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Females gain survival benefits from immune-boosting ejaculates

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Females in many animal taxa incur significant costs from mating in the form of injury or infection, which can drastically reduce survival. Therefore, immune function during reproduction can be important in determining lifetime fitness. Trade-offs between reproduction and immunity have been extensively studied, yet a growing number of studies demonstrate that mated females have a stronger immune response than virgins. Here, we use the Texas field cricket, *Gryllus texensis*, to test multiple hypotheses proposed to explain this postmating increase in immune function. Using host-resistance tests, we found that courtship, copulation, and accessory fluids alone do not affect female immunity; rather, only females that acquire intact ejaculates containing testes-derived components exhibit significant increases in survival after exposure to bacterial pathogens. Our data suggest that male-derived components originating from an intact ejaculate and transferred to females during sex are required for the increased immune function characteristic of mated female crickets to occur.

KEY WORDS: ***Gryllus texensis*, cricket, immunity, host resistance.**

Mating can result in physical injuries and can drastically increase rates of parasitism, predation, and disease (Arnqvist and Nilsson 2000). All of these costs can result in premature death, making the ability of the body to fight off infection or repair wounds an important aspect of reproduction. This is especially true for females, where fitness is often increased by prolonging survival to afford additional time to produce and care for more offspring (Trivers 1972). Although the ties between reproduction and immunity have been extensively investigated (Morrow and Innocenti 2011), most studies focus on how these two life-history traits trade-off with one another, where an increased investment in one results in a decreased investment in the other (Bascuñán-García et al. 2010; Kelly 2011).

Alternatively, considerable evidence indicates that mated females have stronger immune systems than virgin females across a number of taxa (Morrow and Innocenti 2011). For example, field crickets (*Gryllus*) experience increased survival in host resistance tests after mating (Shoemaker et al. 2006) and mating multiple times increases female lifespan (Wagner et al. 2001). Several hypotheses have been proposed to explain increased immunocompetence after mating, yet they remain untested. One possibility is that because mating increases the rates of infection (Knell and Webberley 2004), natural selection has favored females that preemptively upregulate their immune response when exposed to males that are potentially laden with longevity-reducing pathogens (Rolff 2002). In addition, females can incur significant injuries or acquire deadly pathogens from males during sex (Crudginton and Siva-Jothy 2000), such that increased immunity is a direct response to these challenges (Lawniczak et al. 2007). In both cases, the initial upregulation of the immune system would allow females to combat subsequent infections (e.g., host resistance tests) much more efficiently.

An alternative explanation could be that elements comprising the ejaculate cause changes in female physiology after mating. Accessory gland proteins (Acps) can upregulate antimicrobial gene expression (McGraw et al. 2004) or have intrinsic antimicrobial activity (Mueller et al. 2007), as demonstrated in *Drosophila*. Acps have known roles in immune function in *Gryllus* crickets (Braswell et al. 2006) and if responsible, then females that receive Acps might exhibit increased immune function even when other

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ejaculate components (i.e., testes-derived compounds) are absent. Finally, sperm or other components derived from the testes could activate female immunity (McGraw et al. 2004). For example, sperm could be treated as a foreign body, therefore causing females to upregulate their immune system in defense (Morrow and Innocenti 2011), or seminal products with important roles in reproduction could have dual roles in immunophysiology (Stanley and Kim 2015). In some cases, testes-derived components may even work in concert with Acps, as seen in *Drosophila* (Chapman et al. 2003; Liu and Kubli 2003), such that only fully intact ejaculates will induce an immune response in the female.

Here, we use the Texas field cricket, *Gryllus texensis*, to manipulate the physical and ejaculate-derived cues females receive during mating to investigate the proximate mechanism responsible for the increased disease resistance exhibited by mated female crickets. We therefore experimentally manipulated courtship, copulation, and ejaculate composition to test whether the increased disease resistance exhibited by mated female *G. texensis* is due to a self-regulated increase in immune response prior to mating, direct response to physical contact with males, or the result of ejaculatory compounds derived from the accessory glands (i.e., Acps) and/or testes.

Methods

EXPERIMENTAL ANIMALS

The lab-reared experimental crickets used in this study were second-generation descendants of 250 wild-caught females collected in Austin, TX (USA) in August 2013. Crickets were reared in communal bins (73 × 41 × 46 cm) until their penultimate instar, after which they were isolated in clear plastic 250 ml containers and checked daily for eclosion. All crickets (>10,000) were housed in an environmentally controlled room (27°C; 12:12 h light/dark cycle) and supplied with cotton-plugged water vials and dry cat food (Special Kitty Premium Cat Food) ad libitum.

PREPARATION OF MALES

We randomly assigned female and male adult crickets to one of four treatments: (1) courtship (females experience calling song and mounting of the male but without genital contact), (2) copulation (females experience courtship and genital contact but without ejaculate transfer), (3) accessory fluids (females mate a castrated male and receive an ejaculate lacking testes-derived components), or (4) an intact ejaculate (females mate an unmanipulated male and receive an ejaculate containing both accessory fluids and testes-derived components).

In preparation for the experimental mating trials, we removed the testes-derived components from the ejaculates of males assigned to the "accessory fluid" treatment by castration. To do this, we cold-anesthetized males and surgically removed the testes via an incision between the 2nd and 3rd abdominal segments, then sealed the incision using Vetbond™ Tissue Adhesive (3M, St. Paul, MN). All castrated males made a full recovery from surgery and preliminary studies demonstrated that castrated males did not differ in survival or behavior from control or sham-castrated males (AMW personal observation). Two days after recovery, males were housed individually and allowed to mate ad libitum with receptive virgin females to deplete testes-derived components stored within the seminal vesicles. We regularly examined spermatophores from each male under a compound microscope (Leica DM 2500, Leica Microsystems) until sperm were absent from three consecutive ejaculates. This process takes ~14 days, ensuring that any infection resulting from surgery was cleared prior to the experimental mating (Shoemaker et al. 2006). This method successfully depletes sperm and other testes-derived seminal products from the ejaculate (Worthington et al. 2015) while leaving the seminal fluid proteins of the accessory fluids intact. Using two-dimensional electrophoresis, Larson et al. (2012) confirmed that the spermatophore contents of castrated *Gryllus* males did not differ from those of normal males with respect to seminal fluid proteins and that all 630 protein isoforms derived from the accessory glands remained in the ejaculate following the castration methods described above. To control for the effects of handling, males used in the "courtship," "copulation," and "intact ejaculate" treatments were also cold-anesthetized 5 days after eclosion and handled in a similar manner as the castrated males, but without incision and testes removal. These males were also paired with receptive virgin females for approximately 12 days and we regularly collected spermatophores from them to control for any effects that mating experience, previous sexual contact, or human handling could have on the postmating immune activity of experimental females.

EXPERIMENTAL MATING

Pairs of male (15–19 days post eclosion) and female (10 days post eclosion) crickets were transferred to clear plastic mating arenas under a 25-W red light. Females in the courtship treatment were monitored until the female mounted her partner, then we immediately removed the male prior to genital contact ($n = 25$). Females in the copulation treatment were allowed to mount their partner, make genital contact, and accept a spermatophore, but the male and spermatophore were immediately removed before ejaculate transfer ($n = 18$). To test the effect of accessory fluids alone within the ejaculate, we paired females with castrated males. Once experimental females mated a castrated male, we immediately removed the male and standardized the duration of ejaculate transfer for each female by manually removing the spermatophore 45 min after attachment ($n = 20$). Finally, to test the

effect of an intact ejaculate, we allowed each female to copulate with an unmanipulated male. After spermatophore transfer, the male was immediately removed and again spermatophore attachment time was standardized to 45 min ($n = 24$). After all females had been paired with their respective males, females were transferred to individual containers (16.5 × 10.5 × 7 cm) and supplied with food and water.

HOST RESISTANCE

Three days after mating, we subjected females to a host resistance test to determine each female's relative disease resistance, a proxy for immune competence (Adamo 2004). Crickets were cold-anesthetized and their pronotum length was measured. We then injected an LD_{50} dose (1.0×10^4 cells/2 μl) of the bacterium, *Serratia marcescens*, into the abdomen using a sterile glass microcapillary needle. *S. marcescens* is a Gram-negative bacterium lethal to *G. texensis* (Kelly and Tawes 2013) and is commonly found in their natural environment. After injection, we transferred females to clean containers supplied with water and food. Individuals were observed until they fully recovered from the anesthesia, then monitored for mortality every 12 h for the next five days. Preliminary studies verified that females injected with 2 μl of sterile water experienced 100% survival after 5 days, demonstrating that all female mortality measured in this study was indeed the result of *S. marcescens* and not from complications in recovering from the injection itself.

STATISTICS

Statistical analyses were conducted using R (R Development Core Team Version 3.2.1, 2009) with $\alpha = 0.05$ and visualized using *ggplot2*. We used a Cox regression and log-rank statistics to test the effect of treatment on survival.

Results

We found a significant effect of treatment on survival (log rank test statistic = 26.15, $P > 0.0001$, df = 3; Fig. 1). Further analyses using Cox regression ($n = 88$) revealed that females that received an intact ejaculate survived significantly longer than females that experienced only courtship ($z = 3.789$; $P = 0.0001$), copulation ($z = 4.086$; $P < 0.0001$), or accessory fluids from a castrated male ($z = 4.303$; $P < 0.0001$). No other treatment comparisons were significantly different from each other.

Discussion

Our data are consistent with the hypothesis that ejaculates enhance disease resistance in mated females, as demonstrated by the fact that only females that received an intact ejaculate containing components derived from the testes experienced increase survival from a bacterial pathogen relative to other treatments. These treatment differences in female susceptibility to infection are unlikely due to any detrimental effects that low oviposition rates might have on female condition, as a previous study on *G. texensis* demonstrated that mated females forced to retain eggs do not exhibit decreased survival rates (Shoemaker et al. 2006). We found that females do not preemptively upregulate their immune response when exposed to physical contact or courtship by mature males, as demonstrated by the fact that virgin females prevented from copulating in our study experienced survival rates similar to females housed with mature females or immature males from a replicated study on the same species (Shoemaker et al. 2006), This result is not surprising as immune activation in insects requires significant resource investment (McKean et al. 2008; Ardia et al. 2012), such that the cost of upregulating it in the absence of disease is likely to outweigh the fitness benefits of fighting off a future pathogen more quickly.

The act of copulating without receiving an ejaculate also did not confer enhanced disease resistance to females, ruling out the hypotheses that genital trauma from mating elicits a wound-healing response or that direct male contact results in the transfer of pathogens, either of which could subsequently affect female immunocompetence. Previous research in *D. melanogaster* supports these findings, where expression of immune-related genes due to physical trauma (Wigby et al. 2008) and response to a microbial challenge (De Gregorio et al. 2001) differed from those expressed by females immediately after mating. Similar gene expression studies have yet to be done in crickets; however, they

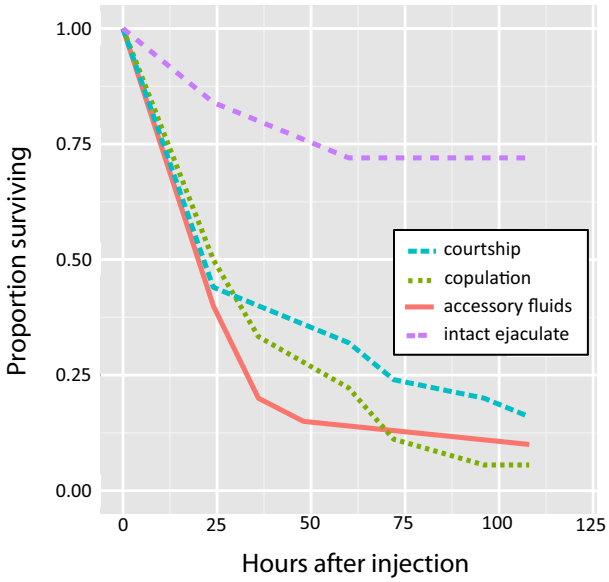

Figure 1. Treatment-specific survival after infection of female crickets experienced courtship ($n = 25$), copulation ($n = 18$), accessory fluids ($n = 20$), or intact ejaculate ($n = 24$).

would be useful in determining how different immune challenges and mating stimuli contribute to the overall activation of the female's immune system.

Although the acquisition of Acps alone did not confer enhanced disease resistance to mated females, there are several potential mechanisms that might be responsible for why intact ejaculates enhance female immunity. First, females could react to sperm as nonself by eliciting an immune response that subsequently primes the body to fight off a secondary infection, although evidence for this is currently limited to placental mammals (Morrow and Innocenti 2011). If sperm is responsible, perhaps a more likely role for it is that it might act as a carrier and reservoir for Acps. In *Drosophila*, Acp70A is bound to sperm and continually released to influence oviposition and female receptivity (Chapman et al. 2003; Liu and Kubli 2003). It is unknown if Acps affecting immunity in crickets (Braswell et al. 2006) also have this close association with sperm, but it remains a plausible explanation for the effect the intact ejaculate has on increasing female disease resistance. It is important to note, however, that female *Drosophila* mated to spermless males still exhibited a weak, short-term increase in oviposition after mating and that the presence of sperm merely strengthened and prolonged this effect. We did not detect any noticeable increase in disease resistance in females mated to castrated males, providing some evidence that Acps are not fully responsible for these physiological changes in mated female crickets.

Alternatively, an uninvestigated mechanism is a hormone-like compound called prostaglandin, which is synthesized in the testes of *Gryllus*, incorporated into the seminal fluid, and transferred to the female during mating (Worthington et al. 2015). Prostaglandin is a major mediator of both insect reproductive physiology and the invertebrate immune system (Stanley and Kim 2015) and therefore has the potential to affect female immunocompetence after mating. Inhibiting prostaglandin synthesis in crickets infected with *S. marcescens* significantly reduces their ability to clear the pathogen, but this effect is reversed by treating individuals with the prostaglandin-precursor arachidonic acid (Miller et al. 1999). Interestingly, the quantity of arachidonic acid required rescue their immune function corresponds to the amount contained within a single spermatophore (Worthington et al. 2015), demonstrating that there is enough prostaglandin and arachidonic acid within the seminal fluid to significantly influence the immune response. This hypothesis has yet to be empirically tested, but it suggests an intriguing mechanism to explain the enhanced immune response exhibited by mated individuals and could offer a more general explanation of the postmating increase in female immunity across taxa (Morrow and Innocenti 2011) as prostaglandin is a known component of ejaculates across taxa, from insects (Stanley-Samuelson and Loher 1986) to mammals (Nomura and Ogata 1976; Templeton et al. 1978).

Although our results bring us closer to understanding the proximate mechanism mediating the postmating increase in female disease resistance, the evolutionary causes and implications of this physiological change remains unknown. It is possible that both males and females produce greater numbers of offspring when females survive better after mating. In such cases, male ejaculates may have evolved to help females offset the extreme survival costs of mating by providing resources that strengthen the female immune system and enhance survival, thereby increasing both male and female lifetime reproductive fitness. However, unnecessary deployment of the immune response is metabolically costly in crickets (Ardia et al. 2012), so females would only benefit if the ejaculate-derived substances helped to strengthen the immune response when activated but did not automatically initiate an immune response upon transfer to the female.

Another possible explanation is that increased disease resistance in mated females could be due to the effects of sexual conflict with males over reproductive decisions, whereby ejaculates force females to increase short-term investment in survival at expense of long-term fitness. For example, short-term activation of the immune response could increase female survival immediately after mating, a point at which mating-induced oviposition rates are the highest (Worthington and Kelly 2016), and therefore guarantee female sperm usage of a recent mate prior to dying. Alternatively, activation of the immune system could cause females to terminally invest in reproduction (Clutton-Brock 1984), increasing the rate of oviposition and sperm usage during the period when the female immune response is active at the expense of future reproduction. Previous research on the effect of immune activation on oviposition in *Gryllus*, however, has demonstrated that bacterial infection does not have a significant effect on oviposition rates (Shoemaker et al. 2006; Shoemaker and Adamo 2007), and infection by a parasitoid actually decreases oviposition (Adamo et al. 1995). Broadly, analyses in other insect taxa agree with the findings that females most often respond to immune upregulation in a manner that compromises the reproductive interests of males (Morrow et al. 2003), so the evolution of ejaculates that adaptively result in physical damage to females or increase female immune investment is unlikely.

Finally, the effect that mating has on female disease resistance could be attributed to the pleiotropic effects of ejaculates, as components that are beneficial to male fitness (e.g., help during sperm competition) may simultaneously have negative effects on female fitness (Parker 1979). It is possible, therefore, that enhanced postmating immune activity is not directly selected for, but is yet another cost of mating that females must endure (Morrow et al. 2003). Crickets are polyandrous (Bretman and Tregenza 2005) and exhibit high mating rates (Worthington and Kelly 2016), so the evolution of ejaculate traits that increase a male's fertilization success is likely, especially considering that

seminal protein genes in crickets evolve more rapidly than genes encoding proteins not involved in reproduction (Andrés et al. 2006). Further, pleiotropic effects of ejaculates is not uncommon in crickets (Green and Tregenza 2009), yet additional studies are required to fully understand the effects of ejaculate components on cricket postmating behavior and physiology. Evidence of the pleiotropic effects of ejaculates is abundant in other insect taxa, such as *Drosophila* (Chapman 2001; Gioti et al. 2012), where ejaculate components with negative fitness consequences for females have primary functions in protecting sperm (Lung et al. 2002), delaying remating (Civetta and Clark 2000; Wigby and Chapman 2005, Fricke et al. 2009), and increasing egg laying (Civetta and Clark 2000). Pleiotropic negative effects of sexual conflict would likely be absent in monandrous species since they would reduce both male and female fitness, so a comparative analysis examining the postmating immune activation in both monandrous and polyandrous species may shed light on this topic if a similar proximate mechanism is responsible across taxa.

In conclusion, we demonstrate that ejaculate components derived from the testes are required to elicit the increased immune activity of mated female crickets; however, whether this is through their own direct action or by assisting Acps to function properly has yet to be determined. Future work will address the specific proximate mechanism underlying this postmating investment in immunocompetence and will examine the ultimate function of ejaculate-derived immune benefits. That female field crickets frequently mate when they apparently receive no direct benefits from males remains an evolutionary puzzle, yet our results suggest that mating does provide females with testes-derived components that boost immunity, which could prolong survival and improve longevity-related fitness benefits. Enhanced disease resistance may be a direct benefit that helps females offset the high risk of infection or injury during the breeding season, or alternatively, it could result from the direct or indirect negative effects that male ejaculates often have on females due to the evolution of sexual conflict over reproductive decisions.

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DATA ARCHIVING

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