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E-Article

Disease Epidemiology in Arthropods Is Altered by the Presence of Nonprotective Symbionts

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abstract: Inherited microbial symbionts can modulate host susceptibility to natural enemy attack. A wider range of symbionts influence host population demography without altering individual susceptibility, and it has been suggested that these may modify host disease risk through altering the rate of exposure to natural enemies. We present the first test of this thesis, specifically testing whether male-killing symbionts alter the epidemiology of a sexually transmitted infection (STI) carried by its host. STIs are typically expected to show female-biased epidemics, and we first present a simple model which indicates that male-biased STI epidemics may occur where symbionts create female-biased population sex ratios. We then examined the dynamics of a STI in the ladybird beetle *Adalia bipunctata*, which is also host to a male-killing bacterium. We present evidence that male-biased epidemics of the STI are observed in natural populations when the male-killer is common. Laboratory experiments did not support a role for differential susceptibility of male and female hosts to the STI, nor a protective role for the symbiont, in creating this bias. We conclude that the range of symbionts likely to alter parasite epidemiology will be much wider than previously envisaged, because it will additionally include those that impact host demography alone.

Keywords: epidemiology, symbiosis, sexually transmitted infection, *Spiroplasma*, demography.

* Author contributions: J. Ryder ran the field studies on which this study was based, with off-site advice from G. D. D. Hurst. Field observations were made by Ryder, M.-J. Hoare, M. Bottery, and D. Pastok. Statistical analysis of field data was designed by R. J. Knell and conducted by Ryder. Polymerase chain reaction assays for infection presence were conducted by Pastok, under the supervision of Hurst, and measures of parasite transmission, virulence, and latent period were made by Pastok, Hurst, and D. Atkinson and analyzed by Hurst. The model was constructed by A. Fenton, Hurst, and Ryder. The concept for the project derived from Hurst, who led the writing of the article. The article was written by Hurst, Atkinson, Knell, Fenton, M. Boots, and Ryder, and the project was funded by a grant to Hurst, Atkinson, Knell, and Boots.

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Introduction

We have gained tremendous insight into the epidemiology of infectious disease by studying binary interactions between a single host and a single parasite, both theoretically and empirically (Hudson et al. 1998; Lively and Dybdahl 2000; Grenfell et al. 2001; Rigaud et al. 2010). However, in cases where infection of a host individual with one parasite modifies susceptibility to another, the epidemiology and disease risk deriving from one parasite cannot be understood in isolation from that of the others circulating in that host species (Holt and Dobson 2005). Indeed, field studies have recently demonstrated the importance of "community context" in understanding disease epidemiology (Abu-Raddad et al. 2006; Ezenwa et al. 2010; Telfer et al. 2010).

Many species of arthropods carry inherited symbionts—bacteria, viruses, and protists that pass from parent (commonly the female parent) to their offspring. Recent work in arthropods has indicated that inherited symbionts are commonly an important determinant of susceptibility to parasites and pathogens. Some symbionts protect their hosts from attack by natural enemies (Haine 2008), while others have the opposite effect and increase susceptibility (Fytrou et al. 2006; Graham et al. 2012). The range of parasites and pathogens affected by symbiont infection is broad and includes parasitic wasps, nematodes, fungi, and viruses (Oliver et al. 2003; Scarborough et al. 2005; Hedges et al. 2008; Teixeira et al. 2008; Jaenike et al. 2010; Xie et al. 2010; Graham et al. 2012), with effects observed in a range of insect taxa. The high frequency with which insect species are infected with symbionts (Duron et al. 2008; Hilgenboecker et al. 2008) and the apparently quite widespread effects on susceptibility (Osborne et al. 2009) produce a compelling argument that the community context for arthropod disease epidemiology requires incorporation of

symbiont effects (Fenton et al. 2011; Jaenike and Brekke 2011).

The interaction between heritable symbionts and disease has to date concentrated on these strong individual impacts of symbionts on susceptibility to infection. However, the impact of disease within a population is a combination of individual susceptibility and rates of disease transmission. Opportunities for disease transmission are commonly related to host demography, such that the demographic impact of one agent can then determine transmission opportunities of others circulating in that host species (Dobson 1985; Holt and Dobson 2005; Fenton 2008). Likewise, any symbiont that affects host demography may also impact on the dynamics of other parasites/pathogens through affecting transmission opportunities. Many heritable symbionts have strong demographic effects, for instance, causing cytoplasmic incompatibility, shortening life span, producing sex ratio distortion, or altering host dispersal (Min and Benzer 1997; Engelstadter and Hurst 2009; Goodacre et al. 2009). Inherited symbionts that modify host sex ratio may modify mating systems and alter the dynamics of sexually transmitted infections (STIs) circulating in their host (Hurst et al. 1997), those that change host density (Dobson et al. 2002) may alter the transmission of parasites that have density-dependent transmission, those that alter age structure may change transmission of vector borne disease (Rasgon et al. 2003), and ones that affect dispersal (Goodacre et al. 2009) may alter the spatial spread of disease, all purely through demographic impacts on the host.

The disease burden imposed upon arthropods by natural enemies is thus very likely to be modified through both the protective effects of symbionts and the wider range of symbionts that alter host demography. However, there are few or no data that link symbiont infection to disease epidemiology in the field (Jaenike and Brekke 2011). We investigated the impact on disease dynamics of an inherited symbiont that is known to alter host demography through killing male embryos: a male-killing bacterium. Male-killing bacteria are found widely in insects (Engelstadter and Hurst 2009) and can reach sufficiently high prevalence that they produce highly female-biased host population sex ratios that are associated with modification of their host's mating system (Jiggins et al. 2000; Charlat et al. 2007). This ability to modify host mating system makes them good candidates for altering the dynamics of STIs carried by the host species. STIs are widespread in vertebrate and invertebrate hosts and are themselves likely to be an important influence in the ecology and evolution of many species (Lockhart et al. 1996; Knell and Webberley 2004).

Previous work on the interaction between mating systems and STI dynamics has emphasized impact of variance in mating rate in determining disease dynamics (Thrall et al. 1997, 2000). Sex differences in the variance in mating rate are common and are associated with intrasexual competition between males. Where males have a higher variance in mating rate than females, models predict the STI has a slower epidemic on male hosts than on female hosts (Thrall et al. 2000). This effect derives from very sexually active males acting as super-spreaders. Females will most commonly mate with promiscuous males, who by virtue of their previous contacts have increased likelihood of carrying the STI. Thus, female hosts have high per-mating exposure to infectious partners. The "majority" of less active males, in contrast, have low mating rate and low exposure, slowing the overall exposure of males to STIs. The pattern of sexual selection modelled is quite widespread. It is also notable that "female-first" pattern of STI spread is observed in some insect-STI systems, notably the sexually transmitted mite *Parobia* on the eucalypt beetle *Chrysopharta* (Seeman and Nahrung 2004).

The models that predict that females are infected first during STI epidemics assume that males and females are equally common and thus have identical mean mating rates. The presence of strongly biased population sex ratio, as commonly created by sex ratio distorting symbionts, is likely to alter the pattern of epidemic spread, as the bias will both increase the fraction of males that mate commonly and the mean frequency with which males mate. As noted verbally, symbionts that distort the sex ratio have the capacity to produce male-biased epidemics of STIs (Hurst et al. 1997).

We examined the impact of male-killer presence on STI epidemiology in natural populations of the two spot ladybird beetle, *Adalia bipunctata*. The symbiont whose effects we examined was a male-killing *Spiroplasma*. This infection passes from a female ladybird to her sons and daughters and kills sons during embryogenesis. *Spiroplasma* frequency is spatially variable in *A. bipunctata*, with infection being common in Stockholm, Sweden (Zakharov and Shaikevich 2001), but rare in mainland continental Europe (Hurst et al. 1999*b*; Majerus et al. 2000).The STI that we studied, *Coccipolipus hippodamiae*, is a hematophagous mite that lives under the wing cases of adult *A. bipunctata* (Hurst et al. 1995). The adult mite is sessile but lays 1–3 eggs per day, which hatch into motile larvae that transmit between partners during host copulation and then metamorphose on the new host individual. *Coccipolipus hippodamiae* infection imposes fertility, fecundity, and mortality costs but does not affect host mating rate (Hurst et al. 1995; Webberley and Hurst 2002; Webberley et al. 2002, 2004; Ryder et al. 2007). The importance of sexual (as opposed to classically infectious) transmission observed in the laboratory (Hurst et al. 1995) is reflected in both the epidemic spread of the mite in the field that

occurs immediately following the onset of host mating activity (Ryder et al. 2013*b*) and correlations between mating rate and disease prevalence (Webberley et al. 2004). Further, models that only include transmission during mating produce a good fit to the dynamics of this mite in natural populations, making it a canonical STI (Webberley et al. 2006*b*).

We first model epidemic spread of the STI through *Adalia* populations under different conditions of male-killer prevalence, as reflected in the biased population sex ratio they create. We then examine whether the pattern predicted—male rather than female-biased epidemics—is observed in nature. Finally, we examine whether other aspects of symbiont biology, such as modification of susceptibility to infection, could explain the observed pattern. The model predicts that the male-killing action of the symbiont is expected to create male rather than female-biased epidemics. We noted that this pattern is observed repeatably in the Stockholm population of *A. bipunctata*, which carries the male-killer at high prevalence, but not in serial observations of a Polish population where the male-killer is absent. We further find no evidence for symbiont-modification of host susceptibility in the population where male-biased epidemics are observed and thus conclude that male-killer impacts on population sex ratio represent the best explanation for the male-biased epidemics observed.

Material and Methods

Modeling the Impact of Male-Killers on STI Epidemiology

STI epidemiology during spring/summer is simplified in this system by the lack of recovery from infection in natural populations (once infected, the individual remains infectious until it dies; see above) and the time-lagged birth of susceptible adult hosts. This gives rise to the epidemic spread from low prevalence (commonly <10%) to high (often all individuals infected) within the overwintered cohort.

We aimed to quantify the degree of sex-biased prevalence expected during epidemic spread for different male-killer prevalence values. To this end, we modified an existing simple deterministic model of mite spread through the spring/summer cohort of *Adalia* (Webberley et al. 2006*a*) to permit the effect of sex ratio bias on epidemic spread to be investigated. We iterated this model for different prevalence values of the male-killer in R and tested the sensitivity of our conclusions to variation in other epidemiological parameters.

The model comprises a series of coupled differential equations describing the within-generation dynamics of uninfected (U), exposed but uninfectious (E) and infectious (I) male (M) and female (F) classes:

$$\frac{dF_U}{dt} = -F_U\left(a + \mu_F \times \nu \times \frac{M_I}{M_N}\right),$$

$$\frac{dF_E}{dt} = F_U \times \mu_F \times \nu \times \frac{M_I}{M_N} - F_E(\sigma + a + b),$$

$$\frac{dF_I}{dt} = F_E \times \sigma - F_I(a + b),$$

$$\frac{dM_U}{dt} = -M_U\left(a + \mu_F \times \frac{F_N}{M_N} \times \nu \times \frac{F_I}{F_N}\right),$$

$$\frac{dM_E}{dt} = M_U \times \mu_F \times \frac{F_N}{M_N} \times \nu \times \frac{M_I}{M_N} - F_E(\sigma + a + b),$$

$$\frac{dM_I}{dt} = M_E \times \sigma - M_I(a + b).$$

The model is broadly equivalent to that of Webberley et al. (2006*a*), but without staged aged classes, and with the addition of sex ratio bias. Here M_N and F_N are the total number of males and females. Females mate at rate μ_F with males, and a proportion M_I/M_N of male partners are infectious for the STI, resulting in transmission with probability ν. The equivalent male mating rate is adjusted by the female-to-male sex ratio, F_N/M_N (i.e., if there are twice as many females as males, then overall male mating rate will be twice that of females). Once infected, individuals pass through an exposed class which they exit at rate σ, which produces a mean latent period (I_P) for the disease of $1/\sigma$. All stages are subject to background mortality at rate a, and the infected stages (the E and I stages) are potentially subject to disease-induced mortality at rate b.

We established an initial population size $N = 1{,}500$, and the impact of male-killers was assessed by altering the sex ratio in this initial population, such that $M_N(0) = 1{,}500/(2 - p_{MK})$ and $F_N(0) = 1{,}500 \times (1 - p_{MK})/(2 - p_{MK})$, where p_{MK} is the prevalence of the male-killer in female hosts. We also compared our model behavior to one with staged age classes as presented by Webberley et al. (2006*a*), and found it performed very similarly in terms of predicting the degree of sex-biased prevalence during epidemic spread but accelerated the epidemic (data not shown). Because our interest is in the degree of sex-biased prevalence attained, we retained the more simple formulation for clarity.

Our model assumes that (*a*) total population size was not affected by male-killing activity, (*b*) females had a fixed mating rate that was independent of the sex ratio, (*c*) male-killing was complete and male-killer frequency was unaffected by the STI, and (*d*) males and females had the same variance in mating rate in populations with a 1 : 1 sex ratio. Lack of effect of male-killing on total population size is

justified when there is density-dependent larval mortality, a scenario very likely in ladybirds that show high levels of cannibalism in preadult phases (Majerus 1994; Schellhorn and Andow 1999). Fixed female mating rates are justified from experimental data indicating mating rate in ladybirds is limited by levels of female willingness to mate rather than male libido (Perry and Rowe 2008). This assumption will, of course, fail at very female-biased sex ratios, where females become limited by male capacity. However, we would expect the assumption to be satisfactory for male-killer prevalence below 80%, which is above the value seen in natural populations. The assumption that all male progeny of an infected female die is approximately true (see fig. S1, in supplementary material that is available online). The effect of survival of males can, if need be, conceptualized as a modest diminution of male-killer prevalence.

The model was implemented in R using the odesolve package. Parameters are described in table 1, alongside the range of values these parameters are likely to take in natural populations, derived from laboratory study and field measurement. The population was seeded with the STI at a prevalence of 10% in males and females in all cases with half of infected beetles being infectious for the STI (both typical of early spring populations). We summarized the speed and sex bias of the epidemics by extracting from the simulation the number of days before mite prevalence in male and females reached 50% and 90%. We examined the sensitivity of sex bias in mite epidemiology to varying (i) male-killer prevalence, (ii) female mating rate, (iii) disease latent period, and (iv) parasite-induced mortality.

Disease Epidemiology in Populations with and without Male-Killing Bacteria

Adalia bipunctata adults emerge from overwintering in April. Epidemic spread of the mite through the overwintered cohort follows the onset of mating activity between May and July. Infection prevalence can rise from 10% to 20% on exit from overwintering to more than 90% after 10 weeks (Webberley et al. 2006*a*). We compared the epidemiology of the mite over two spring epidemics in a region of low male-killer prevalence (Toruń, Poland) with one of high male-killer prevalence (Stockholm, Sweden).

In 2009, the epidemic in Toruń, Poland, was monitored between May 1 and July 27. Samples were taken from three different locations, which were scored and treated separately because they were heterogeneous in mating rate and thus the mite epidemics. These were city-center lime trees (53°01′19.95″N, 18°57′ 75.38″E), suburban lime trees (53°018′ 39.8″N, 18°56′ 96.41″E), and suburban rose bushes (53°01′90.56″N, 18°57′45.55″E; grid references refer to approximate center of sampling area). These data were augmented with previously published epidemic data from Poland in 1999 (Webberley et al. 2006*a*).

In 2010, the epidemic in Stockholm, Sweden, was monitored between May 17 and August 15. Beetles were taken from Stockholm suburbs, north (Kista: 59°24′14.17″N, 17°56′32.38″E and Akalla: 59°24′52.48″N, 17°54′55.31″E), and from Stockholm, center (Strandvägen, Östermalm: 59°19′53.76″N, 18°5'3.78″E and Ringvagen, Södermalm: 59°18′31.01″N, 18° 4'52.61″E). Suburb and city-center epidemics were different from each other, but the sampling sites within the city center and within the suburbs were not heterogeneous and were therefore pooled. Supplementary sampling was carried out at a Stockholm suburb, south (Hagerstensasen : 59°17′44.13″N, 17°58′50.16″E; data from this suburban location were treated separately from the other suburban locations, which were approximately 13 km north).

In 2011, the epidemic in Stockholm, Sweden, was monitored between May 22 and July 27. Beetles were taken

Table 1: Variables in the model of sexually transmitted infection epidemiology, with range observed in natural populations

Parameter	Description	Range in natural populations	Source
p_{MK}	Male-killer prevalence (fraction of females infected)	0%–70%	This article
σ	Rate at which individuals move from being exposed to being infectious (day^{-1})	Latent period = $1/\sigma$ = 16–45 days	Webberley et al. 2006*b*
ν	Probability of an uninfected host acquiring an infection following copulation with an infectious host	.8–1.0	Hurst et al. 1995; Webberley and Hurst 2002; Webberley et al. 2002, 2004; Ryder et al. 2007; this article
μ_F	Female mating rate (day^{-1})	0–.30	Webberley et al. 2006*b*; Haddrill et al. 2008; Ryder et al. 2013*b*
a	Uninfected mortality rate (day^{-1})	.01–.02	Complete death of overwintered cohort is observed by 90 days in field (Webberley et al. 2006*b*; this article)
b	Additional mortality rate associated with infection with parasite (day^{-1})	0–.05	Some evidence of weak mortality effect in laboratory (Ryder et al. 2007)

from a Stockholm suburb, north (Tensta: 59°23′39.72″N, 17°54′9.63″E), a Stockholm suburb, south (Hagerstensasen: 59°17′44.13″N, 17°58′50.16″E), and from Stockholm city center (Ringvagen and Södermalm). In 2011, the sites were all heterogeneous with respect to the timing of mating and of the mite epidemic (though not in prevalence of the male-killer), and so were all treated separately.

Monitoring of epidemics followed the protocol of (Webberley et al. 2006*a*). In brief, beetles were collected from the field weekly by beating *Tilia* lime trees, and by eye from *Rosa* bushes, with the aim of collecting over 100 beetles in each site sampled in each week. On collection, single individuals were confined individually in Eppendorf tubes to prevent contagion before scoring, and individuals that were mating were confined in their mating pair. On return to the laboratory, the individuals were sexed using characteristics in Randall et al. (1992), and mite infection status scored by examining the subelytral surfaces for mite presence under a binocular microscope. Individuals were partitioned into overwintering generation and new cohort on the basis of depth of elytral color (Webberley et al. 2006*a*). They were scored as either uninfected (no mites), latent infected (mites present at varying stages of development, but no motile larval mites observed), and infectious (adult mites, eggs and infectious larval mites all visible). Beetles were then returned to the point in the field from which they were collected within 24 h.

Testing the effect of host sex in each population is made more difficult because of the different trajectories of the various epidemics. To account for this we used generalized additive models (GAM; Zuur et al. 2009), which allow both a nonparametric smoother and other terms to be included in the model, and examined whether model fit for each epidemic was improved by inclusion of sex as a factor. For each epidemic, we used the gam() function in the mvcv package (Wood 2011) in R (R Core Development Team 2012) with the default option for a smoother (thin-plate spline) and binomial errors to fit models describing the relationship between the fraction of animals infected and time. We used likelihood ratio tests to compare models with and without sex included as an explanatory factor. The code for this process, alongside the data on STI frequency on male and female hosts over time, is available in the supplementary material.

Male-killer prevalence in these populations was ascertained post hoc through polymerase chain reaction (PCR) assay of field-collected female beetles. DNA was created from whole female beetles using the Promega Wizard system. Template quality was determined using a PCR assay based on the cytochrome oxidase I gene of insect mtDNA (Brunton and Hurst 1998). Templates passing this quality control were then tested for the three known male-killing bacteria resident in this species. *Spiroplasma* presence was assayed by PCR assay using primer pair HaIn1/MGSO, which amplifies a part of the 16S rRNA gene of group VI Spiroplasma as described by Hurst et al. (1999*b*). *Wolbachia* presence was assayed using primer pair 81F/691R that amplifies the *wsp* gene of Wolbachia as described by Zhou et al. (1998). *Rickettsia* presence was assayed using primer pair R1/R2 that amplify part of the 17-kDa outer membrane protein gene (see Werren et al. 1994 for protocol). Positive amplicons were sequenced for 1–5 individuals to ensure these represented strains identified as male-killers in previous studies.

Susceptibility of Male and Female Beetles to Mite Infection and Evaluation of Spiroplasma*-Mediated Protection*

Male-biased prevalence of a parasite has three possible causes. First, male and female hosts may be differentially susceptible to acquiring an infection. For a sexually transmitted infection, this would follow from a female sex ratio bias altering per capita mating rate in each sex. In our population, the 4 female : 1 male population sex ratio will give male individuals a fourfold-higher mating rate than females. Differential susceptibility to acquiring infection could also occur if a female mating with an infectious male is more likely to pick up infection than a male mating with an infectious female or if male hosts became infectious more quickly than females. Second, male and female could differentially retain infection. If female hosts were more likely to clear infection than male, male-biased prevalence would be observed. Third, the parasite might differ in virulence on male and female beetles. Female-biased virulence would create male-biased prevalence.

We examined these alternate explanations for the observed male-biased prevalence in Stockholm beetles. We additionally directly examined whether the *Spiroplasma* altered the ability of host females to acquire and retain infection (symbiont-mediated protection). We conducted experiments in two blocks; in the first block (beetles from Stockholm and Gävle), both sexes were exposed to infection through mating to an infectious partner; in the second block (beetles from Stockholm), female beetles alone were tested to produce greater power to analyze any effects of *Spiroplasma* on the persistence and acquisition of infection.

To this end, infectious '"donor" individuals were offered a "recipient" uninfected member of the opposite sex for 1 h in a clean petri dish, and the presence/absence of mating was scored. Where mating was observed, the pairs were allowed to copulate to completion, and then the two parties were sexed and separated to new clean dishes to avoid any postcopulation transmission. Subsequent to mating, the previously uninfected recipient beetle was placed in an incubator at 22°C on a 20L : 4D light cycle with reduced nighttime temperature (10°C). They were

then scored for the presence of larval mites after 24 h. Beetles found to have become infected were then fed pea aphids daily, and the progression of mite infection monitored. This involved (*a*) checking any beetles that died for the presence of viable mites and (*b*) checking surviving beetles from day 14 onward for the presence of infectious larval female mites. Infection was deemed to be successful if infectious larval mites were produced from the infected host. In block 2 (female beetles only) we also measured the intensity of infection (number of reproducing adult mites) 30 days after initial infection. The *Spiroplasma* infection status of females was obtained post hoc by PCR assay (above).

From this experiment, the following metrics were calculated: (i) transmission efficiency during copulation: the proportion of recipient individuals in the above experiments that bore larval mites 24 h postmating; (ii) recovery from infection: the proportion of recipient individuals that initially became infected but went on to lose the infection during the experiment; (iii) mortality effects on hosts: the proportion of hosts dying before becoming infectious; and (iv) latent period of infection: the interval between copulation and the recipient individual becoming infectious. Latent period, alongside transmission efficiency during copulation between infectious individuals, determines the rate of male-to-female and female-to-male onward transmission.

We first compared whether male and female hosts differed in these properties. For categorical data (transmission efficiency, recovery, mortality) this was achieved through tests of association between host sex and effect. Latent period was compared using a *t*-test. We then examined whether *Spiroplasma*-infected and *Spiroplasma*-uninfected females differed in these properties. This utilized data across two experimental blocks. For categorical data, we tested for association between *Spiroplasma* and the metric in question using a generalized linear model (GLM) with binomial errors, with *Spiroplasma* infection status and sample collection as explanatory variables. For latent period, we conducted an analysis of variance with block as a factor.

In addition, we examined whether there was evidence of *Spiroplasma*-mediated protection of female hosts in the field. Field samples were obtained during early or mid-epidemic phases from three locations, Stockholm (as before), Gävle (60°40′N, 17°10′E), and Malmö (55°35′N, 13°2′E) during June and July 2011. Females were typed for mite infection status in the field and for *Spiroplasma* infection status in the lab, and association between mite and *Spiroplasma* status then tested. We tested for an association between Spiroplasma and mite infection status using a GLM with binomial errors, with *Spiroplasma* infection status and sample collection as explanatory variables.

Results

Models of STI Epidemiology under Different Male-Killer Prevalence

We first iterated a basic model of STI epidemiology for three male-killer prevalence values (0, 50%, 75% representing a 1 : 1, 2 female : 1 male, and 4 female : 1 male population sex ratios, respectively) for a set of typical parasite and host parameters (mean latent period = 18 days; mean mating rate of female = 0.1/day; no parasite-induced mortality). Sex-biased epidemiology, with more rapid spread through male than female hosts, was observed at both of the higher male-killer prevalences (fig. 1).

We then summarized the effect of male-killer prevalence on disease dynamics by calculating the time taken to achieve 50% and 90% STI prevalence for different male-killer frequencies. The degree of sex-biased prevalence increases with increasing male-killer frequency in an accelerating fashion (fig. 2). For the male-killer prevalence observed in the field (ca. 70%; see below), males are predicted to hit 50% prevalence approximately 12 days before females and 90% prevalence 18 days before females. Notably, the model predicts that the speed of the epidemic in females is also accelerated by presence of the male-killer, as high prevalence in male hosts transfers to high exposure of females later in the epidemic. This acceleration will of course be sensitive to the assumption that female mating rate is fixed (the epidemic in females is decelerated if female mating rate declines with male scarcity).

We examined the sensitivity of male-killer driven sex-bias in STI prevalence to other parameters that affect STI epidemiology for our particular value of male-killer prevalence (70%). We examined each of these factors individually, and each of them had only a minor effect on the degree of sex bias observed. We then used this analysis to determine the least propitious natural conditions for sex-biased prevalence (high transmission efficiency, high mating rate, low parasite -induced mortality, and long latent period, all within realistic ranges of parameter values in table 1), and examined epidemiology under these conditions. Sex-biased prevalence remained strong, implying that we would expect to see sex-biased epidemics wherever male-killer prevalence is high (fig. 3).

Epidemics of Coccipolipus hippodamiae *Are Male Biased in a Population with High Male-Killer Prevalence.* We first confirmed the frequency of male-killer infection and the sex ratio in these two *Adalia bipunctata* populations. Three male-killing bacteria are known in this species, a *Spiroplasma*, a *Wolbachia*, and a *Rickettsia* (Hurst et al. 1999*a*, 1999*b*; Werren et al. 1994). PCR assay indicated that 76 of 102 female beetles collected from lime trees across Stockholm were infected with a male-killing bacterium,

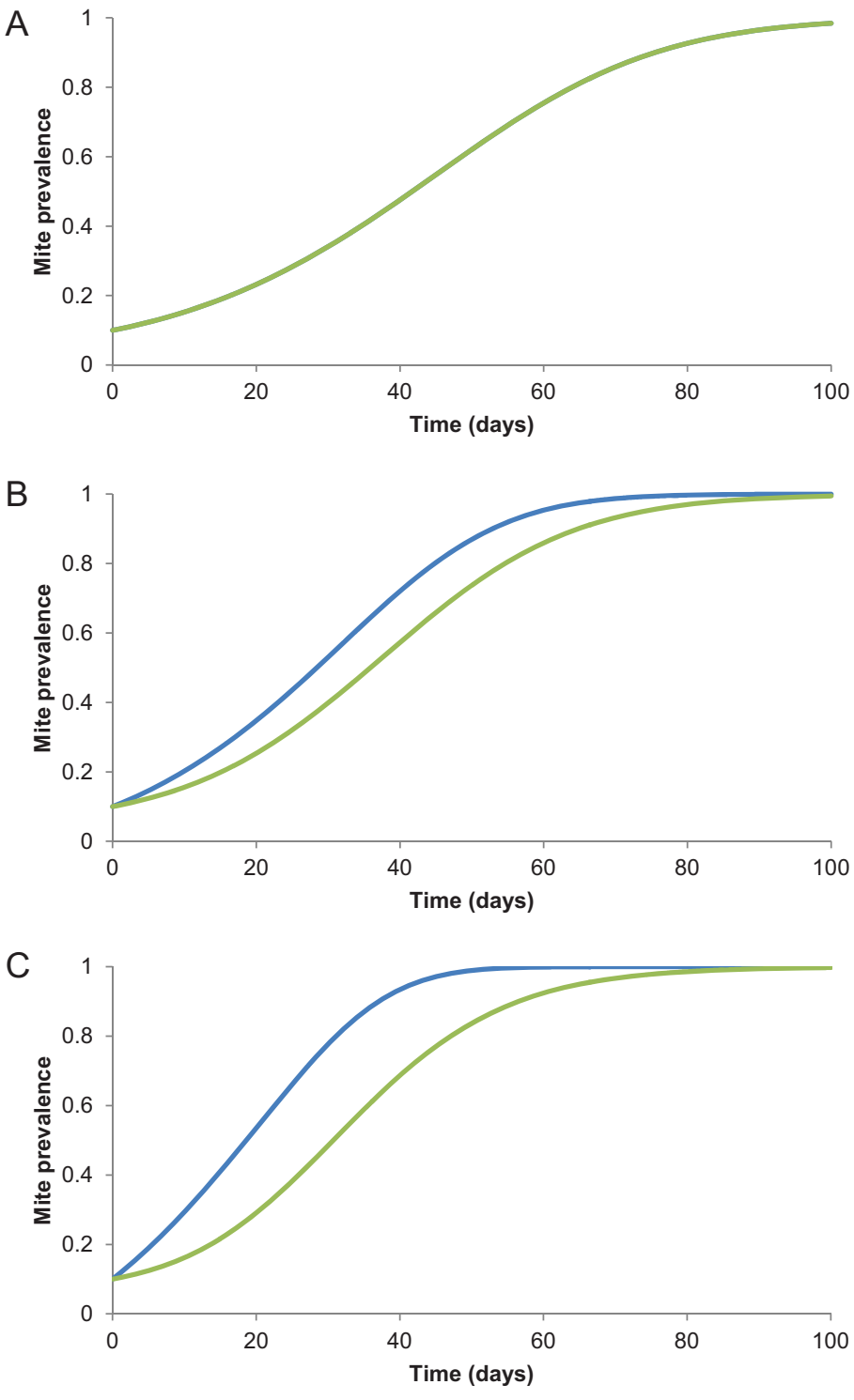

Figure 1: Simulation of epidemic spread of the sexually transmitted infection (STI) through populations with 0% (*A*), 50% (*B*), and 75% (*C*) male-killer prevalence, through male (blue) and female (green) hosts. Male and female STI prevalence over time are identical at 0% male-killer prevalence. Other parameters: $a = 0.01$; $\mu_F = 0.1$; $I_P = 18$; $\nu = 0.9$; $b = 0$. Note that at 0% male-killer prevalence, male and female epidemics are coincident.

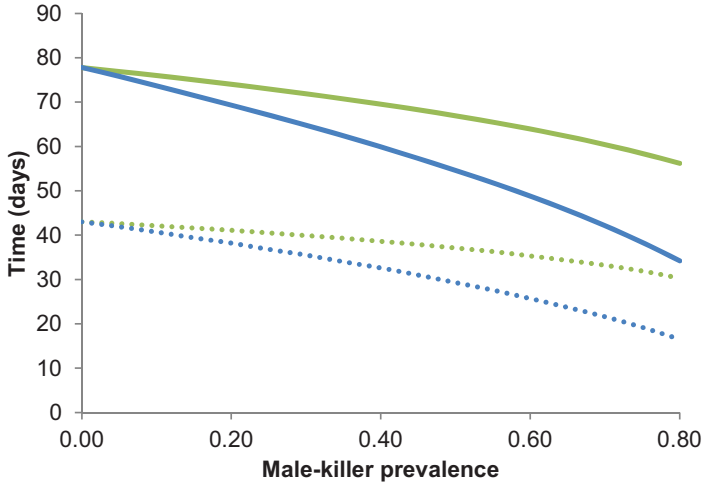

Figure 2: Effect of male-killer prevalence on time taken for sexually transmitted infection to reach 50% and 90% prevalence in male and female beetles, derived from model output. Data for time until 50% prevalence represented with dashed lines, data for time until 90% prevalence represented with solid lines, data for females represented with green, and data for males represented with blue. Other parameters: $a = 0.01$; $I_p = 18$; $\mu_F = 0.1$; $\nu = 0.9$; $b = 0$.

with no heterogeneity between sampling locations in the city (74.5% of female beetles infected; binomial confidence interval: 65%-83%). *Spiroplasma* infection represented the dominant male-killing infection, with 71 individuals carrying this infection, 5 carrying *Wolbachia*, and none carrying *Rickettsia*. Breeding data indicated that *Spiroplasma*-infected beetles gave rise to either all-female or strongly female-biased broods as previously described and that uninfected beetles produced normal sex ratios (see fig. S1). The symbiont is thus expected to produce population sex ratio bias between 71% and 80% female. In accordance with this, the population sex ratio in Sweden measured at pupation in 2010 was strongly female biased (74% female, $n = 732$), such that we can conclude the symbiont is the principle determinant of population sex ratio. In contrast, 3.6% of beetles in the Polish populations were infected with a male-killer. The sex ratio at pupation in Toruń, Poland, in 2009 was 52.5% male ($n = 150$). Thus, there was variation in the prevalence of a male-killing bacterium between the two locations, and this generated the expected demographic impact, variation in population sex ratio.

We then tested whether the mite epidemic pattern followed the prediction that high male-killer prevalence would drive a male-biased STI epidemic (Hurst et al. 1997). The epidemiology of the mite in the overwintered cohort from the high male-killer frequency/female-biased Swedish population was compared to epidemic data for the low male-killer frequency/equal sex ratio, Polish population. Epidemic spread of the mite was observed through the overwintered cohort in both locations and in each season (fig. 4). However, the two locations differed in the nature of the epidemic in male and female hosts. In the Swedish population, the rise in frequency of mite infection on male beetles occurred before the rise in female beetles (fig. 4, bottom two rows). Infection prevalence in females then lagged behind that in males until a plateau was reached approximately 7 weeks later, at which point nearly all the adult overwintered beetles had become infected. In contrast, mite epidemiology in the Polish population was not male biased in either the 1999 epidemic reported in Webberley et al. (2006a) or in our data from 2009, where we monitored three sampling locations (fig. 4, top row). Data underlying figure 4 are deposited in the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.1b2f0 (Ryder et al. 2013*a*).

We then examined the epidemics for evidence of sex bias using GAMs (see "Methods"). Models without host sex included gave substantially worse fits to the data in all six Swedish epidemics, with the highly significant differences between the fit of these models and those with sex as an explanatory factor confirming that male hosts were more commonly infected in these populations. GAM model output estimates the mite reached 50% prevalence on male hosts on average 12 days before female hosts in the Stockholm epidemics ($n = 6$, range: 5–21 days), which is consistent with model predictions (a 12-day lag). In the case of the Polish epidemics, by contrast, the differences in fit between models with and without sex included did not approach significance for any of the four epidemics (table 2), indicating that there were no appreciable differences in infection rates between male and female beetles in this population.

We also examined some data for prevalence of mite

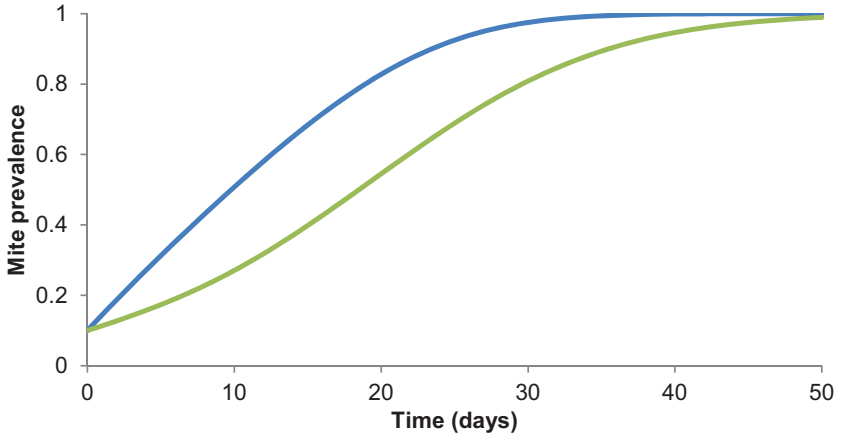

Figure 3: Epidemiology of the *Adalia* sexually transmitted infection in male and female hosts under parameter regime producing weakest sex-biased prevalence at 70% frequency of the male-killer. Blue line traces predicted spread through male hosts and green line through female hosts. Parameters: $a = 0.01$; $\mu_F = 0.3$; $v = 1.0$; $b = 0$; $I_p = 45$.

infection from early/midepidemic points in Malmö (55°35′N, 13°2′E; 500 km west-northwest of Toruń, 612 km southwest of Stockholm) and Gävle (60°40′N, 17°10′E; 175 km north of Stockholm), both populations where the male-killer is common and the population sex ratio is female biased (>40% prevalence, 2 females per male). In Malmö, 3.8 % of females and 11.0% of males were infected in June 2002 ($n = 185$ and 91, respectively; population sex ratio 2.03 females/male, $n = 276$), and 50% of females and 62% of males infected in June 2011 ($n = 126$ and 74, respectively; population sex ratio 1.7 females/male). In Gävle in June 2011, 38% of females and 44% of males were infected ($n = 138$ and 61, respectively; population sex ratio 2.26 females/male). We examined the null hypothesis of even mite prevalence between the sexes by testing for an association between host sex and mite infection status, using a GLM with binomial errors, with sex and sample collection as explanatory variables. The sex by sample interaction term was not significant and was dropped from the model. In the simplified model, sex was a significant explanatory variable for mite presence (deviance associated with factor "sex" = 8.498, df = 1, Akaike Information Criterion (AIC) = 42.17, likelihood ratio test (LRT) = 6.19, $P = .013$), as was "sample collection" (deviance associated with factor = 161, df = 2, AIC = 193, LRT = 159, $P < .001$).

Susceptibility of Male, Female, and Spiroplasma*-infected Females to Infection*

We then analyzed whether host sex prevented acquisition of mite infection, development of infection, or onward transmission. All classes of female were included together in this analysis, representing the "wild" population that was followed during epidemic spread. We observed no evidence of a male-female difference in mite acquisition, retention, and latent period for any of the four metrics (acquisition following mating, recovery from infection, latent period until infectious, mortality; table 3). Of the four metrics, only mortality showed any tendency to be affected by host sex, but the bias was in this case the reverse of that which would explain male-biased prevalence (males were more likely to die from infection, which would remove infected males from the population rather than produce their accumulation; this reflects previous work: Webberley and Hurst 2002). These data provide evidence against the obvious alternative complementary hypotheses for sex-biased prevalence, that biases are produced by differences in male and female biology following exposure to an infected partner.

We also examined evidence for symbiont-mediated protection directly, through testing whether mite infection parameters differed between *Spiroplasma*-infected and *Spiroplasma*-uninfected females. There was no evidence that *Spiroplasma* affected female acquisition of the mite following mating (*a*; df = 1, $P > .4$), retention of the mite (*b*; df = 1, $P > .2$), mortality of the host (*c*; df = 1, $P > .66$) or latent period of infection (*d*; GLM: no significant variance associated with *Spiroplasma* infection status: $F = 0.27$, df = 1, 38, $P > .85$; evidence of heterogeneity between blocks $F = 14.46$, df = 1, 38, $P > .001$). We also examined whether the number of reproducing adult mites that developed differed between *Spiroplasma* infected and uninfected females at 30 days post initial infection and found no evidence of association between *Spiroplasma* presence and intensity of infection (mean number of reproducing

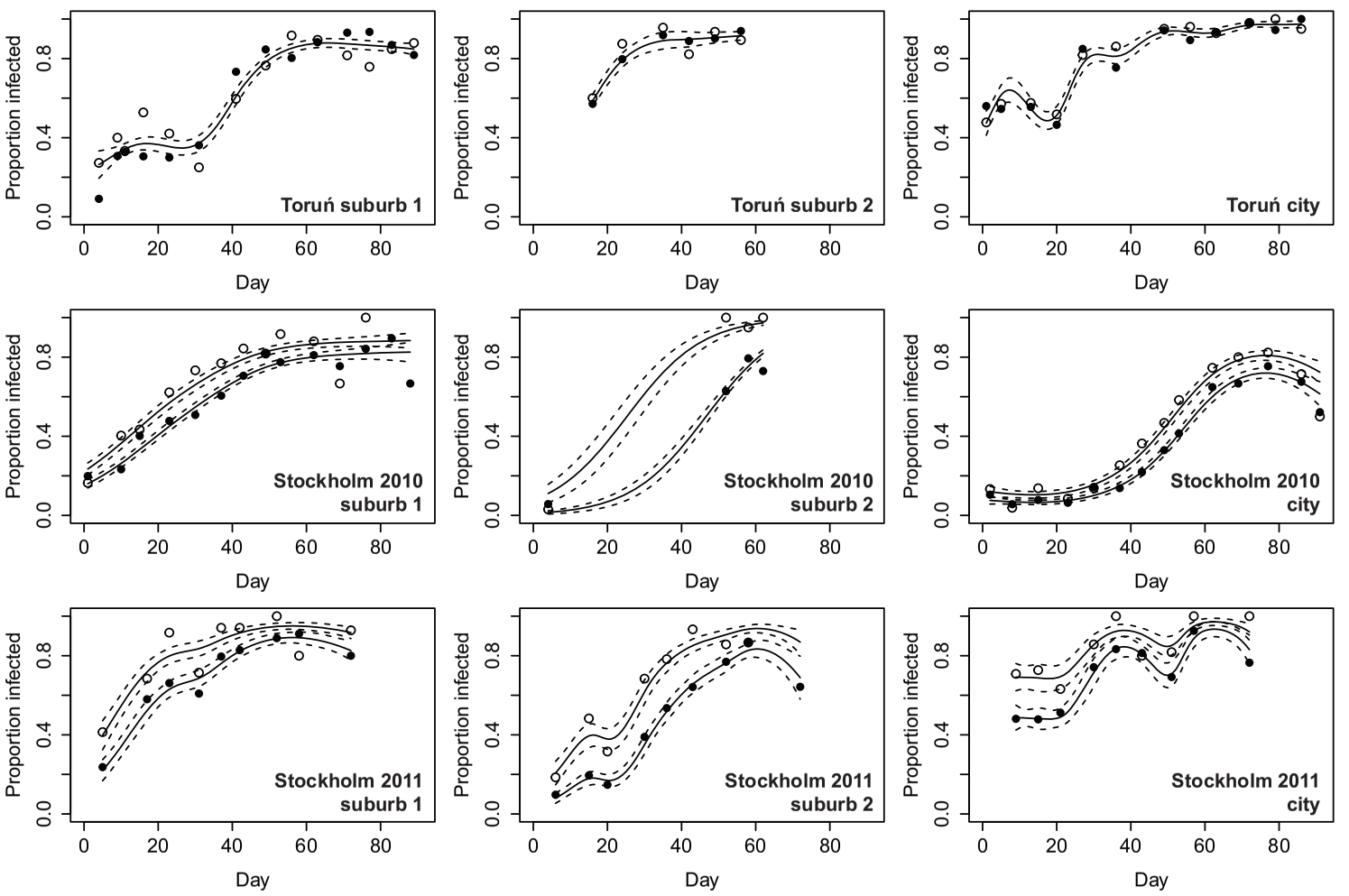

Figure 4: The prevalence of *Coccipolipus hippodamiae* on the overwintered cohort of male (open circles) and female (filled circles) *Adalia bipunctata* in Sweden (male-killing bacteria common, 2010, 2011) and Poland (male-killing bacteria rare, 2009) over the course of the spring/summer epidemic. Time is given as days since first observation, with day 0 corresponding to May 1 in all cases. Generalized additive model–fitted smoothers for the data are given (solid line), including confidence limits to the epidemics (dashed line). Smoothers are for all data in the Toruń epidemics (no sex bias of infection) and for male and female separately in Stockholm epidemics (male-biased infection).

Table 2: Effect of removing the factor "host sex" on model fit in a general additive model of factors affecting *Coccipolipus hippodamiae* presence/absence

Population	df	Change in deviance associated with removing "host sex" as factor in model	Significance (P) of model difference
2011 Stockholm suburb, north	−.98	−13.36	<.0001
2011 Stockholm suburb, south	−3.42	−34.21	<.0001
2011 Stockholm city, center	−.85	−10.49	<.001
2010 Stockholm suburb, north	−.80	−13.2	<.001
2010 Stockholm suburb, south	−1	−38.10	<.0001
2010 Stockholm city, center	−.94	−16.93	<.0001
1999 Toruń city	−.1	−.75	.39
2009 Toruń suburb, lime trees	−.98	−.09	.76
2009 Toruń suburb, roses	−1.01	−.08	.79
2009 Toruń city, lime trees	−1	−.25	.62

mites on S+ females = 7.08 ± 1.30 mean number of reproducing mites on S− females = 6.80 ± 1.30; t = 0.16; df = 21, $P > .8$).

We then examined whether mite infection status in field-collected female beetles showed an association with *Spiroplasma* infection status, as would be expected if the symbiont either protected the host against initial transmission or produced resistance against the mite leading to its death; table 4). GLM model fit was not significantly affected by deletion of the term "*Spiroplasma* infection status" (deviance associated with factor infection status = 0.95; df = 1, AIC = 32.009, LRT = 0.6329, $P = .426$), but was affected by "sample collection" (deviance associated with factor = 13.338; df = 2, AIC = 42.397, LRT = 13.02, $P = .0015$). There is thus no evidence of an association between *Spiroplasma* presence and mite infection status in field data.

Discussion

Empirical study of the impact of symbionts on other parasites has generally focussed on the effect of infection on individual host susceptibility. We present a case study testing the hypothesis that the presence of one associate of ladybirds, a male-killing bacterium, alters the epidemiology of another, an STI, through altering the demography of the host (specifically, sex ratio), and that this demographic change alters the transmission opportunities of a second parasite, a sexually transmitted infection. Because every mating couple comprises one male and one female, any population sex ratio bias associated with reproductive parasitism will necessarily be accompanied by an elevation in the mating rate of individuals of the rare sex over individuals of the common sex, and this produces differential exposure to sexually transmitted parasites. This has the capacity to alter STI dynamics from the "expectation" of female-biased epidemics of disease to a male-biased epidemic.

We first presented a simple model of this process to quantify the size of epidemiological effect expected under different male-killer prevalence conditions in our system. This model confirmed the previously made verbal concept and indicated that epidemiological impacts should be readily observable for moderate sampling effort (e.g., 100 beetles per sample point) at male-killer prevalence values above 50%. We then ran this model for the observed male-killer prevalence in Swedish populations, and it predicted a substantial (2-week) lag between the epidemic in male and female beetles.

We then assessed whether this dynamic was observed in field populations. In Sweden, male-killing *Spiroplasma* symbionts are common, such that population sex ratios are strongly female skewed, with 3–4 times as many females as males. Individual males in these populations must therefore on average mate 3–4 times as often as females. This was reflected in the STI epidemic occurring earlier on male compared to female hosts within Stockholm populations of the beetle, a pattern observed in distinct epidemics across locations in the city and in different years. The data was consistent with other "spot" measures of higher mite prevalence on male hosts (compared to female) in other Swedish populations where the male-killer was common and contrasted with the lack of host sex-bias in STI epidemics observed in Poland, where the male-killer was rare.

The data represent the first "male-biased" STI epidemic recorded and present a contrast with the biologically very similar *Parobia* mite/*Chrysopharta* beetle interaction, in which female-biased epidemics are observed (Seeman and Nahrung 2004). Our data recapitulate the expected pattern for a female-biased population. It would be premature to derive causality, as the epidemics sampled are from geographically restricted, creating the potential for other "environmental" correlating features to cause the pattern. However, the predicted symbiont common/ female-biased

Table 3: Impact of host sex and *Spiroplasma* infection status on acquisition, persistence and transmission of mite infections in Swedish *Adalia bipunctata*

		Females			
Metric, block	Males	*Spiroplasma* infected	*Spiroplasma* uninfected	Unknown	Analysis, male vs. all females
Proportion of individuals acquiring infection following copulation:					
1	.94 (54)	.941 (17)	.875 (8)	.885 (26)	Fisher, $P > .45$
2		.823 (17)	.75 (16)		
Proportion of infected individuals recovering in first 20 days:					
1	0 (34)	0 (16)	0 (7)	0 (2)	Fisher, $P = 1$
2		0 (16)	.071 (14)		
Latent period of infection (days, ± 1 SE):					
1	21.45 ± .62 (20)	21.58 ± .633 (12)	22.33 ± .95 (6)	19.5 ± 1.5 (2)	$t = .85$, df = 38, $P > .5$
2		24.50 ± .59 (13)	23.70 ± .42 (10)		
Proportion beetles dying in 20 days postinfection:					
1	.382 (34)	.1875 (16)	.25 (8)	0 (2)	Fisher, $P = .12$
2		.133 (15)	.214 (14)		

Note: Female hosts are subdivided by infection status, but analysis is completed on males versus all females. Block 2 represents data comparing infection properties on infected and uninfected *Spiroplasma* females. Sample size is given in parentheses; errors for latent period are ± 1 SE.

population sex ratio/male-biased epidemic pattern was observed, consistent with hypothesis. We would predict that wherever there are significant biases in the sex ratio of reproductively mature adults, caused by sex ratio distorters, meiotic drive elements or other biological factors, male rather than female-biased epidemics of STIs are likely.

The sexual transmission of this parasite means that the presence of a sex ratio bias associated with a male-killer must elevate the exposure of male hosts above that of female hosts. We experimentally analyzed the degree to which other factors may have contributed to the observed male-biased mite epidemics in Sweden. In terms of exposure, wild-collected females carrying infection transmitted the mite to male beetles during copulation at a rate comparable to that with which males transmitted to females, and infected individuals became infectious at a comparable rate. Thus, males and females are similar in terms of exposure outside of their relative copulation rate. Retention of the parasite was very similar—recovery was only seen on 1 beetle in 89. Further, mortality was, if anything, higher in male than in female beetles, which would create the opposite pattern to that observed. We therefore conclude that the major driver of the male-biased epidemic observed is the male-killer-induced sex ratio skew, elevating the copulation rate of males above that of females. We additionally examined whether the *Spiroplasma* provided any form of protection against developing a mite infection. We conclude that it did not: *Spiroplasma*-infected and -uninfected females were equally likely to be infected during copulation with an infectious male; both developed infections to the point of being infectious in similar time and with similar intensity of infection. Thus, the male-killing symbiont has an impact on disease epidemiology through demographic effects alone.

In sum, we conclude that STI epidemiology in this system cannot be understood without the strongly female-biased population sex ratio that is caused by the demographic effects of the symbiont infection. Reciprocally, the "total" impact of the male-killing infection requires incorporation of its effects on the STI. At first inspection, the impact appears subtle—an altered pattern of epidemic spread but one in which all individuals nevertheless become infected in the end. However, it should be recognized that the mite epidemic occurs in a cohort that is progressively diminishing in size over time, with the last individuals in the cohort dying in early August. Thus while our data superficially present a scenario where the subtlety of the epidemic dynamic is altered (speed, sex bias), the per-individual chance of catching the disease before dying will be very different, because only a small fraction (20%) of individuals actually

Table 4: Mite and *Spiroplasma* infection status of female *Adalia bipunctata* beetles collected during epidemic spread in Gävle June 2011 and July 2011 and in Malmö June 2011

	Spiroplasma positive	Uninfected with *Spiroplasma*
Gävle June 2011:		
Infected with mite	13	18
Uninfected with mite	36	49
Gävle July 2011:		
Infected with mite	10	11
Uninfected with mite	20	15
Malmö June 2011:		
Infected with mite	20	41
Uninfected with mite	24	38

survive to the point where all individuals are infected. Thus, the chance of an individual suffering the impact of infection changes with the speeding/slowing of the epidemic. Further, the length of time over which the individual suffers the impact is also altered. This is especially significant for a sterilizing STI—any changes in the length of time for which females are infected will affect both the timing and level of recruitment of the next cohort.

Future work will need to examine the precise impact of the *Spiroplasma* on *Coccipolipus hippodamiae* disease burden more precisely. The impact will depend crucially on how the host mating system changes under population sex ratio bias. Three patterns are possible. A much more rapid epidemic of the STI through males and a subtly faster epidemic through females are expected when female mating rate is unchanged by population sex ratio bias (our model). If, on the other hand, female mating rate declines when males are rare (if, e.g., they become limited by contact), then the epidemic through females slows. At the limit where male mating rate is constant, the epidemic through males is subtly slowed from the pattern seen in a 1 : 1 sex ratio population and that in females greatly slowed. Finally, female-biased population sex ratios may be associated with increased female mating rate and speed the epidemic through both male and female hosts. This occurs when female mating rate is partly determined by male mating history—for instance, when males that mate commonly make females less refractory. This is exemplified in the butterfly *Hypolimnas bolina*, where female mating rate is highest at intermediate male-killer prevalence (Charlat et al. 2007). Overall, it is likely that male *Adalia bipunctata* mating rate is normally limited by female availability (Perry et al. 2009). We thus predict that the sex ratio skew associated with male-killing would increase female availability for the remaining males, such that the mating rate of male ladybirds not only exceeds that of females but is also elevated above that which is found in the absence of the male-killer. If this is the case, then the male-killer will increase overall STI disease burden in the species.

Our study presents a case study of symbiont-induced effects on disease epidemiology mediated through demographic effects on the host. The data establish two previously unverified principles. First, inherited symbionts (which are commonly cryptic and require careful analysis to uncover) can alter the epidemiology of parasites and pathogens of their host. Second, the influence of symbionts on disease epidemiology includes effects mediated through host demography, not just the more obvious effects mediated through host susceptibility. This broadens the range of symbionts that are likely to influence the dynamics of natural enemies beyond those that affect individual susceptibility to the wider range of symbionts that affect host demography. Our study has focused on transmission through mating, but any effects on host density will likely affect the transmission of many pathogens and parasites, and those that affect individual movement (e.g., Goodacre et al. 2009) will affect spatial patterns of disease spread. Thus, understanding of the dynamics of parasites circulating in a host species requires us to understand both alteration of susceptibility of individuals following infection, and alteration of transmission rates/dispersal biology associated with demographic effects of infection on the host population.

We conclude that attempts to understand natural enemy dynamics in arthropods require us to ascertain the presence/absence of symbionts. Previous work has emphasized the importance of infection on individual susceptibility. Our study indicates that effects on host demography require additional consideration. While it is now thought the majority of arthropod species are infected with symbionts (Duron et al. 2008; Hilgenboecker et al. 2008), symbiont presence is often not obvious in the field, and their presence is often only revealed only by PCR assays. The phenotypes of inherited microbes are also subtle. Changes

in sex ratio commonly require comparison of the sex ratio produced by individual females in the laboratory, and alteration of dispersal requires even more defined observation. Understanding alteration of susceptibility to natural enemies likewise requires careful laboratory study. The frequency with which symbiont-mediated protection, and symbiont alteration of demography occur, suggest nevertheless that it is important that studies of disease epidemiology in arthropods establish the presence and effects of symbionts in their target host species.

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APPENDIX

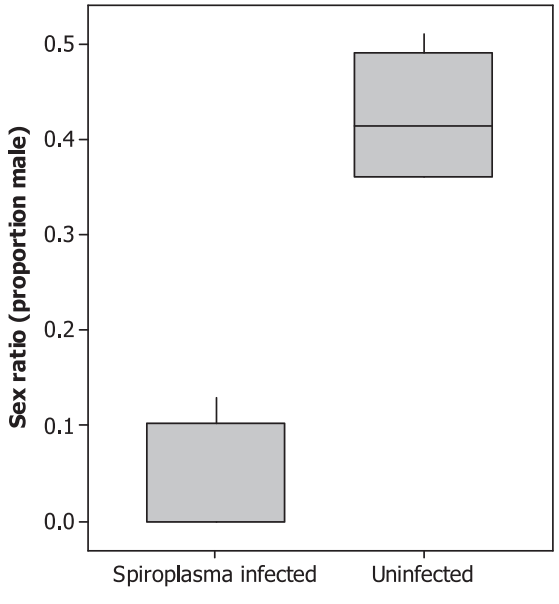

Figure A1: Boxplot of the sex ratio produced by *Spiroplasma*-infected and uninfected female *Adalia bipunctata* from Stockholm, Sweden.

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