Evaluating starvation resistance in *Drosophila melanogaster*

By

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A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biology

Memorial University of Newfoundland

May 2016

St, John’s, Newfoundland and Labrador

Abstract

Feeding is a complex behaviour that must be regulated to maintain an appropriate energy balance and avoid death by starvation. Adjustments to either the homeostatic regulation, including complex signaling cascades and neuropeptides, or to the post-feeding reward systems could substantially affect the reserve of energy and thus survivorship in times of nutrient adversity. This thesis uses *Drosophila melanogaster* to model the biological basis of starvation and determine how to enhance survivorship upon amino acid starvation media via manipulating components that control feeding. The conserved insulin receptor pathway and its endpoint effector the foxo transcription factor are pivotal for survival during nutritional stress. The Akt1 kinase and the Sir2 deacetylase are modifiers of foxo activity. Novel *Akt1* hypomorphs show a significant increase in survival on amino acid deprived media, yet have decreased lifespan and growth when aged upon standard media. When these mutants are combined with null *foxo* mutants, biometric analysis and longevity evaluation indicate a phenotype similar to the original *foxo* mutant signifying its necessity in the *Akt1* phenotype. Investigation of mutant *Sir2* heterozygotes showed that they do not have altered growth when raised upon standard conditions, yet exhibit a greatly extended lifespan when reared on both a standard diet and when starved of amino acids. The neuropeptide NPF, a homologue of mammalian NPY, acts to induce feeding within the homeostatic regulation of the behaviour. Drosophila also bear a shorter form of NPF known as short NPF (sNPF) that can influence feeding. Overexpression or reduced expression of *NPF* or *sNPF* increased sensitivity and diminished survivorship upon amino acid starvation media. The neural hormone regulator, the dopamine transporter (DAT) works to clear dopamine from the synapses. This action may manipulate the post-feeding reward circuit in that lowered dopamine levels depress feeding and excess dopamine can encourage feeding. When *DAT* is either over-expressed or reduced via mutation, Drosophila have an increased sensitivity

to amino acid starvation. Taken together these results indicate that subtle variations in the expression of key components of these systems impacts survivorship during reduced nutrient conditions. These findings may advance the understanding of the biological response to starvation to aid in treatment of eating disorders or malnourishment.

Acknowledgements

I would like to thank my supervisor, Dr. Brian Staveley, for providing his advice, financial support and unwavering patience throughout the duration of my degree. I would also like to thank Drs.Helene Volkoff and Andrew Lang for their advice as members of my supervisory committee. Thanks to Drs. Liqui Men, formerly of the Bio-molecular Imaging Cluster at Memorial University of Newfoundland, and Michael Shaffer, formerly of the Microanalysis facility of Memorial University of Newfoundland for technical assistance with scanning electron microscopy. Thanks to members of the Genomics and Proteomics facility at Memorial University of Newfoundland for assistance with sequencing. For Drosophila lines I would like to thank Drs. Martin Junger and Ernst Hafen for providing the *foxo*^{W124X} line, Dr. Howard Lipshitz for providing the w^{1118} line, Dr. Norbert Perrimon for providing the original w^{1118} *; P[FRT ;*w^{+}*]*^{2A} *P[ry*$^{+}$ *neo*^{R} *FRT]*^{82B} line, Dr. P. Shen (University of Georgia) for the NPF lines, Dr. K. Yu (Korea Research Institute of Bioscience and Biotechnology) for the sNPF lines, Dr. R. Jackson (Tufts University) for the DAT mutant line, and the Bloomington Drosophila Stock Center (University of Indiana, Bloomington, USA) for the other Drosophila lines. I'd like to thank the biology department staff as well as all members of the Staveley lab, past and present, for providing any assistance when required. Funding for this work was provided by Natural Sciences and Engineering Research Council of Canada Post-Graduate Scholarship, the School of Graduate Studies of Memorial University of Newfoundland, and a number of CUPE contracts to teach undergraduate biology labs. Finally I would like to thank my family, especially my parents, for giving me words of encouragement during stressful times, and all the love and support I needed to finish this degree.

Co-Authorship Statement

The following statement clarifies the roles played by the authors in the manuscript chapters of this thesis, 2, 3, 4 and 5. In accordance with the requirements of the School of Graduate Studies, my role in the completion of these manuscript chapters is defined with respect to the following categories*: i) design and identification of the research proposal, ii) practical aspects of the research, iii) data analysis,* and *iv) manuscript preparation.*

i) design and identification of the research proposal and ii) practical aspects of the research: Chapters 2 and 3: BES initiated the research project by generating the novel Akt1 hypomorphs, and assisting with the generation of recombinant double mutants. JDS completed the generation of the recombinant double mutants. All biometric analysis, longevity and survivorship assays were carried out by JDS. Chapters 4 and 5: All experiments were conceived of through joint discussion between JDS and BES. All experiments were carried out by JDS.

iii) data analysis: JDS collected and analyzed all data.

iv) manuscript preparation: All manuscripts were prepared by JDS with critical review from BES

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List of Abbreviations

4E-BP- eIF4E binding protein
aa- amino acid
ABA – activity based anorexia
ADHD – attention deficient hyperactivity disorder
AGRP – Agouti related peptide
Akh – adipokinetic hormone
AN – anorexia nervosa
Bcl-2 –
Bim – Bcl-2 interacting mediator of cell death
CART – cocaine and amphetamine regulated transcript
CCK – cholecystokinin
Cdks – cyclin dependent kinases
cGMP – cyclic GMP
CNS – central nervous system
CRF – corticotropin releasing factor
DAT – dopamine transporter
DH – diuretic hormone
DR – dietary restriction
FaRPs – FMRFamide-like peptides
FG – frontal ganglion
FLP – flippase recombinase
Fmn – fumin (DAT mutant)
for – foraging
Foxo – forkhead box "O"
FRT – flippase recombination target
GFP – green fluorescent protein
GLP – glucagon-like peptide
GPCR – G-protein coupled receptor
GPCR –G-protein coupled receptor
HCG – hypocerebral ganglion
ICV – intracerebroventricular
IGF – insulin like growth factor
Ilp – insulin-like peptide
INR – insulin receptor
INRS – insulin receptor substrate
IRS – insulin receptor signalling
MCH – meanin-concentrating hormone
MCH – melanin-concentrating hormone
MnSOD – Manganese superoxide dismutase
MSH – α-melanocyte stimulating hormone
NPF – neuropeptide F
NPY – neuropeptide Y
OPE – ommatidia per eye
OrR – Oregon R
PGC-1 - peroxisome proliferator-activated receptor (gamma) coactivator 1 (alpha)

PH – pleckstrin homology
PI3K – phosphatidylinositol-3-kinase
PKG – cGMP-dependent kinase
ppl – pumpless
PVG – proventricular ganglion
RNAi – RNA interference
rpr – reaper
Ry – rosy
SEM – scanning electron micrograph
Sir2 – silent information regulator 2
sNPF – short neuropeptide F
SNS – stomatogastric nervous system
SR – starvation resistance
Tb – tubby
TH – tyrosine hydroxylase
UAS – upstream activation sequence
Y1-Y6 – NPY receptors

Chapter 1:

Introduction and Overview

Animals must consume food in order to acquire sufficient energy to satisfy the cellular demands for processes such as growth, reproduction and metabolism. Feeding behaviour includes both the frequency and size of meals, and defines how, when, what, and how much an animal eats. The understanding of feeding behaviour is not only of a basic biological interest, but may also have medical implications. A current epidemic related to feeding behaviour in humans is over-eating leading to obesity. While obesity is preventable, there is an increasing prevalence of this condition in children (Barness et al., 2007). Obesity is considered a leading cause of death due to health problems associated with being overweight, such as increased risk of cardiovascular disease, diabetes, and certain types of cancer (Barness et al., 2007; Vaag, 2009). In addition to over-eating, there are also diseases associated with under-eating. These include bulimia and anorexia nervosa, which often arise from a fear of becoming overweight and result in either a lack of eating, binge-eating, or some routine consisting of both. These diseases also lead to secondary problems such as malnourishment, and cardiovascular problems (Herpertz-Dahlmann, 2008) and in extreme cases, can induce starvation, potentially resulting in compounded long-term detrimental effects, or early fatality. Understanding the molecular mechanisms involved in the control of feeding may aid in the understanding of how diseases and problems associated with feeding occur. Recognizing the biological basis of starvation often associated with under-eating and discovering how to extend survivorship during such conditions could lead to the ability to prolong treatment of individuals with eating disorders to achieve greater success in rehabilitation.

Feeding behaviour in mammals

In mammals, feeding behaviour is primarily controlled by a homeostatic method whereby complex interactions between the central nervous system (CNS) and peripheral organs (Ahima and Osei, 2001; Arora and Anubhuti, 2006; Dhillo, 2007; Gruninger et al., 2007; Magni et al., 2009; Meister, 2007; Morton et al., 2006) rely on the brain to receive, integrate and respond to signals to indicate the nutritional state and energy level of the organism. There are both short-term and long-term signals sent from the periphery to the brain. The short-term satiety signal lets the brain know when the animal feels 'full', thereby controlling the intake of food on a short-term basis. The long-term signal, also known as the adiposity signal, is sent to the brain in relation to body-fat stores (Cameron and Doucet, 2007). Both types of signals act at sites within the CNS, particularly the hypothalamus, which secretes a large number of peptides that influence food intake. The hypothalamus has been implicated as the essential center of feeding regulation, and thereby controls appetite in a manner for the organism to maintain a weight 'set-point'. Mammals tend to maintain a range of body weight and size throughout their lifetime, based on a balance of energy production and usage. Animals that experience weight-loss due to a restricted diet will increase food consumption when re-introduced to free-feeding in order to return to their previous weight. Conversely, when animals gain weight due to forced administration of excess nutrients, they will consume less food when returned to an *ad libitum* diet (Ahima and Osei, 2001; Schwartz and Seeley, 1997). The ability of animals to carry out this type of feeding behaviour suggests the presence of physiological signals that interpret energy balance within the body, and the effectors of this control are neuropeptide hormones secreted from the hypothalamus.

The hypothalamus can receive a wide range of signals (Arora and Anubhuti, 2006; Shioda et al., 2008; Valassi et al., 2008), for example, in the form of neuropeptides or neurotransmitters secreted from the pituitary gland, including the neurohypophysis, and the adenohypophysis. Signals from the periphery may cross the blood-brain barrier at areas where neurons are in contact with both blood and cerebral spinal fluid. In response to these signals, neurons of the hypothalamus may secrete orexigenic hormones that stimulate feeding behaviour. Important examples of these include NPY, AGRP, orexins, melanin-concentrating hormone (MCH), galanin and endocannabinoids. In contrast to this, the hypothalamus can secrete anorexigenic hormones to decrease feeding, including CART, α-melanocyte stimulating hormone (MSH), glucagon-like peptide (GLP), corticotropin-releasing factor (CRF), neurotensin and serotonin. Peptide hormones secreted in the gut, such as ghrelin and cholecystokinin (CCK), can relay either a hunger or satiety signal, respectively. The peptide hormone leptin from adipose tissue, and the hormones amylin and insulin secreted from the endocrine pancreas, can relay to the CNS the level of body fat stores, indicating to the organism that it is time to stop eating.

Beyond homeostatic control, the feeding behaviour of mammals can also be regulated by a post-satiation reward system though the use of neurotransmitters, of which dopamine is one of the most well-studied examples. In mammals, after hedonic feeding, or ingesting something pleasurable or sweet, dopamine is released into the synapses and results in positive reinforcement. This signaling is regulated by transporter proteins that can reuptake the neurotransmitter and clear it back into the cytoplasm of the neuron, or destroy it. Although the aforementioned homeostatic method works well when animals are required to forage, when there is an excess of food, the post-satiation reward can override the homeostatic signals (Palmiter,

2007). As such it is important to consider both means of control when evaluating feeding behaviour.

Feeding behaviour in Drosophila and other insects

As in vertebrates, feeding in invertebrates is controlled homeostatically via a number of hormones and peptides. While there are homologous versions of certain vertebrate hormones in a few invertebrate species, there are a number of peptides specific to invertebrates. Insects, in particular, possess an array of different peptides that stimulate or inhibit feeding (Audsley and Weaver, 2009; Hartenstein, 2006; Konopinska et al., 1992). Vertebrate peptides are found in the hypothalamus and peripheral organs, but in insects the brain and stomatogastric nervous system (SNS) is the site of peptide action (Audsley and Weaver, 2009). The SNS varies in different insects, but generally consists of a number of interconnected ganglia, that also connect to the CNS. The frontal ganglion (FG) lies anterior to the brain, but is linked to it via two frontal connective nerves. The FG is also connected to the hypocerebral ganglion (HCG), which has axons extending into nervous tissue that lies posterior to the insect brain, known as corpora cardiaca. Lastly, the HCG is connected to the proventricular ganglion (PVG), which is positioned anterior to the midgut. The SNS has been shown to affect feeding behaviour via the control of gut motility.

Neuropeptides associated with the SNS in insects include FMRFamide-like peptides (FaRPs), tachykinin related peptides, proctolin, allatoregulatory peptides, and myoinhibiting peptides (Reviewed in Audsley and Weaver, 2009). The FaRPs have an inhibitory effect on visceral muscles in insects, including the foregut. Peptide members of FaRPs include

FMRFamides, FLRFamides and HMRFamides. FMRFamides are found throughout the SNS, and cause an increase in the contractions of the foregut, and an increase in feeding (Audsley and Weaver, 2009). FLRFamides, sometimes referred to as myosuppressions, are also located throughout the SNS and have been shown to reduce feeding in various insects when injected (Audsley and Weaver, 2009). The HMRFamides, also called sulfakinins, are similar to the vertebrate hormone CCK. In a number of insects, injections of sulfakinins lead to an inhibition of feeding. In contrast, when sulfakinins are blocked, an accumulation of food in the foregut, midgut and hindgut is observed, indicating an increase in feeding (Audsley and Weaver, 2009). Tachykinin peptides are found in vertebrates where they rapidly induce contraction of gut muscle. The insect tachykinin peptides, similar to the vertebrate tachykinins, are found in the FG, HCG and PVG of the SNS in most insects, and stimulate contractions of the hindgut to allow for an increase in feeding (Audsley and Weaver, 2009). Proctolin is a myotropic peptide that is located in each of the ganglia of the SNS. Proctolin has been shown to stimulate contractions of the foregut and increase feeding, potentially via modulation by FMRFamides (Audsley and Weaver, 2009). The allatoregulatory peptides include both allatostatins and allatotropins, which inhibit or stimulate feeding in insects, respectively. The allatostatins are found mainly in the FG of the SNS, and decrease feeding through the inhibition of foregut contractions. Conversely, the allatotropins stimulate feeding by inducing contractions of the foregut (Audsley and Weaver, 2009). Lastly, the insect myoinhibitory peptides are characterized as having 9 amino acids, with a tryptophan on either end. They are localised in all the ganglia of the SNS, and inhibit contractions of the foregut, and thus feeding, when present (Audsley and Weaver, 2009). Clearly the control of feeding behaviour in insects is a complex process that requires a number of SNS peptides.

In addition to the peptides of the SNS, insects have neural and peripheral peptides that contribute to the control of feeding. One neuropeptide, NPF, a structural homologue of NPY in vertebrates, is often expressed in the brain and gut. Immunostaining of starved and fed insects suggests the NPF is released into the haemolymph after feeding (Audsley and Weaver, 2009; de Jong-Brink et al., 2001; Maule, 1995). The peripheral diuretic hormones (DH) of insects are related to the vertebrate hormone CRF, and function to stimulate fluid secretion from the Malpighian tubules. When exogenous DH is injected, insects eat less and experience significant weight loss (Audsley and Weaver, 2009). These peptides function in unison with the SNS peptides, and appear to have a similar outcome as their vertebrate counterparts in the control of feeding behaviour in insects.

Aside from peptides, research of invertebrate feeding behaviour has also extended to the search for genes that control these actions. The *foraging* (*for*) gene has been identified in the social honeybee and harvester ant insects, and in the solitary *Drosophila melanogaster* and *Caenorhabditis elegans* (Tan and Tang, 2006). Drosophila larvae, in the presence of food, will frequently move around and through the food while eating. An overexpression of *for* enhances this behaviour, while larvae that have a lower expression of *for* tend to travel less through food patches (Tan and Tang, 2006). In the social honeybee, individuals begin to forage for the colony at a particular age. Expression of *for* is increased in bees when they reach this age, and an overexpression of *for* in bees prior to this age is sufficient to induce foraging behaviour (Tan and Tang, 2006). The *for* gene encodes a cyclic-GMP (cGMP)-dependent protein kinase (PKG) that phosphorylates downstream targets in the presence of cGMP (Tan and Tang, 2006). Another gene found to be associated with feeding in invertebrates is *pumpless* (*ppl*), which has been studied in Drosophila (Zinke et al., 1999). The *ppl* gene is expressed in the fat body, an organ

that functions in a manner analogous to that of the mammalian liver and adipose tissues. When mutated, *ppl* leads to a cessation of food intake that extends into conditions of starvation where mutants continue to not feed. In addition to this, loss of *ppl* induces the wandering behaviour normally observed in third-instar larvae that are ready to pupate (Zinke et al., 1999). These two invertebrate genes demonstrate the presence of an underlying mechanism in the control of feeding behaviour.

The control of feeding in insects, and in invertebrates in general, is not well understood. A multitude of peptides have been discovered in a wide range of species, but their mechanisms of action, their interactions and their release are still not clear. Further studies conducted on an increased number of different invertebrates are required in order to fully understand how these peptides work together to control the complex behaviour of feeding.

Regulatory components of feeding behaviour

Homeostatic control: The Insulin Signaling Pathway Components Akt1 and foxo

Insulin is a prominent peptide hormone in humans, and has been extensively studied due to its involvement in carbohydrate metabolism and progression of diseases such as diabetes. Insulin is a member of a peptide family that includes insulin-like peptides, and insulin-like growth factors (Garofalo 2002; Jackson 2006). In mammals, insulin is required for the conversion of glucose to glycogen, as well as the inhibition of glycogen breakdown and gluconeogenesis (Farese 2001). *Drosophila melanogaster* have seven insulin-like peptides and of these, dILP2 has been shown to interact with the Drosophila insulin receptor and is thought to

be the most similar to human insulin (Brogolio et al. 2001; Edgar 2006). The insulin signaling pathway is associated with several functions including metabolism, cell size and proliferation, longevity and reproduction (Brogiolo et al. 2001; Barbieri et al. 2003; Cheng et al. 2005; Dionne et al. 2006), and is comprised of a number of components.

Insulin receptor signaling begins when the ligand binds to the insulin receptor on the cell membrane. In Drosophila, a strong loss-of-function mutation in the insulin receptor results in an inability to produce adult flies; therefore, the receptor is required for insulin signaling and survival (Poltilove et al. 2000; Whitehead et al. 2000). Following ligand binding, insulin receptor substrates (INRS) and the phosphoinositide kinase-3 (PI3K) are recruited to the membrane. The synthesis of second messenger phosphoinositides by PI3K directs the kinase Akt1 to the membrane, where it is activated to initiate the downstream effects of insulin signaling.

Mutations in insulin signaling components affect the longevity and growth of organisms (Gems and Partridge 2001; Bartke 2001; Cheng et al. 2005). Overexpression of the components of the pathway upstream of PI3K, including the insulin-like peptides (ILPs), the insulin receptor (InR) and the INRS (chico), in Drosophila results in larger than normal flies, while mutation or loss of function of these components results in size reduction and developmental delay (Wu and Brown 2006). A mutant form of the Drosophila homologue of the INRS, chico (Clancy et al. 2001), results in both a reduced size of the overall organism and an extended lifespan. The effects on cell growth and longevity obtained through altering components of insulin signaling resemble those that are seen through various nutritional stresses. When growing Drosophila larvae are starved of nutrients, development halts, and is resumed when nutrients are returned (Britton et al. 2002; Kramer et al. 2003). When larvae are raised under nutrient limited

conditions, the adults are smaller than those eclosing from well-fed larvae, a phenotype similar to that observed in flies with an inhibition of insulin signaling (Kramer et al. 2003). When insulin signaling is inhibited through an overexpression of dominant negative versions of PI3K, developmental arrest similar to that seen during starvation in first instar larvae occurs (Britton et al. 2002). It is clear that this pathway functions to coordinate nutrient intake with growth and development.

The central component of the insulin signaling pathway, the serine/threonine kinase Akt1, is highly conserved between mammals and *Drosophila melanogaster* (Scheid and Woodgett, 2001; Franke et al., 2003). In Drosophila, A*kt1* is expressed throughout the organism during embryogenesis (Cavaliere et al., 2005; Scanga et al., 2000; Staveley et al., 1998). A role for *Akt1* in the control of cell growth and survival is established as overexpression of *Akt1* in Drosophila leads to an increase in cell size, although there is no effect on cell growth rates or division. Underexpression or loss of *Akt1* can result in the production of smaller animals or, if severe enough, can be lethal (Kennedy et al., 1997; Staveley et al., 1998; Verdu et al., 1999). The role of *Akt1* in cellular survival was suggested by its inhibition of several pro-apoptotic genes (Kennedy et al., 1997; Nunez and del Peso, 1998). As expected for a maternally expressed gene, *Akt1* female mutants are sterile, and Drosophila *Akt1* mutant germline clones undergo apoptosis (Staveley et al., 1998). This demonstrates that the activities of the Akt1 kinase are of great importance to the survival of the cell, and to the overall organism.

A key downstream target of Akt1, the transcription factor foxo, mediates the transcriptional regulation of the insulin pathway and controls several important cellular functions including metabolism, cell cycle regulation, DNA repair, apoptosis and protection of the cell against oxidative stress (Burgering and Kops, 2002; Barthel et al., 2005; Puig and Tijian, 2005;

Gershman et al., 2007). Through these diverse functions, the transcription factor foxo can facilitate the end result of Akt1 activity on the regulation of cell growth and survival. A reduced insulin signaling level is required for maximal survival during starvation, as overexpression of insulin signaling components, such as PI3K or InR, leads to the inability of Drosophila to survive during nutritional stress (Britton et al. 2002). Overexpression of *foxo*, similar to its activation in the absence of insulin signaling, results in the same phenotypes that arise when larvae are starved (Kramer et al. 2003). Previous studies of *foxo*'s role during nutritional stress, by inducing ubiquitous expression of *foxo* in the first instar larvae, showed a complete arrest of growth, yet the larvae survived for several days (Kramer et al. 2003; Junger et al. 2003). Analysis of endogenous foxo activity and foxo loss-of-function demonstrated a role of *foxo* in the organism's response to amino acid starvation. Newly hatched Drosophila larvae require nutrients in order to increase their body mass via replication of cells in mitotic tissues. In contrast, when larvae are hatched into conditions of amino acid starvation, they live in a state of developmental arrest until nutrients become available (Beadle et al. 1938). Loss of *foxo* resulted in an increased sensitivity to starvation due to low levels of amino acids (Junger et al. 2003). When measuring the amount of foxo present in various nutrient conditions, it has been shown that when nutrient levels are low, *foxo* expression is increased. Upon return of nutrients, *foxo* levels return to basal levels (Kramer et al. 2003). Ectopic expression of Drosophila *foxo* leads to inhibition of growth and generation of small adults. In addition, when there is an excess of foxo activity in larvae, feeding behaviour is altered. These findings suggest that insulin signaling, through Akt1 and foxo, is involved in mediating the developmental response of larvae to nutrient intake, and thus starvation. In the absence of adequate nutrition, insulin signaling is reduced, allowing foxo to enhance the transcription of downstream targets to aid in the overall survival of the organism.

Homeostatic Control: The deacetylase Sir2 and foxo

In addition to the highly conserved insulin receptor pathway, sirtuin deacetylases mediate transcription of genes involved in metabolic homeostasis (Rodgers et al, 2005; Bordone et al, 2006; Rodgers et al, 2007). The first sirtuin was identified in yeast and named silent information regular-2 (Sir2) (Frye, 2000). Mammals possess seven sirtuins that occupy different subcellular compartments and control the activity of stress-responding transcription factors such as p53, foxo and NF-kB (Longo 2009). The role of sirtuins in metabolic homeostasis was validated when it was observed that small molecule activators of mammalian SIRT1 are active in the same pathways that are induced during caloric restriction (Wang, 2014). Extra copies of *Sir2* in yeast 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). 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. Clearly, the expression of these proteins can influence survival.

Interestingly, sirtuins augment longevity through overexpression regardless of adequate nutrition or nutritional stress. A number of the physiological pathways active during dietary restriction are shared with active *Sir2* expression (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), and the mRNA expression, protein levels and activity of Sir2 are induced during starvation in control flies (Banerjee et al., 2012). Gene profiles of 782 genes altered in Drosophila with extended lifespans due to an overexpression of Sir2 show that 72% of the upregulated genes and 61% of the downregulated genes are also observed to be altered in Drosophila undergoing dietary restriction (Antosh et al., 2011). The ability of extra Sir2 to enhance lifespan in Drosophila undergoing starvation is logical as Drosophila Sir2 plays a crucial role in fat metabolism and systemic insulin signaling (Banerjee et al., 2012) as it acts as a critical factor in fat mobilization from the fat body during starvation. In fact, only a moderate increase in the expression of Sir2 in the fat body of adult Drosophila is required to extend their lifespan by 13% (Hoffman et al., 2013) and weak overexpression of Sir2 in the fat body is sufficient to increase the survival in both female (12%) and male (13%) flies compared to the controls that are not undergoing nutritional stress (Hoffman et al., 2013). Taken together, these findings clearly show that the action of Sir2 is essential in the survival of Drosophila ingesting fewer nutrients.

Homeostatic Control: Neuropeptide Y, Neuropeptide F and Short Neuropeptide F

In vertebrates, neuropeptide Y (NPY) has been studied extensively as a central enhancer in the control of food intake. NPY is a peptide hormone consisting of 36 amino acids, beginning and ending with a tyrosine (Y) residue, giving it its name (Arora and Anubhuti, 2006). Invertebrate homologues of the NPY hormone have been found in major phyla (de Jong-Brink et al., 2001; Garczynski et al., 2005; Maule, 1995) but are named neuropeptide F (NPF) for having a conserved phenylalanine (F) residue at the carboxyl terminus in place of tyrosine. NPF has

been consistently detected in the brain of invertebrates, and is often expressed in the midgut, suggesting its role in feeding regulation.

The receptor protein that NPY operates on is a G-protein coupled receptor (GPCR) that spans the phospholipid membrane seven times (Larhammar et al., 2001; Lindner et al., 2008). Five subtypes (Y1, Y2, Y4, Y5 and Y6) have been identified in most vertebrates. In mammals, the Y2 receptor is predominantly expressed in the brain and hippocampus and has been shown to affect circadian rhythm and memory retention. The Y4 receptor is predominantly expressed in the gastrointestinal tract. Subtypes Y1 and Y5 are located in the hypothalamus and appear to mediate the NPY anabolic effects. Receptors resembling the vertebrate NPY receptors have been identified in a number of invertebrates, including snails, the nematode *Caenorhabditis elegans*, and *Drosophila melanogaster* (de Bono and Bargmann, 1998; Tensen et al., 1998; Garczynski et al., 2002). The first insect NPF receptor was identified in *Drosophila* and found to be a G-protein coupled receptor that is 481 residues long (Garczynski et al., 2002; Nassel and Wegener, 2011). Experiments using the Drosophila NPF receptor showed its ability to cross-react with human NPY, suggesting the functional conservation between NPF and NPY (Garczynski et al., 2002). Expression of invertebrate NPF receptors is generally located in both the central and peripheral nervous systems, akin to vertebrates. In Drosophila, it was found to be expressed in 26 neurons of male fly brains, and 20 in female brains (Nassel and Wegener, 2011; Lee et al., 2006). The conserved function and distribution of both peptides and their receptors indicate the importance of NPF in insects.

As an orexigenic hormone, NPY stimulates feeding behaviour. NPY synthesis and secretion in mammals have been observed to be upregulated in situations of low energy stores, such as during periods of starvation (Arora and Anubhuti, 2006; Chee and Colmers, 2008).

Injection of NPY into the rodent hypothalamus stimulates feeding (Beck, 2001; Pedrazzini, 2004). NPY regulates feeding in fish, as both central and peripheral injections of either mammalian or fish NPY increase food intake (Matsuda, 2009; Volkoff, 2006). Expression of NPY is upregulated in fasted fish, and returns to basal levels upon re-feeding. Fish have homologues of Y1 and Y2 receptors expressed in the brain and the peripheral tissues of the gastrointestinal tract. Central administration of Y1 receptor antagonists decrease feeding in fish, while injections of Y1 agonists increase feeding (Volkoff, 2006). These observations suggest that NPY is an important modulator of feeding behaviour in vertebrates.

The invertebrate NPF has also been well studied, and has been shown to play a role in foraging, feeding, motivation, ethanol sensitivity, stress response, aggression and learning (Nassel and Wegener, 2011). Most of the studies of NPF in insects have been carried out in Drosophila. Early studies indicated that when Drosophila were exposed to sugar, an increase of NPF expression was observed (Shen and Cai, 2001). This same response was not observed when flies were exposed to sugar-free yeast or aspartame. In Drosophila, young larvae feed excessively to obtain the weight requirement for pupation, while older larvae do not feed as much and begin to wander to find a place to pupate (Melcher et al., 2007). In young larvae, NPF expression is upregulated, while in the older larvae, there is less NPF expression. When transgenic larvae are created so that they lack NPF signalling, young larvae exhibit the phenotypes of wandering and lack of feeding normally seen in older larvae. When NPF was overexpressed, older larvae displayed the phenotypes of younger larvae, which included increased feeding (Wu et al., 2003; Wu et al., 2005). With regard to motivational behaviours associated with feeding, less NPF reduces the motivation to eat noxious food, even when the larvae have been starved, while an increase in NPF encourages larvae to feed on the same

noxious food when already well fed and even in unfavourable conditions, such as cold. Although an NPY homologue has not yet been identified in *C. elegans*, a genetic study has implicated a conserved NPY-like signalling system in regulating food-conditioned foraging behaviour in the worm (de Bono and Bargmann, 1998). These findings indicate that NPF appears to play a similar role in the regulation of feeding behaviour in invertebrates as it does in vertebrates. Other potential roles of NPF have been suggested in studies showing that lowered NPF signaling in the male specific neurons of male Drosophila results in delayed courtship and increased aggressive behaviour, suggesting NPF expression in male flies contributes to decreasing aggression (Dierick and Greenspan, 2007). With regard to learning behaviours, it is known that hungry flies typically respond better when offered a food reward. However, when NPF expression is increased in well-fed flies, they perform to the same level as the hungry flies (Krashes et al., 2009). Clearly this peptide can function in a number of complex behaviours, but is most prominently know for controlling food intake.

After the first invertebrate NPF peptide was identified in the tapeworm *Monieza expansa* (Maule et al., 1991), homologues that were 36 amino acids in length, similar to NPY, were discovered in a number of other invertebrate species. However, when NPFs were first identified in insects they were much shorter at only 8-10 amino acids. It was not until Drosophila were investigated that both a long NPF peptide (36 amino acids) and shorter version (subsequently named sNPF) of the peptides were identified (Vanden Brojeck, 2001). Although the long and the short NPF peptides share the same name and appear to have some of the same functions, the peptides themselves do not appear to be related to each other (Nassel and Wegener, 2011). Their C-terminus regions are similar, indicating that both peptides might be able to interact with the same receptor, yet they each have distinct N-terminal regions and prepropeptide sequences

suggesting they may not be closely related. While both peptides appear to function in the control of feeding behaviour, only sNPF links the regulation of feeding to the control of growth. The longer NPF peptide is likely the homologue of NPY in mammals while sNPF is a different peptide, which appears to associate with other neuropeptides and neurotransmitters in a number of functions.

There are four known short neuropeptide F (sNPFs) sequences in Drosophila that are encoded by a single gene (Vanden Broeck, 2001). Analysis of these small peptides in Drosophila indicates they are expressed in a large number of neurons in both the brain and the stomatogastric system, and are often co-expressed with other neuropeptides such as insulin and classical neurotransmitters (Nassel and Wegener, 2011). The number of cells expressing *sNPF* increases from larval to adult stage as 700 neurons, 1000-1200 Kenyon cells in the brain and 120 ventral nerve cord cells express *sNPF* in the larvae, but 280 neurons, and 4000 Kenyon cells express *sNPF* in the adult brain. The first sNPF receptor was identified and characterized in Drosophila, and was found to be activated by the four Drosophila sNPF proteins (Vanden Broeck, 2001; Mertens et al., 2002; Feng et al., 2003; Garczynski et al., 2006). The entire distribution of these receptors is not yet fully known, but sNPF receptors have been localized on insulin-producing cells, indicating a relationship between insulin and sNPF signaling.

Experiments on Drosophila with manipulated expression patterns of *sNPF* confirm its control of initiating or stopping feeding. An increase in *sNPF* promoted food intake in both larvae and adult Drosophila (Lee et al., 2004). This was associated with an increase in growth and overall size. As the insulin receptor signaling pathway is known to control growth in Drosophila, this reinforces a link between the two signaling systems. The opposite also holds true as a reduction in *sNPF* leads to decreases in feeding and growth, resulting in smaller flies

(Lee et al., 2008). However, it has not yet been confirmed that an increase in *sNPF* expression can induce feeding in starved flies. Molecular analysis of insulin signaling with *sNPF* expression shows that when there is less *sNPF*, there is less Drosophila insulin-like peptides being produced, therefore less growth (Lee et al., 2008), resulting in a similar phenotype to insulin signaling pathway mutants. In addition to feeding, flies with lowered *sNPF* expression are also shown to retain less water and therefore become more sensitive to desiccation (Kahsai et al., 2010*a*). Flies with less *sNPF* also wander or walk further distances at a faster pace than control flies indicating sNPF may play a role in locomotion (Kahasai et al., 2010*b*). Similar to the longer NPF, sNPF can play intricate roles in other behavioural responses, but appears to be primarily associated to feeding.

Post-Satiation Reward Control: Dopamine and the dopamine transporter

Homeostatic control can either initiate or halt feeding behaviour. In response to this, after hedonic feeding, the brain can induce a post-satiation reward response that can encourage feeding beyond homeostatic control. A major component of this system, dopamine, was first identified in 1957 (Hornykiewicz, 2006). Dopamine is a monoamine neurotransmitter regulated by the enzymes that lead to its production and the transporters that relocate it between the cytoplasm and synapses of neurons (reviewed in German et al., 2015). The dopamine transporter (DAT) is a membrane-bound protein that regulates the levels of extracellular dopamine through rapid reuptake of the neurotransmitter to clear the synapses. DAT was first sequenced and cloned in 1991 as a member of the solute carrier 6 (SC6) family of transporters (Giros et al., 1991; Kilty et al, 1991; Shimada et al 1991). Drosophila DAT was originally identified as a

single 3-4kb mRNA via homology-based cloning (Porzgen et al 2001) and then isolated in a screen for genes that affect sleep (Kume et al 2005). A mutant containing an altered DAT protein, named fumin (*fmn*), which is Japanese for sleepless, is active for nearly 24 hours of the day. Expression patterns of Drosophila DAT were found to be concentrated in the location of known dopaminergic neurons within larvae and the adult head (Kume et al 2005), analagous to the expression of DAT in mammalian brains.

Dopamine is transported via DAT through conformational changes in the DAT protein. Located in the neuronal membrane, DAT can either face "outward" or "inward" with regard to the synapse and cytoplasm (Shan et al., 2011; Vaughan and Foster, 2013). After dopamine has been released from the synapses, it can bind to DAT proteins that are in the "outward" conformation, projecting into the synapse. The addition of Na^+ and Cl^- bound to this DAT protein allows it to change shape to the "inward" conformation, facing the cytoplasm, thereby carrying the dopamine molecule into the cytoplasm. Upon release of dopamine, Na^+ and Cl^-, the DAT protein reverts back to the outward conformation. The activity of DAT can be regulated by post-translational modifications and protein interactions. The conserved structure of DAT includes twelve transmembrane regions and N- and C-terminal regions that are located within the cytoplasm (Vaughan and Foster, 2013). The N-terminal region can become phosphorylated at serine residues by protein kinase C, or ERK to inhibit or exert its activity, respectively (Ramamoorthy et al., 2011). The ubiquitinylation of the N-terminal region can also inhibit the actions of DAT, by preventing the conformational change (Jiang et al., 2004; Miranda et al., 2007). Palmitoylation of the C-terminal region enhances the down-regulation of DAT by PKC and can also initiate the degradation of DAT (Foster and Vauhgan, 2011). These modifications can therefore lead to either hyper- or hypo-dopaminergic signaling conditions.

Manipulation of the extracellular levels of dopamine, through the dopamine transporter, can result in a number of diseases (Palmiter, 2007; Yamamoto and Seto, 2014). Lower levels of dopamine are associated with ADHD, depression and undereating (Cannon et al., 2004; Sotak et al., 2005; Gunaydin and Deisseroth, 2014). Individuals diagnosed with ADHD have been found to have coding polymorphisms in DAT, which could potentially explain abnormal dopamine signaling. Psychostimulant drugs are often used as a treatment for ADHD. These drugs interact with DAT such that initially DAT increases in number on the neuronal membrane in order to clear the excess dopamine released from the drugs. However, over extended period of time, these drugs can lead to a reduction in the levels of DAT thereby increasing risks of addiction to these drugs. An excess of dopamine signaling, which could occur when DAT is not functioning at it full level, can lead to overfeeding and obesity. Hyperdopaminergic mice are found to overfeed (Pecina et al., 2003; Cagniard et al 2006), yet mice without any DAT expression are smaller than controls with a decrease of roughly 44% of body size (Pecina et al., 2003). Studies in rats show that upon feeding or drinking there is a rapid firing of dopamine in the brain, whereas animals with lowered or no dopamine activity are hypoactive, apathetic and could die from starvation or dehydration (Schulz, 2006). Scheduled ingestion of sucrose has been shown to increase the expression of DAT in food-restricted rats, which is not observed in free-fed rats (Bello et al., 2003). During the times dopamine levels are lowered, there is an increase in the affinity for more palatable food, such as sucrose. As such impaired dopamine signalling can enhance the affinity for sweets therefore contributing to obesity. Alteration of the transporter which regulates the strength and duration of dopamine signaling can lead to a number of intricate outcomes, which may either heighten or debilitate survivorship during low nutrient intake.

Starvation

Any organism may face conditions of limiting food resources; therefore it is pertinent that they develop mechanisms to avoid starvation-induced mortality. Starvation is considered a biological condition wherein an organism, while willing and capable of ingesting nutrients, is unable to do so due to some extrinsic limitation. Fasting, on the other hand, is when an organism foregoes ingesting nutrients through some intrinsic mechanism. Fasting may be chosen due to a requirement of exerting energy reserves alternatively, such as in predator avoidance, thermoregulation, or reproductive efforts. Forced calorie restriction, as is often the method to induce starvation in model organisms, is considered to be a mild form of starvation.

While starvation is considered to be a continuous process, several researchers have defined physiological phases based on levels of energy reserves. In general, a body's response to starvation includes changes in body mass and physiological fuels. Many fishes and invertebrates do not lose body mass, even during prolonged periods of starvation (McCue, 2010). Also, even if a large amount of total body mass is lost, survival mostly depends on how much lipid is retained. Therefore one animal could lose a greater amount of total body mass but still retain a greater lipid storage amount and therefore survive starvation better (McCue, 2010). There are several types of physiological fuels utilized by all animals. The first to be accessed during the initial stages of starvation is the catabolism of carbohydrate stores. Circulating levels of glucose decrease initially but levels can be restored by secretion of glucagon from the pancreas in most animals (Young and Scrimshaw, 1971). Once these have been used up, the next response is to catabolize lipids. Most animals are able to tolerate anywhere from 20 to 70% loss of their total body lipid content during starvation (McCue, 2010). As starvation progresses, increased

secretion of lipases mobilize glycerol and unesterified fatty acids from triglyceride stores (Clarenburg, 1992, McCue, 2010). The lipid metabolites of glycerol, ketone bodies and free fatty acids are elevated in response to starvation so that they may be utilized as fuel. As a last resort, in prolonged starvation, protein stores are catabolized. This occurs once the lipid stores have been depleted to a preset threshold. An animal, when adequately nourished, will typically maintain a balance between protein synthesis and protein degradation. However, once proteins become a source of fuel during undernourishment, then the degradation outweighs the synthesis. In particular, certain amino acids serve as gluconeogenic substrates, including alanine and glutamine (Felig et al., 1970; Ruderman and Berger, 1974). Therefore the quantity of stored protein is crucial in determining the length of survival during starvation in many organisms.

The ability to tolerate starvation varies in vertebrate species. Small birds or mammals can survive roughly 24 hours of total starvation (Baggott, 1975; Mosin, 1984; Blem, 1990) while snakes and frogs can survive nearly two years (Grably and Piery, 1981; de Vosjoli et al, 1995). A record of 1594 days of total starvation belongs to the European eel, *Anguilla anguilla* (Boetius and Boetius, 1985). Tolerance of induced periods of starvation can be achieved by different mechanisms. When thought of as a "supply and demand", the supply would refer to the energy stores (carbohydrate, lipid, protein) and the demand would refer to the amount of energy used by the organism during the period of starvation. From a supply standpoint, starvation tolerance could be increased by enhancing both the quality and quantity of energy stores. This may be achieved by engineered diets of varying fatty acid compositions. Different types of free fatty acids and amino acids are mobilized and catabolized at different rates during starvation, therefore modulating the diet to suit these needs may maximise starvation resistance. With regard to demand, the resting metabolic rate, including changes in hormone levels, rates of protein

turnover, enzyme activity and mitochondrial density could be manipulated in order to make energy usage more efficient during times of low nutrition.

Studies of Starvation Resistance in Drosophila

The majority of studies of starvation resistance in Drosophila focus on acute or complete food deprivation as opposed to reduction of any one particular nutrient (Rion and Kawecki, 2007). Genes that are associated with acute starvation resistance include *methuselah*, which encodes a membrane receptor and where loss of function extends longevity by 35% and enhances SR (Lin et al., 1998). GLaz, a lipid-binding protein, when overexpressed, is able to extend longevity by 29% and SR by 60% (Walker et al., 2006). The hormone Akh (adipokinetic), which modulates lipid and sugar metabolism, also enhances SR but does not affect longevity when the neurons that produce the hormone are ablated (Lee and Park, 2004). It therefore appears that insects are able to employ similar modes of surviving starvation, with regard to the physiological fuels that are used under conditions of low nutrition, as for vertebrates. Drosophila with increased acute starvation resistance have an increase in the activity of enzymes associated with lipid biogenesis (Harshman et al., 1999), and ablation of the insulin-producing cells in the late third instar produces more lipid stores, an extended longevity and a great resistance to starvation and oxidative stress (Broughton et al., 2005). Interestingly, a protein-poor diet in adult Drosophila induces an increase in lipid reserves (Simmions and Bradley, 1997; Piper et al., 2005). Reducing the amount of protein in the diet can increase acute starvation resistance up to two-fold (Chippindale at al 1993, Piper et al 2005, Burger et al 2007).

The lifespan of Drosophila under the extreme conditions of total starvation is exceptionally short, thereby denying the researcher the ability to observe any subtle differences in survival ability. Therefore investigating survivorship of Drosophila undergoing dietary restriction, either through total overall calorie restriction or based on one dietary component, can subtly implicate several genes and pathways that can enhance survivorship. The insulin signalling pathway mutant *chico* has an increased longevity, but this extension in longevity is not observed when *chico* mutants are raised on DR (Clancy et al., 2002). This may be due to the fact that as the insulin pathway regulates nutrient intake, the *chico* mutants already eat less and are therefore already dietary-restricted. Therefore adding additional DR to this condition will be harmful rather than beneficial. Drosophila typically feed at least once every hour, and while they could increase their food intake on dietary restriction, this does not appear to be the case (Partridge et al., 2005). Both the histone deacetylase Rpd3 (Jiang et al., 2002) in heterozygous mutants, and the protein deacteylase Sir2, can extend longevity during a normal diet, and we have found a heterozygous mutant of *Sir2* is able to increase survival when undergoing DR of amino acids (Rogina and Helfand, 2004, Slade and Staveley, 2016*b*). However, null mutants do not extend lifespan in either condition. It is evident that manipulation of the homeostatic control of feeding can enhance survivorship when the diet is restricted.

In Drosophila, the effects of DR are more effective when fed as an adult as opposed to being fed as a pre-adult, during development (Tu and Tatar 2003). It is thought that DR extends longevity by changing the RNA transcript profile characteristic of normal ageing. 23% of the genes in the fly genome show a change in transcript representation with age when exposed to DR compared to controls (Pletcher et al., 2002). These changes were similar in relation to the physiological age of the cohort as opposed to chronological age. In Drosophila, DR can increase

longevity by lowering the mortality rate over time (Mair et al., 2003). The effect on mortality rates is acute; when DR is onset later in the lifespan of an organism, it can switch mortality rate to those flies that have been permanently on DR within 48 hours (Mair et al., 2003). However, alterations of metabolic rate do not appear to mediate the effects of DR on lifespan in Drosophila.

The two major components of a complete diet for Drosophila are a carbohydrate source, such as sugar, and a protein source, usually yeast. Flies exposed to diet restriction through a reduction of yeast alone, as opposed to sugar, are more sensitive to mild starvation (Min and Tatar, 2006). While in rodents it is the reduction in overall calories rather than alteration of specific dietary components that leads to an extension in lifespan (Masoro et al 1989), this is not the case for Drosophila, which show varying phenotypes based on whether protein (casein) or sugar is manipulated (Van Herrewege, 1974). Comparisons of survivorship between flies on diets of sugar alone and those with sugar and varying amounts of the protein casein indicated the addition of protein improved the longevity of the flies. Therefore, a phenotype signifying starvation resistance on amino acid depleted media could be more noteworthy than that same phenotype in other types of starvation assays.

Animal Models and Eating Disorders

Eating disorders in humans are presently defined according to the American Manual of Psychiatry DSM-5 to include three main subtypes: anorexia, bulimia and binge eating disorder. Characteristics of *anorexia nervosa* (AN) include symptoms of a distorted body image and

excessive dieting (Garcia et al., 2011). Co-morbidities of this disease include anxiety, depression, addiction and phobias (Erdur et al., 2012). Bulimia and binge eating disorder have similar symptoms but also include recurrent episodes of binge eating, and inappropriate purging behaviour. Anorexia has one of the highest mortality rates of all psychiatric diseases with 16% of those suffering from the disorder dying due to the consequences of the disease (Lowe et al., 2001). AN presents genetic etiological components for 33-84% of the patients (Boraska et al., 2014). The neural profile of AN corresponds to a predominant imbalance between the reward (meso-cortico-limbic system) and inhibition (prefrontal cortex) systems of the brain (Phillipou et al., 2014). Thus, dissecting the mechanisms of action of the different neuropeptides and neurotransmitters involved in the regulation of food intake, as well as the motivational aspects of feeding, becomes a necessity to open new perspectives for an efficient therapy of this disease complementary to the psychological approaches.

Current animal models to study the biological basis of AN can be sorted into two groups, the genetic models and environmental models (reviewed in Mequinion et al., 2015). Within the genetic models, there are engineered models and models that arose due to spontaneous mutation. The most popular model is the *anx/anx* mice model. This model emerged in 1976 and consists of mice that are emaciated, eat less and die early (Maltais et al., 1984; Nilsson et al., 2013). They have a 50% reduction in the *Ndufaf1* gene in the hypothalamus, which is a gene that encodes a protein required for assembly of the mitochondrial complex 1 (Lindfors et al., 2011). They also have variations in NPY expression. Genetically engineered models include manipulation of various genes encoding peripheral hormones (leptin, ghrelin, PYY), the neuropeptidergic system (NPY, *etc*.), neurotransmitters (dopamine, serotonin) and other genes (reviewed extensively in Mequinion et al., 2015). The peripheral hormone models alter the expression of hormones that

either stimulate or inhibit feeding behaviours and include leptin, PYY, ghrelin, pancreatic polypeptide and cholecystokinin, but none of these is able to appropriately mimic the main alterations observed in AN patients (Mequinion et al., 2015). The neuropeptide and neurotransmitter models are able to mimic one or more of the phenotypes of AN, but none is able to mimic all the phenotypes. Those studied include alterations in NPY, the NPY receptors, agouti-related peptide, MCH and cannabinoid receptors for the neuropeptides, and serotonin receptors and tyrosine hydroxylase, an enzyme required for the production of dopamine. These models are mostly able to mimic the weight decrease and voluntary food restriction. Environmental models focus mainly on altering the pattern or distribution of food and nutrition or exposure to chronic and acute stress (reviewed in Mequinion et al., 2015). This includes mild to severe food restriction, restriction of specific dietary components, such as amino acids, and exposure to dehydration, extreme temperatures, and social stresses. The levels of body weight and hormones in these models are compared to those in AN patients. In particular, the amino acid restricted models have a similar weight loss (30% body weight compared to 20-25% for AN patients) and have reduced IGF/insulin and serotonin levels (Cheng et al., 2010; Goto et al., 2010; Narita et al., 2011; Anthony and Gietzen, 2013). AN patients have a decrease in IGF/insulin, dopamine and serotonin and an increase in NPY expression.

Of the current models, the one that appears to mimic AN the best is the activity-based anorexia (ABA) model in rats (de Rijke et al., 2005; Boakes, 2007; Verhagen et al., 2008; Duclos et al., 2009; Filaire et al., 2009; Adan et al., 2010; Gutierrez 2013). This model is also known as food restriction-induced hyperactivity model and produces rapid weight loss of close to 25% of the body mass, reduced food intake or "self-starvation" resembling that observed in AN patients, and hyperactivity. In addition, these rats exhibit a decrease in IGF/insulin and

serotonin expressions and an increase in NPY expression as seen in AN patients, but interestingly show an increase in dopamine levels upon feeding.

The complexity of these diseases makes them difficult to recapitulate in their entirety. Therefore, studying the biological basis of these conditions could be beneficial in uncovering the subtle yet significant mechanisms of enhancing survivorship. Typically, individuals diagnosed with an eating disorder do not respond well to psychological treatments while being nutritionally rehabilitated, therefore the ability to sustain these patients until their psychological constraints can be dealt with could be advantageous. Developing Drosophila as a model to better understand the biological mechanism of starvation resistance is one method that could be used for applicable therapy to these instances and other related human conditions.

The Intent of This Research

Drosophila melanogaster can serve as an uncomplicated model to study the molecular basis of starvation during nutrient deprivation to uncover the potential mechanisms of metabolic disease. Several organ systems of Drosophila function analogously to those in vertebrates; for example the fat body in Drosophila is utilized to store triglycerides akin to the liver in vertebrates (Owusu-Ansah and Perrimon, 2014). Drosophila contain a specialized group of cells, the oenocytes, which function similar to that of hepatocytes in vertebrates to mobilize stored lipids during periods of low food intake. In addition, Drosophila possess homologous hormones to regulate feeding behaviour. These similarities allow for experiments in Drosophila to be applicable to human health research.

In this work, we have set out to characterize novel *Akt1* mutants (Slade and Staveley, 2015) aged upon amino acid starvation media (Slade and Staveley, 2016*a*). We expected this phenotype to be foxo-dependent, and therefore investigated ifthe novel *Akt1* hypomorphs show the same longevity phenotypes when combined with null *foxo* mutants. In addition, we have investigated the effects of an additional homeostatic control of feeding, heterozygous and homozygous alleles of the deacetylase Sir2, upon the survivorship of Drosophila when deprived of amino acids (Slade and Staveley, 2016*b*). Lastly, we have investigated the survival of Drosophila during amino acid starvation with regard to the neuropeptide homeostatic control, and the DAT post-satiation reward control of feeding, through both the overexpression and loss-of-function of *NPF*, *sNPF* and *DAT* (Slade and Staveley, 2016*c*). The significance of these findings could lead to a better understanding of the biological consequences of malnutrition that exists as a result of eating disorders.

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Chapter 2:

Compensatory growth in novel Drosophila *Akt1* mutants

A version of this chapter is published as:

Slade, J.D., and Staveley, B.E. 2015. Compensatory growth in Drosophila *Akt1* mutants. BMC Research Notes **8:** 77.

Abstract

Background: Organisms, tissues and cells are genetically programmed to grow to a specific largely pre-set size and shape within the appropriate developmental timing. In the event of mutation, cell death, or tissue damage, the remaining cells may increase their rate of growth to compensate and generate an intact, potentially smaller, tissue or organism in order to achieve the desired size. A delay in the developmental timing could aid in this process. The insulin receptor signalling pathway with its central component, the Akt1 kinase, and endpoint regulator, the transcription factor foxo, plays a significant role in the control of growth. *Drosophila melanogaster* is an excellent model organism with a well-studied life cycle and a consistently developing compound eye that can undergo analysis to compare changes in the properties of adult ommatidia as an indicator of growth.

Results: Imprecise excision of a *PZ* P-element inserted in the upstream region of *Akt1* generated several novel hypomorphic alleles with internally deleted regions of the P element. These mutations lead to small, viable Drosophila that present with delays in development. Suppression of this phenotype by the directed expression of $Akt1^{+}$ indicates that the phenotypes observed are *Akt1* dependent. Somatic clones of the eyes, consisting of homozygous tissue in otherwise heterozygous organisms that develop within a standard timeframe, signify that more severe phenotypes are masked by an extension in the time of development of homozygous mutants. Generation of Drosophila having the hypomorphic *Akt1* alleles and a null allele of the downstream target foxo result in a phenotype very similar to that of the *foxo* mutant and do not resemble the *Akt1* mutants. **Conclusion:** The developmental delay of these novel *Akt1* hypomorphs results in a latent phenotype uncovered by generation of somatic clones. The compensatory growth occurring during the extended time of development appears to be

implemented through alteration of foxo activity. Production of clones is an effective and informative way to observe the effects of mutations that result in small, viable, developmentally-delayed flies.

Background

The cell is the basic structural unit of all living organisms. The overall size of a cell can either augment or limit its ability to perform essential functions. Consequently size homeostasis is pertinent for the fitness and function of cells. Even slight disruptions of this homeostasis can lead to disease, thus it is critical to understand the complex mechanisms that control cell growth. *Drosophila melanogaster* develops quickly through a sequence of three feeding and growing larval stages followed by pupation and eclosion (Robertson, 1936) and is an ideal model system in which to study cell growth.

A crucial point in the control of growth in Drosophila is the achievement of the critical mass, the minimum weight required for transition from larvae to pupae, upon which any further feeding, or lack of feeding, will not prevent this change (Beadle et al., 1938; Tennessen and Thummel, 2011). Drosophila larvae, when fed generously, can grow to, or past, the critical weight within four days. Restriction of dietary proteins slows this process, while total absence can halt growth completely (Tatar, 2007). Once larvae have reached the critical weight required for pupation, they may continue to feed for a period of time before undergoing the transition (Mirth and Riddiford, 2007). Several factors can influence the rate of growth during the larval stages including nutrition, temperature, density of organisms present in the environment, and underlying genetic mechanisms (Oldham et al., 2000; Clancy et al., 2002; Kramer et al., 2003; Arking, 2005; Germinard et al., 2006). Slowed growth, due to genetic mechanisms or nutrient

conditions, characteristically results in larvae that develop into smaller adults. While many mutations can influence growth; some alter the growth of individual organs, some retard overall growth without changing the final adult size, the mutations which slow growth and lead to a reduction in the overall organ and body size may be the most intriguing.

The conserved insulin receptor (InR) signalling pathway is implicated in the management of final adult size. In Drosophila, this highly conserved pathway has been shown to control cell size and growth, and to regulate body size and nutrient usage (Coelho and Leevers, 2000; Aoyama et al., 2006). When any of the seven Drosophila insulin-like peptide (Ilp) genes are overexpressed, growth rates in larvae and adults are greatly increased, and ablation of the medial neurosecretory cells in the brain (the main producer of Ilps) leads to a decrease in the growth rate and final size (Edgar, 2006). Overexpression of the upstream components of the pathway, including the ligand (Ilps), the insulin receptor (Inr) and the insulin receptor substrate (chico), in Drosophila results in larger than normal flies, while mutation or loss of function of these components results in size reduction and developmental delay (Wu and Brown, 2006). This reinforces the pivotal role of insulin receptor signalling in the control of growth.

The Akt1 kinase is a central component of the insulin receptor signalling pathway. When Drosophila *Akt1* is overexpressed, it is shown to increase cell size but not proliferation, or number of cells, by overriding the control mechanisms that are responsible in determining the final size of cells (Verdu et al., 1999). Loss of *Akt1* can result in lethality (Staveley et al., 1998) while hypomorphic activity can result in the production of smaller adults (Verdu et al., 1999). A key downstream target of Akt1, the transcription factor foxo, mediates the transcriptional regulation of the insulin pathway and controls several important cellular functions including metabolism, cell cycle regulation, DNA repair, apoptosis and protection of the cell against

oxidative stress (Burgering and Kops, 2002; Barthel et al., 2005; Puig and Tijian, 2005; Gershman et al., 2007). Through these diverse functions, the transcription factor foxo can facilitate the end result of Akt11 activity upon the regulation of cell growth and survival.

In order to explore the influence of *Akt1* activity upon cell growth, a series of novel *Akt1* hypomorphs were generated through imprecise excision of a P-element situated in the control region upstream of the gene's coding region. A subset of these hypomorphs was selected, based upon reduction in adult size, and were further characterized with replacement analysis to confirm the reduction in size was due to altered *Akt1* activity. Due to the extended time required to reach eclosion by the homozygotes, somatic clones of the eye were generated to produce a mutant phenotype less influenced by a developmental delay. Finally, to further investigate the dynamic interaction between *Akt1* and *foxo*, Drosophila lines with novel hypomorphic alleles and a null version of the downstream target foxo gene were created and the potential for epistasis was evaluated. Our intent through these experiments is to better understand the effect of extended development time upon the overall phenotype of the novel *Akt1* hypomorphs.

Methods

Drosophila stocks, media and culture

The initial P-element insertion line $ry^{506}\ P\{PZ\}Akt11^{04226}/TM3,\ ry^{RK},\ Sb^{1},\ Ser^{1}$ ($Akt1^{04226}$) was obtained from the Bloomington Drosophila Stock Center. This line contains a P-element inserted within the 5' untranslated region of the *Akt1* gene on the third chromosome. Initial reports of this allele state that it is semi-lethal (Gao et al., 2000), but we, in addition to other researchers (Stocker et al 2002; Lee and Chung, 2007) have found the homozygotes to be viable. The control line $w^{1118}\ ;\ P[FRT\ ;w^{+}]^{2A}\ P[ry^{+}\ neo^{R}\ FRT]^{82B}\ Akt1^{+}$ was derived from lines obtained

from Norbert Perrimon of Harvard University. The *PΔ2-3, ry*$^{+}$ line was utilized to generate the novel *Akt1* mutants (Robertson et al., 1988). The *foxo* null mutant line *w; foxo*^{W124X} was obtained from Drs. E. Hafen and M. Junger (Juner et al., 2003) of the University of Zurich. 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. Stocks and crosses were maintained on a standard medium containing cornmeal, molasses, yeast, agar and water. Routinely, stocks were kept at room temperature (22 ± 2°C) while crosses and experiments were carried out at 25°C.

Generation of Drosophila lines

Hypomorphic alleles of *Akt1*^{04226} were generated via P-element excision by crosses to a line containing a stable source of transposase, *PΔ2-3*. The critical class offspring of the dysgenic males and *Ly/TM3, Sb ry* females were selected based upon loss of the *PZ* P-element by the presentation of the *rosy* eye colour phenotype. These novel alleles were expected to differ from the *Akt1*$^{+}$ line and *Akt1*^{04226} by the resultant alterations of the PZ P-element and/or the adjacent *Akt1* sequences. To allow for clonal analysis, recombinants of *w; P[FRT ;w*$^{+}$*]*^{2A} *P[ry*$^{+}$ *neo*^{R} *FRT]*^{82B} and the novel derivatives of *Akt1*^{04226} were generated and balanced over *TM6B, Hu Tb e*. Of the derivatives generated, a subset of these recombinants were selected for analysis based on the appearance of non-*Tubby* homozygotes.

Replacement studies were carried out by generating independent lines of *w*^{1118}*; UAS-Akt1*$^{+}$*/CyO; Akt1*^{m}*/TM6B* and *w*^{1118}*; arm-GAL4/CyO; Akt1*^{m}*/TM6B* where *m* represents each of the novel *Akt1* mutant alleles. Crosses between these lines generated the critical class of *w*^{1118}*; UAS-Akt1*$^{+}$*/arm-GAL4; Akt1*^{m}*/Akt1*^{m} to be analyzed.

The presence of FRT sites near the centromere of the 3R chromosome arm in the *Akt1*^{04226} derivative stocks allowed for somatic clones to be generated. The Drosophila line *y w; P{w*$^{+m}$*=GAL4-ey.H}*$^{3-8}$ *P{w*$^{+mC}$*=UAS-FLP1.D}*^{JD1}*; P{ry*$^{+t7.2}$*=neoFRT}*^{82B} *P{w*$^{+mC}$*=GMR-hid}*^{SS4} *l(3)CL-R*^{1}*/TM2* possesses *eyeless*-driven *FLP* and a distal recessive lethal allele (Stowers and Schwarz, 1999) which, when crossed to each of the *Akt1*^{04226} derivatives generated the critical class of *y w; P{w*$^{+m}$*=GAL4-ey.H}*$^{3-8}$ *P{w*$^{+mC}$*=UAS-FLP1.D}*^{JD1}*/+*; *P[FRT ;w*$^{+}$*]*^{2A} *P[ry*$^{+}$ *neo*^{R} *FRT]*^{82B} *Akt1*^{m}*/ P{ry*$^{+t7.2}$*=neoFRT}*^{82B} *P{w*$^{+mC}$*=GMR-hid}*^{SS4} *l(3)CL-R*^{1} where *m* represents the allele of *Akt1* derived from *Akt1*^{04226}. The distal lethal allele resulted in the death of any homozygous *Akt1*$^{+}$ cells thereby making the eye almost completely composed of homozygous *Akt1*^{m} cells.

Generation of flies bearing both the novel mutant *Akt1* alleles and a null *foxo* allele was performed via standard recombinant methods. As *Akt1*^{52} and *Akt1*^{57} exhibited the greater developmental delay, these alleles were selected for recombination with the null allele of *foxo*. From these combinations, a series of lines were selected based upon adult phenotypes and confirmed through PCR and sequencing.

Molecular Characterization of the novel hypomorphs

Homozygous wild type, novel hypomorphic and double mutant Drosophila samples were collected from crosses of adult heterozygous female virgins to heterozygous males of each genotype. Ten homozygous male flies were collected upon eclosion and aged three to five days before being flash-frozen at -70°C. DNA was extracted from each sample via a standard phenol-chloroform protocol. The Flybase database (*http://flybase.org*) includes the complete sequence for the PZ P-element positioned within the *Akt1*^{04226} line, and the National Center for

Biotechnology Information (NCBI) database (*http://www.ncbi.nlm.nih.gov/*) includes the complete gene sequence for *Akt1*$^{+}$. To design oligonucleotides indicating the breakpoint region of each novel mutant, a series of oligonucleotides both flanking and spanning the P element insertion site was carried out via Primer3. PCR analysis via primers spanning the PZ P element revealed forward primer sites present and positioned near the breakpoints. A reverse primer positioned near the end of the PZ P-element allowed for generation of breakpoint fragments using HotStart Taq Polymerase (Qiagen Inc.) in an Eppendorf Mastercycler gradient thermal cycler through standard methods. Gels were photographed with a ChemiImageTM Ready 4400 v5.5 photo-documentation system. Purification and sequencing of the PCR products was completed at the Genomics and Proteomics (GaP) facility, Memorial University of Newfoundland.

Analysis of Developmental Timing

Heterozygotes are identified based upon the presence of *Humeral (Hu)*, an allele of *Antennapedia* carried by the *TM6B, Hu Tb e* balancer chromosome that results in extra bristles on the outer edges of the prothorax, while homozygous mutant flies lack this marker. Heterozygous females and males of the novel *Akt1* hypomorphs were transferred to fresh media, incubated at 25°C for six hours to allow for egg-laying then removed. Vials were returned to the incubator immediately after removal of adult flies and examined each morning for fifteen days. Observations included days until pupation and eclosion. Pupae and adult flies were scored as *Tubby* heterozygotes or non-*Tubby* mutants and used to generate developmental delay line graphs in GraphPad Prism Version 5.03.

Biometric analysis of Drosophila eyes

Critical class males of the homozygous mutant *Akt1* alleles, the transgenic rescues, the somatic clones and the double mutant lines were collected and aged for three days. Flies were then flash-frozen at –70°C before preparation for scanning electron microscopy. Preparation included mounting upon aluminum SEM studs, desiccated and sputter coating in gold. Images were taken with either a Hitachi S-170 or S-570 Scanning Electron Microscope as per standard methods and analyzed through counting and measuring the ommatidia of each eye using NIH Image J software (Abramoff et al., 2004).

Results

Three novel Akt1 mutants retain portions of inserted PZ P-element

Molecular characterization of the three small viable *Akt1* mutants revealed internally deleted versions of the *PZ* P-element at the original point of insertion (Figure 1). Analysis indicates that the retained sections are from both ends of the *PZ* P-element. $Akt1^{87}$ possesses the largest deleted region (11029 base pairs) between nucleic acids 2184 (within *lacZ*) and 13213 (within ry^{+}) of the *PZ* P-element sequence. The next largest deletion (9532 base pairs) is in $Akt1^{52}$ between nucleic acids 2754 (within *lacZ*) and 12307 (within ry^{+}). Lastly $Akt1^{57}$ has the smallest deletion of the three mutants of 3259 base pairs between nucleic acid 4315 (within the *HSP70* poly-adenylation control region) and 7574 (within ry^{+}). Each deletion includes a part of the ry^{+} gene, responsible for the phenotype (ry^{-}) upon which these mutants

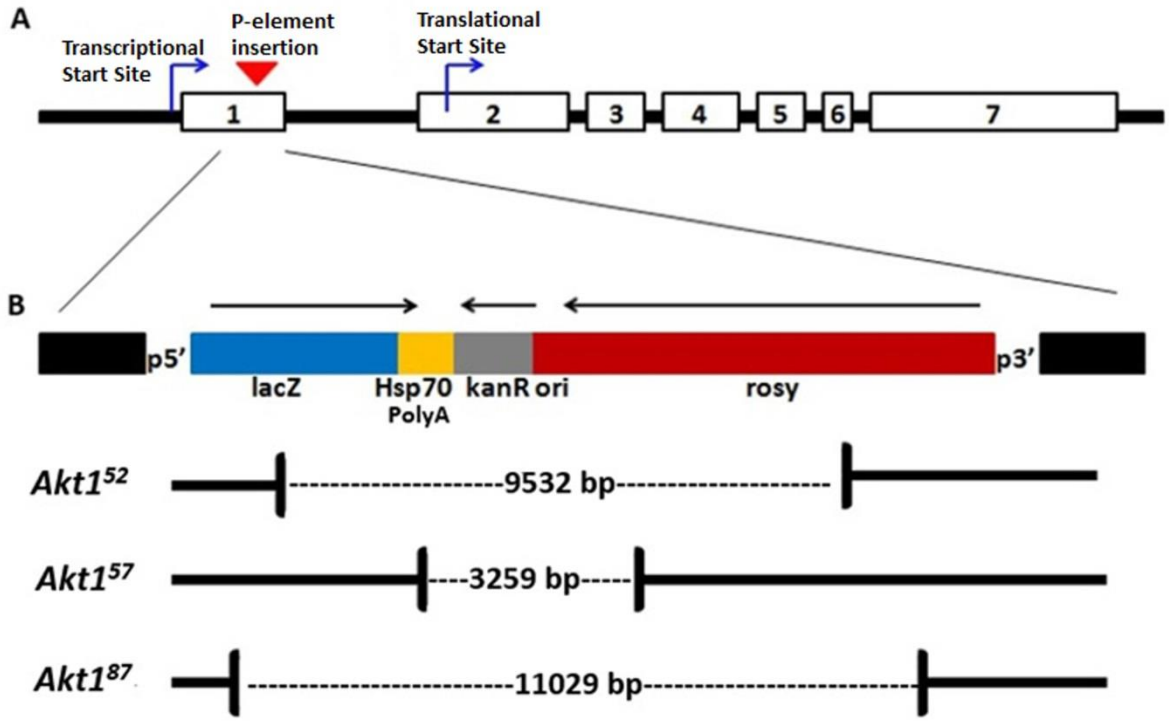

Figure 1: Novel hypomorphic alleles of *Akt1*$^{+}$ retain PZ P-element sequences within exon 1. A) Gene map of *Akt1*$^{+}$, transcript variant B (Genbank accession: NM_169707.2) White boxes represent each of the seven exons. The P-element insertion site is located within exon 1 between the transcriptional and translational start sites. B) Molecular characterization confirms the base pair sizes of the internally deleted sections for each hypomorphic allele. Analysis indicates that each allele retains various portions of both the *lacZ* and *rosy* genes located either end of the PZ P-element.

were selected. No alteration to the *Akt1* coding region sequence was detected in the three mutants.

Three novel Akt1 mutants are developmentally delayed

In the development from embryo to adult, *Akt1* mutant heterozygotes are similar to controls (Figure 2). The formation of heterozygous (*Tubby*) pupae occurs in a similar time-frame to the control lines (data not shown). The time required to eclose by adult *Akt1* heterozygotes (*Humeral*) and control flies is nine to ten days (Figure 2). Emergence of the homozygous adult flies is delayed by two to four days. One allele, $Akt1^{87}$, is delayed until day 12 while the other alleles are delayed until day 14 (Figure 2). The extended time of development of homozygotes may be required for the production of the adult mutants.

The eyes of novel Akt1 mutants are reduced in ommatidia size and number

Biometric analysis of homozygous *Akt1* mutant eyes indicates there is an overall decrease in both ommatidia number and size when compared to controls (Table 1, Figure 3). The control eyes had 676.4 ± 13.2 ommatidia per eye (OPE) and an ommatidia area of 222.6 (± 4.3) um^2, which was the largest overall. The original P element insertion mutant $Akt1^{04226}$ has the smallest ommatidia area of 185.6 ± 2.4 um^2, but with an ommatidia number of 604 ± 2 OPE. The three novel *Akt1* hypomorphs were all smaller than the control in ommatidia area and significantly reduced in ommatidia number when compared to both the control and the original P-element insertion mutant. Of these three, $Akt1^{52}$ is the smallest with a count of 544.8 ± 14.1 OPE and an area of 191.1 ± 2.4 um^2. The mutant $Akt1^{87}$ is slightly larger with an ommatidia area

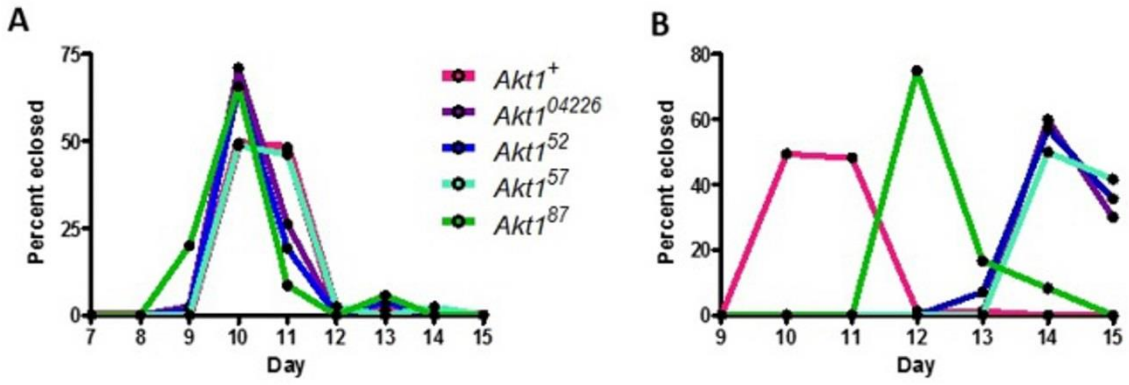

Figure 2: Novel *Akt1* mutants are developmentally delayed. Data of developmental delay experiment was plotted as percent eclosed versus day as a line graph. This method allows the peaks of each line to clearly represent the day of which most adult flies eclosed for each genotype. N = 85 ($Akt1^{+}$), 48 ($Akt1^{04226}$), 40 ($Akt1^{52}$), 53 ($Akt1^{57}$) and 47 ($Akt1^{87}$). **A)** Heterozygotes eclose by day ten along with the control. B**)** The homozygous mutants are delayed in growth and do not eclose until two to four days later than the controls.

Figure 3: Novel *Akt1* mutants are reduced in both ommatidia size and number, enhanced in somatic clones and partially rescued by expression of *Akt1*$^{+}$. Scanning electron micrographs illustrate the phenotypes of the homozygote, somatic clone and transgenic rescue of the control, original P-element insertion mutant and each of the three novel *Akt1* mutants. Top row images (A-E) of homozygous mutants signify that hypomorphic alleles of *Akt1* are reduced in size. The middle row images (F-J) of somatic clones of these alleles reveal an increase in severity of this phenotype. The bottom row of images (K-O) express wild type *Akt1* in the background of the hypomorphic alleles and validate these phenotypes are *Akt1* dependent. Full genotypes are **A:** *w; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{+}/Akt1^{+}* **B:** *w; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{04226}/Akt1^{04226}* **C:** *w; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{52}/Akt1^{52}* **D:** *w; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{57}/Akt1^{57}* **E:** *w; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{87}/Akt1^{87}* **F:** *y w; P{w^{+m}=GAL4-ey.H}$^{3-8}$ P{w^{+mC}=UAS-FLP1.D}^{JD1}/+; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{+}/ P{ry$^{+t7.2}$=neoFRT}^{82B} P{w^{+mC}=GMR-hid}^{SS4} l(3)CL-R^{1}* **G:** *y w; P{w^{+m}=GAL4-ey.H}$^{3-8}$ P{w^{+mC}=UAS-FLP1.D}^{JD1}/+; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]82B Akt104226/ P{ry+t7.2=neoFRT}82B P{w+mC=GMR-hid}SS4 l(3)CL-R1* **H:** *y w; P{w^{+m}=GAL4-ey.H}$^{3-8}$ P{w^{+mC}=UAS-FLP1.D}^{JD1}/+; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{52}/ P{ry$^{+t7.2}$=neoFRT}^{82B} P{w^{+mC}=GMR-hid}^{SS4} l(3)CL-R^{1}* **I:** *y w; P{w^{+m}=GAL4ey.H}$^{3-8}$ P{w^{+mC}=UAS-FLP1.D}^{JD1}/+; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{57}/ P{ry$^{+t7.2}$=neoFRT}^{82B} P{w^{+mC}=GMR-hid}^{SS4} l(3)CL-R^{1}* **J:** *y w; P{w^{+m}=GAL4-ey.H}$^{3-8}$ P{w^{+mC}=UAS-FLP1.D}^{JD1}/+; P[FRT ;w^{+}]^{2A} P[ry^{+} neo^{R} FRT]^{82B} Akt1^{87}/* P{ry$^{+t7.2}$=neoFRT}^{82B} P{w^{+mC}=GMR-hid}^{SS4} l(3)CL-R^{1} **K:** w^{1118}; UAS-Akt1^{+}/arm-GAL4; *Akt1^{+}/Akt1^{+}* **L:** *w^{1118}; UAS-Akt1^{+}/arm-GAL4; Akt1^{04226}/Akt1^{04226}* **M:** *w^{1118}; UASAkt1^{+}/arm-GAL4; Akt1^{52}/Akt1^{52}* **N:** *w^{1118}; UAS-Akt1^{+}/arm-GAL4; Akt1^{57}/Akt1^{57}* **O:** *w^{1118}; UAS-Akt1^{+}/arm-GAL4; Akt1^{87}/Akt1^{87}* . Scale bar = 100 um. Biometric analysis of both the number, (**P**) and area, (**Q)** of ommatidia quantifies the subtlety of these size differences. N values can be found in table 1. Green bars represent the homozygotes; purple bars represent the clones and blue bars represent the transgenic rescues. The original P-element insertion mutant *Akt1^{04226}* is smaller than the control but larger than the novel mutants in both size and number. All three novel *Akt1* mutants are reduced in both the area and number of ommatidia as both homozygotes or clones but reach almost comparable measurements compared to the control when rescued by a transgene. Error bars represent standard error of the mean ($p = <0.05$). Further statistical analysis can be found in table 1.

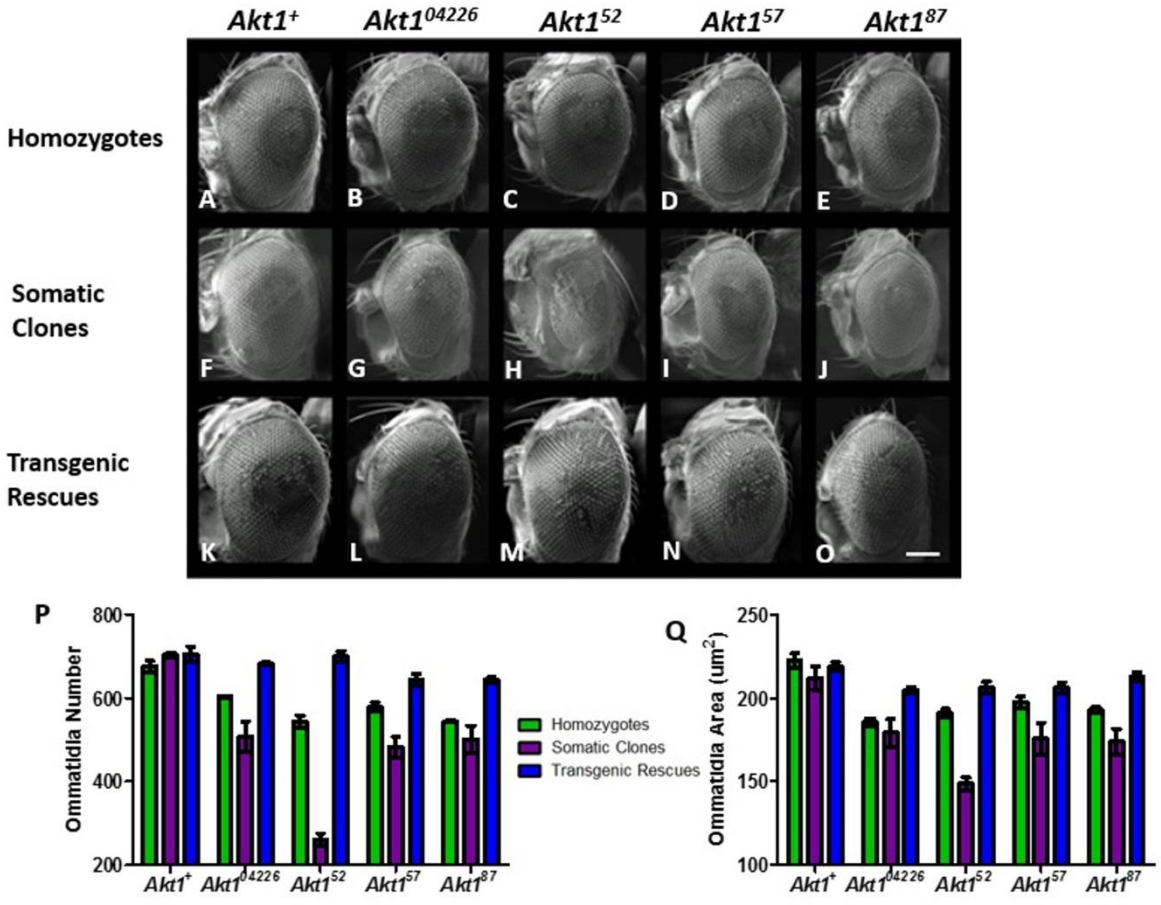
Akt1+
Akt1 04226
Akt1 52
Akt1 57
Akt1 87
Homozygotes
Somatic Clones
Transgenic Rescues
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Ommatidia Number
800
600
400
200
Homozygotes
Somatic Clones
Transgenic Rescues
Q
Ommatidia Area (um2)
250
200
150
100

Table 1: Biometric analysis of ommatidia area and number in homozygous mutant, transgenic rescue and somatic clones of novel *Akt1* mutant alleles

Allele	a) Homozygotes									
	N	**OA**	**P1**	**P2**	**P3**	**N**	**ON**	**P1**	**P2**	**P3**
Akt1^{+}	15	222.6 ± 4.3	N/A	0.5286 NS	<0.0001 S	5	676.4 ± 13.2	N/A	0.2179 NS	0.1671 NS
Akt1^{04226}	15	185.6 ± 2.4	<0.0001 S	<0.0001 S	<0.0001 S	5	604 ± 2	0.0006 S	<0.0001 S	0.0007 S
Akt1^{52}	15	191.1 ± 2.4	<0.0001 S	0.0019 S	<0.0001 S	5	544.8 ± 14.1	0.0001 S	<0.0001 S	<0.0001 S
Akt1^{57}	15	197.6 ± 3.5	0.0002 S	0.0741 NS	<0.0001 S	5	579.4 ± 11	0.0005 S	0.0057 S	0.0017 S
Akt1^{87}	15	192.8 ± 2.1	<0.0001 S	<0.0001 S	<0.0001 S	5	545.4 ± 2.5	<0.0001 S	<0.0001 S	0.0214 S
Allele	**b) Transgenic Rescues**									
	N	**OA**	**P1**			**N**	**ON**	**P1**		
Akt1^{+}	15	218.9 ± 2.7	N/A			5	705.8 ± 17.6	N/A		
Akt1^{04226}	15	204.7 ± 1.8	0.0002 S			5	683.2 ± 3.9	0.2445 NS		
Akt1^{52}	15	206.3 ± 3.6	0.0086 S			5	701 ± 12.6	0.8297 NS		
Akt1^{57}	15	206.2 ± 3.2	0.0052 S			5	644.6 ± 7.1	0.0246 S		
Akt1^{87}	15	212.9 ± 2.5	0.1112 NS			5	643.6 ± 7.1	0.0112 S		
Allele	**c) Somatic Clones**									
	N	**OA**	**P1**			**N**	**ON**	**P1**		
Akt1^{+}	9	212.1 ± 7.1	N/A			3	704.3 ± 5.5	N/A		
Akt1^{04226}	9	179.3 ± 8.3	<0.0001 S			3	508.3 ± 35.4	0.0007 S		
Akt1^{52}	9	148.7 ± 3.9	<0.0001 S			3	261 ± 14.3	<0.0001 S		
Akt1^{57}	9	175.7 ± 9.6	<0.0001 S			3	483 ± 24.5	0.0001 S		
Akt1^{87}	9	173.8 ± 7.9	<0.0001 S			3	501 ± 33.2	0.0005 S		

** OA = Ommatidia Area (um^2), ON = Ommatidia Number, P1 = P-value when compared to *Akt1^{+}* control, P2 = P-value when compared to transgenic rescue counterpart, P3 = P-value when compared to somatic clone counterpart, S = significant, NS = not significant

of 192.8 ± 2.1 um^2 and 545.4 ± 2.5 OPE. The largest of the mutants is $Akt1^{57}$ with an ommatidia area of 197.6 ± 3.5 um^2 and an ommatidia number of 579.4 ± 11 OPE.

Transgenic replacement partially rescue the phenotype of mutant homozygotes

Ubiquitous expression of wild-type *Akt1* in the background of the homozygous mutants results in a partial rescue of both ommatidia size and number (Table 1, Figure 3).
The transgenic control $Akt1^+$ is the largest eye overall having an ommatidia area of 218.9 ± 2.7 um^2 and a total number of 705.8 ± 17.6 OPE. The transgenic expression of $Akt1^+$ in the background of the original P-element insertion mutant results in eyes that are only slightly smaller than the control having an ommatidia area of 204.7 ± 1.8 um^2 and a total of 683.2 ± 3.9 OPE. In all cases the size of the ommatidia and the total count of ommatidia for the mutants with transgenic replacement of wild-type $Akt1^+$ does not differ significantly from the control (Figure 3). The average ommatidia area is very similar for $Akt1^{52}$ and $Akt1^{57}$ being 206.3 ± 3.6 um^2 and 206.2 ± 3.2 um^2 respectively; while the area for $Akt1^{87}$ is larger at 212.9 ± 2.5 um^2. The ommatidia number for the partially rescued mutants is similar for $Akt1^{57}$ and $Akt1^{87}$ being 644.6 ± 13.5 OPE and 643.6 ± 7.1 OPE respectively, with $Akt1^{52}$ having a few more ommatidia at 701 ± 12.6 OPE.

Somatic clones of the eye have a more severe phenotype than the homozygotes

Given the developmental delay of the *Akt1* homozygotes, analysis of somatic clones of the eye was carried out. The FLP recombinase was driven by the *eyeless* promoter to direct expression in the developing eye tissue. In the presence of FLP, homologous chromosomes undergo mitotic recombination between the FRT sites located on chromosome pairs.

Heterozygous parent cells can produce both homozygous *Akt1* mutant cells containing two copies of the mutant allele, and cells containing two copies of $Akt1^{+}$. In this system, the $Akt1^{+}$ daughter cells are lost due to the presence of a linked recessive cell lethal mutation located on the same arm of the chromosome bearing the $Akt1^{+}$ allele. Thus, in the eyes of clone bearing flies, the surviving cells bear two copies of the *Akt1* mutant allele under investigation. The clone of the control is the largest and most consistent in size when compared to its homozygous and transgenic rescued counterpart having an ommatidia area of 212.1 ± 7.1 um^2 and number of 704.3 ± 5.5 OPE. The cloned original P-element insertion mutant $Akt1^{04226}$ is reduced in both size and number compared to its original homozygous version with an average area of 179.3 ± 8.3 um^2 and ommatidia number of 508.3 ± 35.4 OPE, yet is comparable in size and number to two of the cloned novel mutants, $Akt1^{57}$ and $Akt1^{87}$, which have an ommatidia area of 175.7 ± 9.6 um^2 and 173.8 ± 7.9 um^2 and a count of 483 ± 24.5 OPE and 501 ± 33.2 OPE respectively. The measurement and count of ommatidia for both of these mutants is significantly smaller than that of their homozygous versions. Of all the mutants, $Akt1^{52}$ exhibits the most severe phenotype with the greatest decrease in ommatidia area (148.7 ± 3.9 um^2) and number (261 ± 14.3 OPE) when compared to both its homozygous counterpart as well as with the other cloned mutants.

Akt1-foxo double mutant lines reveal an epistatic relationship

Drosophila lines having both the novel *Akt1* mutant alleles in combination with a null *foxo* mutant allele resemble the original *foxo* mutant more closely than the *Akt1* mutants (Figure 4; Table 2). The controls *OrR* and w^{1118} have an ommatidia area of 189.4 ± 1.43 um^2 and 185.5 ± 1.34 um^2 and an ommatidia count of 675.7 ± 4.4 OPE and 665.3 ± 9.6 OPE respectively. In

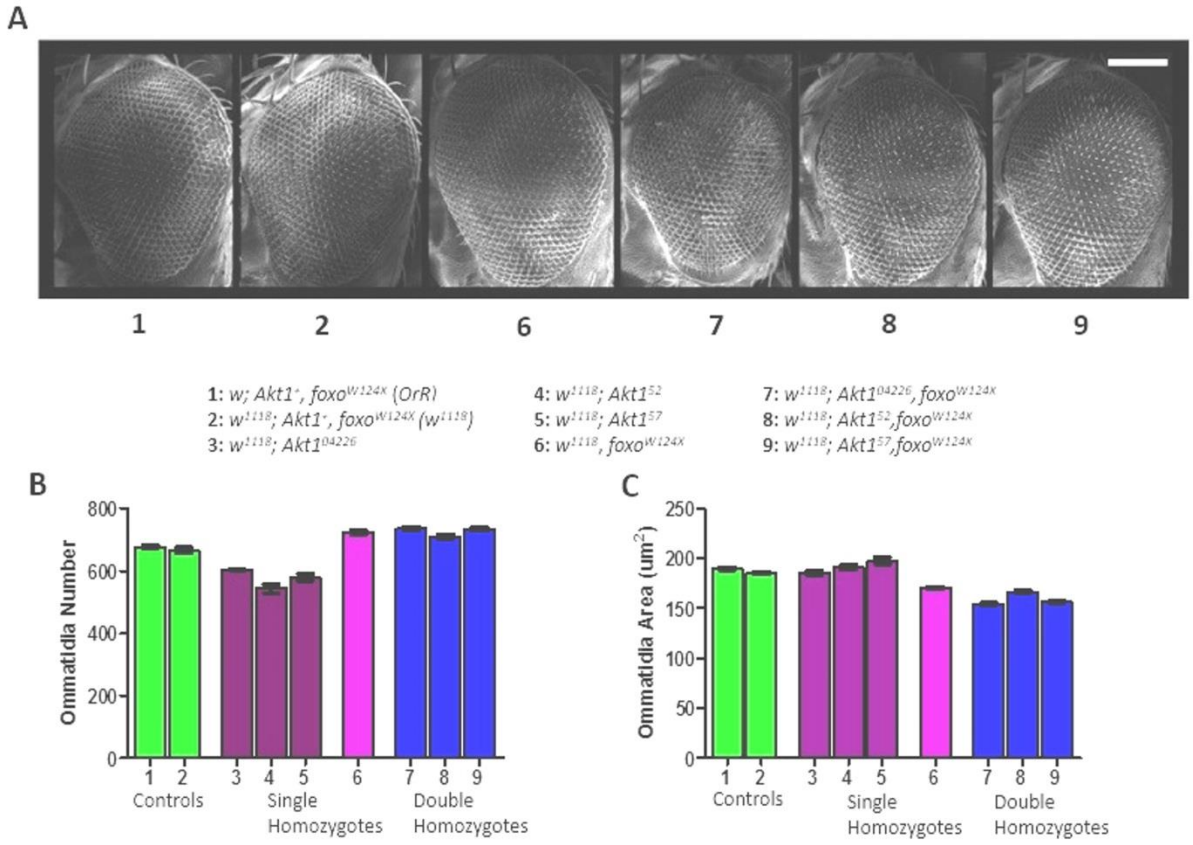

Figure 4: Double *Akt1/foxo* mutants demonstrate an epistatic effect upon growth. A. Scanning electron micrographs of Drosophila eyes bearing hypomorphic alleles of *Akt1* and null mutations of the *foxo* gene indicates that the double mutants more closely resemble the phenotype of the null *foxo* mutation. Representative images of genotypes 3, 4 and 5 can be found in figure 3. Scale bar = 100 um. Biometric analysis quantifies this similarity in terms of ommatidia number (**B**) and size (**C**). N values can be found in table 2. Green bars represent the controls, purple bars represent the novel *Akt1* mutants and the null *foxo* mutant, blue bars represent the double mutant homozygotes. In analysis of both the ommatidia size and number, the double mutants have a larger number of smaller ommatidia in comparison to the original *Akt1* mutants, but are comparable in both size and number to the null *foxo* mutant. Error bars represent standard error of the mean ($p = <0.05$). Further statistical analysis can be found in table 2.

Table 2: Biometric analysis of ommatida area and number of Drosophila eyes bearing both a novel *Akt1*mutant allele and a null *foxo* mutant allele

Genotype	**N**	**OA**	**P1**	**P2**	**P3**	**P4**
w; Akt1^{+}, foxo^{+} (OrR)	48	189.4 ± 1.43	N/A	N/A	N/A	N/A
w^{1118}; Akt1^{+}, foxo^{+} (^{w1118})	36	185.5 ± 1.34	N/A	N/A	N/A	N/A
w^{1118}; foxo^{w124x}	42	170.4 ± 1.42	<0.0001 S	<0.0001 S	N/A	N/A
w^{1118};Akt1^{04226},foxo^{W124x}	45	154.4 ± 1.19	<0.0001 S	<0.0001 S	<0.0001 S	<0.0001 S
w^{1118};Akt1^{52}, foxo^{W124x}	39	166.3 ± 1.68	<0.0001 S	<0.0001 S	<0.0001 S	0.0630 NS
w^{1118};Akt1^{57}, foxo^{w124x}	54	156.3 ± 1.12	<0.0001 S	<0.0001 S	<0.0001 S	<0.0001 S
Genotype	**N**	**ON**	**P1**	**P2**	**P3**	**P4**
w; Akt1^{+}, foxo^{+} (OrR)	16	675.7 ± 4.4	N/A	N/A	N/A	N/A
w^{1118}; Akt1^{+}, foxo^{+} (^{w1118})	12	665.3 ± 9.6	N/A	N/A	N/A	N/A
w^{1118}; foxo^{w124x}	14	723 ± 5.6	<0.0001 S	<0.0001 S	N/A	N/A
w^{1118};Akt1^{04226},foxo^{W124x}	15	735 ± 5.2	<0.0001 S	<0.0001 S	<0.0001 S	0.1282 NS
w^{1118};Akt1^{52}, foxo^{W124x}	13	709.1 ± 8.2	0.0008 S	0.0020 S	<0.0001 S	0.1686 NS
w^{1118};Akt1^{57}, foxo^{w124x}	18	733.9 ± 6.3	<0.0001 S	<0.0001 S	<0.0001 S	0.2212 NS

** OA = Ommatidia Area (um^{2}), ON = Ommatidia Number, P1 = P-value when compared to *OrR* control, P2 = P-value when compared to the *w^{1118}* control, P3 = P-value when compared to the *Akt1* homozygote counterpart, P4 = P-value when compared to *w^{1118}; foxo^{W124X}* , S = significant, NS = not significant

comparison, the null *foxo* mutant eye has a smaller average ommatidia area of 170.4 ± 1.42 um^2 and a higher ommatidia count of 723 ± 5.6 OPE. The double mutant lines are smaller than both the controls and the null *foxo* mutant in size of ommatidia, but have counts of ommatidia that are not significantly different from the null *foxo* mutant. Both the double mutants bearing $Akt1^{04226}$ and $Akt1^{57}$ alleles have similar ommatidia areas of 154.4 ± 1.2 um^2 and 156.3 ± 1.1 um^2, and ommatidia numbers of 735 ± 5.2 OPE and 733.9 ± 6.3 OPE respectively. The double mutant bearing the $Akt1^{52}$ allele is closer to the null *foxo* mutant in ommatidia size (166.3 ± 1.7 um^2) but has slightly fewer ommatidia with 709.1 ± 8.2 OPE. The ommatidia size for each of the double mutants is considerably smaller than the original homozygous mutant versions of each *Akt1* mutant allele, while the counts of ommatidia are much higher, exhibiting the same trend as the null *foxo* mutant in comparison to the novel *Akt1* mutants.

Discussion

Viable novel *Akt1* hypomorphs were generated via imprecise P-element excision and were found to retain internally deleted versions of the original *PZ* P-element upstream of the *Akt1* gene's protein coding region (Figure 1). Three selected *Akt1* hypomorphs were characterized phenotypically as small in size and delayed in terms of developmental time. In Drosophila, the development from egg to adult involves three larval stages plus pupation before the non-growing sexually mature adult fly arises. The timing of transition between these stages is dependent upon the rate of growth. The insulin receptor signalling pathway is a major contributor in the control of growth and has been implicated in the control of the onset of metamorphosis in Drosophila (Walkiewicz and Stern, 2009). Ablation of insulin producing cells within the larval brain decreases the growth rate and delays metamorphosis in Drosophila, as does a loss-of-

function mutation of the insulin receptor (Rulfison et al., 2002; Shingleton, 2005). As Akt1 is a central component of the insulin receptor signalling pathway, it is not surprising that these novel hypomorphic alleles result in a delay of development and overall smaller adult organisms.

Due to the extension in the time for the novel mutants to undergo eclosion, a comparison of the phenotypes of the eye for both homozygous mutants and somatic clones was undertaken. The clone eyes are comprised of homozygous mutant tissue in a heterozygous organism that develops within a relatively normal timeframe. Biometric analysis of the eyes of these mutant clones revealed enhanced severity of the decreased growth phenotype. Adult organisms, as well as their organs and tissues, have a tendency to develop within a range of normal overall size, such that the cellular composition may vary from a large number of small cells, to a small number of large cells. Cell growth includes an increase in cell number and cell size, and while not mutually exclusive, both can be regulated by distinct extracellular processes (Coelho and Leevers, 2000; Day and Lawrence, 2000; Stocker and Hafen, 2000; Johnston and Gallant, 2002), including the insulin receptor signalling pathway, which is highly conserved between invertebrates and mammals (Brogiolo et al., 2001). Reduced expression or loss of *Akt1*, the central component of insulin receptor signalling, can result in the production of smaller animals or, if severe, lethality (Kennedy et al., 1997; Staveley et al., 1998; Verdu et al., 1999). The smaller eyes observed in the homozygotes is expected with the lower expression of *Akt1* in these novel mutants.

Compensatory growth is widespread and occurs in the surviving cells of damaged tissues to generate final structures of near normal overall size (Gerhold et al., 2011; Worley et al., 2012). This growth consists of remodeling the existing tissue to regenerate the full body plan in response to tissue damage leading to the development of a smaller but still complete and intact

organism. In order to maintain tissue homeostasis, cells that survive the tissue damage can compensate for those that are lost by increasing their rate of proliferation and cell divisions. Cells in Drosophila that have experienced an increase in cell death via radiation showed an increase in proliferation by the surviving cells (Schweizer, 1972). Compensatory proliferation has been shown to lead to the development of normal-sized adult wings even when 40-60% of cells in the wing disc of Drosophila are either killed or rendered incapable of further proliferation (Haynie and Bryant, 1977). The novel *Akt1* hypomorphs examined in this study have been shown to be developmentally delayed and result in the formation of small adult flies. The mutant clone eyes show a more severe phenotype due to the reduced replacement of missing tissue without the extended time during development. Clearly, the extended period of time required for these mutants to develop allows compensatory proliferation to generate smaller but intact adults.

In order to begin to understand the mechanisms responsible for the observed compensatory growth, double mutant lines of the novel *Akt1* hypomorphs and an amorphic allele of *foxo*, a gene encoding a key downstream target of *Akt1*, were generated. The transcription factor foxo is known as a major effector of insulin receptor signalling and has been implicated in the control of cell growth. Overexpression of the mammalian homologues of *foxo*, as well as Drosophila *foxo*, leads to growth arrest (Kramer et al., 2003; Neufeld, 2003) which can be suppressed with increased insulin receptor signalling. This suppression is ineffective when the foxo transcription factor has been made incapable of phosphorylation, and thus nuclear exclusion, by Akt1. In addition to this, *foxo* governs the expression of target genes that encode factors that regulate cell growth such as the eukaryotic initiation factor 4E-binding protein (4E-BP) gene and cell cycle regulators including $p27^{kip1}$. The *4E-BP* product is a negative regulator of protein synthesis and has been shown to strongly influence the regulation of cell growth

(Miron et al., 2001). When foxo is upregulated, so is 4E-BP, which binds to the messenger RNA 5' cap-binding protein eIF4E to inhibit protein synthesis and cell growth. In humans, $p27^{kip1}$ inhibits cyclin-dependent kinases (cdks) (Dijkers et al., 2000), which aid in promoting the transitions between cell-cycle phases. Overexpression of $p27^{kip1}$ in human cells leads to cell-cycle arrest in the G1 phase, and when *foxo* is upregulated, subsequently $p27^{kip1}$ is upregulated (Miron et al., 2001; Van Der Heide et al., 2004). Co-expression of *foxo* and constitutively active *Ras2*, which can induce G1/S progression and cell proliferation, is able to partially rescue the phenotype in the eye that is observed with an overexpression of *foxo* alone (Kramer et al., 2003). An increase in *foxo* activity appears to result in a decrease in cell proliferation. The double mutants, having both hypomorphic alleles of *Akt1* and null alleles of *foxo*, more closely resemble the *foxo* mutants. Analysis of ommatidia number shows an epistatic effect whereas an argument could be made in the comparison of ommatidia area for a slight synergistic enhancement of the phenotype. Regardless this suggests that without the presence of the *foxo* gene product, the hypomorphic alleles of *Akt1* do not cause the same reduction of growth and strongly suggests that *foxo* is necessary for the processes that lead to compensatory growth.

Through the generation of clones, we were able to uncover a more severe effect of these *Akt1* hypomorphs upon the control of growth. Originally, the generation of somatic clones was utilized to study homozygous tissue in a heterozygous organism when the homozygotes themselves were not viable. However, when used to study homozygous tissues of viable, yet small and developmentally delayed organisms, this system can expose a subtle phenotype previously obscured by compensatory proliferation. Developmental delay is a common phenomenon associated with many genetic mutations and could potentially play a significant role in the final phenotype. Generation of somatic clones would eliminate this developmental timing

factor, thereby clarifying the impact a genetic mutation has on cellular processes including growth.

List of abbreviations foxo: forkhead box subgroup "O"; Ilp: insulin-like peptide; InR: insulin receptor; OPE: ommatidia per eye

Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

JDS generated some recombinant lines, performed the molecular characterizations, the developmental timing evaluations, the scanning electron microscopy and the biometric analysis of the mutants, performed the statistical analyses and drafted the initial manuscript. BES generated the mutants and some recombinant lines, 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 number: 14-09-BS).

Acknowledgments

The authors would like to thank Drs. Liqui Men, formerly of the Bio-molecular Imaging Cluster at Memorial University of Newfoundland, and Michael Shaffer, formerly of the Microanalysis facility of Memorial University of Newfoundland for technical assistance with scanning electron microscopy. Thanks to members of the Genomics and Proteomics facility at Memorial University of Newfoundland for assistance with sequencing. Thanks also to Drs. Martin Junger and Ernst Hafen for providing the *foxo*^{W124X} line, Dr. Howard Lipshitz for providing the w^{1118} line, and to Dr. Norbert Perrimon for providing the original w^{1118} *; P[FRT ;*w^{+}*]*^{2A} *P[ry*$^{+}$ *neo*^{R} *FRT]*^{82B} line. This project was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Post-Graduate Scholarship to JDS and NSERC Discovery Grants to BES. JDS was also partially funded by a Department of Biology Teaching Assistantship and a School of Graduate Studies Fellowship from Memorial University of Newfoundland.

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Chapter 3:

Enhanced survival of *Drosophila Akt1* hypomorphs during amino acid starvation requires *foxo*

A version of this chapter is published as:

Slade, J.D., and Staveley, B.E. 2016. Enhanced survival of Drosophila *Akt1* hypomorphs during amino acid starvation requires *foxo*. Genome **59**(2): 87-93.

Abstract

Disordered eating includes any pattern of irregular eating that may lead to either extreme weight loss or obesity. The conserved insulin receptor signalling pathway acts to regulate energy balance and nutrient intake, and its central component Akt1 and endpoint effector foxo are pivotal for survival during nutritional stress. Recently generated *Akt1* hypomorphic mutant lines exhibit a moderate decrease in lifespan when aged upon standard media, yet show a considerable increase in survival upon amino acid starvation media. While the loss of *foxo* function significantly reduces the survival response to amino acid starvation, a combination of these *Akt1* hypomorphs and a null *foxo* mutation reveal a synergystic and severe reduction in lifespan upon standard media, and an epistatic relationship when undergoing amino acid starvation. Evaluation of survivorship upon amino acid starvation media of these double mutants indicate a phenotype similar to the original *foxo* mutant demonstrating the role of *foxo* in this *Akt1* phenotype. These results indicate that the subtle manipulation of foxo through Akt1 can enhance survival during adverse nutrient conditions to model the ability of individuals to tolerate nutrient deprivation. Ultimately, we believe that a Drosophila model of disordered eating could generate new avenues to develop potential therapies for related human conditions.

Key words: *Drosophila melanogaster*, starvation, Akt1, foxo

Introduction

A number of eating disorders, including the medically diagnosable forms of anorexia and bulimia, or any irregular eating patterns leading to extreme weight loss or gain, can be life threatening (Neilson, 2001; Fairburn and Harrison, 2003). The psychiatric illness with the highest mortality rate is anorexia, with 10% of diagnosed individuals dying within 10 years of the onset of the disease (Sullivan 2002). Irregular eating and periods of malnutrition may be common, as during the lifetime of most organisms there is a fluctuation in the availability of nutrition. In order to deal with situations of low nutrient availability, complex signalling cascades, such as the insulin receptor signalling pathway, combine both the control of nutrient intake and the control of cell growth during development to appropriately manage energy stores.

Drosophila melanogaster can serve as a simple animal model system to study the molecular basis of starvation during nutrient deprivation. In Drosophila there are seven insulin-like peptides (Ilps) produced by 14 neurosecretory cells (Broeck 2001; Cao and Brown 2001; Ikeya et al., 2002). The insulin receptor signalling (IRS) pathway in flies reduces the levels of trehalose in the hemolymph (Rion and Kawecki 2007). Flies that lack Ilps develop into small adults with low fertility and metabolic defects similar to mammalian diabetes including elevated sugar levels and initiation of the starvation response, one aspect being lowered amount of triglycerides stored in the fat body (Zhang et al., 2009). The central component of the insulin receptor signalling pathway, the serine/threonine kinase Akt1, is highly conserved between mammals and *Drosophila melanogaster* (Scheid and Woodgett 2001; Franket et al., 2003). In Drosophila, A*kt1* is expressed throughout the organism during embryogenesis (Staveley et al., 1998; Scanga et al., 2000; Cavaliere et al., 2005). A role for *Akt1* in the control of cell growth

and survival is established as overexpression of *Akt1*in *Drosophila* leads to an increase in cell size, although there is no effect on cell growth rates or division. Under-expression or loss of *Akt1* can result in the production of smaller animals or, if severe, in lethality (Kennedy et al., 1997; Staveley et al., 1998; Verdu et al., 1999). The role of *Akt1* in cellular survival was suggested by its inhibition of several pro-apoptotic genes (Kennedy et al., 1997; Nunez and del Peso 1998). As expected for a maternally expressed gene, *Akt1* mutant females are sterile, and Drosophila *Akt1* mutant germline clones undergo apoptosis (Staveley et al., 1998). This demonstrates that the activities of the Akt1 kinase are of great importance to the survival of the cell, and to the overall organism.

The negatively regulated downstream target of Akt1, the transcription factor foxo (Brunet et al., 1999; Burgering and Medema 2003; Puig et al., 2003; Barthel et al., 2005; Puig and Tijan 2005), mediates the transcriptional response of the insulin receptor pathway to fluctuating nutrient conditions. As such, it controls a number of downstream targets involved in several important cellular functions. These include control of metabolism, cell cycle regulation, DNA repair, apoptosis and protection against oxidative stress (Burgering and Kops 2002; Barthel et al., 2005; Puig and Tijan 2005; Gershman et al., 2007). Through these many functions, the transcription factor foxo can affect cell growth and survival. Overexpression of *foxo*, such as its activation in the absence of insulin receptor signalling, results in phenotypes that resemble starved larvae (Kramer et al., 2003). A reduced insulin receptor signalling level is required for optimal survival during starvation, as overexpression of insulin receptor signalling components leads to the inability of Drosophila to survive during nutritional stress (Britton et al., 2002). Excess insulin receptor signalling results in a decrease in foxo activity, and loss of foxo function results in a decrease in survivorship upon amino acid starvation media (Kramer et al.,

2008). These findings suggest that insulin receptor signalling is involved in mediating the developmental response of larvae to starvation, and lead to the conclusion that in the absence of adequate nutrition, insulin receptor signalling is reduced, allowing foxo to enhance the transcription of downstream targets to aid in the overall survival of the organism.

The majority of studies of starvation resistance in Drosophila focus on complete food deprivation as opposed to reduction of any one major dietary component (Rion and Kawecki 2007). However, the lifespan of Drosophila under these conditions is extremely short, thereby limiting the ability to observe any subtle differences in survival. The largest parts of a complete diet for Drosophila include a carbohydrate source, such as sugar, and an amino acid/ protein source, usually yeast. Flies exposed to dietary restriction through a reduction of yeast alone, rather than sugar, are more sensitive to starvation (Min and Tatar 2006). In addition, when survivorship of flies was compared from diets of sugar alone to those with sugar and varying amounts of the protein casein, the addition of protein improved the longevity of the flies. Age-specific mortality in Drosophila is recognized to be related to dietary conditions (Good and Tatar 2001). Upon a full diet, including sufficient protein typically in the form of yeast, survival of Drosophila is considered to be optimal. However, Drosophila exposed to a diet lacking yeast show an increase in age-specific mortality, which decreases upon reintroduction of yeast. Taking these findings into consideration, we believe a phenotype signifying starvation resistance on amino acid depleted media could be more enlightening than that same phenotype in other types of starvation assays. We have characterized novel *Akt1* mutants (Slade and Staveley 2015) that, upon amino acid starvation media, show an increased starvation resistance when compared to wild type controls. When combined with a null *foxo* mutation, the

novel *Akt1* hypomorphs do not show the same survivorship to suggest this phenotype is *foxo* dependent.

Materials and Methods

Generation and Source of Drosophila Strains and Culture

The initial P-element insertion line ry^{506} *P{PZ}Akt1*^{04226}*/TM3,* ry^{RK}, Sb^{1}, Ser^{1} (*Akt1*^{04226}) was obtained from the Bloomington Drosophila Stock Center (Perrimon et al., 1996; Spradling et al., 1999). This line contains a P-element inserted within the 5' untranslated region of the *Akt1* gene on the third chromosome. The control line *w; +/+; P[FRT;* w^{+}*]*^{2A} *P[*ry^{+}*neoR FRT]*^{82B} *Akt1*$^{+}$ *(Akt1*$^{+}$*)* was derived from lines obtained from Dr. Norbert Perrimon, Harvard University. The novel mutants of *Akt1*^{52}, *Akt1*^{57}, and *Akt1*^{87}, as previously described (Slade and Staveley 2015), were generated by imprecise excision of the P-element via crossing to a line with a stable source of transposase, *PΔ2-3,* ry^{+} (Roberston et al., 1988). These novel mutants are viable, developmentally delayed and reduced in size. The *foxo* null mutant line *w;* $foxo^{W124X}$ was obtained from Drs. E. Hafen and M. Junger (Junger et al., 2003) of the University of Zurich. 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 stocks required for transgenic replacement, *w; arm-Gal4* and *w; UAS-Akt1* were previously described (Staveley et al., 1998) and complex lines were generated for each *Akt1* allele (*w; arm-Gal4;* $Akt1^{m}$ and *w; UAS-Akt1;* $Akt1^{m}$ where *m* is the allele). Transgenic replacement studies were carried out by generating independent lines of w^{1118}*; UAS-Akt1*$^{+}$*/CyO;*

Akt1^{m}*/TM6B* and *w*^{1118}*; arm-GAL4/CyO; Akt1*^{m}*/TM6B* where *m* represents each of the novel *Akt1*mutant alleles. Crosses between these lines generated the critical class of *w*^{1118}*; UAS-Akt1*$^{+}$*/arm-GAL4; Akt1*^{m}*/Akt1*^{m} to be analyzed. Generation of flies bearing both the novel mutant *Akt1* alleles and a null *foxo* allele was performed via standard recombinant methods as previously described (Slade and Staveley 2015). Stocks and crosses were maintained on a standard medium containing cornmeal, molasses, yeast, agar and water. Routinely, stocks were kept at room temperature (22 ± 2°C) while crosses and experiments were carried out at 25°C.

Longevity Assay

Experiments were carried out on standard media containing cornmeal, molasses, yeast, agar and water at 25^{o}C. Adult heterozygous virgin females of *w; FRT*^{2A} *FRT*^{82B} *Akt1*$^{+}$, *w; FRT*^{2A} *FRT*^{82B}*Akt1*^{04226} and each of the mutants were mated to heterozygous males of the same genotype. Several single vial matings of three to five females plus two males were made of each genotype which were transferred onto new food every two days. A cohort of adult homozygous male flies were collected upon eclosion. Only male flies were observed to avoid the effect of reproduction investment associated with female flies. Approximately between 120 and 340 critical class males were 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 5.00 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

Crosses as in the longevity assay were performed and homozygous males of the desired phenotype were collected within 24 hours of eclosion. As in the longevity assay, male flies were observed to avoid the effect of reproduction investment associated with female flies. Between one hundred and nearly four hundred male flies were collected per genotype, and maintained in non-crowded conditions by a maximum number of 20 flies per vial. Adults were aged on fresh amino acid starvation medium, consisting of 5% sucrose in phosphate buffered saline solution and 3% agar, and replenished every four to six days. Studies of survival upon the starvation media was 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. This is a test statistic 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.

Results

Novel Akt1 mutants have a reduced lifespan on standard media

Longevity assays comparing the lifespan of the mutant homozygotes to the wild-type controls indicate there is a slight decrease in lifespan (Figure 1). The control had a median lifespan of 60 days, while $Akt1^{52}$ had median lifespan of 52 days, and $Akt1^{57}$ and $Akt1^{87}$ both had a median lifespan of 57 days. This corresponds to between a 13% and 16% decrease in lifespan.

Novel Akt1 mutants outlive controls on amino acid starvation media

Starvation assays ageing mutant homozygotes and wild-type controls resulted in between 33% and 67% increase in survivorship of the *Akt1* hypomorphic mutants (Figure 2A). The $Akt1^{+}$ line, with the shortest length of survival, has a median survivorship of 18 days. In comparison, the $Akt1^{04226}$ line had a median survival time of 24 days, $Akt1^{57}$survived for a median of 28 days, and both $Akt1^{52}$and $Akt1^{87}$ have a median time of survival of 30 days. When wild type *Akt1* was expressed under the control of the *armGal4* transgene in the genetic background of the *Akt1* mutant homozygotes, the extension in survival upon the starvation medium was suppressed (Figure 2B). This indicates the alteration of *Akt1* activity in the mutants is responsible for the extension in lifespan upon amino acid starvation media.

Combination of novel Akt1 hypomorphs and a null foxo mutation show a complex relationship

To determine if *foxo* was required for the observed longevity phenotypes of the *Akt1* mutants, Drosophila bearing mutations in both genes were generated and aged upon standard and amino acid starvation media. Lifespans of either the *Akt1* mutants or the null *foxo* mutants upon

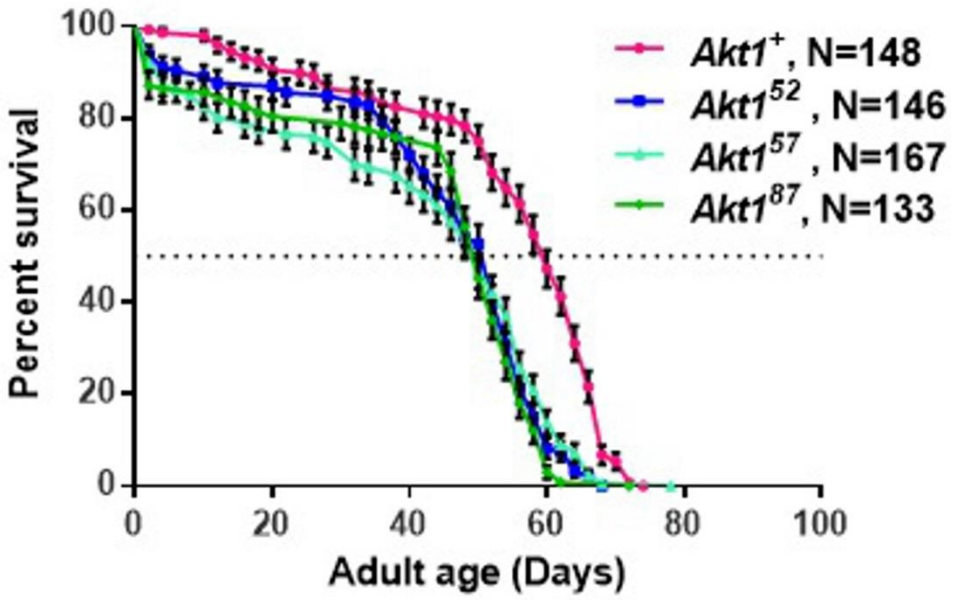

Figure 1: Longevity assay of novel *Akt1* hypomorphs display a shortened lifespan when compared to controls. Longevity is shown as percent survival ($P < 0.05$ as determined by log rank). The dotted line represents the median survival of the flies. The median lifespan of novel hypomorphic mutants falls between 8-10 days behind the wild type control. Full genotypes are Akt1^{+}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{+}* (N=148); Akt1^{52}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{52}* (N=146); Akt1^{57}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{57}* (N=167); Akt1^{87}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{87}* (N=133). Error bars represent the standard error of the mean.

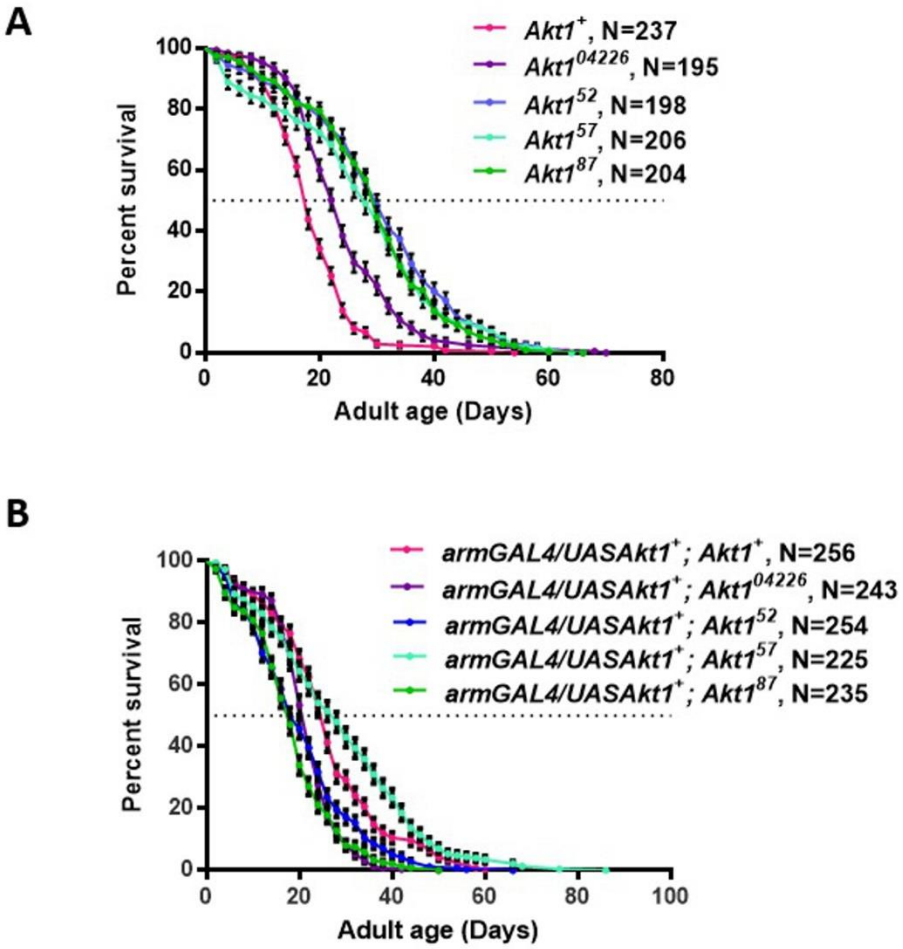

Figure 2: Amino acid starvation survivorship assays of novel *Akt1* hypomorphs indicate a long life phenotype that is suppressed when wild type *Akt1* is expressed. A: Amino acid starvation survivorship of novel hypomorphs compared to a wild type control. Full genotypes are Akt1^{+}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{+}* (N=237); Akt1^{04226}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{04226}* (N=195); Akt1^{52}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{52}* (N=198); Akt1^{57}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{57}* (N=206); Akt1^{87}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{87}* (N=204). **B:** Amino acid starvation survivorship of hypomorphs with background expression of wild type *Akt1+*. Full genotypes are Akt1^{+}: *w; arm-GAL4/UASAkt1^{+}; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{+}* (N=256); Akt1^{04226}: *w; armGAL4/UASAkt1^{+}; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{04226}* (N=243); Akt1^{52}: *w; arm-GAL4/UASAkt1^{+}; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{52}* (N=254); Akt1^{57}: *w; arm-GAL4/UASAkt1^{+}; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{57}* (N=225); Akt1^{87}: *w; arm-GAL4/UASAkt1^{+}; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{87}* (N=235). Survivorship is shown as percent survival ($P < 0.05$ as determined by log rank). The dotted line represents the median survival of the flies. Error bars represent the standard error of the mean.

standard media is decreased, with median days of survival for the *Akt1* mutants ranging between 52-57 days (Figure 1) and the median lifespan of the null *foxo* mutants was 26 days (Figure 3A). The double mutant lines also exhibited a decrease in lifespan when compared to the control with a median lifespan of 10 to 12 days (Figure 3A). When starved of amino acids and protein, the null *foxo* mutants are sensitive with a median lifespan of 12 days (Figure 3B). The novel *Akt1* mutants have an extended lifespan with median survival of 24 to 30 days (Figure 2A). The double mutant resembles the *foxo* mutants with a median survival by day 14 (Figure 3B). This suggests that in the absence of *foxo* signalling, the *Akt1* mutants do not outlive the controls when starved of amino acids.

Discussion

Ageing can be defined as the progressive degeneration of somatic tissues to the point that leads to the eventual termination or death of the organism. Clues to the basis of the molecular control of ageing were revealed in *C. elegans* when it was observed that certain mutants would undergo formation of the dauer larvae stage, a type of starvation-induced developmental arrest, in the presence of free access to food (Tatar 2004). In Drosophila, a similar diapause occurs with the manipulation of insulin receptor pathway associated genes, and this diapause retards both ageing and growth. The underlying mechanism may be instrumental in the increased longevities seen with mutants of some genes encoding components of the insulin receptor signalling pathway. We have shown that hypomorphic *Akt1* mutant homozygotes have a reduced lifespan when compared to controls with an 8 to 10 day difference in median lifespan. While alterations to insulin receptor signalling components have shown an increase in lifespan, the same is not true

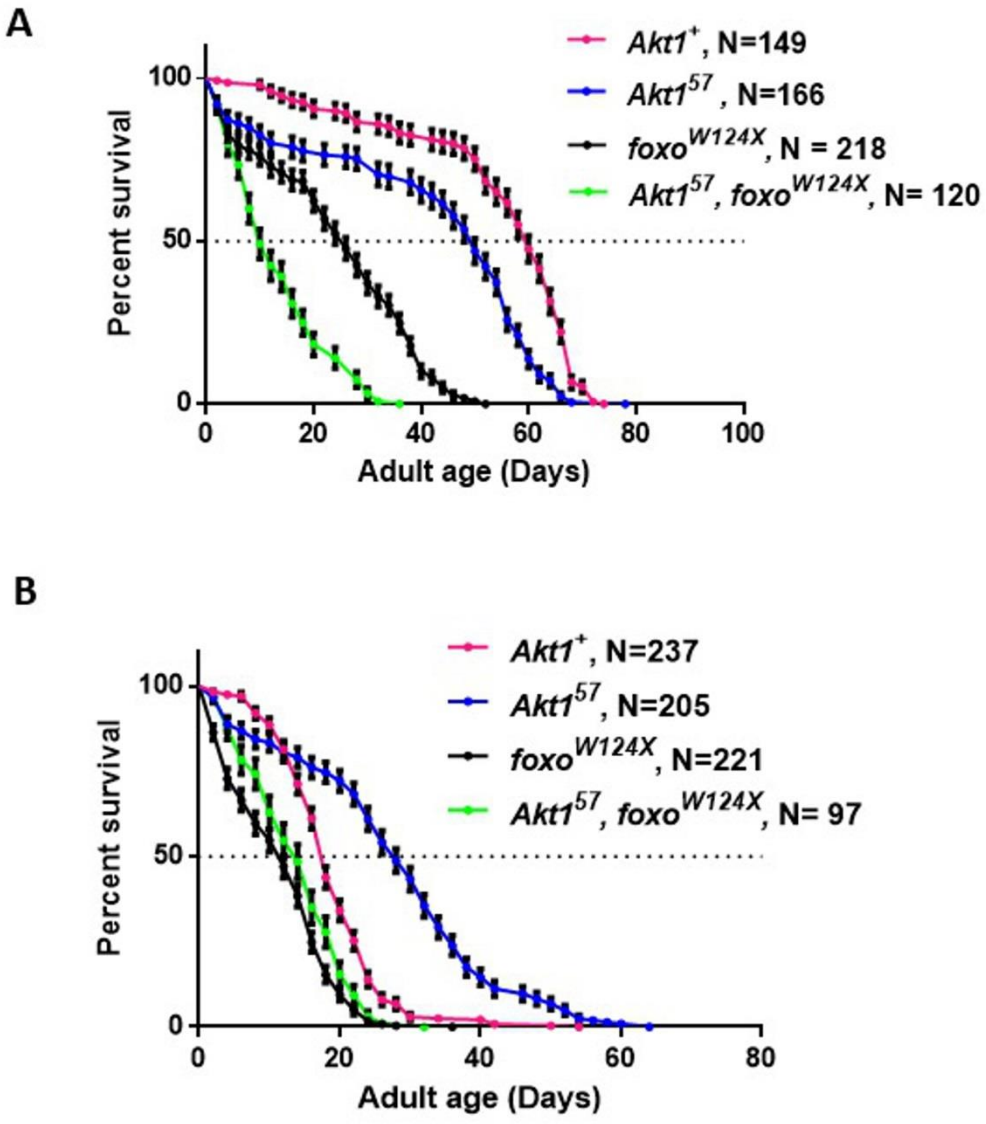

Figure 3: Longevity and amino acid starvation survivorship assays of Drosophila bearing both novel *Akt1* and a null *foxo* mutations: a synergistic effect upon standard media and an epistatic relationship when starved of amino acids. A: Longevity assay of double and single mutants. **B:** Amino acid starvation survivorship assay of single and double mutants. Full genotypes are Akt1^{+}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{+}* (N=149 or 237); Akt1^{57}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{57}* (N=166 or 205); foxo^{W124X}: *w; +/+; foxo^{W124X}/foxo^{W124X}* (N=218 or 221); Akt1^{57}, foxo^{W124X}: *w; +/+; P[FRT; w^{+}]^{2A} P[ry^{+}neoR FRT]^{82B} Akt1^{57}/foxo^{W124X}* (N=120 or 97). Survivorship is shown as percent survival ($P < 0.05$ as determined by log rank). The dotted line represents the median survival of the flies. Error bars represent the standard error of the mean.

for these novel mutants. The Akt1 kinase is an important cell survival protein, and expression of *Akt1* occurs throughout embryogenesis (Staveley et al., 1998). Null mutations in *Akt1* carried by germline clone females result in production of cuticle-deprived embryos and the induction of ectopic apoptosis. Subtle reduction in *Akt1* influenced activities could certainly impede Drosophila development.

Interestingly, when aged upon amino acid starvation media, these novel mutants show an extension in lifespan. A method for enhancing starvation resistance is increasing energy stores such as accumulated fats. A protein-poor diet in adult flies induces an increase in lipid reserves. Drosophila deficient in insulin receptor signalling maintain an increase in lipid stores which may aid in starvation resistance (Rion and Kawecki 2007). As these mutants are hypomorphs, similar to conditions of reduced insulin receptor signalling, this might contribute to the extension in lifespan.

This phenotype may also be a result of a mechanism that shares some aspects with dauer formation in *C. elegans* in response to low nutrients in which their growth is halted, and resumed again once nutrients are replenished. Newly hatched Drosophila larvae require nutrients in order to increase their body mass via replication of cells in mitotic tissues. In contrast, when larvae are hatched into conditions of amino acid starvation, they live in a state of developmental arrest until nutrients become available (Beadle et al., 1938; Kramer et al., 2003). When insulin receptor signalling is inhibited, developmental arrest similar to that seen during starvation in first instar larvae occurs (Britton et al., 2002). When larvae are amino acid starved, their overall growth is impeded. In previous studies, dominant-negative mutations of PI3K, and therefore inactivation of *Akt1*, has been shown to result in flies which phenocopy the effects of amino acid starvation (Kramer et al., 2003). In addition, previous studies of *foxo*'s role during nutritional stress, by

inducing ubiquitous expression of *foxo* in the first instar larvae, caused a complete arrest of growth, yet the larvae survived for several days (Junger et al., 2003; Kramer et al., 2003). As foxo is a significant downstream target of Akt1, it is possible that this is responsible for the phenotypes we have observed.

Removing *foxo* activity from the *Akt1* hypomorphs showed both synergistic and epistatic results. Analysis of longevity upon standard media indicates that while both Akt1 and foxo may overlap one another through sharing control over genes that regulate survival, reduction in activity or loss of both severely impacts lifespan. This additive effect is evidently beyond the Akt1-foxo pathway and most likely dependent upon one or more of the other downstream targets of either gene. Comparison of the *Akt1* hypomorphs, null *foxo* mutants and the double mutants show that the double mutant lines much more closely resemble the null *foxo* mutants. As the double mutants do not recapitulate the starvation resistance of the *Akt1* hypomorphs, this demonstrates the necessity of *foxo* in this process. Loss of *foxo* has been shown to result in an increased sensitivity to starvation caused by low levels of amino acids (Kramer et al., 2008). When nutrient levels are low, *foxo* activity is increased and upon return of nutrients, *foxo* levels return to normal. Ectopic expression of Drosophila *foxo* leads to inhibition of growth and generation of small adults. In addition, when there is an excess of *foxo* activity in larvae, feeding behaviour is altered (Kramer et al., 2003). This highlights the importance of *foxo* in times of nutritional stress, and taken together, these results show the importance of insulin receptor signalling and *foxo* in the survival of organisms during malnutrition. This may be due to a number of the genes that foxo transcriptionally activates. In mammalian tissue culture studies, *foxo* induces programmed cell death, or apoptosis, through the withdrawal of cytokines, up-regulation of pro-apoptotic genes, such as Bcl-2 family member Bim, cytochrome

c release and caspase activation (Dijkers et al., 2002). In addition, *foxo* induces the expression of genes associated with stresses including *MnSOD* and *4EBP* (Zinke et al., 2002; Puig et al., 2003). An increase in *MnSOD* aids in the reduction of life threatening free radicals, while induction of *4EBP* decreases protein synthesis, an unnecessary process during famine. The extended lifespan of *Akt1* mutants on amino acid starvation media may act through *foxo* to induce the expression of pro-survival genes.

Investigation of the ability of these novel *Akt1* mutants to survive amino acid starvation may provide an excellent basis to build an understanding of how organisms counteract the stress of an inadequate diet. The ability to endure such conditions, if evolutionarily conserved, may be quite relevant to the comprehension the basic biology of individuals living with eating disorders. Typically patients do not respond well to psychological treatments while being nutritionally rehabilitated, therefore the ability to sustain them until their psychological constraints can be alleviated could be very beneficial. Developing Drosophila as a model to better understand the biological mechanisms underpinning resistance to starvation may provide an approach that could eventually lead to the development of therapies for these and other related human conditions.

List of abbreviations 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 generated lines, performed the longevity and starvation assays, carried out the statistical analyses and drafted the initial manuscript. BES generated mutants and lines, 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 number: 14-09-BS).

Acknowledgments

The authors would like to thank members of the Genomics and Proteomics facility at Memorial University of Newfoundland for assistance with sequencing. Thanks also to Drs. Martin Junger and Ernst Hafen for providing the $foxo^{W124X}$ line, Dr. Howard Lipshitz for providing the w^{1118} line, and to Dr. Norbert Perrimon for providing the original w^{1118}*;* $P[FRT;w^{+}]^{2A}$ $P[ry^{+}\ neo^{R}\ FRT]^{82B}$ line. We thank Frankie Amanda Slade for critical review of the manuscript. This project was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Post-Graduate Scholarship to JDS and NSERC Discovery Grants to BES. JDS was also partially funded by a Department of Biology Teaching Assistantship and a School of Graduate Studies Fellowship from Memorial University of Newfoundland.

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Chapter 4:

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

A version of this chapter is published as:

Slade, J.D., and Staveley, B.E. 2016. Extended longevity and survivorship during amino acid starvation in a Drosophila Sir2 mutant heterozygote. Genome **22:** 1-8 [epub ahead of print].

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; Rodgerset al. 2007). Mammalian sirtuins control the activity of several transcription factors, such as p53, foxo and NF-kB, 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 increases 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 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). Accordingly, 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 investigated. $Sir2^{17}$ 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, $Sir2^{2A\text{-}7\text{-}11}$, 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 ± 2°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^{o}C. Critical class males of the control and *Sir2* mutant alleles were collected upon eclosion and aged, per genotype, at a density of $\leq$ 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 ($Sir2^{17}$/+, 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 ± 2 um^2 (Figure 1, Table 1). The range of mean ommatidium area for the mutants was 177 ± 1.49 um^2($Sir2^{17}$, N = 33) to 207.8 ± 1.8 um^2 ($Sir2^{17}$/+, 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;

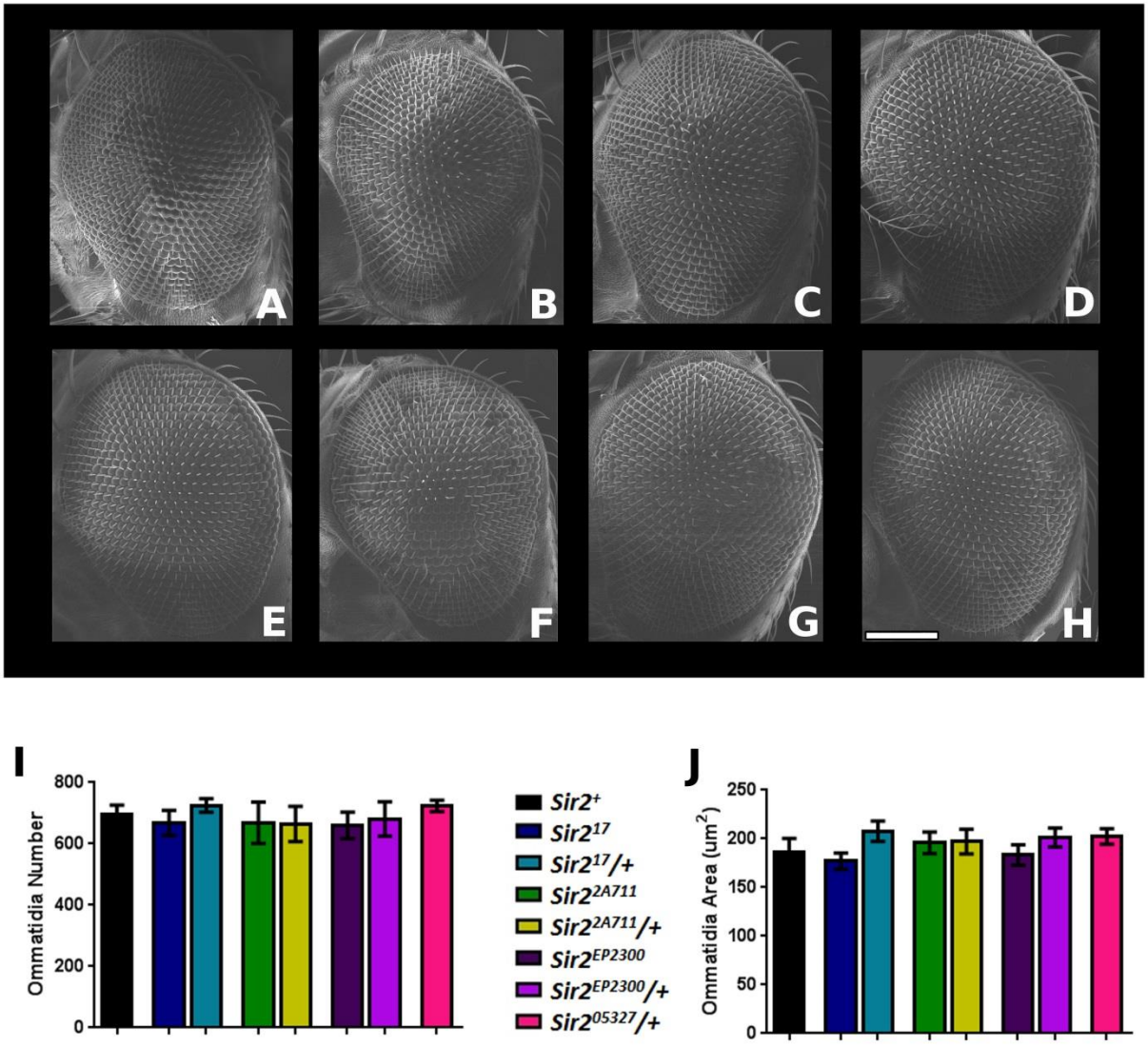

Figure 1: *Sir2* mutant homozygotes and heterozygotes do not differ in size or number of ommatidia from the control. Scanning electron micrographs illustrate the phenotypes of the control and*Sir2* mutant homozygotes and heterozygotes. Corresponding genotypes are **A:** $Sir2^{+}$, **B:** $Sir2^{17}$, **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.

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 (um^2)
$Sir2^{+}$	N/A	15	697.7 ± 7.45	45	186.8 ± 2
$Sir2^{17}$	Null	11	668.5 ± 12.52	33	177 ± 1.49
$Sir2^{17}$/+	Heterozygous Null	11	725.3 ± 6.76	33	207.8 ± 1.81
$Sir2^{2A711}$	Null	10	668.9 ± 21.39	30	195.8 ± 2.03
$Sir2^{2A711}$/+	Heterozygous Null	11	664.9 ± 17.32	33	197 ± 2.19
$Sir2^{EP2300}$	Insertional	15	660.3 ± 11.12	45	183.4 ± 1.55
$Sir2^{EP2300}$/+	Heterozygous insertional	10	681.2 ± 17.6	30	201.3 ± 1.8
$Sir2^{05327}$/+	Heterozygous insertional	6	723.7 ± 7.6	22	202.5 ± 1.7

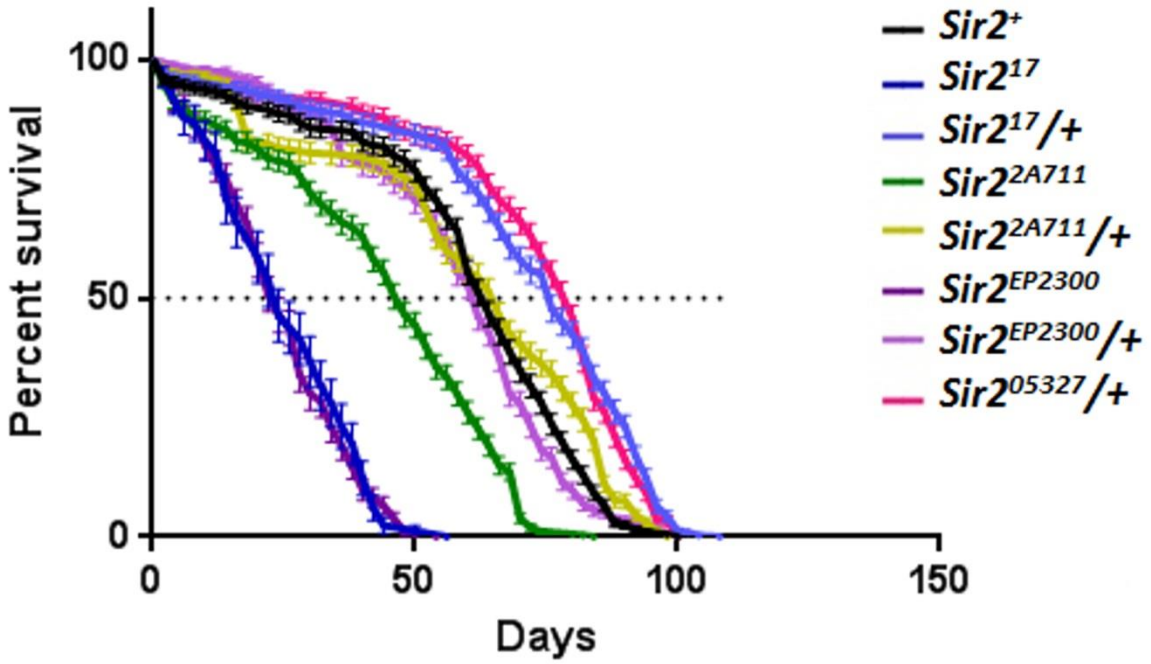

Figure 2: Selected *Sir2* mutant heterozygotes show extended longevity when compared to the control. Longevity is shown as percent survival ($P < 0.05$ as determined by log rank). The dotted line represents the median longevity of the flies. All homozygous *Sir2* mutants were decreased in longevity compared 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.

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
$Sir2^{+}$	N/A	377	64	369	24
$Sir2^{17}$	Null	82	24	308	20
$Sir2^{17}$/+	Heterozygous Null	324	76	398	22
$Sir2^{2A711}$	Null	361	48	381	14
$Sir2^{2A711}$/+	Heterozygous Null	322	66	434	18
$Sir2^{EP2300}$	Insertional	183	24	354	22
$Sir2^{EP2300}$/+	Heterozygous insertional	301	62	363	22
$Sir2^{05327}$/+	Heterozygous insertional	322	80	384	38

Figure 2, Table 2). The other heterozygous mutants had median longevities that were not significantly different from the control (66 days for $Sir2^{2A\text{-}7\text{-}11}$/+ , 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. $Sir2^{2A\text{-}7\text{-}11}$ 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 longevities and can be capable of surviving for extended periods of amino acid starvation. This

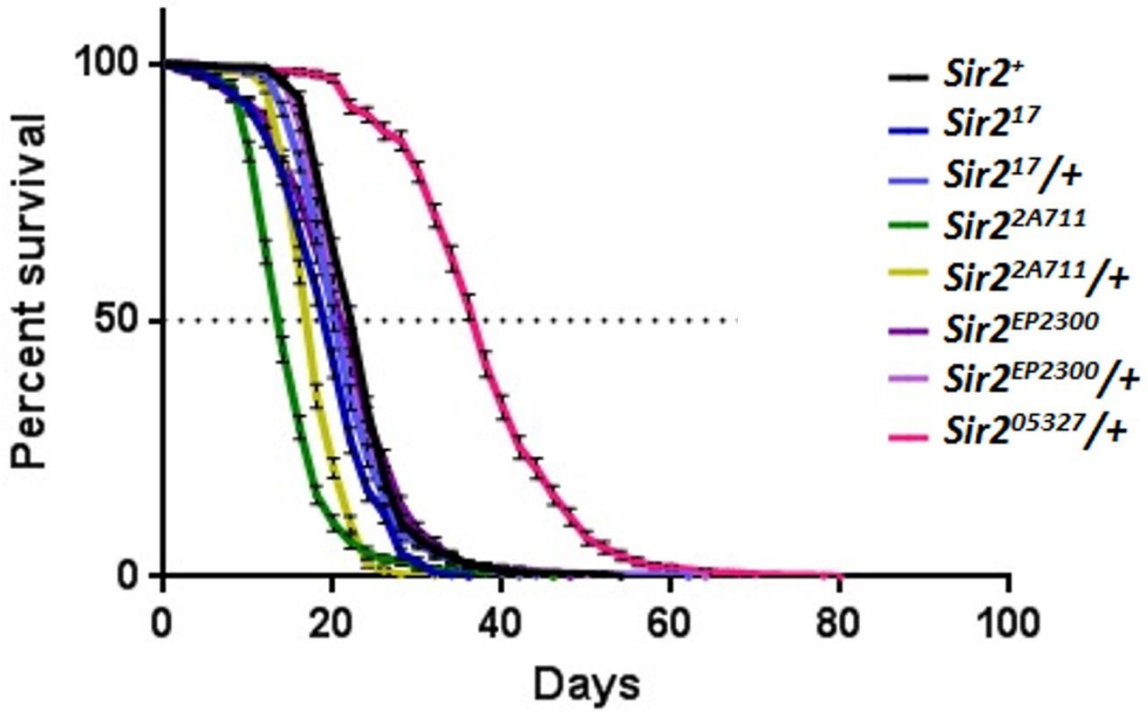

Figure 3: The *Sir2*^{05327}/+ mutant heterozygote is able to endure amino acid starvation. Survivorship is shown as percent survival ($P < 0.05$ as determined by log rank). The dotted line represents the median survivorship of the flies. All *Sir2* mutant homozygotes and heterozygotes were either similar 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.

intermediate reduction is functionally significant, as both the complete loss of *Sir2* and 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 interestingthat 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 with loss-of-function of *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).

Acknowledgments

The authors would like to thank Drs. Liqui Men, formerly of the Bio-molecular Imaging Cluster at Memorial University of Newfoundland, and Michael Shaffer, formerly of the Microanalysis facility of Memorial University of Newfoundland for technical assistance with scanning electron microscopy. Thanks to the Bloomington Drosophila Stock Center. This project was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Post-Graduate Scholarship to JDS and NSERC Discovery Grants to BES. JDS was also partially funded by a Department of Biology Teaching Assistantship and a School of Graduate Studies Fellowship from Memorial University of Newfoundland.

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Chapter 5:

Manipulation of components that control feeding behavior in *Drosophila melanogaster* increases sensitivity to amino acid starvation

A version of this chapter is published as:

Slade, J.D., and Staveley, B.E. 2016*c*. Manipulation of components that control feeding behavior in Drosophila melanogaster increases sensitivity to amino acid starvation. Genet Mol Res **15**(1).

Abstract

Feeding is a complex behaviour that must be regulated by several internal mechanisms. Neuropeptides are able to survey the quantity of energy stores and relate to the organism if intake of nutrients is required. In addition to this homeostatic regulation, a post-feeding reward system positively re-enforces feeding. Slight adjustments to either system can tilt the balance to affect the reserve of energy and thus survivorship in times of nutrient adversity. The neuropeptide NPF, a homologue of mammalian NPY, acts to induce feeding within the homeostatic regulation of the behavior. Drosophila, and other insects, bear a shorter form of NPF known as short NPF (sNPF) that can influence feeding. A neural hormone regulator, the dopamine transporter (DAT) works to clear dopamine from the synapses. This action may manipulate the post-feeding reward circuit in that lowered dopamine levels depress feeding and excess dopamine can encourage feeding. We have over-expressed and impaired the activity of each NPF, sNPF and DAT in Drosophila and examined their ability to survive during conditions of amino acid starvation. Too much or too little NPF or sNPF, key players in the homeostatic regulation of feeding, leads to an increased sensitivity to amino acid starvation and a diminished survivorship when compared to controls. When DAT, a member of the post-feeding reward system, is either over-expressed or reduced via mutation, Drosophila have an increased sensitivity to amino acid starvation. Taken together these results indicate that subtle variation in the expression of key components of these systems impacts survivorship during adverse nutrient conditions.

KEY WORDS: Starvation, Drosophila, NPF, sNPF, DAT

Introduction

The act of feeding by any organism must be modified based on the environment and available nutrition in order to maintain an appropriate energy balance. A disruption in this balance may lead to metabolic disease, including both obesity and eating disorders such as anorexia (Neilson, 2001; Fairburn and Harrison, 2003). A myriad of genes aid in maintaining this balance by prompting foraging and feeding or by encouraging the cessation of feeding. Expression of these genes is often triggered by internal mechanisms that monitor the level of peripheral energy stores. When there is sufficient energy stored, anorexic genes can signal satiety to cause the organism to stop feeding. When internal energy stores are lowered, the orexic genes can signal to the organism that feeding is necessary to restore energy. Typically this homeostatic process, which in mammals measure energy stores and relate signals between the hypothalamus and the periphery (Hoebel, 1971), works efficiently but in an environment where nutrients are readily available an additional post-satiation reward system can regulate feeding patterns. Although the homeostatic response works well when animals are required to forage, when there is an excess of food the post-satiation reward can override the homeostatic signals (Palmiter, 2007). As such it is important to consider both means of control when evaluating feeding behaviour in the understanding of eating disorders.

Drosophila melanogaster serves as a simple model organism to uncover the potential mechanisms of metabolic disease. The organ systems of Drosophila include members that function analogously to those in vertebrates; for example the fat body in Drosophila is utilized to store triglycerides akin to the liver and adipose tissue in vertebrates (Owusu-Ansah and Perrimon, 2014). Drosophila contain a specialized group of cells, the oenocytes, which function similarly to the hepatocytes in vertebrates to mobilize stored lipids during periods of low food

intake. In addition to this, Drosophila possess homologous hormones to regulate feeding behaviour. These similarities allow for experiments in Drosophila to be applicable to human health research.

In vertebrates, the neurotransmitter neuropeptide Y (NPY) has been studied extensively as a central enhancer in the control of food intake. NPY is a peptide hormone consisting of 36 amino acids, beginning and ending with a tyrosine (Y) residue, giving it its name (Arora and Anubhuti, 2006). First identified in the tapeworm *Monieza expansa* (Maule *et al*. 1991), invertebrate homologues of NPY have been found in major phyla (de Jong-Brink *et al.*, 2001; Maule, 1995) but encode a 36 amino acid neuropeptide F (NPF) named for having a conserved phenylalanine (F) residue at the carboxyl terminus. NPF has been consistently detected in the invertebrate brains, and is often expressed in the midgut, which points to its role in feeding regulation. Receptors resembling the vertebrate NPY receptors have been identified in a number of invertebrates, including *Caenorhabditis elegans* and *Drosophila melanogaster* (de Bono and Bargmann, 1998; Garczynski *et al.*, 2002). The structure of the NPF receptor resembles that of vertebrates and the NPF receptor from *Drosophila* was shown to cross-react with human NPY, to suggest the functional conservation between NPF and NPY (Garczynski *et al.*, 2002). Akin to vertebrates, expression of invertebrate NPF receptors is generally localized to both the central and peripheral nervous systems.

Interestingly, the initial identification of NPF peptides in insects revealed shorter peptides of only 8 to 10 amino acids in length. Both long NPF peptide (36 amino acids) and short versions of the peptides, that share similarity to the carboxyl-terminus of the longer and were subsequently named short NPFs (sNPFs), were found in Drosophila (Vanden Broeck 2001). The long NPF peptides are considered the functional homologues of vertebrate NPYs and the sNPFs

are believed to be independent as each have their own distinct precursor genes and receptors. There are four known short neuropeptide F (sNPFs) sequences in Drosophila encoded by a single transcription unit (Vanden Broeck, 2001). Analysis of these small peptides in Drosophila indicates they are expressed in a large number of neurons in both the brain and the stomatogastric system and are often co-expressed with other neuropeptides such as insulin and other neurotransmitters (Nassel and Wegener, 2011). Studies in Drosophila that either reduce the expression of sNPF or over-express the peptide confirms it controls the initiation or stopping of feeding. While both peptides appear to function in the control of feeding behaviour, only sNPF links this to the control of growth.

The dopamine transporter (DAT) is a membrane-bound protein central to the regulation of the levels of extracellular dopamine through the rapid reuptake of the neurotransmitter to clear the synapses. The dopamine neurotransmitter functions in reward-motivated behaviours, and as such changes to the extracellular levels of dopamine can result in a number of diseases (Palmiter, 2007; Yamamoto and Seto, 2014). Low levels of dopamine are associated with ADHD, depression and under-eating (Cannon *et al.*, 2004; Sotak *et al.*, 2005). Studies in rats show that upon feeding or drinking there is a rapid firing of dopamine in the brain whereas animals with lowered or no dopamine activity are hypoactive, apparently apathetic and prone to die from starvation or dehydration (Schulz, 2006). Hyper-dopaminergic mice, on the other hand, are found to overfeed, and eventually become obese (Pecina *et al.*, 2003; Cagniard *et al.*, 2006). Therefore manipulation of the transporter which regulates the strength and duration of dopamine signalling can lead to similar outcomes. The Drosophila DAT gene was re-isolated in a screen for genes that alter sleep (Kume *et al.*, 2005). The DAT mutant, named fumin (*fmn*) [Japanese for sleepless], is active for nearly 24 hours of the day. Expression patterns of Drosophila DAT

were found to be concentrated in the location of known dopaminergic neurons within larvae and the adult head. Overall, it is evident that the activity of dopamine could be an effective regulator of feeding.

The prevalence of eating disorders and obesity in modern societies has generated a great deal of interest in elucidating the pathways and mechanisms that control feeding behaviour. A better understanding of how these systems work to control feeding could lend itself to enhancing survival during conditions of nutritional adversity. We have investigated the impact of survival of Drosophila during amino acid starvation with both an overexpression and loss-of-function of each *NPF*, *sNPF* and *DAT*.

Material and Methods

Drosophila stocks and culture

The *UAS-NPF* and *NPF-Gal4* lines (Wu *et al.*, 2003) were received from the laboratory of Dr. P. Shen (University of Georgia). The *UAS-sNPF* and *UAS-sNPF-RNAi* lines (Lee *et al.*, 2004) were received from the laboratory of Dr. K. Yu (Korea Research Institute of Bioscience and Biotechnology). The *DAT* mutant line, *fmn* (Kume *et al.*, 2005) and a control subset line of the standard w^{1118} stock, selected due to lowered activity, was obtained from the laboratory of Dr. Rob Jackson (Tufts University, Boston). The *UAS-DAT* line was obtained from Bloomington Stock Center (Indiana University) as were the pan-neural expressing *elav-Gal4* transgene and the *UAS-GFP*, *UAS-lacZ* and *UAS-rpr* responding lines. All stocks were maintained upon standard corn-meal/yeast/molasses/agar media in plastic culture vials.

Amino acid Starvation Ageing Assays

All crosses were performed upon standard media and maintained at 25^{o}C until eclosion. Adult male flies of the critical class were collected daily and placed upon amino acid starvation media in groups of 20 or less. Amino acid starvation media consists of 5% sucrose in PBS and 3% agar in plastic culture vials. Flies were assessed daily for the presence of any dead flies until no flies remained. Media was replenished as necessary. Data was analyzed using GraphPad Prism version 5.0 (GraphPad Software, Inc. in San Diego, California, USA) with statistical analysis carried out as a Log-Rank test.

Results

Manipulation of NPF expression reduces survivorship upon amino acid starvation media

To reduce the presence of NPF in Drosophila, the experimental approach made use of a Gal4 transgene with expression specific to the specific neurons that produce the neuropeptide. This "driving transgene" was used to over-express the benign control protein GFP (N=353 males observed) which resulted in a median survivorship of 30 days when aged upon the nutrient deprived media (Table 1, Figure 1A). In comparison, when the *NPF-Gal4* driver was used to over-express a cell death protein reaper (*rpr*, N= 371 males observed), the ablation of NPF producing neurons resulted in a median survivorship of 24 days (Table 1, Figure 1A). This difference (6 days) indicates a significant sensitivity to amino acid starvation.

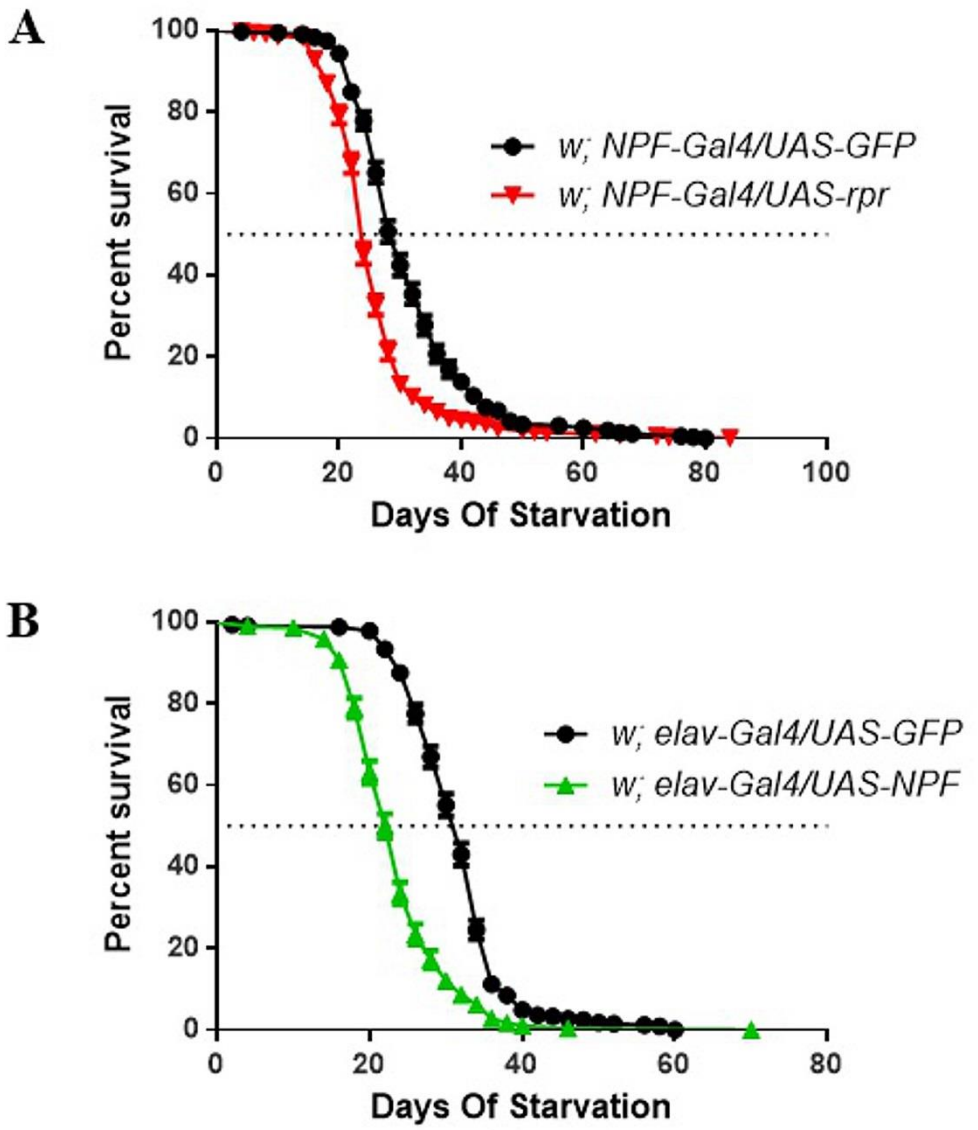

Figure 1: Survivorship of Drosophila is reduced with either excess or reduced expression of NPF when starved of amino acids. A: Cell death mediated loss of function of *NPF*. Genotypes are: *w; UAS-GFP/+; NPF-Gal4/+*, N= 353 and *w; UAS-rpr/+; NPF-Gal4/+*, N=371. **B:** Overexpression of *NPF*. Genotypes are: *w; elav-Gal4/UAS-GFP*, N = 330, and *w; elav-Gal4/UAS-NPF*, N= 290. Survivorship is shown as percent survival ($P < 0.05$ as determined by log rank). The dotted line represents the median survival of the flies. Error bars represent the standard error of the mean.

Table 1: Median day of survival and total number of deaths observed of Drosophila with an overexpression or loss of function of *NPF*

Genotype	**Condition**	**Number of Deaths Observed**	**Median Day of Survivorship**
w, elav-Gal4/UAS-GFP	Control	330	32
w, elav-Gal4/UAS-NPF	Overexpression	290	23
NPF-Gal4/UAS-GFP	Control	353	30
NPF-Gal4/UAS-rpr	Cell death mediated loss of function	371	24

Overexpression of *NPF* was achieved via the pan nervous system driver *elav-Gal4*. The combination of this transgene and the benign responding transgene *UAS-GFP* as a control (N= 330 males observed) resulted in a median survivorship of 32 days when aged on the amino acid starvation media (Table 1, Figure 1B), comparable to the control described above. When *elav-Gal4* was used to overexpress *NPF* in *elav-Gal4*/*UAS-NPF* flies (N= 290 males observed) the median length of survivorship decreased to 23 days (Table 1, Figure 1B). This difference of 7 days indicates a significant sensitivity to amino acid starvation media.

Manipulation of sNPF expression increases sensitivity to amino acid starvation

The nervous system specific driving transgene, *elav-Gal4,* was used to either inhibit or increase the expression of *sNPF*. As a control this transgene was combined with *UAS-GFP*, the benign responder transgene (N= 330 males observed) resulted in a median survivorship of 32 days when aged upon amino acid starvation media (Table 2, Figure 2A). To inhibit the expression of *sNPF*, the *elav-Gal4* driver was combined with *UAS-sNPF-RNAi* (N= 310 males observed). Ageing of these flies upon amino acid starvation media lead to a median survivorship of 22 days (Table 2, Figure 2A), a significant difference of 10 days fewer than the control.

To enhance the expression of *sNPF*, the *elav-Gal4* transgene was combined with *UAS-sNPF* (N= 280 males observed). These flies exhibited a median survival time of 24 days (Table 2, Figure 2B), a significant 8 day reduction in survivorship.

Manipulation of DAT expression decreases survivorship upon amino acid starvation media

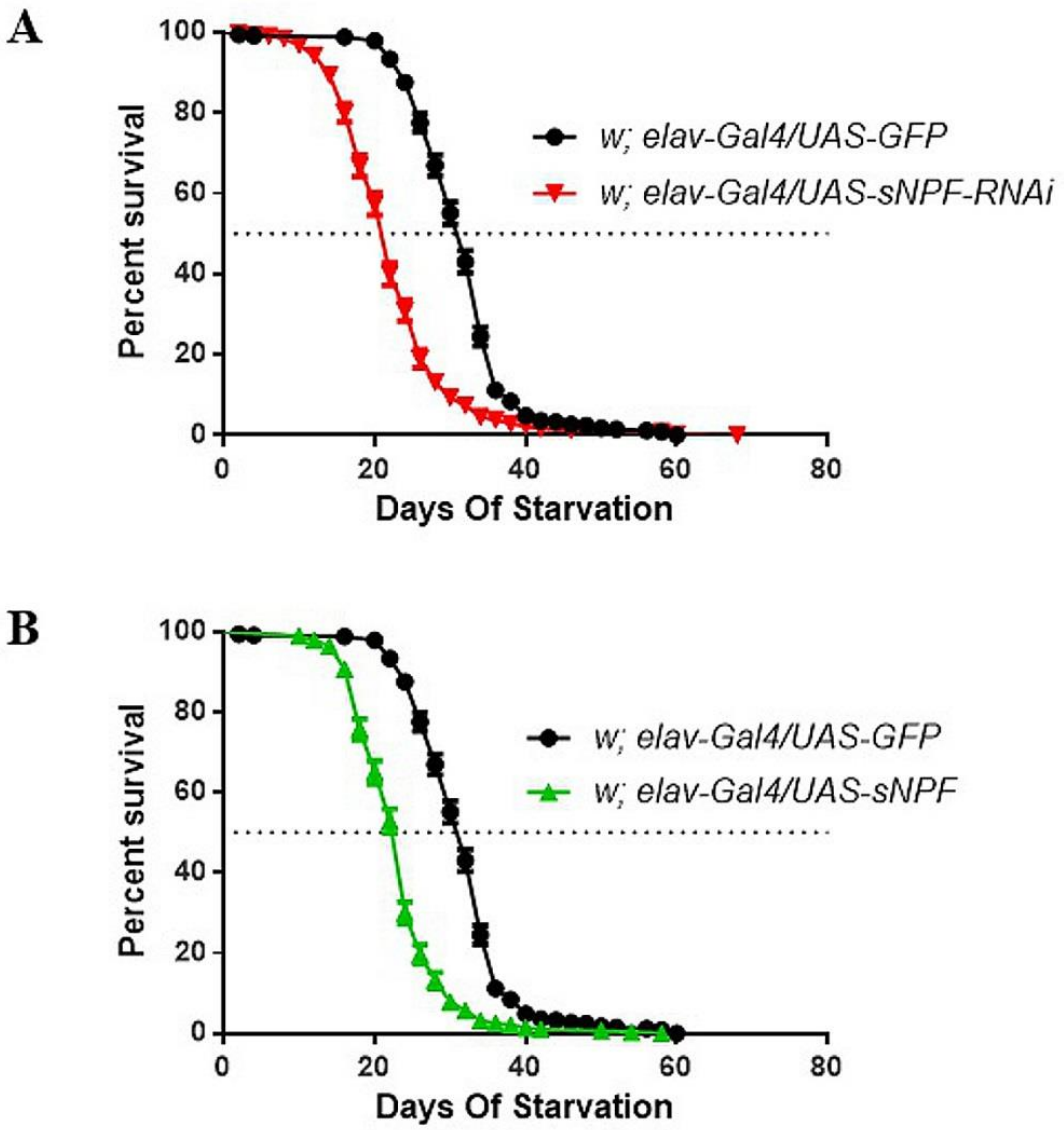

Figure 2: Survivorship of Drosophila is reduced with either excess or reduced expression of *sNPF* when starved of amino acids. A: RNA interference mediated loss of function of *sNPF*. Genotypes are: *w; elav-Gal4/UAS-GFP*, N= 330, and *w; elav-Gal4/UAS-sNPF Ri*, N= 310. **B:** Overexpression of *sNPF*. Genotypes are: *w; elav-Gal4/UAS-GFP*, N= 330, and *w; elav-Gal4/UAS-sNPF*, N= 280. Survivorship is shown as percent survival ($P < 0.05$ as determined by log rank). The dotted line represents the median survival of the flies. Error bars represent the standard error of the mean.

Table 2: Median day of survival and total number of deaths observed of Drosophila with an overexpression or loss of function of *sNPF*

Genotype	**Condition**	**Number of Deaths Observed**	**Median Day of Survivorship**
w, elav-Gal4/UAS-GFP	Control	330	32
w, elav-Gal4/UAS-sNPF	Overexpression	280	24
w, elav-Gal4/UAS-sNPF-RNAi	RNA interference mediated loss of function	310	22

Reduction in *DAT* expression was observed in the *fmn* mutant produced by the insertion of a ~ 2 kb transposon within the *DAT* gene which has been demonstrated to have little to no DAT activity (Kume *et al.*, 2005). The control for this experiment, a w^{1118} line, the genetic background in which the *fmn* mutant has been placed (N=508 males observed), survives for a median of 24 days when challenged with the amino acid starvation media (Table 3, Figure 3A). In comparison, the *fmn* mutant flies (N= 444 males observed) had a median survivorship of 18 days aged upon the same media (Table 3, Figure 3A). This reduction of 6 days represents a significant decrease in survivorship.

Overexpression of *DAT* was achieved by the nervous system specific driver *elav-Gal4* controlling the expression of *UAS-DAT*. The control experiment of the benign *UAS-GFP* driven by *elav-Gal4* (N= 515 males observed) survived for a median of 26 days upon the nutrient deprived media (Table 3, Figure 3B). When *DAT* was over-expressed under the influence of *elav-Gal4* (N= 463 males observed), the median survival time was 20 days (Table 3, Figure 3B). This difference of 6 days is a significant decline in survivorship.

Discussion

The importance of a regulated internal mechanism, dependent upon neuropeptides and hormones, to control foraging and feeding of animals is evident. Here we have found that increases or decreases in the expression of the key neuropeptides NPF and sNPF, and the neural hormone regulator DAT, can severely debilitate survivorship during conditions of nutritional adversity, in particular amino acid starvation.

NPF in insects is a structural homologue of the vertebrate NPY, considered to be the most powerful neuropeptide responsible for the enhancement of appetite (Valassi *et al.*, 2008). In

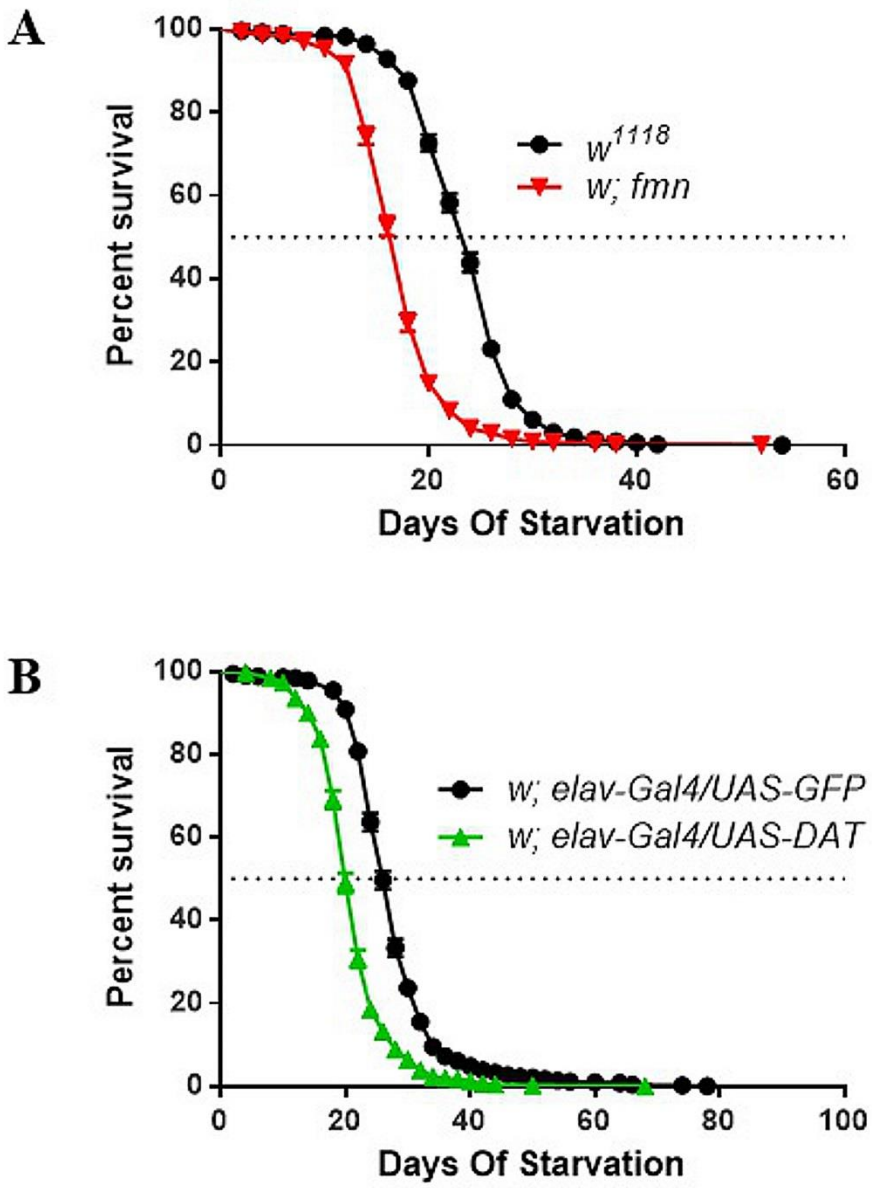

Figure 3: Survivorship of Drosophila is reduced with either excess or reduced expression of *DAT* when starved of amino acids. A: Mutant loss of function of *DAT*. Genotypes are: w^{1118}, N = 508, and *w; fmn*, N= 444 **B:** Overexpression of *DAT*. Genotypes are: *w; elav-Gal4/UAS-GFP*, N= 515 and *w; elav-Gal4/UAS-DAT*, N= 463. Survivorship is shown as percent survival ($P < 0.05$ as determined by log rank). The dotted line represents the median survival of the flies. Error bars represent the standard error of the mean.

Table 3: Median day of survival and total number of deaths observed of Drosophila with an overexpression or loss of function of *DAT*

Genotype	**Condition**	**Number of Deaths Observed**	**Median Day of Survivorship**
w^{1118}	Control	508	24
w; elav-Gal4/UAS-GFP	Control	515	26
w; fmn	Mutant loss of function	444	18
w, elav-Gal4/UAS-DAT	Overexpression	463	20

Drosophila melanogaster, young larvae feed intensively to attain the weight requirement to undergo pupation, while older larvae do not feed to the same extent and begin to wander to eventually find a place to pupate (Melcher *et al.*, 2007). In young larvae, *NPF* expression is up-regulated, while in the older larvae, there is less *NPF* expression (Maule, 1995; de Jong-Brink *et al.*, 2001). Young transgenic larvae that lack NPF signalling exhibit the phenotypes of wandering and lack of feeding normally seen in older larvae (Wu *et al.*, 2003). When larvae are starved they will "lower their standards" and eat foods they might otherwise avoid and forage during sub-optimal conditions (Wu *et al.*, 2005, Lingo *et al.*, 2007). Alteration of *NPF* expression affects these behaviours in that larvae with diminished *NPF* expression will continue to avoid noxious food even while being starved, and when the NPF receptor has been impaired, fasted larvae will not forage. Certainly a lessened appetite and reduced amount of feeding could be expected to impair survivorship, especially under adverse nutrient conditions; which we observed when the function of NPF was reduced in Drosophila maintained upon amino acid depleted media.

Typically *NPF* expression is lower in older larvae, but overexpression of NPF in these larvae leads to phenotypes similar to those observed in younger larvae, including increased feeding (Wu *et al.*, 2003; Wu *et al.*, 2005) continuing well past the normal developmental time-frame to result in delayed wandering and delayed pupariation. Drosophila will not forage for food in colder conditions, but fasted larvae in a cold environment will forage when the expression of the NPF receptor is high (Lingo *et al.*, 2007), and well-fed larvae with an overexpression of *NPF* will feed on noxious foods (Wu *et al.*, 2005). While the amount of food ingested by the Drosophila over-expressing *NPF* was not measured in our experiments, the

excess *NPF* expression and potential overfeeding did not increase survivorship of the flies on the amino acid depleted media.

Variation of the expression of *sNPF* decreased the survivorship of Drosophila during the amino acid starvation assays. This shorter neuropeptide is implicated in the control of feeding as it has been observed that loss of expression in both larvae and adults results in organisms that do not feed, but an abundance of *sNPF* expression in both larvae and adult Drosophila initiates the feeding behaviour (Lee *et al.*, 2004). However, sNPF appears to function in the control of feeding in a different manner than NPF, particularly in larvae that have transitioned into the wandering stage. Both neuropeptides can either enhance (through overexpression) or inhibit (through loss of function) feeding in larvae prior to this stage, but alteration in *sNPF* expression does not enhance feeding beyond this point as overexpression of *NPF* has been demonstrated to do (Wu *et al.*, 2003, Lee *et al.*, 2004). Although it is not yet known if sNPF can affect feeding in malnourished larvae or adults similar to NPF (Wu *et al.*, 2005), our findings suggest *sNPF* expression is pertinent to the survival of Drosophila exposed to depleted nutrients as elevation or reduction of *sNPF* limits the ability to survive under conditions of starvation.

In vertebrates, feeding behaviour may also be adjusted through the activity of the dopamine reward system that is known to be activated post-ingestion. Feeding increases release of dopamine, a response that becomes blunted in obese individuals. If tyrosine hydroxylase (TH), an enzyme necessary for the production of dopamine, is removed from dopaminergic neurons in mice, they do not feed (Zhou and Palmiter 1995). The dopamine transporter functions to clear dopamine from the synapses, therefore reducing its expression will increase levels of dopamine in the hypothalamus. Individuals suffering from eating disorders may undergo lengthy periods of dietary restriction, leading to lowered dopamine levels in the hypothalamus (Kontis and

Theochari, 2012). Animals which have been feed-restricted typically have less dopamine transporter expressed than free-fed animals (Bello *et al.*, 2003). When DAT is actively reducing the levels of dopamine in the brain, hedonic feeding, which is feeding after being sated on sweet or high-fat foods, is reduced (Mebel *et al.*, 2012). Mice with a 10% reduction of DAT activity have about 70% higher dopamine levels (Pecina *et al.*, 2003): they eat roughly 21% more, drink 15% more, gain roughly 5% body weight and work harder for a sweet reward. Upon consumption of sucrose after fasting, an exaggerated release of dopamine is observed (Bello *et al.*, 2003; Polivy and Herman, 1985; Hagan and Moss, 1997). Given this, it is expected that Drosophila with impaired DAT function should experience higher levels of dopamine signalling, and eat more while Drosophila over-expressing *DAT* should eat less. This may affect the levels of energy stores and therefore affect their ability to survive amino acid starvation. In our study both instances lead to an increased sensitivity and decreased survivorship.

The majority of studies of the regulation of feeding behaviour involve the insulin receptor signalling (IRS) pathway, which is well conserved between mammals and Drosophila (Britton *et al.*, 2002). During conditions of low nutrient availability, as in amino acid starvation the IRS signalling pathway is muted. With less insulin there is more trehalose present in the haemolymph coupled with an increase in lifespan (Broughton *et al.*, 2008). A main downstream effector that is negatively regulated by insulin receptor activity, the transcription factor foxo, is more active when IRS is lessened. The foxo transcription factor has been identified as important in survival during starvation of amino acids (Kramer *et al.*, 2008). Drosophila exposed to amino acid starvation media show an increase in expression of *foxo* while the loss of *foxo* activity results in a decreased survivorship when only fed sucrose without protein or amino acids. The neuropeptide regulation of feeding is associated with this mechanism as the cell surface of

specific neurons in Drosophila contain both the NPF receptor and the insulin receptor (Wu *et al.*, 2005). When IRS is lowered in these neurons, larvae will overeat, including foods they would normally avoid, yet when there was an increase in IRS in these neurons, the larvae would eat far less than expected. This suggests that the IRS negatively regulates the action of the NPF receptor, and hence NPF signalling in Drosophila. Therefore, one would expect that lowered *NPF* expression should share a similar outcome to what is observed with increased IRS during amino acid starvation, and *vice versa*. However, our study reveals that both overexpression and impaired function of *NPF* results in a sensitivity to amino acid starvation suggesting that the mechanism is more complex that originally hypothesised.

In *Drosophila melanogaster*, sNPF has been linked to IRS, as the neurons that express sNPF are situated in close proximity to the insulin producing cells (Lee *et al.*, 2008). Characterization of Drosophila with alterations to *sNPF* expression bear similar phenotypes to flies with abnormal IRS levels. Hypomorphs of *sNPF* are 23% smaller than controls and express decreased levels of the Drosophila insulin-like peptide 2 (Dilp2), and *sNPF* mutants have down-regulated Akt1, increased levels of foxo located within the nucleus, an increase in *4EBP* expression and an increased lifespan (Lee *et al.*, 2008) suggesting that sNPF negatively regulates IRS. Consequently lowering sNPF should increase foxo activity, and therefore, enhance survival during nutrient deprived conditions. However, we find that a decrease in sNPF function does not enhance survival during amino acid starvation. Alternatively, overexpression of *sNPF* leads to 24% larger flies showing an increase of *Dilp2* expression (Lee *et al.*, 2008). An increase in *sNPF* expression and binding to the sNPF receptor activates the IRS and demonstrates Dilp2 to be downstream of sNPF signalling. This increase in IRS should increase Akt1 activity and

decrease the transcriptional activity of foxo to sensitize the flies to amino acid starvation, which was observed in this study.

Dopamine signalling, strongly influenced by DAT activity, is linked to IRS as the insulin receptor is expressed in the dopaminergic neurons of the midbrain of Drosophila (Palmiter, 2007; Mebel *et al.*, 2012). Inhibition of the receptor reduces IRS and *DAT* expression and thus dopamine clearance and the administration of insulin directly to the rat brain (via intracerebroventricular administration) leads to an increase in *DAT* mRNA and activity to clear the synapses and decrease dopamine levels (Figlewicz et al 1994). Rats fed a high-fat diet have a decreased level of Akt1 activity in the brain and require a longer period of time to clear the synapses of dopamine (Speed *et al.*, 2011). Upon addition of the insulin receptor substrate, Akt1 levels are restored as is the expression of DAT on the cell surfaces in the striatum. Other hormones that homeostatically control feeding behaviour have been shown to interact with receptors on neurons that secrete dopamine as well. Leptin, an anorexigenic hormone, inhibits dopamine activity while grelin, an orexin, stimulates dopamine activity to influence the reward potential of feeding (Palmiter, 2007). As lowered IRS and increased foxo activity are pertinent to the maximised survival of Drosophila undergoing amino acid starvation (Kramer *et al.*, 2008), it would be expected that reduced DAT would result in the same phenotype while overexpression of *DAT* would impair survival. However we have found that both up-regulation and reduced activity of DAT decreased survivorship when starved of amino acids.

It is clear the manipulation of components of the homeostatic and reward-based control of feeding behaviors, namely the principal neuropeptides NPF and sNPF, and the dopamine regulator DAT, affects survival during adverse nutrient conditions. While it is expected that overexpression should yield opposite results than lowering or loss of function experiments, this

was not observed. These results show that it is important to maintain a sensitive balance in order to appropriately regulate the complex behavior of food intake so that survivorship during adverse nutrient conditions can be maximised.

Acknowledgements

The authors would like to thank Dr. P. Shen (University of Georgia), Dr. K. Yu (Korea Research Institute of Bioscience and Biotechnology), Dr. R. Jackson (Tufts University) and the Bloomington Drosophila Stock Center (University of Indiana, Bloomington, USA) for the Drosophila lines. We thank Frankie Amanda Slade for helpful comments on the manuscript. JDS was funded by a Natural Sciences and Engineering Research Council of Canada Post-Graduate Scholarship and the School of Graduate Studies of Memorial University of Newfoundland. This project was funded by a Natural Sciences and Engineering Research Council of Canada Discovery Grant to BES.

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Chapter 6:

General Discussion and Conclusion

The goal of this research was to gain a better understanding of the control of starvation resistance in Drosophila. Through application of this knowledge it may be possible eventually to prolong longevity of individuals undergoing eating disorders so that they may have a better chance at rehabilitation. A number of eating disorders, including the diagnosable forms of anorexia and bulimia, or any irregular eating behaviour, can be life threatening (Neilson 2001; Fairburn and Harrison, 2003). The etiologies of complicated disorders are multifactorial, but there is evidence in recent advances in neurobiology to suggest that genetic factors may play a particularly strong role.

Irregular eating and periods of malnutrition may be common with fluctuation in the availability of nutrition throughout the lifetime of most organisms. Evolution has responded to this problem through the formation of complex signalling cascades to combine both the control of nutrient intake and cell growth during development to manage energy stores (Brown-Borg, 2003; Bishop and Guarente, 2007; Broughton and Partridge, 2009; Longo, 2009). Homeostatic hormones and neuropeptides regulate the balance through the prompting or cessation of foraging and feeding. This homeostatic process works efficiently but when nutrients are readily available, a post-satiation reward system can regulate feeding (Palmiter, 2007). Therefore it is important to consider both means of control when evaluating feeding behaviour in the understanding of eating disorders.

Drosophila melanogaster was utilized as a simple animal model system to study the molecular basis of starvation and starvation resistance during amino acid deprivation. The majority of studies of starvation resistance in Drosophila focus upon complete food deficit and not reduction of any single dietary component (Rion and Kawecki, 2007). However, the lifespan of Drosophila under these conditions is extremely short, thereby limiting the ability to observe

any subtle differences in survival. 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 amino acids (Yamada et al., 2013). Taking this into consideration, investigation into the homeostatic control of feeding through novel mutants of the insulin receptor pathway kinase Akt1, a deacetylase Sir2, the neuropeptides NPF and sNPF, and the post-satiation reward control dopamine transporter (DAT) were conducted to determine the effects upon survivorship of Drosophila when deprived of amino acids.

Homeostatic Control: The Insulin Receptor Pathway Kinase Akt1

The initial investigation focused upon the highly conserved insulin receptor signalling (IRS) pathway and its central component, the Akt1 kinase. In Drosophila, there are seven insulin-like peptides (Ilps) produced by 14 neurosecretory cells (Broeck, 2001; Cao and Brown, 2001; Ikeya et al., 2002). The IRS pathway in flies reduces the levels of trehalose in the hemolymph (Rion and Kawecki, 2007). Flies that lack Ilps develop into small adults with low fertility and metabolic defects similar to mammalian diabetes including elevated sugar levels and initiation of the starvation response, including low amount of triglycerides stored in the fat body (Zhang et al., 2009). Therefore it can be said that the IRS pathway and the regulation of energy stores in Drosophila is conserved and functions in a manner comparably to vertebrates.

Hypomorphic Akt1 mutants are developmentally delayed and show compensatory growth

Given the importance of the insulin pathway and its conservation, we began the investigation of starvation resistance by the characterization of novel *Akt1* mutants (Slade and Staveley, 2015). These novel *Akt1* hypomorphs were generated via an imprecise excision of a PZ P element in the upstream control region of the Akt1 gene. Molecular analysis indicates that portions of the PZ P element were retained in various amounts in each gene. Initial analysis of these mutnats show that they are developmentally delayed, a phenotype similar to dauer formation in *C. elegans.* Growth is halted in response to low nutrients and resumes once nutrients are replenished (Riddle, 1988). When larvae are amino acid starved, their overall growth is impeded, and larvae with a dominant-negative mutation of PI3K (and lowered Akt1 activity) phenocopy the effects of amino acid starvation (Kramer et al., 2003). Slowed growth, due to genetic mechanisms or nutrient conditions, characteristically results in larvae that develop into smaller adults. As Akt1 is a central component of the IRS pathway, it is not surprising that these novel hypomorphic alleles result in a delay of development and overall smaller adult organisms. In future work, RT-qPCR analysis of these mutants could be carried out to identify the level of reduction of Akt1 activity in each mutant.

To determine the weight of the developmental delay upon cell growth, somatic clones of the eye were generated to produce a mutant phenotype less influenced by the extended time in development. The clone eyes are comprised of homozygous mutant tissue in a heterozygous organism that develops within a relatively normal timeframe. Biometric analysis of homozygous *Akt1* mutant eyes indicated an overall decrease in both ommatidia number and size when compared to controls (Slade and Staveley, 2015), but in comparison, analysis of somatic clones

revealed a more severe decrease in these measurements. Reduced expression or loss of *Akt1* results in the production of smaller animals or, if severe, lethality (Beadle et al., 1938; Staveley et al., 1998; Verdu et al., 1999), therefore the smaller eyes observed in the homozygotes is expected with the lower expression of *Akt1* in these novel mutants. The mutant clone eyes show a more severe phenotype due to the removal of developmental delay, and a reduction in time for compensatory growth to replace missing tissue. Compensatory growth is widespread and occurs in the surviving cells of damaged tissues to generate final structures of near normal overall size (Gerhold et al., 2011; Worley et al., 2012). Clearly, the extended period of time required for the *Akt1* hypomorphs to develop allows compensatory proliferation to generate smaller but intact adults. This method of analyzing homozygous tissue in a heterozygous organism to remove delay in development allows for exposure of a subtle phenotype previously obscured by compensatory proliferation. Therefore this technique could be advantageous in a number of situations to help clarify the impact a genetic mutation has on cellular processes including growth.

To determine the mechanisms responsible for the observed compensatory growth, the double mutant lines of the novel *Akt1* hypomorphs and the amorphic allele of *foxo* were analyzed. Analysis of ommatidia number shows an epistatic effect whereas an argument could be made in the comparison of ommatidia area for a slight synergistic enhancement of the phenotype. Regardless, this suggests that without the presence of the *foxo* gene product, the hypomorphic alleles of *Akt1* do not cause the same reduction of growth and strongly suggests that *foxo* is necessary for the processes that lead to compensatory growth. As a transcription factor, *foxo* governs the expression of target genes that regulate cell growth such as the eukaryotic initiation factor 4E-binding protein (4E-BP) gene and cell cycle regulators including

$p27^{kip1}$(Dijkers et al., 2000; Miron et al., 2001). The *4E-BP* product is a negative regulator of protein synthesis and has been shown to strongly influence the regulation of cell growth (Miron et al., 2001). When foxo is upregulated, so is 4E-BP, which binds to the messenger RNA 5' cap-binding protein eIF4E to inhibit protein synthesis and cell growth. In humans, $p27^{kip1}$ inhibits cyclin-dependent kinases (cdks) (Dijkers et al., 2000), which aid in promoting the transitions between cell-cycle phases. Overexpression of $p27^{kip1}$ in human cells, as would be seen with upregulated *foxo*, leads to cell-cycle arrest in the G1 phase (Miron et al., 2001; Van Der Heide et al., 2004). An increase in *foxo* activity appears to result in a decrease in cell proliferation, suggesting that the smaller eyes of the *Akt1* hypomorphs may work through foxo as the double mutants do not show the same reduction in size.

Novel Akt1 mutants show increased survivorship when aged upon amino acid deprived media

Interestingly, subjecting these novel *Akt1* hypomorphs to amino acid starvation resulted in increased resistance to amino acid starvation when compared to controls (Slade and Staveley, 2016*a*). When *Akt1* was expressed in the genetic background of the *Akt1* mutant homozygotes, the extension in survival upon the starvation medium was suppressed. This indicates that the alteration of *Akt1* activity in the mutants is indeed responsible for the extension in lifespan upon amino acid starvation media. Surviving starvation requires increased energy stores such as accumulated fats. Drosophila deficient in IRS maintain an increase in lipid stores that may aid in starvation resistance (Rion and Kawecki, 2007). As the *Akt1* mutants are hypomorphs, similar to conditions of reduced IRS, this might contribute to the extension in lifespan. A suggestion of further analysis would be to quantify the lipid stores in the control and each mutant initially and

at a predetermined point (or several points) later in the amino acid starvation experiment in order to determine if there are indeed increased lipid stores that may be contributing to the extended survival observed.

To elucidate a potential mechanism for the starvation resistance, the novel *Akt1* mutants were combined with a null *foxo* mutation. However, these double mutants did not show the same survivorship, which implies this phenotype of starvation resistance is dependent upon *foxo*. When starved of amino acids, the null *foxo* mutants and the double mutants died earlier, unlike the hypomorphic *Akt1* mutants. This demonstrates the necessity of foxo in the extended survival phenotype. Loss of *foxo* has been shown to result in an increased sensitivity to starvation caused by low levels of amino acids (Britton et al., 2002; Junger et al., 2003; Kramer et al., 2008). *foxo* induces the expression of genes associated with stresses including *MnSOD* and *4EBP* (Zinke et al., 2002; Puig et al., 2003). An increase in *MnSOD* aids in the reduction of life threatening free radicals, while induction of *4EBP* decreases protein synthesis, an unnecessary process during famine. The *Akt1* mutants may act through *foxo* to induce the expression of these pro-survival genes to extend their survivorship during amino acid starvation.

Homeostatic control: The deacetylase Sir2

Given that the IRS pathway is a complex signaling cascade known to have implications in development and growth, an alternative mechanism to extend survivorship during amino acid starvation was investigated. Sirtuins are protein deacetylases that mediate transcription of genes involved in metabolic homeostasis through transcription factors, such as foxo, (Rodgers et al.,

2005; Bordone et al., 2006; Rodgers et al., 2007), therefore *Sir2* mutants were chosen for investigation.

Typically it is the overexpression of sirtuins that increase longevity during conditions of adequate and inadequate nutrition, 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). 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). Additionally the activity of *Sir2* is induced in control flies during starvation (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. 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 how much is necessary?

A heterozygous Sir2 mutant shows extensive survivorship upon amino acid starvation

A number of homozygous null and heterozygous *Sir2* mutants were subjected to amino acid starvation. One heterozygous *Sir2* mutant in particular, the $Sir2^{05327}$ insertional mutant heterozygote, had a generous extension in survivorship (Slade and Staveley, 2016*b*). A physiological response to starvation in aid of survival is an increase in lipid storage (Stout et al., 1976), and RT-qPCR shows that genes involved in in fat storage are up-regulated in *Sir2* mutants (Banerjee et al., 2012). Given that the *Sir2* mutants observed here are heterozygotes, it

is possible that they may have elevated levels of stored fats that aid in their survival. Future direction may be to quantify the lipid stores in the mutants as previously mentioned for the *Akt1* hypomorphs.

In addition to a physiological response, a sufficient level of *Sir2* activity is required during starvation for the regulation of downstream targets responsible for surviving stresses. 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 (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). In the amino acid starved *Akt1* mutants, foxo is required to generate the extension in survival phenotype (Slade and Staveley, 2016*a*). *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 excess 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. To confirm the extension in survival of this heterozygote may work through foxo, a series of double mutants bearing both the heterozygous $Sir2^{05327}$ mutation and the null *foxo* mutation should be generated via recombinant crosses and analyzed.

The heterozygous Sir2 mutant lives longer and has eyes that are not smaller in size

Further characterization of the *Sir2* mutants indicates that they do not have the same disadvantageous influence upon organismal growth and development as the *Akt1* mutants, and

also enhance longevity upon a regular diet. Biometric analysis of eyes, including ommatidia number and area, resulted in values that were all within the standard error of the mean when compared to the control, indicating there was no significant difference in the size or number of ommatidia when *Sir2* is altered (Slade and Staveley, 2016*b*). Therefore slightly reducing the expression level of *Sir2* should have no significant impact upon development.

Importantly, these *Sir2* mutants, in addition to enhancing survivorship during starvation, also enhanced longevity (Slade and Staveley, 2016*b*). The slight reduction in *Sir2* activity is not only beneficial during conditions of nutritional stress, but may also be advantageous to standard longevity. 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), while a Drosophila null mutant of *Sir2* is more sensitive to starvation (complete starvation) when compared to controls (Banerjee et al., 2012). Interestingly, the subtle reduction of *Sir2* activity, as in these mutant heterozygotes, results in Drosophila that are not strongly affected during growth or development, but have slightly increased longevities and can be capable of surviving for extended periods of amino acid starvation (Slade and Staveley, 2016*b*). Although total loss of *Sir2* is detrimental, it is interesting that reducing the gene dosage of *Sir2* through an insertional mutant heterozygote can result in extensions in both longevity and survival. In the future, generation of double mutants with null *foxo*, or any other candidate targets of Sir2, and the heterozygous *Sir2* allele, may elucidate a potential mechanism for this extension.

Homeostatic control: Neuropeptides

A considerable amount of research conducted on feeding behavior is associated with the homeostatic control via neuropeptides, therefore an eminent neuropeptide regulator of the behaviour was selected and investigated. NPF in insects is the structural homologue of NPY, which in vertebrates is considered to be the dominant neuropeptide responsible for initiating feeding (Valassi *et al.*, 2008). Drosophila contain both NPF, and additionally four known short neuropeptide F (sNPFs) sequences in Drosophila encoded by a single transcription unit (Vanden Broeck, 2001). Studies in Drosophila that alter the expression of *NPF* or *sNPF* confirm their role in either the initiation or stopping of feeding. Given their role in the regulation of feeding, the expression levels of both neuropeptides was manipulated to see if enhancing feeding could extend survivorship during amino acid starvation. It was expected that overexpression would enhance survival while reduction of expression would debilitate survival during low nutrient conditions.

Both the overexpression and reduction in expression of NPF and sNPF increase sensitivity to amino acid starvation

Drosophila with either an overexpression or reduced expression of *NPF* or *sNPF* showed increased sensitivity to amino acid starvation (Slade and Staveley, 2016*c*). In *Drosophila melanogaster*, young larvae feed intensively (Melcher *et al.*, 2007) and *NPF* expression is up-regulated, while in the older larvae, there is less *NPF* expression (Maule, 1995; de Jong-Brink et al., 2001). Young larvae that lack NPF signalling wander before necessary and do not feed (Wu

et al., 2003), and continue to avoid noxious food even when starved (Wu et al., 2005). Certainly, a lessened appetite and reduced amount of feeding could be expected to impair survivorship, especially under adverse nutrient conditions. Conversely, *NPF* expression is lower in older larvae, but overexpression of *NPF* in these larvae leads to phenotypes similar to those observed in younger larvae, including increased feeding (Wu et al., 2003; Wu et al., 2005) continuing well past the normal developmental time-frame to result in delayed wandering and delayed pupariation. Additionally, well-fed larvae with an overexpression of *NPF* will feed on noxious foods (Wu et al., 2005). It was expected that encouraging excess feeding through overexpression of *NPF* would increase survivorship during amino acid starvation. The cell surfaces of specific neurons in Drosophila contain both the NPF and the insulin receptors (Wu et al., 2005), and it was found that IRS negatively regulates the action of the NPF receptor, and hence NPF signalling in Drosophila. Therefore, one would expect that lowered *NPF* expression should share a similar outcome to what is observed with increased IRS during amino acid starvation, and *vice versa*. We observed survivorship was impaired when the function of NPF was reduced or enhanced in Drosophila maintained upon amino acid depleted media, thereby suggesting that the mechanism is more complex than originally hypothesized.

Similarly, overexpression and reduced expression of *sNPF* decreased the survivorship of Drosophila during the amino acid starvation assays. This shorter neuropeptide is implicated in the control of feeding as it has been observed that loss of expression in both larvae and adults results in organisms that do not feed, but an abundance of *sNPF* expression in both larvae and adult Drosophila initiates the feeding behaviour (Lee et al., 2004). Although it was expected that overexpression would enhance survival while loss of expression would reduce survival, as in the NPF experiments, the findings in this research instead suggest a sensitive balance of *sNPF*

expression is pertinent to the survival of Drosophila exposed to depleted nutrients. Characterization of Drosophila with alterations to *sNPF* expression bear similar phenotypes to flies with abnormal IRS levels (Lee et al., 2008) and suggests that sNPF negatively regulates IRS. Consequently lowering sNPF should increase foxo activity, and therefore, enhance survival during nutrient deprived conditions, but this was not observed. Alternatively, overexpression of *sNPF* should increase Akt1 activity and decrease the transcriptional activity of foxo to sensitize the flies to amino acid starvation, which was observed.

The alteration in expression for *NPF* and *sNPF* was carried out using the UAS-Gal4 system, the cell death gene reaper (rpr), and RNA interference. These lines were obtained from other researchers (Wu et al., 2003; Lee et al., 2004) who had tested the functionality of the techniques, however in the future it may be beneficial to test the lines to determine if the functionality remains the same. This could include analysis of the Drosophila brain to see if rpr was indeed killing the NPF specific neurons, and carrying out RT-qPCR to determine if the overexpression of *sNPF-RNAi* was lowering the level of *sNPF* expression. Another method that could be implemented would be to measure the amount of food ingested by the flies in each case. As expectations were based on the alteration of these neuropeptides causing a change in feeding behaviours, quantification of the behaviour would be beneficial to see if this was the actual outcome. This could be accomplished by a number of methods, including feeding the flies dyed food and measuring the dye absorption in the resulting fecal matter, as was used previously (Min and Tatar, 2006) and determined that Drosophila ingesting a diet with less protein consumed less food. This could be occurring here, adding to the potential for decreased survivorship, which is observed.

Post-satiation control: Dopamine and the dopamine transporter

In vertebrates, feeding behaviour may also be adjusted through the activity of the dopamine reward system, which is known to be activated post-ingestion (Palmiter, 2007). Feeding increases release of dopamine, so individuals suffering from eating disorders may undergo lengthy periods of dietary restriction, leading to lowered dopamine levels in the hypothalamus (Kontis and Theochari, 2012). The dopamine transporter functions to clear dopamine from the synapses, therefore reducing its expression will increase levels of dopamine in the hypothalamus. Given this, it is expected that Drosophila with impaired DAT function should experience higher levels of dopamine signalling, and eat more while Drosophila over-expressing *DAT* should eat less. This could affect the levels of energy stores and therefore affect the ability to survive amino acid starvation.

Overexpression or reduction of the dopamine transporter increases sensitivity to amino acid starvation

Both the overexpression of *DAT* in Drosophila and the Drosophila DAT mutant, named fumin (*fmn*), resulted in a decrease in survivorship when compared to the control (Slade and Staveley 2016*c*). Dopamine signalling, strongly influenced by DAT activity, is linked to IRS as the insulin receptor is expressed in the dopaminergic neurons of the midbrain of Drosophila (Palmiter, 2007; Mebel *et al.*, 2012). Inhibition of the receptor reduces IRS and *DAT* expression and thus dopamine clearance. As lowered IRS and increased foxo activity are pertinent to the maximized survival of Drosophila undergoing amino acid starvation (Kramer *et al.*, 2008), it

would be expected that reduced DAT would result in the same phenotype while overexpression of *DAT* would impair survival. However both up-regulation and reduced activity of DAT decreased survivorship when Drosophila were starved of amino acids. This indicates that a sensitive balance of DAT and dopamine signaling is required for optimal survivorship, and additional analysis is required to determine potential mechanisms.

Concluding Statements

Past research has indicated that nutrient sensing mechanisms, such as the IRS pathway, sirtuin proteins, and neuropeptides and transmitters can play a pivotal role in the mediation of food intake/energy usage in order to maximize longevity. The majority of studies on the regulation of feeding behaviour involve the IRS pathway, which is well conserved between mammals and Drosophila (Britton *et al.*, 2002). During conditions of low nutrient availability, as in amino acid starvation, the IRS pathway is muted. With less insulin there is more trehalose present in the haemolymph coupled with an increase in lifespan (Broughton *et al.*, 2008). A main downstream effector that is negatively regulated by insulin receptor activity, the transcription factor foxo, is more active when IRS is lessened. The foxo transcription factor has been identified as important in survival during starvation of amino acids (Kramer *et al.*, 2008). Drosophila exposed to amino acid starvation media show an increase in expression of *foxo* while the loss of *foxo* activity results in a decreased survivorship when only fed sucrose without protein or amino acids.

This research has demonstrated that manipulation of the foxo regulators Akt1 and Sir2 can extend survivorship during amino acid starvation. However, the novel *Akt1* hypomorphs are

developmentally delayed and display decreased standard longevity. The *Sir2* heterozygous mutation not only induces a longer extension in survivorship, but also does not result in smaller organisms and increases standard longevity. Beyond signalling cascades, it is clear that the manipulation of components of the homeostatic and reward-based control of feeding behaviours, including the principal neuropeptides NPF and sNPF, and the dopamine regulator DAT, affects survival during adverse nutrient conditions. While it is expected that overexpression of the neuroregulators should yield opposite results than lowering or loss of function experiments, this was not observed. These results show that it is important to maintain a sensitive balance in order to appropriately regulate the complex behaviour of food intake so that survivorship during adverse nutrient conditions can be maximized.

The prevalence of eating disorders and obesity in modern societies has generated a great deal of interest in elucidating the pathways and mechanisms that control feeding behaviour. A better understanding of how these systems work to control feeding could lend itself to enhancing survival during conditions of nutritional adversity. The neural profile of an individual with anorexia corresponds to one of imbalance between the reward and inhibition systems of the brain (Phillipou et al., 2014). Therefore dissecting the mechanisms of action of the different neuroregulators and peripheral genes and pathways involved in the regulation of food intake and motivational aspects of feeding is necessary to discover potentially new therapies for related conditions. Through the use of Drosophila, these experiments have investigated both the homeostatic control of food intake through components of the IRS pathway, Sir2 and the neuropeptides NPF and sNPF, as well as the motivational DAT. This serves as a beginning to determine the biological consequence of malnutrition and develop potential gene therapies to enhance starvation resistance. The ability to enhance survival of organisms during nutrient

deprivation can aid in prolonging the treatment of individuals with eating disorders to achieve greater success in rehabilitation.

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