Title: Senescence of the cellular immune response in *Drosophila melanogaster*.

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Abstract

Immune system effectiveness generally declines as animals age, compromising disease resistance. In *Drosophila*, expression of a variety of immune-related genes elevates during ageing; however how this is linked to increasing pathogen susceptibility in older flies has remained unclear. We investigated whether changes in the *Drosophila* cellular immune response might contribute to immunosenescence. Experiments studied fly cohorts of different ages and compared the numbers and activity of the circulating haemocytes involved in pathogen defence. In female wildtype Samarkand and Oregon R flies the haemocyte population fell by 31.8% and 10.2% respectively during the first four weeks of adulthood. Interestingly we detected no such decline in male flies. The impact of ageing on the phagocytic activity of haemocytes was investigated by injecting flies with fluorescently labelled microbes or latex beads and assessing the ability of haemocytes to engulf them. For all immune challenges the proportion of actively phagocytosing haemocytes decreased as flies aged. Whilst 24.3% ± 1.15% of haemocytes in one-week-old flies phagocytosed *Escherichia coli* bacteria or *Beauveria bassiana* fungal spores, this decreased to 16.7% ± 0.99% in four-week-old flies. This clear senescence of the *Drosophila* cellular immune response may underpin increased disease susceptibility in older flies.

Key words: ageing, immunosenescence, phagocytosis, haemocytes, immunity, *Drosophila*

1. Introduction

Ageing has a profound effect on immune system performance in many organisms [1]. Both the cellular basis and clinical relevance of immunosenescence are increasingly well characterised in humans [2]. In contrast, for invertebrates the mechanisms underpinning immunosenescence are poorly understood, even for the best-characterised invertebrate model, *Drosophila melanogaster.* Although invertebrates lack the acquired immune responses that are the hallmark of vertebrate immune defence, many innate immunity genes and signalling pathways are functionally conserved between invertebrates and vertebrates [3]. Unravelling the processes which degenerate in the senescence of the *D. melanogaster* immune system is important to allow exploitation of *D. melanogaster* as a model for human ageing conditions.

For humans, ageing is accompanied by major alterations in both acquired and innate immune defence. Decreasing abundance of naive B cells and an altered T cell repertoire result in a declining ability to form and retrieve immunological memories, leading to poor clinical responses to vaccination and infection late in life [4]. The major ageing-dependent pattern in innate immune defence is one of increasing dysregulation; one consequence may be heightened inflammatory responses, termed 'inflamm-aging' [5]. Chronic inflammatory states underlie many degenerative diseases of the elderly: arthritis, dementia and type II diabetes have all been linked to inflamm-aging [6]. In *Drosophila*, patterns of increasing disease susceptibility with age largely mirror those found in vertebrates [7]. However, the immune system changes that cause this remain unclear.

The *Drosophila* immune system is made up of multiple defences [8]. Pathogens entering the fly are detected as 'non-self' by pattern recognition receptors that bind to conserved microbial molecules, stimulating a coalition of immune defences [9]. Pathogen detection is followed by cellular and humoural responses. The principal component of the humoural immune response is the systemic and local production of antimicrobial peptides (AMPs) [10]. Production of melanin by the enzyme phenoloxidase also contributes to the humoural response: melanin and its reactive synthesis intermediates are toxic to microorganisms and play a role in wound healing [10]. An additional immune defence component is RNA interference (RNAi) which defends against RNA virus infection [8].

Phagocytosis is the main component of the *Drosophila* adult cellular immune response and is carried out by plasmatocytes [11]. Two other haemocyte classes are present in

Drosophila: lamellocytes encapsulate foreign objects (such as parasitoid eggs) and crystal cells store phenoloxidase for release following immune activation [12]. However, lamellocytes and crystal cells are found only in larvae [13], and are therefore not relevant to immune system ageing in adults. *Drosophila* haemocytes either circulate freely throughout the haemolymph or are sessile [11]. Phagocytosis is initiated by binding of pathogen recognition receptors such as Eater [14] and PGRP-LC [15], and may be mediated by opsonin molecules that target microbes for engulfment [10]. Once haemocytes encounter a microbe the cytoskeleton is remodelled to enable endocytosis, before it is destroyed by lysosomal enzymes and reactive oxygen species [12].

Although the *Drosophila* immune system is well characterised, relatively little is known about how ageing affects its function. As is the case for vertebrates, mortality following infection elevates in older flies [7], but we have limited knowledge of the senescent mechanisms underpinning these changes. A range of immune-related genes becomes upregulated as flies age [16-18], which perhaps mirrors vertebrate inflamm-aging. However, the bacterial burden associated with flies also increases with age [19], therefore whether this elevated transcriptional activity reflects immune senescence directly or if it results from heightened activation is not known.

A small number of studies in *Drosophila* have investigated the mechanisms underlying age-related changes in immune defence. Ramsden et al., [7] suggested that increases in pathogen susceptibility during ageing were not due to impaired ability to clear infection, but perhaps resulted from reduced tolerance of infection pathology. Similarly, Lesser et al., [20] investigated how the ability of 25 *D. melanogaster* genotypes to clear *E. coli* infection changed with age and found considerable variation in both the direction and magnitude of age-dependent changes. Zerosky and colleagues [21] demonstrated that following infection expression of the AMP *diptericin* was higher and more prolonged in older compared to younger flies; however the significance of this result to overall immune competence is unclear.

We know more about invertebrate immune senescence from studies in non-model organisms. Ageing was associated with increased pathogen susceptibility and reduced phenoloxidase levels in the cricket *Gryllus texensis* [22]. In scorpion flies (*Panorpa vulgaris*), whilst haemocyte numbers remained unchanged during ageing, the phagocytic activity of those cells declined [23]. Hillyer et al., [24] showed that older mosquitoes (*Aedes aegypti*) had reduced survival following infection with *E. coli*, a lower ability to clear the infection, and

fewer phagocytic haemocytes compared with younger mosquitoes. However they only assayed mosquitoes across the first five days of life, so it is questionable whether these trends truly represent senescence or some other life-history shift. Doums et al., [25] found older bumblebees (*Bombus terrestris*) had impaired ability to encapsulate foreign implants, but haemocyte numbers were unaffected by ageing. Similarly Whitehorn and colleagues [26] found that in the wild older bumblebees (*Bombus muscorum*) had lower phenoloxidase titres. These studies each assayed varying suites of immune effectors. Whether differences between species in the immune system components shown to senesce represent true species-specific variation in the effects of ageing or whether this simply reflects differences in experimental power and design has not been determined.

Here we investigate the senescent mechanisms underpinning increased pathogen susceptibility in aged *D. melanogaster* and focus on changes in the cellular immune response. We test whether the number of circulating haemocytes declines with age and also whether the haemocytes of older individuals have impaired phagocytic activity. Vertebrate macrophages are less efficient at phagocytosing larger particles [27]. With the expectation that the same would be true for *D. melanogaster* haemocytes, we investigate whether the rate at which phagocytosis ability senesces is faster for larger, more challenging targets.

2. Materials and Methods

2.1. Fly culturing

We used Oregon R and Samarkand wildtype genotypes (obtained from Bloomington Stock Centre). Flies were reared on Lewis medium [28] at 25 °C, 70% RH, 12hr L/D. Both genotypes were initially bred at low density for two generations (five females oviposited in each bottle for three days). Cultures of both genotypes were set up weekly in bottles. To keep larval density constant eggs were collected on apple juice agar plates seeded with yeast, then washed and 13µl of eggs transferred to each bottle using a pipette [29]. When offspring eclosed they were transferred to 11 litre demography cages (~400 mixed-sex flies per cage). Flies were fed Petri dishes of Lewis medium every other day and left to age for between one and four weeks. Cages were set up every week so that flies of all ages were simultaneously available for experiments. A total of 10 independent demography cages were represented in the experiment, flies were removed from each cage at weekly intervals as each population aged. Flies removed for experiments or that died were counted, and constant density was maintained by topping up cages from separate, similarly aged, populations maintained for that purpose.

2.2. Fluorescent material for assessing phagocytosis

Escherichia coli (strain BB4) was grown overnight in LB broth at 37°C. 1 ml of culture was washed four times by centrifuging and resuspending in 1ml of sodium carbonate buffer (0.1M $NaHCO_3$, pH 9). The final suspension was mixed with its own volume of Fluorescein isothiocyanate (FITC) (Sigma) (1mg ml^{-1} in $NaHCO_3$ buffer) and incubated for one hour at room temperature in the dark with continual shaking. Bacteria were then washed six times in the same manner, then resuspended in 1ml of PBS (pH 7) and diluted to 5×10^6 cells ml^{-1}. *Beauveria bassiana* spores originated from a strain used previously [30]. Fungal material was grown for two weeks on potato dextrose agar with chloramphenicol (5×10^{-5}g ml^{-1}), then left to sporulate and dry at room temperature. Spores were FITC-labelled as for *E. coli* and diluted to the same concentration. We also assessed haemocyte activity using Fluoresbrite® Fluorescein labelled carboxylate-modified latex microspheres (Polysciences) of a range of sizes (0.5, 1.0, 2.0, 3.0 and 4.5µm) suspended in PBS at 5×10^6 beads ml^{-1}.

2.3. The influence of fly age on haemocyte phagocytic activity

A Drummond Scientific oocyte microinjection pipette was used to inject one to four-week old Samarkand flies with 0.05µl of the fluorescent microbe or bead suspensions prepared above. Flies were injected into their lateral thorax in single sex five-fly batches, then incubated at 25^{o}C for 30 min. The combined haemolymph of five flies was bled into a pool of 20µl Hoechst anticoagulant buffer (0.01mg ml^{-1} Hoechst (Invitrogen) in 10 mM sodium cacodylate, 10mM $CaCl_2$, 280mM sucrose, pH 7.4). The haemolymph mixture was transferred to a 1.5 ml microcentrifuge tube, and a further 20µl cacodylate buffer added, then left for 5 min to allow fat droplets to separate. The bottom 20µl was then transferred to a polylysine-coated well on a 96-well plate. Plates were centrifuged at 30.7 *g* for 3 min, then 10µl of 11.1% formaldehyde in PBS and a drop of VectaShield^{R} containing DAPI (Vector Laboratories) were added. Plates were viewed on an Axiovert 135 epifluorescence microscope; 100 cells were inspected in each well and the number of fluorescent included particles per cell recorded. 187 biological replicates (wells containing pools of five flies) were used for this experiment.

2.4. The effect of age on numbers of circulating haemocytes

One to four-week-old Samarkand and Oregon R flies were anesthetised on ice in single sex batches of three flies and haemolymph was extracted from each batch using a standardised pulled-glass capillary needle inserted into the thorax. The length of haemolymph in the capillary was measured using an eyepiece graticule before adding the haemolymph to a 1.5 ml microcentrifuge tube containing 25µl of cacodylate buffer (as described above). The cell density was then counted using a Neubuer haemocytometer. In total 263 biological replicates (three fly batches) were counted. The order in which fly batches were bled was random with respect to age and sex. The calibration between the fill-length of capillaries and the haemolymph volume they contained was calculated by filling 72 pulled needles to a range of lengths with a 2.0µm bead suspension of standard concentration. This fluid was mixed with buffer as before, beads counted on a haemocytometer and the corresponding capillary volume calculated. A fourth order polynomial curve was fitted through the data (R^2 = 0.973), which was then used to calculate the haemolymph volume extracted from each fly batch above.

2.5. Statistical Methods

Data were analysed using the Lme4 package [31] for R statistical software, version 2.11.1 [32]. We constructed linear mixed-effects models with two random effects: the cage in which flies were housed whilst ageing, and the Julian day on which experiments were carried out. Our fixed effects included fly age, sex, weight, time of day for the assay, and immune treatment (injections of beads, bacteria or fungal spores) in addition to the two-way interaction between age and treatment as well that between age and sex. We used models with Gaussian errors to determine how these factors influenced the mean number of particles that haemocytes phagocytosed. Binomial errors were used for assessing the proportion of active haemocytes employing the cbind function in R to analyse a two-vector response including the number of active and inactive cells. For analyses of the haemocyte numbers in flies of each age, we used the same model structure including fly genotype as a fixed factor and the log10 transformation of cell number as response. Models were sequentially simplified by selecting terms for which the parameter estimate was closest to zero and eliminating them if their presence did not improve the explanatory power of the model by 2 AIC units. Significance of terms was determined using likelihood ratio tests. Means are given ± their standard errors throughout.

3. Results

3.1. The proportion and activity of phagocytically active cells declined with age

To test whether haemocyte phagocytic activity is affected by age, Samarkand flies aged 1, 2, 3 and 4 weeks post-eclosion were injected with fluorescently labelled bacteria (*E. coli*) or fungal spores (*B. bassiana*). As flies aged there was a dramatic reduction in the proportion of haemocytes that phagocytosed these microbes (χ^2_1 = 10.88, *P* = 0.001) (Table 1a). In one-week old flies, around 24.3% ± 1.15% of haemocytes phagocytosed microbes, whereas by four weeks old this had fallen to 16.7% ± 0.99%. This represents a ~30% decline during the first four weeks of life in the fraction of cells that were phagocytically active in this assay. This trend occurred both for fungal spores and bacteria (Fig 1A); there was no difference between these microbes in the rate of phagocytosis decline with age (age x microbe interaction: χ^2_1 = 0.03, *P* = 0.861). However, when other fixed effects were taken into account, there was a small but significant difference between the two microbes in the proportion of active haemocytes: slightly more haemocytes phagocytosed bacteria than fungal spores (χ^2_1 = 6.38, *P* = 0.012).

Marginally more of the cells in males phagocytosed microbes than in females (χ^2_1 = 3.68; *P* = 0.055); however both genders had the same rate of decline in haemocyte activity as they aged (age x sex interaction: χ^2_1 = 0.67, *P* = 0.413). The proportion of active haemocytes increased with fly mass (χ^2_1 = 6.52, *P* = 0.011) and flies injected later in the day had more active haemocytes than those injected earlier (χ^2_1 = 7.35, *P* = 0.007).

After comparing the proportion of haemocytes that were phagocytically active in flies of different ages, we next investigated cellular immune senescence by assessing how the number of included particles per cell changed as flies aged. When Samarkand flies were injected with *E. coli* or *B. bassiana*, the mean number of inclusions per circulating haemocyte decreased significantly as flies aged (χ^2_1 = 8.52, *P* = 0.004) (Table 1b); in one-week-old flies each haemocyte phagocytosed a mean of 0.30 particles (± 0.03), whereas this fell by 33.33% to 0.21 particles (± 0.02) by week four. Furthermore, cells in flies challenged with *E. coli* had 4.5% more inclusions compared those receiving *B. bassiana* (χ^2_1 = 7.18, *P* = 0.007). Cells in heavier flies phagocytosed more particles (χ^2_1 = 11.42, *P* = 0.001). Those in males had a greater number of inclusions (χ^2_1 = 10.14, *P* = 0.001), and cells of flies injected later in the day phagocytosed more (χ^2_1 = 10.56, *P* = 0.001). Again, the gradient of age-related decline was the same between microbes and between sexes (age x microbe interaction: χ^2_1 = 0.87, *P* = 0.352; age x sex interaction: χ^2_1 = 1.36, *P* = 0.243).

3.2. Phagocytic ability of a subset of haemocytes is unaffected by ageing

We finally analysed the data for how the number of included microbes per cell changed as flies aged considering only those cells that were phagocytically active in our assay, ignoring those which had not engulfed particles. Interestingly, there was no age-related change in the number of inclusions for these active cells (χ^2_1 = 0.40, P = 0.526); the mean for one week old flies was 1.33 inclusions per cell (± 0.05), whereas that for four week old flies was 1.39 (± 0.06). In these analyses of active haemocytes, cells phagocytosed slightly more fungal spores than they did bacteria (χ^2_1 = 6.15, P = 0.013 (table 2a)).

3.3. The rate that phagocytic ability senesces was unaffected by particle size

We predicted that the ability of cells to phagocytose would be reduced for larger particles and that the rate of age-dependent decline might be faster for these more challenging targets. Samarkand flies from one to four weeks old were challenged with fluorescent beads ranging from 0.5µm to 4.5µm in size (Fig 1B). Clear and significant differences existed in the ability of haemocytes to phagocytose different sized beads (χ^2_1 = 91.84, P < 0.001) (Table 3a): fewer than half as many cells were able to phagocytose the large beads (4.5µm) as were able to phagocytose the smaller beads (0.5-2.0µm) (Fig 1B). As before, there was a marked senescent decline in the proportion of phagocytosing haemocytes (χ^2_1 = 14.60, P < 0.001). However, our hypothesis that the rate of senescence in phagocytosis ability might be faster for more challenging phagocytosis targets was not supported: the age-dependent decrease in phagocytosis ability was consistent for the five beads sizes (age x bead size interaction: χ^2_1 = 0.72, P = 0.398). Again, more cells phagocytosed beads in flies injected later in the day (χ^2_1 =1.53, P = 0.040). We found no influence of fly mass (χ^2_1 = 0.02, P = 0.216), there was no difference between males and females (χ^2_1 = 0.02, P = 0.888), and cell activity senesced at similar rates in both sexes (age x sex interaction: χ^2_1 = 0.12, P = 0.731).

3.4. Haemocyte number declined as females aged

The efficacy of the cellular immune system may be influenced by the number of cells as well as by their activity. We investigated how the density of cells circulating in the haemolymph changed with age. In this case we compared two wildtype fly genotypes, Oregon R and Samarkand. Cell number declined significantly as flies aged (χ^2_1 = 8.53, P = 0.0035) (Table 4a) and the rate of decline was consistent for the two genotypes (age x genotype interaction: χ^2_1 = 2.59, P = 0.107) (Fig 2). However, the age-dependent trend in cell number was very different for the two sexes (age x sex interaction: χ^2_1 = 6.85, P = 0.009). For both Samarkand and Oregon R, whilst cell number in females fell markedly with age (χ^2_1 = 11.18, P = 0.001) (Table 4b), no such change occurred for males (χ^2_1 = 0.36, P = 0.547). Female Samarkand

flies had 3060 (± 303) cells μl^{-1} when one week old but only 2086 (± 197) cells μl^{-1} at four weeks; for Oregon R females cell number fell from 4024 (± 362) cells μl^{-1} to 3614 (± 446) cells μl^{-1} over four weeks. These represent 31.8% and 10.2% declines in cell number respectively. In contrast, one week old Samarkand males had 4514 (± 273) cells μl^{-1} and this remained approximately constant through to 4374 (± 367) cells μl^{-1} at week four. The same was true for Oregon R males for which the week one and four cell densities were 4439 (± 368) and 4964 (± 557) cells μl^{-1} respectively. Males had on average 25.9% more haemocytes than females ($\chi^2_1 = 31.47$, $P < 0.0001$). Oregon R flies had slightly higher cell density than Samarkand (Fig 2), however this difference was not significant ($\chi^2_1 = 3.32$, $P = 0.069$). Cell number was not affected by time of day ($\chi^2_1 = 0.98$, $P = 0.322$).

4. Discussion

For the first time in *D. melanogaster* we demonstrate that the cellular immune response undergoes senescence. Age-dependent degeneration occurred in two aspects of cellular immunity. Firstly the haemocyte population, which is responsible for clearing microbes from the haemocoel, became less able to phagocytose microbes: fewer cells were phagocytically active in older flies. Secondly, in female flies the number of haemocytes circulating in the haemolymph declined during ageing.

By four weeks of age, only 70% of the haemocytes that had been active in young flies were still able to phagocytose microbes. In addition, whilst cell numbers in males did not change, in females there was an average fall of about 21.0% in haemocyte density between week one and week four. These combined effects mean that, at least for females, the phagocytic capacity of the haemocyte population reduced by ~46% over the first four weeks of life. These immunological changes must be due to senescent deterioration in individual flies, because the average total mortality in our fly cages between one and four weeks of age was too low (7.2% ± 0.8%) for non-random mortality at the population level to explain this decline. Our phagocytosis assay investigated the ability of cells to clear particles from the haemolymph within 30 minutes; if we had left flies for longer more particles would have been engulfed. However, the rate of activity of the immune response is important: whether and when an insect dies from infection may be determined by the relative rates at which a pathogen can grow and at which it is cleared. Previous work indicated that flies in which phagocytosis was impaired by a null mutation in the phagocytosis receptor *Eater* succumbed to infection with *Serratia marcescens* two days before wild-type flies [14]. Our assay investigated changes in the free haemocyte population which circulates within the haemolymph. A proportion of haemocytes are sessile and bound to tissues [13,33]; further work will be required to investigate how these sessile cells are affected by ageing.

Haemocytes in older flies on average phagocytosed fewer microbes and fluorescent beads than those in young flies. This was due to a decline in the proportion of cells which were phagocytically active, rather than a decline in phagocytosis rate in all cells. Considering only those cells that were active (containing at least one phagocytic inclusion), there was no change in the number of included particles between one and four weeks of age. This suggests that there may be heterogeneity in the haemocyte population. Either haemocytes have specialised roles and the relative frequency of cells specialising in phagocytosis declines, or senescence may affect the phagocytic activity of some cells but not others. We

used microbes and beads labelled with FITC to assess phagocytosis; FITC can modulate the efficacy of phagocytosis [34] meaning our estimates may not directly equate with the ability of cells to engulf pathogens. However, labelling was consistent across age classes and particle types, and therefore could not have influenced our comparisons.

The cellular response is important for pathogen defence: both domino mutant larvae (which lack haemocytes) and *Drosophila* adults in which phagocytosis has been blocked have compromised immunity [33,35]. A quantitative comparison of humoural and cellular responses revealed that phagocytosis contributes a considerable amount to overall pathogen defence [36]. This leads to the prediction that as flies age and the cellular immune response declines their ability to clear microbial infection should decrease. However, this contrasts with the results of Ramsden et al., [7] who found that although mortality following infection increased in older flies, their ability to suppress *E. coli* growth was unchanged. Ramsden et al., [7] hypothesised that age-dependent increases in infection mortality resulted from decreased tolerance to infection rather than decreased resistance to microbial replication.

In addition to phagocytosis, haemocytes have other immune defence roles. They produce hemolectin and other factors involved in blood clotting [37], and synthesise small quantities of AMPs besides those produced by the fat body and epithelia [38]. Haemocytes also produce opsonins, such as thioester-containing proteins (TEPs), which promote phagocytosis [39]. Furthermore, these cells do not act in isolation, but produce signalling molecules that interact with other parts of the immune system, such as the Toll ligand Spätzle [38,40] and Upd3 which triggers JAK/STAT pathway activity [41]. Thus declining haemocyte numbers during senescence may have several immunological consequences.

Whilst circulating haemocyte numbers declined strongly with age in female flies, those in males did not. Functional senescence of some behavioural and physiological traits is known to differ between the sexes and can vary according to genetic background [42]. Notably, flies in the current study were aged in mixed-sex populations and therefore mated throughout their lives. The immune systems of both males and females are influenced by mating [43,44]. It is therefore also possible that sexually antagonistic interactions or adaptive post-mating responses may have contributed to the decline in female cell density. As females experience copulatory wounding [45], haemocytes may be sequestered for wound repair and so removed from general circulation. In contrast to these trends in cell number, the age-

dependent decline in phagocytic activity of cells occurred at similar rates in both males and females.

The phagocytic ability of mammalian macrophages is impaired for larger phagocytic targets [27]. Here we demonstrate that the same phenomenon occurs in *D. melanogaster.* Flies were injected with beads ranging from 0.5µm to 4.5µm in diameter; above 2µm diameter the proportion of the haemocyte population that achieved phagocytosis decreased by around half. We hypothesised that if engulfing larger particles is more challenging, then the rate of phagocytosis senescence might be faster for larger targets. This was not the case; the rate of senescence was consistent for all five bead sizes, and indeed was not different for the two microbes tested (*E. coli* and *B. bassiana*). Although an element of pathogen specificity exists for some phagocytosis receptors [46,47], the consistency of these age-dependent declines suggests that phagocytosis senescence results from changes in general aspects of the phagocytic machinery. Phagocytosis may be modulated by opsonins and requires recognition and attachment of the haemocyte to the foreign body; remodelling of the cytoskeleton and particle engulfment then follows [12]. Senescence of phagocytic ability could result from any of these processes being compromised. The age-dependent decline in haemocyte numbers could be due to increased apoptosis rates in the haemocyte population of older flies, as occurs in other tissues [48]. Alternatively, if haemocytes are lost when they clear microbes from the haemolymph, cumulative exposure during a fly's life may result a reduced cell population.

This study demonstrates clear senescent declines in the cellular immune response. Previous ageing studies focussing on the expression of humoural immune response genes have shown transcript levels to increase, indicating heightened activity [16-18,21]. Our findings suggest that age-dependent increases in humoural immunity may relate to the observed senescence of the cellular immune response. A compromised cellular response could result in increased microbial susceptibility thus intensifying stimulation of the humoural response. Increased humoural activity can offset reduced cellular activity; when phagocytosis is experimentally inhibited overexpression of the AMP Defensin restored pathogen resistance [36].

A higher proportion of cells phagocytosed particles in those flies injected later in the day. This could reflect circadian rhythms in the immune response as has been shown in previous studies [49,50], however in the absence of a controlled experiment to investigate this, we refrain from drawing strong conclusions.

Little information about how long *Drosophila* lives in the field exists to put the age-dependent immunological changes we have observed into ecological context. However, it seems unlikely that many flies naturally survive to the oldest ages studied here. Nevertheless, senescence of phagocytosis ability was roughly linear from one through to four weeks of age. Thus, cellular immune senescence begins at an early age and may influence pathogen defence from week one onwards, an age relevant to wild populations.

Finally we note that plasmatocyte haemocytes in adult *Drosophila* have closest similarity to the monocyte lineage that gives rise to macrophages in vertebrates [11]. Vertebrate macrophages have a plethora of roles including antibacterial defences, chemotaxis and wound repair but they specialise in phagocytosis; most of these functions decline with age in humans, mice and rats [51]. Other vertebrate phagocytic cells such as dendritic cells and granulocytes also suffer impaired function in aged individuals [52,53]. The age-related changes we observed in *D. melanogaster* haemocyte phagocytosis and abundance mirror senescent changes known from vertebrate cellular immunity. That the phenomena underlying immunosenescence, if not the mechanisms themselves, may be conserved between flies and humans opens up novel possibilities to address questions of clinical relevance.

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Table 1. Factors influencing the phagocytic activity of haemocytes challenged with microbes during ageing.

(a) Data analysed as the proportion of active haemocytes.

Factors Estimates	Parameter	SE	χ^2	P-value
Age (weeks)	-0.105	0.029	10.88	0.001
Mass (mg)	0.086	0.015	6.52	0.011
Microbe (*B. bassiana* spores)	-0.167	0.067	6.38	0.012
Sex (male)	0.124	0.065	3.68	0.055
Time of day (hrs)	0.120	0.021	7.35	0.007

Output of terms from the model analysing the proportion of phagocytosing haemocytes in adult *D.melanogaster* between one and four weeks of age. Flies were injected with flourescently labelled *E. coli* or *B. bassiana* spores. Parameter estimates with reference to female flies injected with *E. coli*.

(b) Data analysed as the mean number of inclusions per haemocyte.

Factors Estimates	Parameter	SE	χ^2	P-value
Age (weeks)	-0.018	0.006	8.52	0.004
Mass (mg)	0.025	0.003	11.42	0.001
Microbe (*B. bassiana* spores)	-0.027	0.014	7.18	0.007
Sex (male)	0.038	0.014	10.14	0.001
Time of day (hrs)	0.031	0.004	10.56	0.001

Output of terms from the model analysing the mean number of included particles per haemocyte in adult *D.melanogaster* between one and four weeks of age. Flies were injected with flourescently labelled *E. coli* or *B. bassiana* spores. Parameter estimates with reference to female flies injected with *E. coli*.

Table 2. Factors influencing the number of included particles per phagocytically active haemocyte during ageing

(a) Flies challenged with microbes.

Factors Estimates	Parameter	SE	χ^2	P-value
Microbe (*B. bassiana* spores)	0.096	0.038	6.15	0.013

Output of terms from the model analysing the mean number of included particles per phagocytically active haemocyte in adult *D.melanogaster* between one and four weeks of age. Flies were injected with flourescently labelled *E. coli* or *B. bassiana* spores. Parameter estimates with reference to flies injected with *E. coli*.

(b) Flies challenged with latex beads.

Factors Estimates	Parameter	SE	χ^2	P-value
Bead size (µm)	-0.046	0.010	17.35	<0.001
Time of day (hrs)	-0.016	0.007	4.86	0.027

Output of terms from the model analysing the mean number of included particles per phagocytically active haemocyte in adult *D.melanogaster* between one and four weeks of age. Flies were injected with one of five sizes of flourescent beads (0.5µm, 1.0µm, 2.0µm, 3.0µm, 4.5µm).

Table 3. Factors influencing the phagocytic activity of haemocytes challenged with latex beads during ageing.

(a) Data analysed as the proportion of active haemocytes.

Factors Estimates	Parameter	SE	χ^2	P-value
Age (weeks)	-0.145	0.025	14.60	<0.001
Bead size (µm)	-0.227	0.024	91.84	<0.001
Time of day (hrs)	0.038	0.018	4.21	0.040

Output of terms from the model analysing the proportion of phagocytosing haemocytes in adult *D.melanogaster* between one and four weeks of age. Flies were injected with one of five sizes of flourescent beads (0.5µm, 1.0µm, 2.0µm, 3.0µm, 4.5µm).

(b) Data analysed as the mean number of inclusions per haemocyte.

Factors Estimates	Parameter	SE	χ^2	P-value
Age (weeks)	-0.029	0.006	11.70	0.001
Bead size (µm)	-0.044	0.004	76.95	<0.001
Mass (mg)	-0.009	0.004	5.27	0.022
Time of day (hrs)	0.009	0.003	6.70	0.010

Output of terms from the model analysing the mean number of included particles per haemocyte in adult *D.melanogaster* between one and four weeks of age. Flies were injected with one of five sizes of flourescent beads (0.5µm, 1.0µm, 2.0µm, 3.0µm, 4.5µm).

Table 4. Factors influencing the number of haemocytes circulating within the haemolymph during ageing.

(a) Both sexes.

Factors Estimates	Parameter	SE	χ^2	P-value
Age (weeks)	-0.063	0.016	8.53	0.004
Genotype (Samarkand)	-0.101	0.053	3.32	0.069
Sex (male)	0.007	0.058	31.47	<0.001
Age x Sex Interaction	0.055	0.021	6.85	0.009

Output of terms from the model analysing the number of haemocytes circulating within the haemolymph in adult *D.melanogaster* for two genotypes: Oregon R and Samarkand, between one and four weeks of age. Parameter estimates with reference to Oregon R females.

(b) Female flies.

Factors Estimates	Parameter	SE	χ^2	P-value
Age (weeks)	-0.052	0.016	11.18	0.001
Genotype (Samarkand)	-0.116	0.041	4.88	0.027

Output of terms from the model analysing the number of haemocytes circulating within the haemolymph in adult female *D.melanogaster* for two genotypes: Oregon R and Samarkand, between one and four weeks of age. Parameter estimates with reference to Oregon R flies.

(c) Male flies.

Factors Estimates	Parameter	SE	χ^2	P-value
Genotype (Samarkand)	-0.997	0.045	4.18	0.041

Output of terms from the model analysing the number of haemocytes circulating within the haemolymph in adult male *D.melanogaster* for two genotypes: Oregon R and Samarkand, between one and four weeks of age. Parameter estimates with reference to Oregon R flies.

Figure Legends

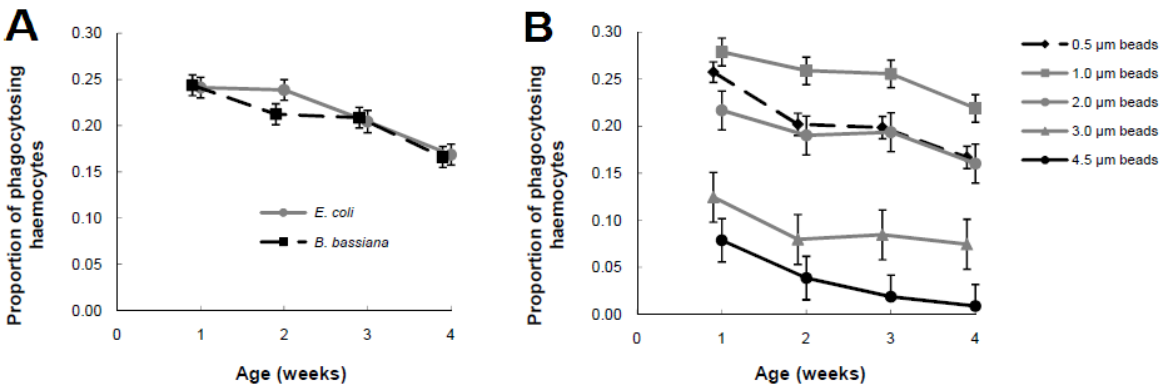

Figure 1. Changes in the proportion of actively phagocytosing haemocytes in adult *D. melanogaster* between one and four weeks of age. (A) Flies were injected with fluorescently-labelled *Escherichia coli* or *Beauveria bassiana* spores. (B) Flies were injected with one of five sizes of fluorescent beads (0.5µm, 1.0µm, 2.0µm, 3.0µm, 4.5µm). Although investigated, no significant difference was detected between the sexes (see text). Points are staggered slightly along the x-axis for clarity. Bars represent standard errors.

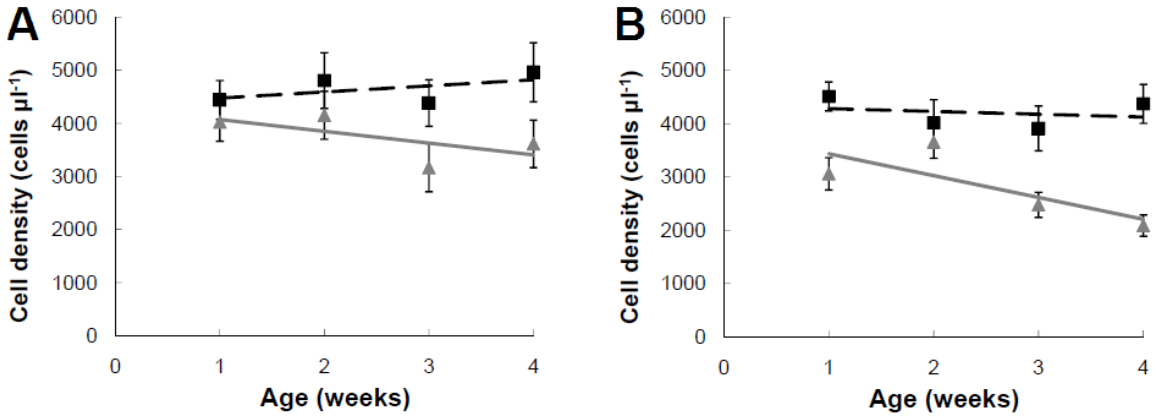

Figure 2. Age-dependent variation in the number of haemocytes circulating within the haemolymph for two fly genotypes: Oregon R (A) and Samarkand (B), divided into males (squares, black dashed line) and females (triangles, grey solid line). The sexes differed significantly in both haemocyte number when young and the pattern of age-dependent change (see text). Bars represent standard errors.