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Thorax Injury Lowers Resistance to Infection in *Drosophila melanogaster*

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The route of infection can profoundly affect both the progression and outcome of disease. We investigated differences in *Drosophila melanogaster* defense against infection after bacterial inoculation into two sites—the abdomen and the thorax. Thorax inoculation results in increased bacterial proliferation and causes high mortality within the first few days of infection. In contrast, abdomen inoculation results in minimal mortality and lower bacterial loads than thorax inoculation. Inoculation into either site causes systemic infection. Differences in mortality and bacterial load are due to injury of the thorax and can be recapitulated by abdominal inoculation coupled with aseptic wounding of the thorax. This altered resistance appears to be independent of classical immune pathways and opens new avenues of research on the role of injury during defense against infection.

Injury commonly provides opportunity for infection. In clinical contexts, burn victims, patients suffering from stab and gunshot wounds, and other victims of traumatic breaches of barrier epithelia all face the prospect of complications due to infection (1, 2). Many factors associated with traumatic injury potentially affect defense against infection, including local tissue damage, immune system activation, and bacterial access to nutrients. Some of these factors are likely to impact resistance—the ability to control pathogen load—while others may impact tolerance—the ability to withstand the damage and consequences at a given pathogen load (3–6). Classification of host responses into these categories is often complicated because activation of the immune system can cause collateral host damage, which in turn reduces tolerance of infection.

Host damage is thought to primarily affect tolerance of infection; however, there is emerging evidence that wounding may influence resistance as well (6). In mice, mortality due to coinfection with pneumonia bacteria and influenza virus is independent of both bacterial load and induction of the immune system but is dependent on tissue damage—a failure of tolerance (7). Infection after severe burn trauma is affected by changes in both tolerance and resistance. Loss of skin lowers tolerance of infection by compromising the patient's ability to stay hydrated and maintain body temperature (8); systemic immune suppression lowers the resistance of burn patients to infection (1). In *Drosophila melanogaster*, wound healing sequesters bacteria at the wound site and improves survival through an unknown mechanism potentially affecting both tolerance and resistance (9). Sequestration of bacteria may generate a more localized immune response, which might improve resistance by more efficiently killing the bacteria or might improve tolerance by limiting host damage.

Although we know little about how *D. melanogaster* becomes infected with bacteria in nature, it is reasonable to suppose that cuticle breaches are a common route of infection. Melanization spots, which are indicative of healed wounds, are frequently observed on wild-caught *D. melanogaster* flies (unpublished observation). It has been demonstrated in the laboratory that ectoparasitic mites can transmit bacterial infection between flies (10), and wounds left by mites can become secondarily infected by bacteria in honey bees (11). Since insects possess an open circulation system, introduced bacteria should circulate freely. However, the suggestion that there may be a difference between infection of the thorax and infection of the abdomen was raised by the recent finding that *Drosophila* mutants with genetically disrupted flight muscle are more susceptible to bacteria delivered to the thorax than they are to bacteria delivered into the abdomen (12). The abdomen of *Drosophila* is dominated by the fat body, the liver equivalent and organ of systemic humoral immunity in insects, and also houses the digestive tract, heart, and reproductive organs. The thorax is filled with flight muscle and tracheae, the tissues responsible for oxygen exchange. Both segments contain hemocytes, with a large number of hemocytes clustered around the heart. The potential for differences in systemic infections that originate in either segment has remained largely unexplored.

The *Drosophila* immune system is well studied, which allows us to probe the relationship between injury and infection using characterized immune system mutants and previously described pathogens (13–16). Insect antibacterial immunity can be broken down into three main components: production of extracellularly secreted antimicrobial peptides (AMPs), cellular phagocytosis, and melanization. The *Toll* and *Imd* pathways are the two primary signaling cascades responsible for AMP production, and mutations that block these pathways result in flies with severely impaired resistance to infection (14, 15). The JAK-STAT pathway also regulates immune induction of genes during septic injury through release of a cytokine (upd3) from the hemocytes to the fat body (17). Phagocytosis of bacteria by hemocytes helps clear bacteria from the hemolymph (insect blood), killing the bacteria in an acidifying lytic vacuole (18–20). In defensive melanization, invading pathogens are bound in a pigmented

Received 29 July 2014 **Accepted** 30 July 2014

Published ahead of print 4 August 2014

Editor: A. J. Bäumler

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.02415-14.

doi:10.1128/IAI.02415-14

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capsule and bombarded with cytotoxic reactive oxygen species. Defensive melanization can be impaired through genetic manipulation of crystal cells and blocking or elimination of proteases in the activation cascade (21–25).

In this paper, we use *D. melanogaster* to investigate the impact of the inoculation site on disease progression, contrasting delivery of bacteria into the thorax with delivery into the abdomen. We find that inoculation into the thorax causes increased bacterial loads and mortality relative to abdomen inoculation. This decrease in resistance results from the injury to the thorax and is independent of the location where the bacteria are actually introduced since abdominal inoculation also results in low resistance when coupled with aseptic injury of the thorax. The effect of thorax injury does not depend on any known immune pathways and is surprising because there is no prior reason to expect remote wounding to influence the level of bacterial proliferation. This unexpected finding holds practical implications for experimental methodologies and indicates a previously unknown role of tissue-specific injury in shaping defense against infection.

MATERIALS AND METHODS

***D. melanogaster* strains and husbandry.** Unless otherwise noted, all experiments were done using wild-type strain Canton-S (CS; Bloomington stock no. 1). Immune system mutants deficient for the Imd pathway (OR; imd^{10191};OR [26]), the Toll pathway (spz^{rm7}/TM6C [27]), and melanization processes (Bc^{1} [22]) were compared to wild-type Oregon R (OR; Bloomington stock no. 5). The melanization mutant 6326;$sp7^{KG0180}$;6326 (24) was compared with its w^{1118} background line (Bloomington stock no. 6326). The JAK-STAT mutant *upd3* (Bloomington stock no. 55728 [17]) was compared with its *w** background line (28). The CS, OR, *upd3* mutant, and w* lines do not carry *Wolbachia pipientis*; the remainder of the lines do.

For each line, approximately 15 males and 15 females were placed in glass bottles with 50 ml of glucose-yeast medium (100 g/liter yeast, 100 g/liter glucose, 1% *Drosophila* agar) and allowed to lay eggs for 2 days. Adults were cleared, and offspring took approximately 10 days to develop. Eclosed adults were transferred to a fresh bottle, kept overnight to allow mating, and sorted on the subsequent day into vials of 20 males or 10 females. Flies were aged for four additional days before injection, such that all experiments were performed with flies from day 5 to day 7 post-eclosion. Males were flipped to new vials every 7 days and females were flipped every 2 days to preserve food quality. Experiments were performed with male flies unless otherwise specified. All flies were kept at 25°C in an incubator with a 12-h-light-12-h-dark cycle unless otherwise noted.

Bacterial strains and infection conditions. The low-virulence pathogens used were *Serratia marcescens* (strain BPL, an attenuated strain derived from type strain ATCC 13880 [29]) and *Salmonella enterica* serotype Typhimurium (strain S5 520, obtained from Martin Wiedmann, Cornell University). The moderate-virulence pathogens used were *Providencia rettgeri* (strain Dmel, isolated as an infection of field-caught *D. melanogaster* [30]) and *Enterococcus faecalis* (isolated as an infection of field-caught *D. melanogaster*). The high-virulence pathogens used were *Providencia sneebia* (strain Dmel, isolated as an infection of field-caught *D. melanogaster* [30]), *Providencia alcalifaciens* (strain Dmel, isolated as an infection of field-caught *D. melanogaster* [30]), and *Pseudomonas aeruginosa* (strain PAO1, ATCC 15692). Bacterial stocks were stored at −80°C in Luria Bertani (LB) broth containing 15% glycerol. Bacteria were first streaked on an LB agar plate from the stock and grown overnight at 37°C and subsequently stored at 4°C for up to a month. For infection, liquid bacterial cultures were inoculated from a single bacterial colony from the plate and grown in 2 ml LB broth at 37°C with shaking. The cultures were then diluted in phosphate-buffered saline (PBS; pH 7.4) to the desired optical density (OD) (A_{600}). Either 23 or 50 nl of bacterial suspension was injected into each fly using a Nanoject II injector (Drummond). The standard density used was an OD (A_{600}) of 0.1, and this corresponds to a dose of about 4,000 to 8,000 CFU per fly. Sterile PBS was injected as a control. Flies were anesthetized with light CO_2 for less than 5 min during the infection procedure. All controls were exposed to CO_2 for the same amount of time, and no CO_2-induced mortality was observed. Infected flies were kept in groups of about 20 per vial at 25°C (except for experiments explicitly measuring the effect of postinfection temperature, in which flies were kept at 18°C, 25°C, or 29°C) in an incubator with a 12-h-light-12-h-dark cycle.

Survival experiments. After inoculation, death was recorded daily. A few flies were homogenized immediately after inoculation to determine the initial bacterial load. For long-term survival assays, flies were transferred every 5 to 7 days to fresh vials. Survival curves are plotted as Kaplan-Meier plots, and statistical significance was determined using Cox Proportional Hazards (*coxph*) with the package "Survival" in R (http://www.r-project.org/). Significant factors are reported with complete models outlined below. A typical replicate contained 2 to 3 vials of 20 flies per condition. Homozygous mutants in *sp7* were difficult to rear in large numbers, and 2 vials of approximately 5 to 10 flies per condition were used in these experiments.

Wounding experiments. In some experiments, individual flies were both inoculated with bacteria and aseptically wounded. Aseptic wounds were generated using glass capillary needles matching those used for infection. No bacterial inoculation or liquid was injected into the aseptic wounds.

Bacterial load. CFU were determined using both spot-plating and a WASP II autoplate spiral plater (Microbiology International). *E. faecalis* bacterial loads were determined using spot-plating; in these experiments, 8 to 16 individual flies were collected at each time point. Each fly was separately homogenized, diluted serially, and plated onto LB agar, and the culture was grown overnight at 37°C. Spots containing 30 to 300 colonies were counted and used to calculate CFU per fly. *P. rettgeri* bacterial loads were determined using the spiral plater; in these experiments, six or more flies were individually homogenized in 250 μl and diluted to the appropriate concentration (at 8 h postinoculation, 1:100; at 24 h postinoculation, 1:100; at 48 and 96 h postinoculation, 1:10; all others, no dilution). Fifty microliters of diluted homogenate was plated exponentially on an LB plate and grown overnight at 37°C, and then colonies were counted using an EZ-Count automated colony counter (Microbiology International), which calculates the number of CFU per fly based on the number of colonies and their positions along a spiral. CFU counts were $\log_{10}$ transformed to approximate a Gaussian distribution. The results of bacterial load experiments performed under only two sets of conditions were compared using a two-tailed *t* test with Welch's correction to take the heteroscedasticity of the data into account. Bacterial load data were assessed for sources of variation by analysis of variance (ANOVA) using the linear modeling (*lm*) process in R (http://www.r-project.org/). Pairwise comparisons between conditions were assessed using Tukey's honestly significant difference (Tukey's HSD) analysis post-ANOVA. The complete linear models are described in detail below.

Quantitative RT-PCR. Flies were inoculated in either the abdomen or thorax with *P. rettgeri* or left unmanipulated. Following injection, the flies were placed in vials and left at 25°C for 6 or 12 h. Groups of 15 flies were homogenized in TRIzol (Life Technologies) and stored at −80°C until further processing. RNA was isolated using a standard TRIzol extraction procedure, RNA samples were treated with DNase (Promega), and cDNA was generated using murine leukemia virus reverse transcriptase (MLV-RT) (Promega). Quantitative PCR was performed using an SSO Advanced SYBR green kit (Bio-Rad) following the kit's protocol scaled down for 15-μl reaction mixtures. Quantitative PCR primers are listed in Table S1 in the supplemental material. AMP gene expression was assessed for sources of variation by ANOVA using the *lm* procedure in R (http://www.r-project.org/). The complete linear model is described in detail below. AMP gene expression levels in infected flies were scaled to expression levels of CO_2 controls for graphical representation.

Phagocytic index. Phagocytic assays were performed based on methods described by Stone et al. (31). Briefly, 10 to 12 flies were injected with 23 nl of 1 mg/ml pHrodo-labeled *Escherichia coli* (Molecular Probes; catalog no. P35361) in either the abdomen or thorax and allowed to phagocytose at room temperature for 30 to 60 min in order to allow bacterial probes access to hemocytes further from the inoculation site. These tagged bacteria fluoresce intensely in the low-pH phagolysosome, allowing easy imaging of phagocytic uptake through the dorsal cuticle. The wings of the flies were removed, and the flies were glued with a tiny drop of Loctite Super Glue (Extra Gel Control) to microscope slides. Fluorescent images of the dorsal surface were taken using epifluorescent illumination with a Leica M165 FC microscope fitted with a Leica DFC 450 camera. Images were captured with Leica Application Suite V4.0, and exposures were set so that the brightest images showed no saturated pixels. The images were further processed in ImageJ. First they were switched to 8-bit (black and white), and then a threshold was set to capture fluorescing cells. The function "analyze particles" was then used to calculate the total fluorescent area of injected flies. Results were obtained from three independent replicates and then pooled. To account for variation between replicates, values were scaled to the median fluorescent area of abdomen-injected flies. Statistical significance was determined using a two-tailed *t* test.

Analysis of variance. Statistical analyses of survival, bacterial load, and AMP gene expression were performed using R (http://www.r-project.org/). Survival curves are plotted as Kaplan-Meier plots, and a model reflecting the contribution of experimental factors was built using the Cox Proportional Hazards (*coxph*) process within the package "Survival" in R (32). Each factor is incorporated in sequence, and factors are listed in order of inclusion in result tables. Sources of variation were assessed by subsequent ANOVA. Pairwise comparisons of survival curves were calculated using the log-rank test in Graphpad Prism.

Bacterial load and AMP gene expression were assessed for sources of variation by ANOVA on linear models built using the *lm* procedure in R. Tukey's honestly significant difference (Tukey's HSD) test was used to assess pairwise comparisons. The factors in each model below are defined for the first model in which they appear. Interactions between factors are represented as factor 1 × factor 2.

Model A tested the effect of site of infection on survival. Factors included were infection state (*I*; medium control or bacterial infection), inoculation site (IS; abdomen or thorax), replicate (*R*), and vial (*V*). For each experiment, *V* is nested sequentially within each factor, finishing with *R*. Model A was as follows:

$$\text{coxph(status,time)} = R + I + \text{IS} + (I \times \text{IS}) + (R \times I) + (R \times \text{IS}) + R\{I[\text{IS}(V)]\}$$

Model B determined the impact of infectious dose (ID) on survival after thorax inoculation. The infectious-dose variable is a $\log_{10}$ transformation of the CFU/fly value determined immediately after inoculation. Model B was as follows:

$$\text{coxph(survival,time)} = R + \text{ID} + (R \times \text{ID}) + R[\text{ID}(V)]$$

Model C assessed the impact of temperature on thorax-mediated mortality. An additional factor included in this model was housing temperature postinfection (*T*; 18°C or 25°C or 29°C). Model C was as follows:

$$\text{coxph(survival,time)} = R + \text{IS} + T + (\text{IS} \times T) + (\text{IS} \times R) + (T \times R) + R\{\text{IS}[T(V)]\}$$

To determine the effect of wounding on both survival and pathogen load, we built three models. Model D is based on inoculation and aseptic wound site, model E is an alternative model based on wound locations and numbers, and model F is a combination of the D and E models. Model F was specifically used to assess the contribution of other factors after damage to thorax had already been taken into account. Additional factors included in these models were the aseptic wound site (WS; abdomen or thorax), damage to thorax (DT; yes or no), and the total number of wounds to each fly (NW; 1 or 2). Damage to thorax (DT) was assessed as any injury to the thorax whether through site of infection or sterile injury. As pathogen load (CFU) was not assessed across vials, *V* is not used as a factor where the pathogen load was the response variable. Model D was as follows:

$$\text{coxph(survival,time) or lm(CFU)} = R + \text{IS} + \text{WS} + (\text{IS} \times \text{WS}) + R\{\text{IS}[\text{WS}(V)]\}$$

Model E was as follows:

$$\text{coxph(survival,time) or lm(CFU)} = R + \text{NW} + \text{DT}$$

Model F was as follows:

$$\text{coxph(survival,time) or lm(CFU)} = R + \text{NW} + \text{DT} + \text{IS} + \text{WS} + (\text{IS} \times \text{WS}) + R\{\text{IS}[\text{WS}(V)]\}$$

Model G assessed whether timing of the aseptic wounding (TW) affected the injury-induced mortality. The only new factor in this model is timing of the aseptic wounding relative to that of the bacterial inoculation (TW at 1 day before, simultaneously with, or 1 day after the bacterial inoculation). Model G was as follows:

$$\text{coxph(survival,time)} = \text{WS} + \text{TW} + (\text{WS} \times \text{TW}) + \text{WS}[\text{TW}(V)]$$

Model H assessed whether AMP gene induction was affected by the inoculation site. The new factors in this model are the level of *rp49* gene expressed as an indicator of RNA quantity (RQ) and the time postinfection (*T*). Model H was as follows:

$$\text{lm(AMP gene)} = \text{RQ} + R + T + \text{IS} + (T \times \text{IS})$$

Model I assessed whether mutations in immune system genes affected the thorax-induced mortality. An additional factor included in this model was genotype (*G*; wild type or mutant). As factors were analyzed sequentially, in this model IS assessed whether there was a significant effect of inoculation site once line was controlled for and (G × IS) assessed whether the mutant altered the magnitude of the relationship between abdomen inoculation and thorax inoculation. Data are shown (see Fig. 5); the complete statistical output is shown in Table S8 and Table S9 in the supplemental material. Model I was as follows:

$$\text{coxph(survival,time)} = R + G + \text{SI} + (G \times \text{SI}) + R\{L[\text{IS}(V)]\}$$

RESULTS

Inoculation site impacts acute mortality. We injected the Gram-negative bacterial pathogen *Providencia rettgeri* or the Gram-positive species *Enterococcus faecalis* into either the thorax or the abdomen of male *Drosophila melanogaster* flies. We found that inoculation of either bacterium into the thorax resulted in marked (over 50%) mortality within the first 3 days after inoculation, while inoculation of either microbe into the abdomen caused a minimal death rate (Fig. 1A and B) (infection determined by inoculation site; *P* of <0.0001). Injection of sterile media into either the abdomen or thorax did not cause acute mortality (Fig. 1). There was no significant difference in the rates of mortality after 3 days postinfection (Fig. 1C and D). Thorax inoculation with low doses of more-virulent pathogens (*Pseudomonas aeruginosa*, *Providencia sneebia*, and *Providencia alcalifaciens*) caused significantly faster mortality than inoculation of the bacteria into the abdomen (see Fig. S1 in the supplemental material). Injection of heat-killed *P. rettgeri* or the less potent pathogens *Salmonella enterica* serovar Typhimurium and *Serratia marcescens* into the thorax did not induce acute mortality (see Fig. S2), indicating that the simple introduction of bacteria into the thorax is not sufficient to cause death. Female flies also experienced increased mortality after thorax inoculation (Fig. S3), but for simplicity, further work was pursued solely in male flies. Thorax inoculation increased mortality during virulent infection but did not affect survival during avirulent infections or after injection of dead bacteria.

In order to test whether mortality induced by thorax inocula-

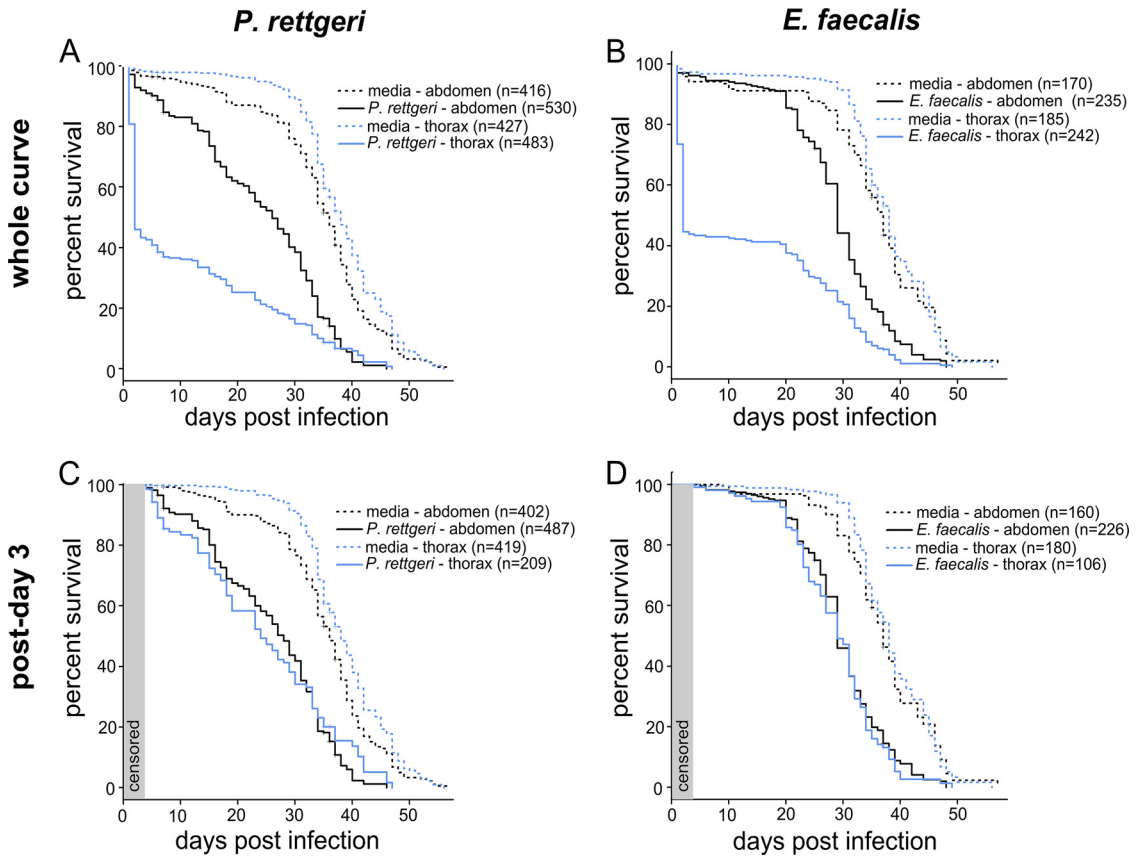

FIG 1 Thorax inoculation induces acute mortality. Flies were inoculated in either the abdomen or thorax with *P. rettgeri* (A and C) or *E. faecalis* (B and D) or a medium control and monitored daily for survival. Flies inoculated in the thorax with bacteria suffered acute mortality (site of injection P of <0.0001); the difference in the rates of mortality of flies injected with *P. rettgeri* in the abdomen and flies injected in the thorax disappeared after the first 3 days (C and D). See model A and Table S2 in the supplemental material for complete statistical analysis.

tion was dose dependent in a moderately virulent infection, we injected *P. rettgeri* flies across a thousand-fold range of optical densities and measured both infectious dose and survival over the first week (see Fig. S4 and Table S3 in the supplemental material). Mortality from thorax inoculation increased with dose from 20 percent mortality at an infectious dose of ~10 CFU per fly to over 90 percent mortality at an infectious dose of $\sim 5 \times 10^4$ CFU per fly. At every dose, there was a significant difference in mortality between abdomen and thorax inoculations (P of <0.0001). Abdominal inoculation induced relatively low mortality at comparable doses, with only 25% mortality even with an infectious dose of 2×10^4 CFU per fly (see Fig. S4C). Subsequent experiments were performed with a midrange infectious dose of 2×10^3 CFU per fly, which resulted in ~60% mortality after thorax injection and allowed the opportunity to detect both increased survival and decreased survival in the experiments presented below.

Since ambient temperature can often exacerbate or retard infection in poikilotherms, we hypothesized that lower temperature might rescue mortality in thorax-injected flies and that higher temperature might induce death in abdomen-injected flies. To test this, we infected flies in either the abdomen or thorax and then housed them at different temperatures postinfection. While increasing temperature did have the anticipated overall effect of decreasing survival and increasing pathogen load after infection, temperature differences did not alter the difference in mortality between abdomen-injected and thorax-injected flies (see Fig. S5 and Table S4 in the supplemental material). Overall, the increased mortality after thorax inoculation relative to abdomen inoculation was a very robust phenotype and did not depend on specific kinetics of infection.

Both injection sites cause systemic infection. One hypothesis to account for the difference in the rates of mortality of abdomen and thorax inoculations is that injection into either site might result in a highly localized infection. To test whether both inoculation sites cause systemic infection, we bisected flies at the abdomen-thorax junction or at the thorax-head junction approximately 30 min after injection with *P. rettgeri* and assayed bacterial load in the thorax and head versus the abdomen. This time point is expected to be prior to bacterial replication. Total bacterial loads per fly were equal (two-tailed t test; P of >0.05), regardless of the site of inoculation. Neither did the inoculation site influence the proportion of injected bacteria found in the thorax versus the head of the flies (see Fig. S6) (two-tailed t test; P of >0.1). These data suggest that the differences in resistance to thoracic versus abdominal inoculation were not due to differential distribution of bacteria immediately after injection.

Acute mortality can be induced by damage to the thorax independently of inoculation. To test whether the difference in mortality was due to tissue-specific damage, we tested whether aseptic injury of the thorax or abdomen could induce acute mor-

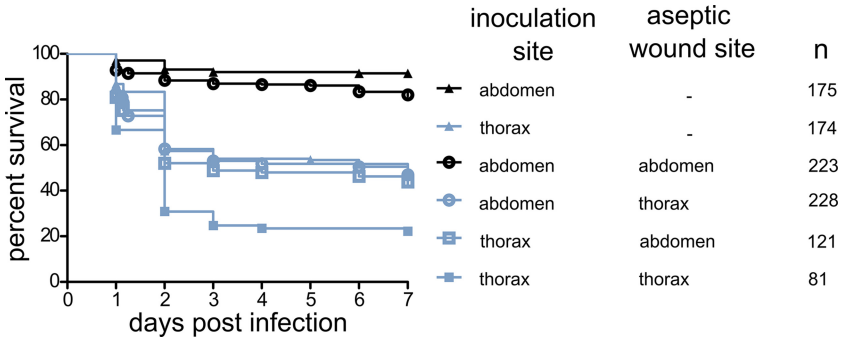

FIG 2 Injury to thorax sufficient to induce acute mortality during infection. Flies were first wounded by an aseptic prick to either the abdomen or the thorax and then inoculated with *P. rettgeri* in either the abdomen or the thorax within 5 min. Flies were monitored for survival daily until 1 week postinoculation. Blue lines indicate survival of flies where any damage was done to the thorax. See models D to F and Table S5 in the supplemental material for complete statistical analysis.

tality when followed by subsequent bacterial inoculation into either site. We first gave an aseptic wound to either the abdomen or thorax and then injected bacteria into one of the two sites within 5 min. Any fly damaged in the thorax exhibited a high proportion of acute mortality when the injury was coupled with bacterial inoculation to either the thorax or abdomen (Fig. 2, injured thorax curves in blue; see also Table S5 in the supplemental material). Even a simple pinprick to the thorax was sufficient to cause high levels of mortality in conjunction with injection of bacteria into the abdomen. Aseptic injuries to the abdomen and thorax coupled with control injection of sterile media caused no mortality (see Fig. S7A).

In order to determine whether damage to the thorax would cause increased mortality when administered on different days than infection, we sterilely wounded flies in the thorax either 1 day before or 1 day after abdominal inoculation with *P. rettgeri*. Thorax injury 1 day before or after abdominal inoculation did result in increased mortality from infection (P of <0.0004), but the degree of mortality was considerably less than when the thoracic injury and abdominal inoculation were delivered within minutes of each other (see Fig. S7B in the supplemental material). In order to determine whether damage to the thorax would induce mortality after the infection had persisted for several days, we aseptically wounded flies that were carrying week-old infections with pathogen burdens of 10^3 to 10^5 bacteria per fly (see Fig. S8). There was no significant increase in mortality from the thorax wound delivered a week after inoculation (see Fig. S7C), suggesting that thorax wounding is not lethal once the infection has stabilized. There was no increased mortality observed when an abdominal injury was coupled with an abdominal inoculation regardless of timing. These data collectively demonstrate that injury to the thorax is lethal only in the presence of bacterial infection and only when the wound is delivered proximally in time to the infection.

Thorax wounding causes an acute defect in resistance. The increased mortality due to thorax wounding may stem from tissue damage that is directly or indirectly lethal when coupled with infection—a defect in tolerance—or it may be that thorax wounds somehow allow the bacteria to establish better and proliferate to higher levels—a defect in resistance. To discriminate between these two possibilities, we assessed bacterial loads longitudinally after *P. rettgeri* injection (see Fig. S8 in the supplemental material). Flies injected with *P. rettgeri* or *E. faecalis* in the thorax had significantly higher bacterial loads at 24 h postinfection, coinciding with the time of higher mortality (Fig. 3A and B). Flies aseptically wounded in the thorax and simultaneously inoculated with *P. rettgeri* in either the abdomen or thorax also exhibited higher bacterial loads 24 h postinfection (Fig. 3C). The difference in resistance was robust with respect to ambient temperature and occurred regardless of whether the flies were housed at 18°C, 25°C, or 29°C postinoculation (P of <0.05 at every temperature; see Fig. S5B in the supplemental material). While flies infected with *P. rettgeri* carried bacterial loads of 10^3 to 10^5 CFU/fly for weeks after inoculation, the difference between flies inoculated in the abdomen versus the thorax did not persist beyond 72 h postinoculation (see Fig. S8), which is consistent with the equilibration of the rate of mortality 3 days after infection (Fig. 1). The combined data clearly show that thorax wounds cause a defect in resistance to infection, allowing the bacteria to proliferate to higher levels.

The canonical immune system is not responsible for the difference in the rates of acute mortality. To assess the contribution of immune processes to the increased mortality induced by thorax wounding, we used immune assays and available genetic mutants to query individual components of the immune system. We assessed induction of five representative AMP-encoding genes at 6 and 12 h postinfection to determine whether the levels of AMP gene induction differed between the flies inoculated in the abdomen and those inoculated in the thorax. These time points were chosen because the inducible immune system is active by 6 h postinfection, but both time points were prior to any observed differences in bacterial load between treatments, insulating the experiment from the feedback effect that significantly different bacterial loads could have on immune system activity. The inoculation site did not have a significant effect on the expression levels of any of the AMP genes (Fig. 4A; see also Table S7 in the supplemental material). We additionally infected flies deficient in the two major signaling pathways that regulate AMP production and monitored survival after infection through the abdomen versus thorax. Although deficiencies in the Toll and Imd pathways caused overall increased susceptibility to infection, the elevated mortality of thorax-inoculated flies relative to abdomen-inoculated flies was preserved in both *spz* mutants (Toll pathway) and *imd* mutants (Imd pathway) (Fig. 5A, B, F, and G; see also Table S8 and S9) (inoculation site P of <0.0001). Thus, we found that neither component of humoral immunity is necessary for the difference in mortality between flies infected in the thorax and those infected in the abdomen.

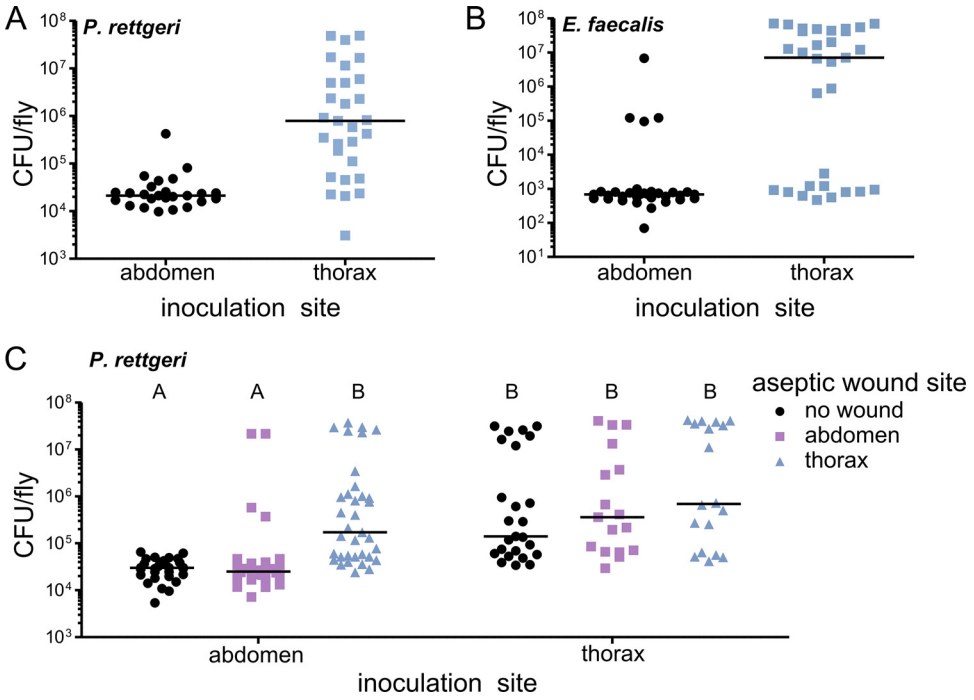

FIG 3 Thorax injury causes defect in resistance. (A and B) Flies were inoculated with *P. rettgeri* (A) or *E. faecalis* (B) (approximately 2,000 CFU/fly) through either the abdomen or the thorax, and bacterial load was assayed at 24 h postinoculation (summary of four replicates and two replicates, respectively) (two-tailed *t* test, *P* of <0.0001). (C) Flies were first aseptically wounded by pricking in either the abdomen or thorax or left unwounded and then inoculated with *P. rettgeri* in either site. Bacterial load was assessed 24 h postinoculation. Data represent a summary of the results determined with three replicates; see models D to F and Table S5 in the supplemental material for complete statistical analysis. Different letters above the columns indicate significant differences at *P* of <0.05 by Tukey's HSD.

We next sought to determine whether phagocytosis was less effective when flies are inoculated in the thorax versus in the abdomen. We assessed the phagocytic activity in flies by injecting dead *E. coli* labeled with a pH-sensitive fluorescent tag and then quantifying their accumulation in the sessile hemocytes visible through the dorsal plate of the thorax. There was no significant difference in the phagocytic index results between flies injected in the thorax and those injected in the abdomen (Fig. 4B and C) (two-tailed *t* test; *P* of >0.1).

To test the potential impact of melanization, we monitored survival after infection of both *Black cells* (*Bc*) and *sp7* mutants, which have impaired melanization and production of reactive oxygen. The difference in the rates of mortality induced by abdomen infection versus thorax infection was preserved in both *Bc* and *sp7* mutants (Fig. 5C, D, H, and I; see also Table S8 and S9 in the supplemental material) (inoculation site *P* of <0.0001).We found no evidence that melanization is necessary for the difference in survivorship between abdomen inoculation and thorax inoculation.

To test the potential impact of the JAK-STAT pathway, we monitored the survival of flies that were deficient in the immune-induced cytokine upd3. This cytokine is normally produced by the hemocytes and alters gene transcription in the fat body (17). The difference in mortality between abdomen inoculation and thorax inoculation was preserved in *upd3* mutants during both *P. rettgeri* infection and *E. faecalis* infection (Fig. 5E and J) (inoculation site *P* of <0.0001). These results indicate that JAK-STAT signaling does not underlie the increased mortality driven by thorax injury.

DISCUSSION

In this paper, we uncover a novel phenomenon in which a remote wound impacts resistance and survival during systemic infection. Wounding of the thorax causes increased host mortality and approximately 20-fold-higher levels of bacterial proliferation by 24 h after inoculation with either of two moderate-virulence pathogens. We have excluded the role of canonical immunity in this altered resistance as all mutants tested exhibited increased mortality after thorax inoculation. This phenomenon offers a new perspective on the role of injury during defense against infection.

One initial hypothesis upon observing the difference in mortality between flies inoculated in the abdomen and flies inoculated in the thorax was that the respective inoculations caused localized infections with different mortality profiles. However, when we tested the distribution of the bacteria within the host at 30 min after inoculation, we found that the infections were equivalently systemic regardless of the site of inoculation. Although this experiment cannot exclude the possibility that there remain quantitatively modest but physiologically important differences in the establishment of local infection, we do not believe that differences in local infection underlie the difference in the rates of mortality of flies inoculated in the thorax and those inoculated in the abdomen. Rejection of this hypothesis is further supported by the observation that coupling of an aseptic wound to the thorax with an inoculation into the abdomen yields the same mortality profile as a septic inoculation into the thorax alone. Instead, it appears that damage done to the thorax causes flies to become more susceptible to infection.

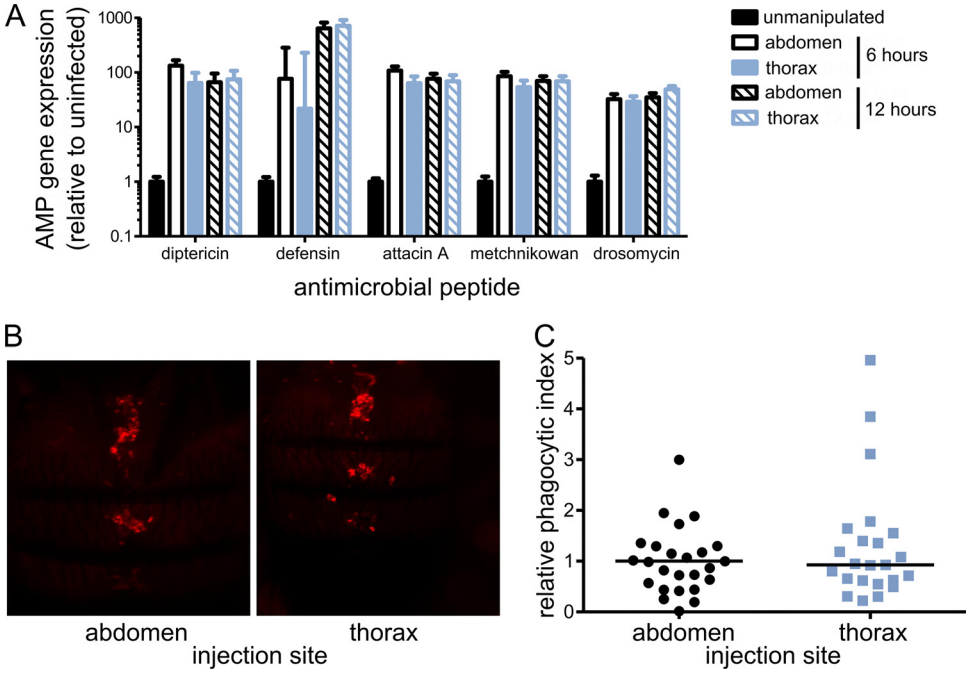

FIG 4 Effect of thorax inoculation on antimicrobial expression and phagocytic index. (A) Expression of antimicrobial peptide (AMP) genes was determined 6 and 12 h after inoculation of *P. rettgeri* in either the abdomen or the thorax. AMP gene expression data represent the least square means derived from the results determined with model H. There was no significant difference in the levels of expression of AMPs due to inoculation site; for complete statistical analysis, see Table S7 in the supplemental material. (B and C) The phagocytic index was measured and images were taken 1 h after injection of 1 mg/ml pHrodo-labeled *E. coli*. (B) Representative images 1 h after injection. (C) Relative phagocytic index data for each fly after processing in ImageJ. There was no significant difference between injection sites in phagocytic index values (two-tailed *t* test, $P > 0.05$).

We noted that flies with bacteria inoculated into the thorax sustained higher pathogen loads than flies with bacteria inoculated into the abdomen, indicating a defect in resistance to infection. We tested whether the observed difference in resistance was mediated by major *Drosophila* immune pathways but saw no differences with respect to the induction of AMPs or phagocytic activity, and mutants in immune pathways still exhibited increased mortality after infection in the thorax versus in the abdomen. We can speculate that there are three possible alternative mechanisms by which a thorax wound might facilitate bacterial proliferation and increase host mortality independently of host immunity. One is that the thorax wound might trigger release of a signal that promotes or enables bacterial growth. Wounding in adult *Drosophila* activates responses in other tissues (e.g., damage to cuticle signals to the gut [33]). A signal released by a thorax wound could, for example, increase circulation of key nutrients that are intended to help fund repair to the thoracic flight muscle but that could also be hijacked by infecting bacteria for their own benefit. Abdominal wounds in adult *Drosophila* result in scars containing large, polyploid cells, but the abdominal muscle tissue is never repaired (34). However, since the thoracic muscle is important for both flight and courtship, the fly may have a greater incentive to repair thoracic muscle. The erosion of the effect of thorax injury over time is also consistent with a diffusible signal or with the release of nutrients to facilitate wound healing. A second possible mechanism is that wounding of the thorax might uncover a profitable niche for the bacteria. Wounded thoracic flight muscle may be a nutrient-rich reserve that is well protected against infection when intact but that becomes accessible to bacteria when injured. This interpretation is consistent with our observation that temporal separation between the thoracic injury and the infection limits or eliminates the associated increase in host mortality and is consistent with a previous observation that mutants with genetically disrupted flight muscle are more susceptible to infection delivered by pricking to the thorax than to that delivered by pricking to the abdomen (12). A third hypothesis is that healing of the thorax wound might recruit resources that would otherwise be used to fight infection but that healing an abdominal wound is less costly. If this hypothesis is true, however, the mechanism must be largely independent of AMP transcript induction or phagocytosis, as we found no difference in the levels of AMP gene expression or phagocytic activity between abdomen- and thorax-inoculated flies. Our phagocytosis assay measures only phagocytosis by sessile hemocytes, and our AMP induction assay looks at transcript and not peptide abundance, so there remains the possibility that we failed to detect a difference in other phagocytic cell populations or in translation of antimicrobial genes. However, we believe it is unlikely that such a nuanced alteration of immune function would be sufficient to result in such a dramatic difference in mortality. We saw that the elevated difference in mortality associated with inoculation of the thorax persisted in every mutant with a mutation of the immune system that we tested (P of <0.0001), including in mutants that are extremely susceptible to infection, such as *imd* mutants infected with *P. rettgeri* or *spz* mutants infected with *E. faecalis*. While there were significant genotype-inoculation site interactions in a few cases, indicating that the magnitude of the difference in the levels of mortality might have been altered in some mutants,

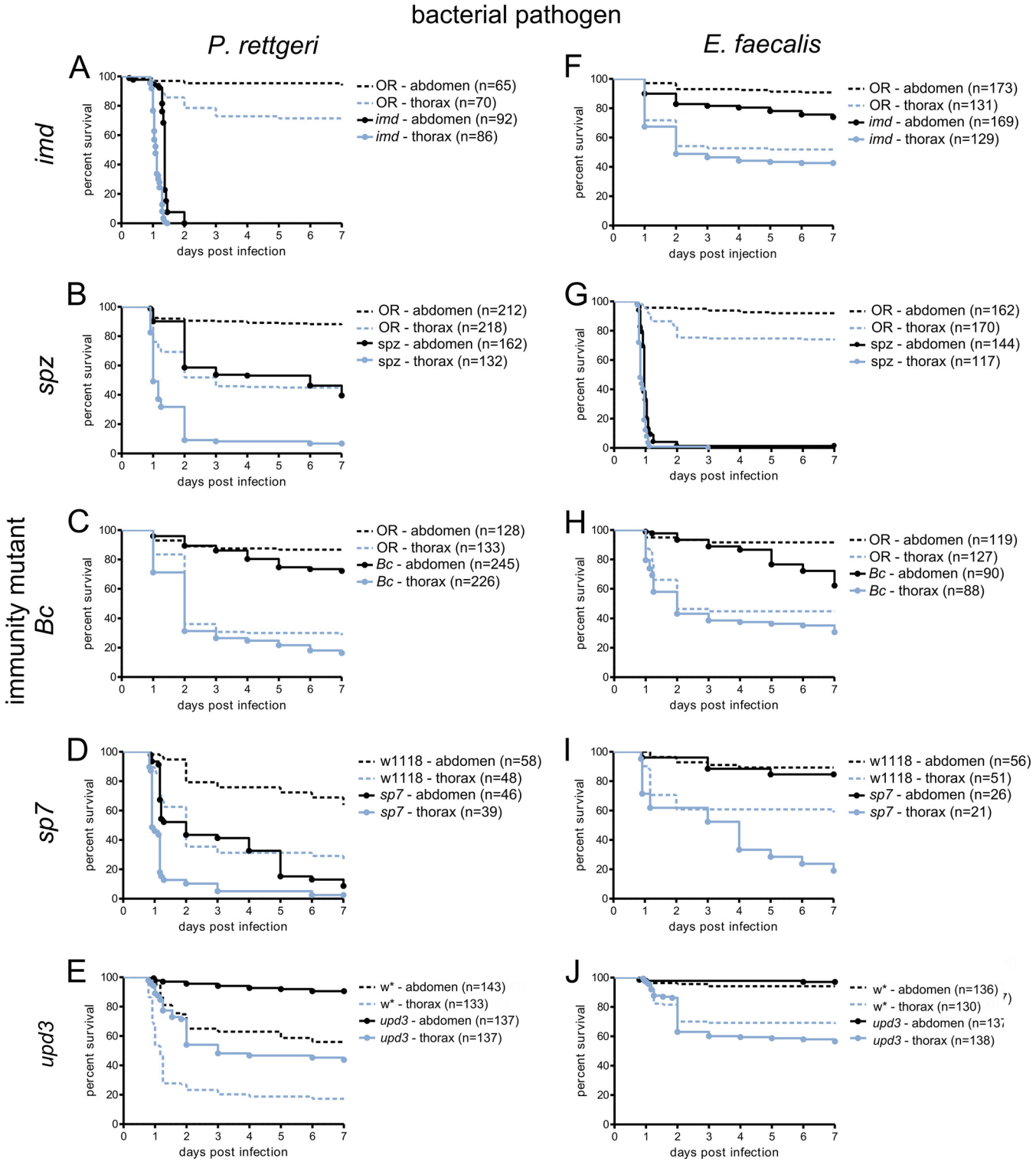

FIG 5 Canonical immune pathways are not responsible for the mortality induced by thorax injury. *imd* (A and F), *spz* (B and G), *Bc* (C and H), *sp7* (D and I), and *upd3* (E and J) mutants, alongside a wild-type control, were inoculated with *P. rettgeri* (A to E) or *E. faecalis* (F to J) in either the abdomen or thorax and monitored daily for survival for 1 week postinjection. Pairwise comparison showed highly significantly increased mortality in thorax-inoculated versus abdomen-inoculated flies for all mutants tested (P of <0.0001 in all cases). For A, $\chi^2 = 114.0$; for B, $\chi^2 = 86.9$; for C, $\chi^2 = 184.6$; for D, $\chi^2 = 21.8$; for E, $\chi^2 = 70.91$; for F, $\chi^2 = 34.4$; for G, $\chi^2 = 29.2$; for H, $\chi^2 = 30.7$; for I, $\chi^2 = 21.7$; and for J, $\chi^2 = 61.9$. For complete statistical analysis, see model I and Tables S8 and S9 in the supplemental material.

these effects were an order of magnitude smaller than the absolute effect of the inoculation site.

We observed some heterogeneity in pathogen loads among individual infected flies, especially at 24 and 48 h postinoculation. Since measurement of bacterial load is a destructive assay, we cannot directly determine what the survival fate of the flies would be with proportionally higher bacterial loads. However, we hypothesize that the thorax inoculation creates two classes of flies—those with the highest bacterial loads that die during acute infection and those with lower loads that survive. This is supported by the fact that there were individual flies infected in the thorax that had pathogen loads comparable to those of flies infected in the abdomen and that the proportion of the population bearing these lower loads matched the overall proportion of flies that survived. This interpretation is also consistent with the observation that the variance in pathogen load among individuals became very much smaller at the time postinoculation when the risks of mortality for the flies infected in the thorax and the flies infected in the abdomen became equivalent. Identifying the signal or signals that shift a subset of the thorax-inoculated flies into the higher bacterial load and mortally doomed state remains an issue for future study.

Our findings have important implications for consistency in research and interpretation of results across laboratories studying *Drosophila* immunity. While a recent review of infection methods suggested that abdomen inoculation might be riskier due to imagined potential puncturing of the intestines, our data demonstrate that thorax inoculation is more detrimental (35). *Drosophila* immunity studies are somewhat divided between the two inoculation sites. In some studies pathogens are injected into the abdomen (24, 26, 36–39), and in others they are injected into the thorax (16, 40–43), while many published papers do not report the site of infection at all. This procedural variance illustrates the general belief that site of inoculation is relatively unimportant to output phenotypes—a belief that is strongly contradicted by our current data. Differences in the route of infection could play a role in conflicting studies in the *Drosophila* literature and in the failure of some studies to replicate findings.

Conclusion. We have uncovered a phenomenon in which remote injury results in lowered resistance to infection that seems to be independent of canonical immunity. Not only does this work challenge us to think more broadly about the role that general biological processes such as wound healing play in defense against infection, it also reveals *Drosophila* to be a potential model for studying complex injury and infection scenarios.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 AI083932 and R01 AI064950.

We thank Nicolas Buchon and Mimi Shirasu-Hiza for giving us *Drosophila* stocks. We also thank Virginia Howick for help writing R scripts and the entire Lazzaro laboratory for helpful comments and discussion.

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