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Extended longevity and survivorship during amino-acid starvation in a *Drosophila Sir2* mutant heterozygote

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Abstract:

The regulation of energy homeostasis is pivotal in order to survive periods of inadequate nutrition. A combination of intricate pathways and proteins are responsible for maximizing longevity during such conditions. The sirtuin deacetylase Sir2 is well conserved from single-celled yeast to mammals, and controls a number of downstream targets that are active during periods of extreme stress.

Overexpression of *Sir2* has been established to enhance survival of a number of model organisms undergoing calorie restriction, during which insulin receptor signalling (IRS) is reduced; a condition that itself can enhance survivorship during starvation. Increased *Sir2* expression and reduced IRS result in an increase in the activity of the transcription factor foxo; advantageous during stress but lethal when overly active. We have found that a lowered gene dosage of *Sir2*, in mutant heterozygotes, can extend normal longevity and greatly augment survivorship during amino-acid starvation in *Drosophila*. Additionally these mutants, in either heterozygous or homozygous form, do not appear to have any disadvantageous effects upon development or cell growth of the organism unlike IRS mutants. These results may advance the understanding of the biological response to starvation, and allow for the development of a model organism to mimic the ability of individuals to tolerate nutrient deprivation.

Key words: *Drosophila melanogaster*, Sir2, longevity, starvation survival

Introduction

Irregular eating patterns and periods of malnutrition are often the consequence of psychological disorders that affect the standard behaviour of eating. The prevalence of such disorders are estimated to be 0.3% (anorexia) to 1% (bulimia) among adolescent and young women, with the frequency believed to increase during the transition between adolescence and young adulthood in some western European populations (Hoek 2007). The etiology of complicated disorders can be multifactorial, but there is some evidence in the recent advances in neurobiology to suggest that genetic factors may play a particularly strong role. Given the fluctuation in the availability of nutrition during the lifespan of any organism, the evolution of biological pathways allows for balancing of caloric restriction with energy homeostasis to maximise longevity. Key components of nutrient sensing, like the well-conserved insulin receptor signalling (IRS) pathway and sirtuins (Brown-Borg 2003; Bishop and Guarente 2007; Broughton and Partridge 2009; Longo 2009), are significant in the regulation of metabolic homeostasis.

The first sirtuin was identified in the yeast *S. cerevisiae* (Klar et al. 1979; Ivy et al. 1985; Ivy et al. 1986) and named silent information regular-2 (Sir2) (Frye 2000). Mammals possess seven sirtuins (SIRT1 through 7) that occupy different subcellular compartments such as the nucleus (SIRT1, SIRT2, SIRT6, and SIRT7), cytoplasm (SIRT1 and SIRT2) and the mitochondria (SIRT3, SIRT4 and SIRT5). These proteins are deacetylases that mediate transcription of genes involved in metabolic homeostasis (Rodgers et al. 2005; Bordone et al. 2006; Rodgers et al. 2007). Mammalian sirtuins control the activity of several transcription factors, such as p53, foxo and NF- κ B, that influence the expression of a number of genes that respond to various stresses (Brunet et al. 2004, Longo 2009). The role of sirtuins in metabolic homeostasis was further validated when it was observed that small molecule activators of SIRT1 are active in the same pathways that are induced during caloric restriction (Wang 2014). In yeast extra copies of *Sir2* extend lifespan by 30% but shorten lifespan by 50% when deleted (Kaeberlein et al. 1999). In *C. elegans*, an extra copy of the *Sir2* homologue, *Sir2.1*, extends lifespan by 50% (Tissenbaum and

Guarente 2001; Hoffmann et al. 2013). Both ubiquitous overexpression and pan neural overexpression of the homologue of *Sir2* in adult *Drosophila* extends lifespan (Rogina and Helfand 2004; Bauer et al. 2009). In mice with no copies of SIRT1, a shorter median lifespan is observed, but elevated SIRT1 activity results in mice that are more metabolically active, more glucose tolerant and have lower levels of cholesterol and insulin (Wang 2014). These changes are akin to those seen in mice that are fed calorie-restricted diets, and show an extension in lifespan.

Overexpression of sirtuins increase longevity during conditions of adequate nutrition and during nutritional stress. A number of the physiological pathways active during dietary restriction are also active when *Sir2* is overexpressed (Antosh et al. 2011). An increase in neuronal expression of *Sir2* in *Drosophila* undergoing dietary restriction has been shown to increase lifespan (Rogina and Helfand 2004; Bauer et al. 2009). Microarray analysis of *Drosophila* with extended longevity caused by an overexpression of *Sir2* reveals that a total of 782 genes are altered in expression (Antosh et al. 2011). Of those, 72% of the genes that are up-regulated and 61% of the genes that are down-regulated are similarly altered in *Drosophila* undergoing dietary restriction. Additionally the activity of *Sir2* is elevated in control flies when starved (Banerjee et al. 2012). *Drosophila Sir2* plays a crucial role in fat metabolism and systemic insulin signalling as it produces a critical factor in fat mobilization from the fat body during starvation, therefore it is expected that elevated *Sir2* expression enhances survival in *Drosophila* undergoing starvation. A moderate increase in the expression of *Sir2* in the fat body of adult *Drosophila* is sufficient to extend lifespan by 13% (Hoffman et al. 2013). However, weak overexpression of *Sir2* in the fat body is sufficient to increase the survival in both female (12%) and male (13%) flies when compared to the controls that are not undergoing nutritional stress. It is clear that the action of *Sir2* is essential in the survival of *Drosophila* that are ingesting fewer nutrients, but to what extent?

Overexpression, or extra copies, of *Sir2* in a number of experimental organisms enhance both longevity and survival during suboptimal nutrient conditions while complete loss of *Sir2* is detrimental to

the survival of organisms in any condition (Li et al. 2008; Brunet et al. 2011; Banerjee et al. 2012). Conversely, IRS pathway (i.e. *Akt1*) mutants can enhance survival during starvation (Slade and Staveley 2016). As Sir2 acts as a nutrient sensing protein similar to that of the IRS pathway, we were interested in investigating the effect of a less active Sir2 upon survivorship. Presently the optimal nutrient composition to maximize lifespan but avoid malnutrition has not yet been established but in particular, it seems to be more effective to restrict certain types of amino acids (Yamada et al. 2013). In *Drosophila*, it is the reduction of amino acids rather than sugar that leads to a “dietary restriction” extension in lifespan (Grandison et al. 2009, Wang 2014). In response, we have investigated the effects of Sir2 heterozygous and homozygous alleles upon the survivorship of *Drosophila* when deprived of amino acids.

Materials and Methods:

Drosophila stocks, media and culture and generation of Drosophila crosses

The wild-type Oregon R (*OrR*) stock was obtained from the Bloomington *Drosophila* Stock Center and *w*¹¹¹⁸ was obtained from Dr. Howard Lipshitz from the University of Toronto. The *Sir2* mutant stocks were obtained from the Bloomington *Drosophila* Stock center (Indiana). Two null mutants and two insertional mutants were observed. *Sir2*¹⁷ is a null mutant generated from an imprecise excision of P(lacW)7223 P element inserted within the 5' UTR of the *Sir2* gene that resulted in a deletion of the majority of the *Sir2* coding sequence and does not produce a protein (Astrom et al. 2003). The other null mutant, *Sir2*^{2A-7-11}, is a targeted knock-out that deletes the entire *Sir2* coding sequence (Furuyama et al. 2004). The insertional mutant *Sir2*^{EP2300} reduces the protein levels of Sir2 by at least 5-fold due to the insertion of an EP transposon 427 base pairs 5' of the start codon in the *Sir2* transcription unit (Furuyama et al. 2004). *Sir2*⁰⁵³²⁷ is the result of a nearby insertion of a PZ P element within the 5' UTR 460 base pairs from the *Sir2* initiation codon (Furuyama et al. 2004). All mutants are viable as

homozygotes with the exception of *Sir2*⁰⁵³²⁷. Control flies were generated through outcrossing of wild-type (OrR) to *w*¹¹¹⁸ to minimize second site effects and maximize longevity. The *Sir2* mutants were outcrossed to *w*¹¹¹⁸ for comparison. Stocks and crosses were maintained on a standard medium containing cornmeal, molasses, yeast, agar and water. Routinely, stocks were kept at room temperature ($22 \pm 2^\circ\text{C}$) while crosses and experiments were carried out at 25°C .

Biometric analysis of Drosophila eyes

Critical class males of the control and *Sir2* mutant alleles were collected and aged for three days. Flies were then quickly frozen at -80°C before preparation for scanning electron microscopy according to standard procedures (Slade and Staveley 2015). Preparation included mounting upon aluminum SEM studs, desiccated and sputter coating in gold. The *Drosophila* eye normally consists of 700 to 800 ommatidia that consistently develop in a highly regulated manner (Baker 2001) and is thus ideal for the study of cell growth. Images of the eyes were taken with either a Hitachi S-170 or S-570 Scanning Electron Microscope as per standard methods and analyzed using NIH ImageJ software.

Longevity Assay

Experiments were carried out on standard media at 25°C . Critical class males of the control and *Sir2* mutant alleles were collected upon eclosion and aged, per genotype, at a density of ≤ 20 flies per vial. Adults were kept on fresh media, which was replenished every four to six days. Flies were observed and scored every two days for presence of deceased adults. Flies were considered dead when they did not display movement upon agitation. Longevity data was analyzed using the GraphPad Prism 5 program. Survival curves were compared using the log-rank test, a statistical test that compares the actual and expected number of failures (death) between survival curves at each individual failure event. Significance was determined at 95%, at a P-value less than or equal to 0.05.

Starvation Assay

As in the longevity assay, male flies were observed to avoid the potential effects of reproduction investment associated with female flies. Critical class male flies were collected per genotype, and maintained in non-crowded conditions by a maximum number of 20 flies per vial according to standard procedures (Slade and Staveley 2016). Adults were aged on fresh amino-acid starvation medium, consisting of 5% sucrose in phosphate buffered saline solution and 3% agar, which was replenished every four to six days. Studies of survival upon the starvation media were carried out and analyzed as described previously for the longevity assay. Flies were scored every two days for presence of deceased adults. Adults were considered dead when they did not display movement upon agitation. Results were analyzed using the GraphPad Prism 5.00 program. Survival curves were compared using the log-rank test. Significance was determined at 95%, at a P-value less than or equal to 0.05.

Results:

Sir2 mutant homozygotes and heterozygotes grow normally

To determine if altering the expression levels of *Sir2* affected cell growth, ommatidia number and area was measured for *Sir2/Sir2* homozygous and *Sir2/+* heterozygous null and insertional mutants and compared to a wild type outcross control. The control (N= 15) had a mean value of 697.7 ± 7.45 ommatidia (Figure 1, Table 1). None of the ommatidia counts for the mutants were greatly different from the control with mean values ranging between 660.3 ± 11.12 (*Sir2^{EP2300}*, N=15) to 725.3 ± 6.76 (*Sir2¹⁷/+*, N=11) (Figure 1, Table 1). Similar results were observed when comparing the size of ommatidia. The control (N = 45) had a mean ommatidium area of $186.8 \pm 2 \text{ um}^2$ (Figure 1, Table 1). The range of mean ommatidium area for the mutants was $177 \pm 1.49 \text{ um}^2$ (*Sir2¹⁷*, N = 33) to $207.8 \pm 1.8 \text{ um}^2$ (*Sir2¹⁷/+*, N = 33) (Figure 1, Table 1). These values were all within the standard error of the mean

when compared to the control, indicating there was no significant difference in the size of ommatidium when *Sir2* is altered.

Sir2 mutant heterozygotes have extended longevity

The longevity of the *Sir2*⁺/*Sir2*⁺ outcrossed control (N = 377) was 64 days (Figure 2, Table 2). In comparison, two of the mutant heterozygotes outlived the control with *Sir2*¹⁷/+ (N = 324) exhibiting a median longevity of 76 days (additional 12 days), and the insertional mutant heterozygote *Sir2*⁰⁵³²⁷/+ (N = 322) having a median longevity of 80 days (additional 16 days; Figure 2, Table 2). The other heterozygous mutants had median longevitys that were not significantly different from the control (66 days for *Sir2*^{2A-7-11}/+ , N = 322, and 62 days for *Sir2*^{EP2300}/+, N = 301). All homozygous mutants had a diminished longevity (24-48 days) (Figure 2, Table 2).

*Sir2*⁰⁵³²⁷ insertional mutant heterozygotes are able to endure amino-acid starvation

The median day of survivorship for the wild type *Sir2*⁺/*Sir2*⁺ outcrossed control upon amino-acid starvation media (N = 369) was 24 days (Figure 3, Table 2). Predominantly the heterozygous and null *Sir2* mutants exhibited either comparable or slightly reduced survivorship in comparison. *Sir2*^{2A-7-11} was reduced (median survivorship of 14 days as a homozygote, N = 381, and 18 days as a heterozygote, N = 434) while *Sir2*^{EP2300} was on par with the control (median survivorship of 22 days as either a homozygote, N = 354, or heterozygote, N = 363) (Figure 3, Table 2). The null mutant *Sir2*¹⁷, which had an extension in longevity compared to the control, was either on par (as a heterozygote, 22 days, N = 398) or reduced (as a homozygote, 20 days, N = 308) in survivorship upon amino-acid starvation media when compared to the control (Figure 3, Table 2). However, the *Sir2*⁰⁵³²⁷/+ insertional mutant heterozygote (N = 384) was demonstrated to have a noteworthy survivorship with an extended median of 38 days (Figure 3, Table 2), 14 days greater than that of the control.

Discussion:

The subtle alteration of *Sir2* activity, as in mutant heterozygotes, results in *Drosophila* that are not strongly affected during growth or development, but may have slightly increased longevity and can be capable of surviving for extended periods of amino-acid starvation. This intermediate reduction is functionally significant, as the complete loss of *Sir2*, as in the heterozygous null mutants, are significantly reduced in longevity. This finding is parallel to analysis of longevity in mammalian models as SIRT1 knockout mice die early, regardless of being either free fed or on caloric restriction (Li et al. 2008). While caloric restriction has been shown to enhance survival of a number of organisms, deleting the *Sir2* homologue in yeast abolishes the increase in survivorship observed with calorie restriction (Brunet et al. 2011). In addition, a *Drosophila* null mutant of *Sir2* is more sensitive to starvation (complete starvation) when compared to controls (Banerjee et al. 2012). Although total loss of *Sir2* is detrimental, it is interesting that reducing the gene dosage of *Sir2* through null and insertional mutant heterozygotes can result in extensions in longevity and survival.

A sufficient level of *Sir2* activity is required during starvation for the regulation of downstream targets responsible for surviving stresses. SIRT1 interacts with PGC-1, which is responsible for inducing the activity of gluconeogenic genes and regulating hepatic glucose output. Activation of these genes would be beneficial during periods of low nutrition (Longo 2009). In addition to this, SIRT1 has been shown to increase the release of insulin and therefore the sensitivity to insulin receptor signalling (Moynihan et al. 2005; Bordone et al. 2006) aiding in extending the survival of mice fed a high fat diet (Bauer et al. 2006). As both sirtuins and the IRS pathway act as nutrient sensing regulators, it is not unexpected that they may interact with each other, especially during conditions of altered nutritional availability or intake.

The link between sirtuins and the well-conserved nutrient sensing IRS pathway has been established as a knockdown of *Sir2* in the fat body leads to increased *insulin-like peptide 5 (ilp5)* mediated IRS (Banerjee et al. 2012). In *Drosophila* that are starved, *ilp5* levels are decreased (Taguchi and White 2008) yet this decrease is not observed in *Drosophila* with a knock-down of *Sir2* in the fat body (Banerjee et al. 2012). Reduced IRS is associated with enhanced survival during starvation. Hypomorphic mutants of the serine/threonine kinase *Akt1*, a central component in insulin signalling, outlive control *Drosophila* when aged upon amino-acid deprived media (Slade and Staveley 2016). Reduced insulin/IGF signalling works through the PI3K/Akt1/foxo mechanism to protect the cell against oxidative stress, among other types of stresses, in model organisms such as *C. elegans* and *Drosophila*, and in mice (Kenyon 2001; Holzenberger et al. 2003; Longo and Finch 2003). The homologue of *Sir2* in *C. elegans* interacts with 14-3-3 proteins to activate DAF-16, the protein homologue of the transcription factor foxo (Brunet et al. 2004; Berdichevsky et al. 2006; Wang and Tissenbaum 2006). In mammals, it has been shown that SIRT1 reverses the acetylation of FOXO1 to aid in its activation (Calnan and Brunet 2008). The acetylation of FOXO1 enhances its phosphorylation by Akt1 (Matsuzaki et al. 2005), and conversely the deacetylation by SIRT1 can override this phosphorylation (Frescas et al. 2005) to allow FOXO1 to enter the nucleus and activate its downstream targets. In the amino-acid starved *Akt1* mutants, foxo is required to generate the extension in survival phenotype (Slade and Staveley 2016). As *Akt1* activity is lowered, *foxo* activity is elevated. *Sir2* can enhance the activation of foxo, and expression of both genes are elevated during starvation (Kramer et al. 2008; Banerjee et al. 2012) to aid in protecting cells from oxidative stress, which may account for the extension in survival observed when *Sir2* is overexpressed. However, an abundance of *foxo* expression and activity is lethal (Kramer et al. 2003). Therefore slightly reducing foxo through the subtle reduction of *Sir2* may allow for enhancing survival in a similar manner as the *Akt1* mutants, which is observed.

A physiological response to starvation in aid of survival is an increase in lipid storage as lipid droplets are of central importance to fat utilization (Stout et al. 1976; Neumann-Haefelin et al. 2004; Kuhnlein 2011; Hoffman et al. 2013). The gene expression profiles of fat bodies overexpressing *Sir2* in *Drosophila* demonstrate quite notable intersections with the lipid droplet proteome. Thirty-nine corresponding genes out of 254 lipid droplet associated proteins were found to be differently regulated in fat bodies overexpressing *Sir2* – equivalent to 15% of the lipid droplet proteome. These include genes for lipid storage, fatty acid metabolism, and stress-response chromatin-assembly (Hoffmann et al. 2013). RT-qPCR shows that genes involved in fat metabolism are down-regulated and genes involved in fat storage are up-regulated in *Sir2* mutants (Banerjee et al. 2012) and the opposite is observed with the overexpression of *Sir2*. In *Drosophila* loss-of-function *Sir2*, there is an increase in triglyceride (TAG) levels and deregulated fat metabolism, and an increase in glucose levels (Banerjee et al. 2012). Overexpression of *Sir2*, on the other hand, decreases the level of stored fat. Simple reduction of the level of *Sir2* expression does not show a similar increase in glucose, but does lead to an increase in TAG levels. Irrespective of dietary regime, there was no significant difference in the weight of flies with increased TAG levels compared to controls, although mutants appear to be unable to breakdown the amassed TAGs due to the expression of genes required for fat breakdown not becoming elevated as observed in control flies undergoing starvation (Banerjee et al. 2012). It is possible that the heterozygous mutants in this study may have an elevated level of stored fats that could aid in their survival.

Past research has indicated that nutrient sensing pathways, such as IRS, and proteins, like sirtuins, can play a pivotal role in mediation of food intake/energy usage in order to maximize longevity. Typically, it is the reduction in insulin signalling or the overexpression of *Sir2* that has led to the extended survivability. Here we show that the potential reduction in *Sir2* activity via null and insertional heterozygous mutants allows for a similar outcome as reduced insulin signalling, potentially through the

subtle increase in *foxo* activity. This extension in survivorship may be consequential in the search for therapeutic treatments of disordered eating.

List of abbreviations

Sir2: silent information regulator 2; foxo: forkhead box subgroup “O”; Ilp: insulin-like peptide; IRS: insulin receptor signalling

Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

JDS performed the scanning electron microscopy, biometric analysis of the eyes, longevity and starvation assays, carried out the statistical analyses and drafted the initial manuscript. BES conceived and participated in the design and supervision of the study and contributed significantly to the final draft of the manuscript. Both authors have read and approved the final manuscript.

Animal Ethics

This study was conducted under the approval of the Animal Care Committee of Memorial University of Newfoundland as a Category of Invasiveness Level A protocol under the project title of “Genetic, biochemical and molecular analysis of cell survival and cell death in *Drosophila melanogaster*” (protocol 277 number: 14-09-BS).

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Table 1: Mean values of ommatidia number and ommatidium area of null and insertional *Sir2* mutants

Genotype	Type of Mutant	N	Mean Value of Ommatidia Number	N	Mean Value of Ommatidium Area (μm^2)
<i>Sir2</i> ⁺	N/A	15	697.7 $\pm$ 7.45	45	186.8 $\pm$ 2
<i>Sir2</i> ¹⁷	Null	11	668.5 $\pm$ 12.52	33	177 $\pm$ 1.49
<i>Sir2</i> ¹⁷ /+	Heterozygous Null	11	725.3 $\pm$ 6.76	33	207.8 $\pm$ 1.81
<i>Sir2</i> ^{2A711}	Null	10	668.9 $\pm$ 21.39	30	195.8 $\pm$ 2.03
<i>Sir2</i> ^{2A711} /+	Heterozygous Null	11	664.9 $\pm$ 17.32	33	197 $\pm$ 2.19
<i>Sir2</i> ^{EP2300}	Insertional	15	660.3 $\pm$ 11.12	45	183.4 $\pm$ 1.55
<i>Sir2</i> ^{EP2300} /+	Heterozygous insertional	10	681.2 $\pm$ 17.6	30	201.3 $\pm$ 1.8
<i>Sir2</i> ⁰⁵³²⁷ /+	Heterozygous insertional	6	723.7 $\pm$ 7.6	22	202.5 $\pm$ 1.7

Table 2: Median days of longevity and survivorship upon amino-acid starvation media for null and insertional *Sir2* mutants

Genotype	Type of Mutant	N	Median Day of Longevity	N	Median Day of Survival on Amino-Acid Starvation Media
<i>Sir2</i> ⁺	N/A	377	64	369	24
<i>Sir2</i> ¹⁷	Null	82	24	308	20
<i>Sir2</i> ¹⁷ /+	Heterozygous Null	324	76	398	22
<i>Sir2</i> ^{2A711}	Null	361	48	381	14
<i>Sir2</i> ^{2A711} /+	Heterozygous Null	322	66	434	18
<i>Sir2</i> ^{EP2300}	Insertional	183	24	354	22
<i>Sir2</i> ^{EP2300} /+	Heterozygous insertional	301	62	363	22
<i>Sir2</i> ⁰⁵³²⁷ /+	Heterozygous insertional	322	80	384	38

Figure 1: *Sir2* mutant homozygotes and heterozygotes do not differ in size or number of ommatidia

from the control. Scanning electron micrographs illustrate the phenotypes of the control and *Sir2* mutant homozygotes and heterozygotes. Corresponding genotypes are **A:** *Sir2*⁺, **B:** *Sir2*¹⁷, **C:** *Sir2*¹⁷/+, **D:** *Sir2*^{2A711}, **E:** *Sir2*^{2A711}/+, **F:** *Sir2*^{EP2300}, **G:** *Sir2*^{EP2300}/+, **H:** *Sir2*⁰⁵³²⁷/+. Scale bar = 200 um. Biometric analysis of both the number (**I**) and area (**J**) of ommatidia indicates there is no significant difference between the mutants and the control. N values can be found in Table 1. Error bars represent the standard error of the mean.

Figure 2: Selected *Sir2* mutant heterozygotes show extended longevity when compared to the control.

Longevity is shown as percent survival (P < 0.05 as determined by log rank). The dotted line represents the median longevity of the flies. All homozygous *Sir2* mutants were decreased in longevity compared to the control. *Sir2* mutant heterozygotes were either comparable to the control, or had an extension in median longevity of between 12 (*Sir2*¹⁷/+) and 16 days (*Sir2*⁰⁵³²⁷/+). N-values can be found in Table 2. Error bars represent the standard error of the mean.

Figure 3: The *Sir2*⁰⁵³²⁷/+ mutant heterozygote is able to endure amino-acid starvation. Survivorship is shown as percent survival (P < 0.05 as determined by log rank). The dotted line represents the median survivorship of the flies. All *Sir2* mutant homozygotes and heterozygotes were either similar or diminished in survivorship compared to the control with the exception of one. *Sir2*⁰⁵³²⁷/+ mutant heterozygotes revealed a considerable extension in median survivorship of 38 days, 14 days longer than that of the control. N-values can be found in Table 2. Error bars represent the standard error of the mean.

Figure 1: *Sir2* mutant homozygotes and heterozygotes do not differ in size or number of ommatidia from the control.
152x152mm (300 x 300 DPI)

Figure 2: Selected *Sir2* mutant heterozygotes show extended longevity when compared to the control.
354x224mm (300 x 300 DPI)

Figure 3: The *Sir2*^{05327/+} mutant heterozygote is able to endure amino-acid starvation.
365x264mm (300 x 300 DPI)