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The Aging of the Immune Response in *Drosophila melanogaster*

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Senescence of functional immunity in invertebrates has been a topic of recent interest. Results from previous studies have been inconsistent with older adults exhibiting wide variation in response to infection. In the present study, we assayed the senescence of functional immune response using a large outbred population of *Drososphila melanogaster* as the model host and *Serratia marcescens* as the model pathogen. We assessed the effect of an individual's age, parental age, sex, and mating status on overall antibacterial immunity. We found an improvement of immunity with the progression of age with 13-day-old flies exhibiting lower bacterial load compared with 3-day-old flies. Parental age did not show consistent effects on the antibacterial immunity of the offspring. Neither mating status nor the sex of an individual had any significant effect on immune response.

Key Words: *Drosophilia melanogaster*—Immune response—Parental age—Senescence—*Serrati marcescens*.

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THEORY and experiments predict a general decline in immune response with age in several taxa including humans (1–4), which potentially can lead to increased morbidity and mortality associated with old age. Few studies have looked at the functional response of the immune system during aging in *Drosophila* (5–9). Further, the experimental results from these studies are fairly variable. Older adults of *Drosophila melanogaster* have been associated with higher bacterial load (7). Zefrosky and coworkers (2005) found a decline in the ability to induce antimicrobial peptide genes with age (5). Ramsden and coworkers (2008) found no change in the ability to clear a bacterial infection with age, but aging significantly reduced the ability of the fly to survive an infection (8). In contrast, Lesser and coworkers (2006) found an overall improvement in the ability to clear bacterial infection with age (6). Hence, the effect of age on the immune response in flies is as yet not well understood. Several studies have shown that important life-history traits such as longevity and fecundity are affected by parental age. In one of the reports (10), reproductive success of males was found to be negatively correlated with paternal age. Priest and colleagues suggested a general decrease in offspring longevity with the maternal age (11). Paternal age also influenced the offspring longevity but effect was lesser compared with mother's age (11). Another life history–related trait, larval viability has also been negatively correlated to maternal age (12,13). However, immunity being a fundamental component of fitness of an individual has not ever been tested in the earlier context.

Thus, given the importance of immunity in aging (2,14), the aim of the present study is to evaluate senescence of immune system and the role of parental aging on immunity of the offspring. We used a large, outbred LH population (named after Larry Harshman, who founded the population in 1991 from 400 wild-caught inseminated females from central California [15]) of *D melanogaster* as the host (15,16). We chose *Serratia marcescens* as the model infecting agent because it is a natural pathogen to wide range of hosts and has also been isolated from D melanogaster (17–19). Further, it is one of the few bacteria that are known to kill the adult flies (20) and commonly used in the studies of evolutionary ecology of immune response (21–23). LH population has been previously shown to undergo reproductive senescence in males due to greater mutational load (10) and in females (Nandy and Prasad, unpublished data) within 13 days posteclosion. Therefore, in the present study, we considered 3-day-old and 13-day-old flies as "young" and "old," respectively. Three-day-old and 13-day-old posteclosed males and females were orthogonally crossed to generate offspring, which were evaluated for their overall antibacterial immunity across the sex and mating status (virgin and mated). Offspring bacterial load was measured on the 3rd, 8th, and 13th day posteclosion to address the senescence of functional immunity.

Materials and Methods

Fly Stock and Maintenance

We used a large, outbred laboratory population of *D melanogaster* called LH for this study. This population has been maintained on a 14-day discrete generation cycle at 25°C and 12:12 hours light:dark cycle on cornmeal–molasses food. For details of the maintenance protocol, see (15).

Microorganism and Infection Protocol

The strain of bacteria used for the experiment was *S marcescens* ATCC 13880. This strain has been shown to grow equally well in males and females and is pathogenic, causing about 80% mortality in males and females over a 11-day period (23). On the evening before infection, we inoculated a fresh culture of the bacteria and allowed it to grow overnight to an optical density of 1.0. We centrifuged the resulting population of bacteria and made it into slurry. Flies under light CO_2 anesthesia were infected by pricking at the lateral side of the thorax with a 0.1-mm minutien pin (Fine Science Tools, Foster City, CA) dipped into the bacterial slurry. As a control, sham infection was carried out by pricking flies with a pin dipped in *Drosophila* Ringer solution (23,24). Infections (and sham infections) were done in the light phase of the 12:12 hours light:dark cycle (lights on at 8:30 AM; lights off at 8:30 PM) between 11 AM and 1 PM. All treatments were completely randomized within 2-hour window during infection (and sham infection) to account for the small differences in the timing of infections. Within the time window of the experiment that we examined (24 hours postinfection), none of the flies died. We only witnessed mortality in flies due to bacterial infection after about 48 hours postinfection (23).

Generation of Experimental Males and Females

All "experimental" males and females used in the experiment came from vials with an egg density of 150/vial. They were isolated as virgins and held in groups of 10/vial until the day of experiment as described below. Females of either 3 (young) or 13 (old) days after adult emergence were orthogonally crossed with males of either 3 (young) or 13 (old) days after adult emergence (see Table 1). Adults from all the vials were combined into fresh food vials at a density of 10 pairs/vial to give rise to four different parental mating combinations. Ten such vials were set up per mating combination. The vials were provided with a limiting quantity of live yeast supplement. After 2 days of interaction, the flies were transferred to fresh food vials and allowed to oviposit for 18 hours. The flies were then discarded. Eggs were collected at density of 150 eggs/vial and incubated at standard laboratory conditions of 25°C and 12:12 light dark cycle. Adults emerging in these vials were isolated as virgins and held at a density of 5 flies/vial for the experiment described below.

Table 1. Summary of Parental Age Combinations Used to Generate Progeny for Experiments

Young (3 days posteclosion) females × Young (3 days posteclosion) males	old (13 days posteclosion) females × Young (3 days posteclosion) males
Young (3 days posteclosion) females × Old (13 days posteclosion) males	old (13 days posteclosion) females × Old (13 days posteclosion) males

Note. Ten parental flies of each sex were combined in vial with food. Ten vials were set up per combination. The entire combination was replicated independently for each of the three blocks.

Experimental Outline

Assay for the antibacterial immune defence was performed with experimental offspring of three age groups, such as 3 (young), 8 (middle), and 13 (old) days posteclosion from each of four parental combinations (see Figure 1 for the experimental design). All the adults were randomly assigned to two types of mating status, "virgin" and "mated," to create two subsets of equal number of flies. For the virgin subset, males and females were held separately at a density of 5 flies/vial until the day of infection on the 3rd, 8th, or 13th day posteclosion with transfer to fresh food vials every alternate day. For the mated subset, males and females were held separately at a density of 5 flies/vial until the 1st, 6th, or 11th day postemergence. This was followed by pairing with 5 "mates" (per vial). Mates were 3-day-old posteclosion (males and females collected as virgins) and went through similar rearing condition as that of experimental males and females. After 2 days of interaction, mates were discarded. On the 3rd, 8th, or 13th day posteclosion (day of infection), three experimental females (or males) out of five were assayed for bacterial load/sham infection. Flies were subjected to light carbon dioxide anesthesia followed by infection ($N = 240$) or sham infection ($N = 144$) as described earlier. After infection/sham infection, flies were put back in a fresh food vial and held for 24 hours. On the next day, females were homogenized in groups of three with a micropestle in 300 µL of Luria Broth, and 50 µL aliquots of 1:100 dilution were spread on Luria Broth agar plates, which were incubated overnight at 37°C. The number of colonies that appeared overnight was assumed to be negatively correlated with the strength of immune response mounted against bacterial infection.

Replicates and Data Analysis

The experiment was replicated thrice, resulting in three independent blocks. In each block, five and three replicate vials were set up in each Parental mating combination × Sex × Mating status combination for infection and sham infection, respectively. Bacterial load data were subjected to a natural log transformation before analysis. Data were analyzed with six-factor analysis of variance with male age, female age, sex, mating status, and day of infection as fixed factors crossed with random blocks.

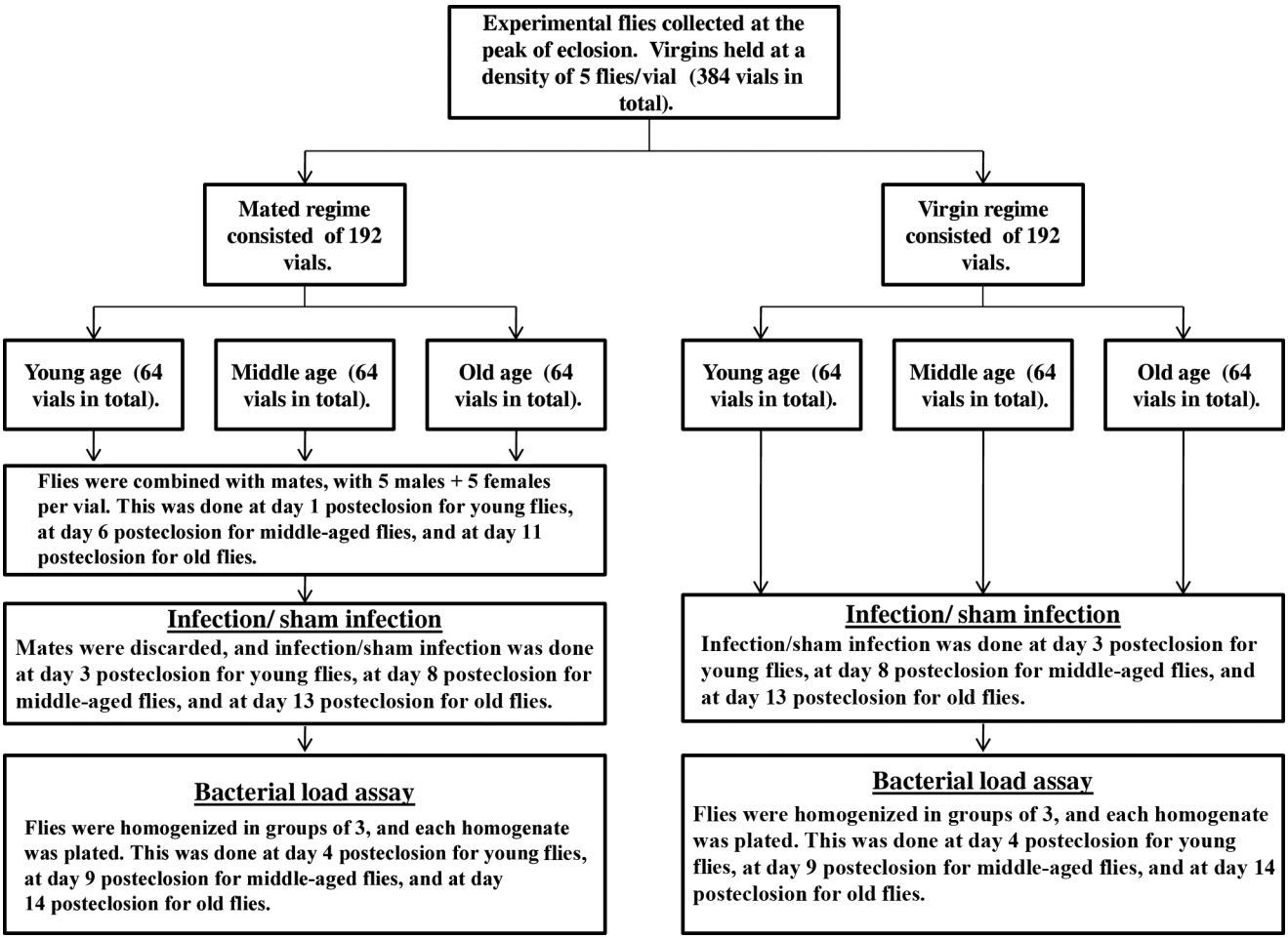

Figure 1. The design of the experiment. The entire structure was replicated independently for each of the four parental age combinations within each of the three random blocks. In each of these blocks, five and three replicate vials were set up in each Parental mating combination × Sex × Mating status combination for infection and sham infection, respectively. Flies were maintained under standard lab conditions and were transferred into fresh food vials on alternate days (see Materials and Methods section for details).

Results

Analysis of variance results indicated a significant effect of day of infection, two-way interaction of Block × Day of infection, and three-way interaction of Block × Male age × Female age. None of the other factors were significant. Given the interaction between block and day of infection, we wanted to ensure that our results were consistent between blocks. Therefore, we analyzed individual blocks separately after pooling data across sex and mating status (separate analysis without pooling data yielded similar results). Within each block, there was a significant effect of day of infection (three-way analysis of variance, all $p < .001$, see Table 2 and Supplementary Table 1 for details). In block 1, multiple comparison indicated that bacterial load on all 3 days were significantly different from each other (Figure 2A). Bacterial load was lowest on day 13 followed by day 8 and day 3 (Tukey's HSD, all $p < .05$). In block 2, bacterial load on day 13 and day 8 was significantly lower than that of day 3 (Tukey's HSD, all $p < 0.05$, Figure 2B). Although Bacterial load on day 13 was lower than day 8, multiple comparison revealed no significant difference between them (Tukey's HSD, all $p > .05$). Bacterial load on day 13 was significantly lower

Table 2. Summary of Three-Way Analysis of Variance on Bacterial Load From Individual Blocks With Male Age, Female Age, and Day of Infection as Fixed Factors

	p Values		
Trait	Block 1	Block 2	Block 3
Male age	.898	.162	.438
Female age	.12	.454	.957
Day of infection	<.0001	<.0001	<.0001
Male age × Day of infection	.876	.414	.292
Female age × Day of infection	.537	.927	.312
Male age × Female age	.016	.013	.601
Male age × Female age × Day of infection	.864	.367	.794

Note. Analysis of variance result indicated a significant effect of day of infection in all the blocks and two-way interaction of Male age × Female age in blocks 1 and 2.

than that on day 8 and day 3 (Tukey's HSD, all $p < .05$, Figure 2C) in block 3. However, day 8 and day 3 were not significantly different from each other in their bacterial load (Tukey's HSD, all $p > .05$). Taken together, bacterial load was always significantly lower in old flies (13 days old) than in younger ones (3 days old; Tukey's HSD, all $p < .05$, Figure 2). Sham-infected flies failed to produce colonies in Luria Broth agar plates.

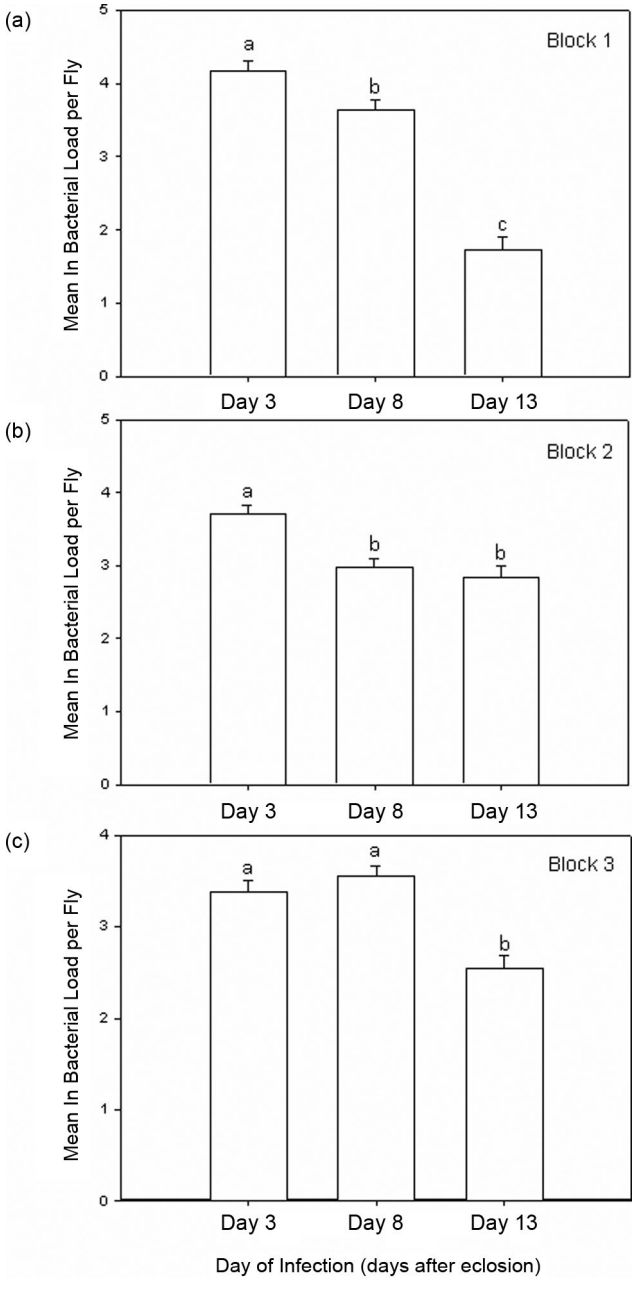

Figure 2. Bacterial load (mean ± *SE*) 1-day postinfection in flies on three different days of infection within individual blocks. Bacterial loads on different days of infection that are not connected by the same letter are significantly different. We found lower bacterial load in older flies, indicating an improvement of immunity with progression of age.

In blocks 1 and 2, there was a significant interaction of Male age × Female age (see Table 2 and Supplementary Table 1). In block 1, offspring of the Young male × Young female had significantly lower bacterial load than the offspring of Young male × Old female (Tukey's HSD, $p < .05$). However, in block 2, offspring of the Old male × Young female had significantly lower bacterial load than the offspring of the Young male × Young female (Tukey's HSD, $p < .05$). No significant interaction of Male age × Female age was found in block 3. To summarize, within blocks there were wide variations in the direction of the Male age × Female age interaction.

Discussion

This study was specifically designed to address three questions: (1) How does aging influence antibacterial immunity of the adult fruit flies? (2) Do sex and mating status of an individual affect antibacterial immunity?, and (3) Does parental age affect progeny immune response? We clearly demonstrate that with age bacterial load was less in both sexes. Bacterial load has been widely assumed to be negatively correlated with the strength of immune response (6,21,22,25). Further, exponential growth of bacteria has been observed in mutant *D melanogaster* deficient in immune machineries (24,26). Therefore, older flies in our study exhibited better immunity compared with younger flies. Sex and mating status of an individual did not significantly affect its antibacterial immunity. We do not see any consistent effect of parental age on offspring antibacterial immunity.

Antibacterial Immune Response With Aging

In contrast to several other studies (5,7,9) addressing senescence of functional immunity in *D melanogaster*, our study found that older flies exhibited a better immunity in terms of lower bacterial load. Only one earlier study, using much older flies (4 weeks old) compared with our flies, has found that immunity improved at later age (6). The study by Ramsden and colleagues involved flies that were comparable in age to the flies used in our study and reported no difference in bacterial load between 3- and 10-day-old flies (8). Overall, our study shows that antibacterial immunity improves with age within the window of age that we looked at.

There are at least two major points that can be potentially attributed to the differences between our results and those of the other studies (5,7–9). First, all the previous studies were carried out either with inbred population or with chromosomal substitution lines, and we used a large outbred population-LH, which harbors considerable amount of genetic variation (15). Second, the age group of flies that we used is also very different from the previous studies. Importantly, mean life span and mortality varies considerably across different *D melanogaster* populations

(27). Hence, the definition of young and old flies is also variable among different studies (6,8). Lesser and colleagues and Ramsden and colleagues designated flies that were 28 days old and 40 days old as old flies (6,8). However, as mentioned earlier, we used LH population that were 13 days old as old flies because males and females of this population show features of senescence (10; Nandy and Prasad, unpublished data) even within 13 days posteclosion.

There are atleast three potential explanations for our observation of increased antibacterial immunity with age. First, Y model of resource allocation predicts a trade-off between life-history traits (28) such as immunity and reproduction. Life history–based approaches suggest that the energetic needs of maintaining a mechanism of immune defence and its deployment when necessary might drive resources away from (29–34) other processes such as growth, somatic maintenance, and reproduction (35). Because organisms are often constrained by the availability of resources, the optimal investment in immunity is likely to vary across life stages. *D melanogaster* population used in the present study is strictly maintained at 14-day discrete generation cycle. Hence, there is likely to be intense selection to invest in reproduction (at the cost of immunity) over the first 4 days after eclosion although such selection is likely to weaken beyond 4 days after eclosion. Thus, an evolved resource allocation pattern across ages can potentially explain the observed difference in antibacterial immune response between 3-day and 13-day-old flies. Second, several earlier studies suggested an upregulation of immune-related genes with the progression of age (5,36,37). Such upregulation of immunity genes can potentially contribute to the improvement of antibacterial response in our study. However, direct empirical evidence for such immune activation results in improved antibacterial immunity at old age is missing to best of our knowledge and warrants future investigation. Third, an alternative possibility is that tolerance of the flies to bacterial infection (38) varies with age. It is quite possible that young flies are capable of maintaining higher fitness in spite of higher levels of bacterial load (ie, higher tolerance) compared with older flies. This would again explain the pattern of results observed in our study. However, the present study was not designed to elucidate the fitness by bacterial load curve within each age class (the slope of which quantifies tolerance), and it would be interesting to further investigate this possibility.

Antibacterial Immune Response in Relation to Sex and Mating Status

Most studies addressing age-specific changes in functional aspect of immunity primarily involve females and therefore rarely address the sexual dimorphism in the context of aging of immunity (8,9). Several studies suggest that investment in immunity is likely to be sexually dimorphic, with a lower immunocompetence in males compared with females (39–43). In one particular study by Imroze and Prasad (2011), effect of bacterial infection has even been found to affect components of adult fitness differentially in both sexes (23). Additionally, age-specific mortality is sexually dimorphic across the taxa (44–46). In the present study, males were not different from females in terms of bacterial load, confirming the results from our earlier study (23). Moreover, this pattern did not change across different age groups in the present study. Our result is also consistent with one previous report that tested males and females of comparable age group separately for their postinfection immune response but failed to detect any sex-specific difference in their bacterial load with the progression of age (8).

We also considered mating status as one of the factors that can potentially affect the aging of immunity. Mating has been shown to extract a cost in terms of mortality (47–49) and immunity in both sexes (24,50). However, several other studies find no immunity cost to mating (51) or increased immunity with mating as well (52,53). Despite of all these, functional senescence of immunity in relation to mating effort has not been evaluated. In our study, we did not find any difference in bacterial load between virgin and mated flies across all the age groups indicating that there is no immunity cost to mating. Our results are in agreement with one recent study by Short and Lazzaro (2010), which shows that the magnitude and the direction of the effect of mating on immunity can potentially vary widely depending on pathogen used, host genotypes, and experimental conditions (54). Taken together, results from our study and that of Short and Lazzaro (2010) indicate that one needs to be careful before assuming immunity costs of reproductive activity, given the fairly variable outcomes from different model host–pathogen systems (54).

Effect of Parental Age on Antibacterial Immune Response in Progeny

Several evidences indicate that parental age can potentially affect offspring fitness components. Progeny of older parents have been shown to have reduced fitness in terms of preadult viability (12), adult longevity (11), and reproductive success (10). Such negative effects of parental age are thought to be mediated through nongenetic inheritance and mutational load accumulated in the parents (55,56). For the same reasons, it is quite possible that parental age can negatively correlate with progeny immune function—an important component of fitness. Alternatively, good genes arguments would suggest that an individual that survives to a late age is likely to possess a superior immune response. By this argument, on an average, one would expect that the progenies of older parents are likely to have better immune response compared with progenies of young parents.

In the present study, we did not find any effect of the maternal age and paternal age individually on the immune response across the various age groups of offspring. This can well be a differential effect of parental aging on various

life history–related traits affecting survivorship or larval viability but not immunity. However, we had maternal age and paternal age significant interaction in two of the blocks, which were of two different directions (see Results). Given such variable interaction between parental age combinations, we cannot comment further on the same.

In summary, we studied the aging of immune system in both sexes and a possible role of maternal and parental age on antibacterial immune response of the offspring. Though, we did not find an effect of parental age on the antibacterial immunity of the offspring, we found an improvement of immunity with progression of age. Neither mating status nor the sex of an individual had any effect on the immune response.

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Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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