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Sexual healing: mating induces a protective immune response in bumblebees

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Abstract:

The prevalence of sexual, as opposed to clonal, reproduction given the many costs associated with sexual recombination has been an enduring question in evolutionary biology. In addition to these often discussed costs there are further costs associated with mating, including the induction of a costly immune response, which leaves individuals prone to infection. Here we test whether mating results in immune activation and susceptibility to a common, ecologically important, parasite of bumblebees. We find that mating does result in immune activation as measured by gene expression of known immune genes, but that this activation improves resistance to this parasite. We conclude that while mating can corrupt immunity in some systems, it can also enhance immunity in others.

Introduction

Sexual reproduction is bi-parental reproduction where, previously successful genotypes are essentially dismantled by splitting the genome in half and then, only one half is passed on to recombine with another previously successful genome dismantled in the same way. As many have noticed, this mode of reproduction is therefore not particularly efficient (Williams, 1975, Maynard Smith, 1971). Despite the obvious costs of sexual reproduction, this mode of reproduction is nearly ubiquitous among eukaryotes (Bell, 1982), and a great many prokaryotes also occasionally exchange genes through other means (Koonin et al., 2001, Gibson & Stevens, 1999). Unsurprisingly, there are many hypotheses that have postulated benefits of sexual reproduction that could counteract these costs. Among those benefits, sex is thought to clear deleterious mutations more rapidly (Kondrashov, 1988), or sex allows for fast adaptation of hosts to coevolving parasites by generating novel host genotypes to which parasite may not be adapted (Bell, 1982, Hamilton, 1980). Adding to the disadvantages of

sexual reproduction, the need for physical mating, as is prominent among species with internal fertilization, carries additional costs (Daly, 1978). For example, finding and courting mates is dangerous and energetically expensive. For instance, close physical contact can increase the risk of contracting infections, perhaps most obviously to sexually transmitted infections (Knell & Webberley, 2004). Mating itself can furthermore cause physical damage, and can induce a strong immune response (Fedorka et al., 2007, Castella et al., 2009, McGraw et al., 2008), which reduces available resources for defending against subsequent infections (Fedorka et al., 2007, Rolff & Siva-Jothy, 2002, Fedorka et al., 2004, Fedorka & Zuk, 2005, McKean & Nunney, 2001). On the other hand, males often transfer immunologically active compounds to their mates, such as anti-microbial peptides in the case of *Drosophila*, which help to protect the female and the sired offspring (Avila et al., 2011).

Social insects, such as some bees, wasps, and all ants, may represent unusual cases in mating induced immune activation, as many - but not all - species mate multiply, yet during a short mating period after which queens of some species can live for many years without further mating. Honeybee queens, which mate multiply, increase expression of immune genes after mating, even in distant tissue such as the brain (Kocher et al. 2008, 2010, Manfredini et al. 2015). Wood ant queens have lower phenoloxidase activity immediately after mating, but over time it increased to exceed that of virgin queens (Castella et al., 2009). These mated queens also have higher antimicrobial responses one week after mating than comparably aged virgin queens (Castella et al., 2009). Leaf-cutting ant queens similarly increase their encapsulation response after mating but the intensity of encapsulation decreases with the amount of stored sperm and the number of males she mated with (Baer et al., 2006) suggesting a trade-off between immune protection and reproductive potential. While all of these studies suggest that there is some form of immune activation after mating, it still

remains to be tested whether this activation corrupts or enhances immunity to natural parasites.

Over the past few decades, the bumblebee *Bombus terrestris* has become a prominent system for the study of natural host-parasite interactions and evolution. Like most bumblebees, queens of *B. terrestris* mate singly (Schmid-Hempel & Schmid-Hempel, 2000), the male transferring a plug (containing linoleic acid) that prevents the queen from mating again (Baer et al., 2001). After mating queens hibernate for up to eight months before emerging and establishing colonies. Young queens - the gynes - emerge into a highly risky environment, as the prevalence of many parasites increases over the colony cycle and is at its highest when daughter queens leave the colony (Popp et al., 2012). Similarly, parasite transmission is high when the young queens emerge from hibernation, as all individuals concentrate on the same few flowers at the beginning of the season. When such daughter queens become infected, for example, by the common trypanosome parasite *Crithidia bombi,* they have dramatically reduced fitness (Brown et al., 2003). As a consequence, misdirected or costly immune responses that leave queens more vulnerable to infection at this stage would be highly damaging. Mating in bumblebees, as it also interferes with further copulations, could be one factor that compromises the immune system and therefore renders young queens more susceptible to infection. Here we test these ideas by investigating the influence of mating on the gene expression and effective defense against infection to a natural parasite in the bumblebee *Bombus terrestris*.

Materials and Methods

We collected queens as they emerged from hibernation in the spring of three years (2010, 2011, 2013) in northern Switzerland and maintained them under conditions conducive to colony establishment (as in (Barribeau et al., 2014)). All of the colonies used for this experiment were inspected carefully for common pathogens twice and found to be clear of infection. Young queens (gynes) and males that were produced towards the end of the colony cycle were removed and given the opportunity to mate in an enclosure (mating cage) by adding three males to a single gyne per cage. The gynes and males were paired from different colonies to avoid inbreeding. After mating, we hibernated the fertilized queens for three months at 4˚C. After taking them out from hibernation, the queens were allowed to establish colonies in the lab. All experiments used the sexual offspring (males, daughter queens) from the colonies derived from these lab-reared queens. Hence, we tested all questions by using the F2-generation of field-caught spring queens to standardize history and remove carry-over effects from the field.

We conducted three experiments. (1) In a preliminary experiment, gynes and males were randomly allocated to either a 'mated' or 'unmated' condition. In the 'mated' condition males and gynes from different colonies were paired and allowed to mate. We kept the individuals in the 'unmated' condition individually isolated for the same amount of time as those in the 'mated' condition. We snap froze the bees in liquid nitrogen two hours after mating finished, along with the individuals that had been kept in isolation for the 'unmated' condition (numbers of unmated gynes: 8; mated gynes: 11; unmated males: 5, mated males: 8).

(2) In a second experiment we altered this protocol. Whilst keeping a 'mated' condition, we also allowed unmated queens access to males but prevented mating by sealing the males' terminal abdominal tergites with wax ('frustrated' condition). This allowed the males to

attempt copulation but prevented genital clasping and intromission. We snap froze the gynes from these conditions again 2 h after mating (n = 7 queens for 'frustrated', and n = 4 queens for the 'mated' condition). In addition, we had groups snap frozen at additional time points, this is, at 6 h (n = 6 frustrated, and n = 7 mated) and 24 h (n = 14 frustrated, and n = 18 mated) after mating. We now also exposed a subset (n = 23) of the gynes - destined to be frozen at 24 h - with *C. bombi* six hours after mating with a cocktail of 20,000 cells from four clones (equal amounts each; strain IDs were: 08.068, 08.075, 08.161, 08.261) of the parasite. Individuals that did not eat the inoculum were excluded from the experiment. All bumblebees were housed individually in boxes and fed with pollen and sugar water *ad libitum*. Finally, we exposed in the same way, and monitored the infection success in an additional 74 gynes (37 of which were from the 'mated' and 37 of which were from the 'frustrated' condition) after one week by visually checking the feces for the presence/absence of *C. bombi* cells. All gynes were randomly assigned to an experimental condition and sampling time point. As the infection success in the previous (2) experiment was modest, we repeated the experiment as before (3) but only to monitor infection success with a higher dose of 40,000 cells to ensure that any differences in infection outcome found earlier was not due to dose limitation (n = 77 bees infected; mated: n = 37, frustrated: n = 40).

Molecular methods

We extracted total RNA from whole bumblebee abdomens with RNeasy plus mini kits (Qaigen, UK). We checked RNA integrity with a 2100 Bioanalyzer (RNA 6000 Nano Kit, Agilent Technologies) and synthesized cDNA with QuantiTect reverse transcription kits (Qiagen, UK). We measured gene expression as in (Barribeau et al., 2014, Brunner et al., 2013, Brunner et al., 2014) using a Fluidigm 96.96 dynamic array IFCs on the BioMark

system with EvaGreen DNA binding dye (Biotium) with three technical replicates according to the advanced development protocol 14 (PN 100-1208 B). We measured the expression of 27 target genes, as informed by the analysis of the immune genes in the bumble bee genome (Barribeau et al., 2015), relative to the invariant geometric mean of five housekeeping genes (dCt-values, Table S1). The details of these primers can be found in Table S1.

We targeted genes that span multiple classes of immune function and pathways including recognition (BGRP, BGRP2, dscam, PGRP-LC, PGRP-S3), signal transduction (calcineurin, hopscotch, pelle, relish), effectors (abaecin, apidaecin, defensin, ferritin, hymenoptaecin, lysozyme, TEP-A, transferrin), melanization (peroxidase, PPO, punch, serpin 27a, yellow), reactive oxygen species regulation (jafrac, peroxiredoxin 5), and metabolic and lipid transfer (apolipophorin III, cytochrome P450, vitellogenin). We preferentially included genes where interesting transcriptional responses upon infection had already been found (Barribeau et al., 2015, Barribeau et al., 2014, Brunner et al., 2013, Brunner et al., 2014, Erler et al., 2011, Radyuk et al., 2010, Riddell et al., 2009, Riddell et al., 2011, Roditi, 2008, Schlüns et al., 2010, Vogel et al., 2011). The choice of the reference gene set was based on previous studies (Hornáková et al., 2010) and our own expression stability tests (Brunner et al., 2013). Gene details, primer sequences and NCBI accession numbers are summarized in the electronic supplementary material, Table S1.

Statistical methods

In the first experiment we analysed how expression differed according to sex and whether they mated or did not mate, in a MANOVA where each gene's expression added as a component to the overall multivariate response (R, stats package). To improve multivariate normality, expression-values (dCt) of five genes was transformed using Yeo-Johnson power

transformations (R, car package; apolipophorin III: -0.01, BGRP2: 0.22, CYP4GII: -0.19, dscam: log). We also used a linear discriminant analysis (LDA) to assess how individuals grouped according to treatment and sex based on their gene expression and assessed the predictability of these groupings using leave-one-out cross validation. We analysed gene expression similarly in the second experiment, with time and mating condition as fixed factors. One sample was dropped because of high variation across technical replicates. Five genes required Yeo-Johnson power transformations to meet assumptions of multivariate normality (Apidaecin: 0.5, CYP4GII: 4.28, ferritin: 4.16, vitellogenin: log, yellow: log). In both analyses, if the MANOVA revealed a significant effect of any fixed effect or interaction, we examined the univariate ANOVA results for each gene (Bray & Maxwell, 1982). While the univariate analyses of MANOVA protects against *P* value inflation to some extent (Bray & Maxwell, 1982), we also tested for statistical significance after several multiple testing corrections using p.adjust (R, stats package: Benjamini & Hochberg false discovery rate, Benjamini & Yekutieli false discovery rate, Holm and Bonferonni adjustments, Table S2-3). We analyzed how infection varied by mating condition using a generalized mixed model with a binomial distribution in R (stats package) with block (expt 2 or 3) and mating as fixed, crossed effects (infection ~ block*mating).

Results

Gene expression. Males and gynes from our first experiment, where individuals were allowed to mate, or were kept singly, differed remarkably in their overall gene expression profile (for gene and primer details see Table S1, MANOVA $F_{2,27} = 115.54$, $p = 0.009$, Table S2). The genes that were either male- or gyne-biased in expression can be found in Fig 1A.

Whether or not individuals mated did significantly influence the overall gene expression (MANOVA $F_{2,27} = 23.71$, $p = 0.041$), with the significantly altered genes shown in Fig 1B. There was a trend suggesting that males and gynes responded differently upon mating (sex*mating interaction, $F_{2,27} = 15.98$, $p = 0.061$). The linear discriminant analysis revealed that gynes that mated were more different in their expressed genes from their non-mated counterparts than the respective groups were different in the males (Fig 1C). Jackknifed leave-one-out validation was able to accurately predict membership to a group 62.5% of the time (considerably better than the 25% accuracy predicted by chance). Predictive power was better with gynes, accurately predicting unmated gynes 87.5% and mated gynes 75% of the time, vs. 37.5% for unmated males and 50% for mated males. All of these values, however, exceed the 25% random prediction.

In our second experiment, we focused on gyne expression when they were allowed to mate, or were in the presence of a male who was unable to physically transfer sperm, termed the 'frustrated' condition. Here we also explored how immune gene expression changed in gynes in response to mating and parasite exposure over time. We detected no significant effect of the parasite 18 h after infection (which is 24 h after mating; MANOVA $F_{2,27} = 0.66$, $p = 0.11$), so we excluded the factor 'exposure' from the full model. But, both, the time point sampled and mating strongly influenced expression, and expression varied depending on the interaction of these factors (MANOVA, time: $F_{26,27} = 6.373$, $p < 0.0001$; mating $F_{26,27} = 18.952$, $p < 0.0001$; time*mating: $F_{26,27} = 3.744$, $p = 0.00060$, Table S4). The genes that individually varied in their expression, either according to mating or time as the main effect, or according to the interaction of the two factors, are shown in Fig 2A. Genes that only varied by mating or time are shown in Fig 2B-C.

Resistance. In two separate experiments, we tested mated and 'frustrated' queens, which were allowed access to males but prevented actual sperm transfer, for their resistance against infections by the parasite *C. bombi*. Contrary to the expectation from an immune cost of mating, we found that mated gynes were almost three times *less* likely to become infected than unmated 'frustrated' gynes (testing all cases from both experiments: 11% vs 27% infected, $\chi^2_{1,148} = 6.91$, $p = 0.0086$). The different experiments had different average infection rates but there was no interaction between experimental block (experiment 2 vs. 3) and mating condition ($\chi^2_{1,147} = 0.74$, $p = 0.39$), suggesting that the effect of mating was consistent across both experiments.

Discussion

Contrary to a number of previous studies in other insects (McKean & Nunney, 2001, Rolff & Siva-Jothy, 2002, Fedorka et al., 2004, 2007, Fedorka & Zuk, 2005), we find that mating reduced the likelihood of infection by two thirds - although, consistent with other work (Avila et al., 2011), we see that mating induced an immune response as measured by a change in expression of a number of key immune genes for up to 24 h post-mating. These included genes responsible for the recognition of pathogens (BGRP1, BGRP2, PGRP-LC), for signaling (relish, hopscotch), for melanization (SPN27a, punch), as well as effectors that damage parasites (the anti-microbial peptides apidaecin, abaecin, defensin, and hymenoptacin) (Fig1B, 2A-B). The expression of anti-microbial peptides is extraordinarily high in the mating condition. For instance, the expression of defensin is more than 120 times higher in mated queens than in frustrated queens 24 h after mating (Fig 2A). This high expression of anti-microbial peptides may explain the reduced infection of mated queens. Infection with *C. bombi* results in increased expression of anti-microbial peptides (Barribeau

& Schmid-Hempel, 2013, Barribeau et al., 2014, Brunner et al., 2013, 2014, Riddell et al., 2009, 2011). One study found that *C. bombi* genotypes that induced high anti-microbial peptide expression was least likely to establish successful infection, indicating that higher expression of these peptides may explain reduced infection after mating (Barribeau et al., 2014). Richter et al. (2012) found that social context influences *B. terrestris* immune expression. When workers were housed with other workers they expressed anti-microbial peptides, lysozymes, and components of the melanization response (SPN27a, PPO) more strongly than if they were housed singly (Richter et al., 2012). We find that many of these same genes (abaecin, defensin, hymenoptaecin, SPN27a) and other functionally linked genes like the antimicrobial peptide apidaecin and upstream recognition and signaling genes (BGRP1&2, PGRP-LC, relish), and another melanization response gene, punch, are higher expressed in mated queens relative to unmated queens that had a similar social condition. This suggests that mating *per se*, rather than social context, influenced immune gene expression in these queens. Mating-induced defenses perhaps make the most sense in terms of defending against sexually transmitted diseases. Peng *et al.* (2016) recently demonstrated that honeybee seminal fluid has antimicrobial activity which can inhibit the sexually transmitted microsporidian parasite *Nosema apis*. While mating-induced defenses may be very important in defense against sexually transmitted infections, such kinds of parasites arguably are generally rare in social insects (Knell & Webberley, 2004, Schmid-Hempel, 1998), and other kinds of pathogens or other transmission pathways might therefore be more relevant. Regardless of the induced function in the reproductive tract, our results demonstrate that mating can itself activate the immune response, and which in turn can protect queens from non-sexually transmitted parasites, such as *C. bombi*, that infects other tissue, *i.e.* the gut in this case. Previous work with honeybees that explored gene expression associated with behavioral shifts after mating also found increased expression of immune genes, including

anti-microbial peptides and recognition genes such as BGRPs and PGRPs, even in the brain (Kocher et al 2008, 2010, Manfredini et al. 2015). These studies suggested that increased immune gene expression may help to protect queens from subsequent infection but did not directly test their defense after mating. Some genes in our study had a distinct male or queen bias in expression (Fig1A) but none of these showed an interaction between sex and mating, suggesting common responses of these genes even if they differ in expression between males and queens. Our discriminant analysis suggests that queens respond much more strongly and distinctively to mating than males (Fig. 1C). The expression of many genes also changed with time according to mating condition. In fact, the expression of AMPs, recognition, and melanization genes increased over time in the mated queens but remained relatively constant in unmated queens. Apolipophorin III and vitellogenin, which are involved in lipid movement and yolk provisioning decreased in mated queens (Fig. 2A). Interestingly, decreases in apolipophorin III also leads to immunosuppression in crickets (Adamo et al., 2008), which might suggest that the observed changes reflect a regulation of the immune response. Further recent work in Texas field crickets (*Gryllus texensis*) demonstrated that transfer of the intact ejaculate, rather than courtship, copulation without spermatozoa transfer, or the transfer of accessory gland fluids without sperm, protects females against the generalist bacterial pathogen, *Serratia marscesens* (Worthington & Kelly, 2016).

Bumblebees queens from most species mate singly, even when polyandry confers tangible benefits by increasing their colony defense against infection (Baer & Schmid-Hempel, 1999), but see (Baer & Schmid-Hempel, 2001). During mating in bumblebees, males will clasp the female and maintain copulation for over an hour in some species (Goulson, 2010) during which time he will transfer sperm and a mating plug that prevents multiple mating by the queen (Baer et al., 2001). A mating plug that discourages sperm competition and multiple paternity of the colony are advantageous for males but not necessarily for the female. In

social insects, females often store sperm for extended periods of time. In ants sperm storage leads to a cost for immune function (Baer et al., 2006). In bumblebees, queens mate before the diapause and store the sperm until use the next season. In this system, too, the insemination of queens can reduce her hibernation success even though only males from certain colonies are 'harmful' (Korner & Schmid-Hempel, 2003, Baer & Schmid-Hempel, 2001) although the mechanism behind this effect remains unknown. This cost could be outweighed by the induced immune response - as shown here - that may serve to protect the male's investment. It may also protect the female from becoming infected around the mating period. More importantly, an enhanced readiness of the immune system would benefit the young queen when she comes out from hibernation the next spring, as she would suffer considerable fitness losses if she became infected (Brown et al., 2003); as yet, there is no evidence for this effect.

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Figure Legends.

Figure 1 Mean log2 fold expression (ddCt) ± 1 SEM of target genes relative to housekeeping genes for the main effects of A sex and B mating condition. A) Positive values indicate higher expression in queens while negative values indicate higher expression in males regardless of mating condition. B) mated and unmated bees, ignoring sex of the bee. Here positive values indicate genes that are more highly expressed in mated bees and negative values indicate genes more highly expressed in unmated bees. All shown genes are significantly different among their respective groups based on univariate analyses of MANOVA which, to some degree protects against *P* value inflation (Bray & Maxwell, 1982), we also denote genes that were significantly different after additional multiple testing correction (* < 0.05, ~ < 0.1, Table S2-3)C: Linear discriminant analysis of all genes according to sex and treatment group. Full statistics can be found in Tables S2-3.

Figure 2 A: Mean log2 fold expression (dCt) ± 1 SEM of queens where there were significant main effects of both mating and time effects (apidaecin, BGRP2, Punch, SPN27a) or a significant interaction between mating and time (abaecin, apolipophorin III, BGRP1, defensin, vitellogenin). B: boxplots of log2 fold expression of queens that differ according to mating treatment across all timepoints. Full statistics can be found in Tables S4-5.