RESEARCH NOTE

Time to death in the presence of *E. coli*: a mass-scale method for assaying pathogen resistance in *Drosophila*

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Introduction

There is now increasing realization that tradeoffs between parasite or pathogen resistance and other life-history related traits could play an important role in shaping life-history evolution (Sheldon and Verhulst 1996; Zuk and Stoehr 2002), and a few experimental studies have examined such tradeoffs in *Drosophila* (Hoang 2001; Kraaijeveld *et al*. 2001; McKean and Nunney 2001) and other insects (Moret and Schmidt-Hempel 2000; Hosken 2001; Adamo *et al*. 2001; Cotter *et al*. 2004). *D. melanogaster* has a well-developed innate immune system that is well characterized genetically (Rutschmann *et al*. 2000; De Gregorio *et al*. 2001; Irving *et al*. 2001) and involves phagocytosis, melanization and production of antimicrobial peptides (Elrod-Erickson *et al*. 2000; Hoffmann 2003). Given that it is also one of the best studied model systems for life-history evolution (Prasad and Joshi 2003), *D. melanogaster* is an attractive choice for studies on life-history related tradeoffs involving the immune system (Sharmila Bharathi *et al*. 2004). A potential limitation in using *D. melanogaster* for rigorous evolutionary experimentation on immune function tradeoffs with life-history related traits is that neither the commonly used infection technique (pricking the fly with a contaminated needle: Elrod-Erickson *et al*. 2000; Rutschmann *et al*. 2000; De Gregorio *et al*. 2001; McKean and Nunney 2001), nor the typical assays for immune competence (e.g. rate of clearing of pathogen: McKean and Nunney 2001; level of antimicrobial peptides induced: Rutschmann *et al*. 2000; extent of phagocytosis: Elrod-Erickson *et al*. 2000) are easy to implement with large numbers of flies (Sharmila Bharathi *et al*. 2004).

We had earlier suggested that time to death of freshly eclosed flies kept in a vial containing LB agar seeded with *Escherichia coli* could form a convenient measure of pathogen resistance in *Drosophila* (Sharmila Bharathi *et al*. 2004). This assay can easily be done with very large numbers of flies and, moreover, directly links infection with mortality, an important fitness component. *E. coli* is known to elicit an immune response in *D. melanogaster* (Elrod-Erickson *et al*. 2000; Rutschmann *et al*. 2000), as well as cause increased mortality in infected *rel*$^{-}$ flies with a compromised immune response (McKean and Nunney 2001). In that study we showed that keeping flies in vials with *E. coli* reduced the mean time to death, compared to that of flies in control vials, and that this reduction was more severe in the case of a long-term laboratory population, compared to populations of recently wild-caught flies (Sharmila Bharathi *et al*. 2004). We also showed that rearing the flies in vials with *E. coli* did result in bacteria entering the body of the flies (Sharmila Bharathi *et al*. 2004).

One possible ambiguity in interpreting the results of Sharmila Bharathi *et al*. (2004) is whether the reduced time to death in the presence of *E. coli* is due to pathogenicity, or due to the diversion of resources into mounting an immune response which, in turn, leads to faster death under the quasi-starving conditions of the LB agar vials. If the latter be the case, one might expect even an exposure to killed or otherwise attenuated *E. coli* to induce a reduction in time to death over that in control vials. Here, we report results from studies on time to death in the presence of heat-killed or streptomycin-treated *E. coli* in *D. melanogaster* and

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D. ananassae. We also confirm, using an improved technique over that used by Sharmila Bharathi *et al*. (2004), that *E. coli* are indeed taken into the body cavity of *Drosophila* when the flies are kept in vials with *E. coli*.

Materials and methods

Experimental populations

Time to death in the presence of heat-killed *E. coli* was assayed on five populations of *D. melanogaster* (MGB-1. . .5), derived by multiway crossing among the four JB populations first described in Sheeba *et al*. (1998). At the time of this assay, the MGBs had completed 101 generations since their derivation, and were maintained on cornmeal food, on a 21-day discrete-generation cycle at 25°C, under constant light and ~90% relative humidity. For each replicate population, 60–80 eggs were collected into each of 40 vials (9 cm × 2.4 cm) and all eclosed adults were transferred to a Plexiglas cage (25 cm × 20 cm × 15 cm) on the 12th day after egg collection, and given a generous smear of live yeast–acetic acid paste, in addition to food medium, for about three days prior to egg collection for initiating the next generation.

Time to death in the presence of streptomycin-attenuated *E. coli* was assayed on two sets of populations: (i) the four JB populations of *D. melanogaster* described in detail by Sheeba *et al*. (1998), which had been in our laboratory for 142 generations at the time of this study, and (ii) four populations of *D. ananassae* (AB-1. . .4), maintained in a manner identical to that of the MGBs described above, that had been in the laboratory as independent populations for about 38 generations at the time of this assay. The four AB populations were derived, after 34 generations of laboratory rearing as a single population, from the laboratory population of *D. ananassae* initiated from ~300 females collected from orchards and domestic garbage dumps in different parts of Bangalore during May and June 2001, first described in Sharmila Bharathi *et al*. (2003).

For the fluorescence microscopy study, we also used four populations of *D. nasuta nasuta*, in addition to the JBs and ABs. The four *D. n. nasuta* populations (NB-1. . .4) were derived, after 24 generations of laboratory rearing as a single population, from a laboratory population established using about 70 females collected from orchards and domestic garbage dumps in different parts of Bangalore during October and November 2001 and first described in Sharmila Bharathi *et al*. (2003). The NBs were maintained in a manner identical to the MGBs and ABs described above, and had been in the laboratory as independent populations for 24 generations at the time of this assay. At the time of this assay, the JBs and ABs had been in our laboratory as independent populations for 129 and 24 generations, respectively.

For generating flies for the assays, eggs were collected at a density of about 60–80 per vial in 24 vials per replicate population of each species. From these vials, freshly eclosed adults were randomly collected and used for the various assays.

Assays

We used the DH5α strain of *E. coli*, which is resistant to ampicillin and susceptible to streptomycin. Five ml of LB broth was inoculated with *E. coli* and the culture was allowed to grow till it reached 0.6 optical density. The heat-killed *E. coli* cultures were obtained by autoclaving such cultures at 121°C for 20 min. Each experimental vial had either 3 ml of LB agar medium with ampicillin, LB agar medium containing ampicillin to which 20 μl of 50% (v/v) diluted culture of live *E. coli* was added, or LB agar medium containing ampicillin with 20 μl of 50% (v/v) diluted culture of heat-killed *E. coli*. These vials were then incubated at 37°C for 24 h. To set up the assay, either five freshly eclosed males or five freshly eclosed females were placed into every experimental vial. Five control vials, five vials with *E. coli*, and five vials with heat-killed *E. coli* were set up for each replicate population × sex combination, and kept at ~25°C under constant light. The flies were transferred into fresh vials of their respective media every 8 h, till death. The vials were checked every 2 h, and the death of any fly during the previous 2 h period recorded. This process was continued until all the flies had died.

For the assay in which streptomycin-attenuated *E. coli* was used, a similar 0.6 optical density culture of *E. coli* in LB broth was used. Each experimental vial had 3 ml of LB agar medium with ampicillin, LB agar medium with ampicillin in which 20 μl of 50% (v/v) diluted culture of *E. coli* was added, or LB agar medium with both streptomycin and ampicillin in which 20 μl of 50% (v/v) diluted culture of *E. coli* was added. The vials were kept for incubation at 37°C for 24 h. Thereafter, either five freshly eclosed males or five freshly eclosed females were placed into each vial. Eight control vials, eight vials with *E. coli*, and eight vials with attenuated *E. coli* were set up for each replicate population × species × sex combination, and kept at ~25°C under constant light. The flies were transferred into fresh vials of their respective media every 8 h, till death. The vials were checked every 2 h and death of any fly was recorded. This process continued till all the flies had died.

For the study involving fluorescence microscopy, freshly eclosed flies from the MGBs, ABs and NBs were introduced into either vials containing LB agar medium with ampicillin (control) or LB agar medium with a strain of *E. coli* (treatment) expressing a fluorescent protein DsRed. Five flies of each sex were introduced into five vials for each replicate population of each species. The flies were kept in the vials for about 8 h, taken out and washed in 70% ethanol thrice, and finally washed with distilled water, a procedure earlier shown to result in effective surface sterilization of the flies (Sharmila Bharathi *et al*. 2004). The washed flies were then viewed under a fluorescence microscope at 10× magnification.

Statistical analyses

All analyses were performed using STATISTICA™ for Windows release 5.0b (Statsoft 1995). In case of the heat-killed *E. coli* assay, a three-way mixed-model analysis of variance (ANOVA) was done with replicate MGB populations treated as random blocks, and treatment (control, live *E. coli*, heat-killed *E. coli*) and sex as fixed factors. The data from the streptomycin-attenuated *E. coli* assay were subjected to four-way mixed-model ANOVA in which pairs of replicate JB and AB populations assayed together were treated as random blocks, whereas species, treatment (control, live *E. coli*, streptomycin attenuated *E. coli*) and sex were treated as fixed factors.

Results and discussion

The mean time to death of MGB flies varied significantly across treatments ($F_{2,8} = 146.31, P < 0.0001$), and was significantly lower ($P < 0.05$; Tukey's HSD) in vials with live *E. coli* than in either control vials or vials with heat-killed *E. coli* (figure 1). Mean time to death did not differ significantly ($P > 0.05$; Tukey's HSD) between control vials and vials with heat-killed *E. coli*. The ANOVA main effect of sex was also significant ($F_{1,4} = 71.8, P < 0.005$): as expected, females lived longer than males (figure 1). The difference between male and female mean time to death was greatly reduced in the vials with live *E. coli*, compared to the other two treatments, and this was reflected in a significant sex × treatment interaction ($F_{2,8} = 30.1, P < 0.0005$).

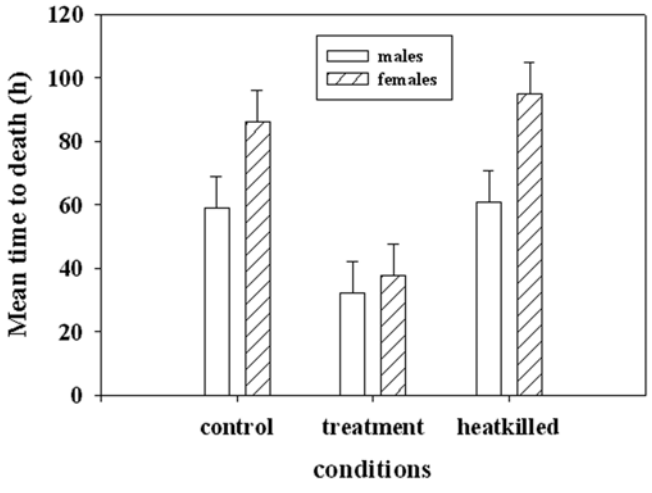

Figure 1. Mean time to death of males and females of MGBs in control vials, vials with live *E. coli* (treatment), and vials with heat-killed *E. coli* (heatkilled). Error bars are Tukey's HSD based confidence intervals derived from the appropriate mean squared error term in the ANOVA.

The mean time to death in the streptomycin-attenuated *E. coli* assay was significantly greater in ABs than in JBs ($F_{1,3} = 59.4, P < 0.005$; figure 2), and greater in females than in males ($F_{1,3} = 26.3, P < 0.05$). The mean time to death also varied significantly across treatments ($F_{2,6} = 33.9, P < 0.001$), and was significantly lower ($P < 0.05$; Tukey's HSD) in vials with live *E. coli* than in either control vials or vials with streptomycin-attenuated *E. coli* (figure 2). Mean time to death did not differ significantly ($P > 0.05$; Tukey's HSD) between control vials and vials with streptomycin-attenuated *E. coli*. The difference between JB and AB mean time to death was greatly reduced in the vials with live *E. coli*, compared to the other two treatments, and this was reflected in a significant species × treatment interaction ($F_{2,6} = 17.1, P < 0.01$).

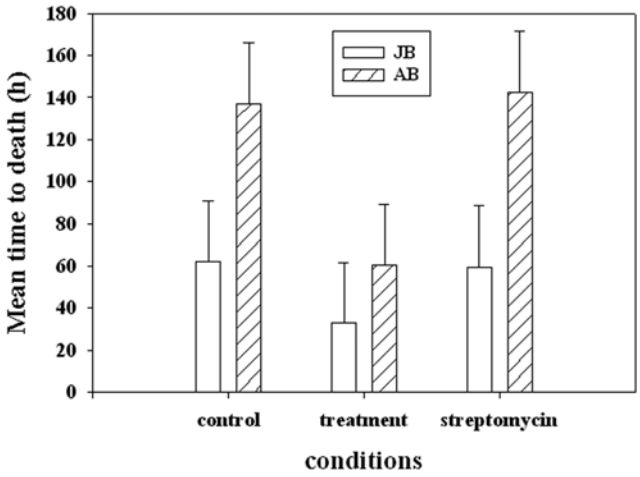

Figure 2. Mean time to death of JB and AB flies (pooled over sexes) in control vials, vials with live *E. coli* (treatment), and vials with streptomycin-attenuated *E. coli* (streptomycin). Error bars are Tukey's HSD based confidence intervals derived from the appropriate mean squared error term in the ANOVA.

The observation that mean time to death does not significantly differ between control vials and vials with either heat-killed or streptomycin-attenuated *E. coli* strongly suggests that the increased mortality rate in vials with live *E. coli* is largely due to pathogenicity rather than a reflection of resource expended in mounting an immune response to foreign antigens. It could be argued that heat-killed bacteria do not elicit an immune response due to denaturation of surface antigens, although the immunogenic potential of particular regions within peptidoglycans (Dziarski 2004) suggests that even many denatured surface antigens would suffice to provoke an immune response. However, streptomycin-attenuated bacteria are not expected to have severely denatured or degraded surface antigens, and should be expected to elicit an immune response. We note that, although *E. coli* appeared to increase mortality only in immune-compromised *rel*$^{-}$ flies, and not wild-type flies, in a previous study (McKean and Nunney 2001), that study involved a one-time infection with *E. coli* by pricking flies with a contaminated needle.

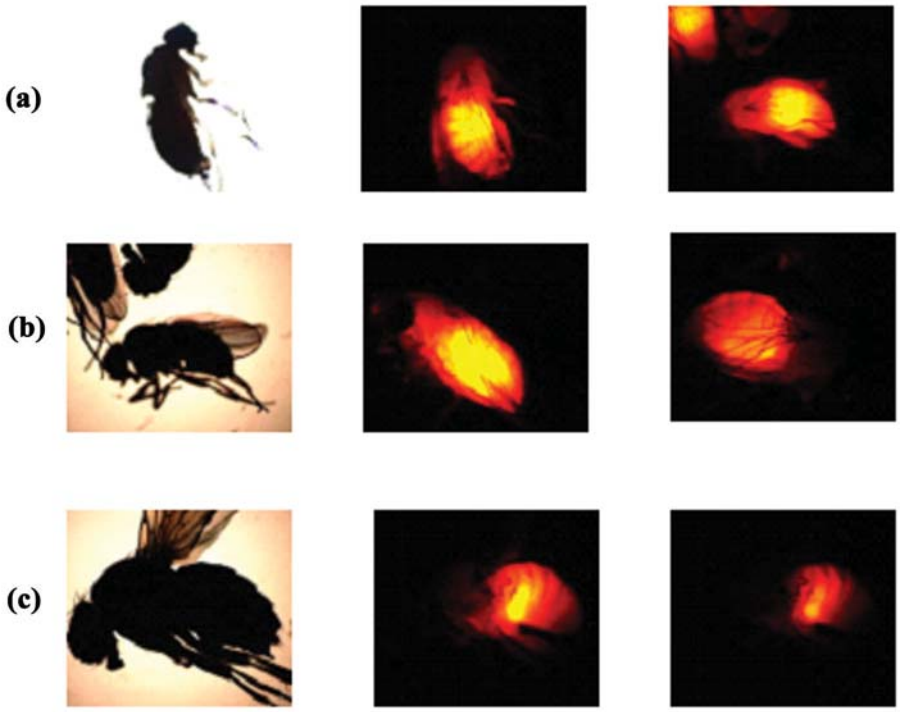

Figure 3. Representative fluorescence microscope pictures of males and females of treatment and control flies from (a) JBs, (b) ABs and (c) NBs. The first column shows control flies, whereas the second and third columns show fluorescing treatment flies that were grown for 8 h in vials with live *E. coli*.

Wild-type flies were able to rapidly clear bacteria from their body, and did not appear to suffer increased mortality as a result of the infection. In our assay, there is a constant supply of bacteria for infection and the LB agar appears to be nonnutritive for flies. Thus, the flies are probably under some degree of starvation stress (Sharmila Bharathi *et al*. 2004), and also continuously exposed to *E. coli*, which likely explains why exposure to *E. coli* in our assay results in increased mortality rates.

We also observed a reduction in pathogen resistance of the ABs compared to data from the study of Sharmila Bharathi *et al*. (2004), at which time the pathogen resistance of population AB-1, which had been in the laboratory for about 40 generations, was not significantly different from that of a recently (eight generations old) wild-caught population of *D. ananassae*, but significantly greater than the long-term laboratory population of *D. melanogaster*, JB-1. Indeed, if we use the measure of pathogen resistance (time to death in treatment vials scaled by mean time to death in control vials) used in Sharmila Bharathi *et al*. (2004) on the present data, it is clear that the pathogen resistance of the ABs has reduced substantially (from about 67% to about 44%) during the 32 generations of further laboratory rearing between the present assay and that reported in Sharmila Bharathi *et al*. (2004), supporting the view that laboratory adaptation in the medium to long term involves loss of pathogen resistance.

Finally, the fluorescence microscopy revealed that all treated JB, AB and NB flies showed strong fluorescence in their body cavity, whereas none of the control flies showed such fluorescence (figure 3). This result reconfirms the earlier observation that rearing flies in vials with live *E. coli* results in the intake of bacteria into the body of the flies (Sharmila Bharathi *et al*. 2004). Thus, taken together, the results of this study clearly suggest that time to death in the presence of live *E. coli* provides a convenient measure of pathogen resistance in *Drosophila* that can be used easily with very large numbers of flies, and relates pathogen resistance directly to mortality, making it especially useful for assessing tradeoffs between immune competence and life-history related traits.

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