A BLUEBERRY EXTRACT-SUPPLEMENTED DIET RESCUES PHENOTYPES IN *DROSOPHILA MELANOGASTER* MODELS OF PARKINSON DISEASE

by

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ABSTRACT

Parkinson disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta. As a result, affected individuals have impaired motor function typically coupled with several non-motor symptoms that arise from extra-nigral damage. Mutations in the human α-synuclein (SNCA) gene have been linked to heritable autosomal dominant PD and duplications of its gene locus result in a more severe early onset form of the disease. Post-mortem analysis of patient brains reveals increased levels of oxidative stress biomarkers in individuals with PD. I investigated the potential therapeutic effects of a diet high in antioxidants both in a Drosophila melanogaster model of PD and on α-synuclein-induced developmental defects of the neuron-rich eye. Longevity assays, climbing trials, and biometric analyses were performed to test the effects of blueberry extract (BBE) on several α-synuclein-induced phenotypes. My results suggest that a diet supplemented with BBE rescues α-synuclein-induced degeneration in D. melanogaster. Both reduced lifespan and disruption to the external morphology of the eye induced by α-synuclein were improved in individuals fed a diet supplemented with BBE. These findings demonstrate that BBE counteracts PD-like phenotypes in an animal model of protein toxicity and suggest that dietary antioxidants may alleviate some of the cellular stress caused by excess α-synuclein. Diets rich in sources of antioxidants, like blueberries, could become a useful tool in treating PD and other similar neurodegenerative disorders if this relationship is conserved in humans.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS AND SYMBOLS

aa - amino acid
AD - autosomal dominant
ADPD - autosomal dominant Parkinson disease
ALS - amyotrophic lateral sclerosis
ANOVA - analysis of variance
ApIbm - apolipoprotein lipid-binding motif
AR - autosomal recessive
AT - acidic tail
ATP13A2 - ATPase type 13A2
BBE - blueberry extract
C - carboxyl terminus
CANSIM - Canadian Socioeconomic Information Management
CAT - catalase
CI - confidence interval
cm - centimeter
CO2 - carbon dioxide
DA - dopaminergic
Ddc - dopa decarboxylase
DNA - deoxyribonucleic acid
EOPD - early-onset Parkinson disease
FBXO7 - F-box protein 7
GAK - cyclin G associated kinase

Gal4 - yeast transcriptional activator for galactose-inducible genes

GBA - glucocerebrosidase

GFP - green fluorescent protein

GIGYF2 - GRB10 interacting GYF protein 2

GMR - glass multiple reporter

GOI - gene of interest

H2O - water

H2O2 - hydrogen peroxide

HLA-DRA - major histocompatibility complex, class II, DR alpha

HSP70 - heat shock protein 70

HTRA2 - HtrA serine peptidase 2

iPSC - induced pluripotent stem cell

k - slope

kbp - kilo-base pairs

LB - Lewy body

LN - Lewy neurite

LOPD - late-onset Parkinson disease

LRRK2 - leucine-rich repeat kinase 2

MAO - monoamine oxidase

mg - milligram

ml - milliliter

MND - motor neuron disease
MSRA - methionine sulfoxide reductase A

n - number of individuals

N - amino terminus

N/A - information not available

NAC - Non-Αβ component

NO - nitric oxide

O₂⁺ - superoxide radical

OCT - hypochlorite

OS - operating system

PARK - Parkinson disease-associated gene locus

PD - Parkinson disease

PINK1 - PTEN-induced putative kinase 1

PLA2G6 - phospholipase A2, group VI

PTEN - phosphatase and tensin homolog

qRT-PCR - quantitative reverse transcriptase polymerase chain reaction

RNA - ribonucleic acid

ROS - reactive oxygen species

SE - standard error

SEM - scanning electron microscopy

SNC - substantia nigra pars compacta

SOD - superoxide dismutase

SOD1 - copper-zinc (Cu-Zn) superoxide dismutase

SOD2 - manganese (Mn) superoxide dismutase
TSP - tissue specific promoter

TRAP1 - tumor necrosis factor receptor-associated protein 1

UAS - upstream activating sequence

UCH-L1 - ubiquitin carboxyl-terminal hydrolase L1

UPS - ubiquitin proteasome system

WHO - World Health Organization

WT - wild type

α-syn - α-synuclein/SNCA

μm - micrometer

°C - degree Celsius
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INTRODUCTION

Parkinson disease: frequency and symptoms

Parkinson disease (PD) is the second most common progressive neurodegenerative disorder surpassed in frequency by Alzheimer disease alone (Weintraub et al., 2008; de Moura et al., 2010). Incidence and prevalence rates of affected individuals increase with age from 0.3% of the general population to 1 - 2% of individuals over the age of 65 years, with rates reaching as high as 4% in the 80-plus age category (de Rijk et al., 2000; Van Den Eeden et al., 2003; McNaught and Olanow, 2006). In Canada, PD affects close to 100,000 individuals and is the 13th leading cause of death, accounting for over 13,000 deaths in the years 2000 - 2007 (Tables 1 and 2). With more people surviving well into old age, the burden to both families and society caused by late-onset disorders such as PD is likely to increase in the future.

First described in 1817 by James Parkinson, PD is a slowly progressive neurodegenerative disorder that is characterized clinically by bradykinesia (i.e. slowed movements), resting tremor, muscular rigidity, and gait abnormalities (Parkinson, 1817). There are also several non-motor symptoms that are exhibited by PD patients, including depression, dementia, obsessional behaviour, and olfactory dysfunction (Weintraub et al., 2008). The hallmark pathological event in PD is the loss of dopaminergic (DA), or dopamine producing, neurons in the substantia nigra pars compacta (SNC), however,
Table 1 - Annual deaths from Parkinson disease for both sexes and all age groups in Canada for the years 2000 - 2007

<table>
<thead>
<tr>
<th>Year</th>
<th>Parkinson disease</th>
<th>Secondary parkinsonism</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>1471</td>
<td>9</td>
</tr>
<tr>
<td>2001</td>
<td>1600</td>
<td>9</td>
</tr>
<tr>
<td>2002</td>
<td>1651</td>
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<td>2006</td>
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</tr>
<tr>
<td>2007</td>
<td>1869</td>
<td>21</td>
</tr>
</tbody>
</table>

1 Missing data on sex of the deceased were imputed based on death registration number.
2 Source: Statistics Canada, Canadian Vital Statistics, Death Database
3 World Health Organization (WHO), International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10)
4 The cause of death tabulated is the underlying cause of death. This is defined as (a) the disease or injury which initiated the train of events leading directly to death, or (b) the circumstances or the accident or violence which produced the fatal injury. This underlying cause is selected from a number of conditions listed on the death registration form.
5 Counts in this table exclude deaths of non-residents of Canada.
6 To reduce the size of the table, only causes of death with a frequency of one or more in Canada are reported.

Modified from: Statistics Canada. Table 102-0526 - Deaths, by cause, Chapter VI: Diseases of the nervous system (G00 to G99), age group and sex, Canada, annual (number) (table), CANSIM (database), http://www.statcan.gc.ca (accessed: January 7, 2011).
Table 2 - Annual ranking of Parkinson disease among the leading causes of death\textsuperscript{1-3} for both sexes\textsuperscript{4} and all age groups in Canada\textsuperscript{5,6} for the years 2000 - 2007\textsuperscript{7,8}

<table>
<thead>
<tr>
<th>Year</th>
<th>Rank of leading causes of death\textsuperscript{9}</th>
<th>Number of deaths</th>
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<tbody>
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<td>2000</td>
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</tr>
<tr>
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<td>13</td>
<td>1609</td>
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<td>2002</td>
<td>13</td>
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</tr>
<tr>
<td>2007</td>
<td>13</td>
<td>1890</td>
</tr>
</tbody>
</table>

1 The cause of death tabulated is the underlying cause of death. This is defined as (a) the disease or injury which initiated the train of events leading directly to death, or (b) the circumstances of the accident or violence which produced the fatal injury. The underlying cause is selected from the conditions listed on the medical certificate of cause of death.
2 World Health Organization (WHO), International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10).
3 The list for ranking leading causes of death that is used in this table is based on the list that was developed and that is been used by the National Center for Health Statistics of the United States in their annual report on leading causes of death.
4 Missing data on sex of the deceased were imputed based on the death registration number.
5 Counts in this table exclude deaths of non-residents of Canada.
6 The category 'Canada' includes deaths with unknown province or territory of residence in Canada.
7 Death refers to the permanent disappearance of all evidence of life at any time after a live birth has taken place. Stillbirths are excluded.
8 Sources: Statistics Canada, Canadian Vital Statistics, Death Database and population estimates
9 The ranking of the leading causes of death is based on the number of deaths.

Modified from: Statistics Canada. Table 102-0563 - Leading causes of death, total population, by sex, Canada, provinces and territories, annual (table), CANSIM (database), \url{http://www.statcan.gc.ca} (accessed January 7, 2011)
considerable extranigral damage also occurs (Jellinger, 1991; Braak and Braak, 2000). Additionally, proteinaceous intraneuronal inclusions known as Lewy bodies (LB) and Lewy neurites (LN) are found in the perikarya (cell body) and processes, respectively, of surviving neurons in PD patient brains (Lewy, 1912; Forno, 1996). Secondary forms of parkinsonism with similar symptoms can be caused by medications, toxins, infections to the central nervous system, and vascular/metabolic disorders (Weintraub et al., 2008). The multi-symptom etiology of PD, caused by irreparable damage to neurons, highlights the importance of cell survival in neurodegenerative disorders.

Parkinson disease: genetics and α-synuclein

The occurrence of PD was initially believed to be entirely sporadic in nature. Though the majority of cases seem to be idiopathic, 10 - 30% of affected subjects reported a positive family history in recent epidemiological studies (Shulman et al., 2011). Eighteen Parkinson-associated (PARK) loci have been identified to-date through a combination of linkage, segregation, and sequence analyses; though several require validation by independent studies (Table 3). In addition to the eighteen PARK loci, the Gaucher's locus has been linked to PD. This locus contains the glucocerebrosidase (GBA) gene whose product catalyzes the breakdown of glucocerebroside to glucose and ceramide. Loss of function mutations in GBA were first associated with Gaucher's disease, an autosomal recessive lysosomal storage disorder. Despite not being classified
Table 3 - Genetic loci linked to Parkinson disease susceptibility in humans

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosome</th>
<th>Gene</th>
<th>Inheritance</th>
<th>Clinical phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1/4</td>
<td>4q21</td>
<td>SNCA</td>
<td>AD</td>
<td>EOPD</td>
</tr>
<tr>
<td>PARK2</td>
<td>6q25.2-q27</td>
<td>Parkin</td>
<td>AR</td>
<td>Juvenile and EOPD</td>
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<td>PARK3</td>
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<td>LOPD</td>
</tr>
<tr>
<td>PARK5</td>
<td>4p14</td>
<td>UCH-L1</td>
<td>AD</td>
<td>LOPD</td>
</tr>
<tr>
<td>PARK6</td>
<td>1p35-p36</td>
<td>PINK1</td>
<td>AR</td>
<td>EOPD</td>
</tr>
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<td>PARK7</td>
<td>1p36</td>
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<td>AR</td>
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</tr>
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<td>Gaucher's locus</td>
<td>1q21</td>
<td>GBA</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

AD: autosomal dominant; AR: autosomal recessive; EOPD: early-onset Parkinson disease; LOPD: late-onset Parkinson disease; N/A: information not available

**Bold** text indicates monogenic forms that are well validated

Table adapted from (Kumar et al., 2011)
as a PARK locus, Gaucher's disease patients can develop parkinsonian features and mutations in GBA are frequently found in clinical populations with sporadic PD (Neudorfer et al., 1996; Tayebi et al., 2001; Clark et al., 2007; Gan-Or et al., 2008). Familial cases occur less frequently than idiopathic PD, however the numerous genetic linkages and increased disease susceptibility caused by gene mutations makes the genetic study of PD worthwhile.

Less than two decades have passed since the discovery of the first gene associated with PD. Studies of an inherited form of autosomal dominant PD (ADPD) in an Italian kindred and three unrelated families of Greek origin identified SNCA (PARK1/4), herein referred to as α-synuclein (α-syn), as the first gene linked to PD (Polymeropoulos et al., 1997). Both point mutations and multiplications of its gene locus result in ADPD, the latter causing a more severe early-onset form of the disease (Kruger et al., 1998; Singleton et al., 2003; Chartier-Harlin et al., 2004; Farrer et al., 2004; Zarranz et al., 2004). One of the major protein components of LBs and LNs in the SNc of patients with idiopathic PD was discovered to be α-Syn (Spillantini et al., 1997). This discovery implicated α-syn in sporadic disease cases and suggested that two distinct mechanisms may exist for α-syn-induced PD pathology. Although familial cases associated with this gene are relatively rare, it is evident that α-syn plays a critical role in PD etiology.
α-synuclein structure, function, and toxicity in Parkinson disease

Human α-Syn, along with β- and γ-Syn, belongs to the synuclein family of proteins that are abundantly expressed in the brain. The α-syn gene consists of a 114 kbp sequence and encodes a 140 aa peripheral membrane protein that localizes to the pre-synaptic region of neurons (Maroteaux et al., 1988). The protein structure of α-Syn contains three distinct regions: i) an amino terminus with apolipoprotein lipid-binding motifs, ii) a central hydrophobic region known as the non-Aβ component (NAC) that is involved in aggregate formation, and iii) a highly negative and often unstructured carboxyl terminus (Figure 1). Although an exact physiological function is unknown, mice overexpressing α-syn have impaired neurotransmitter release and recycling (Nemani et al., 2010). Additionally, increased amounts of α-Syn impaired dopamine release in neurons isolated from mice that typically express α-syn at low levels (Larsen et al., 2006). Taken together, these results suggest that α-Syn acts at the pre-synapse and controls neurotransmitter release.

The toxicity of α-Syn appears to depend on its conformation and solubility in cells. Conway et al. discovered that both wild type (WT) and disease-related mutants of α-Syn form amyloid-like fibrils after prolonged incubation in solution (Conway et al., 2000). Aggregation consists of a series of events beginning with the natively unfolded protein and culminating in mature fibril formation and is believed to be the main pathogenic mechanism of α-Syn (Stefanis, 2012). In support of this view, WT α-Syn with a disrupted NAC domain were neither found to aggregate nor affect the survival of Drosophila DA neurons (Periquet et al., 2007). It was also discovered that Heat Shock
Figure 1 - Schematic of α-Synuclein protein structure. ApoBM: apolipoprotein lipid-binding motif; NAC: non-Aβ component; AT: acidic tail; N: amino terminus; C: carboxyl terminus. Arrows indicate location of mutations linked to ADPD.
Protein 70 (HSP70), a molecular chaperone that prevents protein misfolding and aggregation, protects DA neurons against α-Syn toxicity and reduces aggregation (Auluck et al., 2002; Klucken et al., 2004). Conversely, other studies question the proposed protective effects of HSP70 (Shimshek et al., 2010). Some speculate that the final aggregated form of α-Syn is less toxic than premature, soluble oligomers. Protein accumulation is widely implicated in both idiopathic and familial PD pathogenesis and the formation of LBs and LNs is believed to confer protection during neurodegeneration (McNaught and Olanow, 2006). Additionally, preventing phosphorylation of α-Syn at Ser129 promotes aggregate formation and reduces toxicity (Chen and Feany, 2005). Understanding the pathogenic mechanisms involved with α-Syn toxicity could unveil several new therapeutic targets in the treatment of PD.

Oxidative stress and its consequences

Highly reactive endogenous atoms or molecules can cause cellular damage. Reactive oxygen species (ROS) are chemically reactive cellular by-products that contain oxygen. Due to their unpaired electrons, ROS are highly electrophilic and attack nitrogen containing compounds (e.g. nucleic acids, proteins, and amino acids) and carbon-carbon double bonds, like those found in polyunsaturated fatty acids and phospholipids in the lipid bilayer of the cell membrane, as they are sites of increased electron density. Though they are potentially harmful, ROS mediate cell growth, migration, and differentiation when present in low to moderate amounts (Palmer and Paulson, 1997; Valko et al., 2007).
Cells are equipped with endogenous antioxidant enzyme defenses that keep ROS levels in check. However, excess generation of ROS can exhaust this system causing the cell to experience oxidative stress whereupon deleterious effects occur and pathways leading to apoptosis and senescence are triggered (Chandra et al., 2000). The majority of endogenous ROS seem to be generated from four sources: i) normal aerobic respiration, which sequentially reduces molecular oxygen and releases ROS by-products; ii) phagocytic cells that fend off bacteria and viruses with a mixture of nitric oxide (NO), superoxide (O$_2^•$), hydrogen peroxide (H$_2$O$_2$), and hypochlorite (OCl$^{-}$); iii) peroxisomes, organelles that produce H$_2$O$_2$ as a by-product during the degradation of fatty acids; and iv) cytochrome p450 enzymes in animals that protect against ingested toxic plant chemicals but produce DNA-damaging ROS during the process (Ames et al., 1993). External stimuli can contribute to the amount of ROS in a cell as pollution, radiation, and xenobiotics can induce ROS-mediated damage (Ziech et al., 2010; Grosicka-Maciag, 2011). Oxidative stress is a complex process and can be triggered from many different angles.

Oxidative stress has long been implicated in ageing and chronic pathologies. Harman first addressed the issue when he proposed the Free Radical/Oxidative Stress Theory of Ageing in the 1950's (Harman, 1956). This theory states that ageing is a consequence of the accumulation of free radical-induced damage to cellular macromolecules that occurs over an organism's lifespan. Since that time, evidence of oxidative stress has been found in many common human diseases including hypertension, cancer, and neurodegenerative disorders (Calabrese et al., 2005; Bonora et al., 2006; Culmsee and Landshamer, 2006; Manrique et al., 2009; Schapira and Jenner, 2011).
Antioxidant therapies may have a future in modern medicine given the ubiquity of ROS and oxidative stress in disease etiology.

Evidence of oxidative stress in Parkinson disease

Whether it is a causative factor or the result of other cellular insults, oxidative stress is consistently associated with PD pathogenesis. Both expression and activity of mitochondrial complex I, a member of the electron transport chain, were found to be reduced in PD patient brains (Schapira et al., 1989). Other oxidative stress biomarkers such as decreased levels of reduced glutathione, as well as increased levels of Mn superoxide dismutase (SOD2) and iron have been detected in the SNc of PD individuals (Dexter et al., 1987; Saggu et al., 1989; Sofic et al., 1992). Long before any genetic linkages were discovered for PD, oxidative stress was known to be involved in its etiology.

A relationship appears to exist between α-Syn toxicity and oxidative stress. Recent studies suggest that α-Syn localizes to the mitochondria and may be involved in organelle maintenance (Parihar et al., 2008; Shavali et al., 2008; Parihar et al., 2009). Human DA neuroblastoma cells overexpressing α-syn show signs of increased oxidative stress, including increased ROS production and lipid peroxidation, believed to be the result of mitochondrial dysfunction. Increased expression of oxidative stress biomarkers was also evident in pluripotent stem cell (iPSC)-derived DA neurons from PD patients with an α-syn triplication (Byers et al., 2011). Studies in Drosophila have revealed that
mutant α-syn-induced toxicity is enhanced under conditions of hyperoxia (Botella et al., 2008). The combination of excess α-Syn and oxidative stress appears to play an important role in the progression of PD, although, the pathogenic mechanism remains unclear.

The Drosophila α-synuclein model of Parkinson disease

*Drosophila melanogaster* has been used extensively as a model organism over the past century due in large part to its short generation time (roughly 10 days from egg deposition to adult) and low cost. Many genetic tools have been developed for this tiny insect. One such tool used frequently in Drosophila disease models is the UAS/Gal4 directed expression system (Brand and Perrimon, 1993). Gal4 is a potent activator of transcription in yeast whose DNA binding and transcriptional activation have been well characterized (Ptashne, 1988). Endogenous binding sites for Gal4 are not found in the Drosophila genome, however it can be used to activate transcription in flies when Gal4 binding sites are inserted within their transcription control regions (Fischer et al., 1988). Gene expression can be manipulated spatially and temporally when Gal4 fused to a Drosophila promoter and its target upstream activating sequence (UAS) are both present in the fly genome. The first step towards achieving directed expression is to construct two stable lines: one containing Gal4 fused to a tissue-specific promoter, and a second with UAS elements inserted upstream of a gene of interest. Crosses are designed with the goal of collecting progeny with at least one copy of each gene construct. When
combined, Gal4 produced in the designated tissue can bind to the UAS and activate transcription of the gene under study (Figure 2). The UAS/Gal4 system functions at standard temperatures (i.e. does not require a heat shock) and facilitates the study of toxic gene products as stable lines can be created prior to Gal4 introduction. The same cannot be said for previously used Drosophila expression systems making the UAS/Gal4 system a superior choice.

The first Drosophila model of PD was developed in 2000, 3 years after the characterization of α-syn as a disease risk factor. Feany and Bender made use of the UAS/Gal4 system to overexpress both WT and mutant human α-syn in Drosophila neurons (Feany and Bender, 2000). Their α-syn model recapitulated several key PD symptoms in flies, including progressive loss of DA neurons, locomotor dysfunction, and the presence of LB-like inclusions containing α-Syn. Additionally, enhanced α-syn expression in the eye, another Drosophila tissue rich in neurons, caused retinal degeneration. Studies using this model from our laboratory have demonstrated that α-syn overexpression at elevated temperatures disrupts the external morphology of Drosophila eyes (Todd and Staveley, 2008). Such Drosophila overexpression models provide a versatile framework for the study of diseases involving increased gene copy number or protein aggregation. When investigating neurodegenerative disorders like PD, a researcher can easily quantify cell survival and rapidly evaluate the therapeutic value of new treatments with a Drosophila model.
Protein

Figure 2 - Directed gene expression in Drosophila via the UAS/Gal4 system. UAS-controlled transcription remains inactive when the two transgenes are isolated in the parental generation (P1). The Gal4 transcription factor binds to the UAS responsive DNA elements in progeny with at least one copy of each transgene (F1) activating tissue-specific transcription of the gene under study. TSP: tissue specific promoter, UAS: upstream activating sequence, GOI: gene of interest. Blue ovals represent Gal4 protein.
Antioxidants prolong lifespan and improve Parkinson disease-related phenotypes in Drosophila

Studies using Drosophila as a model organism have revealed a great deal concerning the effects of antioxidants upon lifespan. Both intrinsic and supplemented antioxidants have been found to prolong lifespan in flies. SOD and catalase (CAT) are examples of endogenous antioxidant enzymes in Drosophila that function together to convert $O_2^\cdot -$ into water via an $H_2O_2$ intermediate (Figure 3). Increased expression of both Sod1 and Sod2, typically located in the cytoplasm and mitochondria, respectively, in Drosophila motor neurons dramatically increased lifespan (Parkes et al., 1998; Phillips et al., 2000). In striking contrast to Sod, a similar result was not found for targeted expression of Cat in the same cells. In addition to revealing that intrinsic antioxidant enzymes protect against ageing, these results suggested that enzymes like SOD and CAT are of varying importance in neurons despite functioning in the same pathway.

Exposing Drosophila to a supplemented diet is relatively easy considering they live and breed on their food source in the laboratory setting. Extracts of many common dietary sources of antioxidants have prolonged survival in Drosophila including: i) whole food extracts from apple, nectarine, and cocoa (Bahadorani and Hilliker, 2008; Boyd et al., 2011; Peng et al., 2011), ii) tea extracts including green tea catechins and black tea (Li et al., 2007; Peng et al., 2009), iii) the traditional herbal medicines Stachys lavandulifolia and Aloe vera (Altun et al., 2010; Chandrashekara and Shakarad, 2011), and iv) individual chemical compounds such as ascorbic acid and resveratrol (Bahadorani et al., 2008). Thanks to its rapid generation time, established genetic tools,
Figure 3 - Schematic of the superoxide dismutase/catalase antioxidant pathway. Superoxide (O$_2$•⁻) is converted to water (H$_2$O) via a hydrogen peroxide (H$_2$O$_2$) intermediate. SOD: superoxide dismutase, CAT: catalase. Red text denotes reactive oxygen species.
and simple food delivery, Drosophila has played an instrumental role in uncovering the effects of antioxidants on lifespan.

Increased antioxidant protection has been found to improve PD-related phenotypes in Drosophila. Co-expression of antioxidant enzymes like methionine sulfoxide reductase A (MSRA) and human Sod1 improve DA neuron survival and protect against premature decreases in climbing ability induced by excess a-Syn in Drosophila DA neurons (Wassef et al., 2007; Botella et al., 2008). The protective effect of mitochondrial chaperone tumor necrosis factor receptor-associated protein 1 (TRAP1) against oxidative stress is dependent on phosphorylation by PTEN-induced putative kinase 1, or PINK1 (Pridgeon et al., 2007). Coincidentally, directed co-expression of the two aforementioned genes with a-syn in Drosophila improves DA neuron survival, eye degeneration, and locomotor deficiencies (Todd and Staveley, 2008; Butler et al., 2012). Supplementing Drosophila food medium with dietary antioxidants has shown promise as well. Medium supplemented with grape extract improved both early mortality and the premature decline in locomotion in a Drosophila model of PD (Long et al., 2009).

Though the literature is relatively new, the interest in potential antioxidant therapies for treating PD has increased thanks in large part to studies using Drosophila as a model organism.

In this study, I hypothesized that increased protection against oxidative stress in Drosophila DA neurons could both prolong lifespan and protect against a-syn-induced degenerative phenotypes. I used a combination of longevity assays, locomotion assays, and biometric analyses to obtain the results. First, I produced additional evidence that directed expression of intrinsic antioxidant enzymes in Drosophila neurons extends
lifespan. Secondly, I investigated the protective properties of blueberry extract (BBE) supplementation in the Drosophila \( \alpha\)-syn model of PD. Blueberries are an excellent source of dietary antioxidants. High-performance liquid chromatography analysis has identified 18 phenolic compounds in blueberries, which combined had the second highest total antioxidant activity in a cohort of tested berries including cranberries, raspberries, black currants, and red currants (Borges et al., 2010). Here I report that supplementation with Webber Naturals’ 36:1 concentrate BBE improves severe cases of early mortality and eye degeneration caused by directed expression of \( \alpha\)-syn in the DA neurons and developing eye, respectively, of \textit{D. melanogaster}.

\section*{MATERIALS AND METHODS}

\textit{Drosophila stocks and culture}

The \textit{UAS-\( \alpha\)-synuclein} (Feany and Bender, 2000) and \textit{Ddc-Gal4} (Li \textit{et al.}, 2000) flies were generously provided by Dr. M. Feany (Harvard Medical School) and Dr. J. Hirsh (University of Virginia), respectively. Each of the \textit{UAS-Sod1} (Parkes \textit{et al.}, 1998), \textit{UAS-Sod2} (Anderson \textit{et al.}, 2005), and \textit{UAS-Cat} (Anderson \textit{et al.}, 2005) stocks were kind gifts from Dr. John P. Phillips (University of Guelph). The \textit{GMR-Gal4} (Freeman, 1996), \textit{UAS-lacZ} (Brand and Perrimon, 1993), \textit{UAS-GFP} (Dickson, 1996), and \( w^{1118} \) flies were
obtained from the Bloomington Drosophila Stock Center at Indiana University. Directed expression of the transgenes in DA neurons and during early eye development was accomplished by crossing homozygous Ddc-Gal4 and GMR-Gal4 females, respectively, to males homozygous for a UAS-controlled transgene. For the antioxidant enzyme overexpression experiments, homozygous Ddc-Gal4 III (on the third chromosome) females were crossed to homozygous UAS-a-synuclein (PD model), UAS-lacZ (control), UAS-GFP (control), UAS-Sod1, UAS-Sod2, and UAS-Cat males. For the BBE-supplementation experiments, homozygous UAS-a-synuclein (PD model) and UAS-lacZ (control) males were crossed to females homozygous for Ddc-Gal4 II (on the second chromosome), Ddc-Gal4 III, or GMR-Gal4. Two additional control lines lacking a UAS-regulated transgene were generated by crossing homozygous Ddc-Gal4 or GMR-Gal4 females to w^{118} males. All crosses were performed as per standard methods. The resulting genotypes were: 1) w^{118}; UAS-a-synuclein/Ddc-Gal4, 2) w^{118}; UAS-a-synuclein/++; Ddc-Gal4/+, 3) w^{118}; UAS-lacZ/Ddc-Gal4, 4) w^{118}; UAS-lacZ/++; Ddc-Gal4/+, 5) w^{118}; +/Ddc-Gal4, 6) w^{118}; +; Ddc-Gal4/+, 7) w^{118}; UAS-a-synuclein/GMR-Gal4, 8) w^{118}; UAS-lacZ/GMR-Gal4, 9) w^{118}; UAS-GFP/++; Ddc-Gal4/+, 10) w^{118}; UAS-Sod1/++; Ddc-Gal4/+, 11) w^{118}; UAS-Sod2/++; Ddc-Gal4/+, and 12) w^{118}; UAS-Cat/++; Ddc-Gal4/+. Flies were fed either a standard cornmeal-yeast-molasses-agar medium (65 g/L cornmeal, 15 g/L nutritional yeast extract, 5.5 g/L agar, 50 ml/L fancy grade molasses in water supplemented with 0.1 g/ml methyl paraben in ethanol and 2.5 ml propionic acid) or standard medium supplemented with Webber Naturals' 36:1 concentrate BBE (WN
Pharmacuticals® Ltd., Coquitlam, B.C., V3K 7B5, www.webbernaturals.com). BBE-supplemented media was produced by adding 1 or 5 g/L BBE to the above recipe during media preparation.

Longevity assay

Flies were collected under gaseous carbon dioxide (CO₂) every 24 hours until a minimum of two hundred adult males of each genotype were obtained. They were then transferred to upright standard plastic shell vials containing standard cornmeal-yeast-molasses-agar medium (control), or standard medium supplemented with either 1 mg/ml BBE or 5 mg/ml BBE. Each group was maintained at 25 °C and kept in non-crowded conditions (≤20 individuals initially per vial). Flies were scored for viability every 2 days and transferred to fresh medium without anesthesia according to established protocol (Staveley et al., 1990). Survival fractions were calculated in Prism version 5.0b for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com) using the product limit (Kaplan-Meier) method.
**Locomotion assay**

Fifty adult males of each genotype were assayed for climbing ability as described previously (Todd and Staveley, 2004). Flies were collected every 24 hours under gaseous CO\textsubscript{2} and transferred to upright standard plastic shell vials containing standard cornmeal-yeast-molasses-agar medium (control), or standard medium supplemented with either 1 mg/ml BBE or 5 mg/ml BBE. Flies were maintained and assayed in groups of 10 individuals. Flies were given 48 hours to recover from the anesthesia before first being assayed. Climbing ability was measured again when flies reached 8 days old and repeated every 7 days following this point. Climbing ability was determined using an apparatus consisting of a 30 cm long clear glass tube with a diameter of 1.5 cm (Figure 4). The bottom of the tube was marked off into five 2 cm sections with the remaining 20 cm of the tube acting as a buffer zone that limits interference between individuals during climbing. A funnel was used to transfer flies to the apparatus and also doubled as a base. Sponges were inserted into both ends of the glass tube to both prevent flies from escaping and allow gas exchange to occur. During analysis, flies were gently tapped to the bottom of the apparatus and given 10 seconds to climb. Each individual was given a score based on the highest section they reached. Flies were scored 10 times per trial and a climbing index was calculated with the following formula:

$$\text{Climbing index} = \frac{\sum (\text{nm})}{N}$$
Figure 4 - Schematic representation of the graded climbing apparatus. The structure consists of a 30 x 1.5 cm glass tube sealed at both ends by sponges. The bottom of the tube is divided into five 2 cm sections with an ascending score (1-5) and the remainder of the upper portion serves as a buffer zone. A funnel acts as both a base for the tube and a means of transferring flies to and from the apparatus (modified from Todd and Staveley, 2004).
where \( n \) is the total number of flies at a given level, \( m \) is the score for that level (1 - 5), and \( N \) is the total number of flies assayed for that trial. Data were analyzed in Prism version 5.0b for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com). To compare climbing ability, climbing indices were subtracted from 5, to compensate for inverting the y-axes of the graphs, followed by a non-linear curve regression analysis. The slopes of curves with non-overlapping 95% confidence intervals (CI) were deemed significantly different.

**Scanning electron microscopy and biometric analyses**

Flies were reared and aged 3 to 5 days post-eclosion on either standard or BBE supplemented medium at 25 or 29 °C. Surviving flies were preserved at -80 °C before being mounted on metal studs under a dissecting microscope. Prepared flies were desiccated overnight and gold coated prior to photography at 170 times magnification with a Hitachi S-570 scanning electron microscope.

All biometric measurements were conducted with the aid of the software package ImageJ64 version 1.42q (Abramoff et al., 2004). The area of a single ommatidium was determined by measuring the average area of a "floret" of ommatidia, consisting of a central unit surrounded by six others, then dividing by 7. These numbers were used to distinguish between normal and atypical ommatidia when measuring percent disruption. A disrupted or atypical ommatidium was defined as having an area 50% smaller or 150% larger than a typical ommatidium for that condition. An oval with an area between
35000-40000 μm² was overlaid on the apex (flattest portion) of each analyzed eye with Paintbrush version 2.1.1 for Mac OS X (Copyright © 2007-2010 Soggy Waffles). Individual areas of disruption within the oval were measured in triplicate and a percent value was obtained by dividing the summed average values into the average area of the oval (also measured in triplicate). This protocol was modified from similar measures previously performed in our lab (Todd and Staveley, 2008). Ten individuals were evaluated in the percent disruption analysis, whereas 15 individuals from each condition were analyzed for ommatidium counts, bristle counts, and measurements of ommatidium area. Bar graphs were produced using Prism version 5.0b for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

Directed expression of catalase in D. melanogaster dopaminergic neurons slightly prolongs lifespan

The damage caused by excess ROS is implicated in senescence and chronic pathologies. Previous findings have shown that directed expression of both Sod1 and Sod2 in Drosophila motorneurons prolongs adult lifespan, whereas Cat has been demonstrated to have no effect (Parkes et al., 1998; Phillips et al., 2000). As both oxidative stress and DA neuron survival have strong connections with PD pathology, I
investigated the effect of increasing intrinsic antioxidant defenses in DA neurons on $D. melanogaster$ lifespan. Enhanced expression of $Cat$ in the DA neurons slightly prolonged lifespan in flies as compared to both control lines (Figure 5A). Flies expressing $Cat$ had a median survival of 80 days whereas $lacZ$- and $GFP$-expressing flies had values of 76 and 74 days, respectively. In contrast to the results found in motorneurons, neither $Sod1$ nor $Sod2$ were found to significantly extend lifespan when their expression was enhanced in DA neurons (Figure 5C & D). Additionally, increased $\alpha$-syn expression in $D. melanogaster$ DA neurons resulted in a median survival of 70 days and did not differ significantly from either control line (Figure 5B). Median survival times for each genotype are presented in Table 4. The effect of $Cat$, $Sod1$, and $Sod2$ expression on Drosophila lifespan appears to be cell-specific as results differ between varying groups of neurons.
Figure 5 - Directed expression of catalase in the dopaminergic neurons slightly extends lifespan in *D. melanogaster*. A The longevity of flies expressing *Cat* is significantly longer than both the *lacZ* and GFP-expressing controls (*p* < 0.05). B - D Flies expressing α-synuclein, *Sod1*, or *Sod2* in their DA neurons have a median survival time comparable to the control lines when expression is driven by *Ddc-Gal4* on the third chromosome. Genotypes are *w*^{1118}; *UAS-Cat/+; Ddc-Gal4/+ (CAT, *n* = 240), *w*^{1118}; *UAS-α-synuclein/+; Ddc-Gal4/+ (*αSYN*, *n* = 257), *w*^{1118}; *UAS-Sod1/+; Ddc-Gal4/+ (SOD1, *n* = 269), *w*^{1118}; *UAS-Sod2/+; Ddc-Gal4/+ (SOD2, *n* = 225), *w*^{1118}; *UAS-lacZ/+; Ddc-Gal4/+ (LACZ, *n* = 241), and *w*^{1118}; *UAS-GFP/+; Ddc-Gal4/+ (GFP, *n* = 303). Error bars represent standard error of the mean. *p*-values were calculated by the log-rank (Mantel-Cox) test and multiple comparisons were corrected for using the Bonferroni method.
Table 4 - Median survival values of *D. melanogaster* with elevated neuronal levels of enzymatic antioxidants or *α*-synuclein

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w^{1118}; UAS-lacZ/+; Ddc-Gal4/+ )</td>
<td>76</td>
</tr>
<tr>
<td>( w^{1118}; UAS-GFP/+; Ddc-Gal4/+ )</td>
<td>74</td>
</tr>
<tr>
<td>( w^{1118}; UAS-α-syn/+; Ddc-Gal4/+ )</td>
<td>70</td>
</tr>
<tr>
<td>( w^{1118}; UAS-Sod1/+; Ddc-Gal4/+ )</td>
<td>78</td>
</tr>
<tr>
<td>( w^{1118}; UAS-Sod2/+; Ddc-Gal4/+ )</td>
<td>70</td>
</tr>
<tr>
<td>( w^{1118}; UAS-Cat/+; Ddc-Gal4/+ )</td>
<td>80*</td>
</tr>
</tbody>
</table>

* indicates a value found to be significantly different \((p < 0.05)\) than both the *lacZ* and *GFP* control
The severity of α-synuclein-induced reductions in lifespan depends on Ddc-Gal4

Differing results have been documented concerning the effect of increased neuronal amounts of α-Syn on Drosophila lifespan. Initially, pan-neural expression of α-syn was reported to not alter lifespan in flies, whereas a later study described an α-syn-induced decrease in lifespan in a similar PD model (Feany and Bender, 2000; Wassef et al., 2007). The results presented herein agree with the latter study as enhanced expression of α-syn in Drosophila DA neurons produced a reduction in survival times (Figure 6). The severity of the aforementioned effect appears to be dependent on the genomic location of the Ddc-Gal4 transgene. Flies expressing α-syn with the Ddc-Gal4 transgene located on the second chromosome (Ddc-Gal4 II) had a severely reduced lifespan compared to control lines; their median survival time being 52 days as compared to 82 in flies without a responsive transgene (w1118) and 74 days in control lacZ-expressing flies (Figure 6A). A less pronounced reduction in survival time was observed when Ddc-Gal4 on the third chromosome (Ddc-Gal4 III) was used to drive α-syn expression (Figure 6B). Flies containing Ddc-Gal4 III combined with either UAS-α-syn, UAS-lacZ, or no UAS transgene (w1118 control) had median survival times of 70, 82, and 78 days, respectively. Table 5 contains the median survival time values for both categories of Ddc-Gal4 flies. Positioning appears to have a more significant effect on transgene expression than initially believed as α-syn-induced mortality differs greatly between Ddc-Gal4 II- and Ddc-Gal4 III-containing flies.
Figure 6 - α-synuclein reduces lifespan in D. melanogaster when expressed in the dopaminergic neurons. A Flies have a severely reduced lifespan when α-synuclein expression in the DA neurons is driven by Ddc-Gal4 II (p < 0.05). n = 227, 218, and 229 for αSYN/Ddc II, LACZ/Ddc II and w^{1118}; Ddc II, respectively. B α-synuclein expression in the DA neurons driven by Ddc-Gal4 III produces a less severe, yet significant, reduction in D. melanogaster lifespan (p < 0.05). n = 229, 254, and 240 for αSYN; Ddc III, LACZ; Ddc III, and w^{1118};+; Ddc III, respectively. Genotypes are w^{1118}; UAS-α-synuclein/Ddc-Gal4 (αSYN/Ddc II), w^{1118}; UAS-lacZ/Ddc-Gal4 (LACZ/Ddc II), w^{1118}; +/Ddc-Gal4 (w^{1118}; Ddc II), w^{1118}; UAS-α-synuclein/+/ Ddc-Gal4/+ (αSYN; Ddc III), w^{1118}; UAS-lacZ/+/ Ddc-Gal4/+ (LACZ; Ddc III), and w^{1118};+; Ddc-Gal4/+ (w^{1118}; Ddc III). Error bars represent standard error of the mean. p-values were calculated by the log-rank (Mantel-Cox) test and multiple comparisons were corrected for using the Bonferroni method.
Table 5 - Median survival values for *D. melanogaster* with *a*-synuclein expression directed to their dopaminergic neurons

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(w^{1118}), +/Ddc-Gal4</td>
<td>74</td>
</tr>
<tr>
<td>(w^{1118}), UAS-lacZ/Ddc-Gal4</td>
<td>82</td>
</tr>
<tr>
<td>(w^{1118}), UAS-a-syn/Ddc-Gal4</td>
<td>52*</td>
</tr>
<tr>
<td>(w^{1118}), +; Ddc-Gal4/+</td>
<td>78</td>
</tr>
<tr>
<td>(w^{1118}), UAS-lacZ/+; Ddc-Gal4/+</td>
<td>82</td>
</tr>
<tr>
<td>(w^{1118}), UAS-a-syn/+; Ddc-Gal4/+</td>
<td>70*</td>
</tr>
</tbody>
</table>

* indicates a value that was deemed significantly different (\(p < 0.05\)) from both the lacZ and \(w^{1118}\) control
Pre-eclosion exposure to a blueberry extract-supplemented diet partially rescues severe 
α-synuclein-induced decreases in D. melanogaster lifespan

The relationship between oxidative stress and several neurodegenerative disorders has invoked interest in the potential therapeutic benefits of dietary antioxidants. Studies of both individual polyphenolic compounds and plant extracts have shown promise in several PD models (Long et al., 2009; Caruana et al., 2011; Kim et al., 2011) and blueberries are known to be a rich dietary source of polyphenols (Borges et al., 2010). Figure 7A shows that α-syn-expressing flies with a severely reduced lifespan (αSYN/Dde II) exposed to food medium supplemented with BBE survived longer than those fed control medium. A diet containing 5 mg/ml BBE significantly extended the median survival time of αSYN/Dde II flies from 52 (control) to 60 days. Similar results were not observed when D. melanogaster food medium was supplemented with 1 mg/ml BBE.

BBE supplementation did not improve α-syn-induced early mortality in flies with a moderate decrease in lifespan (αSYN; Dde III). Neither 1 mg/ml nor 5 mg/ml of BBE extended median survival time in αSYN; Dde III flies (Figure 7B). Median survival time values for all α-syn-expressing flies can be found in Table 6. Extrinsic antioxidants provided via BBE supplementation appear to only benefit individuals with severe α-syn-induced increases in mortality.
Figure 7 - Pre-eclosion blueberry extract supplementation benefits α-synuclein-expressing D. melanogaster with severely reduced lifespans. A Flies expressing α-synuclein in their DA neurons via Ddc-Gal4 II that were reared on a diet containing 5 mg/ml BBE survived significantly longer than those given a standard diet (p < 0.05). A similar effect was not found with flies reared on medium containing 1 mg/ml BBE. n = 227, 267, and 283 for control, 1 mg/ml BBE, and 5 mg/ml BBE medium, respectively. B BBE supplementation had no effect on lifespan in D. melanogaster expressing α-synuclein via Ddc-Gal4 III. n = 229, 238, and 265 for control, 1 mg/ml BBE, and 5 mg/ml BBE medium, respectively. X denotes mg/ml. Genotypes are w^{1118}; UAS-α-synuclein/Ddc-Gal4 (αSYN/Ddc II) and w^{1118}; UAS-α-synuclein/++; Ddc-Gal4/+ (αSYN; Ddc III). Error bars represent standard error of the mean. p-values were calculated by the log-rank (Mantel-Cox) test and multiple comparisons were corrected for using the Bonferroni method.
Table 6 - Median survival values for α-synuclein-expressing *D. melanogaster* fed either a standard or blueberry extract-supplemented medium pre-eclosion

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS-α-syn/Ddc-Gal4</em> (control)</td>
<td>52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS-α-syn/Ddc-Gal4</em> (1 mg/ml BBE)</td>
<td>54&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS-α-syn/Ddc-Gal4</em> (5 mg/ml BBE)</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS-α-syn/+; Ddc-Gal4/+</em> (control)</td>
<td>70&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS-α-syn/+; Ddc-Gal4/+</em> (1 mg/ml BBE)</td>
<td>66&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS-α-syn/+; Ddc-Gal4/+</em> (5 mg/ml BBE)</td>
<td>66&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripted letters indicate a significant difference (p < 0.05) between values for a particular genotype; comparisons were not made between different genotypes.
Pre-eclosion blueberry extract supplementation decreases lifespan in D. melanogaster with enhanced lacZ expression in their dopaminergic neurons

The lacZ gene encodes the enzyme β-galactosidase and is a portion of the lac operon found in *Escherichia coli*. The β-galactosidase protein hydrolyses lactose to galactose and glucose and is perceived as a harmless reporter gene when inserted into other organisms. In this experiment, *D. melanogaster* with enhanced lacZ expression in their DA neurons were used as a control line to compare against results from flies expressing α-syn in the same region. Unexpectedly, lacZ-expressing flies fed BBE-supplemented medium prior to eclosion had significantly reduced lifespans compared to those fed a control diet (Figure 8). *D. melanogaster* containing *Ddc-Gal4 II* (LACZ/Ddc II) and *Ddc-Gal4 III* (LACZ; Ddc III) both experienced a similar reduction in survival. Median survival time for LACZ/Ddc II flies dropped from 82 days for those fed control medium to 72 and 70 days when fed 1 mg/ml and 5 mg/ml BBE-supplemented medium, respectively (Figure 8A). The median lifespan for LACZ; Ddc III flies was also 82 days, however the median survival times produced by 1 mg/ml and 5 mg/ml BBE supplementation for this genotype were 74 and 76 days, respectively (Figure 8B).

The effect of BBE supplementation on lifespan was further tested with *D. melanogaster* lacking a UAS-controlled transgene. Flies administered either concentration of BBE supplementation produced survival curves similar to those of the same genotype fed a control medium (Figure 8C & D). The median survival times for transgene-less *D. melanogaster* with *Ddc-Gal4 II* (w1118; Ddc II) fed either control, 1 mg/ml BBE, or 5 mg/ml BBE were 74, 76, and 76 days; whereas those for *Ddc-Gal4 III*
(w$^{1118}$; Ddc III) were 78, 76, and 76 days, respectively. The median survival values for
lacZ and w$^{1118}$ flies are found in Table 7. The detrimental effects of BBE
supplementation on lifespan are confined to flies with enhanced expression of lacZ in
their DA neurons. Exposure to a dietary source rich in polyphenol antioxidants early in
development combined with excess β-galactosidase in the DA neurons may harm
Drosophila.
Figure 8 - Pre-eclosion blueberry extract supplementation shortens lifespan in *D. melanogaster* expressing lacZ in their dopaminergic neurons. **A, B** Both lines of lacZ-expressing flies had shorter median survival times when reared on a diet containing BBE as compared to those reared on a standard diet (*p* < 0.05). n = 218, 228, and 225 for LACZ/Ddc II flies and 254, 272, and 255 for LACZ; Ddc III flies fed control, 1 mg/ml BBE, or 5 mg/ml BBE medium, respectively. **C, D** BBE supplementation did not affect the longevity of *w*^1118^ control flies. n = 229, 282, and 298 for *w*^1118^; Ddc II flies and 240, 260, and 249 for *w*^1118^; Ddc III flies fed control, 1 mg/ml BBE, or 5 mg/ml BBE medium, respectively. X denotes mg/ml. Genotypes are *w*^1118^; UAS-lacZ/Ddc-Gal4 (LACZ/Ddc II), *w*^1118^; UAS-lacZ/+; Ddc-Gal4/+ (LACZ; Ddc III), *w*^1118^;+/Ddc-Gal4 (*w*^1118^; Ddc II), and *w*^1118^; +; Ddc-Gal4/+ (*w*^1118^; Ddc III). Error bars represent standard error of the mean. *p*-values were calculated by the log-rank (Mantel-Cox) test and multiple comparisons were corrected for using the Bonferroni method.
Table 7 - Median survival values of lacZ-expressing and responsive transgene-lacking control lines fed either standard or blueberry extract-supplemented food medium pre-eclosion

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w^{1118} ); UAS-lacZ/Ddc-Gal4 (control)</td>
<td>82(^a)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ/Ddc-Gal4 (1 mg/ml BBE)</td>
<td>72(^b)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ/Ddc-Gal4 (5 mg/ml BBE)</td>
<td>70(^b)</td>
</tr>
<tr>
<td>( w^{1118} ); +; Ddc-Gal4 (control)</td>
<td>74(^c)</td>
</tr>
<tr>
<td>( w^{1118} ); +; Ddc-Gal4 (1 mg/ml BBE)</td>
<td>76(^c)</td>
</tr>
<tr>
<td>( w^{1118} ); +; Ddc-Gal4 (5 mg/ml BBE)</td>
<td>76(^c)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ/+; Ddc-Gal4/+ (control)</td>
<td>82(^d)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ/+; Ddc-Gal4/+ (1 mg/ml BBE)</td>
<td>74(^e)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ/+; Ddc-Gal4/+ (5 mg/ml BBE)</td>
<td>76(^e)</td>
</tr>
<tr>
<td>( w^{1118} ); +; Ddc-Gal4/+ (control)</td>
<td>78(^f)</td>
</tr>
<tr>
<td>( w^{1118} ); +; Ddc-Gal4/+ (1 mg/ml BBE)</td>
<td>76(^g)</td>
</tr>
<tr>
<td>( w^{1118} ); +; Ddc-Gal4/+ (5 mg/ml BBE)</td>
<td>76(^g)</td>
</tr>
</tbody>
</table>

Different superscripted letters indicate a significant difference (\( p < 0.05 \)) between values for a particular genotype; comparisons were not made between different genotypes.
Post-eclosion blueberry extract supplementation does not ameliorate α-synuclein-induced early mortality in D. melanogaster

As PD is a progressive disorder that typically manifests in older individuals, transferring D. melanogaster to BBE-supplemented food after they had eclosed as adults was used to mimic a treatment plan that could be implemented later in life. Flies were bred on control food and were first exposed to a BBE-supplemented diet within 24 hours of reaching adulthood. BBE supplementation later in D. melanogaster development did not improve the shortened lifespan caused by excess α-Syn in DA neurons. The median survival time of αSYN/Ddc II flies was 46 days for each food medium (Figure 9A), whereas αSYN; Ddc III flies fed control, 1 mg/ml BBE, or 5 mg/ml BBE medium had median survival times of 54, 54, and 52 days, respectively (Figure 9B). Contrary to the results discovered for pre-eclosion supplementation, BBE does not seem to counteract α-syn-induced declines in lifespan when administered following eclosion.

Post-eclosion BBE supplementation in lacZ-expressing flies produced the opposite results to those found for pre-eclosion supplementation. Flies expressing lacZ first exposed to BBE supplementation in adulthood survived slightly longer than those fed a control medium. Only 5 mg/ml BBE significantly prolonged the median survival time of LACZ/Ddc II flies (Figure 10A). Those fed a high concentration of BBE had a survival time of 60 days compared to 58 for individuals fed control medium. In the case of LACZ; Ddc III flies, both concentrations of BBE-supplemented medium prolonged lifespan (Figure 10B). The median survival time increased from 52 days for flies fed control medium to 56 days for individuals that received either 1 mg/ml or 5 mg/ml BBE-
supplemented medium. Table 8 contains median survival values generated from the post-eclosion BBE supplementation experiment. Contrary to pre-eclosion supplementation, delayed exposure to a BBE-supplemented diet improved the longevity of lacZ-expressing D. melanogaster. This data supports the aforementioned hypothesis that the combination of excess β-galactosidase with a dietary source rich in polyphenolic antioxidants is only detrimental to individuals early in development.

Please note that the median survival times obtained during this particular run of the experiment are atypical and may reflect a response to an unknown environmental condition (e.g. temperature change) that is discussed in Appendix 2.
Figure 9 - Post-eclosion blueberry extract supplementation does not affect lifespan in α-synuclein-expressing D. melanogaster. A Longevity curves of flies expressing α-synuclein in their DA neurons via Ddc-Gal4 II fed control (n = 208), 1 mg/ml BBE (n = 221), or 5 mg/ml BBE (n = 197) medium. B Longevity curves of flies expressing α-synuclein in their DA neurons via Ddc-Gal4 III fed control (n = 233), 1 mg/ml BBE (n = 226), or 5 mg/ml BBE (n = 242) medium. X denotes mg/ml. Genotypes are w^{1118}; UAS-α-synuclein/Ddc-Gal4 (αSYN/Ddc II) and w^{1118}; UAS-α-synuclein/+; Ddc-Gal4/+ (αSYN; Ddc III). Error bars represent standard error of the mean. p-values were calculated by the log-rank (Mantel-Cox) test and multiple comparisons were corrected for using the Bonferroni method.
Figure 10 - Blueberry extract fed post-eclosion slightly extends lifespan in lacZ-expressing D. melanogaster. A Flies expressing lacZ fed 5 mg/ml BBE (n = 129) survived significantly longer ($p < 0.05$) than those fed either a control diet (n = 145) or one supplemented with 1 mg/ml BBE (n = 136) when expression was driven by Ddc-Gal4 II. B Diets supplemented with both 1 mg/ml (n = 297) and 5 mg/ml (n = 331) BBE significantly extended lifespan ($p < 0.05$) in flies with lacZ expression in their DA neurons driven by Ddc-Gal4 III as compared to a control diet (n = 274). X denotes mg/ml. Genotypes are $w^{1118}; UAS-lacZ/Ddc-Gal4$ (LACZ/Ddc II) and $w^{1118}; UAS-lacZ/++; Ddc-Gal4/+ (LACZ; Ddc III)$. Error bars represent standard error of the mean. P-values were calculated by the log-rank (Mantel-Cox) test and multiple comparisons were corrected for using the Bonferroni method.
Table 8 - Median survival values for α-synuclein- and lacZ-expressing D. melanogaster fed either a standard or blueberry extract-supplemented diet post-eclosion

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$w_{118}^{118}; \text{UAS-α-syn/Ddc-Gal4 (control)}$</td>
<td>46$^a$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-α-syn/Ddc-Gal4 (1 mg/ml BBE)}$</td>
<td>46$^a$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-α-syn/Ddc-Gal4 (5 mg/ml BBE)}$</td>
<td>46$^a$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-α-syn/++; Ddc-Gal4/+ (control)}$</td>
<td>54$^b$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-α-syn/++; Ddc-Gal4/+ (1 mg/ml BBE)}$</td>
<td>54$^b$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-α-syn/++; Ddc-Gal4/+ (5 mg/ml BBE)}$</td>
<td>52$^b$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-lacZ/Ddc-Gal4 (control)}$</td>
<td>58$^c$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-lacZ/Ddc-Gal4 (1 mg/ml BBE)}$</td>
<td>60$^{cd}$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-lacZ/Ddc-Gal4 (5 mg/ml BBE)}$</td>
<td>60$^d$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-lacZ/+; Ddc-Gal4/+ (control)}$</td>
<td>52$^e$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-lacZ/+; Ddc-Gal4/+ (1 mg/ml BBE)}$</td>
<td>56$^f$</td>
</tr>
<tr>
<td>$w_{118}^{118}; \text{UAS-lacZ/+; Ddc-Gal4/+ (5 mg/ml BBE)}$</td>
<td>56$^f$</td>
</tr>
</tbody>
</table>

Different superscripted letters indicate a significant difference ($p < 0.05$) between values for a particular genotype; comparisons were not made between different genotypes.
*D. melanogaster* with enhanced *α*-synuclein expression in their dopaminergic neurons do not prematurely lose climbing ability

One of the hallmark phenotypes of the Drosophila *α*-syn PD model is the premature loss of climbing ability in older individuals. Though several labs have reproduced this result, their protocols and control lines often differ (Haywood and Staveley, 2004; Todd and Staveley, 2008; Butler et al., 2012; Hillman et al., 2012). Two types of control lines were used in this experiment: one with enhanced expression of a non-detrimental gene (*lacZ*) in the DA neurons regulated by *UAS*, and one lacking a *UAS*-controlled transgene (no upregulated expression). In this experiment, the progressive loss of climbing ability in the *α*-syn PD model was similar to the two control lines for both *αSYN/Ddc II* and *αSYN; Ddc III* flies (Figure 11). The 95% CI of the slopes overlapped in each of the three genotypes indicating that any observed differences are likely due to chance. All statistical values associated with the climbing curves in Figure 11 are found in Table 9. Despite repeated efforts, I was not able to duplicate the premature loss in climbing ability observed in the Drosophila *α*-syn PD model.
Figure 11 - Targeted \textit{a-synuclein} expression in the dopaminergic neurons of \textit{D. melanogaster} does not affect locomotion. A Climbing curves for flies containing \textit{Ddc-Gal4 II}. B The climbing ability of flies expressing \textit{a-synuclein} was similar to both the \textit{lacZ} and \textit{w^{1118}} controls containing \textit{Ddc-Gal4 III}. Genotypes are \textit{w^{1118} UAS-a-synuclein/Ddc-Gal4} (\textit{aSYN/Ddc II}), \textit{w^{1118} UAS-lacZ/Ddc-Gal4} (\textit{LACZ/Ddc II}), \textit{w^{1118} +/Ddc-Gal4} (\textit{w^{1118}; Ddc II}), \textit{w^{1118} UAS-a-synuclein/+\ Ddc-Gal4/+} (\textit{aSYN; Ddc III}), \textit{w^{1118} UAS-lacZ/+\ Ddc-Gal4/+} (\textit{LACZ; Ddc III}), and \textit{w^{1118} +\ Ddc-Gal4/+} (\textit{w^{1118}; Ddc III}). Error bars represent standard error of the mean. Climbing ability was determined via nonlinear curve fit (CI = 95\%).
Table 9 - Locomotion assay statistics used to compare the climbing ability of α-synuclein-expressing *D. melanogaster* to a *lacZ* and responsive transgene-lacking control

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Slope (k)</th>
<th>Standard error (SE)</th>
<th>95% confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w^{1118}; +/Ddc-Gal4 )</td>
<td>0.03376</td>
<td>0.003540</td>
<td>0.02664 - 0.04089</td>
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<tr>
<td>( w^{1118}; UAS-lacZ/Ddc-Gal4 )</td>
<td>0.04015</td>
<td>0.003221</td>
<td>0.03376 - 0.04653</td>
</tr>
<tr>
<td>( w^{1118}; UAS-a-syn/Ddc-Gal4 )</td>
<td>0.03365</td>
<td>0.003373</td>
<td>0.02704 - 0.04026</td>
</tr>
<tr>
<td>( w^{1118}; +; Ddc-Gal4/+ )</td>
<td>0.03931</td>
<td>0.002493</td>
<td>0.03433 - 0.04429</td>
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<tr>
<td>( w^{1118}; UAS-lacZ/+; Ddc-Gal4/+ )</td>
<td>0.03797</td>
<td>0.002566</td>
<td>0.03289 - 0.04305</td>
</tr>
<tr>
<td>( w^{1118}; UAS-a-syn/+; Ddc-Gal4/+ )</td>
<td>0.03928</td>
<td>0.002859</td>
<td>0.03362 - 0.04495</td>
</tr>
</tbody>
</table>

* indicates a value that was deemed significantly different (p < 0.05) from both the *lacZ* and \( w^{1118} \) control
Blueberry extract supplementation does not improve locomotion in D. melanogaster with upregulated α-synuclein expression in their dopaminergic neurons

Treatment with both intrinsic antioxidant enzymes and antioxidants obtained via diet supplementation has improved the premature loss of climbing ability characteristic of the Drosophila α-syn model of PD (Wasef et al., 2007; Botella et al., 2008; Long et al., 2009). Figure 12 and Table 10 contain the climbing curves and their associated statistics discovered for α-syn-expressing flies fed either control medium or a diet supplemented with one of two concentrations of BBE. Neither pre-eclosion (Figure 12A & B) nor post-eclosion (Figure 12C & D) BBE supplementation improved locomotion in either αSYN/Ddc II or αSYN; Ddc III flies. Unlike the results found for S-methyl-L-cysteine and grape extract supplementation, BBE supplementation up to a concentration of 5 mg/ml did not alter climbing ability in the Drosophila α-syn model of PD.
Figure 12 - Blueberry extract supplementation does not affect locomotion in α-synuclein-expressing D. melanogaster. **A, B** Climbing curves for α-synuclein-expressing flies fed BBE-supplemented medium pre-eclosion. **C, D** Climbing curves for α-synuclein-expressing flies fed BBE-supplemented medium post-eclosion. X denotes mg/ml. Genotypes are w^{1118}; UAS-α-synuclein/Ddc-Gal4 (αSYN/Ddc II) and w^{1118}; UAS-α-synuclein/++; Ddc-Gal4/+ (αSYN; Ddc III). Error bars represent standard error of the mean. Climbing ability was determined via nonlinear curve fit (CI = 95%).
Table 10 - Locomotion assay statistics generated from the non-linear curve fit model for a-synuclein-expressing D. melanogaster fed either control or blueberry extract-supplemented medium

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th>Slope (k)</th>
<th>Standard error (SE)</th>
<th>95% confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-eclosion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(w^{1118}; \text{UAS-a-syn/Ddc-Gal4} ) (control)</td>
<td>0.03365</td>
<td>0.003373</td>
<td>0.02704 - 0.04026&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>(w^{1118}; \text{UAS-a-syn/Ddc-Gal4} ) (1 mg/ml BBE)</td>
<td>0.04359</td>
<td>0.005264</td>
<td>0.03299 - 0.05418&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>(w^{1118}; \text{UAS-a-syn/Ddc-Gal4} ) (5 mg/ml BBE)</td>
<td>0.03902</td>
<td>0.004376</td>
<td>0.03019 - 0.04785&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>(w^{1118}; \text{UAS-a-syn/+; Ddc-Gal4/+} ) (control)</td>
<td>0.03928</td>
<td>0.002859</td>
<td>0.03362 - 0.04495&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(w^{1118}; \text{UAS-a-syn/+; Ddc-Gal4/+} ) (1 mg/ml BBE)</td>
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<td>0.002955</td>
<td>0.03111 - 0.04294&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.04209</td>
<td>0.003584</td>
<td>0.03492 - 0.04927&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Post-eclosion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(w^{1118}; \text{UAS-a-syn/Ddc-Gal4} ) (control)</td>
<td>0.03365</td>
<td>0.003373</td>
<td>0.02704 - 0.04026&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(w^{1118}; \text{UAS-a-syn/Ddc-Gal4} ) (1 mg/ml BBE)</td>
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<td>0.003183</td>
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<tr>
<td>(w^{1118}; \text{UAS-a-syn/Ddc-Gal4} ) (5 mg/ml BBE)</td>
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<td>0.02238 - 0.04160&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(w^{1118}; \text{UAS-a-syn/+; Ddc-Gal4/+} ) (control)</td>
<td>0.03928</td>
<td>0.002859</td>
<td>0.03362 - 0.04495&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>(w^{1118}; \text{UAS-a-syn/+; Ddc-Gal4/+} ) (1 mg/ml BBE)</td>
<td>0.05054</td>
<td>0.005597</td>
<td>0.03932 - 0.06176&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(w^{1118}; \text{UAS-a-syn/+; Ddc-Gal4/+} ) (5 mg/ml BBE)</td>
<td>0.05742</td>
<td>0.006762</td>
<td>0.04382 - 0.07102&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripted letters indicate a significant difference (p < 0.05) between values for a particular genotype; comparisons were not made between different genotypes.
Post-eclosion blueberry extract supplementation improves locomotion in D. melanogaster with upregulated lacZ expression in their dopaminergic neurons

I have previously described how a diet supplemented with BBE fed post-eclosion can extend lifespan in D. melanogaster with enhanced expression of lacZ in their DA neurons (Figure 10 and Table 8). LACZ; Ddc III flies first exposed to a BBE-supplemented diet in adulthood also had significantly improved climbing ability compared to those fed a control diet (Figure 13 and Table 11). A similar result was not found for LACZ/Ddc II flies fed post-eclosion or either lacZ genotype fed pre-eclosion. The locomotion of flies fed control medium was also similar to those fed BBE-supplemented medium for both w^{118} control genotypes, though flies fed 5 mg/ml BBE climbed significantly better than those given 1 mg/ml BBE (Figure 14 and Table 12). Post-eclosion exposure to a BBE-supplemented medium appears to improve both survival and locomotion in D. melanogaster with upregulated lacZ expression in their DA neurons. A beneficial interaction likely exists between increased neuronal amounts of β-galactosidase and dietary antioxidants ingested during adulthood as evidenced by the prolonged lifespan and improved climbing ability during old age in LACZ; Ddc III flies.
Figure 13 - Post-eclosion blueberry extract supplementation improves mobility in lacZ-expressing *D. melanogaster*. A, B Climbing curves for lacZ-expressing flies fed BBE-supplemented medium pre-eclosion. C, D Climbing curves for lacZ-expressing flies fed BBE-supplemented medium post-eclosion. D BBE supplementation post-eclosion significantly improved mobility in flies with lacZ expression directed to the DA neurons via *Ddc-Gal4 III* (*p < 0.05*). No significant effect was found with any of the other categories of flies (A, B, C). Genotypes are *w*^{1118}; *UAS-lacZ/Ddc-Gal4* (LACZ/Ddc II) and *w*^{1118}; *UAS-lacZ*+/*; *Ddc-Gal4*+/+ (LACZ; Ddc III). Error bars represent standard error of the mean. Climbing ability was determined via nonlinear curve fit (*CI = 95%*).
Table 11 - Locomotion assay statistics generated from the non-linear curve fit model for \( \text{lacZ}\)-expressing \( D. \text{melanogaster} \) fed either control or blueberry extract-supplemented medium

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th>Slope (k)</th>
<th>Standard error (SE)</th>
<th>95% confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-eclosion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}/\text{Ddc-Gal4} ) (control)</td>
<td>0.04015</td>
<td>0.003221</td>
<td>0.03376 - 0.04653\textsuperscript{a}</td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}/\text{Ddc-Gal4} ) (1 mg/ml BBE)</td>
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<td>( w^{118} ; \text{UAS-lacZ}/\text{Ddc-Gal4} ) (5 mg/ml BBE)</td>
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<tr>
<td>( w^{118} ; \text{UAS-lacZ}+/\text{Ddc-Gal4/+} ) (control)</td>
<td>0.03797</td>
<td>0.002566</td>
<td>0.03289 - 0.04305\textsuperscript{b}</td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}+/\text{Ddc-Gal4/+} ) (1 mg/ml BBE)</td>
<td>0.03570</td>
<td>0.002854</td>
<td>0.02999 - 0.04141\textsuperscript{b}</td>
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<tr>
<td>( w^{118} ; \text{UAS-lacZ}+/\text{Ddc-Gal4/+} ) (5 mg/ml BBE)</td>
<td>0.04309</td>
<td>0.003401</td>
<td>0.03625 - 0.04993\textsuperscript{b}</td>
</tr>
<tr>
<td><strong>Post-eclosion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}/\text{Ddc-Gal4} ) (control)</td>
<td>0.04015</td>
<td>0.003221</td>
<td>0.03376 - 0.04653\textsuperscript{c}</td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}/\text{Ddc-Gal4} ) (1 mg/ml BBE)</td>
<td>0.05240</td>
<td>0.005646</td>
<td>0.04107 - 0.06372\textsuperscript{c}</td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}/\text{Ddc-Gal4} ) (5 mg/ml BBE)</td>
<td>0.05404</td>
<td>0.006958</td>
<td>0.04004 - 0.06804\textsuperscript{c}</td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}+/\text{Ddc-Gal4/+} ) (control)</td>
<td>0.03797</td>
<td>0.002566</td>
<td>0.03289 - 0.04305\textsuperscript{d}</td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}+/\text{Ddc-Gal4/+} ) (1 mg/ml BBE)</td>
<td>0.05459</td>
<td>0.003611</td>
<td>0.04736 - 0.06183\textsuperscript{e}</td>
</tr>
<tr>
<td>( w^{118} ; \text{UAS-lacZ}+/\text{Ddc-Gal4/+} ) (5 mg/ml BBE)</td>
<td>0.06078</td>
<td>0.007640</td>
<td>0.04541 - 0.07615\textsuperscript{e}</td>
</tr>
</tbody>
</table>

Different superscripted letters indicate a significant difference \((p < 0.05)\) between values for a particular genotype; comparisons were not made between different genotypes.
Figure 14 - Blueberry extract supplementation does not affect locomotion in *D. melanogaster* that lack a responsive transgene. **A, B** Climbing curves for flies fed a BBE-supplemented diet pre-eclosion. X denotes mg/ml. Genotypes are *w*¹¹¹⁸; *+/Ddc-Gal4* (*w*¹¹¹⁸; Ddc II) and *w*¹¹¹⁸; *+/Ddc-Gal4/+* (*w*¹¹¹⁸; Ddc III). Error bars represent standard error of the mean. Climbing ability was determined via nonlinear curve fit (CI = 95%).
Table 12 - Locomotion assay statistics generated from the non-linear curve fit model for *D. melanogaster* that lack a responsive transgene fed either control or blueberry extract-supplemented medium pre-eclosion.

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th>Slope (k)</th>
<th>Standard error (SE)</th>
<th>95% confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>w^{1118}; +/Ddc-Gal4</td>
<td>0.03376</td>
<td>0.00354</td>
<td>0.02664 - 0.04089&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w^{1118}; +/Ddc-Gal4</td>
<td>0.03009</td>
<td>0.002380</td>
<td>0.02533 - 0.03485&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(1 mg/ml BBE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w^{1118}; +/Ddc-Gal4</td>
<td>0.04210</td>
<td>0.003804</td>
<td>0.03444 - 0.04975&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(5 mg/ml BBE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w^{1118}; +; Ddc-Gal4/+</td>
<td>0.03931</td>
<td>0.002493</td>
<td>0.03433 - 0.04429&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w^{1118}; +; Ddc-Gal4/+</td>
<td>0.03197</td>
<td>0.002442</td>
<td>0.02709 - 0.03685&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(1 mg/ml BBE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w^{1118}; +; Ddc-Gal4/+</td>
<td>0.04956</td>
<td>0.003346</td>
<td>0.04286 - 0.05625&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(5 mg/ml BBE)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different superscripted letters indicate a significant difference (p < 0.05) between values for a particular genotype; comparisons were not made between different genotypes.
A blueberry extract-supplemented diet does not affect subtle α-synuclein-induced phenotypes in D. melanogaster eyes raised at 25 °C

The adult Drosophila eye comprises a repeating array of roughly 750 to 800 multicellular subunits known as ommatidia. Each ommatidium is a cluster of 20 cells and contains 8 photoreceptor neurons, pigment cells, and lens secreting cone cells. Mechanosensory bristles are located at alternating vertices of the ommatidia and are composed of 4 cells, including a sensory neuron (Kumar, 2012). Thus, the developing eye is another neuron-rich tissue in which α-syn-induced cell death can be evaluated.

The glass multiple reporter (GMR)-Gal4 construct (Freeman, 1996) causes high-level expression in Drosophila eye imaginal discs. Flies heterozygous for GMR-Gal4 have no observable phenotype at 25 °C (Kramer and Staveley, 2003). Though external eye morphology appears normal, α-syn expression at 25 °C via GMR-Gal4 causes retinal degeneration in flies (Feany and Bender, 2000; Haywood and Staveley, 2004). Figure 15 displays SEM images of D. melanogaster eyes with increased expression of either lacZ or α-syn at 25 °C. Similar to previous results, the external morphology of the eye appears normal for both genotypes. Biometric analyses, however, revealed subtle phenotypes present at 25 °C that are induced by α-syn (Figure 16). Both the number of ommatidia and bristles are reduced in α-syn-expressing flies (αSYN). A significant decrease in ommatidium number was found for each food medium; however, the decreased amount of bristles in αSYN flies fed control medium was not significant. Supplementation with either concentration of BBE was unable to improve these subtle phenotypes and the values for both counts are found in Table 13.
BBE extract supplementation also appears to affect cell growth in αSYN flies. Individuals exposed to a diet containing 5 mg/ml BBE had significantly larger ommatidia than those fed either control or 1 mg/ml BBE medium (Figure 16C). LACZ flies given 5 mg/ml BBE in their diet also had larger ommatidia than those fed 1 mg/ml BBE, however a difference was not observed between a control diet and both the aforementioned media. Despite the observed differences in ommatidial area, BBE extract supplementation does not appear to affect subtle α-syn-induced phenotypes in the Drosophila eye.
Figure 15 - Expression of $\alpha$-synuclein during eye development does not produce a visible phenotype at 25 °C. A - F Scanning electron micrographs of D. melanogaster eyes with elevated amounts of either $\alpha$-syn (D, E, F) or lacZ (A, B, C) transcript. Both genotypes were reared on control (A, D), 1 mg/ml BBE (B, E), or 5 mg/ml BBE (C, F) medium. X denotes mg/ml. Genotypes are $w^{1118}$; UAS-$\alpha$-synuclein/GMR-Gal4 ($\alpha$SYN) and $w^{1118}$; UAS-lacZ/GMR-Gal4 (LACZ).
Figure 16 - Blueberry extract supplementation has no effect on moderate α-synuclein-induced phenotypes in *D. melanogaster* eyes at 25 °C. **A** Enhanced α-synuclein expression significantly reduces the mean number of ommatidia in *D. melanogaster* eyes. **B** Increased levels of α-synuclein reduces the mean number of bristles present in *D. melanogaster* eyes. BBE-supplemented media did not rescue either of the aforementioned phenotypes. **C** α-synuclein-expressing flies fed medium supplemented with 5 mg/ml BBE had significantly larger ommatidia than those fed either a control diet or one supplemented with 1 mg/ml BBE (*p* < 0.05). Genotypes are *w*¹¹¹⁸: UAS-α-synuclein/GMR-Gal4 (αSYN) and *w*¹¹¹⁸: UAS-lacZ/GMR-Gal4 (LACZ). *n* = 15 for each analysis. Error bars represent standard error of the mean. *p*-values were calculated via one-way ANOVA followed by Tukey’s Multiple Comparison test.
Table 13 - Biometric analyses of the eyes of *D. melanogaster* expressing *a*-synuclein or *lacZ* raised at 25 °C

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th># normal ommatidia</th>
<th># bristles</th>
<th>Ommatidium area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>w</em>¹¹¹⁸; UAS-<em>a</em>-syn/GMR-Gal4 (control)</td>
<td>651.8</td>
<td>505.4</td>
<td>208.5</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; UAS-<em>a</em>-syn/GMR-Gal4 (1 mg/ml BBE)</td>
<td>644.0</td>
<td>493.8</td>
<td>212.7</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; UAS-<em>a</em>-syn/GMR-Gal4 (5 mg/ml BBE)</td>
<td>632.8</td>
<td>491.7</td>
<td>228.6</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; UAS-<em>lacZ</em>/GMR-Gal4 (control)</td>
<td>695.3</td>
<td>539.7</td>
<td>214.1</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; UAS-<em>lacZ</em>/GMR-Gal4 (1 mg/ml BBE)</td>
<td>704.4</td>
<td>543.4</td>
<td>207.2</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; UAS-<em>lacZ</em>/GMR-Gal4 (5 mg/ml BBE)</td>
<td>708.9</td>
<td>548.9</td>
<td>219.6</td>
</tr>
</tbody>
</table>
Blueberry extract supplementation improves severe α-synuclein-induced degeneration in D. melanogaster eyes raised at 29 °C

Degeneration caused by overexpressing α-syn early in eye development is pronounced at higher temperatures. Flies heterozygous for GMR-Gal4 have developmental defects and increased levels of apoptosis when raised at 29 °C (Kramer and Staveley, 2003). This rough eye phenotype worsens when GMR-Gal4 is used to drive expression of α-syn in the developing eye (Figure 17D) (Todd and Staveley, 2008). Scanning electron micrographs for both αSYN and LACZ flies fed control, 1 mg/ml BBE, or 5 mg/ml BBE are found in Figure 17. Targeted expression of α-syn at 29 °C increased the amount of atypical ommatidia in D. melanogaster eyes (Figure 18A). BBE supplementation completely rescued this phenotype as the results for αSYN flies fed either concentration of BBE were comparable to those found for LACZ individuals fed control medium (Table 14). Biometric analyses also revealed an α-syn-induced decrease in bristle number at 29 °C (Figure 18B). Both concentrations of BBE supplementation were sufficient to partially rescue the reduced bristle number, increasing the mean bristle number from 372.9 in αSYN flies fed control medium to 414.6 and 437.7 for those fed 1 mg/ml and 5 mg/ml BBE, respectively. BBE supplementation had no effect on bristle number in LACZ flies, as the mean values for individuals fed control, 1 mg/ml BBE, and 5 mg/ml BBE medium were 541.3, 543.6, and 538.1, respectively. The mean number of bristles for both genotypes are found in Table 14. These results further support that early exposure to a diet supplemented with BBE improves severe α-syn-induced phenotypes in a Drosophila model of PD.
Figure 17 - α-synuclein expression during eye development produces a rough external eye morphology at 29 °C. A - F Scanning electron micrographs of D. melanogaster eyes with elevated amounts of either α-synuclein (D, E, F) or lacZ (A, B, C) transcript. Both genotypes were reared on control (A, D), 1 mg/ml BBE (B, E), or 5 mg/ml BBE (C, F) medium. D Rough external eye phenotype produced by enhanced expression of α-synuclein in the developing D. melanogaster eye. Genotypes are w^{118}, UAS-α-synuclein/GMR-Gal4 (αSYN) and w^{118}; UAS-lacZ/GMR-Gal4 (LACZ).
Figure 18 - Severe degenerative phenotypes in D. melanogaster eyes caused by a-synuclein expression at 29 °C are suppressed by a blueberry extract-supplemented diet. A Both concentrations of BBE supplementation completely rescued the amount of atypical ommatidia caused by enhanced a-synuclein expression (p < 0.05, n = 10). B BBE supplementation partially rescues the reduced bristle number in a-synuclein-expressing eyes (p < 0.05, n = 15). Genotypes are w^{1118}; UAS-a-synuclein/GMR-Gal4 (αSYN) and w^{1118}; UAS-lacZ/GMR-Gal4 (LACZ). Error bars represent standard error of the mean. p-values were calculated via one-way ANOVA followed by Tukey's Multiple Comparison test.
Table 14 - Biometric analyses of the eyes of *D. melanogaster* expressing *α*-synuclein or *lacZ* raised at 29 °C

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th>Atypical ommatidia (% area)</th>
<th># bristles</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS</em>-α-syn/GMR-Gal4 (control)</td>
<td>73.5</td>
<td>372.9</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS</em>-α-syn/GMR-Gal4 (1 mg/ml BBE)</td>
<td>40.3</td>
<td>414.6</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS</em>-α-syn/GMR-Gal4 (5 mg/ml BBE)</td>
<td>29.2</td>
<td>437.7</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS</em>-lacZ/GMR-Gal4 (control)</td>
<td>28.9</td>
<td>541.3</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS</em>-lacZ/GMR-Gal4 (1 mg/ml BBE)</td>
<td>N/A</td>
<td>543.6</td>
</tr>
<tr>
<td><em>w</em>¹¹¹⁸; <em>UAS</em>-lacZ/GMR-Gal4 (5 mg/ml BBE)</td>
<td>N/A</td>
<td>538.1</td>
</tr>
</tbody>
</table>
DISCUSSION

The longevity-promoting effects of antioxidant enzymes vary between groups of neurons

Antioxidant enzymes may not provide the same protection in all neurons. I have reported that targeted expression of Cat in the DA neurons extends lifespan in D. melanogaster (Figure 5A). A similar effect, however, was not found for either Sod1 or Sod2 despite these enzymes participating in the same pathway as Cat. Studies have shown that both Sod1 and Sod2, but not Cat, extend Drosophila lifespan when their expression is directed to motor neurons (Parkes et al., 1998; Phillips et al., 2000). The importance of SOD and CAT activity appears to vary between different types of neurons. Both PD and motor neuron diseases (MNDs) share oxidative stress as a common feature of disease etiology. The first genetic linkage associated with amyotrophic lateral sclerosis (ALS), the most common adult-onset MND, was determined to be a mutation in Sod1, suggesting its activity is essential for the survival of motor neurons. Though a mutation in Sod has not been associated with PD, ROS are produced during DA neuron metabolism as the breakdown of dopamine by monoamine oxidase (MAO) produces H₂O₂ (Coyle and Puttfarcken, 1993). Excess CAT may be particularly beneficial to DA neurons since this enzyme converts reactive H₂O₂ into water and could help protect cells against H₂O₂-induced oxidative damage. Motor neurons and DA neurons vary in their function, neurotransmitter production, and metabolism. I can, therefore, speculate that
the discrepancies between my results and those previously reported for Sod and Cat are
due to the type of cell in which expression was targeted.

**Dietary antioxidants can prolong lifespan in Drosophila**

Several recent studies have suggested that Drosophila food medium supplemented
with a dietary source of antioxidants can prolong lifespan. Adult Drosophila fed extracts
of nectarine, green tea, black tea, and apple survive longer than those exposed to a
standard food medium (Li et al., 2007; Peng et al., 2009; Boyd et al., 2011; Peng et al.,
2011). I have demonstrated that post-eclosion BBE supplementation extends the lifespan
of LACZ flies (Figure 10). According to the Free Radical/Oxidative Stress Theory of
Ageing, senescence is a consequence of the accumulation of free radical/ROS-induced
damage to cellular macromolecules that occurs over an organism's lifespan (Harman,
1956). Ageing is inevitable and associated with a time-dependent decline in the
biochemical and physiological function of major systems (Doria et al., 2012). For
example, an age-dependent increase in ROS was associated with functional decline of the
mitochondria in rat brains (Benzi et al., 1992). Moderate amounts of dietary antioxidants
are likely most beneficial late in Drosophila lifespan when intrinsic antioxidant defenses
no longer provide sufficient protection against the age-dependent accumulation of ROS.

My results are the first to demonstrate the effects of antioxidant supplementation
at different time points during *D. melanogaster* development. The control used in this
study closest to WT *D. melanogaster* was the responsive transgene-lacking (*w^{1118}*.)
control. As shown in Figures 8 and 14, pre-eclosion BBE supplementation did not affect the longevity or climbing ability of these flies. Unfortunately, post-eclosion BBE supplementation experiments were not performed on this genotype; however, I can hypothesize that BBE extract would prolong lifespan based on my post-eclosion BBE supplementation results with LACZ flies. Additionally, Peng et al. have already documented that post-eclosion BBE supplementation extends lifespan in WT Drosophila (Peng et al., 2012). Despite the detrimental effects observed in LACZ flies, neither pre-eclosion nor post-eclosion supplementation of BBE up to concentrations of 5 mg/ml appears to be harmful. My results support a role for BBE in promoting Drosophila longevity.

Excessive disruption to biological pathways early in development may shorten lifespan in Drosophila

I have found that flies with targeted expression of lacZ in their DA neurons respond negatively to BBE supplementation early in development. LACZ flies exposed to BBE prior to eclosion had significantly reduced lifespans compared to individuals fed a control diet (Figure 8A & B). ROS present at appropriate amounts are regulators of several important cellular processes and it may be that the excess antioxidants provided by BBE are disrupting these pathways during development. For example, nitric oxide (NO) directly regulates gene expression and promotes ecdysteroidogenesis and metamorphosis during Drosophila development (Yamanaka and O'Connor, 2011).
Excess antioxidants available during development could decrease the amount of ROS involved in signaling pathways below what is considered physiologically normal. This is not likely the case, however, as pre-eclosion supplementation of BBE was not found to be detrimental to responsive transgene-lacking control flies (Figure 8C & D). Thus, it appears that the combined presence of excess β-galactosidase and BBE supplementation early in development is likely responsible for shortening lifespan in LACZ flies.

As mentioned, lacZ is often used in Drosophila studies and its gene product, β-galactosidase, is not associated with detrimental effects in flies. Another possible scenario for LACZ/BBE supplementation toxicity involves the interaction between protein accumulation and ROS. Despite β-galactosidase's benign nature in flies and its lack of substrate, I introduced a large quantity of exogenous and unnecessary protein into a sensitive D. melanogaster tissue. The ubiquitin proteasome system (UPS) is involved in maintaining cellular homeostasis via protein degradation. Degradation by the proteasome is signaled when at least four ubiquitin monomers are attached to an unwanted or unnecessary protein (Thrower et al., 2000). The UPS is responsible for degrading up to 60% of unwanted or unnecessary proteins and is expected to be important to neuronal functioning and synaptic plasticity (Dennissen et al., 2012). Thus, it is possible that the excess β-galactosidase present in DA neurons increases the activity of the UPS. Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) belongs to the family of deubiquitinating enzymes and is involved in regulating protein turnover via the UPS. The primary function of UCH-L1 is to remove ubiquitin from protein substrates by hydrolysing carboxyl terminal esters and amides of ubiquitin (Fang et al., 2010). The methionine residues of this protein can be reversibly oxidized and this modification has
been suggested to be involved in regulating protein function (Hoshi and Heinemann, 2001). Excess antioxidant protection conferred by BBE supplementation may reduce ROS to a level below what is considered normal, thereby affecting the regulation of UCH-L1. Drosophila development prior to eclosion is a precisely coordinated and sensitive period involving many tightly regulated pathways. The combination of increased UPS activity alongside reduced regulation of the system may alter development in a manner that results in adults with decreased lifespans. This effect appears to be confined to occurring early in development since post-eclosion BBE supplementation did not harm LACZ flies (Figure 10). Future studies should aim at evaluating differences in the expression and activity of enzymes like UCH-L1 between LACZ flies fed either a control or a BBE-supplemented medium.

The α-synuclein-induced premature loss of climbing ability was not reproduced in this study

One of the characteristic features of the Drosophila α-syn model of PD is the premature loss of climbing ability. I was unable to reproduce this result and report that flies overexpressing α-syn in their DA neurons do not lose their climbing ability earlier than control flies. Several protocols exist for measuring locomotion (i.e. climbing ability) in Drosophila. A common method involves observing the movement of flies through a clear vial or tube for a set amount of time. To obtain my results I used a graded analysis in which different zones were assessed a score that ascended with height. Previous
findings from our laboratory using the same protocol and the same Drosophila $\alpha$-syn model of PD have reported a premature loss of climbing ability (Todd and Staveley, 2008). Feany and Bender used a non-graded analysis and recorded how many flies successfully passed a pre-determined height in a set period of time (Feany and Bender, 2000). Both manual and automated methods were used by Wassef et al. to measure locomotion in Drosophila (Wassef et al., 2007). Reactive locomotion was measured by vortexing tubes of flies at a low setting and measuring the number of flies still on the side of the tube. Spontaneous locomotion measurements were determined by computing how many times flies passed through an infrared laser over a set amount of time. Despite the varied protocols, both of the aforementioned groups reported a premature loss of climbing ability in Drosophila with increased neuronal expression of $\alpha$-syn. A consistent feature of these studies is the lack of a control line with directed expression of a non-detrimental protein in the same tissue. We used targeted expression of lacZ in D. melanogaster DA neurons to control for the effect of enhancing expression via the UAS/Gal4 system. To my surprise, the progressive loss of climbing ability was similar in $\alpha$SYN and LACZ flies and neither differed from a responsive transgene-less control similar to those used by other groups (Figure 11). Our graded method of measuring climbing ability is a sensitive and subjective assay that requires calculated and precise measurements of time and height over a long period of time. As such, it is possible that additional protocols may help to explain ambiguities between my results and those found by other groups.
A blueberry extract-supplemented diet only influences certain α-synuclein-induced phenotypes in D. melanogaster

The severity of α-syn-induced phenotypes depends on both intrinsic and extrinsic factors. I have reported that D. melanogaster with targeted expression of α-syn in their DA neurons have a reduced lifespan (Figure 6). Unexpectedly, a significant difference was observed between αSYN/Ddc II and αSYN; Dde III flies with the former having a markedly reduced median survival time. Although both of the aforementioned αSYN lines model PD, it appears that the position of Ddc-Gal4 in the D. melanogaster genome affects the severity of α-syn-induced phenotypes. Both lines of flies were maintained in the same conditions and subjected to the same analyses with the only difference between the two being the location of Ddc-Gal4. It is, therefore, possible that regulatory elements surrounding the chromosomal insertion site of Ddc-Gal4 affect its expression in D. melanogaster. Molecular analyses like qRT-PCR and Western blotting could be applied in future experiments to test for differential expression between αSYN/Ddc II and αSYN; Ddc III flies. Duplications and triplications of the α-syn gene locus result in a severe, early-onset version of PD in humans (Singleton et al., 2003; Chartier-Harlin et al., 2004; Farrer et al., 2004). A similar expression-dependent effect may result from increased α-syn transcript or protein levels in D. melanogaster DA neurons.

Temperature affects the amount of α-syn-induced degeneration in Drosophila eyes. Directed expression of α-syn in D. melanogaster eyes at 25 °C produces subtle phenotypes. Conversely, flies raised at 29 °C have severely disrupted external eye morphologies. In this case, the same genotype was analyzed and temperature alone
appears to be responsible for the dramatic difference. The UAS/Gal4 system used for targeted expression of α-syn in Drosophila eyes was isolated from yeast. The optimal temperature used to grow this microscopic eukaryote in most laboratories is 37 °C and this system functions better at temperatures approaching this point. Future experiments could not only analyze the amount of α-syn production at both 25 and 29 °C, but also the generation and binding capability of Gal4 at these temperatures.

A BBE-supplemented diet was only capable of suppressing the severe α-syn-induced phenotypes discussed above. Both the increased mortality and severe external eye disruption in αSYN flies were at least partially rescued by a BBE-supplemented diet. I have hypothesized that both genetic and environmental factors cause differential production of α-Syn in the lines that were tested. Recent studies have suggested that excess α-Syn increases oxidative stress by disrupting mitochondrial maintenance (Parihar et al., 2009; Byers et al., 2011). It is possible that secondary antioxidants obtained from food are only beneficial when a cell reaches a certain degree of oxidative stress. A BBE-supplemented diet was only beneficial when fed to αSYN flies pre-eclosion. Drosophila development is sensitive and precisely coordinated and the increased oxidative stress caused by excess neuronal α-Syn may disrupt this process. The increased antioxidant protection conferred by BBE appears to protect against severe developmental damage caused by α-Syn-induced oxidative stress.
Possible mechanisms for blueberry extract-induced protection in neurons

My findings could be the result of a strengthened overall antioxidant defense system in α-syn-expressing flies. BBE extract increases the expression of intrinsic antioxidant defense enzymes like Cat, Sod1, and Sod2 in Drosophila (Peng et al., 2012). Additionally, BBE extends lifespan and partially protects WT flies under conditions of increased oxidative stress; however, a similar effect was not found with either Cat or Sod knockout mutants. Similar results have been documented for several other foods high in dietary antioxidants, including extracts of apple polyphenols, green tea catechins and black tea (Li et al., 2007; Peng et al., 2009; Peng et al., 2011). Although dietary antioxidants likely provide an invaluable secondary support to cells undergoing oxidative stress it appears their protective effects are dependent on intrinsic enzymatic antioxidant defense systems.

Directed expression of α-syn during eye development results in premature degeneration of the retina and abnormal development of the external eye morphology (Haywood and Staveley, 2004; Todd and Staveley, 2008). My results suggest that a diet containing BBE protects neurons in the eye against severe α-syn-dependent defects. Both the amount of atypical ommatidia and total number of bristles were improved in flies fed a BBE-supplemented diet. Although the protective mechanism is not understood, a link exists between BBE supplementation and protein turnover via the UPS. Flies fed BBE have increased levels of Rpn11 messenger RNA (Peng et al., 2012), an essential lid component of the 26S proteasome structure. In humans, the parkin gene (PARK2) encodes a 465 aa protein that functions as an E3 ubiquitin ligase (Shimura et al., 2000).
Expression of parkin in Drosophila eyes suppresses $\alpha$-syn-induced retinal degeneration in older flies (Haywood and Staveley, 2004). Additionally, overexpression of endoplasmic reticulum-associated degradation pathway proteins suppress late onset retinal degradation in a Drosophila model of autosomal dominant retinitis pigmentosa, an age-related degenerative eye disease (Kang and Ryoo, 2009). The authors hypothesized that this restoration is due to increased proteasomal degradation of misfolded proteins in the endoplasmic reticulum. Post-mortem analysis of PD patient brains reveals that ubiquitin is a major component of LBs and LNs present in surviving DA neurons (Bancher et al., 1989) and protein aggregation/turnover is of particular interest in research pertaining to PD etiology. The neuroprotective effects of BBE on $\alpha$-syn-induced damage in the D. melanogaster eye may be due in part to increased activity of the UPS system.

**CONCLUSION**

Recent evidence suggests that a diet rich in blueberries may help slow the age-related degeneration of neurons. In a human study, blueberry juice supplementation improved memory function in older adults with early memory decline (Krikorian et al., 2010). Other groups have demonstrated that short-term blueberry supplementation both increased HSP70-mediated protection against inflammation in aged hippocampal cells and improved object recognition memory in older rats (Goyarzu et al., 2004; Galli et al.,
These studies suggest that the neuroprotective effects of blueberries or BBE are not confined to *D. melanogaster* and may extend to mammals.

I have hypothesized that increased protein turnover and intrinsic antioxidant protection result from BBE supplementation in *D. melanogaster*. α-Syn appears to influence mitochondrial maintenance and its toxicity in cells depends on protein conformation. If BBE supplementation does indeed increase UPS and antioxidant activity simultaneously, this would provide cells with a two-tiered protection system for eliminating both misfolded protein and excess ROS generated from mitochondrial dysfunction. If these results extend to humans, dietary antioxidants could potentially be used as a safe and natural component of future PD treatment plans.

Here, I present the first demonstration of the neuroprotective effects of an extract of blueberries in a Drosophila model of an α-Synucleinpathic disease. Previous findings have demonstrated that grape extract improved both the early mortality and premature decline in locomotion in a similar Drosophila model of PD (Long *et al.*, 2009). Additionally, grape seed extract restored external eye morphology and increased lifespan in Drosophila models of taupathy and Huntington disease, respectively (Pfleger *et al.*, 2010; Wang *et al.*, 2010). Taken together, these results demonstrate the value of using Drosophila to study neurodegenerative disorders. Though the literature is relatively new, studies in this versatile organism have helped develop interest in the potential neuroprotective effects of dietary antioxidants in medical research. Future studies should aim towards unraveling the interaction of dietary antioxidants and the activity of cellular mechanisms, such as the UPS and enzymatic antioxidant pathways.
REFERENCES


APPENDIX 1

The following is a copy of a manuscript that was prepared for publication using the results of this study:

Blueberry extract supplemented diet improves α-synuclein-induced phenotypes in a Drosophila melanogaster model of Parkinson disease

ABSTRACT

Oxidative stress is consistently associated with Parkinson disease (PD) etiology. We investigated the effects of blueberry extract (BBE) supplementation on α-synuclein induced phenotypes in a Drosophila melanogaster model of PD. Enhanced α-synuclein expression in Drosophila dopaminergic (DA) neurons reduces lifespan. We performed longevity assays to measure the effects of BBE on Drosophila lifespan. Flies expressing α-synuclein in their DA neurons fed BBE had a 9% longer median lifespan than those fed a control diet. BBE also improved α-synuclein-induced developmental defects in the Drosophila eye. Our biometric analyses revealed that individuals fed BBE had less atypical ommatidia as well as an increased number of mechanosensory bristle cells than those fed a control diet. We propose that BBE, rich in naturally occurring antioxidants, promotes the survival of neurons in tissues with increased levels of α-Synuclein through a protective mechanism involving other cell signaling pathways.
INTRODUCTION

Parkinson disease (PD) is the second most common progressive neurodegenerative disorder behind Alzheimer's disease (de Moura et al., 2010). The pathophysiological hallmarks of PD include the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and the presence of intraneuronal inclusions known as Lewy bodies (LB) in surviving cells. Affected individuals have both motor and non-motor symptoms ranging from bradykinesia, resting tremor, and muscular rigidity to dementia, depression and olfactory dysfunction. Initially believed to be an entirely sporadic disease, linkage studies identified α-synuclein (PARK1/4) as the first gene related to PD (Polymeropoulos et al., 1997). The human α-synuclein gene (SNCA) encodes a 140 aa peripheral membrane protein that localizes to the pre-synaptic region of neurons (Stefanis, 2012). Both point mutations and duplications of its gene locus result in autosomal-dominant PD (ADPD), the latter causing a more severe early-onset form of the disease. Additionally, both LBs and Lewy neurites, located in the perikarya and neuronal processes, respectively, stain positively for α-Synuclein. α-synuclein is associated with both sporadic and familial PD and seems to play a critical role in its etiology.

Oxidative stress is consistently associated with the pathogenesis of PD however its role in disease progression remains unclear. A cell undergoes oxidative stress when the net balance between the generated reactive oxygen species (ROS) and the available antioxidant defense mechanisms favours the former. Post-mortem analysis of PD patient brains reveals higher levels of oxidative stress biomarkers like dysfunctional mitochondria, decreased levels of reduced glutathione, and deficiencies in antioxidant enzymes in the SN of affected individuals (Schapira and Jenner, 2011). Recent evidence
suggests pluripotent stem cell-derived DA neurons from a PD patient with a SNCA triplication accumulate a-synuclein and are susceptible to oxidative stress (Byers et al., 2011). This data suggests that the combination of oxidative stress and excess α-Synuclein may play a pivotal role in the progression of PD.

The toxicity of excess α-Synuclein appears to be enhanced under conditions of oxidative stress. Studies in Drosophila melanogaster have been especially helpful in elucidating this relationship. Neural expression of a-synuclein in Drosophila brains and DA neurons recapitulates the locomotor dysfunctions, age-dependent degeneration of DA neurons, and formation of LBs characteristic of human PD (Feany and Bender, 2000). Decreased lifespan and retinal degeneration have also been observed in Drosophila with increased neuronal levels of α-Synuclein (Wassef et al., 2007). Co-expression of methionine sulfoxide reductase A (MSRA) and PTEN-induced putative kinase 1 (PINK1), involved in ROS neutralization and damaged mitochondrion turnover, respectively, with a-synuclein improves PD-related phenotypes (Todd and Staveley, 2008). Given their versatility, Drosophila can help unravel the role of oxidative stress in PD and unveil any potential antioxidant therapies.

Blueberries are an excellent source of dietary antioxidants. The therapeutic potential of blueberries in cancer and vascular disease has been described and recent studies in Drosophila suggest that plant extracts may be beneficial to individuals suffering from neurodegenerative diseases (Neto, 2007; Long et al., 2009). In this study we describe the restorative effects of Webber Naturals’ 36:1 concentrate blueberry extract (BBE) on a Drosophila model of PD. Enhanced mortality and eye degeneration caused
by directed expression of α-synuclein in the DA neurons and developing eye, respectively, is improved by supplementing growth media with BBE.

**MATERIALS AND METHODS**

*Fly stocks and culture*

The *UAS-α-synuclein* (Feany and Bender, 2000) and *Ddc-Gal4* (Li et al., 2000) flies were generously provided by Dr. M. Feany (Harvard Medical School) and Dr. J. Hirsh (University of Virginia), respectively. *GMR-Gal4* (Freeman, 1996) and *UAS-lacZ* (Brand and Perrimon, 1993) flies were obtained from the Bloomington Drosophila Stock Center at Indiana University. Directed expression of the transgenes in DA neurons and during early eye development was accomplished by crossing homozygous *Ddc-Gal4* and *GMR-Gal4* females, respectively, to homozygous *UAS-α-synuclein* (PD model) and *UAS-lacZ* (control) males as per standard methods. Flies were fed either a standard cornmeal-yeast-molasses-agar medium (65 g/L cornmeal, 15 g/L nutritional yeast extract, 5.5 g/L agar, 50 ml/L fancy grade molasses in water supplemented with 0.1 g/ml methyl paraben in ethanol and 2.5 ml propionic acid) or standard medium supplemented with either 1 mg/ml or 5 mg/ml Webber Naturals' 36:1 concentrate BBE (WN Pharmaceuticals* Ltd., Coquitlam, B.C., V3K 7B5, www.webbernaturals.com)

*Longevity assay*

Flies were collected under gaseous CO₂ every 24 hours until a minimum of 200 adult males of each genotype were obtained. They were then transferred to upright standard plastic shell vials containing standard (control), or standard medium
supplemented with either 1 mg/ml or 5 mg/ml BBE. Each group was maintained at 25 °C and kept in non-crowded conditions (1-20 individuals per vial). Flies were scored for viability every 2 days and transferred to fresh medium without anesthesia according to established protocol (Staveley et al., 1990). Survival fractions were calculated in Prism version 5.0b for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com) using the product limit (Kaplan-Meier) method.

Scanning electron microscopy and biometric analyses

Flies were reared and aged 3 to 5 days post-eclosion on either standard or BBE supplemented medium at 29 °C. Surviving flies were preserved at -80 °C before being mounted on metal studs under a dissecting microscope. Prepared flies were desiccated overnight and gold coated prior to photography at 170 times magnification with a Hitachi S-570 scanning electron microscope as per standard methods.

All biometric analyses were measured using ImageJ64 version 1.42q (Abramoff et al., 2004). The area of a single ommatidium was determined by dividing the average area of a floret of ommatidia by 7 (data not shown). These numbers were used to distinguish between normal and atypical ommatidia when measuring percent disruption. A disrupted or atypical ommatidium had an area 50% smaller or 150% larger than a typical ommatidium for that condition. An oval with an area between 35000-40000 μm² was overlaid on the flattest portion of each analyzed eye with Paintbrush version 2.1.1 for Mac OS X (Copyright © 2007-2010 Soggy Waffles). Individual disrupted areas within the oval were measured in triplicate and a percent value was obtained by dividing the summed average values into the average area of the oval (also measured in triplicate).
= 15 for each analyzed condition for both bristle counts and ommatidium area measurements, whereas n = 10 for percent disruption analysis. Bar graphs were produced using Prism version 5.0b for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

*Increased concentrations of blueberry extract protects against α-Synuclein-induced early mortality*

Here we report a reduced lifespan in flies when *α-synuclein* expression is enhanced in the DA neurons (Figure 1A). The median survival time of *α-synuclein*-expressing flies was reduced by 37% compared to the *lacZ* cohort when both groups were fed a control diet. A diet rich in BBE partially rescued the reduced lifespan caused by increased neuronal amounts of α-Synuclein in Drosophila (Figure 1B). *α-synuclein* flies fed a diet containing 5 mg/ml BBE had a 9% longer median lifespan than those fed a control diet, whereas a similar result was not found with a concentration of 1 mg/ml BBE. The median survival values for each group are found in Table 1.

*Blueberry extract suppresses α-Synuclein-induced degeneration in the developing eye*

A rough external eye phenotype occurs when *GMR-Gal4* is used to drive expression of *α-synuclein* in the developing Drosophila eye (Figure 2B) (Todd and Staveley, 2008). BBE supplementation restores mean *α-synuclein*-induced disruption back to control levels (Figure 2E). The mean disruption of *lacZ* flies fed a control diet was 29% of the analyzed area. In *α-synuclein* flies, the mean disruption was reduced
from 73\% in those fed a control diet to 40\% and 29\% in flies fed 1 and 5 mg/ml BBE, respectively. Increased \( \alpha \)-Synuclein levels reduced the mean number of bristles per eye to 373 in flies fed a control diet, whereas \( \text{lacZ} \) flies on the same diet had 541 (Figure 2E). The number of bristles per eye was raised to 415 and 438 when the \( \alpha \)-synuclein flies were fed a diet consisting of 1 and 5 mg/ml BBE, respectively. This provides another example of BBE-induced protection against PD-related cell death in a Drosophila tissue that is rich in neurons.

**DISCUSSION**

Recent evidence has suggested that a diet rich in blueberries may help slow the age-related degeneration of neurons. In a human study, blueberry juice supplementation improved memory function in older adults with early memory decline (Krikorian et al., 2010). Furthermore, Galli et al. have demonstrated that short-term blueberry supplementation increased heat shock protein 70 (HSP70)-mediated protection against inflammation in aged rat hippocampal cells (Galli et al., 2006). These studies suggest that the neuroprotective effects of blueberries or BBE are not confined to Drosophila and may translate to mammals.

The Free Radical/Oxidative Stress theory of ageing originated in the 1950's and suggests that an organism ages due in part to the accumulation of free radical-induced damage to its cellular macromolecules. Previous studies have shown that BBE is capable of extending lifespan *Drosophila melanogaster* (Peng et al., 2012). Our results are novel as we have shown that BBE supplementation can extend lifespan in a Drosophila model of a neurodegenerative disease. The additional antioxidants provided by BBE
supplementation may alleviate some of the excess ROS generated during the progression of PD-like cell death resulting in less cellular damage and a longer median survival time in affected flies.

The Drosophila compound eye consists of multiple subunits, or ommatidia, composed of several neurons and peripheral mechanosensory bristle cells. Directed expression of \textit{a-synuclein} during early eye development results in premature degeneration of the retina and the abnormal development of the external morphology of the eye (Haywood and Staveley, 2004; Todd and Staveley, 2008). Our results suggest that a diet containing BBE protects neurons in the eye against \textit{a-synuclein}-dependent defects. Both the amount of atypical ommatidia and total number of bristles were improved in flies fed a BBE-supplemented diet. A link exists between BBE supplementation and protein turnover via the ubiquitin proteasome system (UPS) as flies fed BBE have increased levels of \textit{Rpn11} messenger RNA, an essential lid component of the 26S proteasome structure (Peng et al., 2012). In humans, the \textit{parkin} gene (PARK2) encodes an E3 ubiquitin ligase and expression of \textit{parkin} in Drosophila eyes suppresses \textit{a-synuclein}-induced retinal degeneration in older flies (Haywood and Staveley, 2004). Protein aggregation/turnover is a point of major interest in PD etiology and the neuroprotective effects of BBE on \textit{a-synuclein}-induced damage in the Drosophila eye may be due in part to increased activity of the UPS system.

Our findings could be the result of a strengthened overall antioxidant defense mechanism in \textit{a-synuclein}-expressing flies. BBE extract increases the expression of intrinsic antioxidant defense enzymes like \textit{catalase (Cat)}, \textit{Sod1}, and \textit{Mn superoxide dismutase (Sod2)} in Drosophila (Peng et al., 2012). Additionally, BBE extends lifespan
and partially protects flies under conditions of increased oxidative stress, however no effect was seen with either Cat or Sod knockout mutants. Similar results have been documented for several other foods high in dietary antioxidants, including extracts of apple polyphenols, green tea catechins and black tea (Li et al., 2007; Peng et al., 2009; Peng et al., 2011). Although dietary antioxidants likely provide an invaluable secondary support to cells undergoing oxidative stress it appears their protective effects are dependent on intrinsic enzymatic antioxidant defense systems.

Here we present the first demonstration of the neuroprotective effects of BBE in a Drosophila model of α-Synuclein pathic disease. Previous findings by Long et al. demonstrated that grape extract improved both the early mortality and premature decline in locomotion in a similar Drosophila model of PD (Long et al., 2009). Taken together, these results epitomize the value of using Drosophila to study PD etiology. Though the literature is relatively new, studies in this versatile organism have helped develop interest in the potential neuroprotective effects of dietary antioxidants in medical research. Future studies could aim towards unraveling the interaction of dietary antioxidants and the activity of cellular mechanisms, such as the UPS and enzymatic antioxidant pathways.

REFERENCES


TABLES

Table 1 - Median survival times of Drosophila melanogaster reared on either a standard or blueberry extract (BBE)-supplemented diet

<table>
<thead>
<tr>
<th>Genotype (food medium)</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$w^{1118}$; UAS-lacZ/Ddc-Gal4 (control)</td>
<td>82$^a$</td>
</tr>
<tr>
<td>$w^{1118}$; UAS-$\alpha$-syn/Ddc-Gal4 (control)</td>
<td>52$^b$</td>
</tr>
<tr>
<td>$w^{1118}$; UAS-$\alpha$-syn/Ddc-Gal4 (1 mg/ml BBE)</td>
<td>54$^b$</td>
</tr>
<tr>
<td>$w^{1118}$; UAS-$\alpha$-syn/Ddc-Gal4 (5 mg/ml BBE)</td>
<td>60$^c$</td>
</tr>
</tbody>
</table>

Groups with different superscripted letters were deemed significantly different ($p < 0.05$) by the log-rank (Mantel-Cox) test followed by a Bonferroni multiple comparison correction.

FIGURE LEGENDS

Figure 1 - Blueberry extract (BBE) partially protects Drosophila melanogaster against $\alpha$-synuclein-induced early mortality. A Directed expression of $\alpha$-synuclein ($n = 218$) in DA neurons shortens lifespan in Drosophila fed a standard diet, as compared to a lacZ ($n = 227$) control ($p < 0.05$). B Flies fed diets containing 5 mg/ml BBE ($n = 283$) were partially protected against the $\alpha$-synuclein-induced mortality ($p < 0.05$), whereas 1 mg/ml BBE ($n = 267$) had no significant effect. Genotypes are $w^{1118}$; UAS-lacZ/Ddc-Gal4 (control) and $w^{1118}$; UAS-$\alpha$-synuclein/Ddc-Gal4 (aSYN). Errors bars represent standard error of the mean. p-values were calculated by the log-rank (Mantel-Cox) test and multiple comparisons were corrected for using the Bonferroni method.
Figure 2 - Blueberry extract (BBE) supplementation counteracts α-synuclein-induced developmental defects of the eye. A-D  Scanning electron micrographs of adult eyes. B Overexpression of α-synuclein during early eye development produces a rough external eye morphology. X denotes mg/ml. E Flies supplemented with BBE have disruption levels comparable to the lacZ control (* represents p < 0.05). F BBE supplementation increases mean bristle number and partially rescues the α-synuclein-induced decrease (* represents p < 0.05). Genotypes are w¹¹¹₈; UAS-lacZ/GMR-Gal4 (control) and w¹¹¹₈; UAS-α-synuclein/GMR-Gal4 (αSYN). Error bars represent standard error of the mean. p-values were calculated via one-way ANOVA followed by Tukey's Multiple Comparison test.

FIGURES

Figure 1 - Blueberry extract (BBE) partially protects *Drosophila melanogaster* against α-synuclein-induced early mortality
Figure 2 - Blueberry extract (BBE) supplementation counteracts $\alpha$-synuclein-induced developmental defects of the eye
APPENDIX 2

Possible explanation for the lifespan variability in α-synuclein- and lacZ-expressing *D. melanogaster* fed control medium observed from two independent experiments

Two separate experiments were performed when analyzing the effects of exposing *D. melanogaster* to a BBE-supplemented diet. The lifespans of flies fed a standard (control) medium differed greatly between the pre- and post-eclosion supplementation experiments as can be seen in Table A2.1. The genotypes tested in the two experiments were identical and crosses were executed with the same procedures and equipment. The only difference between the two experiments was the location in which the flies were maintained during testing. The pre-eclosion longevity assays were performed in our laboratory in the basement of the Biotechnology building, whereas the post-eclosion experiments were conducted in an equipment storage room on the first floor of the Science building due to space constraints in the laboratory incubator. The median survival times reported from the latter experiment are significantly shorter than those discovered during the pre-eclosion assays. This difference is likely due to an unknown environmental factor as the food media and fly lines used were the same in each experiment. The incubator in which the flies were maintained during the post-eclosion experiment was checked regularly for temperature changes, however, the incubator was an older piece of equipment and any fluctuations outside of work hours might have gone
unnoticed. The variability reported between the pre- and post-eclosion supplementation experiments is unfortunate, however it does not change the results I have reported for the effects of BBE supplementation on $\alpha$-syn-induced phenotypes.
Table A2.1 - Median survival times of α-synuclein- and lacZ-expressing D. melanogaster fed control media from two independent experiments

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w^{1118} ); UAS-α-syn/Ddc-Gal4 (1)</td>
<td>46(^a)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-α-syn/Ddc-Gal4 (2)</td>
<td>52(^b)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-α-syn+/; Ddc-Gal4+/+ (1)</td>
<td>54(^c)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-α-syn+/; Ddc-Gal4+/+ (2)</td>
<td>70(^d)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ/Ddc-Gal4 (1)</td>
<td>58(^e)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ/Ddc-Gal4 (2)</td>
<td>82(^f)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ+/; Ddc-Gal4+/+ (1)</td>
<td>52(^g)</td>
</tr>
<tr>
<td>( w^{1118} ); UAS-lacZ+/; Ddc-Gal4+/+ (2)</td>
<td>82(^h)</td>
</tr>
</tbody>
</table>

Different superscripted letters indicate a significant difference (\( p < 0.05 \)) between values for a particular genotype; comparisons were not made between different genotypes.

1: post-eclosion supplementation experiment, 2: pre-eclosion supplementation experiment.