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Research Article

Sigma Virus (DMelSV) Incidence in Lines of *Drosophila melanogaster* Selected for Survival following Infection with *Bacillus cereus*

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The immune response of *Drosophila melanogaster* is complex and involves both specific and general responses to parasites. In this study we tested for cross-immunity for bacteria and viruses by scoring the incidence of infection with the vertically transmitted Sigma virus (DMelSV) in the progeny of a cross between females transmitting DMelSV at high frequencies and males from lines subjected to three selection regimes related to resistance to *Bacillus cereus*. There was no significant difference in transmission of DMelSV among selection regimes, though results suggest that the *B. cereus* selected lines had lower rates of infection by DMelSV. We found a significant difference in viral infection with respect to the sex of the progeny, with males consistently less likely to be infected than females. Given a finite energy budget, flies that have experienced immune system challenge may show alterations in other life history traits. Later eclosing progeny were also less likely to be infected than earlier eclosing progeny, indicating a relationship with development time. Finally, there was a significant interaction between the timing of collection and the sex of the progeny, such that later eclosing males were the most resistant group. Increased development time is sometimes associated with increased energy acquisition; from this perspective, increased development time may be associated with acquiring sufficient resources for effective resistance.

1. Introduction

The goal of this study was to test for cross-immunity between bacteria and viruses by scoring the incidence of the vertically transmitted rhabdovirus Sigma (DMelSV) infection in progeny of DMelSV-infected females and males from lines subjected to three selection regimes related to resistance to the Gram-positive bacterium, *Bacillus cereus*. Cross-immunity among viruses has been previously demonstrated for flies selected for survival following infection with DCV [1]. Similarly, if there are overlapping pathways involved in resistance to bacteria and viruses in Drosophila, then selection for resistance to the bacterium might also confer resistance to DMelSV.

The Gram-positive bacterium *Bacillus cereus* might be expected to mount an immune response via the Toll pathway; however, the DAP-type peptidoglycans of the *Bacillus* genus elicit IMD response [2]. There is conflicting evidence with respect to a role for the Toll and IMD pathways in response to infection by DMelSV. One study found that the major resistance gene to DMelSV, *ref(2)P*, was a component of the Toll signaling pathway [3]. In another study, expression of various downstream genes regulated by the Toll pathway was significantly increased in response to DMelSV, and key components of that pathway such as Toll, as well as Relish from the IMD pathway, were upregulated as well; however, neither the Relish nor Toll increases were statistically significant [4]. Yet another study failed to find any evidence of upregulation of either Toll or IMD upon infection with DMelSV [5]. Therefore, we hypothesized that there might be cross-immunity between *B. cereus* and DMelSV infection via either Toll, IMD, or yet another pathway.

Bacillus cereus are an aerobic spore forming Gram-positive bacteria that are closely related to *Bacillus anthracis*. *B. cereus* is commonly found in soil, on vegetable matter, and in human foods (both raw and after cooking). *B. cereus* is a saprophyte in soil and can be an opportunistic pathogen of soil invertebrates. This bacterium can cause medical problems in humans through consumption of bacterial contaminated food, causing several types of self-limiting gastric problems.

DMelSV is a rhabdovirus commonly found in *Drosophila* [6]. DMelSV is transmitted to progeny by a parent of either sex (vertical transmission), but transmission by females is typically more effective [3, 4]. The virus is virulent; DMelSV can result in a reduction in fitness in the laboratory and in field populations [4, 5]. Infection of *Drosophila* by DMelSV results in a characteristic sensitivity to CO_2 [7], such that infected flies will become paralyzed and die when exposed to concentrated CO_2. This sensitivity can be used to infer the presence of the virus in individual flies and thus estimate the prevalence of the virus in fly populations.

We used lines previously selected for resistance to the bacterium *B. cereus* [6, 8] to test the hypothesis that there would be cross-immunity between bacteria and viruses, in this case, DMelSV. As a partial control in the previous selection experiment, some lines were also evolved in response to sterile wounding, which could involve inflammatory processes or other responses connected to the immune system [7, 9, 10], and perhaps similarly result in diminished infection by DMelSV. The test for *D. melanogaster* refractoriness to infection by DMelSV was novel in that the consequences of two regimes of genetic selection on the host flies were investigated in terms of the likelihood of virus transmission. We performed crosses between males from the S, CI, and CN selected lines and females from an unrelated line infected with DMelSV to test for relationships between transmission and selection regime, progeny sex, and development time in these *D. melanogaster* populations that were related by common ancestry but differentiated by selection.

We did not find evidence for cross-immunity; that is, the *B. cereus* selected lines did not have significantly lower rates of infection by DMelSV. There was, surprisingly, a significant difference in viral infection with respect to the sex of the progeny: male progeny were consistently less likely to acquire the virus than were female progeny. Later eclosing progeny were also characterized by higher rates of uninfected flies than earlier ones. Finally, there was an interaction between timing of collection and progeny sex such that late eclosing males were the least likely to be infected. Thus, longer development time appears to be associated with reduced virus acquisition.

2. Materials and Methods

The present study involved progeny from crosses between a stock of female *D. melanogaster* that carried DMelSV with male flies from lines that were selected for survival after infection by *B. cereus* and control lines. The incidence of DMelSV in progeny was assessed by exposing female and male progeny to concentrated CO_2.

2.1. Fly Stock and Lines. Because DMelSV transmission by females is higher than that from males [6], we used infected females in our experiment to explore interactions between resistance to *B. cereus* and transmission of DMelSV. The females used for crosses in our experiment were DMelSV-infected via injection and were effectively isogenic (stock 27, described in detail by Rittschof et al. [11]; this stock is not infected with *Wolbachia*). Flies were cultured under standard light and temperature conditions (12 : 12, light : dark; 25°C) on standard molasses-agar food. Individual vials were set up with a constant density of five females and five males and allowed to lay eggs for five days, for at least three generations prior to the experimental crosses. The experimental crosses were kept under similar light and temperature conditions in standard food vials (described by Rittschof et al. [11]). Flies infected with DMelSV die after concentrated CO_2 exposure [9], which serves as an inexpensive, quick, and reliable way to test for infection. Females were transmitting virus at 100% frequency (i.e., half their progeny were exposed to CO_2 and all of those progeny died) at the start of the study.

Selection was conducted on three replicate lines for each of the three treatments: S (resistance to *B. cereus* infected), CI (response to wounding), and CN (the unperturbed control) for 19 generations prior to the experiments reported here (i.e., selection was relaxed for one generation prior to shipment of flies to UF) [12]. None of the lines were infected with *Wolbachia*, no doubt reflecting the low frequency of *Wolbachia*-infected flies in the original population from which the lines were derived. Following selection, a 3.3-log increase in the number of spores required for 50% mortality was observed [12].

2.2. Experimental Protocol. Nonvirgin, uninfected males from S, CI, and CN lines were crossed to virgin, DMelSV-infected females. Females were held for 12–36 hours prior to crossing. Virginity was verified by checking female holding vials for progeny (larvae or pupae) a week later. No incidents of nonvirginity were discovered. Crosses were performed with a controlled density of five females and five males in each vial. Females were permitted to lay eggs for five days at the standard light and temperature conditions described previously. Females were then assayed for infection via a CO_2 sensitivity assay per the protocol of Wayne et al. [13]; no females recovered from the assay, confirming their positive DMelSV infection status. Nine replicate vials of crosses were used for the CI lines, nine replicated crosses were used for the CN lines, and eight replicate crosses were conducted for the S lines.

Two collections of offspring from the experimental crosses were made: the first was 11 days after the crosses were initiated and the second was 13 days after the crosses were initiated. Thus, day of collection was a proxy for development time. Following collection, progeny were held for 24 hours and then assayed for sensitivity to CO_2 [13].

2.3. Statistical Analysis. The dependent variable was the percentage of flies that did not show CO_2 sensitivity, that is, the percentage of uninfected flies. The percent data was transformed by arcsine square root to improve normality

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Table 1: Percent of uninfected progeny by sex and selective regime. Selective regimes included the following: S (resistance to *B. cereus* infected), CI (response to wounding), and CN (the unperturbed control). Percent was calculated as the total number of uninfected progeny divided by total number of progeny.

	S	CI	CN
Males	50/341 = 14.7%	17/232 = 7.33%	26/343 = 7.58%
Females	16/366 = 4.37%	5/268 = 1.87%	6/396 = 1.52%

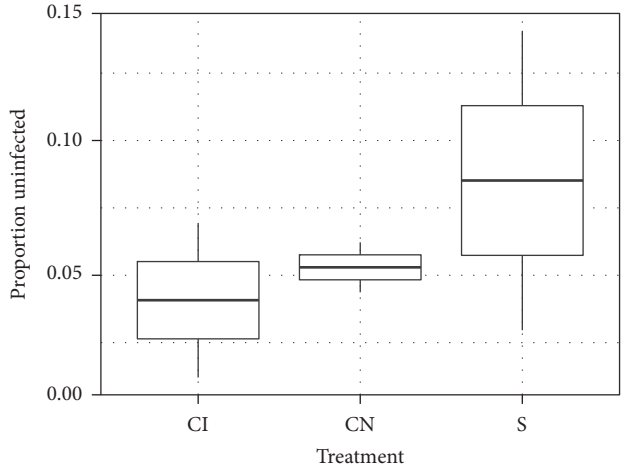


Figure 1: Box plot showing median and interquartile range of uninfected progeny (untransformed data) for each selection regime. Selective regimes included the following: CI (response to wounding), CN (the unperturbed control), and S (resistance to *B. cereus* infected). The median proportion of uninfected progeny was highest for the lines selected for resistance to *Bacillus cereus*.

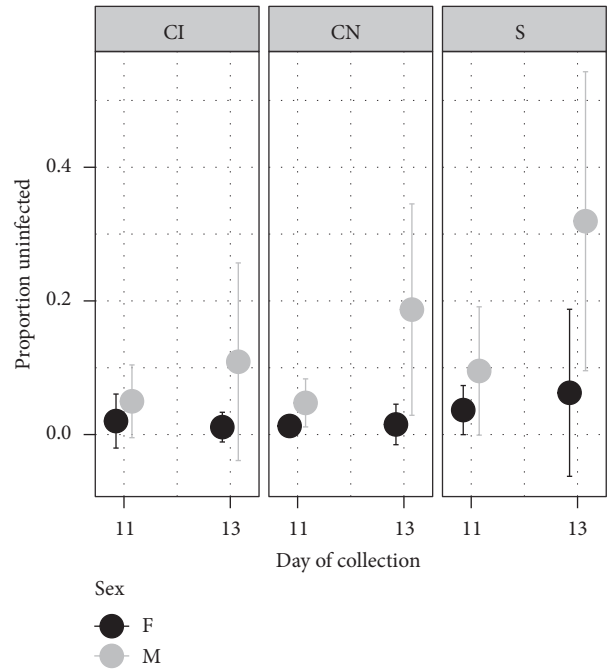


Figure 2: Graph (means ± 2 standard errors) of proportion uninfected progeny (untransformed data) for each selection regime × day × sex combination. Selective regimes included the following: CI (response to wounding), CN (the unperturbed control), and S (resistance to *B. cereus* infected). The significant interaction of day × sex is obvious, with male progeny having higher proportion surviving on the second day of eclosion compared to females across treatments. Again, the lines selected for resistance to *B. cereus* tended to have the highest proportion of uninfected animals, and this trend was notably driven by the male progeny eclosing on the second day of collection.

of the residuals. The data were then analyzed in R using lme4: ANOVA with type III Wald F tests and Kenward-Roger degrees of freedom. The model included the main effects of selection regime, day of collection, and sex of the progeny and main effect interactions.

3. Results

The vast majority (1,826/1,946; >93%) of progeny flies succumbed to CO_2 anesthesia, consistent with infection by DMelSV (Table 1). The number of uninfected male and female progeny from each category of cross between the stock carrying DMelSV and the three types of lines (S = *B. cereus* selected, CI = control wounded each generation, and CN = no perturbation controls) is reported in Table 1. To test for refractoriness to DMelSV infection, we analyzed the proportion of females or males that survived the CO_2 assay (i.e., which were not infected) for each type of cross (Table 2). The main effect of selection regime was not significant (P = 0.518; Table 2; Figure 1) nor were any of its interactions (Table 2). Although a trend in the data (Figure 1) suggests that there were a higher proportion of uninfected animals in the lines selected for resistance to *B. cereus* than in either of the two control treatments, in no case did survival differences between crosses types attain statistical significance.

Males were significantly more likely than females to be uninfected regardless of selection or control regime (Tables 1 and 2 and Figure 2; $P < 2.7\times10^{-4}$). Although transmission by males is generally lower than that by females and mutations blocking male but not female transmission are well known [6], we are unaware of other work demonstrating differences in sex-specific refractoriness to acquisition of DMelSV.

There was a significant effect of development time, as assayed by the day of collection, such that a higher proportion of uninfected flies eclosed by day 13 than that on day 11 (day: $P < 0.027$, Table 2; Figure 2). Moreover, there was a significant interaction between collection date and progeny sex (day × sex: $P < 0.016$, Table 2; Figure 2), such that surviving males were more likely to eclose on the second day of collection than surviving females.

4. Discussion

The present study tested for cross-immunity via differential survival following CO_2 exposure, which is fatal to flies infected with DMelSV, among progeny sired by males of lines

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Table 2: ANOVA of transformed proportion of uninfected progeny. The model included the main effects of selection regime, day of collection, and sex of the progeny and main effect interactions. The proportion was transformed by arcsine square root to improve normality of the residuals.

Source	F	D.f.	D.f. resid.	P
Intercept	21.62	1	2	0.043*
Selection regime	1.36	2	1	0.518
Day	5.83	1	18	0.027*
Sex	20.33	1	18	2.7×10^{-4}***
Selection regime × day	1.62	2	18	0.225
Selection regime × sex	1.05	2	18	0.369
Day × sex	7.13	1	18	0.016*
Selection regime × day × sex	1.00	2	18	0.387

* $P < 0.05$; *** $P < 0.001$.

selected for *B. cereus* resistance and progeny sired by control males, both crossed to females from a stock that transmits DMelSV at effectively 100%. Neither selection regime (i.e., selected versus controls) nor any of its interactions were significant, and so we have no evidence for cross-immunity between *B. cereus* and DMelSV, despite the fact that both parasites could involve the Toll and IMD pathways. Failure to detect a significant effect may be the artifact of the small sample sizes in this experiment, small effect size, or both. Given that the trend in the data is consistent with increased survival following CO_2 exposure in the offspring of males selected for *B. cereus* resistance, it is possible that such cross-immunity between bacteria and viruses does exist and that a greater sample size might provide sufficient power to detect a difference in cross-immunity between selected and control lines. However, the current data do not support such a conclusion.

To the best of our knowledge, a sex bias in DMelSV transmission has not been previously reported. The absolute numbers of male and female S progeny were very similar (341 males, 366 females), so the difference cannot be explained by sample size. One possibility is that the virus we used [11] is peculiar such that it mediates sex bias in infected progeny. However, this outcome has not been observed in other studies using the same virus and the same stock 27 ([11]; other unpublished data).

Infection by DMelSV is known to increase development time [14] in infected *D. melanogaster*, and viral titer is positively associated with development time in infected flies [15]. Thus, we were initially surprised that uninfected animals had increased development times relative to infected flies. However, the data documenting increased development time as a result of DMelSV are either from comparisons of infected and uninfected flies [14] or among flies that are all infected but with variable viral titers [15]. Thus, to the best of our knowledge, the relationship between transmission success versus failure and development time among the progeny of infected flies has not been explored.

The cost of mounting an immune response is relevant to life history trait correlations and evolution in animals. It is generally thought that mounting an immune response is physiologically costly and that standing immunity negatively impacts other fitness traits. Populations of *D. melanogaster* have been used for artificial selection for resistance to infection by microbes or parasitoids, and the evolution of other fitness-related traits is a typical result of such studies (summarized by McKean and Lazzaro [16]). Conversely, artificial selection on life history traits can affect immunity; Modak et al. [17] documented that *D. melanogaster* selected for decreased development time exhibited a shorter time to death following introduction of *E. coli* than unselected controls. In the present study, lines of *D. melanogaster* selected for survival after introduction of *B. cereus* tended to have decreased incidence of progeny that acquired DMelSV. Additionally, selection for survival after exposure to *B. cereus* resulted in constitutively slower development time [12], consistent with a tradeoff between immunity and development time. In the data presented here, the proportion of uninfected progeny, that is, progeny with potentially greater immune investment, increased with time (significant day of collection term), again consistent with a tradeoff.

Transmission of DMelSV to progeny was significantly higher in males in all of the lines used for crosses (Table 2, Figure 2). Progeny male refractoriness to acquisition of virus was particularly notable as an interaction between selected lines and day of collection (Table 2, Figure 2), driven by the relatively high frequency of uninfected males on the second collection day. Although this pattern is seen in all selection treatments, it is most pronounced in the S lines (the lines selected for resistance to *B. cereus*; Figure 2).

Vincent and Sharp [18] demonstrated greater resistance (as well as tolerance) to *P. aeruginosa* in male *D. melanogaster* relative to females. They note that higher male resistance is not uncommon in response to actual parasite challenge (realized immunity), while higher female immunocompetence is often observed in uninduced animals (constitutive immunity). In our experiment, offspring were challenged by DMelSV during development, thus representing realized rather than constitutive immunity. Thus, our observation of greater resistance in males (and of increased development time in resistant animals in general and males in particular) is possibly the result of greater investment in immunity and is consistent with the patterns described by Vincent and Sharp [18].

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There are two general mechanisms, which are not mutually exclusive, that could underlie an association between delayed development time and refractoriness to DMelSV transmission. The first is that delayed development results in an increase in time available for the accumulation of food resources, which could then be used to mount a successful immune response and which in our study could result in resistance to DMelSV. A second possibility is that delayed development time is associated with a molecular process that directly results in increased immunity. As one possibility, ecdysone signaling is an innate immunity maturation or immunity-stimulating hormonal agent [19, 20]. Further investigation of the relationship between host development time and successful transmission of DMelSV will help distinguish between these hypotheses.

5. Conclusions

There was no statistically significant impact of fly resistance to *B. cereus* on virus transmission incidence. However, there was a weak association between resistance to *B. cereus* and diminished transmission of DMelSV. Moreover, there were statistically significant effects of progeny sex, timing of collection, and their interaction: males eclosing later were associated with reduced virus transmission. The selected lines were previously found to develop more slowly, and, in this experiment, animals from the second collection (i.e., slow developers) were more likely to be resistant. If longer development time is generally associated with reduced virus transmission, it would be interesting to investigate the developmental maturation of the innate immune system in *D. melanogaster* and the interaction between nutritional status and maturation of innate immunity. The results of the present study could lead to novel insight into the ontogeny of immunity in this model for genetic and innate immunity research.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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