RESEARCH ARTICLE

Pioglitazone: an anti-diabetic compound with anti-aging properties

**Mahtab Jafari · Behnood Khodayari ·
Jeffrey Felgner · Irvin I. Bussel ·
Michael R. Rose · Laurence D. Mueller**

Abstract Insulin and Insulin-Growth-Factor-like (IGF) signaling pathways are well known longevity pathways in nematodes, insects and mammals. To our knowledge, there are no systematic pharmacological studies evaluating the anti-aging properties of medications that target this pathway in *Drosophila*. Although there are no published data implicating an anti-aging role for these compounds in *Drosophila*, we hypothesized that their promising pharmacological profile might decrease mortality. However, the decrease in mortality could be due to a number of potential artifacts and confounds such as fecundity depression, decrease in metabolic rate, or CNS depression. Therefore, the mere finding that a compound decreases mortality does not qualify it as an anti-aging compound. In this study, we evaluated the anti-aging properties of four compounds that might target the insulin signaling pathway in *Drosophila*. Once it was established that the compound decreased mortality, we proceeded to evaluate possible confounding factors that could have contributed to the mortality reduction. We show that only piolglitazone displayed anti-aging properties. At present, we do not have a mechanistic explanation for this pharmacological disparity.

Keywords Anti-aging · Anti-diabetics · Drosophila · Drug testing · Lifespan · Longevity

M. Jafari (✉)
Department of Pharmaceutical Sciences, University of California, Irvine, CA 92697, USA
e-mail: mjafari@uci.edu

B. Khodayari · J. Felgner · I. I. Bussel
Undergraduate Program, School of Biological Sciences, University of California, Irvine, CA 92697-2525, USA

M. R. Rose · L. D. Mueller
Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2525, USA

Introduction

Aging involves multiple biological pathways. Manipulation of these pathways by pharmaceutical and botanical compounds is an emerging focus of anti-aging research. However, the pharmacology of aging is likely to involve secondary effects, given the multifold pathways that affect fruit fly aging (Fleming et al. 1993; Pletcher et al. 2002; Rose and Long 2002). One of these pathways is the insulin/insulin like growth factor signaling pathway that is highly conserved. Mutations affecting this pathway have resulted in lifespan extension in Caenorhabditis elegans (daf-2 and age-1), fruit flies (Chico and InR), and rodents (Sirt1). (Clayton et al. 2002; Hursting et al. 2003; Lemieux et al. 2005) Although some compounds that have been tested in *Drosophila* increase lifespan, this work has typically lacked evaluation of secondary mechanisms that could have resulted in ostensibly anti-aging effects artifactually (Jafari and Rose 2006). Particular pharmaceuticals or

botanicals that affect aging may do so through multiple pathways, not just the pathway that is of *a priori* interest. Most importantly, it is not appropriate to simply assume that a compound that affects adult survival *only* has such effects. Such compounds might also have adverse secondary effects that would limit their clinical use in the treatment of aging. As discussed in a review by Le Bourg, the potential secondary effects of antioxidants have been evaluated in a few studies (LeBourg 2001). To date, anti-aging studies have not systematically evaluated the secondary adverse effects of potential anti-aging compounds. Another challenge in studying anti-aging effects of compounds that are administered through feeding is the issue of toxicity and starvation. Although, Thompson et al suggests that lethality induced by cyclophosphamide is due to starvation and not drug toxicity (Thompson et al. 2006) we cannot generalize this conclusion since the compounds that we examined in our study are not as toxic as cyclophosphamide. We, on the other hand, examined the impact of the compounds on the fecundity to test for drug induced starvation and toxicity.

This study was designed to test the effect of commonly used anti-diabetic pharmaceuticals on the life span of adult *Drosophila*. Furthermore, we examined any possible life extension confounds and artifacts in cases of significant reduction in adult mortality.

Methods

Drosophila population employed

All *Drosophila melanogaster* stocks used in these experiments were ultimately derived from a sample (called "IV") of the Amherst, Massachusetts, Ives population that was collected in 1975 and cultured at moderate to large population sizes ever since (Rose et al. 1984; Rose et al. 2004). This population has been reared at controlled densities (50–80 eggs per vial) for more than 700 generations with discrete generations cultured every 2 weeks.

Mortality assays

All flies used in these assays were raised as larvae in 5 ml of standard banana-molasses food at densities of between 50 and 80 eggs per 8-dram vial. Populations

were maintained at about 25°C with constant illumination. During the assays, adults were kept in standard 5 ml food vials containing banana-molasses food and 1 mg of yeast paste to promote egg laying.

All compounds were supplied to adults only. The compounds were mixed into the yeast paste; the adults preferentially consume this paste. Adults were transferred to fresh vials and survivors were counted every 2 days. All assays were conducted on flies that had undergone two generations of controlled density rearing. When flies from different treatments were compared, all preliminary rearing was carried out in parallel.

We evaluated the impact of each compound on mortality rate. For each assay, three doses of each compound were compared to a control group. For each dose, 320 males and 320 females were exposed to the compound. There were 4 males and 4 females in each vial, with a total of 80 vials per dose per sex, and the flies were transferred every other day during the aging phase, which lasts 4 weeks in IV stocks (Rose et al. 2002). We attempted to determine a dose-response relationship during the mortality assays. This required repeating testing of the same compound over a range of doses. Compounds with a beneficial effect on mortality rates were subjected to fecundity assays, as described below.

The data were analyzed by examining the number of surviving flies at the end of week four. The statistical evaluation at the end of week 4 is based on data accumulated from the records of survivors obtained every 2 days. Significant differences between the control and each drug treatment were assessed by the Pearson chi-square test.

Pharmacological trials

Stock solutions for each compound were prepared and mixed into the yeast paste. The calculated dose used in our data analyses reflects the final concentration of the compound in the yeast paste that flies consumed during the experiment. Each vial contained 1.5 mg of yeast that was mixed with the drug. Compounds were obtained from the pharmaceutical manufacturer in tablet format. For each trial, three doses of each compound were compared to a control. We screened metformin, glipizide, rosiglitazone, and piolglitazone at the following dosing regimens: metformin (0.4, 0.8, 1.6 mg/ml), glipizide (0.01,

0.1, 1 mg/ml), rosiglitazone (0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 6, 12, 18 mg/ml), and piolglitazone (0.01, 0.02, 0.04, 0.08, 0.1, 1.0, 10 mg/ml). Adult flies, starting at day 1, were fed with these compounds.

Survival probability

Pioglitazone treatment resulted in a beneficial effect on mortality rate; therefore a total survivorship assay was conducted to reaffirm the result. This assay also allowed the determination of mean life span change as a result of the compound. Survival data were obtained from every other day transfers until every fly was dead. Flies receiving the most efficacious dose from the mortality assay, 0.02 mg/ml of pioglitazone, were compared to controls. For each group (control vs. drug treatment), 2,400 males and 2,400 females were used in sets of 8 (4 males: 4 females) per vial. The flies from each group were housed in 15 separate racks.

Gompertz mortality analysis

In this formulation we will let the index i indicate one of the 15 racks, j indicate sex (1 = female, 2 = male), k indicates drug treatment (1 = control, 2 = drug treated), and t indicate age. Then the predicted mortality between ages t and $t + 1$ is y_{ijkt}. The basic nonlinear model is given by,

$$y_{ijkt} = f(\Psi_{ijk}, t) + \varepsilon_{ijkt}, \qquad (1)$$

where Ψ_{ijk} is the vector of parameters, t is the age, and ε_{ijkt} is the within population variation. The function f is the Gompertz model,

$$f(\Psi_{ijk}, t) = 1 - \exp\left\{ \frac{A_{ijk}}{\alpha_{ijk}} \left[\exp(\alpha_{ijk} t) - \exp(\alpha_{ijk}(t+1)) \right] \right\}. \qquad (2)$$

The parameter A is sometimes called the age-independent parameter of the Gompertz and is a reflection of background mortality that does not change with age. On the other hand α is called the age-dependent parameter and measures the rate at which mortality increases with age, e.g. senescence. We assume that the parameters of the Gompertz equation may be affected by the fixed effects, sex and

drug treatment and the random rack environment. These assumptions translate into a system of equations,

$$
\begin{aligned}
A_{ijk} &= \beta_1 + \gamma_1 \delta_j + \phi_1 \delta_k + b_{1i} \\
\alpha_{ijk} &= \beta_2 + \gamma_2 \delta_j + \phi_2 \delta_k + b_{2i},
\end{aligned}
\qquad (3)
$$

where $\delta_i = 0$ if $i = 1$, or 1 otherwise. To test for significant effects of sex on A and α we determine if γ_1 or γ_2 is significantly different from zero respectively. Likewise a test for the effects of drugs on A and α corresponds to a test for whether ϕ_1 or ϕ_2 is significantly different from zero. Model (3) could be expanded to include interactions between sex and drugs. Although we tested for such interactions, they were never significant so we do not include them in the basic model described here.

The variance of mortality is expected to change with the mean value of mortality. The general formulation for the variance of ε_{ijkt} is,

$$Var(\varepsilon_{ijkt}) \cong \sigma^2 g^2(\hat{u}_{ijkt}, t), \qquad (4)$$

where $\hat{u}_{ijkt} = E(y_{ijkt}|\mathbf{b}_i)$. In this analysis we used $g(.) = |y_{ijkt}|^\delta$. The $\mathbf{b}_i$ were distributed as,

$$\mathbf{b}_i \sim N\left(\mathbf{0}, \begin{bmatrix} \sigma_1 & 0 \\ 0 & \sigma_2 \end{bmatrix} \right). \qquad (5)$$

The parameters in Eqs. (3–5) were estimated from a nonlinear mixed effects model as implemented by the *nlme* package of R (r-project.org; version 2.4.0) (Pinheiro and Bates 2000).

Two-stage Gompertz mortality

Large cohorts of fruit flies exhibit departures from the Gompertz mortality dynamics in the form of a leveling off of mortality rates at advanced ages (Carey et al. 1992; Curstinger et al. 1992). We have developed a model, called the two-stage Gompertz, that shows Gompertz dynamics at young ages and then at an advanced age, called the break day, mortality rates plateau at a constant value (Rose et al. 2002). The discontinuity in the two-stage Gompertz model caused by the break day makes it especially difficult to obtain estimates of the parameters for this model (Steinsaltz 2005). As a practical matter the same methods used to infer the drug effects on the

Gompertz model can not be used with the two-stage Gompertz.

To overcome this difficulty we have used bootstrap samples as a means of determining if there were significant differences in the two-stage Gompertz parameters due to drug treatment. For the two-stage Gompertz model the probability of surviving to age t is,

$$p(t) = \begin{cases} \exp\left(A(1 - \exp(\alpha t))/\alpha\right) & \text{if } t \leq bd \\ \exp\left(A(1 - \exp(\alpha bd)/\alpha\right) + A_2(bd - t) & \text{if } t > bd \end{cases} \tag{6}$$

where bd is the break day A and α have similar interpretations as in the Gompertz and A_2 is the plateau mortality rate. The mortality between ages t and $t + 1$ is,

$$1 - \frac{p(t + 1)}{p(t)}. \tag{7}$$

Least squares estimates of the parameters, $\theta = (A, \alpha, bd, A_2)$ are obtained by minimizing the sum of squared differences between the observed and predicted mortality (7). In the discussion below we will also refer to the components of θ as $\theta_1, \theta_2, \theta_3, \theta_4$.

One set of observations consists of a vector of ages of death, $T = (t_1, t_2,...,t_m)$, the number of deaths at each of those ages, $D = (d_{t_1}, d_{t_2}, ..., d_{t_m})$ and a total sample size of $N = \sum d_{t_i}$. To create bootstrap samples we need to define the probability of dying on any one of the m-days in T as $P_i = d_{t_i}/N$. One bootstrap sample then consists of N individuals whose ages of death are equal to one of the days in T. Thus, the chance of choosing day t_i as the day of death is P_i.

This sampling process was used to create B bootstrap samples, $D^{*1}, D^{*2}, ..., D^{*B}$. From each of these samples a least squares, θ^{*i}, estimate was made by the methods described above. The bootstrap parameter estimates are given by,

$$\hat{\theta}^* = \left(B^{-1} \sum_i \theta_1^{*i}, B^{-1} \sum_i \theta_2^{*i}, B^{-1} \sum_i \theta_3^{*i}, B^{-1} \sum_i \theta_4^{*i} \right). \tag{8}$$

We generated B bootstrap samples for the control samples, θ_c^* and the drug treated sample, θ_d^*. From these we computed the difference between the drug treatment parameter values and the control treatment parameter values, $\hat{\theta}_d^* - \hat{\theta}_c^*$. To test for significant differences between the drug and control we created a random permutation, without replacement, of the $2B$ control and drug bootstrap vectors. We then computed B differences between the first B values of the permuted vector and the last B values. This process was repeated 1,000 times to create an empirical distribution to judge the statistical significance of the differences $\hat{\theta}_d^* - \hat{\theta}_c^*$.

Fecundity assays

Some compounds may increase lifespan simply by substantially depressing fecundity; depressed fecundity will increase longevity by and of itself in *Drosophila* (Jafari and Rose 2006; Maynard Smith 1959). Therefore, a fecundity assay is an important check for artifactual lifespan enhancement. We evaluated age-specific fecundities using the same dosing protocols as above, except that the flies were handled with one female and one male in each assay vial. Fecundity was assayed from day 1 to 10 of adult flies. The number of eggs laid each day by each individual female was recorded for a period of 10 days.

We analyzed the total number of eggs laid over 10 days for each female. The impact of drugs on fecundity was assessed by a one-way analysis of variance (ANOVA). We also compared the 10-day fecundity of control females to the pooled data from all three doses using a standard t-test on the mean values.

Metabolic rate assays

As a further test for artifactual effects, compounds that had a beneficial effect on mortality, but did not depress fecundity, were assayed for their effect on metabolic rate. This assay was used to ascertain whether there had been an artifactual decrease in mortality due to hypometabolism. In addition,

metabolic rate is a useful surrogate for a number of physiological and behavioral functions that could potentially be impaired by medications, such as locomotor activity.

CO_2 production in drugged flies was compared to that of a control group handled in parallel and assayed simultaneously. We used flow-through respirometry to measure the rate of CO_2 release from groups of flies following the methods of Williams et al. (2004). Room air was passed through a small chamber containing soda lime, two silica gel chambers and a Drierite/Ascarite/Drierite column. The air was directed by a series of computer-controlled valves (Sable Systems, Las Vegas, NV, USA) that allowed six individual flies in separate chambers to be measured in turn. The flies were measured while in vials in the presence of food and the appropriate pharmacological agent. Measurements of CO_2 were made using a Licor LI-6260 gas analyzer. CO2 levels were averaged and recorded every second using data acquisition software (Sable Systems). Each vial was measured for 20 min. During periods when they were not being measured, the flies were kept in a stream of dry, CO_2-free air by flushing with a separate air stream. The last 5 min of each 20-min recording of CO_2 release was averaged to provide an estimate of relative metabolic rate. The flies were fed with the compound for 10 days and on day 11, the effects of drug treatments on metabolic rates were analyzed with a one-way ANOVA using drug level treated as a fixed effect.

CNS evaluation assays

Male virility was used as an assay for drug-induced CNS depression. In order to administer the drugs to the males, they were maintained in vials containing the drug/yeast solution for 10 days prior to the virility assay, using the same protocol as that for the mortality assays. The control males were maintained in parallel, with all conditions of rearing and maintenance identical except for the absence of the compound in the yeast paste given to the control males.

For each assay of virility, two male flies, one that was exposed to the drug and one that was not exposed to the drug for 10 days, were placed in a mating vial with a virgin female fly, who had not been exposed to the compound. Among the mating vials, half had marked drugged males; the other half had marked control males. The marking procedure used a felt-tip marker to color the tip of one wing.

Each virility assay employed 120 vials, with 40 vials for each dose. Virility was scored according to the number of vials in which the drugged male mated with the test female. The scoring of a successful mating required mounting for at least 30 s.

On the 11th day, each male was scored according to his drug status (control or drugged), marked status (marked or not marked), and mating status (mated or not mated). The data were inserted into a contingency table that was analyzed using a log likelihood model. The basic model included only the main effects (drug status, marked status and mating status). The fit of this model was compared to that of a model with these main effects and interactions between herb status and mating status, as well as marked status and mating status. If the model with interactions provided a better fit, we then determined if this improvement was due to the drug effects, the marking effects, or both.

Results

Negative results

Metformin, glipizide, and rosiglitazone did not result in statistically significant decreases in mortality rate. Although we observed sex dependent change in the number of flies surviving to the end of the aging period with rosiglitazone, this effect was not consistent. These inconsistently positive results led us to check the effect of rosiglitazone on fecundity. Rosiglitazone was associated with significant depressed fecundity in a dose-dependent manner. For this reason, even though there were some, albeit inconsistent, sex dependent mortality benefits from rosiglitazone, we did not test for additional side-effects. As a result, except for piolglitazone, none of these compounds were tested further for confounds or artifacts (Table 1).

Positive results

Mortality and confounds

Pioglitazone resulted in a statistically significant decrease in mortality. Consequently, we proceeded

Table 1 Fraction that died during aging phase with rosiglitazone, metformin, and glipizide

Compound	Dose (mg/ml)	Fraction dying ± SD Male	P-value	Fraction dying ± SD Female	P-value
Rosiglitazone	0 (Control)	0.42 ± 0.03	–	0.42 ± 0.03	–
	0.01	0.49 ± 0.03	0.06	0.44 ± 0.03	0.58
	0.05	0.45 ± 0.03	0.45	0.51 ± 0.03	0.02
	0.1	0.49 ± 0.03	0.10	0.36 ± 0.03	0.18
	0 (Control)	0.46 ± 0.03	–	0.33 ± 0.03	–
	0.5	0.47 ± 0.03	0.86	0.44 ± 0.03	0.007
	1.0	0.41 ± 0.03	0.15	0.33 ± 0.03	0.95
	1.5	0.53 ± 0.03	0.12	0.46 ± 0.03	0.003
	0 (Control)	0.46 ± 0.03	–	0.44 ± 0.03	–
	6	0.49 ± 0.03	0.47	0.37 ± 0.03	0.06
	12	0.53 ± 0.03	0.09	0.40 ± 0.03	0.32
	18	0.58 ± 0.03	0.004	0.48 ± 0.03	0.35
Metformin	0 (Control)	0.56 ± 0.03	–	0.38 ± 0.03	–
	0.4	0.59 ± 0.03	0.46	0.43 ± 0.03	0.24
	0.8	0.57 ± 0.03	0.71	0.39 ± 0.03	0.85
	1.6	0.58 ± 0.03	0.59	0.44 ± 0.03	0.11
Glipizide	0 (Control)	0.51 ± 0.03	–	0.52 ± 0.03	–
	0.01	0.52 ± 0.03	0.64	0.46 ± 0.03	0.11
	0.1	0.50 ± 0.03	0.94	0.48 ± 0.03	0.39
	1.0	0.47 ± 0.03	0.38	0.45 ± 0.03	0.09

P-value refers to a comparison with the control group

with tests of fecundity, metabolic rate, CNS depression, and total longevity. At the higher dose range (0, 0.02, 0.04, 0.08 mg/ml), a significant mortality decrease was observed in males supplemented with 0.02 mg/ml of pioglitazone (Fig. 1). At this dose range no changes in fecundity were observed (Fig. 2). At the lower dose range (0, 0.001, 0.01, 0.1 mg/ml) a significant mortality benefit was observed in both males and females supplemented with 0.01 mg/ml of pioglitazone (Fig. 3). This dose range did not exhibit any changes in fecundity relative to control (Fig. 4). Based on these results, we concluded that piolglitazone at 0.01 and 0.02 mg/ml exhibits anti-aging properties and these doses were used in our further tests.

We proceeded on to a metabolic rate assay. As shown in Fig. 5, pioglitazone did not have a significant adverse effect on metabolic rate at any dose on either sex.

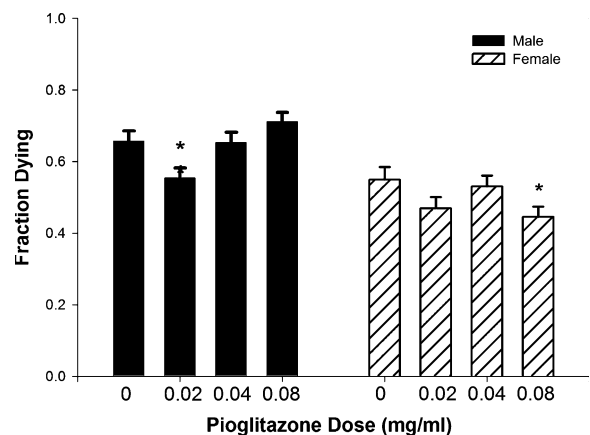Accordingly, we continued to an assay of male mating success, as a test for generalized nervous

Fig 1 Fraction dying with piolglitazone at 0. 0.02, 0.04, and 0.08 mg/ml. Decrease in fraction dying with 0.02 mg/ml in male flies. $P < 0.05$

system depression. As shown in Table 2, 0.01 mg/ml of pioglitazone did not display any significant decreases in CNS function, but a significant
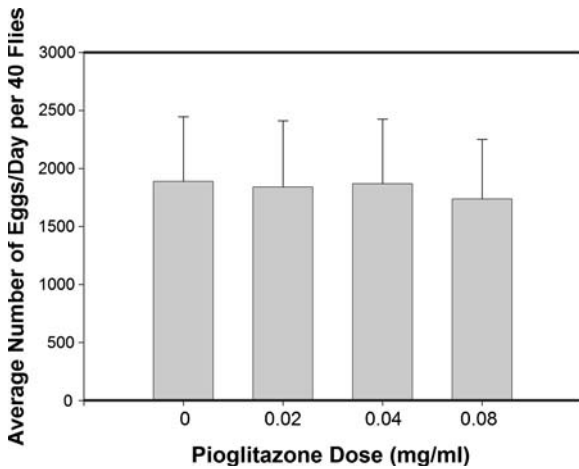
Fig 2 The impact of piolglitazone on the fecundity at 0, 0.02. 0.04. 0.08 mg/ml. Pioglitazone did not decrease fecundity with any dose. $P < 0.05$
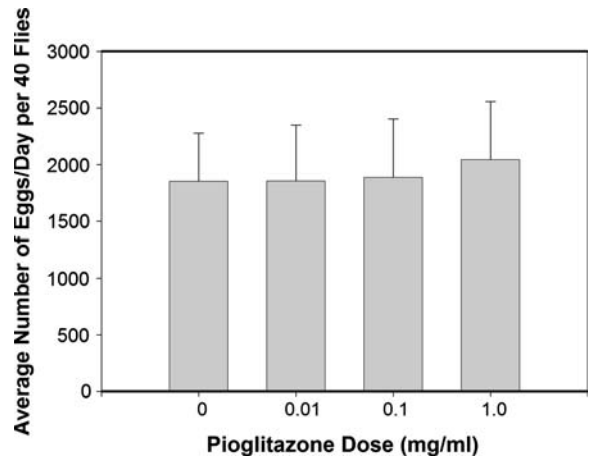

Fig 4 The impact of piolglitazone on the fecundity at 0, 0.01, 0.1, and 1 mg/ml. Pioglitazone did not decrease fecundity with any dose. $P < 0.05$
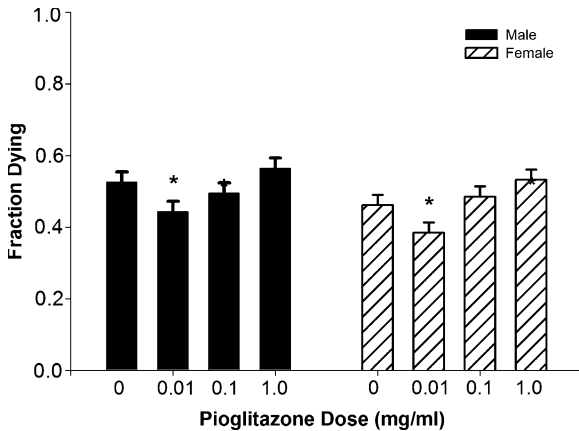

Fig 3 Fraction dying with piolglitazone at 0. 0.01, 0.1, and 1 mg/ml. Decrease in fraction dying with 0.01 mg/ml in both male and female flies. $P < 0.05$
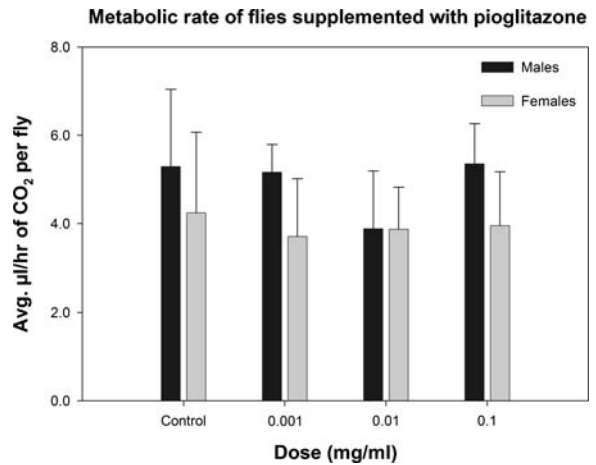

Fig 5 The metabolic rate of adult males and females exposed to three different doses of pioglitazone. The bars are standard errors (s.e.)

incidence of CNS depression was observed at the dose of 1 mg/ml.

Longevity

The mean longevity for the entire experiment was used to determine if there were significant differences between the flies given drugs and the controls (Table 3). Pioglitazone had small but positive effect on longevity. Female longevity was increased by 1.1 days and male longevity by 0.9 days. All differences in longevity were statistically significant

due to the very large number of flies used in these experiments.

If we use the observed variances we can estimate the minimum required sample sizes to detect the observed differences. The smallest difference was 0.87 days and this difference could have been detected with a sample size of 914. From the estimated confidence intervals (Table 3) it is apparent that differences in longevity as small as 0.54 days could have detected. As discussed previously the differences in mean longevity may be caused by a variety of changes in the age-specific mortality

Table 2 CNS assay data with pioglitazone at 0.01, 0.1, and 1 mg/ml

Dose (mg/ml)	Frequency of mating success	P-value
Control	0.486	0.24
0.01	0.514	
Control	0.595	0.10
0.1	0.405	
Control	0.639	0.02
1	0.361	

The P-value is the result of testing for the significant of an interaction between drug dose and mating success in a hierarchical log-linear model

Table 3 The mean longevity and 95% confidence intervals about the differences between flies raised on pioglitazone and controls

|	Mean	Variance	Difference (drug-control)	±95% c.i. on difference
Males			0.87	±0.54
Control	28.7	94.32		
Drug	29.5	85.5		
Females			1.13	±0.62
Control	29.0	108.4		
Drug	30.1	105.0		

patterns. Making inferences about these changes is a statistically more challenging undertaking.

Distribution of deaths

If we plot the quantiles from the drugged flies vs. the quantiles from the control flies (Fig. 6) we see that for both males and females the points are elevated slightly above the $x = y$ line at most ages. This suggests that drugged flies are dieing more slowly even from the start of the experiment. This would be consistent with a smaller age-independent Gompertz parameter for flies treated with Pioglitazone. Neither males nor females shows a tendency to increase the departures from the $x = y$ line at advanced ages. Therefore from this analysis we see no evidence of differences in the rate of ageing parameter.

Sampling units

A single sex, drug-level treatment consisted of 15 separate racks of adult flies, with approximately 160
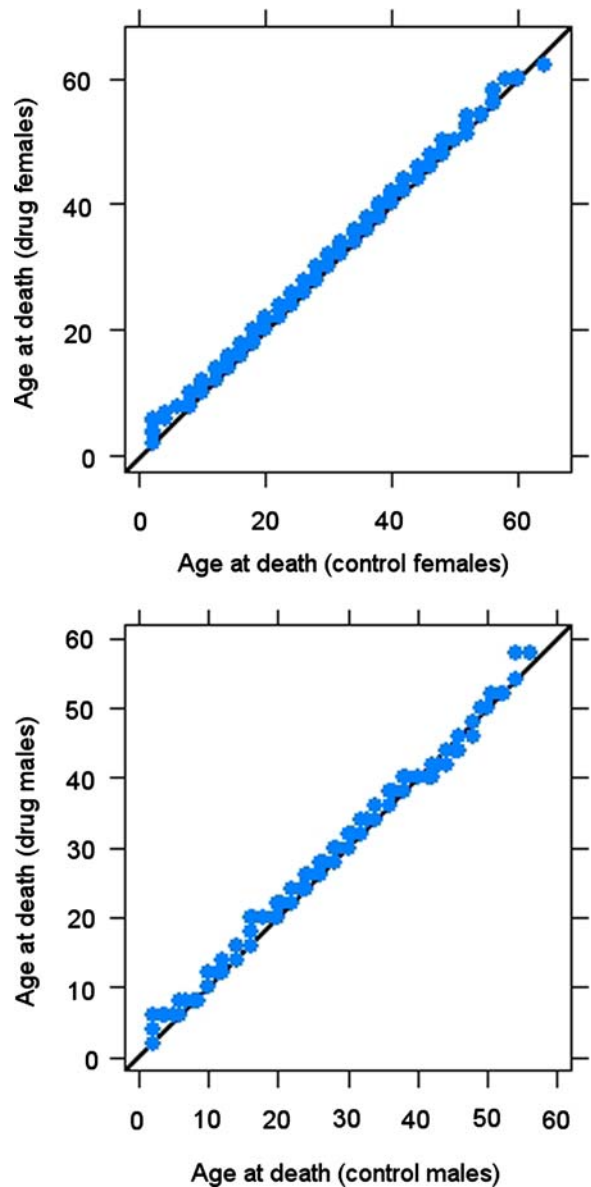
Fig 6 The quantiles of drugged flies plotted against the control flies quantiles

flies per rack. To some extent each rack can be considered its own micro-environment which may differ slightly from one rack to the next. There are two very different ways these data might be analyzed. The observations from all 15 racks can be pooled to form one set of observations which can then be used to estimate age-specific mortality. Alternatively, age-specific mortality can be estimated for each rack separately and then the results from all 15 racks pooled.

The parameters of the Gompertz model were estimated by regression analysis and thus the number of distinct ages and the variability of the underling mortality estimates determine the variance of the parameter estimates. Pooling all 15 racks will roughly increase the total sample size (N) by a factor of 15. We expect that in the pooled sample the variance of the estimated mortalities should decrease since the variance is approximately binomial and thus proportional the N^{-1}. However, the number of different ages will not increase linearly with increasing N, although it should be somewhat higher in the pooled sample than for any individual rack.

The control flies have actually been tested on two separate occasions or blocks: in the pioglitazone experiment and in a separate experiment not reported here testing the effects of *Rhodiola*. Thus, we can analyze these results with two different models. In the first model assume that there is random variation in the values of A and α due to each rack and that this level of variation was nested within the variation from each block. The second model pools the results from all 15 racks to create two samples, one per block. The estimates of A and α are each slightly different with each procedure (Fig. 7) although the differences are not statistically significant. However, the pooling procedure results in substantially higher standard errors for both parameters (Fig. 7).
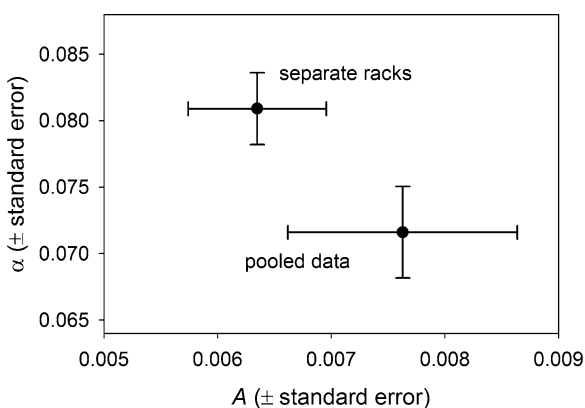
If we examine the fraction of the variance in A due to racks vs. blocks we find that 80% is due to racks. For α the fraction of variance due to racks is virtually 100%. We interpret this to mean that the small sample sizes of the racks leads to uncertainty in the estimated mortality rates and that this uncertainty affects α to a greater extent than A. Presumably if the number of flies in racks was reduced even further the standard error of α estimated from individual racks would be higher than the variance from a pooled estimate. While we have no general rule yet for an optimal size for racks it appears that in our study it is still better to analyze the data by keeping results from individual racks separate. Consequently, we have chosen to analyze the results of the experiments in this study by analyzing each rack separately.

Gompertz mortality dynamics

Males have a significantly lower value of A than do females (γ_1 Table 4) but a significantly higher value of α (γ_2 Table 4). Pioglitazone appears to significantly lower A (ϕ_1 Table 4) but caused a non-significant increase in the value of α (ϕ_2 Table 4). Pioglitazone lowers A by 15% in females and 57% in males Fig. 8).

Partial data set

For a population where mortality is well described by the Gompertz model, it may be possible to get good estimates of the model parameters with just the first few weeks of observations rather than waiting for every fly to die (Mueller et al. 1995). We repeated the analysis of the pioglitazone data set using only the

Fig 7 The estimated parameters of the Gompertz equation for the control population and their standard errors. Two methods of estimation are shown. The first method estimated the parameters separately for each of 15 racks in the experiment while the second method pooled all 15 racks into one population

Table 4 The parameter estimates for pioglitazone with model (3). Each parameter was tested to determine if it differed from zero

Parameter	Value	Standard error	d.f	P-value
β_1	0.0042	0.00028	1537	<0.0001
γ_1	−0.00099	0.00028	1537	0.0003
ϕ_1	−0.00063	0.00030	1537	0.034
β_2	0.093	0.0025	1537	<0.0001
γ_2	0.016	0.0027	1537	<0.0001
ϕ_2	0.0037	0.0031	1537	0.23

The statistical significance of this *t*-test is given in the column labeled *P*-value
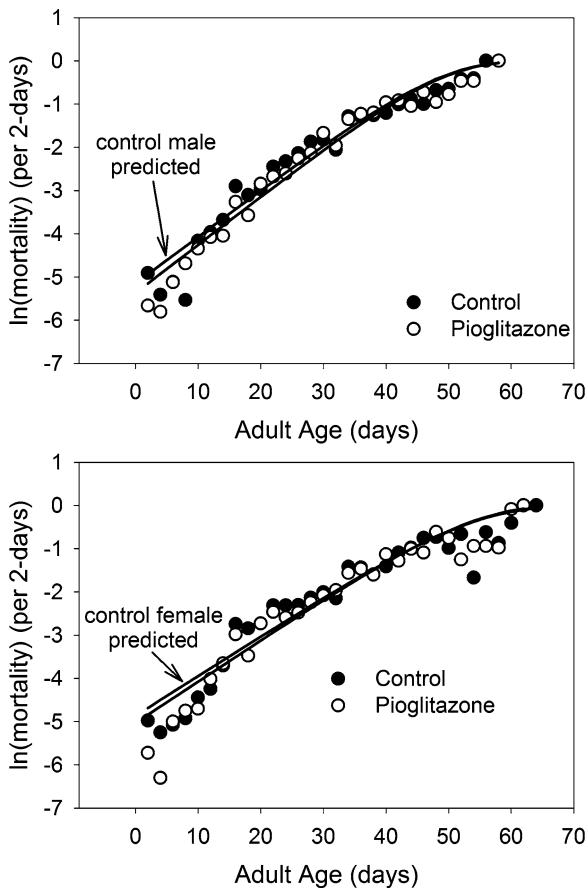
Fig 8 The natural log of mortality vs. age for control and pioglitazone treated flies. The predicted values are not linear since the predictions are for two-day intervals of mortality

first 28 days of data but otherwise using the same techniques as described previously. With the reduced dataset neither sex nor drug has a significant effect on A or α (Table 5).

Table 5 The parameter estimates for the partial (day 28) pioglitazone dataset with model (3)

Parameter	Value	Standard error	d.f	P-value
β_1	0.0021	0.00025	805	<0.0001
γ_1	−0.00024	0.00025	805	0.33
ϕ_1	−0.0002	0.00029	805	0.46
β_2	0.14	0.0056	805	<0.0001
γ_2	0.0058	0.0065	805	0.37
ϕ_2	−0.0039	0.0069	805	0.57

Each parameter was tested to determine if it differed from zero. The statistical significance of this t-test is given in the column labeled P-value

We have already pointed out that the effect of pioglitazone on longevity was near the limit of sensitivity for the full data set. As it turns out the average longevity was also very similar for males and females. Control females lived on average only 0.3 days longer than males and drugged females only 1.1 days longer than drugged males. Therefore, it is not surprising that when we reduce the sample size by almost 50% statistically significant effects become insignificant.

Two-stage Gompertz mortality dynamics

The estimation of the two-stage Gompertz parameters requires observations late in life to get reasonable estimates of the break day and plateau mortality rate. Consequently, we have not followed the previous protocol of obtaining individual estimates for each rack. Additionally, there are now four parameters to estimate rather than two and the estimation is done separately for each sex and drug treatment. We expect that our ability to infer differences between treatments when they exist will decline relative to the power available with the Gompertz model. The estimated parameters for the two-stage Gompertz show no significant differences between the control and pioglitazone treated flies (Table 6). The control

Table 6 The estimated parameters values and the bootstrap tests for significant differences between control and drug flies

Parameter	Control male	Drug male	Control female	Drug female
A	0.0082	0.0087	0.0099	0.0093
Difference	0.00047		−0.00063	
P-value	0.44		0.45	
α	0.079	0.080	0.072	0.072
Difference	0.0012		0.00032	
P-value	0.46		0.49	
A_2	0.78	0.58	0.26	0.065
Difference	−0.20		−0.20	
P-value	0.29		0.21	
Break Day	51.4	49.2	44.9	50.3
Difference	−2.2		5.4	
P-value	0.23		0.24	

The difference is computed as the drug fly value minus the control fly value. The P-values are based on 1,000 bootstrap samples

and pioglitazone treated males have very similar values for all four parameters (Table 6, Figs. 9, 10).

Except for A_2 females from both treatment groups also show very similar parameter values (Table 6, Fig. 10). In light of the very similar distributions of

Fig 9 The mortality rates vs. age for males with predictions from the two-stage Gompertz model. The filled circles are drug treated flies and the open circles are the control flies

Fig 10 The mortality rates vs. age for females with predictions from the two-stage Gompertz model. The filled circles are drug treated flies and the open circles are the control flies

deaths (Fig. 6) for both males and females the lack of significant differences is not surprising.

Discussion

The anti-aging properties of piolglitazone might in principle be explained by a caloric restriction effect on reproduction that secondarily reduces mortality (Chippindale et al. 1993). The mechanism behind CR is not very well understood. However the regulation of insulin and insulin-like growth factor (IGF) hormones are involved in the response of nematodes, insects, and mammals to restricted-calorie diets that increase longevity (Rogina et al. 2000; Tatar et al. 2003). In *Drosophila*, mutations in the insulin receptor gene (*InR*) and the insulin receptor substrate (*chico*) increase average lifespan in females (Lee et al. 2003; Marden et al. 2003; Tu et al. 2005). Similarly, modifications in the *C. elegans* insulin pathway can produce positive longevity effects by regulating downstream targets such as *Daf-2* and *Daf-16* genes (Kenyon 2001; Lund et al. 2002).

Silent mating-type Information Regulation-2 (SIR2) homologs, a conserved deacetylase, have been associated with important related effects of CR. SIR2 is a conserved enzyme across many model organisms. An increase in the activity of this enzyme by dietary restriction has resulted in increased longevity in yeast, worms, flies, and rodents (Haigis and Guarente 2006; Rogina et al. 2002; Tissenbaum and Guarente 2002). It is hypothesized that SIR2 pathways promote stress resistance while playing important roles in DNA repair, gene silencing, and rDNA recombination (Gasser and Cockell 2001; Guarente 2001; Rogina et al. 2002).

Although the anti-aging mechanism of piolglitazone was not evaluated in our study, it is conceivable that piolglitazone acts as a CRM. Pioglitazone is a thiazoldinediones, a class of oral anti-diabetic drugs used in patients with type II diabetes. Its mechanism of action involves activation of the gamma isoform of the peroxisome proliferator-activated (PPAR-γ) which results in transcription of several genes involved in glucose and lipid metabolism. The ultimate clinical outcome of piolglitazone is to sensitize insulin receptors for a more efficient glucose uptake via GLUT4 glucose transporters. Pioglitazone increases glycolysis, glucose oxidation, and lowers
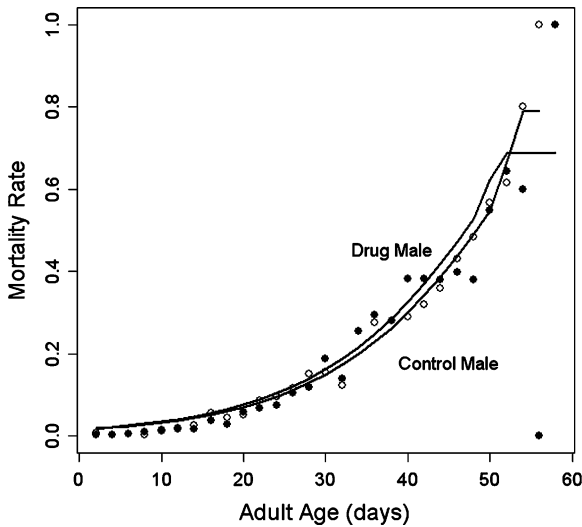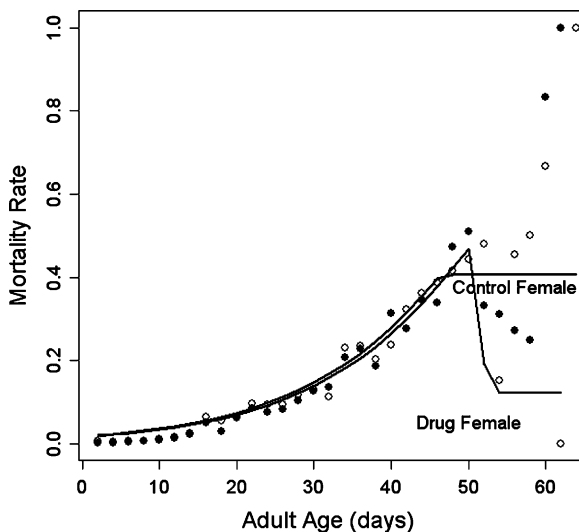
triglyceride levels. The cellular effects of piolglitaz-one mimic the metabolic, hormonal, and physiological effects of CR. As a result, the drug could potentially act as a CRM for pharmacological regulation of insulin/IGF pathways.

Another reason why pioglitazone could act as a good CRM is due to its regulation of SIRT1, a homolog of SIR2. It is hypothesized that CR increases the NAD+ to NAD ratio, a necessary mechanism for controlling SIRT1. The activity of SIRT1 is correlated with the activity of PPAR-γ, suggesting that SIRT1 can mildly suppress PPAR-γ to prevent lipid allocation into adipocytes and prevent diseases such as diabetes and atherosclerosis (Guarente and Picard 2005).

Another theory to explain our observed anti-aging properties with piolglitazone is the fact that this compound is a glycation inhibitor. It is established that the process of glycosylation is an important etiology for aging. Glycosylation results in glycated proteins. These proteins may react with any other proteins resulting in irreversible 'cross-linking'. The ultimate outcome of cross-linking is the formation of clusters of damaged protein products, named AGEs (Advanced Glycosylation End products). AGEs may interact with free radicals and other tissues resulting in oxidation and further tissue injury. By inhibiting the formation of AGEs, piolglitazone may delay the aging process (Rahbar et al. 2000).

Our study showed that piolglitazone has anti-aging properties without significant impact on metabolic rate, fecundity, or CNS. As CR effects are characteristically associated with greatly reduced fecundity (Chippindale et al. 1993), the absence of such effects in our experimental cohorts suggests that pioglitazone does not act as a CR mimetic. Thus the hypothesis that this drug's anti-aging mechanism of action is due to its CRM properties faces significant difficulties.

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