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Post-mating disparity between potential and realized immune response in *Drosophila melanogaster*

Kenneth M. Fedorka[1,*], Jodell E. Linder[2], Wade Winterhalter[1] and Daniel Promislow[2]

[1]*Department of Biology, University of Central Florida, Orlando, FL 32816, USA*
[2]*Department of Genetics, University of Georgia, Athens, GA 30602, USA*

Reproductive costs are an essential component of evolutionary theory. For instance, an increase in reproduction is generally coupled with a decrease in immunocompetence shortly after mating. However, recent work in *Drosophila melanogaster* suggests that the potential to mount an immune response, as measured by the levels of immune gene expression, increases after mating. These data are in contrast to previous studies, which suggest that mating can reduce a fly's ability to survive an actual bacterial challenge (realized immunity). This pattern may be driven by some aspect of mating, independent of resource limitation, which reduces immune function by inhibiting the effective deployment of immune gene products. Though several studies have examined both the potential and the realized immunity after mating, none have examined these immune measures simultaneously. Here, we examined the link between the potential and the realized immunity in a sterile mutant of *D. melanogaster*. Shortly after mating, we found that female immune gene expression was high, but survival against infection was low. Surprisingly, this pattern was reversed within 24 h. Thus, estimates of immunity based on gene expression do not appear to reflect an actual ability to defend against pathogens in the hours following copulation. We discuss the possible mechanisms that may account for this pattern.

Keywords: immune function; gene expression; trade-offs; *Drosophila melanogaster*; mating; sexual conflict

1. INTRODUCTION

Immune systems are presumed to evolve primarily in response to a coevolutionary arms race between a host and a pathogen (Williams 1975; Hamilton 1980; Lively *et al.* 1990). However, other aspects of host biology are known to play a key role in how immune systems are shaped and expressed. For instance, the degree of an immune reaction may be influenced by the individual's current reproductive effort (Sheldon & Verhulst 1996). This common constraint is evident in the ground cricket, *Allonemobius socius*, where multiply mated females exhibit a decreased ability to defend against a simulated parasitoid attack when compared to their singly mated counterparts (Fedorka *et al.* 2004). Similarly, in the pea aphid, *Acyrthosiphon pisum*, a female's susceptibility to parasitoid attack is positively related to her fecundity (Gwynn *et al.* 2005). Thus, genotypes that invest heavily in the production of offspring may incur an increased risk of pathogenic infection due to a reduction in the limited resources available for immune defence.

Over the past several years, the notion of a trade-off between immunity and reproduction has gained wide support from studies in a variety of taxonomic groups, including birds, mammals and insects (Festa-Bianchet 1989; Norris & Evans 2000; McKean & Nunney 2001; Zuk & Stoehr 2002). These immune-related costs have been observed in both males and females of the same species (Rolff & Siva-Jothy 2002; Fedorka *et al.* 2004), and are even more apparent when an individual female mates with a genetically diverse group of males (Fedorka & Zuk 2005; Baer *et al.* 2006). Though the physiological mechanism(s) that underlies the immunity/reproduction trade-off is currently unknown, it may be hormonally based. For example, the deleterious effect of mating on immune function in invertebrate systems may be driven by juvenile hormone, which has been shown to simultaneously initiate gamete production while inhibiting phenoloxidase activity (an important component of invertebrate innate immunity associated with parasitoid defence; Rolff & Siva-Jothy 2002). Regardless of the underlying mechanism, an increase in reproductive effort appears to be coupled with a decrease in immune function.

Recent evidence, however, suggests that an individual's ability to defend against an immune insult may also increase after mating (Johansson *et al.* 2004; Shoemaker *et al.* 2006). This phenomenon has been shown clearly at the level of immune gene expression in *Drosophila melanogaster*, where sperm and other seminal proteins directly upregulate the female genes associated with bacterial defence (e.g. *attacin A* and *B*, *cecropin A1* and *A2*, *metchnikowin*) in the hours after copulation (Lawniczak & Begun 2004; McGraw *et al.* 2004; Peng *et al.* 2005*b*). The simultaneous investment in bacterial defence products and mating appears to run counter to previous work in *D. melanogaster*, showing that an increased mating effort decreases a male's ability to defend against bacterial infection (McKean & Nunney 2001) or

* Author for correspondence (fedorka@mail.ucf.edu).

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that exposure to bacterial pathogens reduces female fecundity (Zerofsky *et al.* 2005).

If there is, in fact, a trade-off between immunity and reproduction, then two possibilities may account for the pattern. First, the discrepancy between gene expression measures of immunity and alternate measures of immunity (such as a functional measure of parasitoid or bacterial susceptibility) may be due to when these measures are taken. For instance, bacterial immune defence may increase shortly after mating (when gene expression is generally measured) at the expense of reproduction in order to defend against sexually transmitted disease. The immune increase may then be followed by a sharp decline in favour of increased reproductive investment (when functional measures of parasitoid or bacterial susceptibility are taken). Second, some aspect of mating outside of energetic demands (e.g. egg production) may reduce immune function by inhibiting potential immunity shortly after copulation. However, no study to date has examined the level of immune gene expression simultaneously with the ability to survive a bacterial infection directly after mating.

Here, we examine the level of gene expression and the ability to survive a bacterial infection after mating in *D. melanogaster* females. Female immune function after mating could decrease if there is a trade-off between the female's investment in egg production and immunity (Sheldon & Verhulst 1996). To alleviate this potential problem, we carried out our experiments using a dominant female sterile mutant, ovo^{D1} (Bloomington Stock Center no. 2121). In insects, oogenesis represents a significant cost of reproduction, considering that eggs generally contain a variety of lipids, proteins, vitellogenin and other compounds that are limiting in nature. The ovo^{D1} mutation inhibits oogenesis prior to vitellogenesis (Mevel-Ninio *et al.* 1991; Granadino *et al.* 1992), which is the process of incorporating these limited components into the maturing oocyte. By reducing trade-offs between egg production and immunity, we were able to focus on other potential mechanism (i.e. resource independent) that may affect immune function after mating.

2. MATERIAL AND METHODS

(a) *Fly stock and maintenance*

Prior to the experiment, the ovo^{D1} mutant was maintained by serial transfer opposite the CyO balancer chromosome in males only, where there is no sterility effect. All individuals were reared on standard cornmeal–yeast–molasses food and maintained on a 12 L : 12 D photoperiod at 24°C. Experimental flies were separated by sex upon eclosion and held at medium density (approx. 20 flies per vial). All flies were 4 ± 1-day-old virgins at the start of the experiment.

(b) *Effect of mating on infection survival*

To address the effect of mating on female immunocompetence, we examined a female's immune gene expression or her ability to survive a bacterial immune challenge directly after mating. Survival provides a holistic assessment of the female's immune status and may have certain advantages over commonly used immunological parameters. Previous work in crickets has shown that direct measures of immune components (e.g. phenoloxidase and lysozyme activity) may not accurately represent an individual's true ability to defend against an immune challenge (Adamo 2004*a*,*b*; Fedorka & Zuk 2005). In addition, bacterial clearance rates (e.g. McKean & Nunney 2001) may not be correlated with the survival cost of infection in *D. melanogaster* (Corby-Harris *et al.* in press).

Twenty-four hours prior to the mating trial, Gram-negative bacteria (*Pseudomonas aeruginosa*) were incubated at 37°C in a sterile broth. At the same time, we placed females in individual food vials under light CO_2 anaesthesia, and then randomly assigned each female to a mated or virgin treatment. For every female that was to be mated, we also set up a separate vial containing two fertile ovo^{D1} males. After 24 h, we serially diluted the log-phase bacterial culture to a 5×10^{-4} concentration, which is close to an LD50 for the ovo^{D1} strain (K. M. Fedorka & J. E. Linder 2004, unpublished data). We then combined each female vial with a male vial without anaesthesia (2 : 1 males to females) and allowed flies to mate for 90 min. Female flies that did not mate were removed from the experiment (mating success per replicate was greater than 95%). We limited female access to males to 90 min in order to minimize any potential cost to the female immune system due to physical harm imposed by males during courtship (Partridge & Fowler 1990; Linder & Rice 2005). After mating, males were aspirated from the female vials and discarded. Virgin females were also transferred during this time, to ensure that both the mated and virgin treatments underwent the same procedures and were held in similar environments. Mated and virgin females were then randomly placed into one of four temporal treatments (3, 9, 21 and 27 h), which corresponded to when they were to be inoculated with bacteria after mating. Such a temporal assessment allowed us to estimate changes in female immune function after mating.

To inoculate females with bacteria, we dipped a fine-tipped needle into the standardized culture dilution. We then inserted the needle into the thorax of flies that had been lightly anaesthetized under CO_2 (methods modified from Lazzaro *et al.* 2004). A subset of the anaesthetized mated and virgin females from the 3 and 27 h treatments was not infected, but instead placed in Trizol regent for future gene expression analysis. A second subset of anaesthetized mated and virgin females from each time point was sham inoculated with sterile broth in lieu of bacteria to assess the mortality consequences of our inoculation method. After inoculation, flies were placed into clean food vials and the surviving number recorded after 72 h (at our bacterial concentration, the majority of mortality occurs by 48 h; K. M. Fedorka & J. E. Linder 2004, unpublished data). It should be noted that, due to the nature of our experimental design, inoculations were done on females of known treatment and therefore does not represent a blind study. In all, four replicates of the experiment were performed with a new bacterial culture created for each replicate, resulting in a total of 1582 females. Of these females, 30 were isolated for gene expression analysis (see §2*c*) and 192 were used for the sham inoculations.

(c) *Effect of mating on immune gene expression*

The humoral immune response in *Drosophila* comprises two separate pathways, *Toll* and *Imd*, which react independently to Gram-positive and Gram-negative bacteria, respectively. To examine immune gene expression, we assayed four immune genes, including *attacin A*, *cecropin A*, *cercropin B* and *metchnikowin*. The first three genes represent antimicrobial peptides for the *Imd* pathway and the latter gene is part of the

Toll pathway. In addition, we examined one of three vitellogenin precursors (yolk protein 1), which probably represents a major reproductive investment. Thus, levels of yolk protein 1 gene expression (hereafter referred to as vitellogenin) provided our estimate of reproductive investment (if any) in the ovo^{D1} mutant.

From the 3 and 27 h treatments, five mated and five virgin flies from each of the first three replicates were separately placed into Trizol (Invitrogen) and their RNA was isolated using a chloroform/isopropanol extraction. Reverse transcription was performed using the Invitrogen Superscript III kit, and the resulting cDNA was diluted to 100 $\mu g\,\mu l^{-1}$ and maintained at $-80°C$. Real-time PCR was performed using a Bio-Rad MyIQ single colour optical detection system and gene amplification was accomplished using the SybrGreen Supermix (Bio-Rad). All primers were designed from the published sequence (available at www.Flybase.org) and all gene expression levels were normalized using the constitutively expressed actin gene, *Act5c*.

(d) *Data analysis*

Survival was recorded as a binary 0/1 (dead/alive) response 72 h after inoculation. Considering that our survival data consisted of a single time point, survival curves could not be constructed. To examine the effect of mating on the female's ability to survive infection, we used a paired *t*-test within each replicate, which represented a mated and virgin pair. In other words, we tested for a difference in survivorship (i.e. the arcsine-transformed proportion of infection survivors) between mated and virgin pairs across the replicates for a given inoculation time (i.e. 3, 9, 21 or 27 h). To control for variation in survival rates between the replicates and to simplify the presentation of our results, we plot the ratio of mated to virgin (control) survival. Thus, a value less than 1 would indicate that mated females have lower survival against bacterial infection.

Gene expression levels were calculated using the ΔΔct method (e.g. Peng *et al.* 2005*b*). Briefly, after normalizing expression levels to an endogenous control gene (*Act5c*), we calibrated the gene expression data of the mated females to that of the virgin females, so that virgin females always had a gene expression equal to 1. A one-sample *t*-test was used to identify significant changes in gene expression. A two-sample *t*-test was used to identify significant differences between the time points (i.e. 3 and 27 h) for a given gene. Statistical analyses were conducted using JMP v. 6.0.

3. RESULTS

(a) *Effect of mating on infection survival*

Sham-inoculated females ($n=192$) suffered no mortality within 72 h of inoculation, indicating that differences in survival were due to the bacteria and not to our method of bacterial delivery. On average, $55.4\pm2.1\%$ of the virgin (control) females died from the bacterial inoculation, indicating that the 5×10^{-4} concentration represents an approximate LD50 for virgins. We found that mated females had lower survivorship than their virgin counterparts when inoculated 3 and 9 h after mating (figure 1; two-tailed paired *t*-test: $t_3=-5.06$, $p=0.0148$; $t_3=-5.26$, $p=0.0133$, respectively). However, no significant difference between mated and virgin females was detected when inoculated at 21 or 27 h ($t_3=0.10$, $p=0.9306$; $t_3=0.81$, $p=0.4754$, respectively).

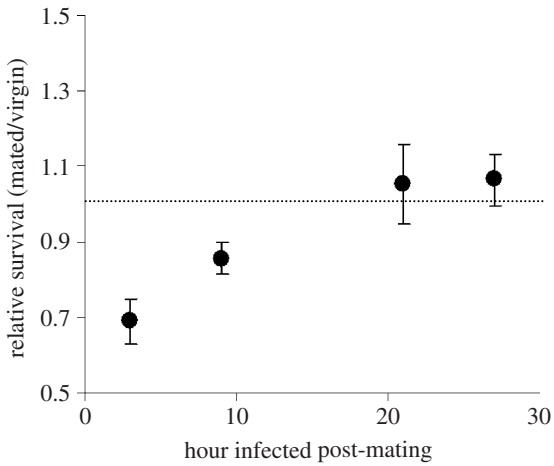

Figure 1. The effect of mating on the female immune system. Mated females exhibited a significant reduction in relative survival compared with the virgins (dotted line) when infected 3 h after mating ($p=0.0148$) and when infected 9 h after mating ($p=0.0133$).

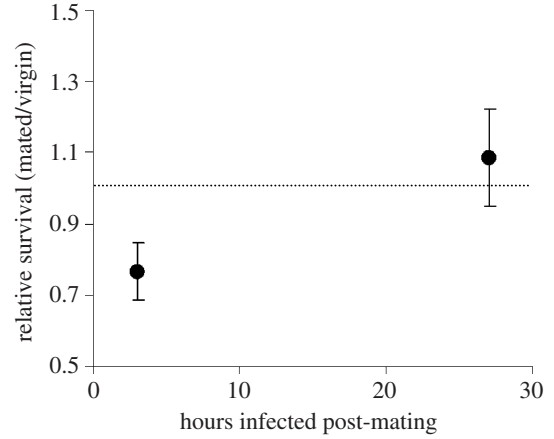

Figure 2. Re-examination of the effect of mating on female immunity. As in figure 1, mated females showed a reduction in relative fitness compared with virgin females (dotted line; $p=0.0083$). Thus, survival appears to be independent of the incomplete penetrance of the sterile phenotype found previously in some females.

Two generations after the experiment was completed, we noted that approximately 18% of the 'sterile' females in the ovo^{D1} stock laid eggs. In this stock (no. 2121), incomplete penetrance of the sterile phenotype is not uncommon, and once established, it tends to increase in the population over time (Kevin Cook, Bloomington Stock Centre 2005, personal communication). Although unlikely, a small proportion of our experimental females may have produced eggs, which could bias our results and lead to the reduced survival that we observed in mated females. Accordingly, we repeated our experiment at 3 and 27 h ($n=300$ females), using a different population of the same stock that exhibited nearly complete penetrance (less than 1% laid eggs). Again, we found that the mated females showed a lower survival rate at 3 h after mating in response to infection (figure 2; one-tailed paired *t*-test: $t_4=-3.95$, $p=0.0083$); however, no difference was detected at 27 h ($t_2=0.77$, $p=0.2604$). These data support the original pattern of survival.

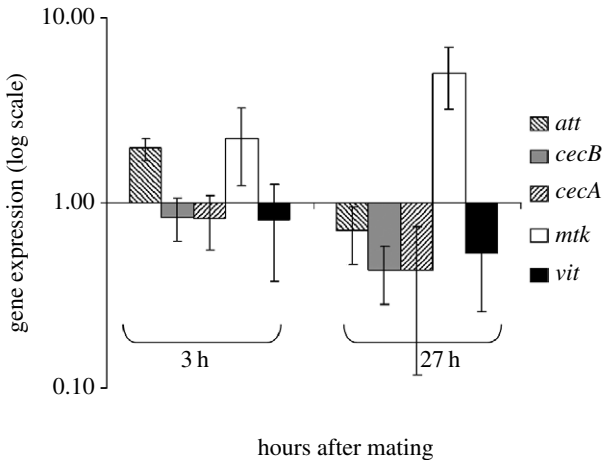

Figure 3. Expression levels of immune genes in mated females. The genes associated with the *Imd* pathway, *attacin* (*att*) and *cecropin A* and *B* (*cecA* and *cecB*, respectively), were either unchanged or upregulated 3 h after mating compared with those in virgin females. At this time, mated females also exhibited a lower survival to bacterial infection (figures 1 and 2). Twenty-four hours later, these same genes were downregulated, at which time mated and virgin females exhibited similar survival rates. *Metchnikowin* (*mtk*), a gene in the *Toll* pathway, was upregulated at both times. *Actin* served as our internal PCR control and vittelogenin (*vit*) provided an estimate of reproductive investment. All gene expression levels are relative to virgins. Error bars represent 95% CI.

(b) *Effect of mating on immune gene expression*

Immune gene expression levels varied according to both mating treatment (mated versus virgin) and time since mating (e.g. 3 versus 27 h). Mated females exhibited an approximate twofold increase in *attacin* 3 h after copulation compared with virgin females (two-tailed *t*-test: $t_2=6.99$, $p=0.0199$; figure 3). Gene expression levels for *cecropin A*, *cecropin B* and *metchnikowin* did not differ between the reproductive treatments during the same time period (all $p>0.1$). Twenty-seven hours after copulation, expression levels of *metchnikowin* were approximately fivefold higher in mated versus virgin females ($t_2=4.41$, $p=0.0478$). In contrast, mated females' expression levels of *attacin*, *cecropin A* and *cecropin B* all exhibited a downward trend compared with their virgin counterparts ($t_2=2.37$, $p=0.1407$; $t_2=4.42$, $p=0.0474$; $t_2=7.59$, $p=0.0169$; $t_2=3.43$, $p=0.0754$, respectively). When gene expression levels were examined across time, we found that both *attacin* and *cecropin B* were significantly downregulated between 3 and 27 h ($t_4=6.77$, $p=0.0025$; $t_4=2.99$, $p=0.0399$; respectively).

When the gene expression data are compared with the survival data, an interesting pattern emerges. When females were infected 3 h after mating, we found that survival was low, even though immune gene expression was normal to high. However, the pattern was reversed when females were infected 27 h after mating, with mated females exhibiting an increased survival coupled with a decreased immune gene expression. Interestingly, levels of vitellogenin production did not increase after mating (figure 3; 3 h: $t_2=0.8531$, $p=0.4835$; 27 h: $t_2=3.43$, $p=0.0754$) or change significantly over the 24 h period ($t_4=1.08$, $p=0.3424$), suggesting that the decrease in survival may not be due to an increase in egg investment.

4. DISCUSSION

Here, we addressed the hypothesis that some aspect of mating outside of energetic demands (e.g. egg production) may reduce immune function by inhibiting immunity shortly after copulation. Three hours after mating, we found that immune gene expression was high in mated females. However, the ability of females to survive a bacterial infection was low compared with their virgin counterparts. Surprisingly, this pattern was reversed within 24 h of mating, with the exception of *metchnikowin*. Furthermore, the effect appeared to be independent of reproductive investment, considering that females did not produce eggs and that levels of yolk protein 1 (a vitellogenin precursor) did not increase after mating. Thus, potential immunity (i.e. gene expression) does not appear to reflect realized immunity (i.e. an individual's ability to defend against a bacterial infection), even though both measures were taken simultaneously.

Several mechanisms exist that may account for our results. First, changes in the level of juvenile hormone may have led to the pattern of reduced immune function just after mating. In *D. melanogaster*, male semen includes sex-peptide (Acp-70), which is passed to females during copulation. Acp-70 has been shown to control oocyte maturation and vitellogenesis through the induction of juvenile hormone (Moshitzky *et al.* 1996; Soller *et al.* 1999). Furthermore, increased levels of juvenile hormone have been shown to inhibit at least one aspect of innate immunity (i.e. phenoloxidase) in the mealworm beetle, *Tenebrio molitor* (Rolff & Siva-Jothy 2002). Thus, a mating-induced increase in juvenile hormone may account for the survival pattern in our experiment. However, juvenile hormone probably operates directly at the level of gene regulation (Harshman & James 1998). If juvenile hormone mediates the trade-off between general immunity and reproduction, as has been suggested (Rolff & Siva-Jothy 2002), then we might expect the female's increased susceptibility to Gram-negative bacteria to be coupled with a downregulation in the Gram-negative immune genes, which we did not find. It may be that the genes associated with the phenoloxidase cascade were indeed downregulated in our experiment. However, what effect this may have on surviving a bacterial immune insult is unknown.

Additionally, we found no evidence for increased vitellogenesis in mated females. These data imply further that juvenile hormone-mediated reproductive trade-offs might not be responsible for our results. However, we did not measure the level of vitellogenin proteins in the haemolymph, nor did we obtain estimates of the two other precursors involved in vitellogenin production (i.e. yolk protein 2 and yolk protein 3). Thus, ovo^{D1} mutants may still be investing in reproduction after mating, especially with regard to other compounds outside of vitellogenesis.

Second, the discrepancy between potential and realized immunity may have been due to an immune refractory period in the mated females. Females in many species exhibit an immune reaction to the male ejaculate (Denison *et al.* 1999; Birkhead 2000; McGraw *et al.* 2004; Peng *et al.* 2005*b*), which is generally assumed to help defend against sexually transmitted disease (Barratt & Pockley 1998; Nunn *et al.* 2000; Nunn 2002). Control females did not receive an ejaculate or risk the sexual transmission of any pathogens. With this in mind, it is

possible that the immune stores of mated females were depleted shortly after mating (due to pathogen transmission) at a faster rate than could be replenished, causing these females to become more vulnerable to our bacterial inoculation treatment. This could have led to the pattern of increased immune gene expression coupled with decreased immunocompetence in the hours following copulation. Once any invading bacteria were neutralized and the immune stores were replenished, immune gene expression levels may have fallen. Again, this would have led to the pattern of increased immunocompetence coupled with a reduced gene expression 27 h after mating.

Third, some aspect of mating (independent of an immune refractory period and the energetics of reproduction) may have reduced immune function by inhibiting potential immunity. In *D. melanogaster*, females release immune genes products (such as attacin and metchnikowin) directly into their reproductive tract after mating (Mack *et al.* 2006). Such an immune reaction may reduce a male's reproductive fitness if these immune products incapacitate sperm (Birkhead 2000). In response, males may pass seminal proteins that reduce female immune function in order to increase sperm viability; a phenomenon that has been observed in several vertebrate taxa (Dostal *et al.* 1997; Maccioni *et al.* 2001). However, how products such as attacin or cecropin would influence sperm viability is unclear, especially considering that these products are assumed to exploit characteristics specific to prokaryotic cells. Alternatively, males may target female immune products designed to incapacitate eukaryotic invaders (pathogenic eukaryotes represent one of the largest sexually transmitted threats in insects; Knell & Webberley 2004), and that the changes in antimicrobial peptides detected here are only correlated to the immune changes that effect sperm viability.

Although ejaculate products would be aimed at suppressing local immunity in the reproductive tract, these products may migrate into the female's systemic system, even though the female's reproductive tract is separated from the haemocoel. Migration of ejaculate products outside of the reproductive tract has previously been documented in *Drosophila*, although no incorporation of these products into the soma or ovaries has been detected in *D. melanogaster* (Pitnick *et al.* 1997). If true, then this would account for our pattern of reduced survival to bacterial infection.

Male manipulation of female physiology is not uncommon. Previous work in *D. melanogaster* has shown that specific male accessory gland proteins (Acps) contained within the ejaculate can increase rates of oviposition (Heifetz *et al.* 2000), decrease a mated female's receptivity to subsequent courting males (Wolfner 1997; Peng *et al.* 2005*a*) and decrease female longevity (Chapman *et al.* 1995; Lung *et al.* 2002). For instance, Chapman *et al.* (1995) showed that increased exposure to Acps led to an increased rate of female mortality in a dose-dependent manner. Likewise, when females were experimentally prevented from coevolving with males, males evolved a more 'toxic' ejaculate that reduced female survival independent of egg production (Rice 1996). It is possible that the male's effect on female survivorship may be mediated by ejaculate-induced changes in the female's immune system.

There are also at least two reasons why natural selection might lead females to mount an immune response in addition to the need to fight-off sexually transmitted pathogens. First, a female may use her immune response to control the amount of sperm used and/or stored by each male, thereby choosing which of two or more mates will fertilize her eggs. Second, females may be selected to mount an immune response to counter the deleterious effects of proteins directly bound to sperm, such as Acp-70, which can decrease female fitness (Liu & Kubli 2003; Wigby & Chapman 2005). In either case, female immunity has the potential to reduce male fitness, creating the opportunity for selection on males to inhibit the female immune response. Thus, there is great potential for an antagonistic coevolutionary dynamic to develop between the ejaculate and the female's immune system.

In summary, our data are consistent with there being an immune refractory period after mating, as well as an immune suppressive effect of the male ejaculate. Both mechanisms can lead to a period of pathogen susceptibility in the female just after mating that is independent of the energetic demands of reproduction. In order to test the male manipulation hypothesis, future experiments should directly manipulate the type of Acps transferred during copulation (e.g. mutant males that produce few Acps), with the hope that we may eventually be able to identify specific immunosuppressive proteins in the male ejaculate.

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