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Partitioning the effects of mating and nuptial feeding on the microbiome in gift-giving insects

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Summary

Mating is a ubiquitous social interaction with the potential to influence the microbiome by facilitating transmission, modifying host physiology, and in species where males donate nuptial gifts to females, altering diet. We manipulated mating and nuptial gift consumption in two insects that differ in nuptial gift size, the Mormon cricket *Anabrus simplex* and the decorated cricket *Gryllodes sigillatus*, with the expectation that larger gifts are more likely to affect the gut microbiome. Surprisingly, mating, but not nuptial gift consumption, affected the structure of bacterial communities in the gut, and only in Mormon crickets. The change in structure was due to a precipitous drop in the abundance of lactic-acid bacteria in unmated females, a taxon known for their beneficial effects on nutrition and immunity. Mating did not affect phenoloxidase or lysozyme-like antibacterial activity in either species, suggesting that any physiological response to mating on host-microbe interactions is decoupled from systemic immunity. Protein supplementation also did not affect the gut microbiome in decorated crickets, suggesting that insensitivity of gut microbes to dietary protein could contribute to the lack of an effect of nuptial gift consumption. Our study provides experimental evidence that sexual interactions can affect the microbiome and suggests mating can promote beneficial gut bacteria.

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Introduction

Social interaction (Archie and Tung, 2015; Smith and Mueller, 2015) and diet (Ley *et al*., 2008; Muegge *et al*., 2011; Yatsunenko *et al*., 2012; David *et al*., 2014) are two key factors that influence the composition of the microbiome. Of the types of social interactions animals engage in, mating is both ubiquitous and among the most likely to influence host microbial communities due to the opportunities it creates for transmission and profound effects on host physiology. Yet scant attention has been paid to the influence of mating on microbial symbiosis beyond the transmission of pathogenic infections (Lockhart *et al*., 1996; Knell and Webberley, 2004) despite the fact that beneficial microbes can also be sexually transmitted during the mating process (Lombardo *et al*., 1999; Smith and Mueller, 2015). Mating also alters the expression of hundreds of genes involved in metabolism, reproduction, and immunity (McGraw *et al*., 2008), which potentially could influence host-microbe interactions. The host immune system in particular plays a critical role in the regulation of the microbiome (Ryu *et al*., 2010; Hooper *et al*., 2012; Engel and Moran, 2013), which in turn influences host immune function (Hooper *et al*., 2012; Engel and Moran, 2013; Levy *et al*., 2015), nutrition (Turnbaugh *et al*., 2006; Engel and Moran, 2013), and behavior (Archie and Theis, 2011; Forsythe and Kunze, 2013).

Sexual interactions can also influence diet, an important determinant of the constitution of the microbiome (Ley *et al*., 2008; Muegge *et al*., 2011; Yatsunenko *et al*., 2012; David *et al*., 2014). In many animals, males provide nuptial gifts that females ingest during courtship or copulation (Yosef and Pinshow, 1989; Vahed, 1998; Gomes and Boesch, 2009). Male crickets and katydids in particular are known for the production of a spermatophylax, a proteinaceous, sperm-free mass that is eaten by females. Consumption of the spermatophylax has varying effects on female fitness, increasing survival and fecundity in some taxa (Gwynne, 1984a; Simmons, 1990; Gwynne, 2008) while producing no apparent benefit in other taxa (Will and Sakaluk, 1994; Vahed, 2007). This has led to extensive debate over spermatophylax evolution. Several lines of evidence suggest that the spermatophylax serves only as an ejaculate protection device to prevent the female from eating the sperm-

laden ampulla (Vahed, 2007), which is transferred with the spermatophylax to females during copulation. These nuptial gifts are not necessarily expected to provide a nutritional benefit, only properties that distract the female long enough for sperm transfer to complete (Vahed, 2007). In contrast, the spermatophylax is expected to be nutritious when it serves as a form of paternal investment that increases the number or quality of offspring sired by the male (Gwynne, 2008). Which of these two explanations is correct is likely to have important implications for how nuptial gifts influence the microbiome, as protein intake can induce rapid changes in the gut microbial communities (Wu *et al*., 2011; David *et al*., 2014).

We manipulated nuptial feeding and mating to measure their effects on the gut microbiome in two insects that differ in the size of their gifts, the Mormon cricket, *Anabrus simplex* (Orthoptera: Tettiginiidae), and the decorated cricket, *Gryllodes sigillatus* (Orthoptera: Gryllidae). Mormon crickets produce a spermatophore six times larger than *G. sigillatus* (19% vs 3% of male body mass; Gwynne, 1984b; Sakaluk, 1985, Fig. 1) and are a well-known example of nutrition-dependent sex-role reversal, with females competing for access to spermatophylax-producing males when food is scarce (Gwynne, 1984b, 1993). In contrast, the *G. sigillatus* spermatophylax is no larger than that required for sperm transfer (Sakaluk, 1984) and does not provide any detectable nutritional benefit to females (Will and Sakaluk, 1994; but see Ivy *et al*., 1999). Given this evidence, we expect that spermatophylax consumption will exert larger effects on the gut microbiome of Mormon crickets than decorated crickets. Whether mating influences the microbiome depends on the potential for sexual transmission, as well as the effect of mating on the physiological state of females. We assessed these alternatives by screening male and female reproductive tissues for bacteria and measuring components of the immune system that are known to change in response to mating in insects.

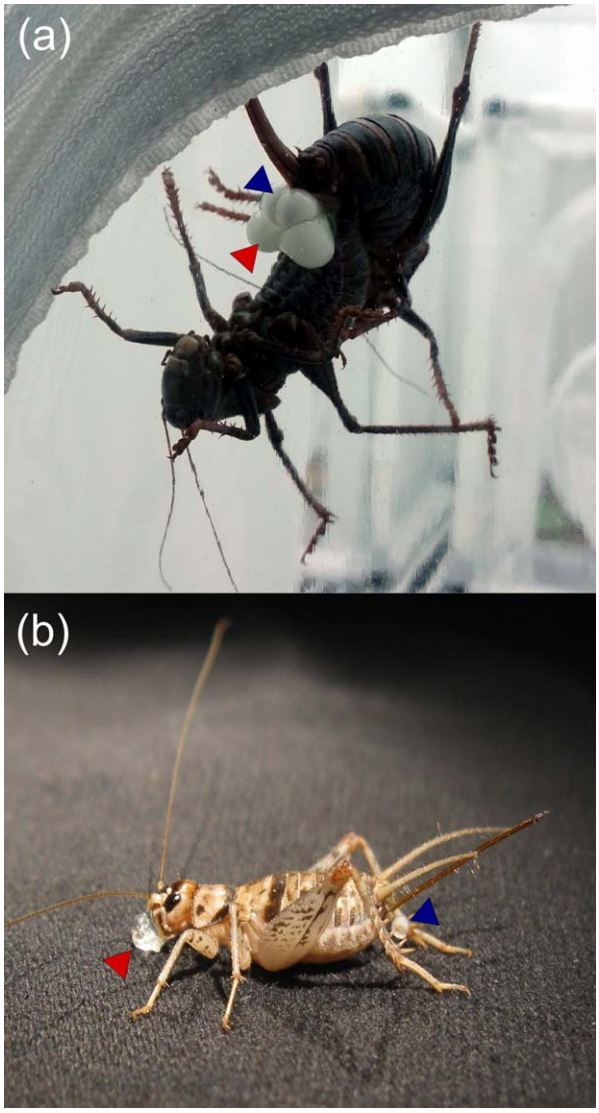

Fig. 1. (a) Mormon cricket *Anabrus simplex* female (top) and male (bottom) in copula and (b) a decorated cricket *Gryllodes sigillatus* female after mating. Red arrow indicates the spermatophylax and blue arrow indicates the ampulla.

Results and Discussion

Mating and the microbiome

We found that mating, but not spermatophylax consumption, influenced the structure of the gut microbiome of Mormon crickets (Fig. 2, Table 1), while neither had an effect in decorated crickets (Table S1, Supporting Information). Among-female variation in microbiome structure collapsed in unmated females (Fig. 2), and ordination of the Mormon cricket OTU scores suggested that five operational taxonomic units (OTUs), all of which were lactic-acid bacteria (Lactobacilliaceae), changed in abundance in response to the mating treatment (Fig. 3). Two of these were among the 15 dominant members of the Mormon cricket microbiome (Figure S1, Supporting Information, *Pediococcus acidilactici* 102222 and *Pediococcus sp*. 17309), while the other three occurred at a lower frequency (*Lactobacillus sp*. 288584, *Pediococcus sp*. 733251, and *Lactobacillus sp*. 1110317).

We compared the abundance of these five lactic-acid bacteria among treatments in univariate analyses and found that three differed depending upon whether females had mated or not, including *P. acidilactici* 102222 and *Pediococcus sp*. 17309 (Fig. 4, Table 2). Comparisons of fecal samples taken before and after the treatments indicated that all three lactic-acid bacteria experienced a precipitous decline in unmated females, but persisted in mated females, resulting in higher abundances in mated females at the end of the experiment (Fig. 4, Table 2).

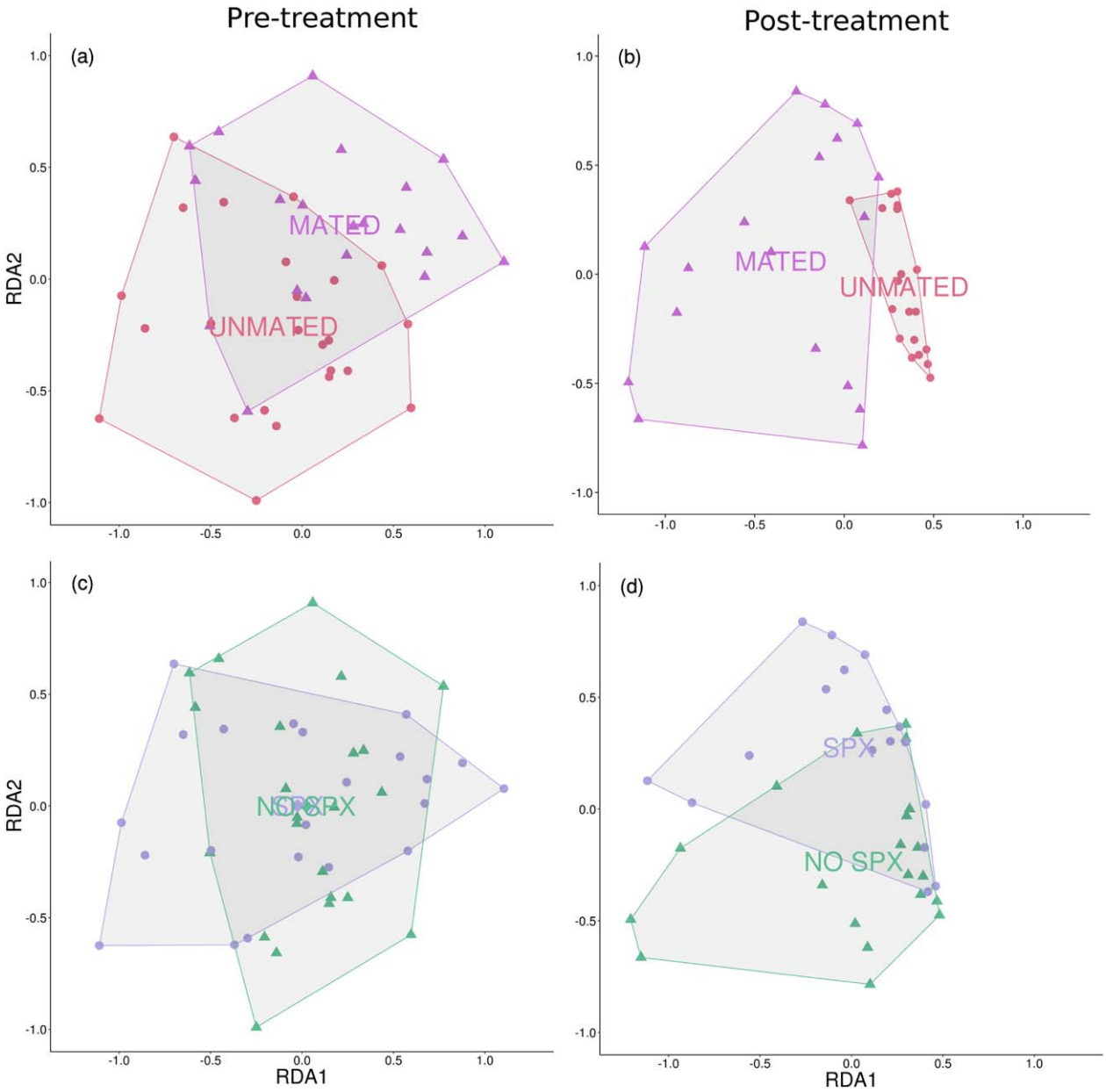

Fig. 2. Ordination of Mormon cricket sample scores from a distance-based redundancy analysis. Points are colored to indicate whether a cricket was mated (triangles) or unmated (circles) (a,b) and whether they were allowed to consume the spermatophylax (circles) or not (triangles) (c,d). Text corresponds to the centroids for samples collected before (a,c) or after (b,d) the treatments were applied. Alpha diversity was not affected by mating or spermatophylax consumption (Tables S2 and S3, Supporting Information).

Lactic-acid bacteria are known for their beneficial associations with the gastrointestinal tract of animals (De Vos *et al.*, 2009; Walter *et al.*, 2011), including insects (Forsgren *et al.*, 2010; Storelli *et al.*, 2011; Vásquez *et al.*, 2012; Erkosar *et al.*, 2015). *P. acidilactici* specifically has been shown to increase digestive efficiency (Castex *et al.*, 2008) and reduce susceptibility to infection (Castex *et al.*, 2009), most likely by priming the innate immune system (Standen *et al.*, 2013) and producing antimicrobial compounds that kill foodborne pathogens (Millette *et al.*, 2007).

One way social behavior can alter the microbiome is by facilitating transmission of microbes between members of the group (Lombardo, 2008; Archie and Tung, 2015). Sexual transmission is unlikely to explain our results, however, because the male spermatophore and female spermatheca were negative in our 16s PCR screens for bacteria, perhaps because of antimicrobial activity in the reproductive tissues. Sexual transmission of both pathogenic and beneficial microbes, however, does occur in insects (Knell and Webberley, 2004; Smith

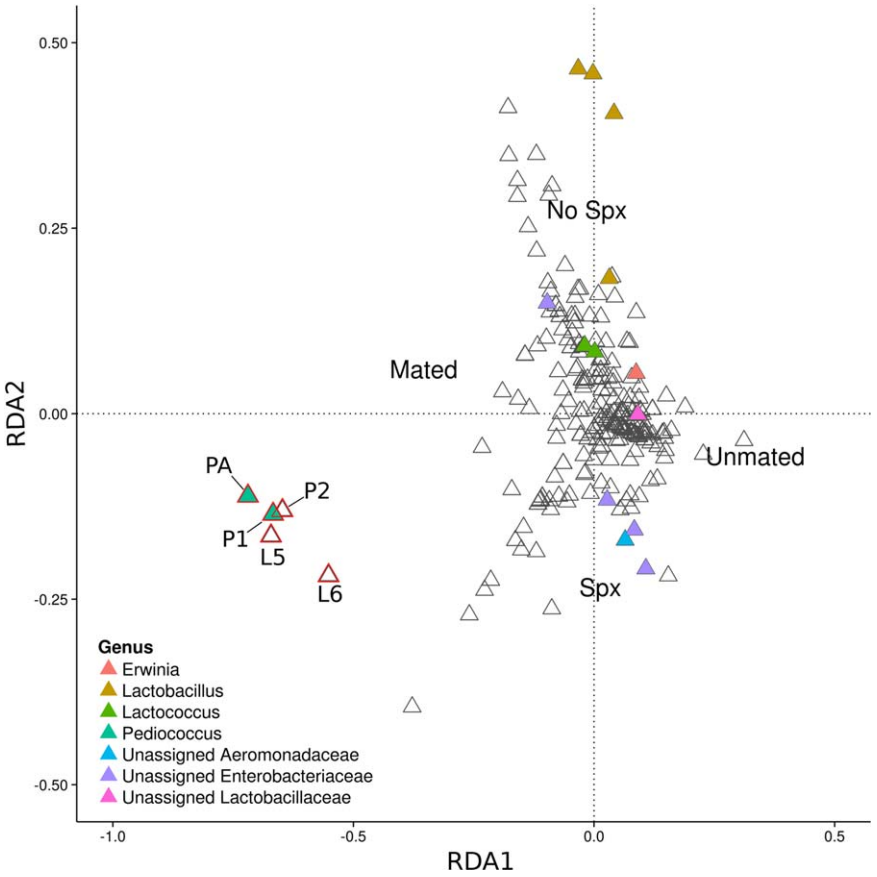

Fig. 3. Ordination of Mormon cricket OTU scores from a distance-based redundancy analysis. Each triangle represents an OTU, with text indicating the centroid of the sample scores from each treatment. Filled triangles are the top 15 most abundant OTUs colored by genus. Labeled OTUs are those displaced along the axis associated with mating and individually analyzed for differences in abundance (see Figure 4, Table 2). PA = *Pediococcus acidilactici* 102222, P1 = *Pediococcus* 17309, P2 = *Pediococcus* 773251, L5 = *Lactobacillus* 288584, L6= *Lactobacillus* 1110317.

Table 1. Permutation tests from distance-based redundancy analysis of female Mormon cricket fecal samples.

		F	*P*
Full model	Mate	0.67	0.66
	Spermatophylax	0.19	0.99
	Time	**3.00**	**0.01**
	Mate * Spermatophylax	1.43	0.20
	Spermatophylax * Time	0.61	0.71
	Mate * Time	**2.39**	**0.04**
Pre-experiment	Mate	0.76	0.74
	Spermatophylax	0.38	0.99
	Mate * Spermatophylax	0.89	0.55
Post-experiment	**Mate**	**1.61**	**0.02**
	Spermatophylax	1.05	0.32
	Mate * Spermatophylax	0.39	0.41

Time refers to whether a sample was collected before or after the treatments were applied

and Mueller, 2015), and more studies are needed to evaluate their prevalence and effects on host fitness and reproductive behavior. Contact with male feces might also have provided an opportunity for the transmission of lactic-acid bacteria to mated females, as coprophagy is a mechanism for microbiome acquisition in other insects (Engel and Moran, 2013). Unmated females, however, had similar levels of lactic-acid bacteria prior to the experiment (Fig. 4) and contact with their own feces in the enclosures. Experiments that cohouse one or both sexes without sexual contact and that explicitly manipulate fecal exposure are required to test whether non-sexual social interactions could also elicit the response of the gut microbiome we observed.

Changes in host physiology in response to social interaction, or lack thereof, could also explain shifts in microbiome structure. Hormones that regulate appetite, energy expenditure, and metabolism are thought to affect the gut microbiome by altering immune function, mucous production in the gut epithelia, and food intake (Spor *et al.*, 2011). Similarly, the stress response (Jašarević *et al.*, 2015; Sandrini *et al.*, 2015) and fluctuations in reproductive hormones (Gajer *et al.*, 2012; Brotman *et al.*, 2014) are associated with changes in the composition of the microbiome. Mating in *Drosophila*

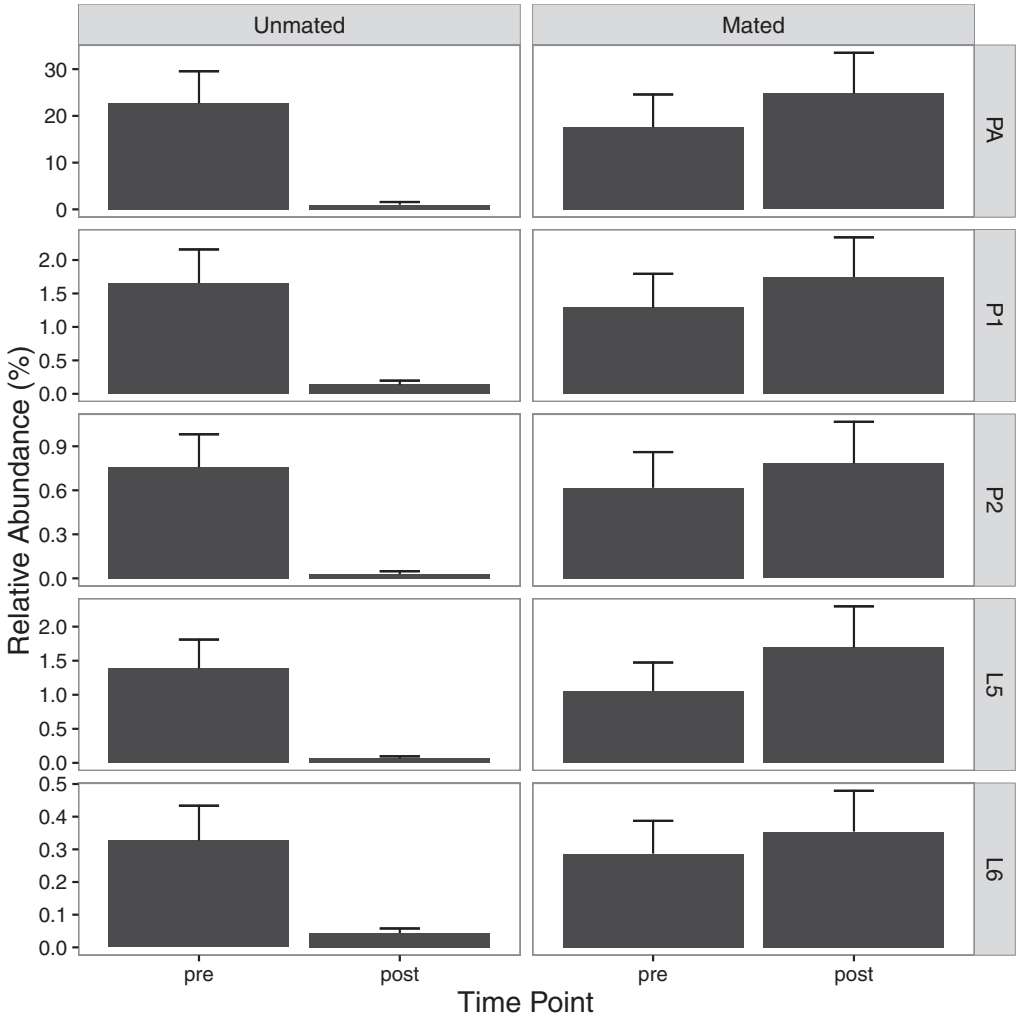

Fig. 4. Relative abundance of five OTUs putatively associated with mating in Mormon crickets. Time point indicates whether samples were collected before or after the treatments were imposed. A significant interaction between mating and time point was detected for the top 3 panels (Table 2).

influences the expression of >1700 genes involved in these physiological processes (McGraw *et al*., 2008), many of which are expressed outside of the female reproductive tract and thus could influence host-microbiome interactions in the gut and other tissues. Whether similar physiological responses to mating can be generalized to other insects, and whether these specific changes do influence host-microbe interactions, remains to be elucidated.

A well-documented physiological response to mating in insects is the suppression of the immune system (Harshman and Zera, 2007), which is a key regulator of the microbiome (Ryu *et al*., 2010; Hooper *et al*., 2012). We measured three components of systemic immunity and found that immunological activity was unaffected by mating (Tables S4 and S5, Supporting Information). This suggests that if the lactic-acid bacteria identified in our study are influenced by the immune system, it likely occurs locally within the gut rather than in response to systemic changes in immunity. This is consistent with experiments in *Drosophila*, where the immune response in gut epithelia is induced by oral introduction of bacteria but not after injection of the same bacteria into the hemocoel (Tzou *et al*., 2000).

Nuptial gift consumption and the microbiome

In contrast to our expectation that larger nuptial gifts should elicit a greater change in microbiome composition, spermatophylax consumption did not affect the gut bacterial communities in either species (Table 1 and Table S1, Supporting Information). At least three non-mutually exclusive possibilities could explain this result. First, it is possible that the spermatophylax is not a highly nutritive meal for the female (Vahed 1998; Vahed 2007), even in Mormon crickets. Hemolymph protein

Table 2. OTU abundance of five taxa putatively associated with mating in Mormon crickets.

		PA	P1	P2	L5	L6
GLM	Mate	3.26 (0.28)	3.26 (0.28)	3.01 (0.28)	2.38 (0.28)	
	Spermatophylax	0.24 (0.84)	0.39 (0.82)	0.25 (0.83)	0.17 (0.85)	
	Time	**13.9 (0.006)**	3.16 (0.28)	3.14 (0.28)	2.42 (0.28)	
	Mate * Spermatophylax	0.01 (0.99)	0.52 (0.77)	0.02 (0.96)	0.07 (0.92)	
	Spermatophylax*Time	0.72 (0.68)	2.42 (0.28)	0.34 (0.83)	0.05 (0.92)	
	Mate * Time	**12.1 (0.007)**	**10.7 (0.01)**	**8.30 (0.03)**	5.20 (0.13)[†]	
Wilcoxon	Pre-experiment samples: Mated vs. unmated females					300 (0.85)
	Post-experiment samples: Mated vs. unmated females					100 (0.40)
	Unmated females only: pre vs. post experiment					200 (0.40)
	Mated females only: pre vs. post experiment					200 (0.99)

Values represent the X^2 (*P*-value) from an analysis of deviance, except for L6, which was analyzed with Wilcoxon signed-rank tests. Significant terms are in bold ($P < 0.05$). PA = *Pediococcus acidilactici* 102222, P1 = *Pediococcus* 17309, P2 = *Pediococcus* 773251, L5 = *Lactobacillus* 288584, L6= *Lactobacillus* 1110317

[†]$P = 0.02$ before FDR correction for multiple tests.

was higher in Mormon crickets that mated and consumed the spermatophylax in our study (Table S4, Fig. S3, Supporting Information). If the increase in hemolymph protein was due to higher protein intake, however, it does not appear to have an overwhelming effect on microbiome structure (Table 1). Although their spermatophylax is relatively large and females compete for spermatophylax-producing males under low nutrient conditions (Gwynne, 1984b, 1993), studies that explicitly measure the composition and nutritional effects of spermatophylax consumption on female Mormon crickets are needed.

Second, nuptial gifts might not influence the gut microbiome because of a lack of sensitivity to dietary protein, irrespective of the nutritional properties of the gift itself. Our experiment supports this hypothesis, as protein supplementation did not significantly influence the gut microbiome, at least in decorated crickets (Table S2, Supporting Information). Cricket gut microbiomes thus might not confer the same degree of plasticity in resource use to the host, as has been suggested for humans (David *et al.*, 2014;; but see Kaufman and Klug, 1991). Experiments measuring the contribution of microbial metabolic activity to host nutrition under different diets and nutrient assimilation (e.g., Kaufman and Klug, 1991) are required to test this hypothesis.

Finally, it is possible that spermatophylax consumption could affect the microbiome under a different dietary regime not tested in our study. Mormon crickets in particular occur in habitats that vary widely in available protein and other nutrients (Gwynne, 1984b), and under these conditions spermatophylax consumption might have a greater effect than observed in our experiments.

Conclusion

Social behavior is emerging as an important factor shaping the diversity of the microbiome (Powell *et al.*, 2014; Smith and Mueller, 2015; Tung *et al.*, 2015; Moeller *et al.*, 2016). Progress in this area requires studies that use experimental manipulations of social interactions to complement surveys that correlate microbiome composition and host traits (e.g., group membership, dominance rank, social interaction networks) to infer their relationship (Archie and Tung, 2015). To our knowledge, our study is the first to use such an experimental approach to show that sexual interactions affect the structure of the gut microbiome. Future studies are required to determine whether the response of the microbiome we observed in Mormon crickets is unique to sexual interaction or could also be generated by intrasexual (Gwynne, 1984b) or non-sexual social interaction (Simpson *et al.*, 2006), the latter of which was not controlled in our experiment. Given the relative simplicity of their gut microbiomes and their long standing as models in the study of sexual behavior, crickets and katydids provide an exciting opportunity to expand our knowledge of host-microbe symbioses.

Data accessibility

Sequences are deposited in Genbank SRA accessions SRP073329 and SRP073374.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Permutation tests from distance-based redundancy analysis of female decorated cricket fecal samples. Diet refers to whether crickets were supplemented with protein and time refers to whether the sample was collected before or after the treatments were applied. We analyzed the data with and after removing *Photorhabdus* to ensure this nematode-vectored insect pathogen did not mask any significant effects. There was no evidence of nematode infection in any of our dissections and survivorship of crickets was high (97%), suggesting that this strain of *Photorhabdus* was not pathogenic in our experiment.

Table S2. Alpha diversity from female Mormon cricket fecal samples. Values are the Wald F-statistic (p-value) for each term from an analysis of deviance. Degrees of freedom were estimated using the Kenward-Roger approximation. Significant terms are in bold ($p<0.05$). Species richness increased over time (mean $\pm$ se: pre-experiment, 26.7 ± 2.4; post-experiment 34.2 ± 3.3).

Table S3. Alpha diversity of female decorated cricket fecal samples. Values are the Wald F-statistic (p-value) for each term from an analysis of deviance. Degrees of freedom were estimated using the Kenward-Roger approximation. See Table S1 legend for justification for removing *Photorhabdus*. A decline in alpha diversity over time was observed for species richness (mean $\pm$ se: pre-experiment: 112 ± 3.4; post-experiment: 101.3 ± 3.0), the Shannon-Wiener index (mean $\pm$ se: pre-experiment, 3.2 ± 0.07; post-experiment: 3.0 ± 0.07), Chao1 (mean $\pm$ se: pre-experiment 326 ± 8.5; post-experiment 293.7 ± 7.6) and Chao1 in the analysis without *Photorhabdus* (mean $\pm$ se: pre-experiment, 325 ± 8.5; post-experiment, 292.7 ± 7.6)

Table S4. Immunological activity and protein concentration of Mormon cricket hemolymph. Values are F-statistics (p-value) for each term. DF=1,39 for lysozyme-like antibacterial activity and DF=1,43 for phenoloxidase, proPhenoloxidase, and protein content. Sample sizes differ because the lysozyme-like activity assay failed for four females. We also found no evidence that community structure was associated with variation in immune activity across females irrespective of treatment (db-RDA: lysozyme, $F=0.95$, $p=0.6$; phenoloxidase, $F=1.21$, $p=0.21$; prophenoloxidase, $F=1.03$, $p=0.37$)

Table S5. Immunological activity and protein concentration of decorated cricket hemolymph. Values are F-stastistics (p-value) for each term. DF=1,38 for lysozyme-like antibacterial activity and DF=1,23 for phenoloxidase, proPhenoloxidase, and protein content. Sample sizes differ because not all decorated crickets produced enough hemolymph to run all of the assays. There were also no significant associations between microbiome composition and our measures of systemic immunity across females irrespective of treatment (db-RDA: lysozyme, $F=0.95$, $p=0.6$; phenoloxidase, $F=1.21$, $p=0.21$; prophenoloxidase, $F=1.03$, $p=0.37$).

Fig. S1. Abundance of the 15 dominant OTUs from Mormon cricket fecal samples, which comprised 99% of the sequences. Post-treatment samples are shown in the columns with treatment assignments for each sample indicated on the x-axis. Data are clustered using Bray-Curtis distance as implemented in the plot_heatmap function in phyloseq. Three orders were represented: Lactobacilliales (9 taxa, 71.6% of sequences), Enterobacteriales (5 taxa, 27.3% of sequences), and Aeromonadales (1 taxon, 1.1% of sequences). *Pediococcus* OTUs associated with mating in the db-RDA analysis (Fig. 3) are in bold (*P. acidilactici* 102222: 18.5% of sequences, *Pediococcus* 17309: 1.3% of sequences). Greengenes (v 13.8) identifiers are in parentheses.

Fig. S2. Abundance of the 20 dominant OTUs from decorated cricket fecal samples, which comprised 99% of the sequences. Post-treatment samples are shown in the columns with treatment assignments for each sample indicated on the x-axis. Data are clustered using Bray-Curtis distance as implemented in the plot_heatmap function in phyloseq. Six orders were represented: Bacteroidales (8 OTUs, 38.8%), Enterobacteriales (5 OTUs, 25.5%), Clostridiales (3 OTUs, 16.9%), Lactobacillales (2 OTUs, 8.6%), Verrucomicrobiales (1 OTU, 8.3%) and Pseudomonadales (1 OTU, 2.0%).

Fig. S3. Protein content in Mormon cricket hemolymph. Bars are mean ± se.