

1 Mating promotes lactic-acid gut bacteria in a gift-giving insect

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5 Smith, Chad C.  
6 Department of Integrative Biology  
7 1 University Station C0990  
8 University of Texas at Austin  
9 Austin, TX 78712  
10 [chadsmith@utexas.edu](mailto:chadsmith@utexas.edu)  
11 <http://chadsmith.wordpress.com>

12  
13 Srygley, Robert B.  
14 USDA-Agricultural Research Service  
15 Northern Plains Area Research Laboratory  
16 1500 N. Central Avenue  
17 Sidney MT 59270

18  
19 Dietrich, Emma I.  
20 Department of Integrative Biology  
21 1 University Station C0990  
22 University of Texas at Austin  
23 Austin, TX 78712

24  
25 Mueller, Ulrich G.  
26 Department of Integrative Biology  
27 1 University Station C0990  
28 University of Texas at Austin  
29 Austin, TX 78712

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## 32 **ABSTRACT**

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

47  
48 Social interaction (Archie and Tung, 2015; Smith and Mueller, 2015) and diet (Ley *et al.*, 2008;  
49 Muegge *et al.*, 2011; Yatsunenko *et al.*, 2012; David *et al.*, 2014) are two key factors that influence the  
50 composition of the microbiome. Of the types of social interactions animals engage in, mating is both  
51 ubiquitous and among the most likely to influence host microbial communities due to its intimacy and  
52 profound effects on host physiology. Yet scant attention has been paid to the influence of mating on  
53 microbial symbiosis beyond the transmission of pathogenic infections (Lockhart *et al.*, 1996; Knell and  
54 Webberley, 2004) despite the fact that beneficial microbes can also be sexually transmitted during the  
55 mating process (Smith and Mueller, 2015). Mating also alters the expression of hundreds of genes

56 involved in metabolism, reproduction, and immunity (McGraw *et al.*, 2008), which potentially could  
57 influence host-microbe interactions. The host immune system in particular plays a critical role in the  
58 regulation of the microbiome (Ryu *et al.*, 2010; Hooper *et al.*, 2012; Engel and Moran, 2013), which in  
59 turn influences host immune function (Hooper *et al.*, 2012; Engel and Moran, 2013; Levy *et al.*, 2015),  
60 nutrition (Turnbaugh *et al.*, 2006; Engel and Moran, 2013) and behavior (Archie and Theis, 2011;  
61 Forsythe and Kunze, 2013).

62 Sexual interactions can also influence diet, an important determinant of the constitution of the  
63 microbiome (Ley *et al.*, 2008; Muegge *et al.*, 2011; Yatsunenکو *et al.*, 2012; David *et al.*, 2014). In  
64 many animals, males provide nuptial gifts that females ingest during courtship or copulation (Yosef  
65 and Pinshow, 1989; Vahed, 1998; Gomes and Boesch, 2009). Male crickets and katydids in particular  
66 are known for the production of a spermatophylax, a proteinaceous (Heller *et al.*, 1998), sperm-free  
67 mass that is eaten by females. Consumption of the spermatophylax has varying effects on female  
68 fitness, increasing survival and fecundity in some taxa (Gwynne, 1984a, 2008; Simmons, 1990) while  
69 producing no apparent benefit in other taxa (Will and Sakaluk, 1994; Vahed, 2007). This has led to  
70 extensive debate over spermatophylax evolution. Several lines of evidence suggest that the  
71 spermatophylax serves only as an ejaculate protection device to prevent the female from eating the  
72 sperm-laden ampulla (Vahed, 2007), which is transferred with the spermatophylax to females during  
73 copulation. These nuptial gifts are not necessarily expected to provide a nutritional benefit, only  
74 properties that distract the female long enough for sperm transfer to complete (Vahed, 2007). In  
75 contrast, the spermatophylax is expected to be nutritious when it serves as a form of paternal  
76 investment that increases the number or quality of offspring sired by the male (Gwynne, 2008). Which  
77 of these two explanations is correct is likely to have important implications for how nuptial gifts  
78 influence the microbiome, as protein intake can induce rapid changes in the gut microbial communities  
79 (Wu *et al.*, 2011; David *et al.*, 2014).

80 We manipulated nuptial feeding and mating to measure their effects on the gut microbiome of

81 two insects that differ in the size of their gifts, the Mormon cricket,  
82 *Anabrus simplex* (Orthoptera: Tettiginiidae), and the decorated  
83 cricket, *Gryllodes sigillatus* (Orthoptera: Gryllidae). Mormon  
84 crickets produce a spermatophore six times larger than *G. sigillatus*  
85 (19% vs 3% of male body mass; Gwynne, 1984b; Sakaluk, 1985,  
86 Fig. 1) and are a well-known example of nutrition-dependent sex-  
87 role reversal, with females competing for access to  
88 spermatophylax-producing males when food is scarce (Gwynne,  
89 1984b, 1993). In contrast, the *G. sigillatus* spermatophylax is no  
90 larger than that required for sperm transfer (Sakaluk, 1984) and  
91 does not provide any detectable nutritional benefit to females (Will  
92 and Sakaluk, 1994; but see Ivy *et al.*, 1999). Given this evidence,  
93 we expect that spermatophylax consumption will exert larger  
94 effects on the gut microbiome of Mormon crickets than decorated  
95 crickets. Whether mating influences the microbiome depends on the  
96 potential for microbial transmission, as well as an effect of mating on the physiological state of  
97 females. We assessed these alternatives by screening male and female reproductive tissues for bacteria  
98 and measuring components of the immune system that are known to change in response to mating in  
99 insects.

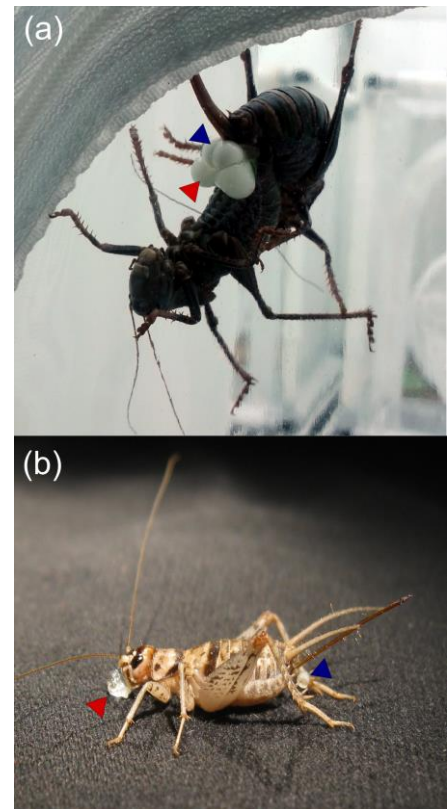


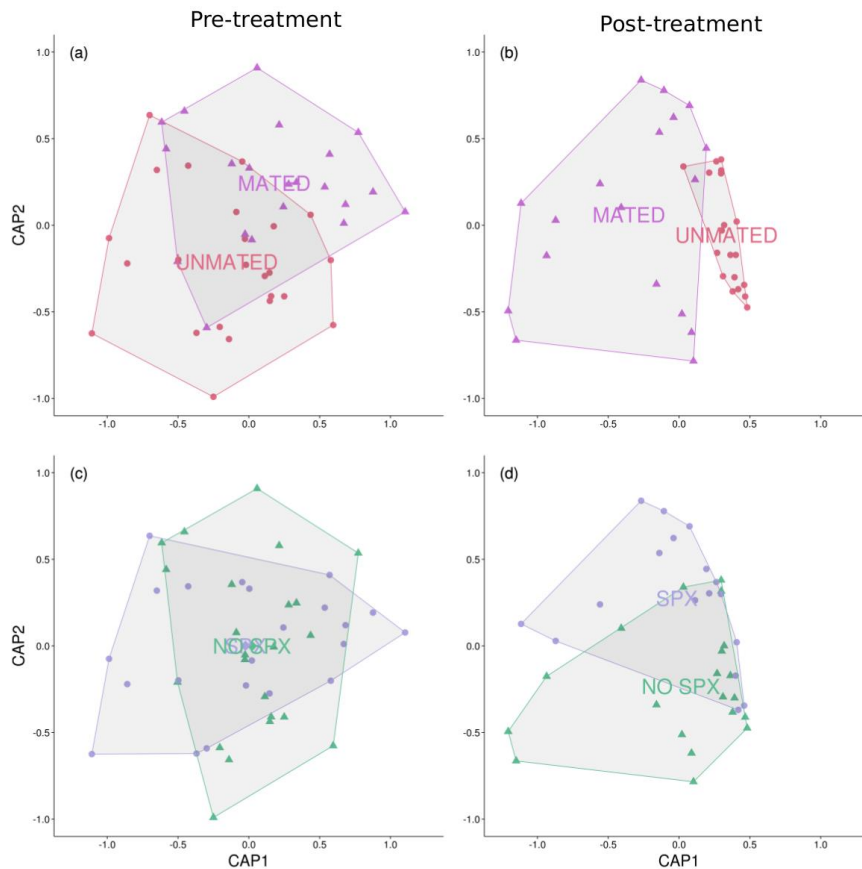
Figure 1. (a) Mormon cricket *Anabrus simplex* female (top) and male (bottom) in copula and (b) a decorated cricket *Gryllodes sigillatus* female after mating. Red arrow indicates the spermatophylax and blue arrow indicates the ampulla.

## 100 RESULTS AND DISCUSSION

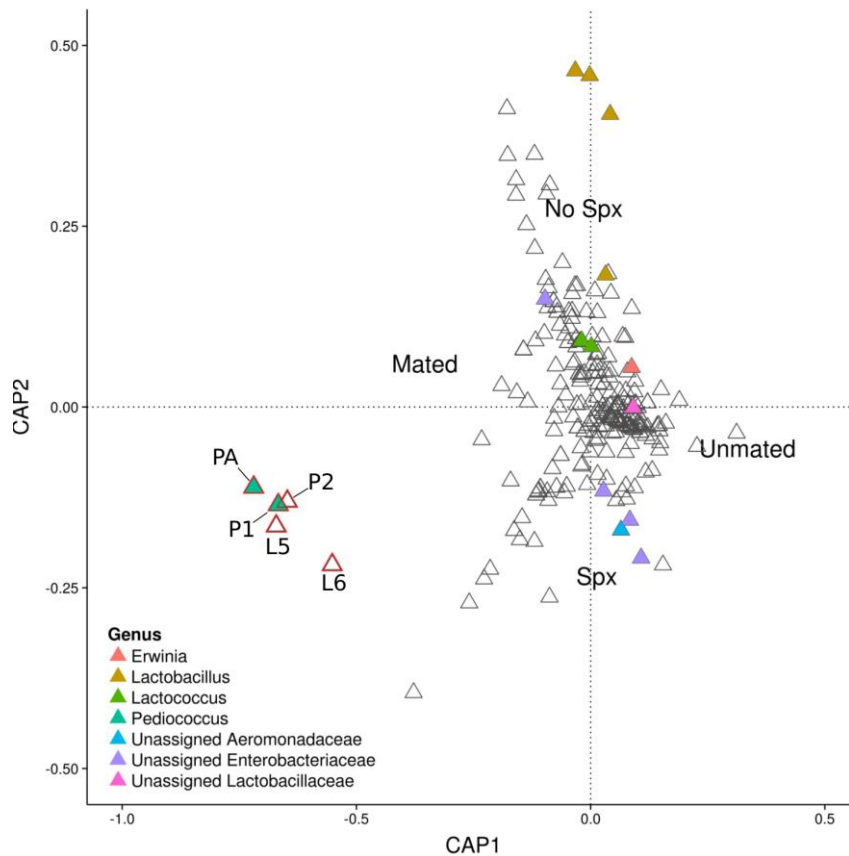
### 101 *Mating and the microbiome*

102 We found that mating, but not spermatophylax consumption, influenced the structure of the gut  
103 microbiome of Mormon crickets (Figure 2, Table 1), while neither had an effect in decorated crickets  
104 (Table S1). Ordination of the Mormon cricket OTU scores suggested that five taxa changed in

105 abundance in response to the mating treatment (Fig. 3), all lactic-acid bacteria (Family  
106 *Lactobacillaceae*). Two of these were among the dominant members of the Mormon cricket  
107 microbiome (Figure S1, *Pediococcus acidilactici* 102222 and *Pediococcus sp.* 17309), while the other  
108 three occurred at a lower frequency (*Lactobacillus sp.* 288584, *Pediococcus sp.* 733251, and  
109 *Lactobacillus sp.* 1110317).



110  
111 Figure 2. Ordination of Mormon cricket sample scores from a distance-based redundancy analysis.  
112 Points are colored to indicate whether a cricket was mated (triangles) or unmated (circles) (a,b) and  
113 whether they were allowed to consume the spermatophylax (circles) or not (triangles) (c,d). Text  
114 corresponds to the centroids for samples collected before (a,c) or after (b,d) the treatments were  
115 applied. Alpha diversity was not affected by mating or spermatophylax consumption (Table S2 and  
116 S3).



117

118 Figure 3. Ordination of Mormon cricket OTU scores from a distance-based redundancy analysis. Each  
119 triangle represents an OTU, with text indicating the centroid of the sample scores from each treatment.  
120 Filled triangles are the top 15 most abundant OTUs colored by genus. Labeled OTUs are those  
121 displaced along the axis associated with mating and individually analyzed for differences in abundance  
122 (see Figure 4, Table 2). PA = *Pediococcus acidilactici* 102222, P1=*Pediococcus* 17309,  
123 P2=*Pediococcus* 773251, L5=*Lactobacillus* 288584, L6= *Lactobacillus* 1110317.  
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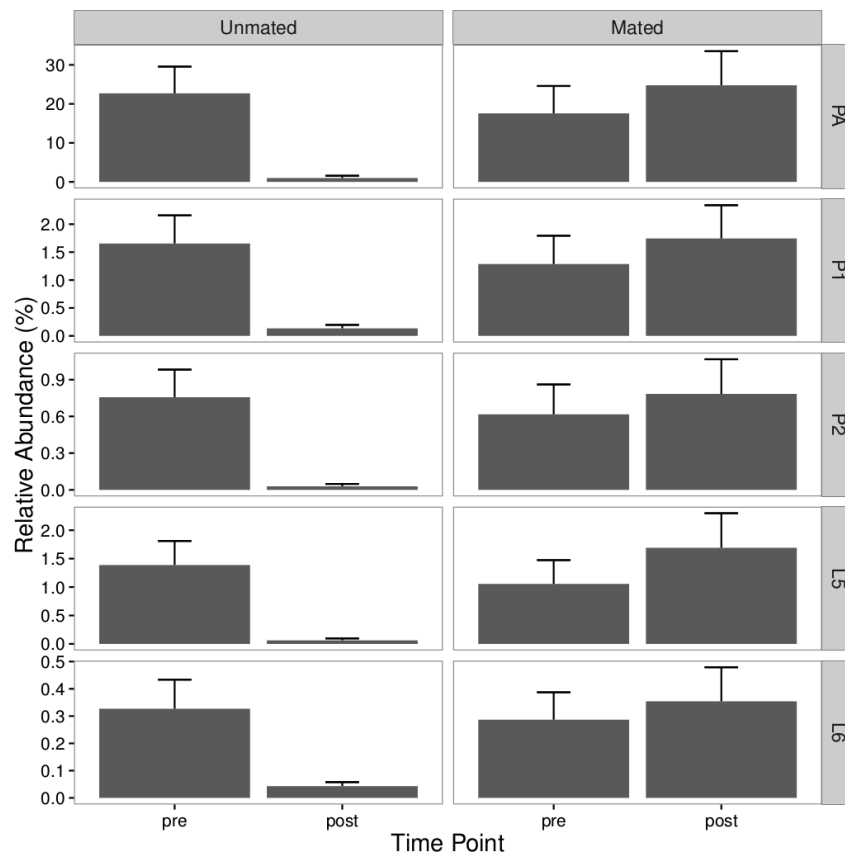
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We compared the abundance of these five lactic-acid bacteria among treatments in univariate analyses and found that three differed depending upon whether females had mated or not, including *P. acidilactici* 102222 and *Pediococcus sp.* 17309 (Fig. 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).



132

133 Figure 4. Relative abundance of five OTUs putatively associated with mating in Mormon crickets.  
134 Time point indicates whether samples were collected before or after the treatments were imposed. A  
135 significant interaction between mating and time point was detected for the top 3 panels (Table 2).  
136

137 Lactic-acid bacteria are known for their beneficial associations with the gastrointestinal tract of  
138 human and non-human animals (De Vos *et al.*, 2009; Walter *et al.*, 2011; Ma *et al.*, 2012), including  
139 insects (Forsgren *et al.*, 2010; Storelli *et al.*, 2011; Vásquez *et al.*, 2012; Erkosar *et al.*, 2015). *P.*  
140 *acidilactici*, for example, has been shown to enhance development and immune function (Neissi *et al.*,  
141 2013), reduce susceptibility to infection (Castex *et al.*, 2009), and produce bacteriocins toxic to food-  
142 borne pathogens (Bhunja *et al.*, 1988). Our study thus shows that sexual interactions can influence the  
143 structure of the microbiome, and suggests that mating can promote the persistence of beneficial  
144 bacteria in the gut.

145 One way social behavior can alter the microbiome is by facilitating transmission of microbes



146 between members of the group (Archie and Tung, 2015). Sexual transmission is unlikely to explain our  
147 results, however, because the male spermatophore and female spermatheca were negative in our 16s  
148 PCR screens for bacteria, perhaps because of antimicrobial activity in the reproductive tissues. Sexual  
149 transmission of both pathogenic and beneficial microbes, however, does occur in insects (Knell and  
150 Webberley, 2004; Smith and Mueller, 2015), and more studies are needed to evaluate their prevalence  
151 and effects on host fitness and reproductive behavior. Contact with male feces might also have  
152 provided a source of lactic acid bacteria to mated females if there are gender differences in the  
153 microbiome. While there is some evidence that gender influences the microbiome in other animals  
154 (Bolnick *et al.*, 2014; Ding and Schloss, 2014), this has yet to be evaluated in Mormon crickets.

155

156 Changes in host physiology in response to social interaction, or lack thereof, could also explain  
157 shifts in microbiome structure. Hormones that regulate appetite, energy expenditure, and metabolism  
158 are thought to affect the gut microbiome by altering (i) immune function, (ii) mucous production in the  
159 gut epithelia, and (iii) behavioral changes in food intake (Spor *et al.*, 2011). Similarly, the stress  
160 response (Jašarević *et al.*, 2015; Sandrini *et al.*, 2015) and fluctuations in reproductive hormones (Gajer  
161 *et al.*, 2012; Brotman *et al.*, 2014) are associated with changes in the composition of the microbiome.  
162 In *Drosophila*, mating influences the expression of >1700 genes involved in these physiological  
163 processes (McGraw *et al.*, 2008). Many of these genes are expressed in tissues outside of the female  
164 reproductive tract and are induced by the transfer of specific male seminal fluid proteins (McGraw *et*  
165 *al.*, 2008). Whether similar physiological responses to mating can be generalized to other insects, and  
166 whether these specific changes do influence host-microbe interactions, remains to be elucidated.

167 Mating in insects can result in the suppression of the immune system due to tradeoffs between  
168 survival and reproduction (Harshman and Zera, 2007), and the immune system is a key regulator of the  
169 microbiome (Ryu *et al.*, 2010; Hooper *et al.*, 2012). We measured three components of systemic  
170 immunity in both species and found that immunological activity was unaffected by mating, and was not



171 associated with variation among crickets in microbiome structure (Table S4 and S5). This suggests that  
172 if the lactic-acid bacteria identified in our study are influenced by the immune system, it likely occurs  
173 locally within the gut rather than in response to systemic changes in immunity. This is consistent with  
174 experiments in *Drosophila*, where the immune response in gut epithelia is induced by oral introduction  
175 of bacteria but not after injection of the same bacteria into the hemocoel (Tzou *et al.*, 2000).

#### 176 *Nuptial gift consumption and the microbiome*

177 In contrast to our expectation that larger nuptial gifts should elicit a greater change in  
178 microbiome composition, spermatophylax consumption did not affect the gut bacterial communities in  
179 either species (Table 1, S1). At least three non-mutually exclusive possibilities could explain this  
180 result. First, it is possible that the spermatophylax is not a highly nutritive meal for the female, even in  
181 Mormon crickets. Hemolymph protein was higher in Mormon crickets that mated and consumed the  
182 spermatophylax in our study (Table S4, Fig. S3); however, if these females did have higher protein  
183 intake, it was not reflected in their microbiome. Although their spermatophylax is relatively large and  
184 females compete for spermatophylax-producing males under low nutrient conditions (Gwynne, 1984b,  
185 1993), the nutritional consequences of spermatophylax consumption has not been explicitly measured  
186 in Mormon crickets.

187 Second, nuptial gifts might not influence the gut microbiota because of a lack of sensitivity of  
188 the microbiome to dietary protein, irrespective of the nutritional properties of the gift itself. Our  
189 experiment supports this hypothesis, as increasing dietary protein did not significantly influence the gut  
190 microbiome, at least in decorated crickets (Table S2). Cricket gut microbiomes thus might not confer  
191 the same degree of plasticity in resource use as has been suggested for humans (David *et al.*, 2014).  
192 Experiments measuring metabolic activity under different dietary regimes are required to test this  
193 hypothesis.

194 Finally, it is possible that spermatophylax consumption could affect the microbiome under a

195 different dietary regime not tested in our study. Mormon crickets in particular occur in habitats that  
196 vary widely in available protein and other nutrients (Gwynne, 1984b), and under some conditions in  
197 nature spermatophylax consumption might have a greater effect than observed in our experiments.

## 198 *Conclusion*

199 Social behavior is emerging as an important factor shaping the diversity of the microbiome (Powell *et*  
200 *al.*, 2014; Smith and Mueller, 2015; Tung *et al.*, 2015; Moeller *et al.*, 2016). Progress in this area  
201 requires studies that use experimental manipulations of social interactions to complement surveys that  
202 correlate microbiome composition and host traits (e.g. group membership, dominance rank, social  
203 interaction networks) to infer their relationship (Archie and Tung, 2015). To our knowledge, our study  
204 is the first to use such an experimental approach to demonstrate that sexual interactions affect the  
205 structure of the gut microbiome. Given the relative simplicity of their gut microbiomes and their long  
206 standing as models in the study of sexual behavior, crickets and katydids provide an exciting  
207 opportunity to expand our knowledge of host-microbe symbioses.

## 208 **DATA ACCESSIBILITY**

209 Sequences are deposited in Genbank SRA accessions SRP073329 and SRP073374.

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341 **TABLES**

342 Table 1. Permutation tests from distance-based redundancy analysis of female Mormon cricket fecal  
 343 samples. Time refers to whether a sample was collected before or after the treatments were applied.

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

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346 Table 2. OTU abundance of five taxa putatively associated with mating in Mormon crickets. Values  
 347 represent the  $X^2$  (p-value) from an analysis of deviance, except for L6, which was analyzed with  
 348 Wilcoxon signed-rank tests. Significant terms are in bold ( $p < 0.05$ ). PA = *Pediococcus acidilactici*  
 349 102222, P1=*Pediococcus* 17309, P2=*Pediococcus* 773251, L5=*Lactobacillus* 288584, L6=  
 350 *Lactobacillus* 1110317.

		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)	
	<b>Time</b>	<b>13.9 (0.006)</b>	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)	
	<b>Mate * Time</b>	<b>12.1 (0.007)</b>	<b>10.7 (0.01)</b>	<b>8.30 (0.03)</b>	5.20 (0.13) <sup>†</sup>	
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)

351 <sup>†</sup>  $P=0.02$  before FDR correction for multiple tests.