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Drosophila melanogaster as a model system for the evaluation of anti-aging compounds

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Understanding the causes of aging is a complex problem due to the multiple factors that influence aging, which include genetics, environment, metabolism and reproduction, among others. These multiple factors create logistical difficulties in the evaluation of anti-aging agents. There is a need for good model systems to evaluate potential anti-aging compounds. The model systems used should represent the complexities of aging in humans, so that the findings may be extrapolated to human studies, but they should also present an opportunity to minimize the variables so that the experimental results can be accurately interpreted. In addition to positively affecting lifespan, the impact of the compound on the physiologic confounders of aging, including fecundity and the health span—the period of life where an organism is generally healthy and free from serious or chronic illness—of the model organism needs to be evaluated. Fecundity is considered a major confounder of aging in fruit flies. It is well established that female flies that are exposed to toxic substances typically reduce their dietary intake and their reproductive output and display an artifactual lifespan extension. As a result, drugs that achieve longevity benefits by reducing fecundity as a result of diminished food intake are probably not useful candidates for eventual treatment of aging in humans and should be eliminated during the screening process.

Drosophila melanogaster provides a suitable model system for the screening of anti-aging compounds as *D. melanogaster* and humans have many conserved physiological and biological pathways. In this paper, I propose an algorithm to screen anti-aging compounds using *Drosophila melanogaster* as a model system.

Rationale

A reprieve from aging and the quest for the fountain of youth has long fascinated man. It has influenced history, played a significant role in literature, and driven major scientific advances. Indeed, it could be argued that the vast majority of medical research, through the curing of disease, is focused on increasing lifespan.

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Accordingly, hundreds of genes have been identified in multiple organisms that influence lifespan. Also, notably, hundreds of compounds have been purported (most of them falsely) to effectively stem the tide of aging. Despite the intense interest and research in this field, no single agent has been definitively shown to increase maximum human lifespan. The probable reason for this seems simple: aging is complex. Genetic heredity, the environment, and changes in metabolism and reproduction will all contribute to determining an individual's lifespan. Further, aging in humans consists of three phases: development, aging and late life. The development phase lasts until puberty, following which aging comprises most of a person's life. Late life is defined by a plateau in mortality and fecundity.[1-4] The late life plateau occurs in humans at very extreme ages, with the majority of people not reaching this phase.[2] These multiple factors create logistical difficulties in the evaluation of anti-aging agents. For aging research to be relevant to humans, a good model system will include genes that exhibit high conservation with those of humans, the ability to be genetically manipulated, multiple life stages and a short enough lifespan and generational time to be effectively monitored over a reasonable period of time.

Drosophila melanogaster fits these qualifications, providing a suitable model system for the screening of anti-aging compounds. Humans and *D. melanogaster* have many conserved physiological and biological pathways. In addition, since fruit flies have a long history of use as a model system in many research fields, there are a wide variety of genetic strains available with different lifespans. This is important not just for validation of compounds in multiple genetic backgrounds, but genetic manipulation of *D. melanogaster* also allows for the development of accelerated assays based on the expression of age-dependent molecular biomarkers coupled to lethal toxins.[5] In addition, the availability of many transgenic models (e.g., flies lacking or with elevated levels of a particular gene function), enables mechanistic studies to evaluate whether the effect of a particular compound depends on a specific pathway. Finally, in an anti-aging research program, using a model organism with a shorter lifespan, such as fruit flies, is a critical factor since lifespan analysis represents the rate-limiting research step.

The outcome of studies of survival in model systems such as Drosophila can often be summarized by the mean and maximum life span. The maximum lifespan is sensitive to sample size and therefore it is difficult to compare this value in different experiments. Mean lifespan is not sample size dependent but does not

convey information about age-specific patterns of mortality. A simple means of summarizing this type of age-specificity is with a simple model called the Gompertz equation.[6] This model is a useful descriptor of mortality in wide array of organisms including humans.[7] Although it is now clear that many organisms show departures from the Gompertz mortality predictions late in life this model can still be successfully used to describe the mortality of the first 90–99% of deaths in model populations.[6] As noted above in organisms like Drosophila, lifespan can be increased by factors that reduce reproduction. These effects are often reflected in the Gompertz model by a reduction in the age-independent parameter of the Gompertz equation.[8] Thus, compounds that increase lifespan through the decrease in the age-independent parameter of the Gompertz certainly suggest that additional research needs to be done on the effects of these compounds on reproduction.

For anti-aging compounds to be useful for humans, though, other parameters also need to be monitored, including the impact of the compound on the physiologic confounders of aging and the health span of the model organism. It is not sufficient that anti-aging compounds increase survival probability alone, they must also do so in a manner that does not compromise quality of life. In addition, to be a true anti-aging compound, it must work independently of effects on known aging confounds such as fecundity. General considerations for the screening of potential anti-aging compounds using the *D. melanogaster* system have been outlined previously.[9] While these considerations are still important, based on our experience in testing the assays that we proposed, and to make the screening process more time efficient, we have since refined this process. In this paper, using *Drosophila melanogaster* as a model system, I propose an algorithm to evaluate anti-aging compounds.

The Identification of Anti-Aging Genes and Compounds

Fruit flies have a long history of use in the identification of genes and pathways that are involved in human diseases. *Drosophila melanogaster* and humans share a large number of key metabolic pathways, including several potential aging modulation pathways such as oxidative stress, DNA repair and insulin-like signaling. There are many known genes involved in the aging process, which have been identified in multiple model systems. For example, in *Caenorhabditis elegans*, more than 100 genes impacting organism aging have been identified.[10] Many mutations in genes encoding members of signaling pathways such as *age-1*,[11] *daf-2*,[12,13] *Indy*,[14] dilp genes,[15] *chico*[16] and others have been found to increase life span in *C. elegans* or *D. melanogaster*. In addition, many genes encoding products directly involved in multiple cellular processes such as glucose metabolism, DNA damage, antioxidant defenses and heat shock response have also been shown to enhance lifespan.[17] Although approximately 40 genes have been identified in the *D. melanogaster* system,[10,15,16,18-20] recent advances suggest that many more genes, possibly as many as 400, are regulated during *D. melanogaster* aging, and could conceivably influence lifespan.[21-23] Paaby and Schmidt have recently published an extensive review of identified genes affecting *D. melanogaster* aging.[24] However, caution is recommended in the interpretation of these findings. On some occasions, increased lifespans due to genetic manipulations are not replicated in independent laboratories, suggesting that the particular modification may be specific to certain diets or environments present, or possibly due to some other cofounding factor, such as mutation in another locus.[25-27]

Histone deacetylases have also been implicated in lifespan in multiple models, including *D. melanogaster*. It has been shown by Lin and colleagues that lifespan extension in *Saccharomyces cerevisae* via caloric restriction requires the Sir2 gene, a nicotinamide adenine dinucleotide (NAD^+) dependent deacetylase of the sirtuin family.[28] Partial reduction in the levels of Rpd3, another histone deacetylase, also resulted in a 30%–50% increase in fly lifespan.[19] Others have previously observed that sirtuin-activating compounds such as resveratrol extend yeast replicative capacity, increase fly lifespan, and can promote human cell survival.[14,18,19,29] Reseveratrol was reported to extend lifespan in flies by activating sirtuins without affecting fecundity.[29] Although based on previous reports, resveratrol-induced increase in lifespan was shown to be Sir2-dependent and to function through pathways related to caloric intake,[29] recent reports using biochemical assays with native substrates, suggested that resveratrol does not directly activate SIRT1.[30] Although, the question of whether resveratrol activate SIRT1 remains controversial, these experiments provide an example of how pharmacologic-genetic interplay can be used to investigate both agent activity and mechanism; and to identify other genes and pathways that influence the aging process. However, it is vital to ensure that any observed anti-aging effects are not due to genetic modifications but result from administration of the pharmacologic agent since genetic modifications are not considered a desired intervention in clinical studies.

In addition to the vast knowledge on *D. melanogaster* aging derived from genetic studies, environmental factors, including various types of diet, have been shown to be key players in the fruit fly aging process, with studies dating back to the early 20th century.[31,32]

While the genetic approach follows the effect of compounds on pathways either known or suspected to be involved in the aging process, there are a number of pharmacologic agents that are believed to influence aging, but the mechanisms have yet to be determined. These putative anti-aging compounds offer the potential to validate the search for anti-aging compounds as well as to further insight into anti-aging pathways. In a screen of 75 pharmaceutical and botanical agents, using *D. melanogaster* as our model system, we identified a handful of potential anti-aging agents. These include extracts of the plants *Rhodiola rosea* and *Rosa damascena* and the anti-diabetic drug pioglitazone. All three decreased the mortality rate in male and female flies without affecting the physiological confounds of aging.[33] Upon further study, *Rhodiola rosea* was found to extend both mean (24% in both sexes) and maximum (16% in males and 31% in females) life span in *D. melanogaster* when compared to controls.[34] Others have studied the antioxidant effects of alpha- and gamma-tocopherol,[35] tocopherol-*p*-chloro-phenoxy acetate,[36] NDGA[36] and Mg-TCA[36] in the *D. melanogaster* system. Notably, while

many of the agents investigated were studied because of their suggested anti-aging effects, several of the investigated compounds have been shown to extend life span at the expense of compromising health. For instance, we found that lamotrigine decreased the mortality rate and increased lifespan in both male and female flies, but it decreased the metabolic rate and compromised the flies' physical performance via locomotor activity.[37] We also observed that metformin decreased mortality rate but it also decreased fecundity significantly (data on file).

The Screening Algorithm

It is possible to find drugs that seem to slow aging in fruit flies, but unfortunately there is a long history of apparent anti-aging responses to toxic or noxious substances by *D. melanogaster* that turned out to be artifacts. Pharmacologic agents in the food may be perceived by flies as toxic or distasteful, which may result in decreased food intake; females that are exposed to toxic substances typically reduce their dietary intake and their reproductive output—effects that are strongly associated with an increase in longevity.[31] Additionally, some pharmacological interventions may result in adverse metabolic effects or affect other physiological systems that can impact lifespan, such as fecundity. Accordingly, these factors should also be evaluated when screening anti-aging agents.[9]

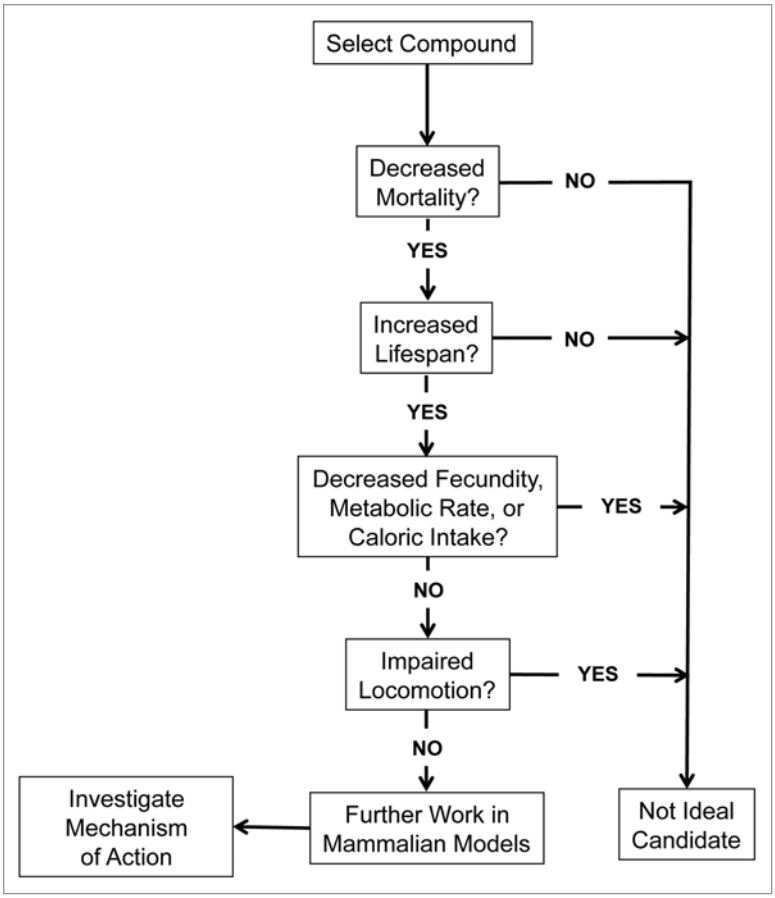

Figure 1. Proposed algorithm to screen anti-aging agents.

An ideal model anti-aging pharmacologic research program should attempt to limit artifacts and not have deleterious effects on health span. Once a compound has displayed such properties, it can be considered for testing in a mammalian system, such as rodents and eventually humans. In an attempt to limit these artifacts and maximize the potential to identify a useful agent for humans, we have developed an algorithm for the assessment of putative anti-aging compounds (**Fig. 1**).

Although lifespan is considered the "gold standard" screening assay, our work suggests that an alternative initial screening assay would be to evaluate the impact of the compound on the mortality rate of flies during their aging phase.[33,38] As described previously, there are three life phases; developmental, aging and late life. For humans, extension of the developmental phase is undesirable and extensions in late life, while not undesirable, may not be useful since many humans do not reach this life phase. Further, we have reported that if the mortality rate in flies is decreased during the aging phase, there is a high chance that the total lifespan will be increased.[33,38] During the screening process, we do not propose to replace lifespan assays with mortality assays but based on our experience, when an agent reduces mortality rate during the aging phase, there is a good chance that this agent will positively impact the lifespan as well.

To demonstrate that a compound has true anti-aging activity, it is necessary to determine that any lifespan increases are due to direct action of the agent on the aging process, and not due to effects on physiological confounds such as fecundity, metabolic rate and dietary restriction.[33,39,40] For example, a reduction in fecundity has been shown to affect fly lifespan,[31,41,42] but fecundity can also be reduced due to dietary restriction. Assaying for female fecundity in the *D. melanogaster* model system is relatively straightforward, and can be accomplished by measuring the number of eggs laid.

As caloric restriction has been shown to increase lifespan in flies, as in other organisms, it is important to determine whether any lifespan increases following agent administration occur through this mechanism. Conveniently, measurement of fly fecundity can also serve as an indicator of caloric intake, since egg production in *D. melanogaster* is proportional to food levels.[43] Chippendale and colleagues have shown that dietary restriction-induced longevity increases in *D. melanogaster* is associated with a several-fold reduction in fecundity.[44] Thus, the fecundity measurement is not only necessary to screen for useful agents; it can serve as a caloric intake surrogate and potentially help delineate a compound's mechanism of action. Metabolic rate is another important potential physiologic confounder that is necessary to follow. While an inverse correlation between metabolic rate and longevity in *D. melanogaster* has been reported in a number of studies, there is still some uncertainty as to whether this variable influences fly longevity.[45] Van Voorhies and colleagues recently evaluated CO_2 production in a large set of recombinant *D. melanogaster* males. The study was conducted over a range of ages and

the authors observed no correlation between longevity and CO_2 production.[46] Regardless, any potential agent that substantially lowers metabolic rate will be unattractive as a potential agent for human use, since humans, as homeotherms, are not capable of adapting to hypometabolic states.

Lifespan extension in the absence of demonstrable physiologic confounders should be considered the baseline for an acceptable anti-aging compound candidate. However, a compound that increases lifespan but has adverse effects on health span is not a desirable candidate. Although not all compounds that have toxic effects on health span in flies will necessarily exert similar effects in humans, health span assays should be monitored. In *D. melanogaster*, this can be accomplished through evaluating the impact of the compound on fly locomotion. We have monitored vertical climbing ability in fruit flies treated with lamotrigine, a drug that extended lifespan in both male and female flies, observing decreases in locomotion at various doses.[37] The results demonstrate that while lamotrigine extends lifespan, it also negatively impacts health span by compromising fly locomotor ability.

Once an agent has shown anti-aging activity without impacting physiologic confounders or adversely affecting health span, it may warrant further evaluation in either other *D. melanogaster* genetic backgrounds or in other model systems. Additionally, determining the mechanism of action may be useful for identifying further candidate agents or evaluating combination therapies. The strength of the *D. melanogaster* system is the availability of a broad range of transgenic and mutant flies that can be used to evaluate the mechanism of action of selected compounds. However, there are still several challenges for the field, which need to be overcome.

Current Challenges

While *Drosophila melanogaster* represents an ideal organism for genetic research and has been studied for many decades, its use as a pharmacologic evaluation tool is still being developed. Since the majority of pharmacological experiments in fruit flies use adult feeding as the preferred mode of drug delivery, the main challenge is the issue of drug bioavailability in the fly. While larval rates of feeding are well understood, adult feeding is not well characterized. For the investigation of anti-aging candidates, it is obviously important to supply candidate compounds at various stages of *D. melanogaster* life, so it is necessary to standardize and quantify adult fly intake in these experiments. Currently, the drug is either directly mixed with the fly food or with yeast paste that is added on the top of the food. A variety of methods might be employed to test for drug intake, including adding dye to the food and drug mixture, administering radiolabeled drugs and measuring drug concentration in fly extracts. We have previously measured drug bioavailability when studying pioglitazone, an anti-diabetic compound that was identified in a screen for compounds that decrease fly mortality.[38] A homogenate of flies that were fed pioglitazone for 3 days was assayed by HPLC/mass spectrometry. Pioglitazone at a concentration of 3 ng/mL was easily detectable in the fly homogenate.[38] While this method was successful, it remains to be determined if this method merits widespread use to evaluate the bioavailability of other agents. It will also be necessary to perform dose-response curves, both as a method of monitoring drug intake, but also as a means of validating compound activity.

Other challenging issues are the drug's molecular site of action and its metabolism. For instance, intake of the drug in food may not lead to delivery of the drug to target tissues beyond the digestive tract, as Drosophila has cytochrome P450s enzymes in the gut that may metabolize pharmaceuticals.[47] In the absence of validated protocols to measure adult fly intake, distribution and metabolism of pharmaceutical candidates, it is important to measure effects in other model systems for which these controls do exist.

Conclusion

Anti-aging drug development needs multiple model systems. No model system is going to be without limitations; this is especially true with aging studies since ideally the results will be extrapolated to human studies. For example, a model system that has a rapid enough generation time to produce sufficient amounts of data may be limited by its ability to show long-term administration effects in longer lived and more complex species, such as humans. Further, simpler organisms may not possess aging phases or aging pathways comparable to humans. In spite of these limitations, *Drosophila melanogaster* represents a good cost and time efficient intersection of organism complexity with a rapid assay potential, making it a valuable system for the evaluation of putative anti-aging compounds. Further, the wealth of strains, as well as 100 years of experimental work in fly genetics and physiological phenotypes, provides valuable tools for the assessment of candidate agents. Using the algorithm proposed here, potential agents can be efficiently screened in *Drosophila melanogaster*, identifying candidates for further study in additional model systems.

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