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Full Length Research Paper

Sex-specific effect of bacterial infection on components of adult fitness in *Drosophila melanogaster*

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We investigated the sex specific effect of *Serratia marcescens* infection through septic injury on two components of fitness, survivorship and adult competitive reproductive fitness in male and female *Drosophila melanogaster.* Under competitive conditions, infected females showed significantly lower reproductive output compared to control females. However, male reproductive fitness was not affected by infection. We did not find any cost of injury and immune deployment in our study indicating that the fitness cost resulted only from sustained infection or virulence. However, the bacteria was found to be pathogenic, growing equally well in males and females and causing about 80% mortality over an eleven day period with no significant difference in mortality between sexes. Thus, such sex specific effects of pathogens on individual components of fitness indicate that the total fitness cost experienced by each sex might be very different, even within a given regime, thereby affecting the evolution of antibacterial immunity.

Key words: Sex-specific effect, fitness, survivorship, *Serratia marcescens, Drosophila melanogaster.*

INTRODUCTION

The fitness cost incurred by a host due to infection defines the virulence of a pathogen. Insects, which possess a sophisticated innate immune system comprised of humoral, cellular and melanisation defences (Hoffmann, 2003; Hultmark, 2003; Lemaitre and Hoffmann, 2007) have been used in several studies to understand the costs of infection. The results of these studies indicate that the cost incurred by the host due to infection (or virulence) depends on various factors such as the genotype of the host and pathogen (Ferguson and Read, 2002; Short and Lazzaro, 2010), host condition (Brown et al., 2000; Jokela et al., 1999) and the environment of host-pathogen interaction (Bedhomme et al., 2004). One of the major factors that can potentially affect the cost of infection is the sex of the host. Life history and sexual selection theories would suggest investment in immunity to be sexually dimorphic (Zuk, 1990; Zuk and Mckean, 1996; Zuk and Stoehr, 2002) with males (which typically experience strong intra-sexual competition and a greater variance in reproductive success compared to females) investing less in immunity compared to females (Bateman, 1948; Rolff et al., 2002; Zuk, 2009). Consequently, males are expected to have higher mortality cost to infection than females. Several studies have indeed found that males have reduced survivorship post infection compared to females (Bedhomme et al., 2004; Gray, 1998; Joop et al*.,* 2006; Moret and Schmid-Hempel, 2000; Sharmila et al., 2007; Ye et al., 2009).

Reproductive output is another important fitness related trait that can potentially be affected by infection. Several studies have shown that female reproductive output declines in response to sustained bacterial infection (McKean et al., 2008; Hurd, 2001; Webb and Hurd, 1996; Webb and Hurd, 1999; Zefrosky et al., 2007). Very few studies have considered the reproductive cost of infection to males, with the exception of studies on inherited pathogens such as *Wolbachia* (Bandi et al., 2001). In one

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of the few studies to consider male reproductive output, Perlmann and Jaenike (2003) using a *Drosophila*-Nematode system found that infection reduced the reproductive output of females much more severely than that of males. However, given the paucity of studies, the reproductive cost of bacterial infection in males is poorly understood.

In the present study, we investigated the possible role of bacterial infection on two important components of fitness, namely, survivorship and adult competitive reproductive output in the two sexes. We used a large, outbred population of *Drosophila melanogaster* as the host and a gram negative bacterium, *Serratia marcescens*, as the pathogen. We chose *S. marcescens* as the model infecting agent because it is a pathogen that has a wide range of hosts. It has also been isolated from *Drosophila* (Flyg et al., 1980; Flyg and Xanthopoulas, 1983; Cox and Gilmore, 2007; Govind, 2008) and is one of the few bacteria known to kill the adult flies (Olcott et al*.,* 2010). Consequently, it is used commonly as a model system in studies of evolutionary ecology of immune response (Lazzaro et al., 2004; Lazzaro et al., 2006). Experimental female and male hosts were allowed to compete for nutrient supplements and mates respectively and the effect of bacterial infection was measured quantitatively in terms of reproductive output. Our study was also designed to dissect out the costs of injury and deployment of an immune response in males and females separately.

MATERIALS AND METHODS

Maintenance of insect populations

Two large, outbred laboratory populations called LH and LH_{st} were used in the present study. The two populations are identical to each other except that flies in the LH population have red eye colour while flies in the LH_{st} population have a recessive scarlet eye colour marker. The maintenance of these flies has been described in detail by Chippindale and Rice (2001). Briefly, the populations are maintained on a 14 day discrete generation cycle, at 25°C temperature and LD 12: 12 cycles. Eggs are collected at a density of 150/vial and incubated. Adult flies were collected as virgins 10 days after egg collection during peak of eclosion (within 4 h of eclosion). On the 12^{th} day post oviposition, adults from all the vials are mixed and redistributed into fresh food vials at a density of 16 pairs per vial. Sixty such vials are set up for the LH population and 30 vials were set up for the LH_{st} population. The vials are provided with a limiting quantity of live yeast supplement. After two days of interactions, the flies were transferred to fresh food vials and allowed to oviposit for 18 h, the flies were then discarded. In these populations, female fitness is greatly dependent on the ability of a female to obtain the yeast in competition with other females, and male fitness depends on its ability to obtain mates in competition with other males over the two day interaction period (Chippindale et al., 2001; Prasad et al., 2007). In all experiments, we collected LH and LH_{st} flies as very young virgins during the peak of eclosion (within 4 h of eclosion) ten days post oviposition. They were then held in single sex vials on cornmeal-molasses food at a density of 7 to 8 flies/vial.

Bacterial Infection

The bacteria *S. marcescens* (strain ATCC 13880) was used for all the experiments. On the evening before infection, we inoculated a fresh bacterial culture in Luria Broth and allowed it to grow overnight to OD 1.0. We centrifuged the resulting population of bacteria and made it into a slurry. Two day old virgin flies under light CO_2 anesthesia were infected by pricking at the lateral side of the thorax with a 0.1 mm minutien dissecting pin (Fine Science Tools, Foster City, CA) dipped into the bacterial slurry. Sham infection was done by pricking flies with a pin dipped in *Drosophila* Ringer solution (McKean and Nunney, 2001). To evaluate the cost of immune deployment, flies were pricked with heat killed bacterial slurry prepared by heating freshly grown culture at 65°C for 45 min. We tested the effect of heat killing by plating the heat killed culture on a LB Agar plate and incubating overnight at 37°C.

Bacterial growth assay

We measured bacterial growth in both sexes. Two day old virgin LH_{st} males and females were infected (n=54 per sex per block) or sham infected (n=30 per sex per block) separately as described earlier. Immediately afterwards, half of the flies that received bacterial infection/sham infection were homogenized in groups of three (under light CO_2 anesthesia in 300 µl of LB medium). Thus, there were 9 homogenates of infected flies and 5 homogenates of sham infected flies in each sex × block combination. Each of these homogenates was then plated with proper dilution (50 µl of the aliquot from 1:100 dilution) on a LB agar plate. The plates were incubated overnight at 37°C.The number of bacterial colonies on each of these plates was then counted as an estimate of the number of bacterial cells that were transferred during infection. The remaining flies were held in groups of three in vials containing cornmeal-molasses food for 24 h. Later, the flies were homogenized in groups of three and the number of bacterial colonies produced was counted as mentioned earlier.

Survivorship assay

Two day old virgin LH_{st} males and females were infected or sham infected as described previously. We then held males and females separately in groups of ten for the subsequent 11 days. Ten such vials were set up per sex per treatment. We transferred flies to fresh food vials on every alternate day and recorded the number of survivors in each vial on the 2^{nd}, 4^{th}, 7^{th}, 9^{th} and 11^{th} day.

Evaluation of male and female reproductive fitness

Our experimental plan for evaluation of reproductive fitness closely resembled the population maintenance protocol described earlier, so as to measure fitness components under “natural” conditions. Target flies came from the LH_{st} population. Flies from the LH population were used as competitors. All flies were collected as virgins as described before and housed in single sex groups at a density of 7 to 8 flies / vial. On the second day after eclosion, virgin male and female LH_{st} flies were randomly subjected to 4 treatments, namely, infection with live bacteria (I), pricked with heat-killed bacteria (HK), sham infection (SI), and noninjected (NI). The flies were then allowed one day to recover. Adult competitive fitness vials were constituted on the 3^{rd} day post eclosion.

For male fitness experiments, we placed 5 LH_{st} males (treated target) with 10 LH males (untreated competitors) and then paired them with 15 LH_{st} females (untreated) in mating vials having 7 mg of live yeast-supplement. Following a 2-day interaction period (during which males competed for access to females), we discarded

Table 1. Summary of two way mixed model ANOVA on bacterial load from individual blocks with sex as fixed factor crossed with random blocks.

Trait	df	MS	F	P
Block	1	63691.9	20.286	0.0001
Sex	1	306.6	0.0977	0.756
Block*Sex	1	778.89	0.2481	0.622

the males and transferred the females singly into test tubes (12 × 75 mm) with corn meal medium and allowed 18 h for oviposition. Thirteen days later, we scored each test tube for the number and eye colour of the progeny. The ratio of the number of scarlet eyed progeny to the total number of progeny in each test tube was taken as a quantitative indicator of the competitive male reproductive output/ fitness. Female fitness evaluation was done similarly. We combined 5 LH_{st} females (treated target) and 10 competitor LH females (untreated competitor) with 15 LH_{st} males (untreated) in food vials with a limiting quantity (7 mg/vial) of live yeast-supplement. Following a 2-day interaction period (during which females competed for the yeast supplement), we transferred the LH_{st} females individually into test tubes to lay eggs for 18 h. After 13 days, we counted the progeny. The number produced by the target females was used as a measure of competitive female reproductive output / fitness.

Data analysis

Bacterial load assay was performed in two independent blocks. We analyzed the data using a two factor mixed model ANOVA treating sex as the fixed factor crossed with random blocks. Since we found a significant block effect (Table 1), we then analyzed data from each block using a one factor ANOVA. The survivorship assay consisted of two blocks. Survivorship was analyzed using the Kaplan Meier method (Bland and Altman, 1998), treating all the flies that had not died by the 11th day as censored data. The male fitness assay consisted of eight replicate vials in blocks 1 and 2 and five replicate vials in blocks 3 and 4. Data were analyzed using a two factor mixed model ANOVA, with infection status as fixed factor and block as a random factor. The female fitness assay consisted of three independent blocks with 10 replicate vials in 2 blocks and 5 replicate vials in the 3rd block. A two factor mixed model ANOVA with infection status as fixed factor and block as a random factor indicated significant block effect on mean progeny number (Table 4). Therefore, data from each individual block was analyzed separately with a one way ANOVA, treating infection status as the fixed factor. Multiple comparisons were carried out using Tukey's HSD. All data were checked for conformity with assumptions of ANOVA using appropriate tests.

RESULTS

Numbers of bacterial cells transferred into the host body were similar for both sexes and approximately 3000 cells were incorporated with the needle. After 24 h of infection, numbers of bacteria retrieved from males and females did not differ significantly (Figure 1, Table 2). Sham infected flies (SI) failed to produce colonies in LB agar plates. Flies pricked with heat-killed bacteria (HK) failed to yield any bacterial colonies.

Survivorship was greatly affected by bacterial infection. While almost all the sham infected flies survived through the 11 days of observation, the survivorship of infected flies was greatly reduced with only about 20% of the infected flies surviving till 11 days post infection (Figures 2A and B). This clearly showed that *S. marcescens* was pathogenic to our flies. However, Kaplan Meier analysis indicated that the survivorship of infected males and females was not significantly different over the eleven days of observation (all $p>0.05$).

There was no significant effect of infection status on competitive reproductive output of the males (Figure 3A, Table 3) indicating that males did not pay a considerable cost (in terms of reproductive output) towards sustained bacterial infection or activation of the immune system. However, female fitness decreased by about ~25% (mean progeny number: infected = 23.9±1.99, sham infected = 31.4±1.43) on infection with live bacteria (Figure 3B, Table 5). Females from the I treatment of each block, produced significantly less progeny compared to females from HK, SI and NI treatments (Tukey's HSD, all $p < 0.05$). However, progeny production did not significantly vary between HK, SI and NI females in any of the three blocks (Tukey's HSD, all $p> 0.05$), indicating that there was little cost to activation of the immune system in the absence of sustained bacterial infection (Figure 3B, Table 5).

DISCUSSION

The major goal of this study was to assess the sex-specific effects of bacterial infection on competitive fitness. Our results clearly show that infecting *D. melanogaster* with the known pathogenic bacteria, *S. marcescens* results in a reduction in the competitive fitness of females (by about 25%) whereas male competitive fitness is not significantly affected by such infection. Our data represent the first clear demonstration of the differential effect of bacterial infection on the competitive fitness of males and females.

Our study was explicitly designed to check the effect of injury (difference in fitness between NI and SI), immune system activation (difference in fitness between HK and SI) and infection (difference in fitness between I and SI) on competitive fitness of females. While the competitive fitness of NI, SI and HK females was not significantly different from each other, the fitness of I females was significantly lower than that of the other three. Thus, we found no cost of injury or immune system activation in females. The only cost was of sustained bacterial infection. This result is in contrast to other studies which found an up-regulation of immunity related gene expression post injury (Wigby et al., 2008) and an associated cost of injury/ immune system activation in terms of reduced fecundity/progeny production (McKean et al., 2008; Zefrosky et al., 2007). There are at least two

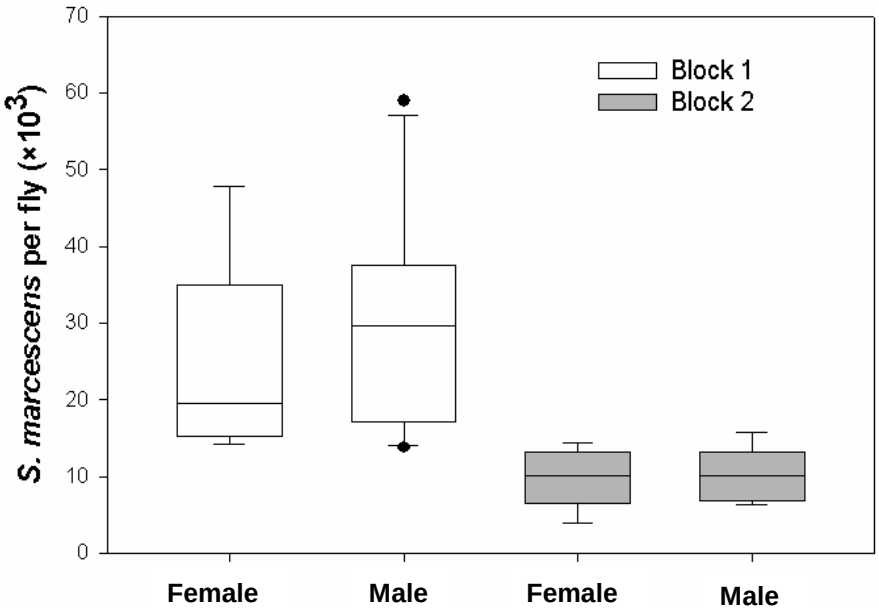

Figure 1. Bacterial load of males and females 24 h post infection (m- male, f- female). Values are from replicate plates within each block. Values are averages across the replicate vials

Table 2. Summary of one way ANOVA on bacterial load from individual blocks with sex as fixed factor.

Block	Trait	df	MS	F	P
1	Sex	1	306.603	0.097	0.756
2	Sex	1	1061.76	0.216	0.648

major differences between our study and the others mentioned before that can potentially explain the observed difference in results- (a) In the previous studies, the measurement of fecundity was typically done immediately after the treatment, whereas in our study fitness was measured three days later. (b) It is quite possible that the yeast supplement provided for two days prior to fitness measurements in our study can potentially ameliorate the costs of injury and immune system activation.

We found no significant effect of treatment (NI, SI, HK and I) on male competitive fitness, clearly indicating that even after infection with live bacteria, the ability of the males to sire progeny under competitive conditions was not significantly reduced. This is in stark contrast to the effect of bacterial infection on female fitness. This effect was not due to the differential growth of bacteria in the two sexes and thus, when infected males and females were put under competitive conditions in the competitive fitness assay, they carried similar bacterial loads.

Post infection survival was not significantly different between males and females indicating that the males did not pay an added survivorship cost. It is to be noted here that the flies used in the survivorship assay were virgins. Thus, it is still possible that under mated conditions, the survivorship of males is more severely affected. Indeed, McKean and Nunney (2001) have shown that male antibacterial immunity is reduced by increased sexual activity. However, McKean and Nunney (2001) used non-pathogenic bacteria and infection followed sexual activity. These conditions are very different from those of our experiment. To summarise, while the potential for infection to affect the longevity of mated males more severely does exist, given our study design, we cannot conclusively address this possibility.

Bacterial contamination of oviposition vials by infected females could compromise larval viability and give rise to the observed pattern of results, independent of the costs of bacterial infection. However, we rule out this possibility because (a) our observations suggest that the number of eggs laid by infected females was lesser than the eggs laid by other types of females. This is also confirmed by a recent study using the *Drosophila-S. marcescens* model system (Brandt and Schneider, 2007). (b) anti-bacterial and anti-fungal agents that are components of the fly food would prevent the growth of any bacteria and (c) in

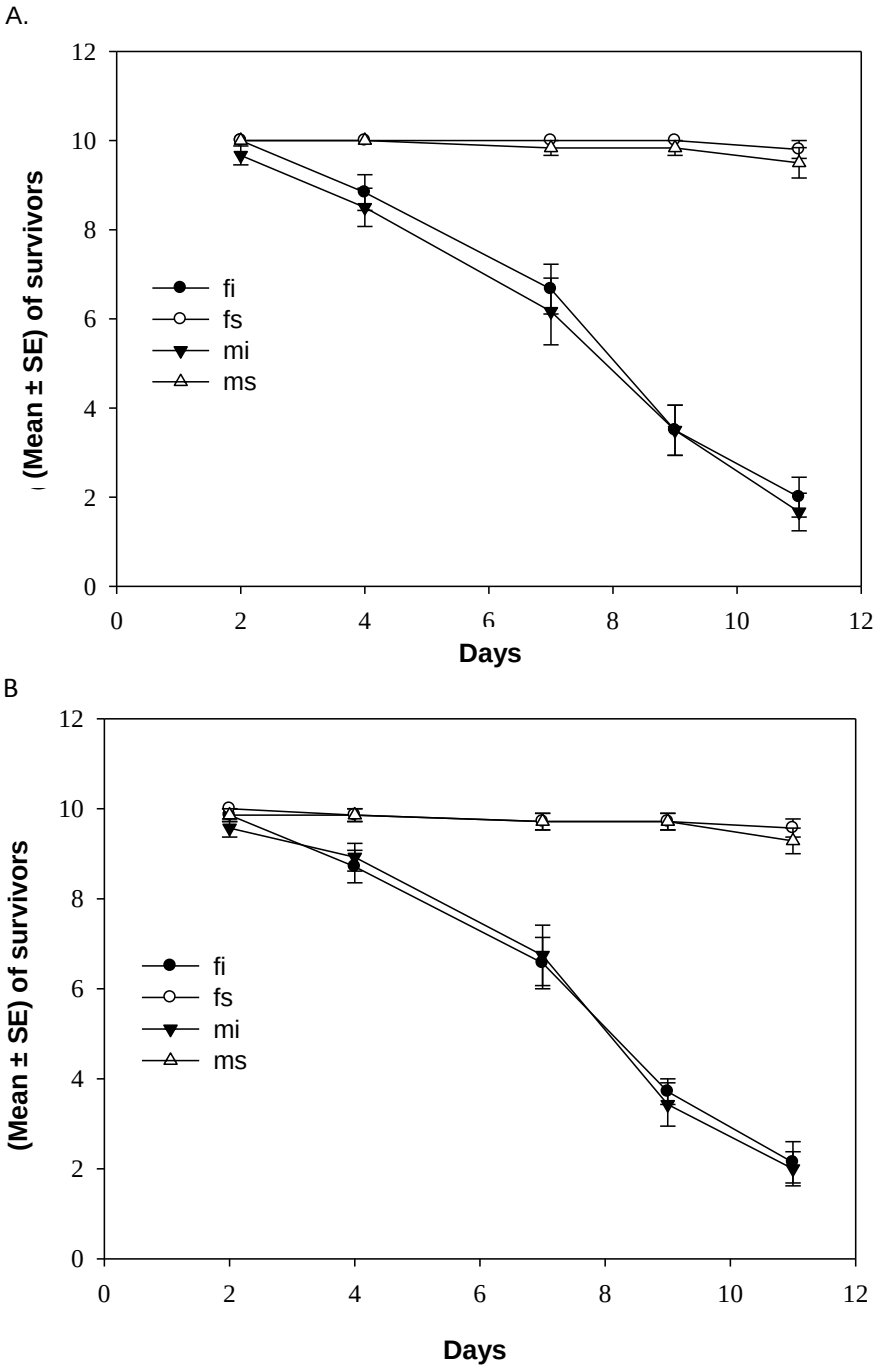

Figure 2. Effect of infection on survivorship of virgin males and females (fi- Infected females, fs- Sham infected females, mi- Infected males, ms- Sham infected males). A) Block-1 and B) Block -2. Values

other experiments in our lab, we have attempted to infect larvae with *S. marcescens* by adding concentrated bacterial suspension into the normal food vials that have larvae. Yet we have failed to infect the larvae.

There are two possible reasons for the observed results; a) previous studies have shown that in females, *S. marcescens* colonises the ovary during infection (Brandt and Schneider, 2007). Further, *Salmonella typhimurium*, a gram negative bacteria like *S. marcescens*, establishes a stable site of infection at the posterior end of the ovary near the oviduct (Brandt and Schneider, 2007) leading to the gradual degradation of the ovary. However, in the males, the site of infection has not been specifically identified. Thus, if in females the

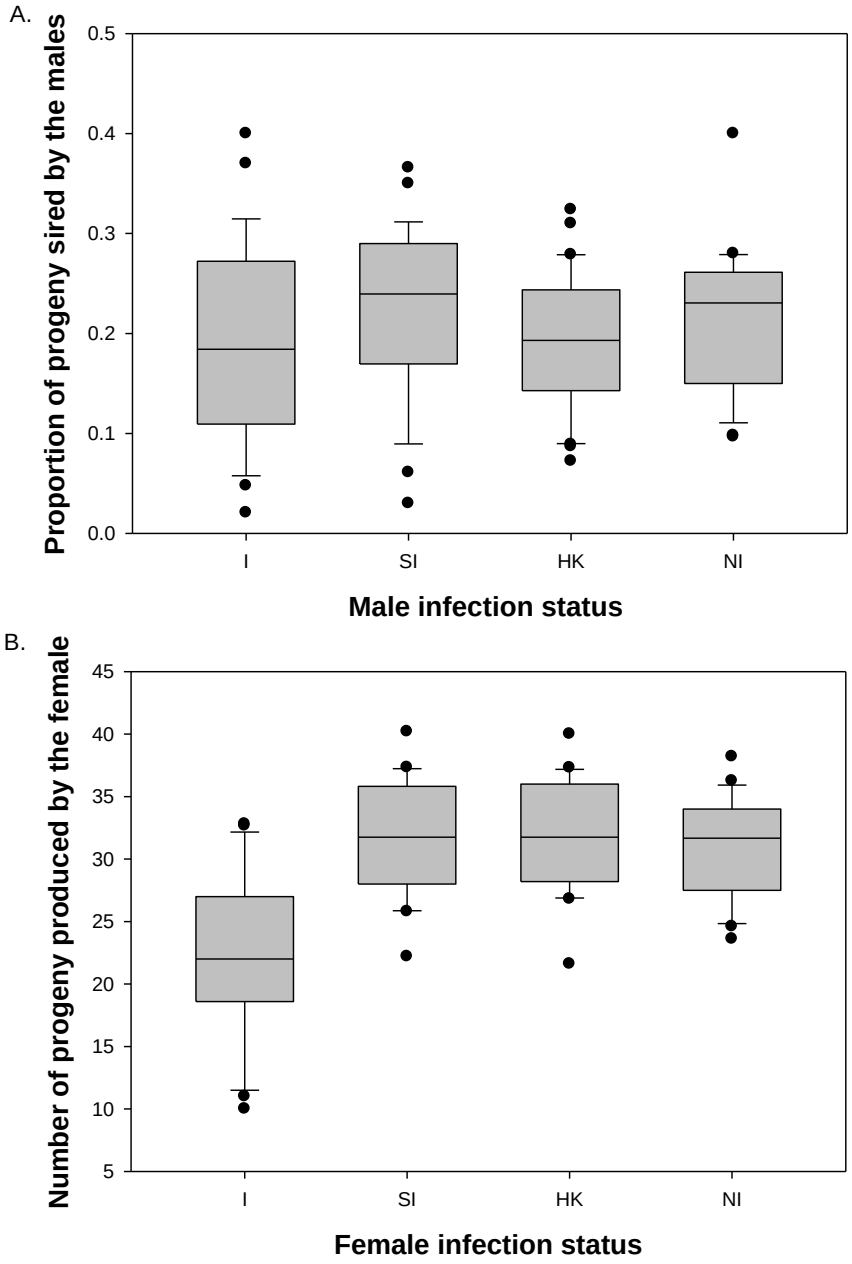

Figure 3. Effect of infection on adult reproductive fitness. A) Males B) Females. Values are from individual vials across all blocks (I- Infected with live bacteria, SI- Sham Infected with Ringer's solution, HK- Infected with heat killed bacteria, NI- Not Infected Controls).

ovaries (among other organs) are a target of bacterial colonization, whereas in males the reproductive structures are not a direct site of colonization, then, tissue degradation along with the systemic response of the reproductive structures to bacterial colonization can reduce female fitness. (b) In *Drosophila*, though males and females become sexually mature by 8 to 12 h post eclosion, the pattern of gamete production (and hence potentially, the timing of investment in gamete production) is very different. Egg production in female *Drosophila* is a continuous process, which is enhanced by the availability of dietary yeast. For the males, there is evidence that components of ejaculate build up over time (Bretman et al., 2010), indicating that males can potentially produce and store ejaculate. Further, male fitness is not affected by dietary yeast-supplement. In our experiment, 3 day old virgin males and females were put under competitive conditions (that is competitors and

Table 3. Summary of two factor mixed model ANOVA on fitness (fraction of progeny sired) of males, with infection status (NI, I, HK and SI) as fixed factor crossed with random blocks.

Trait	df	MS	F	P
Block	3	0.00917	1.3332	0.2680
Infection status	3	0.00564	0.8202	0.4858
Block*Infection status	9	0.00096	0.1400	0.9984

Table 4. Summary of two factor mixed model ANOVA on fitness (progeny number) of females, with infection status (NI, I, HK and SI) as fixed factor crossed with random blocks.

Trait	df	MS	F	P
Block	2	347.94	19.377	<0.0001
Infection status	3	438.934	24.445	<0.0001
Block*Infection status	6	8.438	0.470	0.829

Table 5. Summary of one way ANOVA on fitness (progeny number) of females from individual blocks treating infection status (NI, I, HK and SI) as a fixed factor.

Block	Trait	df	MS	F	P
1	Infection status	3	84.93	8.29	0.001
2	Infection status	3	298.520	16.5239	<0.0001
3	Infection status	3	134.105	5.6614	0.004

limiting yeast supplement) 24 h post infection. Thus, the males in our experiment could invest in and store ejaculate prior to infection, whereas the females had to invest in egg production post infection, thereby leading to the observed sex differences in competitive fitness. This argument assumes that bacterial infection has little or no effect on male mating behaviour, an assumption that warrants further investigation.

Our study has important implications for the understanding of the evolution of immunocompetence. The cost of infection in terms of total fitness is likely to be one of the most important factors that affect the evolution of antibacterial immunity. However, the weighting of the individual components of fitness to total fitness is likely to vary considerably depending on the ecological and demographic contexts (Prasad and Joshi, 2003). Given that infections can affect various components of fitness differentially in a sex-specific way, the total fitness cost experienced by each sex is likely to vary considerably. As an extreme example, consider laboratory populations maintained on, say, 14 day discrete generation cycles. In such conditions, pathogens that reduce early life reproductive success but do not affect survivorship extract a heavy cost compared to pathogens that affect only survivorship beyond 4 to 5 days of adult life. The situation is complicated by the fact that such effects can be sex-specific, as shown by our study. While ours was a laboratory study and used a specific pathogen- *S. marsescens*, it is not unreasonable to expect that situations where the weighting of the fitness components to total fitness is different and pathogens which affect components of fitness in a sex-specific way, could in principle be found in the wild. Thus such sex-specific effects of pathogens on total fitness need to be considered by both experimental and theoretical studies. Further, our study highlights the fact that for experimental studies with evolutionary overtones, quantifying immunity in terms of bacterial load or survivorship might not be always enough. Instead, it is necessary to place these measures in the context of their contribution to total fitness.

In conclusion, it was found that bacteria grew equally well in either sex and reduced one component of total fitness- post infection survival- equally in either sex. However, the same bacterial infection affected another component of total fitness- reproductive success- differentially in either sex. Thus, the total fitness cost paid by each sex towards bacterial infection can potentially be different.

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