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RESEARCH ARTICLE

Physiology declines prior to death in *Drosophila melanogaster*

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Abstract For a period of 6–15 days prior to death, the fecundity and virility of *Drosophila melanogaster* fall significantly below those of same-aged flies that are not near death. It is likely that other aspects of physiology may decline during this period. This study attempts to document changes in two physiological characteristics prior to death: desiccation resistance and time-in-motion. Using individual fecundity estimates and previously described models, it is possible to accurately predict which flies in a population are near death at any given age; these flies are said to be in the "death spiral". In this study of approximately 7,600 females, we used cohort mortality data and individual fecundity estimates to dichotomize each of five replicate populations of same-aged *D. melanogaster* into "death spiral" and "non-spiral" groups. We then compared these groups for two physiological characteristics that decline during aging. We describe the statistical properties of a new multivariate test statistic that allows us to compare the desiccation resistance and time-in-motion for two populations chosen on the basis of their fecundity. This multivariate representation of the desiccation resistance and time-in-motion of spiral and non-spiral females was shown to be significantly different with the spiral females characterized by lower desiccation resistance and time spent in motion. Our results suggest that *D. melanogaster* may be used as a model organism to study physiological changes that occur when death is imminent.

Keywords Death spiral · Fecundity · Desiccation resistance · *Drosophila melanogaster*

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Introduction

Evolutionary biologists recognize three important life stages that arise from Hamilton's forces of natural selection in sexually reproducing organisms (reviewed in Rose et al. 2007; Shahrestani et al. 2009). These three stages are development, aging, and late life. Development refers to the period prior to reproduction in which natural selection strongly selects against genetic variants that reduce survival; individuals that do not survive to reproduce do not contribute genetically to the next generation.

The end of development and the onset of reproduction mark the start of the aging period. During the aging period, selection becomes progressively weaker with advancing age (Charlesworth 1994) and results in worsening of age-specific fitness with age. At late adult ages, the falling force of natural selection reaches very

low levels where its effects are weaker than drift. These late adult ages correspond to a period of stable mortality, fecundity, and virility (Carey et al. 1992; Curtsinger et al. 1992; Rose et al. 2002; Rauser et al. 2006; Shahrestani et al. 2012a). This period of unchanging but insignificant force of selection has been called "late life" by evolutionary biologists (e.g. Rose et al. 2002; Rauser et al. 2006; Shahrestani et al. 2009).

We recently discovered a fourth life history stage that we have called the "death spiral" (Rauser et al. 2005; Mueller et al. 2007, 2009; Shahrestani et al. 2012a). When observing lifetime reproductive patterns of individual female *Drosophila melanogaster*, we found that for a period of 6–15 days prior to death, the fecundity of females that are about to die drops at a much faster rate compared to the fecundity of similarly aged females that are not about to die. The death spiral appears at all ages and thus would not be considered an aging phenotype. The female fecundity death spiral has been independently documented by at least one other laboratory, where it was called the "terminal phase" (Rogina et al. 2007). Similar phenomena have been observed in mice (Wax and Goodrick 1978; Weinert and Weinert 1998). Male *D. melanogaster* also experience reproductive death spirals; specifically, the virility of males within 7 days of death is significantly lower than the virility of similarly aged males that are not near death (Shahrestani et al. 2012a).

Individuals in a population enter the death spiral at different absolute ages (Mueller et al. 2007; Shahrestani et al. 2012a), and the death spiral foreshadows impeding death. It is likely that the observed death spiral in reproductive behavior signals a very general decline in physiological health prior to death. However, since many interesting physiological traits require that the organism be sacrificed, then some means of determining, prior to death, if a female is in the death spiral or not is required. In this study, we used methods described by Mueller et al. (2009) to dichotomize the population into death spiral and non-spiral groups before testing individuals for physiological characteristics, one which results in death of the fly.

Mueller et al. (2009) described methods for accurately identifying females that have entered the death spiral prior to their actual death, using their observed fecundity over 3 days prior to classification. Predicting the death of individual flies allows for the possibility of studying a host of interesting questions about the dying process, including questions about physiological characteristics that are studied through destructive assays. In this five-fold replicated study, we compared two physiological characteristics (desiccation resistance and time spent in spontaneous motion) between death spiral and non-spiral groups of same-aged female fruit flies. We hypothesized that *D. melanogaster* females that are in the death spiral should have lower measures for both of these physiological characteristics compared to non-spiral females.

Materials and methods

Study system

We used *D. melanogaster* from five replicate Baseline, or B, populations (B_{1-5}) derived from the IV laboratory population (Ives 1975; Rose 1984). The B_{1-5} populations have been separated from each other since 1980 (Rose 1984) and have all been maintained under identical conditions in eight-dram vials with 5 mL of banana-molasses food per vial, at 25 °C and constant illumination. These populations were kept on strict 2 weeks generation cycles at densities of 60–80 eggs/vial. The first replicate population was tested in April 2009, the second through fifth replicate populations were tested between March 2011 and July 2011.

Mortality and fecundity assays

For each population, eggs were collected on the same day from the stock population and were kept at densities of ~65 eggs per vial for 14 days. On day 14 from egg, the adult flies were transferred to plexiglass population cages at densities of ~1,000 flies per cage (sexes mixed at approximately equal numbers). Flies were aged in the population cages for 2 weeks. Throughout these 2 weeks, fresh yeasted charcoal food medium was placed in the cages every other day. At 28 days from egg, the flies were transferred to vials in the following manner. We made approximately 1,520 vials for each population, each vial containing one female fly and one male partner. For each replicate population, 240 vials, each containing 8 backup male partners and two females were also collected. Daily mortality was recorded and flies were transferred to fresh yeasted charcoal food vials every other day. If a male partner died, he was replaced by another same-aged backup male.

For at least 3 days before testing the flies for physiological characteristics, we recorded daily fecundity data on the females. Over this period, the flies were transferred to fresh food daily and the eggs laid in the previous day's vial were counted. On the last day of fecundity counts, we tested the flies for time-in-motion and desiccation resistance as described below.

Time-in-motion assay

This assay tests for a fly's spontaneous locomotor activity in a set time interval. Flies were lightly anesthetized using carbon dioxide. Individual flies were placed in eight-dram glass vials and confined to the bottom 1 cm of the vial using a plug. The vial was inverted so that the fly was clearly visible when viewed from above. Each fly was given 10 min to fully recover from the carbon dioxide anesthesia. Then a stopwatch was used to time the spontaneous movement of each fly in a 2-min time interval. To improve the accuracy of the measured time-in-motion, each fly was tested twice back-to-back by two separate experimenters with about 30 s between measurements. The two measurements were averaged to give the time-in-motion for each fly.

Desiccation resistance assay

After the time-in-motion assay was completed, the sponge in the vial was moved up so that there was only $\sim$1 cm of space between the tip of the sponge and the opening of the vial. Three grams of Drierite desiccant was then added on top of the sponge and the vial was sealed with two layers of parafilm to keep moisture from entering the vial. The vials were checked every hour and the number of dead flies was recorded. Death was inferred from a fly's lack of movement upon mechanical provocation.

Statistical analysis

The mortality of females was checked daily until the assay day, t^*. For 3 days prior to t^* the fecundity of each female was recorded. Since t^* is at an age where less than 50 % of the original cohort has died, we expect the trajectory of mortality to be well described by the Gompertz model (Mueller et al. 1995). The deaths prior to age t^* are used to estimate the two parameters of the Gompertz equation which we describe next.

Gompertz parameter estimation

Age-specific mortality can be accurately modeled by the Gompertz equation during the majority of the adult lifespan,

$$u(x) = A\exp(\alpha x) \quad (1)$$

where A is called the age-independent parameter, α the age-dependent parameter and x is the adult age. The two most common methods of estimation are non-linear regression (Gavrilov and Gavrilova 1991) and maximum likelihood (Mueller et al. 1995; Pletcher 1999). Mueller et al. (1995) show that under some circumstances the maximum-likelihood method provides superior estimates relative to non-linear regression. However, there are several ways to implement the maximum likelihood technique. One method described by Pletcher (1999) and implemented in a popular software package, *Winmodest*, has recently been shown to be biased since it treats the age-at-death as known when in fact only rough estimates can be collected (Shahrestani et al. 2012b). Therefore we used the maximum likelihood methods described in Mueller et al. (1995) which utilize the total number of deaths in each census period which is known exactly.

We pooled the survival data from all five populations to arrive at the final estimates since the numbers of deaths for any single population was small which in turn would lead to inaccurate parameter estimates.

Death spiral classification

The death spiral is a period just prior to death that is characterized by a dramatic decline in fecundity for females (Rauser et al. 2005) and virility for males (Shahrestani et al. 2012a). The length of the death spiral is 5–14 days for females and at least 1 week for males. Classification of females as either in the death spiral or not is a two-step process (Mueller et al. 2009). The first step is to determine what fraction of the population should be in the death spiral on the assay day. If the length of the death spiral is ω-days then the chance that a female is in the death spiral (e.g. it will die in the next ω-days) at time t^* is, $P = 1 - \frac{p_{t^*+\omega}}{p_{t^*}}$ where p_{t^*} is derived from the Gompertz equation as, $\exp\{\frac{A[1-\exp(\alpha t^*)]}{\alpha}\}$.

To determine which females are in the death spiral fraction we have measured female fecundity 3 days prior to t^*. The females with the lowest ($P \times 100$) %

mean fecundity are then placed in the death spiral category and the remaining females are placed in the non-spiral category (Mueller et al. 2009). In our analysis in this study we set ω to 10.

Results

Handling correlated traits

Our goal is to compare the desiccation resistance and time-in-motion of the spiral and non-spiral groups. However, if fecundity is correlated with either or both of these traits then the process of picking females with the lowest values of fecundity may also produce a population with low desiccation resistance and activity time. The entire collection of females has an estimated correlation coefficient of 0.089 and 0.052 between fecundity and desiccation resistance and fecundity and time-in-motion respectively. Both of these estimated correlations are significantly greater than zero. The correlation between desiccation and time-in-motion is small and not significantly different form zero.

A biologically relevant null hypothesis would then be that there is a single population of females which show small positive correlations between fecundity and desiccation resistance and time-in-motion. Under the null hypothesis if we did a linear regression of desiccation resistance (or time-in-motion) versus fecundity for the "spiral" females and "non-spiral" females each group should have a significant regression but with the same slope and *y* intercept for both types of females.

A relevant alternative hypothesis is that there are in fact two populations. The two populations have the same correlations between fecundity and starvation resistance and time-in-motion but they have different mean values. When these two populations are mixed together they show the correlations we have reported above. In this case the regression of say desiccation resistance on fecundity would have the same slope for the "spiral" females and "non-spiral" females but their *y* intercepts would differ. Thus, it would appear that one approach to comparing the death spiral and non-death spiral groups is by a simple regression comparing *y* intercepts.

Simulation study

We investigated this approach by creating, on a computer, a collection of fecundity and desiccation time data for 3,379 females which was the number examined in this study. All simulations and data analysis were carried out with R (R Development Team 2012). We assumed that female fecundity and desiccation resistance had a multivariate normal distribution with a correlation of 0.089. Samples from this population would then be a direct test of the null hypothesis. We would expect only 5 % of such tests to show significant differences in their *y* intercepts. We then separated the females into two groups according to their fecundity with the lowest 10 %, 20 %, 30 % or 50 % being placed in the death spiral group and the remainder being placed into the non-death spiral group. We used the *lm* R-function to estimate the regression parameters. We repeated this with 1,000 different samples and then estimated the fraction of those which yielded significant differences between the *y* intercepts of the "death spiral" and "non-death spiral" groups. In Fig. 1 the 0 % line for the *y*-intercept test is close to the nominal 5 % for the four tested combinations of death spiral females. Thus, this test gives the correct type-I error rate. We next tested

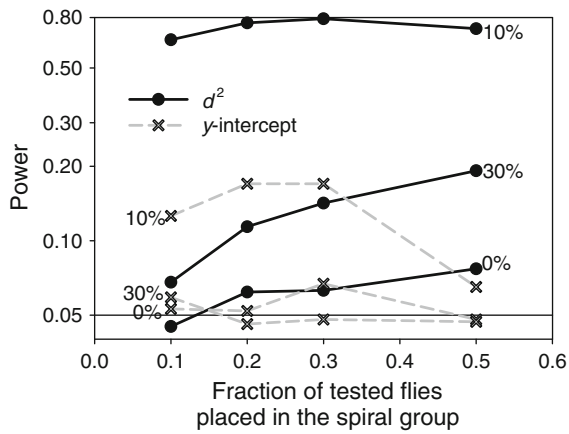

Fig. 1 The power of the linear regression and squared Mahalanobis distance test. In each case the power (fraction of significant tests with *p* values at or below the 0.05 level) was estimated from 1,000 independent simulations. The *x* axis shows the fraction of females that were placed into the death spiral group by virtue of their low fecundity. For each test there are *three lines* that give results for three different synthetic populations (0 %, 10 % and 30 %) that consisted of different numbers of actual death spiral and non-death spiral females. In these synthetic populations death spiral females were assumed to have a lower mean desiccation resistance and time-in-motion than the non-death spiral females as shown in Table 1. The simulation with 0 % death spiral females was just a single population and was used to ensure that the type-I error rate was at the nominal 5 % level

the power of this regression approach to detect differences when they were present, e.g. the statistical power of the *y*-intercept test.

We simulated a relatively simple alternative hypothesis. We assumed that the death spiral group made up 10 or 30 % of the population of tested females. We assumed that there was no correlation between fecundity and desiccation resistance and fecundity and time-in-motion but that the mean value of all three traits was lower in the death spiral group than in the non-death spiral group. We then chose mean values for the death spiral group so that when they were mixed with the non-death spiral group the total population would have the approximate correlations seen in our data. The characteristics of these populations are given in Table 1. We ran 1,000 simulations each having a female population of 3,379.

The 10 %, *y*-intercept test results (Fig. 1) show a power of between 10 and 20 % except when the assumed number of female in the spiral group (50 %) is much greater than the true value (10 %). When the spiral females make up 30 % of the population the difference in the mean desiccation and fecundity is smaller (Table 1). This resulted in a substantially reduced power for the *y*-intercept test (see 30 %, *y*-intercept line in Fig. 1). Next we describe an alternative test with substantially greater power.

Table 1 The mean for the spiral females and correlations (ρ) in the total population (spiral and non-spiral females) used in the power simulations

Fraction (%)	Spiral female mean			Correlation	
	Fecundity	Desiccation	Time-in-motion	ρ(fec, des)	ρ(fec, time)
0	0	0	0	0.089	0.052
10	−1.0	−0.9	−0.9	0.068	0.086
30	−1.0	−0.4	−0.4	0.064	0.077

The non-spiral females were assumed to have a mean of 0 for each of the three traits and unit variance and zero correlation between the traits. Within the spiral population the variances and correlations were the same as the non-spiral females. The "fraction" column indicates the fraction of the total population assumed to be composed of spiral females. The 0 % fraction describes the single population test which assumed that fecundity was correlated with desiccation resistance and time-in-motion

A more powerful test

We describe a method for handling correlated data that (i) is a multivariate approach, and (ii) we tested through computer simulation to determine its efficacy. For each individual, i, in an experiment with a total of n individuals, we have a vector of observations, $y_i = (y_{i1}, y_{i2}, y_{i3})$, where the three y variables are fecundity, desiccation resistance and time-in-motion respectively. We let the matrix of all experimental observations be $\mathbf{Y}$, which has dimensions $n \times 3$. We then took the reduced matrix consisting of columns 2 (desiccation resistance) and 3 (time-in-motion) of $\mathbf{Y}$ and center and scale the variables in each column to produce an $n \times 2$ matrix, $\tilde{\mathbf{Y}}$. The matrix $\tilde{\mathbf{Y}}$ was then used to estimate principal components. Let the 2×2 matrix containing the principal components as columns be $\mathbf{P_c}$. The original desiccation and motion scaled and centered data was then converted to the principal component coordinates via the matrix multiplication, $\mathbf{Z} = \tilde{\mathbf{Y}}\mathbf{P_c}$.

The matrix $\mathbf{Z}$ was then divided into two sub matrices according to which females belong to the spiral group, $\mathbf{Z}_s$, and those belonging to the non-spiral group, $\mathbf{Z}_{ns}$ using the procedure described previously. We then estimated the mean, $\hat{\mu}$, and covariance, $\hat{\Sigma}$, of $\mathbf{Z}_{ns}$. To evaluate whether there is a difference between the members of the spiral and non-spiral populations we then computed the average squared Mahalanobis distance between the spiral females and the mean of the non-spiral populations, $\overline{d_s^2}$, and the average distance between the non-spiral females and the mean of their population, $\overline{d_{ns}^2}$. If the principal component coordinates for a female is z_i, then the squared Mahalanobis distance is defined as, $(z_i - \hat{u})^T \Sigma^{-1} (z_i - \hat{u})$. Although the individual squared Mahalanobis statistic has a beta distribution (Ververidis and Kotropoulos 2008) their mean should be normally distributed and hence a simple t test can be used to compare $\overline{d_s^2}$ to $\overline{d_{ns}^2}$ (the corresponding author can be contacted for copies of the software). We next describe a simulation study to determine if this test statistic yields the nominal type-I error rate under the null hypothesis that the spiral and non-spiral females are drawn from the same population. We also tested the power of this statistic under several simple but realistic scenarios.

In general as the difference in desiccation resistance and time-in-motion gets larger between the death spiral

and non-death spiral groups the power of the test increases (compare the 10 %, d^2 line in Fig. 1 to the 30 %, d^2 line). If the fraction of the population that is in the death spiral is badly overestimated (e.g. $x = 50$ % on the 10 %, d^2 line in Fig. 1) then the power starts to decline. Presumably this happens because there are a large number of non-spiral females "contaminating" the spiral female population. However, in general including too many females in the spiral group does not have a severe effect on the power. Compared to the *y*-intercept test the Mahalanobis test is much more powerful. In addition when the null hypothesis is true the Mahalanobis test gives *p* values close to the nominal 5 % value (see 0 %, d^2 line, Fig. 1).

At this point we have provided evidence that the proposed Mahalanobis test has the expected nominal type-I error rate and that the test has reasonable power for synthetic populations that mimic the characteristics of our actual data. While we might now simply move on to the analysis of the experimental data it would be of interest to know why this test works (as colleagues who have read this paper tell us). To that end we have done some additional work. Presumably the confounding effects of the correlation between fecundity and the other traits are eliminated by this test when we are only dealing with a single population. Thus, we repeated the simulation above that consisted of only a single population with correlations between fecundity and desiccation resistance and fecundity and time-in-motion. This simulation gave rise to the 0 %, d^2 line in Fig. 1. Now we saved the correlation between fecundity and the first principal component, z_1, in each simulation as well as the correlation between fecundity and d^2 in each simulation. We see that the correlation between fecundity and z_1 while on average around zero can be large leading to the possibility that spurious results could arise if we relied on only one principal component when comparing the spiral and non-spiral females (Fig. 2). However, the correlation between fecundity and d^2 is both close to zero and small. The empirical 95 % confidence interval on this correlation is (−0.034, 0.029). Presumably the orthogonality of the principal components and use of a test statistic based on both is essential for this technique.

Experimental results

The mortality data and the predicted mortality from the Gompertz equation are shown in Fig. 3. These results

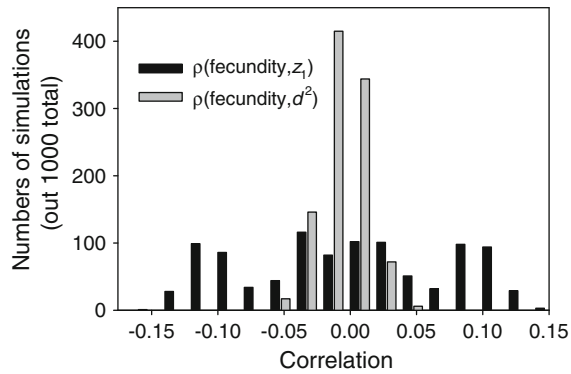

Fig. 2 The distribution of correlations between fecundity and the first principal component, z_1 (*black bars*) and fecundity and the squared Mahalanobis distance statistic (*grey bars*). The correlations are based on 1,000 simulations of 3,379 females from a single population. In this population the correlation between fecundity and desiccation resistance was set to 0.089 and between fecundity and time-in-motion, 0.052

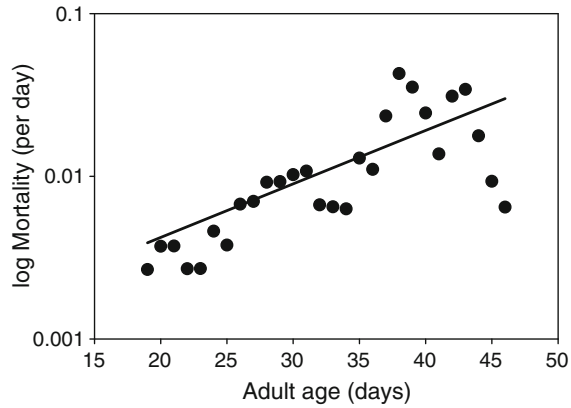

Fig. 3 The observed log mortality (*circles*) from the five B populations and the predicted mortality (*line*) from the Gompertz model (Eq. 1). The Gompertz parameter values were, $A = 0.00278$, $\alpha = 0.0761$

along with each female's 3-day fecundity score were then used to classify females into two groups and carry out statistical tests on the desiccation and time-in-motion data. The actual average daily fecundity shows some overlap between the two groups (Fig. 4). This happens because the assays for each population were not always conducted at the same age and thus the critical fecundity that determined group membership was not exactly the same. The death spiral females had lower desiccation resistance than the non-spiral females (Fig. 5). The death spiral females also had smaller time-in-motion (Fig. 6). However, given the correlation between fecundity and desiccation resistance and

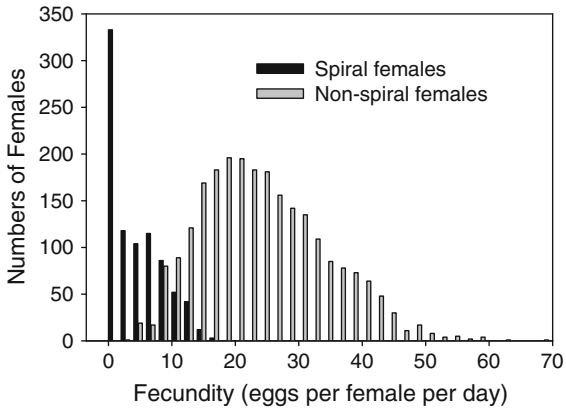

Fig. 4 The distribution of daily egg production for the spiral and non-spiral females. There is overlap due to the use of different test ages for the five different B populations

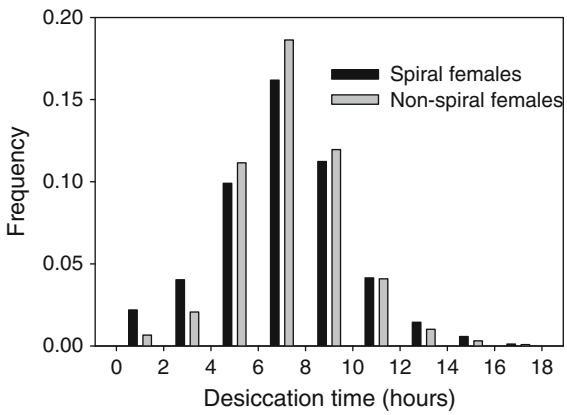

Fig. 5 The distribution of desiccation time in the spiral and non-spiral female groups

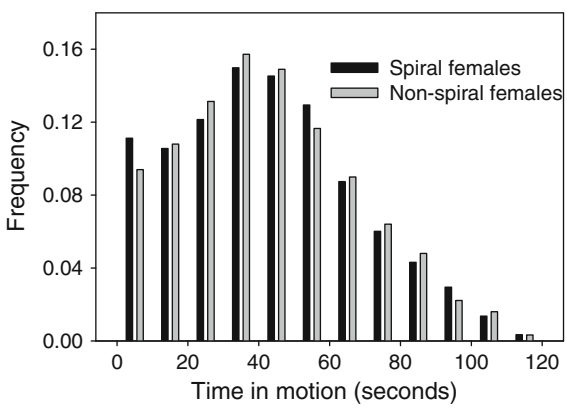

Fig. 6 The distribution of time-in-motion in the spiral and non-spiral female groups

time-in-motion it is unclear how much of this difference can be attributed to the correlation alone. We then computed the average squared Mahalanobis distance between the spiral females and the average non-spiral female and compared this to the average of the non-spiral females to their mean. The average squared distance of the spiral females was 2.71 compared to 2.00 for the non-spiral distance and these means were significantly different ($t_{960} = 3.41$, $p = 0.00069$).

Discussion

In this study, we recorded 3 days of fecundity data on individual female *D. melanogaster* in mid-adulthood and used methods described by Mueller et al. (2009) to dichotomize the population into death spiral and non-spiral groups before testing individuals for physiological characteristics. This allowed us to study physiological changes that occurred during the dying process. We found that both time spent in spontaneous motion and desiccation resistance were lower in the death spiral group compared to the non-spiral group. We selected these two characteristics to study because they have been shown to deteriorate during aging (Ganestzky and Flanagan 1978; Rose et al. 1992, 2004), which suggests that they may be necessary for survival.

The death spiral in *D. melanogaster* can be used to study physiology prior to death, or more specifically to model the disablement process in humans; this process is defined as the period of cumulative disorders and disability prior to death (Verbrugge and Jette 1994). The onset of disability may lead to a downward spiral of new pathologies and lead ultimately to death (Verbrugge and Jette 1994; Morley 2008). The significant drops in fitness components (fecundity and virility) in death spiral flies are closely synonymous to the inexorably declined health in humans who are experiencing the disability period prior to death. Death spiral like characteristics have also been observed in humans. In a study of 2,262 elderly Danish individuals borne in 1905, each individual was assessed every 2 years for their physical and cognitive abilities. Individuals who died within 2 years of the assessment had significantly lower physical and cognitive scores than those individuals who lived. Those individuals who remained in the study for the longest time also showed the slowest rate of decline in physical and cognitive scores (Christensen et al. 2008).

Several other studies have shown that a number of physiological characteristics in flies have possible predictive power for the life span of individuals. For example, Papadopoulos et al. (2002) found that individual medfly that were an average of 2 weeks from death began to show a distinct behavior in which they were upside down for short periods of time. In addition, Carey et al. (2006) found that not only the onset of being upside down (called supine behavior), but also the termination of medfly calling behavior were time-to-death specific. In longitudinal assays of individual flies using real-time video tracking of GFP fluorescence, hsp22 and hsp70 transgenic reporters began to spike in expression $\sim$5 to 10 h before death (Grover et al. 2008, 2009). It has been suggested that hsp gene expression levels could possibly be used to predict remaining life span of individuals (Yang and Tower 2009).

Drosophila melanogaster have already emerged as a powerful model system for investigating the biology that underlies age-related functional decline (reviewed in Grotewiel et al. 2005). Similar functional studies can help identify key systems that fail in the dying process. It would be interesting to determine which functional declines are part of the dying process. Presumably, not all organ systems must fail before death, and not all that fail are necessarily related to survival, allowing for a distinction between causes and symptoms of death. It will be important to study the environmental, genetic, and demographic factors which may affect the onset and duration of the period of disability prior to death, and the *D. melanogaster* model makes such studies feasible.

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