Age-Associated Cardiac Dysfunction in *Drosophila melanogaster*

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Abstract—The fruit fly, *Drosophila melanogaster*, has served as a valuable model/organism for the study of aging and was the first organism possessing a circulatory system to have its genome completely sequenced. However, little is known about the function of the heartlike organ of flies during the aging process. We have developed methods for studying cardiac function in vivo in adult flies. Using 2 different cardiovascular stress methods (elevated ambient temperature and external electrical pacing), we found that maximal heart rate is significantly and reproducibly reduced with aging in *Drosophila*, analogous to observations in elderly humans. We also describe for the first time several other aspects of the cardiac physiology of young adult and aging *Drosophila*, including an age-associated increase in rhythm disturbances. These observations suggest that the study of declining cardiac function in aging flies may serve as a genetically tractable model for genome-wide mutational screening for genes that participate in or protect against cardiac aging and disease. **(*Circ Res*. 2001;88:1053-1058.)**

Key Words: aging ■ genetic screening ■ heart ■ *Drosophila melanogaster*

The genome of *Drosophila melanogaster* was the first to be fully sequenced for an animal possessing a circulatory system.[1] The heart of the fly consists of a tubular structure that contracts spontaneously throughout the lifespan of the insect and has the main function of circulating the endolymph, which transports energy substrates from the abdomen to the thorax and head.[2] The normal lifespan of the fruit fly depends on the temperature at which the flies are kept, being shorter at higher temperatures. The mean lifespan of *D melanogaster* is 45 to 60 days at 25°C.[3]

Several groups have exploited *Drosophila* genetics for identifying genes regulating cardiac development in the fly, and this approach has proved to be useful for guiding research on cardiac development in vertebrates. One of the more notable examples is the identification of the *Drosophila* gene *tinman*,[4] which prompted the cloning of homologues regulating cardiac development in mice (Nkx2-5/Csx).[5,6] The finding of homologous genes that similarly influence development of the heartlike organ of *Drosophila* and the mouse heart suggests that at least some aspects of fly cardiac biology are common to mammals. The relevance of some fly genes to human cardiac pathology is also supported by the finding that mutations in the *HERG* potassium channel gene cause long QT syndrome, a potentially fatal cardiac arrhythmia.[7] HERG stands for "human ether-a-go-go–related gene," and it was first identified by virtue of its homology to the *Drosophila* potassium channel gene "ether-a-go-go."[8]

Several human disease models have been developed in *Drosophila*, particularly models for neurological diseases.[9–11] *Drosophila* is also commonly used as a model organism for studying the genetics of aging, partly because it represents a genetically tractable organism with a short lifespan.[12] For example, genetic screening has allowed the identification of a single gene that controls the lifespan in flies, increasing it by ≈35%.[13] However, essentially nothing is known about cardiac changes that might occur with aging in the fly, and attempts have not yet been made to exploit *Drosophila* genetics for investigations of adult cardiac dysfunction.

The relation between aging and heart disease is clear.[14] The prevalence of heart failure is almost 70 times higher in persons aged ≥65 years than in persons aged 20 to 34 years.[14] Furthermore, cardiac functional reserve declines with age in humans.[15,16] Nearly 80% of hospital admissions in the United States for heart failure involve patients aged >65 years.[17] Cardiac aging and heart disease are 2 distinct but interacting processes.

Therefore, we sought to explore cardiac function in flies during aging, asking whether an age-associated decline in some aspect of cardiac performance occurs and attempting to develop a methodology for studying the heartlike organ in intact adult flies. In humans, impairments in cardiac function are commonly revealed only under stress, inasmuch as resting function is affected only in very advanced forms of cardiac disease.[15,16] For this reason, we explored ways of challenging the fly heart. Two methods were developed that tested the ability of the *Drosophila* heart to sustain an elevated heart rate: (1) elevated ambient temperature, which triggers an endogenous response that results in increased heart rate, and

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(2) external electrical pacing using microelectrodes to drive the heart to contract at higher frequencies. Using these methods, we have documented striking reductions in cardiac function with aging in *Drosophila*, particularly a decline in the maximal heart rate achievable. These observations in flies are remarkably similar to some aspects of the age-related decline in cardiac performance observed in humans, which includes an age-associated decrease in maximum heart rate.[15] The findings may lay a foundation for the eventual application of genome-wide screens for genes that accelerate or retard age-associated heart dysfunction with the use of *Drosophila*. The identification of genes affecting cardiac aging (and their interaction with genes affecting heart disease) is our main aim.

Materials and Methods

Animals

Oregon-R flies were obtained as a kind gift from Dr S. Wasserman (University of California, San Diego, La Jolla). Transgenic flies expressing green fluorescence protein (GFP) under the control of an actin promoter (stock 4533) were produced by Dr J.M. Reichart and Dr D. Ferrandon (Institut de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Strasbourg, France) and were obtained from the Bloomington stock center (http://flystocks.bio.indiana.edu). Flies were maintained at 24°C in continuous light and 50% relative humidity in 120-mL bottles in uncrowded conditions (≈40 flies per bottle). The flies were fed a standard yeast-cornmeal-molasses-agar diet and transferred to new bottles every 4 days. All experiments were carried out with male flies, unless otherwise specified, to avoid the effects of pregnancy on cardiovascular function.

Anesthesia

Triethylamine was used as an anesthetic agent as a 50% solution (Flynap, Carolina Biological Supply Co). This was administered by use of an absorbent wand that was dipped in the anesthetic and then inserted into the vial containing flies. The flies were removed from the vial as soon as they were immobile. Ether and CO_2 were used for preliminary comparison of anesthesia methods. Ether was administered as described for triethylamine, whereas CO_2 was delivered as a continuous gas flow.

Measurements of Heart Rates

Anesthetized flies were mounted on glass slides and observed with a Nikon Diaphot-TMD inverted microscope, with Nomarski (DIC) optics (without analyzer) and a ×10 (numerical aperture 0.25) objective. Images were obtained by closing the diaphragm so that the light beam was concentrated on the first ventricle of the heart. Flies were positioned on their backs, exactly perpendicular to the light path, and fixed in this position by mounting the wings on the glass slide with double-stick tape. Images of the first cardiac ventricle were recorded by using a Sony DXC-101 videocamera on VHS tape, and the heart rate was measured from slow-motion replays. End-diastolic and end-systolic dimensions were measured on still images at the midpoint between the 2 major transversal tracheal tubes passing over the first cardiac ventricle.

Temperature Stress Test

Two different protocols were used for stimulating elevations in heart rate as a result of increased ambient temperature. In protocol 1, the flies were anesthetized, and the temperature was increased progressively from 22°C to 28°C over the course of 1 hour. Measurements were taken at 22°C and at 28°C. A control group was kept at room temperature for the same amount of time under the same conditions of immobilization and did not show any change in heart rate. In protocol 2, the flies were anesthetized and then inserted into an incubator containing the Diaphot microscope at 2 minutes before recording. This was done in separate groups at temperatures of 28°C, 32°C, 35°C, and 38°C. No significant differences in heart rate were noted at 28°C with use of the 2 protocols, regardless of fly age.

External Electrical Pacing

Platinum electrodes and a model 611 square-wave stimulator (Phipps & Bird) were used for external cardiac pacing. The electrodes were positioned on the ventral surface of the abdomen by use of a micromanipulator, and heart images were recorded as described previously. Electrode gel (Signa gel, Parker) was applied on the electrodes. The pacing protocol consisted of pulses of 20-second duration, with each pulse followed by a recovery period of 1 minute. The pacing rate was increased in 1-Hz steps for each fly from 5 Hz (300 bpm) to 8 Hz (480 bpm) for the 22°C experiment. At 28°C, given the higher baseline heart rate values, the pacing was increased from 6 Hz (360 bpm) to 9 Hz (540 bpm). The duration of the pacing stimuli was 30 ms, and the voltage was 40 V. Lower voltages and durations failed to capture consistently during external pacing.

Experiments With GFP Transgenic Flies

Transgenic flies expressing GFP (S65T) under the control of the distal actin 5c promoter were generated by Drs J.M. Reichart and D. Ferrandon (http://www-ibmc.u-strasbg.fr/upr9022/GreenBalancers.html). We used 2 different microscopes, which provided images of similar quality. One was the Heidelberg Retina Angiograph (Heidelberg Engineering), a confocal laser-scanning system developed for digital fluorescein angiography in ophthalmic patients.[18] The other was a Bio-Rad MRC-1024 confocal microscope. Flies were anesthetized as described previously, and the wings were attached to glass slides. Images were recorded in digital form and analyzed with image analysis software (NIH Image). Absolute quantification of ventricular dimensions was obtained for these flies with the use of the Nikon Diaphot-TMD microscope to scale the relative measurements obtained.

Automated Heart Rate Detection

We developed a semiautomated digital image-processing method to measure heart rate and its variation directly from video signals recorded in a single fly. Whereas careful manual counting might be more accurate, it is not practical on a large scale; hence, more automated techniques were required. Automated detection also allows additional parameters such as heart rate variability to be detected in a single fly. Video image sequences were stored in the memory of a Pentium II–based microcomputer with use of a high-resolution video frame grabber (Data Translation DT3155), at a sampling frequency of 30 frames per second. For each fly, we acquired 2-second video sequences (60 frames each) 10 times consecutively, and we developed custom software to construct a time-space image signal representing the time course of image intensity along a line segment of pixels that crosses the ventricular lumen transverse to the heart axis. After applying a low-pass filter to reduce noise in the time-space signal, the heart rate was estimated by automated counting of the peaks in the signal. From the unfiltered time-space signal, we obtained a second measure of heart rate by computing the autocorrelation and then the spectral density (by fast Fourier transformation),[19] confirming the previous calculation. An average and a standard deviation of the 10 repetitions in the same fly were obtained, and the coefficient of variation was calculated.[20]

Statistical Analysis

All results are expressed as mean±SEM. ANOVA was used to analyze age-associated changes in heart rate with the Bonferroni correction for multiple comparisons. The incidence of fibrillation-like rhythm was analyzed by use of the χ^2 statistic. The other comparisons were made by use of the *t* test. Values of $P<0.05$ were considered statistically significant.

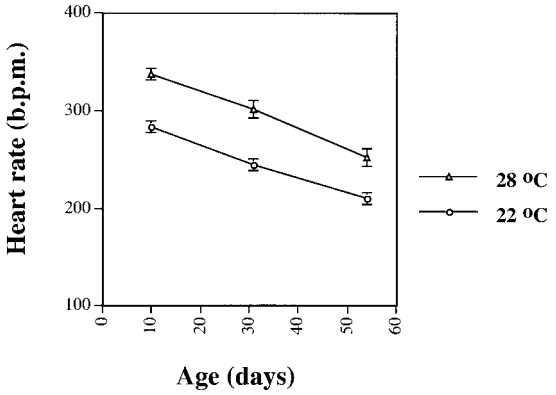

Figure 1. Heart rate declines with age. Heart rate (in bpm) declines with age at both 22°C and at 28°C. Flies were studied at 10, 31, and 54 days of age with protocol 1. Heart rate was measured by use of the Diaphot microscope. Data are mean±SEM.

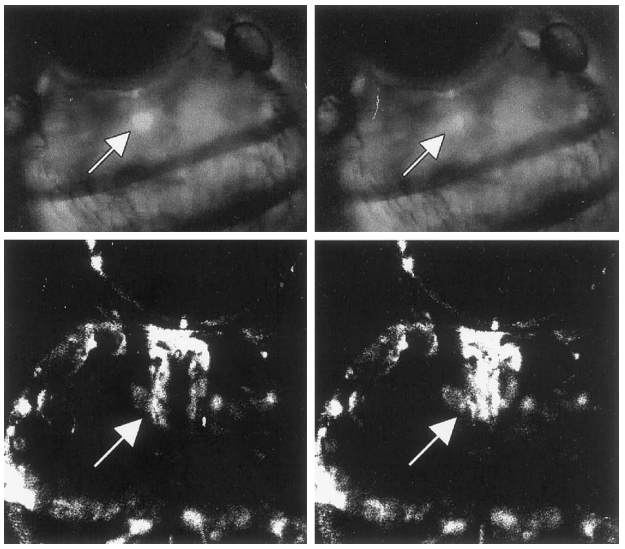

Figure 2. Drosophila heart images. Top, Images were obtained with the Nikon Diaphot microscope. The first ventricle of the heart is indicated by arrows (left panel, diastole; right panel, systole). Bottom, Images were obtained by use of the Bio-Rad MRC-1024 confocal microscope in transgenic flies expressing GFP (S65T) under control of the distal actin 5c promoter. A portion of the fly body similar to that seen in the top panels is depicted. The left panel shows the *Drosophila* heart in diastole, and the right panel shows the heart in systole (arrows point to the first ventricle).

Results

Effects of Anesthesia on Heart Rate

CO_2 is the most commonly used anesthetic for *Drosophila*. This form of anesthesia was shown to cause cardiac arrest within a few seconds in *Drosophila* (n=10), with resumption of the heart beat 20 to 30 seconds after cessation of gas exposure. Ethyl ether was also shown to depress the heart rate in flies. The heart rate was 120±19 bpm immediately after ether anesthesia, but it increased to 248±11 bpm in the same flies after 30 to 40 minutes, when leg movements began to return (n=5, P=0.001). In contrast, triethylamine did not cause significant changes in heart rate under the same conditions, either in young (10 day-old, 283±6 versus 282±4 bpm; n=6) or in older (48 day-old, 213±6 versus 211±11 bpm; n=7) flies. Therefore, triethylamine was used for all subsequent experiments.

Resting Heart Rate Declines With Age in *Drosophila*

The average heart rate measured at room temperature (22°C) decreased progressively with age (Figure 1). In male flies at 10 days of age, the mean heart rate was 286±3 bpm (n=59), compared with 249±5 bpm (n=29) at 31 days of age and 220±3 bpm (n=64) at 54 days of age (P<0.01 for comparisons between all age groups). A similar decrease in heart rate was also observed in female flies: 271±6 bpm in 10-day-old flies (n=14) versus 189±5 bpm in 54-day-old flies (n=26) (P<0.01). The age-related decline in heart rate was also confirmed in the GFP transgenic strain: 275±8 bpm in 15-day-old flies versus 216±6 bpm in 56-day-old flies (P<0.01), measured at 22°C. An example of the images used for measuring heart rate is shown in Figure 2 (top panels).

Temperature Stress Tests Reveal Age-Associated Cardiac Impairment in *Drosophila*

With the use of temperature stress test protocol 1, in which heart rates were measured at 28°C (compared with measurements at 22°C), a more pronounced effect of age on average heart rate was observed. For every age group examined, increased ambient temperature resulted in a faster heart rate. The average heart rate measured in male flies was 339±6 bpm (n=26) at 10 days of age compared with 301±9 bpm at 31 days of age (n=19) and 254±10 bpm (n=14) at 54 days of age (Figure 1). The effects of age and temperature on mean heart rate were statistically significant for all pairwise comparisons of the data (P<0.01).

Using temperature stress test protocol 2, we studied flies at 28°C, 32°C, 35°C, and 38°C (Figure 3). At every age tested, heart rates measured at 28°C with the use of protocol 1 and

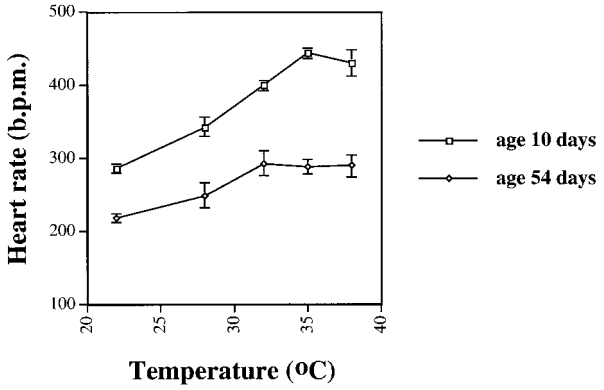

Figure 3. Maximal heart rate during temperature stress in young and old flies. Heart rate (in bpm) was measured in young (10-day-old) and old (54-day-old) flies by the Diaphot microscope, at different temperatures (22°C [n=59 and 64, respectively], 28°C [n=10 and 7, respectively], 32°C [n=10 and 9, respectively], 35°C [n=10 and 9, respectively], and 38°C [n=5 and 5, respectively]), by protocol 2. Age seems to interact with temperature, as indicated by the nonparallel increases of heart rate with temperature. Also, the temperature at which the heart rate plateaus differs with age. Data are mean±SEM.

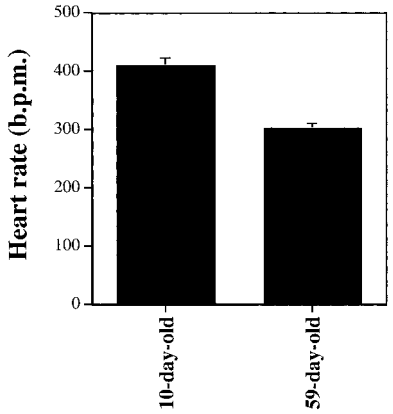

Figure 4. Maximal heart rate during electrical pacing in young and old flies. Heart rate (in bpm) was measured in young (10-day-old) and old (59-day-old) flies during the electrical pacing protocol (at 22°C). Data are mean±SEM (n=15 and 14 for young and old flies, respectively).

protocol 2 were not significantly different, confirming the reproducibility of these results. For every age group examined, temperature-induced increases in heart rate reached a plateau by 35°C. Therefore, using 35°C for comparisons, we observed a decline in the mean heart rate of flies with increasing age, from 440±7 bpm (n=10) at 10 days of age to 372±8 bpm (n=6) at 30 days of age to 288±10 bpm (n=9) at 54 days of age ($P<0.01$ between all groups) (Figure 3).

External Electrical Pacing

External electrical pacing was used to estimate the maximal heart rate achievable in young and old flies at 2 different temperatures. The electrical pulse interval could be decreased to a limit beyond which the heart rate failed to increase further. The maximum frequency of stimulated contractions was recorded as the estimated maximum achievable heart rate. Using this method, we found that the maximum achievable heart rate is substantially lower in older flies: at 22°C, 411±13 bpm (n=15) in 10-day-old flies versus 303±8 bpm (n=14) in 59-day-old flies ($P=0.0001$) (Figure 4).

Electrical pacing often triggered a fibrillation-like rhythm. The heart walls initially displayed very fine and fast tremors rather than full contractions, and the heart subsequently stopped completely. Interestingly, fibrillation occurred (at 22°C) in only 20% of the 10-day-old flies (3 of 15 flies) compared with almost 70% of the 59-day-old flies (12 of 18 flies) ($P=0.02$). Furthermore, all younger flies tested (n=15) returned to normal rhythm within 2 minutes, whereas 40% of the older flies that went into this fibrillation-like rhythm never recovered (5 of 18 flies).

We also performed the pacing experiment at 28°C. The maximum achievable heart rate was again lower in older flies: 498±9.6 bpm in 6-day-old flies (n=12) versus 414±18 bpm in 59-day-old-flies (n=6) ($P=0.001$). Fibrillation occurred in 25% of the 6-day-old flies (3 of 12 flies) compared with 78% of 59-day-old flies (11 of 14 flies) ($P=0.02$). All younger flies tested at 28°C returned to normal rhythm within 2 minutes, but none of the older ones did (P=NS comparing incidence of fibrillation in flies paced at 28°C versus 22°C).

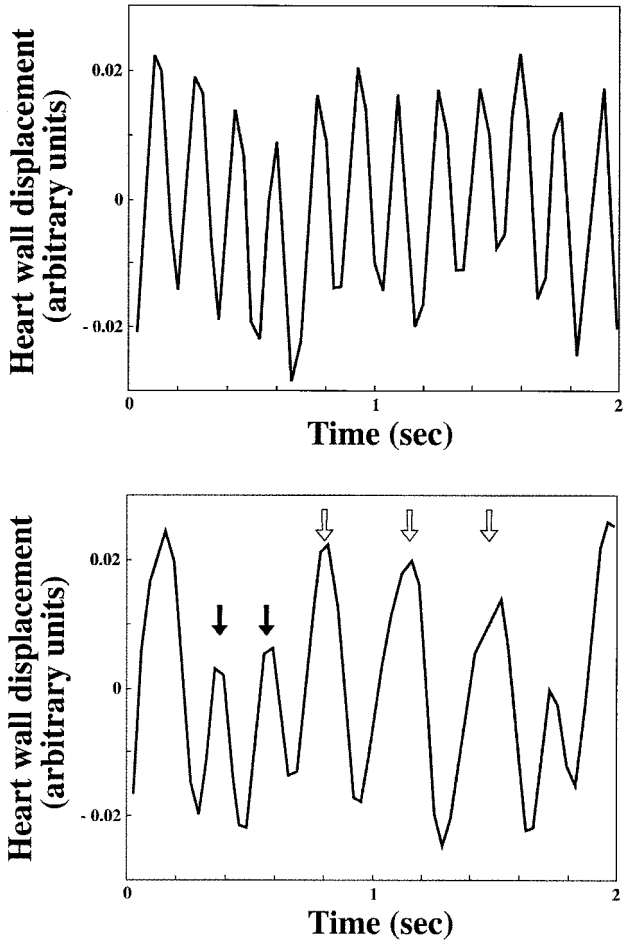

Figure 5. Heart wall displacement plots obtained with automated heart rate detection software. Heart wall displacement plots were obtained in a young fly (6 days old, top) and an aged fly (47 days old, bottom). The software was used to estimate the heart rate and the variability with time. The plot for the aged fly displays a lower number of beats and increased variability in the intervals between beats (compare black and white arrows).

These findings suggest the presence of age-associated electrophysiological defects in the hearts of aged flies.

Heart Rate Variability

Using automated heart rate detection software, we compared heart rate variability (ie, oscillations in heart rate with time in each fly) between young (6-day-old, n=9) and aged (47-day-old, n=8) flies (Figure 5). The coefficient of variation of heart rate was significantly larger in older flies: 14.8±1.4 (aged) versus 9.2±0.4 (young) ($P=0.001$). We also confirmed with the automated image processing method the age-associated decline in heart rate (at 25°C): 310±6 bpm (young) versus 245±9 bpm (aged) ($P<0.01$), thus corroborating our results reported above.

Estimation of End-Systolic and End-Diastolic Heart Dimensions

We used several methods to estimate end-systolic and end-diastolic dimensions but failed to detect significant alterations associated with aging (at least within the resolution of our methods). For example, when we used the same microscope

that was used for heart rate determinations (for which images were recorded and film frames were frozen at the end of systole or diastole), we measured mean end-systolic diameters of 4.4±0.5 versus 4.0±0.7 μm and mean end-diastolic diameters of 34.8±1.15 (n=51) versus 36.8±1.15 (n=58) μm in 10-day-old compared with 54-day-old male flies, respectively. The fractional shortening did not differ between the older (0.9±0.03) and the younger (0.87±0.01) flies.

Measurements of end-systolic and end-diastolic diameter of the fly heart were greatly assisted by imaging GFP-expressing transgenic flies. However, even in this case, by use of 2 different microscopy techniques, no significant age-associated differences in cardiac dimensions were detected: end systole was 8.0±1 versus 7.5±1 μm and end diastole was 33±3 (n=18) versus 36±2 (n=14) μm in 10-day-old compared with 54-day-old flies, respectively. The fractional shortening was also not changed: 0.79±0.03 in 54-day-old flies and 0.75±0.02 in 10-day-old flies. Figure 2 (bottom panels) shows an example of systolic and diastolic images in a fly.

Discussion

The present study was the first investigation of in vivo functional changes in the heartlike organ of *D melanogaster* with aging. A few reports from the early 1970s report abnormalities in cardiac cell ultrastructure in the hearts of aged flies. The changes observed (with the use of electron microscopy) include enlarged mitochondria with glycogen inclusions in the matrix and loss of cristae, appearance of numerous autophagic vacuoles containing cytoplasmic organelles at various stages of degeneration, and degenerating nuclei.[21,22] However, functional assessments have, until now, not been performed.

Our finding of an age-associated reduction in heart rate in flies is consistent with the decrease in exercise capacity observed in *Drosophila* with aging.[23,24] In this regard, it has been documented that aging flies have reduced exercise tolerance compared with young flies, as measured by climbing ability. Thus, our observations reveal intriguing similarities between the decline in cardiac function during aging in flies and humans. For example, data from the Baltimore Longitudinal Study of Aging[15] demonstrated that a significant but limited reduction of resting heart rate occurs with age in humans. However, that study also documented that a much more pronounced decrease in maximum heart rate achieved during exercise is associated with aging.[15] The intrinsic sinus rate in humans (measured in the presence of both sympathetic and parasympathetic blockade) is also significantly diminished with age.[25] Age-related changes in heart rate have also been reported in rats.[26]

Our findings of an increased likelihood of fibrillation during pacing and increased rate irregularity in the hearts of older flies are also consistent with the results obtained in mammals. Aging in humans, even in those apparently free of disease, is accompanied by an increased incidence of cardiac arrhythmias.[27] The occurrence of Ca^{2+}-dependent ventricular fibrillation is also increased in older rats.[28]

It has been reported that in mammals,[29] pacing can elicit equivalent heart rates in senescence, whereas other stimuli do not. There are several possible explanations for the different results we obtained with pacing in flies. The high incidence of fibrillation that we report might impede the effects of pacing in older fly hearts. It is also possible that this might represent a difference between species. In fact, we wish to stress that our expectation is not that the aging fly heart might mimic in every respect the mammalian counterpart, but more realistically, we propose that in our genetically more tractable model, rapid progress might be made and testable hypotheses be formulated that could be investigated further in vertebrates.

The search for genes extending the lifespan in *Drosophila* is actively under way and has recently begun to provide insights into the genetics of aging in this animal.[13] Mutant flies have been screened for variations in lifespan, revealing that single gene mutations can increase the lifespan by as much as 35% in these invertebrate animals. However, the predominant causes of death in aged fruit flies are unknown and might be largely irrelevant to those affecting humans. Even in rats, the main causes of mortality in old animals (kidney disease and certain types of cancer) are not the same illnesses that are the most common causes of death in humans.[30] Therefore, fly screenings based on mortality could lead to the identification of genes affecting a physiological processes that might not necessarily be relevant to human health.

On the other hand, investigating age-associated changes in *Drosophila* with a focus on cardiac function has clear advantages. By directly assessing the status of the heart, the complexity of the object of study is reduced, which might be expected to yield a smaller, more manageable, set of candidate genes for subsequent analysis after initial genetic screens. Furthermore, several of the most promising models used in aging research (yeast[31] and, recently, even bacteria[32]) are only informative for the replicative senescence of actively dividing cells. Thus, a need exists for appropriate models for the study of the aging of tissues that have very limited replicative capacity, eg, the heart; such tissues play an important role in determining human mortality.

A commonly recognized limitation in the search for single gene mutations that can lengthen the lifespan of *Drosophila* is inbreeding depression.[33] To make a recessive mutation homozygous and to analyze its phenotype in *Drosophila* require inbreeding, and this favors the fixation of alleles possessing a deleterious effect on lifespan. Working on a parameter that can be measured throughout life (heart rate) might allow us to individuate beneficial mutations, detecting their effect at early ages, even against an unfavorable genetic background due to inbreeding that could shorten the lifespan nonspecifically.

Another advantage of using the fly as a model pertains to the size of its genome. Whereas the human genome may contain between 30 000 and 40 000 genes, only ≈14 000 genes have been identified in the fly genome.[1] Human genes are often members of extended families with redundant functions, making genetic analysis problematic in higher eukaryotes compared with invertebrates such as *Caenorhabiditis elegans* or *Drosophila*. Thus, approaching the problem of age-associated cardiac deterioration in a genetically trac-

table organism such as the fly can help to avoid genetic redundancy.

Therefore, we speculate that genetic screens based on age-associated differences in heart rates under the stress of elevated temperature could be exploited for identifying evolutionarily conserved genes that either accelerate or retard the rate of age-associated cardiac decline in *Drosophila*. Given that a recent survey has shown remarkable conservation of human genes in the fly genome, including cardiac disease–relevant genes,[34] candidate genes identified by such genetic screens have a strong possibility of being relevant to humans.

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