Manuscript

1 # The effect of mating on immunity can be masked by
2 # experimental piercing in female *Drosophila melanogaster*.

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19 **Abstract**

20 Mating and immunity are two major components of fitness and links
21 between them have been demonstrated in a number of recent investigations. In
22 *Drosophila melanogaster*, a seminal fluid protein, sex-peptide (SP), up-regulates
23 a number of antimicrobial peptide (AMP) genes in females after mating but the
24 resulting effect on pathogen resistance is unclear. In this study we tested 1)
25 whether SP-induced changes in gene expression affect the ability of females to
26 kill injected non-pathogenic bacteria and 2) how the injection process *per se*
27 affects the expression of AMP genes relative to SP. The ability of virgin females
28 and females mated to SP lacking or control males to clear bacteria was assayed
29 using an established technique in which *E. coli* are injected directly into the fly
30 body and the rate of clearance of the injected bacteria is determined. We found
31 no repeatable differences in clearance rates between virgin females and females
32 mated to SP producing or SP lacking males. However, we found that the piercing
33 of the integument, as occurs during injection, up-regulates AMP gene expression
34 much more strongly than SP. Thus, assays that involve piercing, which are
35 commonly used in immunity studies, can mask more subtle and biologically
36 relevant changes in immunity, such as those induced by mating.

37

38 **Keywords:** mating, immunity, sex-peptide, injection, antimicrobial

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40

1. Introduction

Immunity and reproduction are important components of fitness and an increasing number of studies report interplay between the two processes (reviewed in Lawniczak et al., 2007). In some species mating and other reproductive processes appear to suppress aspects of immunity, thus broadly supporting a resource trade-off model (Sheldon & Verhulst, 1996). For example, in the flour beetle, *Tenebrio molitor*, mating suppresses an immune effector system (phenoloxidase) in both sexes (Rolff & Siva-Jothy, 2002), potentially reducing pathogen resistance. Reductions in measures of immunity resulting from mating or reproductive activity have also been detected in female damselflies, *Matrona basilaris japonica* (Siva-Jothy et al., 1998), female ground crickets, *Allonemobious socius* (Fedorka et al., 2004; Fedorka & Zuk, 2005) female pea aphids, *Acyrthosiphon pisum* (Gwynn et al., 2005), female ants, *Atta colombica* (Baer et al., 2006) and male *Drosophila melanogaster* (McKean & Nunney, 2001).

However, in some other species mating apparently increases aspects of immunity. For example, in females of the cricket *Gryllus texensis*, mating increases pathogen resistance (Shoemaker et al., 2006) and in *Drosophila melanogaster* a number of immune genes, particularly antimicrobial peptides (AMPs) are up-regulated for several hours after mating (Lawniczak & Begun, 2004; McGraw et al., 2004; Peng et al., 2005; Domanitskaya et al., 2007). This up-regulation of immune genes results from the actions of male accessory gland proteins (Acps) (McGraw et al., 2004) which are transferred to females in seminal fluid. One Acp in particular (Acp70A, the sex-peptide, SP), up-regulates

66 several AMPs (Peng et al., 2005; Domanitskaya et al., 2007). However, increases
67 in immune gene expression or other proxy measures of immunity do not
68 necessarily result in an increase in pathogen resistance (Adamo, 2004a; Adamo,
69 2004b). McKean & Nunney (2005) found that *D. melanogaster* females showed
70 no difference in their ability to clear injected non-pathogenic bacteria whether
71 they were maintained with males or in single sex groups as virgins.
72 Unexpectedly, Fedorka et al. (2007) found that, when AMPs were up-regulated
73 in mated females (3 hrs post-mating) resistance to an injected pathogenic
74 bacterium was lower than that of virgin females. Moreover, at 27 hrs post-
75 mating, when several AMPs were down-regulated in mated females, pathogen
76 resistance was similar to that of virgin females. Fedorka et al.'s (2007) study
77 shows that there can be a disparity between proxy measures of immunity, such as
78 gene expression, and the real ability of animals to fight infection.
79 There is currently no general pattern in the effects of mating and
80 reproductive effort upon immunity in insects (Lawniczak et al., 2007). One
81 potential reason for this is that a range of different techniques have been
82 employed to measure aspects of immunity in insects: some are proxy measures
83 and others are direct measures of pathogen resistance. Furthermore, several of
84 these techniques involving piercing the integument to inject pathogens, non-
85 pathogenic bacteria or foreign objects into the body (e.g. Siva-Jothy et al., 1998;
86 McKean & Nunney, 2001; McKean & Nunney, 2005; Baer et al., 2006; Fedorka
87 et al., 2007). However, in *Drosophila* it is not known how piercing the
88 integument *per se* affects the expression of AMP genes or how any changes
89 compare to those induced by mating. In this study we addressed this issue. Firstly
90 we investigated whether SP-induced up-regulation of immune genes affects the

91 ability of female *D. melanogaster* to kill injected bacteria. We used the immunity
92 assay developed by McKean and Nunney, in which non-pathogenic bacteria are
93 injected into females and the remaining live bacteria are retrieved after several
94 days (McKean & Nunney, 2001; McKean & Nunney, 2005). We compared
95 females that were virgin, mated to wild-type males or mated to SP knockdown
96 males (which produce no detectable SP). Secondly, to examine whether the
97 injection process *per se* affects female immunity and how any changes compare
98 to those induced by SP we measured the expression of two AMP genes in
99 females that were either virgin and pierced (with nothing injected), virgin and
100 injected with Ringers solution, virgin and injected with synthetic SP solution,
101 virgin and not pierced, mated to SP lacking males and not pierced, or mated to
102 SP producing (control) males and not pierced.
103
104 **2. Materials and Methods**
105
106 *2.1 Fly stocks and husbandry*
107
108 All cultures were maintained at 25°C on a 12:12 h light: dark cycle. Flies
109 for bacterial clearance assays were maintained on sugar-yeast food and flies for
110 gene expression assays were maintained on cornmeal-yeast-agar food. Wild type
111 stocks used were Dahomey, for bacterial clearance assays, and Oregon-R for
112 gene expression assays. SP knockdown males were obtained by RNA
113 interference as previously described (Chapman et al., 2003). These consist of two
114 replicate, genetically matched, knockdown and control lines whereby SP1
115 knockdown is matched with control 1 and SP2 knockdown is matched with

116 control 2 (Wigby & Chapman, 2005). SP^0 and control (SP^+) males were as

117 described in (Liu & Kubli, 2003). SP^0 males contain a mutant non-functional SP

118 allele in place of the wild-type *SP* gene and produce no SP. SP^+ control males

119 contain both the mutant and wild-type genes and produce normal levels of SP

120 (Liu & Kubli, 2003).

121

122 *2.2 Injections and piercings*

123

124 All injections and piercings were performed using pulled glass needles

125 with the flies under ice or CO_2 anaesthesia. Control flies (not pierced or injected)

126 were anaesthetised in the same way to control for fly handling.

127

128 *2.3. Bacterial clearance assay*

129

130 The bacterial clearance assay was based on that used by McKean and

131 Nunney (2001) with minor modifications. On the evening before the bacteria

132 were injected, *E. coli* D21 (which is resistant to both ampicillin and

133 streptomycin) were grown overnight in LB solution. The following morning the

134 resulting population was centrifuged and re-suspended in *Drosophila* Ringers

135 solution. The suspension was diluted and the cell concentration determined using

136 a Helber counter. The suspension was diluted further to a concentration of $\approx 13 \times$

137 10^9 cells/ml. 74nL of the solution was injected into flies which equates to $\approx 10^6$

138 cells per fly. Flies were injected in the thorax. Three days after injection the flies

139 were assayed for the number of surviving *E. coli* D21. Individual flies were CO_2

140 anaesthetized, placed in an Eppendorf and homogenised in 200 µL Ringers

141 solution. The solution was diluted × 75 and 300μL of the resulting solution was
142 spread on LB agar plates containing 50μg/ml streptomycin. The plates were
143 stored overnight at 37°C and the number of colonies were counted manually.
144
145 *2.4. The ability of virgin females and females mated to control or SP knockdown*
146 *males to clear bacteria*
147
148 To test the ability of females to clear bacteria after mating, three
149 experiments were performed. For all bacterial clearance assays, wild-type
150 Dahomey females were reared at standard density (Clancy & Kennington, 2001),
151 collected as virgins within eight hours of eclosion using ice anaesthesia and
152 housed for 4-5 days in groups of 10. Flies were maintained in vials with sugar-
153 yeast food and added live yeast grains. In the first experiment the females were
154 either kept as virgins or mated to wild-type Dahomey males which were derived
155 from the same culture bottles as the females. For the mating treatment one
156 female was aspirated, without anaesthesia, into a vial that already contained 2
157 males. Females were allowed to mate once and any pairs that mated for less than
158 10 minutes were discarded. Females that mated once for more than 10 minutes
159 were aspirated into fresh vials in groups of 10. Mated and virgin females were
160 randomly allocated to one of 2 treatments. One set of flies was injected with
161 bacteria 4 hrs after the matings and the other set of flies was injected 24 hrs after
162 the matings. At both time points a further 10 virgin females were injected with
163 Ringers solution to act as negative controls. After the injections, females were
164 housed, 10 per vial, in fresh vials. Each day after injections, females were

165 transferred to fresh vials. Three days after injections, individual females were
166 assayed for the number of living *E. coli* D21 remaining in them.
167 The second and third experiments were identical to the first except that
168 females were mated to SP knockdown or control males and all injections were
169 performed at 4 hrs post mating (there was no 24 hr treatment). In the second
170 experiment all flies were assayed simultaneously whilst in the third experiment
171 the two replicate knockdown lines were assayed at different times and hence
172 there were two sets of virgin controls. SP knockdown in the males was
173 confirmed by performing Western Blots.
174
175
176 *2.5. Statistical analysis*
177
178 To test for differences between treatments in the ability of females to clear
179 bacteria, colony count data was compared between treatments using Kruskal-
180 Wallis tests. Analyses were carried out using JMP 5.1.2 statistical software (SAS
181 Institute Inc.).
182
183 *2.6. Quantitative real-time PCR*
184
185 Total RNA was prepared using Trizol, followed by DNase treatment to control
186 for amplification of background genomic DNA in the RNA samples (Ambion,
187 DNA-free). Total RNA was quantified with a spectrophotometer (NanoDrop®
188 ND-1000 UV-Vis). 1 μg total RNA was used for cDNA synthesis using the
189 Qiagen reverse transcription system (Qiagen, Cat. No. 205111). Reactions

190 without reverse transcriptase were used to control for amplification of
191 background genomic DNA in the RNA samples. Each QRT-PCR was performed
192 using SYBR Green PCR Core Reagents (Applied Biosystems). Rpl32 (60S
193 ribosomal protein L32), tubulin and actin-were used as reference control genes.
194 The QRT-PCR data were analyzed using the comparative CT method (Livak &
195 Schmittgen, 2001). Briefly, the relative difference in cycle times, ΔCT, measured
196 during the exponential phase of the reactions was standardised to the reference
197 control genes (Rpl32, tubulin or actin). ΔΔCT was obtained by finding the
198 difference between treatments. The fold change was calculated as FC=2-ΔΔCT.
199 We took measurements from 3 replicate QRT-PCRs on each extraction to
200 determine the variability in ΔΔCT arising from the methods we used. Confidence
201 intervals were calculated and converted to the fold-change scale.
202
203 *2.7. The effects of mating, sex-peptide and piercing the integument on*
204 *antimicrobial peptide gene expression*
205
206 To test the relative effects of mating and piercing of the integument on
207 immune gene expression in females QRT-PCRs were performed for *Attacin-A*
208 and *Diptericin*. These AMPs show SP dependent expression in mated females
209 (Fig 2; Peng et al., 2005; Domanitskaya et al., 2007). Wild-type Oregon-R
210 females were collected as virgins within 5 hours of eclosion on ice anaesthesia.
211 Five day-old females were assayed either as virgins or after mating to SP^0 or
212 control (SP^+) males (Liu & Kubli, 2003). Virgin females were allocated to one of
213 6 treatments: 1) pierced in the abdomen (with nothing injected), 2) injected in the
214 abdomen with 50nL Drosophila Ringers solution, 3) injected in the abdomen

215 with 50nL synthetic SP (3pmol) dissolved in Drosophila Ringers solution, 4)
216 injected in the thorax with 50nL Drosophila Ringers solution, 5) injected in the
217 thorax with 50nL synthetic SP (3pmol) dissolved in Drosophila Ringers solution
218 or 6) not pierced or injected. Synthetic SP was prepared as described in Schmidt
219 et al. (1993). QRT-PCRs were performed on RNA extracted from the abdomens
220 of females 4 hours after the injections, piercings or matings. RNA was pooled for
221 10-20 flies per treatment.
222
223 **3. Results**
224
225 *3.1. The ability of virgin females and females mated to control or SP knockdown*
226 *males to clear bacteria*
227 In all bacterial clearance assays control females injected with Ringers solution
228 produced bacterial counts of 0, showing that there was no contamination from
229 non-injected bacteria. In the first experiment, mated females injected at 4 hrs and
230 at 24 hrs had significantly lower bacterial counts than virgin females (4 hrs, $\chi^2_1 =$
231 4.27, $P = 0.039$, 24 hrs, $\chi^2_1 = 7.10$ $P = 0.008$, Fig. 1a). However, in the second
232 and third experiments, we found no significant differences in colony counts
233 between virgin females, females mated to SP knockdown or females mated to
234 control males in either knockdown line (experiment 2, Line 1, $\chi^2_2 = 1.16$, $P =$
235 0.56, Line 2, $\chi^2_2 = 3.70$, $P = 0.158$, Fig. 1b; experiment 3, Line 1, $\chi^2_2 = 0.82$, $P =$
236 0.665, Line 2, $\chi^2_2 = 1.48$, $P = 0.478$, Fig. 1c).
237
238 *3.2. The effects of mating, sex-peptide and piercing the integument on*
239 *antimicrobial peptide gene expression*

240

241 The expression data show, as expected, that mating with SP producing males up-
242 regulated AMP gene expression in females (mean fold-change for *AttA* = 4.67
243 and for *Dpt* = 2.43, Fig 2) and that mating to SP^0 males failed to produce this up-
244 regulation (mean fold-change for *AttA* = 1.48 and for *Dpt* = 0.80, Fig 2).
245 However, injection or piercing of the integument, either in the abdomen and in
246 the thorax, up-regulated *AttA* and *Dpt* considerably more than mating and the
247 presence of SP did not further increase this gene expression (mean fold change
248 for females pierced in the abdomen, *AttA* = 7.31 and *Dpt* = 10.00, for females
249 injected in the abdomen with Ringers solution, *AttA* = 20.69 and *Dpt* = 11.55, for
250 females injected in the abdomen with SP *AttA* = 13.60 and *Dpt* = 9.66, for
251 females injected in the thorax with Ringers solution, *AttA* = 22.34 and *Dpt* =
252 18.69 and for female injected in the thorax with SP, *AttA* = 19.46 and *Dpt* =
253 18.63, Fig 2). In females injected in the abdomen there was a trend for lower
254 AMP expression when SP was injected compared to when Ringers alone was
255 injected, in contrast to the effect seen when SP was delivered by mating.
256
257 **4. Discussion**
258
259 The results of the first part of this study show that a single mating, and
260 specifically the receipt of SP from that mating, has no repeatable effect on the
261 ability of females to clear injected *E. coli*. This is consistent with the findings of
262 McKean and Nunney (2005) who found that females maintained with males
263 (who were therefore likely to have mated at least once) do not clear bacteria at a
264 different rate from virgin females. It is not clear why we found differences

265 between virgin and mated females in the first experiment but not in subsequent
266 experiments. One possibility is that we used males of different genotypes in
267 experiment 1 (wild-type) vs experiments 2 and 3 (SP knockdown and controls).
268 However, the control males used in experiments 2 and 3 are effective at inducing
269 post mating responses (Chapman et al., 2003) so there is no reason to expect
270 these males to be ineffective at inducing changes in immunity in females. It is
271 clear that the effects seen in experiment 1 were not repeatable and are therefore
272 unlikely to be of major biological importance.

273 The second part of our study highlights a potential caveat with immunity
274 assays that involve piercing the integument. We found that the effect of mating,
275 specifically of SP, on the expression of 2 AMP genes, was dwarfed by the effect
276 of piercing with a needle. It was not possible to detect, using SP injection, the
277 up-regulation of *AttA* and *Dpt* that occurs when SP is delivered via the natural
278 method of mating (Peng et al., 2005 Domanitskaya et al., 2007; Fig. 2). Instead
279 there was a trend for lower AMP gene expression in females injected in the
280 abdomen with SP solution compared to females injected with Ringers only.
281 Injection of SP has been shown to successfully stimulate 2 of the other major
282 postmating responses: non-receptivity to mating and an increase in egg laying
283 (Chen et al., 1988). Injected SP must therefore reach at least some of its natural
284 targets. Instead, our results suggest that assays that involve piercing the
285 integument of insects may be a poor method for examining subtle immune traits
286 because of the potentially large effect of the piercing on immunity. Thus, we can
287 not exclude the possibility that the lack of repeatable differences in the ability to
288 clear bacteria between virgin and mated females in this study and in McKean and

289 Nunney (2005) might be a result of any effects being masked by the effect of
290 piercing on immunity.
291 We can also not exclude the possibility that the effects of piercing on
292 AMP gene expression or the effects of mating on bacterial clearance might differ
293 between fly stocks. We used Oregon-R females in the AMP gene expression
294 assays but bacterial clearance experiments have been performed on females from
295 the Dahomey stock (this study) and a stock from California (McKean & Nunney,
296 2005). It is therefore important that future studies examine the relationship
297 between gene expression and phenotypic immunity, using the same flies and in
298 the same experiment. It will also be important to connect gene expression and
299 pathogen resistance to the levels of AMPs circulating in the haemolymph. In
300 *Drosophila*, the upregulation of AMP genes are typically measured over the
301 course of few hours to 1 day following mating or immune challenge but
302 measures of pathogen resistance are taken days later. Levy et al. (Levy et al.,
303 2004) found that the molecules induced by bacterial challenges show peak
304 concentrations at 6 and 24 hrs post insult and most are at decreased
305 concentrations by 2 days. A challenge for future research will be to determine the
306 temporal relationship between changes in gene expression, AMP concentration
307 and pathogen resistance.
308 Mating or reproduction induced changes in immunity have been detected
309 using assays involving piercing the integument (e.g. Siva-Jothy et al., 1998;
310 McKean & Nunney, 2001; McKean & Nunney, 2005; Baer et al., 2006; Fedorka
311 et al., 2007) which clearly shows that such assays are not without value. McKean
312 and Nunney (2001, 2005) detected changes in the bacterial clearance abilities of
313 *D. melanogaster* males in response to sexual behaviour using the assay that we

314 replicated in this study. Changes in male immunity in response to continued
315 mating and reproductive behaviour may therefore be much larger than potential
316 changes in female immunity after a single mating, and are thus not masked by
317 piercing effects. Fedorka et al (2007) detected changes in female immunity after
318 mating using an assay in which pathogenic bacteria were placed directly in the
319 thorax by piercing the integument and measuring female survival times. It is not
320 clear why the method used by Fedorka et al (2007) was able to detect mating
321 induced immunity changes in females whereas the bacterial clearance assay used
322 by McKean and Nunney (2005) and this study failed to. Fedorka et al's (2007)
323 assay is more immunologically challenging to flies (it results in death) than the
324 injection of non-pathogenic bacteria used here and in McKean and Nunney
325 (2001, 2005). It is possible that this difference might account for the contrast in
326 results if stronger immune challenges are more effective at uncovering small
327 differences in immunity. More generally, the use of non-pathogenic agents (e.g.
328 *E. coli*, here and in McKean & Nunney, 2001; 2005) in immunity studies may
329 result in important phenomena being overlooked. Recent studies have
330 highlighted a strong degree of specificity in invertebrate immunity (reviewed in
331 Little et al., 2005). The use of non-pathogenic microbes or general
332 immunoelicitors in immunity studies might therefore yield little information
333 about biologically relevant invertebrate immune responses.

334 In larger insects the effect of piercing in immunity may be ameliorated
335 because the relative size the wound inflicted compared to the size of the insect
336 decreases with increasing body size (given a fixed needle size). However, our
337 finding that piercing produced much higher immune gene expression than mating
338 in *D. melanogaster* suggests that investigators should explore ways of measuring

339 immunity that do not require integument piercing. For example, insects can be
340 exposed to entomopathogenic fungi (e.g. *Metarhizium anisopliae*, Barnes &
341 Siva-Jothy, 2000; Moret & Siva-Jothy, 2003) to investigate immune function.
342 With this type of system infection occurs naturally without the need for manual
343 damage to the integument. Of particular value to investigations into mating and
344 immunity would be to explore the fitness effects of sexually transmitted insect
345 pathogens (reviewed in Knell & Webberley, 2004). For example, it would be
346 interesting to examine the ability of virgin and mated individuals to fight
347 pathogens that are commonly transmitted during mating to determine whether
348 mating induced changes in immunity are adaptations to the risk of disease. This
349 prospect is especially intriguing in light of the recent finding that copulatory
350 wounding occurs in many species of *Drosophila* (Kamimura, 2007), a process
351 that could potentially facilitate pathogen entry into the female haemolymph.
352

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358
359

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457 Fig. 1. The level of *E. coli* infection in female flies 3 days after infection.
458 Median (± inter-quartile range) bacterial colony counts per fly. Sample sizes
459 were A) *N* = 38 and 41 for mated females and virgin controls injected at 4 hours
460 post-mating, *N* = 47 and 48 for mated females and virgin females injected 24
461 hours post-mating; B) *N* = 44, 49, 43, 45 and 44 for virgin females, females
462 mated to SP1 knockdown males, females mated to SP2 knockdown males,
463 females mated to control 1 males and females mated to control 2 males; C) *N* =
464 53, 57, 57, 56, 60 and 57 for virgin 1 females, females mated to SP1 knockdown
465 males, females mated to control 1 males, virgin 2 females, females mated to SP2
466 knockdown males and females mated to control 2 males.
467
468 Fig. 2. Expression (mean ± standard deviations of replicate QRT-PCRs) of A)
469 *AttA* and B) *Dpt* in the abdomen of females. The values shown are the fold-
470 change relative to virgin females (virgin value=1).
471
472

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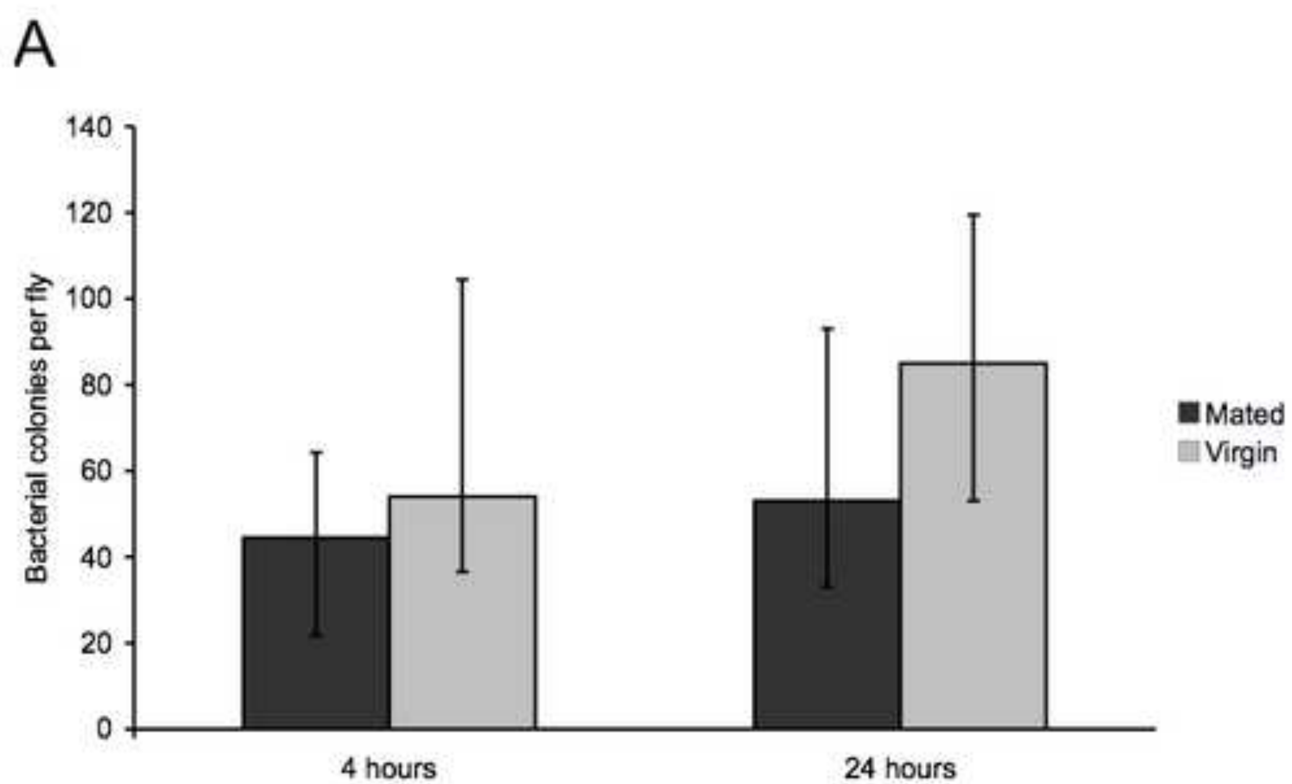

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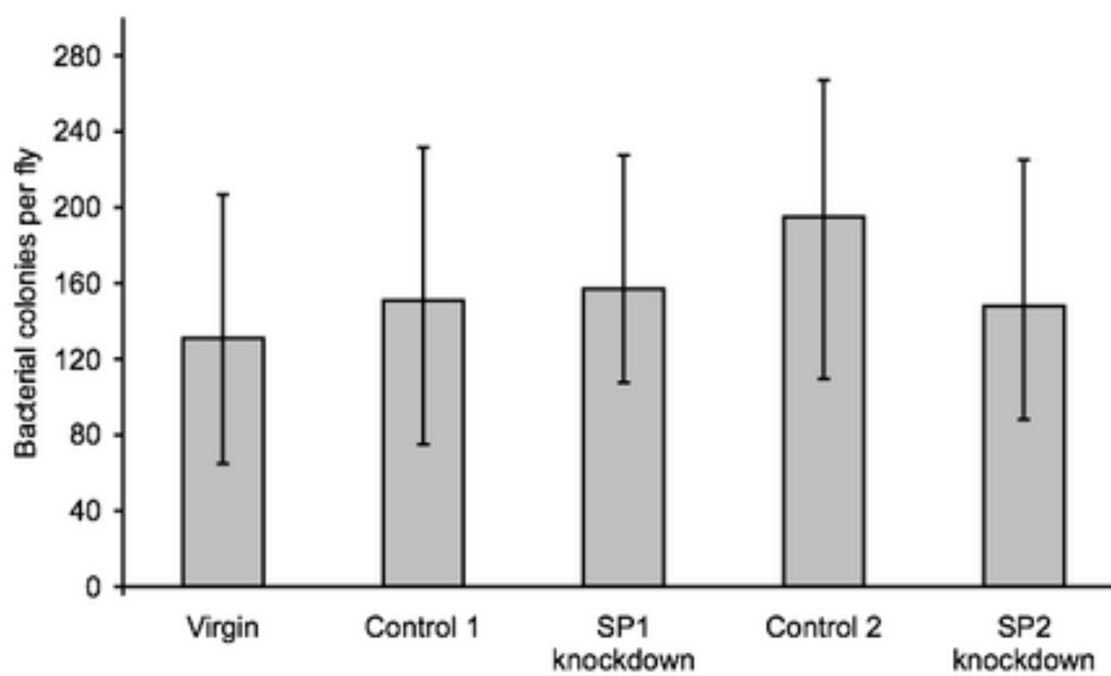

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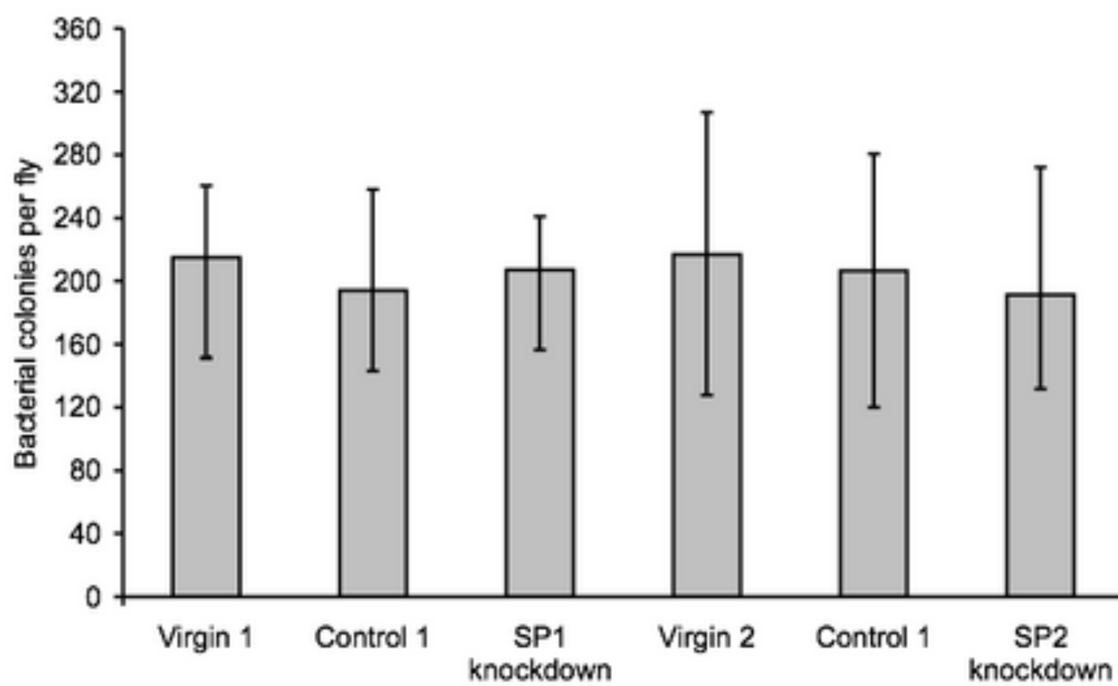

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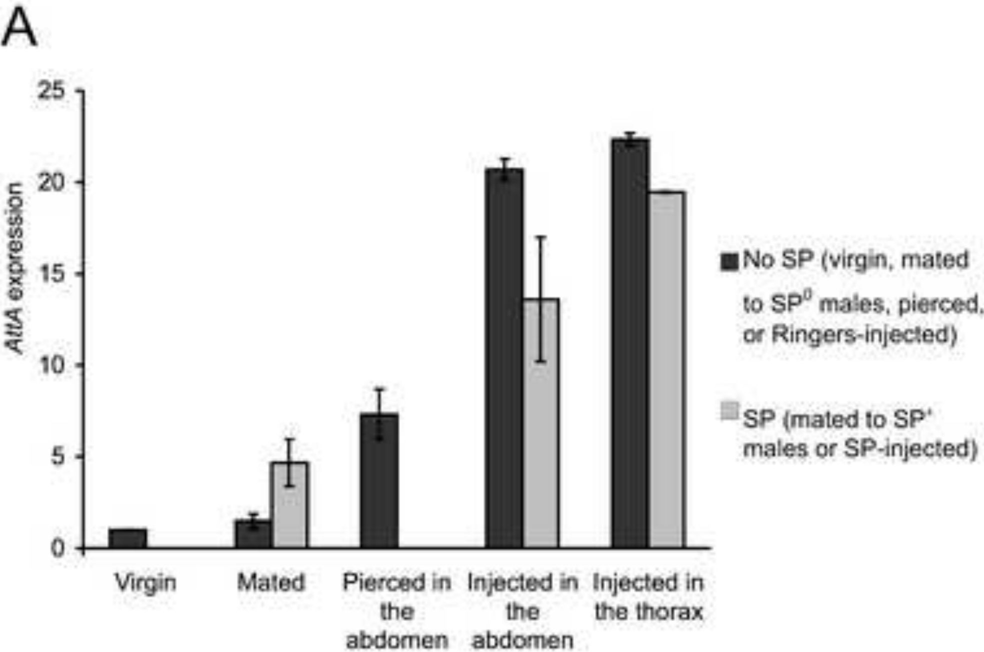

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