P.N., B. B. . (1996). Ancient asexual scandals. Trends Ecol Evol. 11, 41–46.

King, K.C., Jokela, J., and Lively, C.M. (2011). Parasites, sex, and clonal diversity in natural snail populations. Evolution 65, 1474-1481.

Kurz, C.L., Chauvet, S., Andres, E., Aurouze, M., Vallet, I., Michel, G.P.F., Uh, M., Celli, J., Filloux, A., de Bentzmann, S., et al. (2003). Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening. Embo Journal 22, 1451-1460.

Lenski, R.E., and May, R.M. (1994). The evolution of virulence in parasites and pathogens - reconciliation between 2 competing hypotheses. J Theo Biol. 169, 253-265.

Lively, C.M. (1987). Evidence from a new-zealand snail for the maintenance of sex by parasitism. Nature 328, 519-521.

Mallo, G.V., Kurz, C.L., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y., and Ewbank, J.J. (2002). Inducible Antibacterial Defense System in *C. elegans* Current Biology 12, 1209-1214.

Morran, L.T., Schmidt, O.G., Gelarden, I.A., Parrish, R.C., and Lively, C.M. (2011). Running with the Red Queen: Host-Parasite Coevolution Selects for Biparental Sex. Science 333, 216-218.

Otto, S.P., and Nuismer, S.L. (2004). Species interactions and the evolution of sex. Science 304, 1018-1020.
Pradel, E., Zhang, Y., Pujol, N., Matsuyama, T., Bargmann, C.I., and Ewbank, J.J. (2007). Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by Caenorhabditis elegans. Proc Nat Acad Sci USA 104, 2295-2300.
Schulenburg, H., Ewbank, J.J. (2004). Diversity and specificity in the interaction between *Caenorhabditis elegans* and the pathogen Serratia marcescens. BMC Evol Biol. 4, 49
Slowinski, S.P., Morran, L.T., Parrish, R.C., Cui, E.R., Bhattacharya, A., Lively, C.M., and Phillips, P.C. (2016). Coevolutionary interactions with parasites constrain the spread of self-fertilization into outcrossing host populations. Evolution 70, 2632-2639.
Stewart, A.D., and Phillips, P.C. (2002). Selection and maintenance of androdioecy in Caenorhabditis elegans. Genetics 160, 975-982.
Vergara, D., Jokela, J., and Lively, C.M. (2014). Infection Dynamics in Coexisting Sexual and Asexual Host Populations: Support for the Red Queen Hypothesis. Am Nat. 184, S22-S30.
West, Lively, and Read. (1999). A Pluralist Approach To Sex And Recombination. Evol Biol. 12.6, 1003-1012.
Williams, G.C. (1975). Sex and Evolution. Princeton University Press.
Smith, J.M. (1978). The Evolution of Sex. Cambridge University Press.