Functional analysis of the *Drosophila* immune response during aging

Sean Ramsden, Yeuk Yu Cheung and Laurent Seroude
Department of Biology, Queen's University, Kingston, ON K7L 3N6, Canada

Summary

One of the most dramatic changes associated with aging involves immunity. In aging mammals, immune function declines and chronic inflammation develops. The biological significance of this phenomenon and its relationship with aging is a priority for aging research. *Drosophila* is an invaluable tool in understanding the effects of aging on the immune response. Similar to the state of chronic inflammation in mammals, *Drosophila* exhibits a drastic up-regulation of immunity-related genes with age. However, it remains unclear whether immune function declines with age as seen in mammals. We evaluated the impact of aging on *Drosophila* immune function by examining across age the ability to eliminate and survive different doses of bacterial invaders. Our findings show that aging reduces the capacity to survive a bacterial infection. In contrast, we found no evidence that aging affects the ability to eliminate bacteria indicating that the mechanisms underlying immune senescence are not involved in eliminating bacteria or preventing their proliferation.

Key words: bacterial clearance; immunity; infection; survival.

Introduction

It is well established that human aging is accompanied by a decline of immune functions (DeVeale et al., 2004; Finch & Crimmins, 2004). The elderly suffer increased morbidity and mortality associated with infection, have a reduced capacity to generate high-affinity antibodies in response to vaccination and are more likely to develop select cancers and autoimmune disorders. Coincident with these functional declines are systemic changes: the adaptive system undergoes remodelling, the innate system is up-regulated and chronic inflammation develops. Furthermore, genes involved in innate immunity exhibit the most dramatic transcriptional changes with age (Lee et al., 2000; Kayo et al., 2001).

The mechanisms underlying innate immunity have been highly conserved across evolution (Hoffmann et al., 1999; Hoffmann & Reichhart, 2002). The *Drosophila* model has been particularly invaluable to elucidate the molecular components and signalling pathways involved in innate immunity (Akira et al., 2001; Royet et al., 2005). Paralleling the state of chronic inflammation that develops in mammals with age, several studies have shown an up-regulation of immunity-related genes during normal *Drosophila* aging (Zou et al., 2000; Pletcher et al., 2002; Seroude et al., 2002; Landis et al., 2004). These observations indicate that *Drosophila* would be an ideal genetic model to investigate the relationship between aging and innate immunity. However, it remains to be established whether flies also experience immune senescence. A recent study suggested that this is the case because older female flies failed to terminate the expression of an antimicrobial peptide as quickly as did younger flies (Zerofsky et al., 2005). However, this study is not conclusive as it focuses solely on female flies, and does not report on flies older than middle age. Moreover, it relies on measuring mRNA transcripts encoding the antimicrobial peptide diptericin as an indirect indicator of immune function. It remains possible that the persistent expression of antimicrobial peptides does not affect the functionality of the immune system. In other words, while older flies may not terminate the expression of antimicrobial peptides as quickly as younger flies, they may still clear and survive the infection. Finally, *Drosophila* immune response relies on the coordinated expression of at least 15 antimicrobial peptides (Imler & Bulet, 2005). The alteration of the expression of a single peptide is likely to not affect the functionality of the immune system because the constitutive expression of a single antimicrobial peptide is sufficient to restore the ability to survive infection in mutants unable to express any antimicrobial peptides (Tzou et al., 2002).

We evaluated the impact of aging on immune function by examining across age the ability to eliminate and survive different doses of bacterial invaders. Our findings indicate that aging reduces the ability to survive an infection, but does not affect the ability to clear bacteria.

Results

Infection protocol

Two critical outcomes of a successful immune response, the elimination of invaders and the ability to survive them, need to be tested in order to investigate subtle changes in immune function, because aging may affect the ability to clear infections without translating into reduced survival. It is also necessary to test various levels of infection because aging may also affect the
Immune senescence in *Drosophila*, S. Ramsden et al.

capacity of the immune system. We took advantage of the immune-deficient mutants available in *Drosophila* to establish the infection protocol. The absence of immune response in those mutants allows confirmation of a direct relationship between the number of infecting bacteria and the severity of the effects on survival and bacterial clearance.

Different amounts of bacteria were injected into *imd* mutants (Lemaitre *et al.*, 1995), and their survival was monitored. Male mutants showed 50% mortality in less than 1 day and 100% mortality in just 2 days when infected with 40 optical density (OD) of bacteria (Fig. 1A). When infected with 4 and 0.04 OD of bacteria, it took them 3 days and 5 days, respectively, to reach 50% mortality, while it took 8 days for both to reach 100% mortality. All three male populations are significantly different from each other (*P* < 0.01). *imd* females infected with 40 OD of bacteria reached 50% and 100% mortality in less than 1 day and 2 days, respectively. Female flies infected with 4 and 0.04 OD of bacteria displayed 50% mortality after 6 days and 7 days, respectively, and 100% mortality 8 days after infection. The 0.04 and 4 OD treatments are significantly different from the 40 OD treatment (*P* < 0.0001), but not from each other. When compared to male survival, female survival in response to 4 OD appears to be higher. Similar results were obtained with the *kenny* mutants (Rutschmann *et al.*, 2000) (data not shown).

Simultaneously, the internal bacterial titre was measured to confirm that the severity of the effect on survival is indeed because of the introduction of more bacteria, and that the fate of the bacteria within the fly can be monitored after the infection (Fig. 1B). As expected, in both male and female mutants, the internal bacterial titre immediately after infection increases with the bacterial concentration injected. The examination of the bacterial titre at later time-points after infection shows that the

![Fig. 1 Survival and bacterial clearance in male and female *imd* mutants after infection with various amounts of bacteria. Representative experiments of two independent trials. (A) Survival. Sample sizes and the amount of bacteria injected are indicated in parenthesis. (B) Bacterial clearance shown as the percentage of bacteria introduced in the animal remaining at various time-points after infection. The amount of bacteria injected (colony-forming units) is indicated in parentheses. Each time-point consists of at least five individual flies. Error bars correspond to two standard deviations.]
4 and 40 OD treatments resulted in significant bacterial proliferation. Indeed, a greater bacterial concentration is associated with higher proliferation. Consistent with the survival results, the 40 OD treatment yields similar results between male and female mutants, whereas the 4 OD treatment suggests that less proliferation occurs in female mutants. It is worth noting that, neither male nor female mutants infected with 0.04 OD of bacteria showed a significant increase in internal bacterial titre, despite showing a severely decreased ability to survive this level of infection.

Surviving an infection is dependent on age

When 3-day-old \(w^{118}\) flies were infected with 4 OD, no significant difference in survival \(P > 0.2\) is detected relative to the negative injection control (Figs 2A and 4 and Supplementary Table S1), but when compared to the no-injection control, significantly lower survival is observed in only one out of two experiments. However, it should be noted that the negative control survives significantly less well than the no-injection control \(P < 0.01\) most likely as a result of airborne agents, the presence of which cannot be absolutely controlled. Alternatively, it is also possible that the lesion causes septicemia caused by endogenous bacteria that might become mobilized by the process of injection and the resultant physical damage. The 40 OD treatment shows significantly lower survival than all other treatments \(P < 0.0001\). At 10 days old, the 4 OD treatment still shows no significant difference relative to the negative injection control \(P > 0.06\) (Fig. 2B). However, this treatment is now significantly different from the no-injection control in both trials \(P < 0.0001\). Again, the negative control survives significantly less well than the no-injection control \(P < 0.0009\), and the 4 OD treatment is significantly different from all other treatments \(P < 0.0001\). Similar results are observed at 30 days old and 40 days old (Fig. 2C,D and Supplementary Table S1) with the exception of the 4 OD treatment that is significantly different from the negative injection controls in one of two trials (two out of two for 30-day-old female mutants). Overall, the impact of infection on survival appears to be more severe with increasing age. At any age, the severity of the effect is greater in male mutants.

To ensure that these observations do not result from the genetic background, a different strain was examined and produced similar trends (Figs 3 and 4, and Supplementary Table S1). At 3 days old, no significant differences are obtained between the negative, 4 OD and no-injection treatments in both male and female flies \(P > 0.02\) (Figs 3A and 4, and Supplementary Table S1). As observed in the \(w^{118}\) strain, the 40 OD treatment has a significant effect in male trials \(P < 0.02\). The same treatment shows a significant effect only in one female trial. At 10 days old, both male and female 40 OD trials show significantly decreased survival compared to any other treatment \(P < 0.0001\) (Fig. 2B). In male flies, unlike \(w^{118}\), no other treatments show a significant decrease in survival \(P > 0.02\). However, in one of the two female trials, the 4 OD and negative injection treatments are significantly different from the negative injection and no-injection treatments, respectively. At 30 days old and 40 days old, the negative and 4 OD treatments are now significantly different from the no-injection control in both genders and trials \(P < 0.0001\) (Fig. 3C,D). In male flies, the 4 OD is also significantly different from the negative injection in one of the trials. As seen with \(w^{118}\), the decrease in survival appears to be lower in female flies at any age while greater in both genders as age increases.

Overall, both strains display a similar progression with age where significant effects are seen with the 40 OD treatment before the 4 OD treatment.

Bacterial clearance remains largely unchanged through age

Regardless of the age at the time of infection and the amount of bacteria injected, at least 75% of the bacteria injected are eliminated within 48 h post-infection from \(w^{118}\) males and females (Fig. 5). With the exception of 40-day-old male flies, no significant differences can be detected in the magnitude of clearance between the two treatments 48 h and 72 h post-infection.

Canton-S (CS) flies show similar clearance patterns, clearing at least 50% of the bacteria 72 h after infection (Fig. 6). Compared to \(w^{118}\), 3-day-old and 10-day-old CS flies appear to respond initially slower, but ultimately clear to a similar level 72 h after infection (Fig. 6A,B). At later ages, the rate and magnitude of clearance are indistinguishable from \(w^{118}\) (Fig. 6C,D). As observed with \(w^{118}\), the amount of bacteria injected does not affect the magnitude of clearance.

Secreted bacterial factors are sufficient to decrease survival

Our results show that aging impairs survival after infection, while the ability to clear bacteria remains intact, indicating that bacterial proliferation inside the fly is not necessary for them to succumb to infection. This observation led us to hypothesize that immune senescence stems from a declining ability to survive bacterially derived factors rather than a declining ability to clear bacteria. This hypothesis implies that bacterial factors are sufficient to kill adult \(Drosophila\). To test whether secreted bacterial factors have the ability to kill flies, \(imd\) mutants were infected with the media of an overnight culture that has been sterilized by filtration but still contains secreted bacterial factors. The percentage survival after injection from three independent experiments was averaged and is presented in Fig. 7. Male flies injected with filtered media show significantly lower survival in two of three trials \(P < 0.0001; \ P = 0.9; \ P < 0.0001\). Female flies show significantly lower survival in all three trials \(P < 0.0001; \ P < 0.0006; \ P = 0.003\).

Discussion

While several previous studies address some aspects of the functionality of the \(Drosophila\) immune system during aging (Kim et al., 2001; Zerofsky et al., 2005; Lesser et al., 2006; Burger et al., 2007; Corby-Harris et al., 2007), none examined simultaneously...
Fig. 2 Percentage survival of male and female w¹¹¹⁸ flies infected with varying amounts of bacteria at different ages. In all graphs, the Y-axis shows percentage survival and the X-axis shows days after infection. The sample size and the amount of bacteria injected (colony-forming units) are provided in parentheses. (A) Three days old. (B) Ten days old. (C) Thirty days old. (D) Forty days old.
Fig. 3 Percentage survival of male and female CS flies infected with varying amounts of bacteria at different ages. In all graphs, the Y-axis shows percentage survival and the X-axis shows days after infection. The sample size and the amount of bacteria injected (colony-forming units) are provided in parentheses. (A) Three days old. (B) Ten days old. (C) Thirty days old. (D) Forty days old.
the ability to survive infection as well as clear infection across age. Furthermore, those studies neither tested both genders nor controlled for differences in endogenous bacteria. It is well known that many Drosophila strains harbour Wolbachia bacteria, some strains of which cause deleterious effects on longevity (Min & Benzer, 1997). It has also been recently reported that a dramatic increase in bacterial load takes place during Drosophila aging (Ren et al., 2007). Finally, these studies did not measure the real amount of bacteria introduced inside the fly, which must be used instead of the OD of the bacterial solution to compare experiments. Although the bacterial solutions used in this study have the same final OD, the data show that the initial bacterial load can vary several fold when different ages using different bacterial solutions are considered. The most extreme example is seen in Fig. 6 where 40-day-old male flies injected with 4 and 40 OD exhibit 4.8 and 2.9 times less bacteria, respectively, than 10-day-old male flies. In addition, 40-day-old female flies display 3.8 and 4.5 times less bacteria than 10-day-old female flies. In contrast, at any given age when the same bacterial solution is used, the differences between male and female flies vary only between 1.03- and 1.63-fold.

In light of the lack of a thorough study of the immune response during Drosophila aging, we examined simultaneously across age both the survival to and clearance of a consistent, quantifiable infection of adult flies. Our results show that two different Drosophila strains both experience a similar decline in the ability to survive an infection. Both strains display the same chronological responses to the two doses of bacterial challenge where detrimental effects of age are observed earlier with the highest dose. Although the detrimental effect for each dose of bacteria occurs at a later age in the CS strain, this apparent delay can simply be attributed to the longer lifespan of this strain, making it physiologically younger than the w1118 strain at the same chronological age (Helfand et al., 1995). The decline in survival

![Graphs showing percent survival differences between treatments and age at infection.](image-url)

**Fig. 4** The average difference in percent survival between each treatment and the non-injected flies 2 days after infection as a function of the age at which flies were infected. Data points represent the mean values after averaging differences from two independent trials. Error bars represent the range.
with age in response to infection may simply result from the increase in frailty with age. However, the first sign of decline can be seen with the 40 OD infections between 3 days old and 10 days old. Because there is no evidence of deterioration of body functions (protein synthesis, metabolic rate, stress resistance) or behaviours (geotaxis, fertility, locomotion, odour avoidance, shock avoidance) during the first 2 weeks of adult life, it is unlikely that a decline in frailty underlies the observed decline in survival (Arking & Wells, 1990; Arking et al., 1991; Goddeeris et al., 2003; Grotewiel et al., 2005; Simon et al., 2006). In our experimental system, an increase in frailty can be estimated from the number of flies that succumb within an hour post-infection because of injury rather than infection (Hedengren et al., 1999; Corby-Harris et al., 2007). Although we observed that 40-day-old CS flies exhibit higher mortality from injury, no big changes are apparent between 3 days old and 10 days old (Supplementary Fig. S1). Furthermore, an improvement can be seen during this period when the w1118 strain is infected with

Fig. 5 Bacterial clearance following infection in male and female w1118 flies across age. The average number of bacteria injected into infected populations is also indicated (colony-forming units). In all graphs, Y-axis shows percentage remaining of initial infection and X-axis shows hours after infection. Error bars represent two standard deviations.
Immune senescence in Drosophila, S. Ramsden et al.

© 2008 The Authors
Journal compilation © Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland 2008

Finally, the age-dependent decline in survival cannot be attributed to the bacterial strain used in this study. A recent study reported the survival of 7-day-old and 20-day-old flies challenged with Gram-negative Pseudomonas aeruginosa or Gram-positive Lactococcus lactis bacteria, both of which are present in natural populations of flies (Burger et al., 2007). Similarly to the Escherichia coli strain used here, those bacteria require 24 h before mortality is observed, although the effect is more severe because a 0.2 OD concentration was sufficient to elicit, after 48 h in 7-day-old flies, an effect comparable to the 40 OD treatment on 10-day-old flies. At 20 days, a much steeper decline in survival was observed resulting in 100% mortality in less than 30 h. These observations in conjunction with the present study indicate that Drosophila experience increased deterioration with age of ability to survive infections where progression is dependent on the dose and the pathogenicity of the infectious agent.
We found no evidence that the ability to clear bacterial invaders is altered in elderly flies. This finding may appear surprising in the light of a recent study of 25 chromosome II replacement lines derived from a natural population (Lesser et al., 2006). This study reported that the effect of age on bacterial clearance varies dramatically among lines with 11 showing an improvement, five lines declining and nine lines exhibiting no change. However, one must keep in mind that the second chromosome carries at least 141 genes involved in immune response, and natural variation in a subset of these genes is known to produce variation in immune response (De Gregorio et al., 2001; Lazzaro et al., 2004). Therefore, it is not possible to determine unequivocally whether changes are because of age or genotypic variations.

One may raise the issue that the maintenance of clearance ability across age is a result of the experimental protocol. The flies collected at a given time-point during the clearance experiments are a fraction of the original population that survived to that point. This fraction may constitute a subpopulation of flies that survived because they maintain the ability to clear bacteria. Although such cohort effect is extremely difficult to rule out altogether, several observations make it very unlikely. First, the survival curves presented in this study do not include any point between 0 and 1 day because no death was observed during this period. As previously mentioned, the same observation was made with different bacterial species (Burger et al., 2007). Therefore, cohort effects cannot bias the clearance measurements at 6 h and 12 h after infection. Thereafter, if cohort effects had overly affected the results, the different mortality rates between 24 h and 48 h versus 48 h and 72 h should translate into different rates of clearance between those two periods. At 24 h, the higher proportion of flies with high bacterial titres would increase the mean remaining bacteria, whereas at 48 h and later their low proportion is less likely to influence the mean.

Furthermore, a large variance should be observed at 24 h before mortality increases, with the amount of variance decreasing sharply once the mortality has decreased.

Our results suggest that aging does not decrease survival after infection by impairing the ability to recognize and eliminate bacteria because a correlation between decreased survival and decreased clearance does not exist. This finding led us to propose that immune senescence results from a decreasing ability to cope with the factors produced by the bacteria rather than a reduced ability to eliminate them, implying that infected flies do not die simply as a result of bacterial proliferation. This hypothesis is supported by the results obtained with immune-deficient flies. While all of the bacterial concentrations resulted in 100% mortality within 8 days after infection, the 0.04 OD treatment showed no evidence of bacterial proliferation. Furthermore, we observed that imd mutants injected with bacterial supernatant survived significantly less well than those infected with sterile growth media.

**Experimental procedures**

**Drosophila** strains and culture

Two different strains were used to control for the influence of the genetic background. The CS strain is a wild-type strain, whereas the w^1118^ strain is a laboratory strain derived from an Oregon-R wild-type strain in which a recessive mutation conferring white eyes has been introduced. This strain was included because it is the background used in aging experiments involving transgenes. The w^1118^ strain has a shorter lifespan than the CS strain (male median lifespans: 39 vs. 53, P < 0.0001, n > 96; female median lifespans: 49 vs. 56, P < 0.0001, n > 136). The w; imd immune-deficiency strain is a w^1118^ background with a
P-element-induced recessive mutation in the imd gene (Lemaitre et al., 1995). All fly lines were treated with tetracycline before initiating any experiments to ensure none would carry Wolbachia bacteria as previously described (Min & Benzer, 1997).

Two independent collections (different cultures, different anaesthesia) were performed for each strain (collection 1: 2000 male flies, 2000 female flies; collection 2: 2500 male flies, 2500 female flies). The flies were allowed to emerge for 48 h. The flies were anaesthetized with nitrogen for a maximum of 2 min, and were segregated by sex into vials (20–30 individuals per vial). They were then maintained and aged at 25 °C, on standard fly media containing 0.01% molasses, 8.2% cornmeal, 3.4% killed yeast, 0.94% agar, 0.18% benzoic acid and 0.66% propionic acid. Flies were transferred to new vials containing fresh media twice a week. At the appropriate age, around 600 male and 600 female flies were split between the four treatments, and infected. Of the fraction alive 1 h post-infection, around 480 male and 480 female flies were used for the survival experiments, and 60 for the clearance experiments.

Infections

Bacteria were grown to exponential growth phase (OD600nm = 0.50 ± 0.05), and the desired amount of bacteria was centrifuged for 5 min at 4000 RCF. The resulting bacterial pellet was re-suspended in the appropriate amount of 2TY growth media to obtain the desired bacterial concentration. The concentration was independently confirmed by plating. The bacterial solution was mixed periodically to prevent sedimentation of the bacterial cells. The bacteria used were a lab strain of E. coli DH5α containing the plasmid pHC60 conferring tetracycline resistance and expressing GFP (Cheng & Walker, 1998; Elrod-Erickson et al., 2000). During infection, the flies were anaesthetized with nitrogen for no more than 5 min. The flies were injected in the pteropleura of the thorax, in the axial plane. Those flies dead within an hour following infection were excluded as death was caused by needle injury (Hedengren et al., 1999; Corby-Harris et al., 2007). This is observed whether the injection is performed with or without bacteria, and hence cannot be attributed to a rapid toxic effect from bacteria (Supplementary Fig. S1). To verify that each fly was injected with comparable amounts of bacteria, five to eight single flies from the infected population were washed with 70% ethanol and homogenized in 2TY immediately after infection, and a 1/100 dilution of the extract was plated. The needle used for injections was a custom-ordered 33-gauge, 30° tip and 1.75-inch length needle (Hamilton, (Reno, NV, USA) cat#780305) mounted onto a Hamilton 5 µL syringe (Hamilton, cat# 87930). Unlike microinjection systems using capillary glass needles, this set-up can accommodate the viscosity of the bacterial mixture allowing higher bacterial concentrations without clogging. It was determined that a 0.1 µL volume was optimal for the recovery of the animal within 1 h after infection. The flies were always infected at the same time of the day to avoid any influences of circadian rhythms.

Survival

The flies were infected as described earlier. Two controls were used. The negative controls are injected with sterile 2TY without bacteria. The ‘no-injection’ controls were anaesthetized like the injected animals, but not injected in order to discern death from natural aging from death from infection. Every 1–3 days thereafter, the number of dead flies was counted and the surviving flies were transferred to new vials with fresh food media. Cox regression and log-rank statistics were performed with Prism (GraphPad Software, Inc., San Diego, CA, USA) to identify statistically significant differences (P < 0.01) in survival among treatments. Student’s t-tests were used to analyse significant differences in the average difference in survival from the no injection control in Fig. 4. Age-dependent trend was assessed by one-way analysis of variance with post-test for linear trend using Prism built-in functions (Supplementary Table S2).

Clearance

The flies were infected as described earlier. At set time-points after infection, five to eight single flies from the infected cohort were washed with 70% ethanol and homogenized in 2TY. Dilution of the homogenate was plated on 2TY media containing tetracycline (10 µg/mL). After 24 h incubation at 37 °C, the number of colonies was counted. The colonies were also confirmed to be expressing GFP to exclude any tetracycline-resistant bacteria that may be present naturally inside the fly. This number and the dilution factor were used to calculate the actual number of bacteria within each fly at a given time-point. Student’s t-test was used to determine whether the numbers of colonies 48 h and 72 h post-infection were significantly different from the number of colonies at 0 h.

Supernatant infection of imd mutants

The DH5α:pHC60 E. coli strain was cultured overnight (12–16 h). The culture was centrifuged and the supernatant was filtered through a 0.22 µm filter. It was determined by plating that no bacteria remained in the supernatant after filtering the supernatant four times. This filtered supernatant was used to inject imd mutants as described earlier. Concurrently, control populations were treated with anaesthesia only or were injected with sterile bacterial growth media.

Acknowledgments

The authors thank Neil Silverman, Dominique Ferrandon and Bruno Lemaitre for providing Drosophila strains, and David Schneider for providing the bacterial strain. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC, RGP250140-02) and the Institute of Aging of the Canadian Institutes of Health Research (CIHR, MOP64248, MOP79519).

© 2008 The Authors

Journal compilation © Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland 2008
References


Supplementary material

The following supplementary material is available for this article:

Fig. S1 Percent mortality resulting from needle injury. One hour after infection, the number of dead flies was recorded and expressed as a percentage of the total number of flies treated in the same manner. Percentages from replicates were averaged and plotted. Comparison with Student’s t-test indicates that there are no significant differences in percent mortality between the three treatments at any age. Because there is no consistent difference in percent mortality between flies treated with sterile 2TY and those treated with bacteria, the bacteria do not exhibit rapid toxicity and therefore do not influence survival within the first hour after infection. Furthermore, the decline in survival post-infection of flies during aging is unlikely because of an overall decrease in frailty. Dramatic decreases in survival can be seen in response to infection with 40 optical density of bacteria beginning at 10 days old. When percent mortality resulting...
from the infection protocol at 3 days old is compared to those at 10 days old, no significant increase is evident indicating that the overall frailty of the populations does not increase. Error bars represent two standard errors of the mean.

**Table S1** Survival statistics of the w^{118} and CS strains after infection at different ages. In each cell, two values are shown corresponding to the two independent trials.

**Table S2** Statistical analysis of the age-dependent trend. One-way analysis of variance with post-test for linear trend of survival to infection across age.

**Table S3** Sample sizes of three replicate experiments presented in Fig. 7.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1474-9726.2008.00370.x

(This link will take you to the article abstract).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.