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Studying Aging in *Drosophila*

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Abstract

Drosophila melanogaster represents one of the most important genetically accessible model organisms for aging research. Studies in flies have identified single gene mutations that influence lifespan and have characterized endocrine signaling interactions that control homeostasis systemically. Recent studies have focused on the effects of aging on specific tissues and physiological processes, providing a comprehensive picture of age-related tissue dysfunction and the loss of systemic homeostasis. Here we review methodological aspects of this work and highlight technical considerations when using *Drosophila* to study aging and age-related diseases.

Keywords

Aging; degeneration; *Drosophila*; homeostasis

1. Introduction

Aging can broadly be defined as the progressive decline in physiological integrity and function. This decline encompasses all biological systems, including molecular interactions, cellular functions, tissue structure and function, as well as systemic physiological homeostasis [1], [2]. Due to this decline, aging is accompanied by a progressive increase in mortality and by a range of age-related diseases, including cancer, diabetes, obesity, cardiovascular disorders and tissue degeneration. Strikingly, however, studies in genetically accessible model organisms have established that the rate of aging is quite plastic. Studies in yeast, worms and flies have identified evolutionarily conserved genetic pathways and biochemical processes that influence the rate of aging and that can extend lifespan [3], [4]. Based on these studies, aging is increasingly recognized as a complex and multifactorial, but alterable process that includes deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, and loss of homeostasis in regulatory and physiological processes [1].

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Drosophila has emerged as an excellent genetic model to study this complexity of the aging process[5]-[11]. In addition to the ability to efficiently identify and characterize single-gene mutations that extend lifespan, the well-developed genetic techniques that allow precise spatiotemporal control of genetic perturbations [12]-[15] have made flies the premier model system to address questions about tissue-specific functional decline and tissue-tissue interactions during the aging process. A particular strength of flies is the ability to characterize how genes that have a demonstrated role in modulating organismal lifespan specifically influence cell and tissue function, how they interact and how their tissue-specific functions might be linked [16]-[19].

In this review, we will focus on currently employed methods to establish the effects of aging in tissue function and to ask whether genetic perturbations in specific cells or tissues influence lifespan. These methods have already provided significant insight into environmental and genetic factors that influence lifespan in *Drosophila*, as summarized below. In the third section, we will then discuss methodological aspects and discuss concerns and pitfalls of these techniques.

2. Lifespan in *Drosophila*: genetic and environmental effects

Fundamentally, the rate of aging is determined by a combination of genetic and environmental conditions. Studies in *Drosophila* have specifically addressed the interaction between these two parameters by characterizing genetic requirements for the longevity effects of environmental perturbations, including diet, stress and infection [20]-[24]. A comprehensive picture of environmental and genetic conditions that promote longevity is thus emerging.

2.1. Genetic perturbations and pathways that extend lifespan

Among the best understood interventions to extend lifespan in organisms as diverse as worms, flies and mice, is reduction in the activity of the nutrient-sensing insulin/IGF Signaling (IIS) pathway and the associated Target of Rapamycin (TOR) pathway [4], [25], [26]. While initially identified in worms, both *Drosophila* and *C. elegans* have provided evidence for the anti-aging effect of reduced IIS or TOR signaling, which in flies results in a remarkably broad-spectrum improvement in health during middle and old age [7], [8], [18], [27]-[30]. The IIS pathway intersects with a variety of other interventions that influence longevity, including activation of the stress-responsive Jun-N-terminal Kinase pathway, promotion of protein homeostasis, and activation of the mitochondrial unfolded protein response [7], [8], [19], [30], [31]. The tools available in flies for spatiotemporal regulation of gene expression have allowed dissecting the endocrine tissue interactions that influence insulin signaling activity in a range of conditions, establishing endocrine signaling networks that influence longevity [7], [8], [17], [18], [29], [31]-[33].

Other genetic perturbations with significant lifespan effects include over-expression of the NAD+ dependent histone deacetylase Sir2 [34], loss of the G-protein coupled receptor methuselah[35], loss of the mitochondrial co-transporter I'm Not Dead Yet (INDY) [36], [37], as well as perturbations of the mitochondrial electron transport chain [38]. How these

conditions tie into the lifespan regulation by the IIS pathway remains unclear, and the longevity effects of some of these perturbations is not without controversy [39], [40].

2.2. Environmental interventions: diet, stress and inflammation

Environmental parameters that significantly influence lifespan include diet, oxidative stress, and conditions causing inflammation [41]-[43] [21], [44], [45].

Dietary restriction (DR) is perhaps the most famous, evolutionarily conserved, intervention to extend lifespan [24], [42], [46]. In DR, nutrient intake is restricted to roughly 65% of an animal's intake when allowed to feed *ad libitum*. In many organisms, DR maintains most physiological processes in an apparently youthful state, delaying the occurrence and/or progression of age-associated disease [42], [47]. Potentially related to DR are interventions that extend lifespan by perturbing the mitochondrial electron transport chain [38], [48].

Oxidative stress has long been proposed as a major driver of aging, and a number of observations in flies support an important role for oxidative damage in age-related degeneration [23], [49]-[53]. These studies have primarily focused on genetic interventions that promote stress tolerance, scavenge reactive oxygen species, or promote repair.

Drosophila has further been used as a model for studying the role of immune senescence and inflammatory responses in aging [44], [45], [54]-[56]. These processes, which originate from innate immune responses, have been implicated as contributors to chronic age-related diseases and senescence in humans. Mechanisms of innate immunity are highly conserved across species, and it has been established that flies exhibit remarkable up-regulation of innate immune response genes with advancing age [44], [45], [57]. Recent studies suggest that limiting over-activation of the Relish innate immune signaling pathway in the aging intestine is sufficient to extend lifespan [45].

2.3. Tissue-specific interventions that influence lifespan of flies

The availability and ease of use of heterologous expression systems have ensured that *Drosophila* serves as a productive and efficient model system to explore the role of tissue-specific genetic perturbations on lifespan. Detailed characterizations of tissue-tissue interactions in the regulation of lifespan by the IIS pathway have thus been performed [8], [29], [33], [45], [58], [59]. Furthermore, specific genetic perturbations in the muscle [19], [31], [60], intestine [45], [61], [62], fat body [29], [33], neurosecretory cells [8], [32], [58], [63], and germline [64] have been identified that significantly influence longevity. It can be anticipated that further exploration of tissue/tissue interactions in the control of systemic homeostasis will provide important new insight into the aging process.

3. Practical aspects for studying aging in flies

3.1. Demography and the assessment of lifespan

A standard metric to assess the impact of specific interventions or genetic perturbations on aging is the assessment of mean and maximum lifespans within a population. While seemingly a simple assay involving the counting of dead flies, the quantification of fly longevity can be fraught with pitfalls that have contributed to a number of controversies in

the aging field [65]-[67]. Among the most critical aspects to establish is to ensure that matched genetic backgrounds are used when specific genetic perturbations are tested for an effect on lifespan. Furthermore, a variety of environmental factors have to be considered when comparing lifespan between populations. These include diet[41], [68], larval densities, Wolbachia infection status [39], [69], mating status and gender cohabitation [70], [71], temperature, humidity and circadian rhythm. We discuss the practical implications of these genetic and environmental considerations below.

3.1.1. Analyzing demographies—Practical considerations of fly husbandry and culture make it difficult to completely standardize the assessment of longevity within individual *Drosophila* populations. Nevertheless, a number of best practices can be implemented to ensure accuracy and reproducibility of demographic studies. These include careful control of genetic backgrounds (discussed in more detail in the following section), proper maintenance of source cultures (healthy cultures with defined larval densities to avoid overcrowding are essential), and proper establishment of test populations. These populations need to be synchronized (i.e. all flies assessed should have emerged within 1-2 days), controlled for mating status (a common practice is to allow animals to mate freely before separating males and females into separate aging cages), and maintained at low enough density to allow free movement (since large populations are required for statistical power, this means that aging cages of large volumes have to be used). Food has to be changed every 2-3 days to maintain healthy populations, and mortality has to be assessed daily. For a detailed description of such a demographic analysis see [66]. In the following, we list an exemplary setup of a demographic study:

- Crosses and progeny are kept at all times at 25°C.
- Cohorts of about 100 males or females are separated after mating for 2 days after hatching and transferred into fresh vials at defined densities (100 flies per 50 ml food).
- Flies are finally separated according to their sex and genotype into cages (50–100 flies/cage). Plastic cages (175 ml volume, 5 cm diameter from Greiner bio-one) are used for lifespan experiments.
- Food is changed every 2 days, provided in vials inserted into a foam plug (4.9 cm in diameter, 3 cm thick from Greiner bio-one), and dead flies are visually identified (flies not moving, not responding to mechanical stimulation and laying on their side or back) and recorded.
- Cages are replaced after 20 days (flies are transferred into new cages without anesthesia).

Demographic data are analyzed using standard statistical software (such as SAS JMP7), and statistical significance of differences between populations is determined using the log-rank or Wilcoxon-rank tests. An interesting discussion on the interpretation of demography and its use in genetic interaction studies is provided by [65].

While curves based on cumulative survivorship calculations are the most common representation of demographic data, an alternative method for quantitative assessment of

aging phenotypes is to plot the log of the population's mortality against time. This can prevent erroneous interpretation of survivorship differences between cohorts that arise from early mortality in one cohort, reducing its mean longevity and cumulative survivorship [72]. An insightful example for the usefulness of this approach can be found in [22].

3.1.2. Gender—There are significant differences in lifespan between males and females for reasons that are not entirely understood, but that may include (i) asymmetric inheritance of mitochondrial genomes and other cytoplasmic genomes, (ii) hormonal and metabolic differences, and (iii) maternal effects [73]). Analysis of genetic or environmental effects on longevity should therefore ideally be performed in both sexes separately. However, many interventions have been characterized primarily in one sex. For example, females are typically used in DR studies, in studies exploring the role of intestinal regeneration in longevity, as well as in studies on the interaction between reproduction and lifespan.

3.1.3. Genetic background—Due to the need of maintaining flies as live stocks, fly populations are generally inbred. This leads to significant problems when assessing physiological and lifespan effects of specific genetic perturbations. Of particular significance is the heterosis effect, which generally results in longer lifespan in outcrossed animals when compared to the inbred parent populations. A number of strategies have been used to minimize such genetic background effects:

- **i.** mutations and transgenes are backcrossed at a minimum of 10 generations to one or (preferably) two well-characterized, commonly available wild-type inbred strains
- **ii.** large populations of outcrossed genetic backgrounds are maintained into which transgenic animals of interest are crossed in.
- **iii.** the effects of genetic background can be negated by employing inducible Gal4 drivers to knock down and overexpress target proteins in a temporal and tissue-specific manner. The most commonly used approach is the RU486-inducible Geneswitch system [13]. Sibling flies of identical genetic backgrounds can thus be maintained in the presence or absence of RU486 and demographic data can be compared directly. However, caution should be taken when using the Geneswitch system for lifespan studies due to two major problems [74]: (i) the precise genetic perturbation achieved with any Geneswitch driver will depend on the concentration of RU486 used, as well as on the age, genetic background and gender of the analyzed animals, (ii) many Geneswitch strains drive substantial UAS-linked expression of transgenes even in the absence of RU486 during development and/or during adulthood. This ‘leakiness’ of the driver can elicit biological effects that confound the analysis. Comparisons between wild-type controls and experimental genotypes, both carrying the Geneswitch transgene, are thus critical controls for experiments in which sibling experimental cohorts maintained with or without RU486 are compared.

3.1.4. Wolbachia—Infection with the parasitic intracellular bacterium Wolbachia can have significant (positive or negative, depending on genetic backgrounds) effects on lifespan [39], [69], [75]. It is therefore critical to establish the Wolbachia infection status of tested fly

lines (which can be achieved through simple PCR assays or by DAPI staining of selected tissues [76]). To eliminate Wolbachia from fly stocks, Tetracycline treatment has been shown to be effective [39], [69], [75].

3.1.5. Diet—The exact composition of the diet has a major influence on fly longevity. This is problematic, since variations in diet between labs are quite common, leading to difficulties in reproducibility and robustness of lifespan effects. Nevertheless, a better understanding of the specific parameters influencing fly lifespan has allowed standardizing diets for aging research [41], [46], [77], [78]. An important insight gained from this work is the critical role of the yeast concentration (yeast is the main protein source in commonly used fly foods) in determining fly longevity. Importantly, recent efforts have developed chemically defined diets, which should allow standardizing diets between labs even more [68], [79]. It is important to note that in addition to the nutritional value of the food, changes in food hydration have also been shown to impact longevity [80], and the production, handling and storage of the used food is thus critical.

3.1.6. Other environmental factors—Other environmental parameters influencing fly lifespan include temperature, humidity, and circadian light exposure. These parameters can (and should) be well controlled using diurnal and humidity controlled incubators for the course of the flies' lifespan.

As expected for a poikilothermic organism, the ambient temperature strongly influences *Drosophila* aging. In fact, fly lifespan can be extended or shortened significantly by maintaining flies at 18°C or at 29°C, respectively, rather than at 25°C. Similarly, age-related phenotypes (such as intestinal dysplasia [61], [81]) manifest themselves in accelerated fashion at higher temperatures. 25°C is the standard temperature used in fly lifespan studies, and caution should be exerted when interpreting lifespan effects at other temperatures, as these effects may not always be a consequence of perturbations in the 'normal' aging process.

Flies respond strongly to dessication, and care should be taken to maintain aging populations at constant ambient humidity. Commonly, relative humidity between 55 – 65% is ideal.

Since the circadian rhythm can significantly influence longevity, daily light:dark cycles should be well controlled as well [82]. Unless changes are experimentally required, flies are commonly maintained at a constant light : dark cycle of 12:12 hours.

3.2. Monitoring aging: measures of functional senescence

To understand the aging process mechanistically, it is not sufficient to rely on demographic studies alone, but detailed insight into the age-related deterioration of tissue function is required. In flies, a large and growing number of different age-related changes and dysfunctions has been identified and characterized in detail:

3.2.1. Systemic molecular biomarkers of aging—Several molecular alterations have been identified in aging flies that may reflect systemic molecular changes causing age-related pathologies. These include Protein Carbonylation [23], [83], Lipid peroxidation [84],

Protein aggregation [19], and accumulation of advanced glycation end products (AGEs) [85]. These markers provide unique opportunities to estimate the effects of specific mutations or environmental perturbations on the aging process systemically and quantitatively.

3.2.2. Gut homeostasis: characterization of epithelial dysplasia and barrier dysfunction—A separate quantitative measure for aging, based on functional characterization of tissue homeostasis, is emerging from studies in the fly gut. In aging flies, the intestinal epithelium develops dysplasia due to over-proliferation of intestinal stem cells (ISCs) and mis-differentiation of ISC daughter cells. Dysplasia is characterized by a large number of mitotic figures in the intestinal epithelium (reflecting ISC proliferation) and by an accumulation of cells expressing stem cell markers (such as esg and Delta). These changes are caused by chronic activation of JNK signaling, and are a consequence of an age-related innate immune imbalance and resulting commensal dysbiosis [45], [51]. Strikingly, ISC proliferation correlates strongly with lifespan of the organism, highlighting the importance of intestinal homeostasis for the health of the animal [61].

Recent studies have developed a new non-invasive assay to assess intestinal health, providing an important new tool for studying aging in flies [86], [87]: In the ‘Smurf assay’, flies are exposed to a medium containing food dyes that only penetrate the gut when barrier function is impaired (standard medium is supplemented with dyes added at a concentration of 2.5% (wt/vol). Blue dye no. 1 and red dye no. 40 from SPS Alfachem have been used). Flies are counted as ‘Smurfs’ when dye coloration is observed outside of the digestive tract. Strikingly, the ‘Smurf’ phenotype is strongly associated with mortality, and increased ‘Smurf’ incidence can be used to predict an increase in mortality within a population [86]. The age-dependent loss of intestinal integrity is associated with altered metabolic and immune signaling systemically, manifested as an increase in expression of antimicrobial peptides (AMPs), impaired IIS activity, and reduced metabolic stores [86].

Immune senescence and deregulation of innate immune responses in the intestinal epithelium have been identified as causes of the intestinal dysplasia and commensal dysbiosis described above [45]. Using a combination of RNAseq analysis, gene expression marker and RT-PCR analysis, as well as quantification of colony-forming units in the intestine, the age-related decline in intestinal commensal and proliferative homeostasis can be monitored [45].

By characterizing aging of the intestinal epithelium, a comprehensive understanding of age-associated changes in innate immune and inflammatory signaling, of stem cell deregulation, and of barrier dysfunction in a rapidly renewing barrier epithelium is thus possible. Since these changes are closely associated with increased mortality in aging flies, such studies will allow deeper understanding of the aging process and of lifespan-determining processes in metazoans.

3.2.3. Aging of the heart—Cardiac aging, or the age-related decline in cardiac performance, has been characterized in detail in the fly [88], [89]. Methods have been developed for live imaging of the adult heart, allowing the collection of detailed

morphometric and kinetic data characterizing heart function. Using electrical pacing, cardiac performance under stress conditions can further be monitored in varying environmental and genetic conditions. The percentage of flies that exhibit heart failure under stress increases progressively with age from 20-35% in 1-2 week old flies to 65-85% in 5-7 week old flies, suggesting that cardiac performance declines with age in flies [88]-[90. Interestingly, cardiac performance can be preserved in aging flies by specifically reducing the activity of the IIS pathway in the heart. This does not, however, increase overall lifespan of flies, highlighting the importance of comprehensively examining age-related dysfunction of specific tissues and assessing the contribution of such dysfunction to fly longevity [30].

3.2.4. Aging of the muscle—Functional, metabolic, and structural deterioration of skeletal muscle is an age-related process in flies, but the underlying mechanisms and signaling interactions between muscle-intrinsic and muscle-extrinsic factors driving this deterioration are only beginning to be understood [19], [31], [39], [60], [69], [75], [91]-[93]

An important quantitative non-invasive assay for skeletal muscle function has been developed by exploting the natural negative geotactic behavior of flies [94]-[96]. When tapped to the bottom of a vial, flies rapidly climb upwards. This behavior declines progressively, and older flies make short abortive climbs and fall back to the bottom of the vial. The decline in this locomotor behavior is likely a combination of age-related changes in innervation and in muscle tone and function. The use of this assay has been described in detail in [96].

3.2.5. Aging of the brain—While much attention has been paid to *Drosophila* models for neurodegenerative disorders [97]-[100], innate neurological changes in aging flies remain understudied. However, flies exhibit pronounced age-related changes in olfactory acuity and in circadian rhythm and sleep patterns [95], [101], [102], suggesting specific neurological or neurosecretory changes in the aging fly brain that also may affect lifespanc[82], [101], [103]. Morphological characterization of changes in the aging brain has focused on the presence and number of dopaminergic neurons, which can be easily identified by anti-TH staining or using TH-Gal4 drivers [104], [105]. While dopaminergic neurons control locomotor behavior, and while locomotion declines with age, significant changes in the number of dopaminergic neurons have not been observed in aging flies [105]. Nevertheless, the dopaminergic system serves as a powerful model to study the function of Parkinson-related genes [97], [104], [106].

4. Conclusion

Drosophila is developing into a rich model system to address questions regarding genetic and environmental contributions to the functional decline of biological processes and tissues in metazoans. The precise spatiotemporal control of gene function is critical for such studies, and flies offer a number of efficient techniques for such perturbations. Critically, recent studies have focused on identifying and characterizing age-related changes in a tissue-specific manner, focusing on functional senescence across biological processes ranging from locomotion to sleep, from metabolism to tissue regeneration. It can be anticipated that this

focus on functional aging will only intensify in the future, allowing new integrative insight into the aging process and into perturbations that extend lifespan.

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