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.

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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

AplBM - 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

CO_2 - 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

H_2O - water

H_2O_2 - 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-Aβ component

NO - nitric oxide

$O_2^{\bullet -}$ - superoxide radical

OCl^- - 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[1] and all age groups in Canada for the years 2000 - 2007[2]

	Cause of death[3-6]	
Year	**Parkinson disease**	**Secondary parkinsonism**
2000	1471	9
2001	1600	9
2002	1651	13
2003	1662	17
2004	1642	15
2005	1866	24
2006	1664	22
2007	1869	21

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 of 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[1-3] for both sexes[4] and all age groups in Canada[5, 6] for the years 2000 - 2007[7, 8]

Year	Rank of leading causes of death[9]	Number of deaths
2000	13	1480
2001	13	1609
2002	13	1664
2003	13	1679
2004	13	1657
2005	13	1890
2006	14	1686
2007	13	1890

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), 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

Locus	Chromosome	Gene	Inheritance	Clinical phenotype
PARK1/4	**4q21**	***SNCA***	**AD**	**EOPD**
PARK2	**6q25.2-q27**	***Parkin***	**AR**	**Juvenile and EOPD**
PARK3	2p13	Unknown	AD	LOPD
PARK5	4p14	*UCH-L1*	AD	LOPD
PARK6	**1p35-p36**	***PINK1***	**AR**	**EOPD**
PARK7	**1p36**	***DJ-1***	**AR**	**EOPD**
PARK8	**12q12**	***LRRK2***	**AD**	**LOPD**
PARK9	**1p36**	***ATP13A2***	**AR**	**Kufor-Rakeb syndrome**
PARK10	1p32	Unknown	Unknown	Unclear
PARK11	2q36-q37	*GIGYF2*	AD	LOPD
PARK12	Xq	Unknown	X-linked	Unclear
PARK13	2p13	*HTRA2*	AD	Unclear
PARK14	22q13.1	*PLA2G6*	AR	Parkinsonism with additional features
PARK15	22q12-q13	*FBXO7*	AR	EOPD
PARK16	1q32	Unknown	Susceptibility locus	LOPD
PARK17	4p16	*GAK*	Susceptibility locus	LOPD
PARK18	6p21.3	*HLA-DRA*	Susceptibility locus	LOPD
Gaucher's locus	1q21	*GBA*	N/A	N/A

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

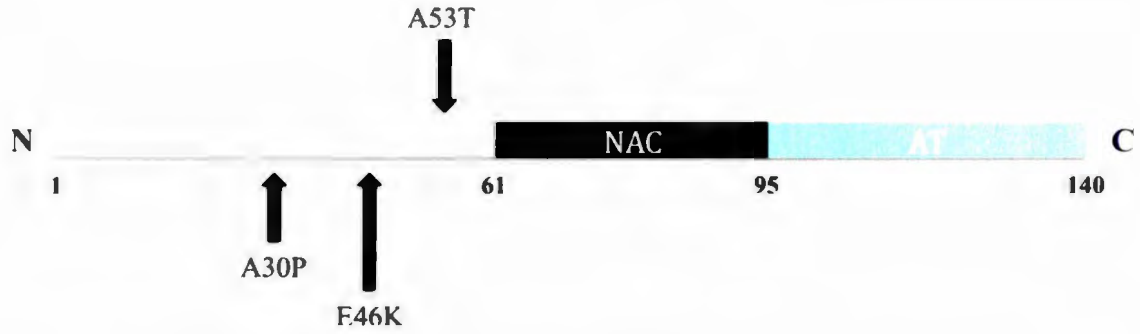

Figure 1 - Schematic of α-Synuclein protein structure. AplBM: apolipoprotein lipid-binding motif; NAC: non-Aβ component; AT: acidic tail; N: amino terminus; C: carboxyl terminus. Arrows indicate location of mutations linked to ADPD.

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 ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hypochlorite (OCl^-); iii) peroxisomes, organelles that produce H_2O_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.

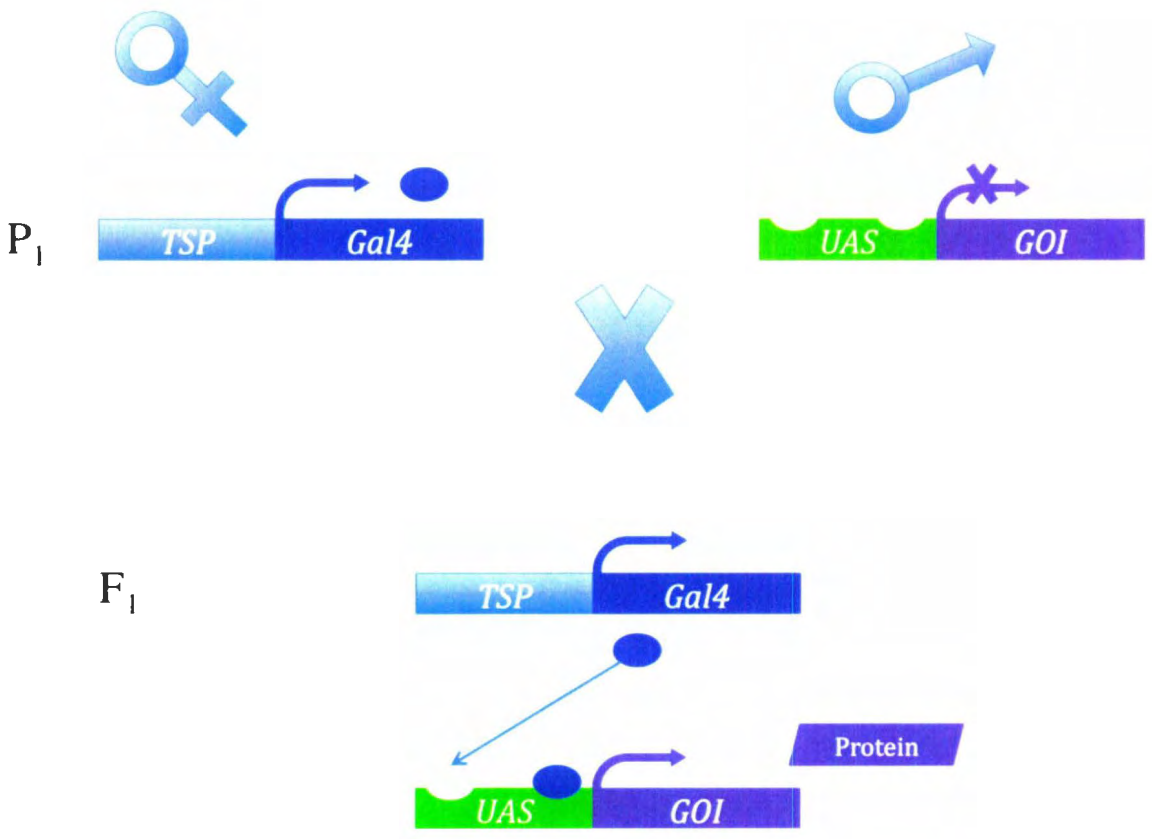

Figure 2 - Directed gene expression in Drosophila via the *UAS/Gal4* system. *UAS*-controlled transcription remains inactive when the two transgenes are isolated in the parental generation (P_1). The Gal4 transcription factor binds to the *UAS* responsive DNA elements in progeny with at least one copy of each transgene (F_1) 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 $O_2^{\bullet -}$ 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,

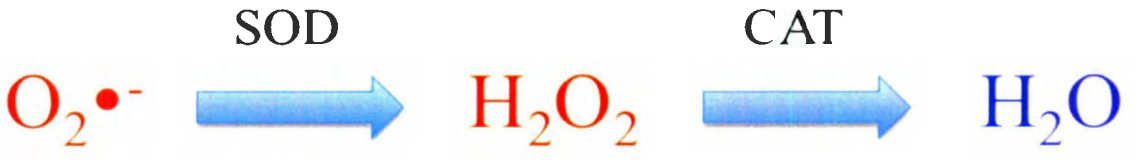

Figure 3 - Schematic of the *superoxide dismutase/catalase* antioxidant pathway. Superoxide ($O_2^{\bullet -}$) is converted to water (H_2O) via a hydrogen peroxide (H_2O_2) intermediate. SOD: superoxide dismutase, CAT: catalase. Red text denotes reactive oxygen species.

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 α-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 *α-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 *α-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 *α-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 *α-syn* in the DA neurons and developing eye, respectively, of *D. melanogaster*.

MATERIALS AND METHODS

Drosophila 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. Each of the *UAS-Sod1* (Parkes *et al.*, 1998), *UAS-Sod2* (Anderson *et al.*, 2005), and *UAS-Cat* (Anderson *et al.*, 2005) stocks were kind gifts from Dr. John P. Phillips (University of Guelph). The *GMR-Gal4* (Freeman, 1996), *UAS-lacZ* (Brand and Perrimon, 1993), *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-α-synuclein* (PD model), *UAS-lacZ* (control), *UAS-GFP* (control), *UAS-Sod1*, *UAS-Sod2*, and *UAS-Cat* males. For the BBE-supplementation experiments, homozygous *UAS-α-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^{1118} males. All crosses were performed as per standard methods. The resulting genotypes were: 1) w^{1118}; *UAS-α-synuclein/Ddc-Gal4*, 2) w^{1118}; *UAS-α-synuclein/+; Ddc-Gal4/+*, 3) w^{1118}; *UAS-lacZ/Ddc-Gal4*, 4) w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+*, 5) w^{1118}; *+/Ddc-Gal4*, 6) w^{1118}; *+; Ddc-Gal4/+*, 7) w^{1118}; *UAS-α-synuclein/GMR-Gal4*, 8) w^{1118}; *UAS-lacZ/GMR-Gal4*, 9) w^{1118}; *UAS-GFP/+; Ddc-Gal4/+*, 10) w^{1118}; *UAS-Sod1/+; Ddc-Gal4/+*, 11) w^{1118}; *UAS-Sod2/+; Ddc-Gal4/+*, and 12) w^{1118}; *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 proprionic acid) or standard medium supplemented with Webber Naturals' 36:1 concentrate BBE (WN

Pharmaceuticals® 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 (CO_2) 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 ºC 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_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:

$$\text{Climbing index} = \sum (nm) / N$$

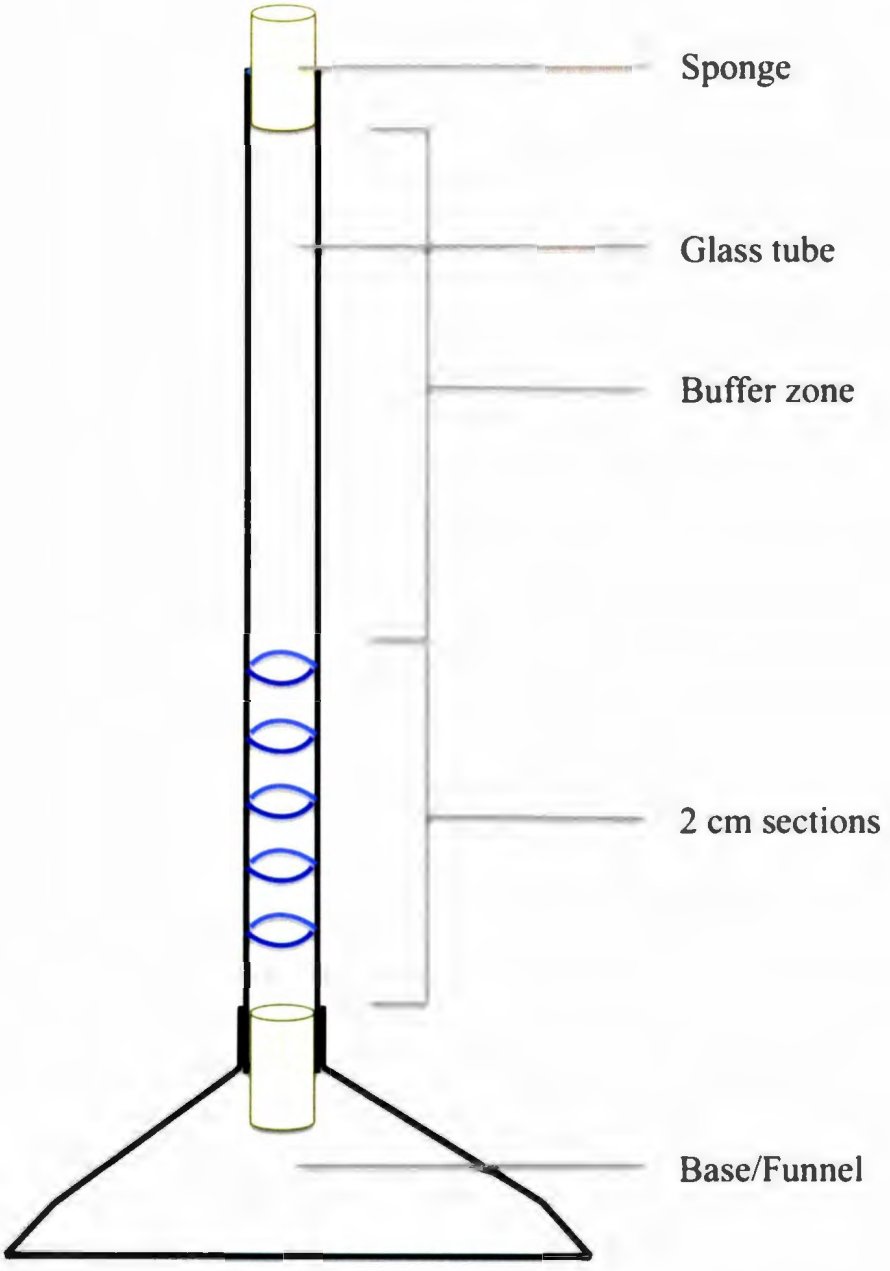

Figure 4 - Schematic representation of the graded climbing apparatus. The structure consists of a 30 x 1.5 cm glass tube sealed at both ends by sponges. The bottom of the tube is divided into five 2 cm sections with an ascending score (1-5) and the remainder of the upper portion serves as a buffer zone. 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 μm^2 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 *α-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.

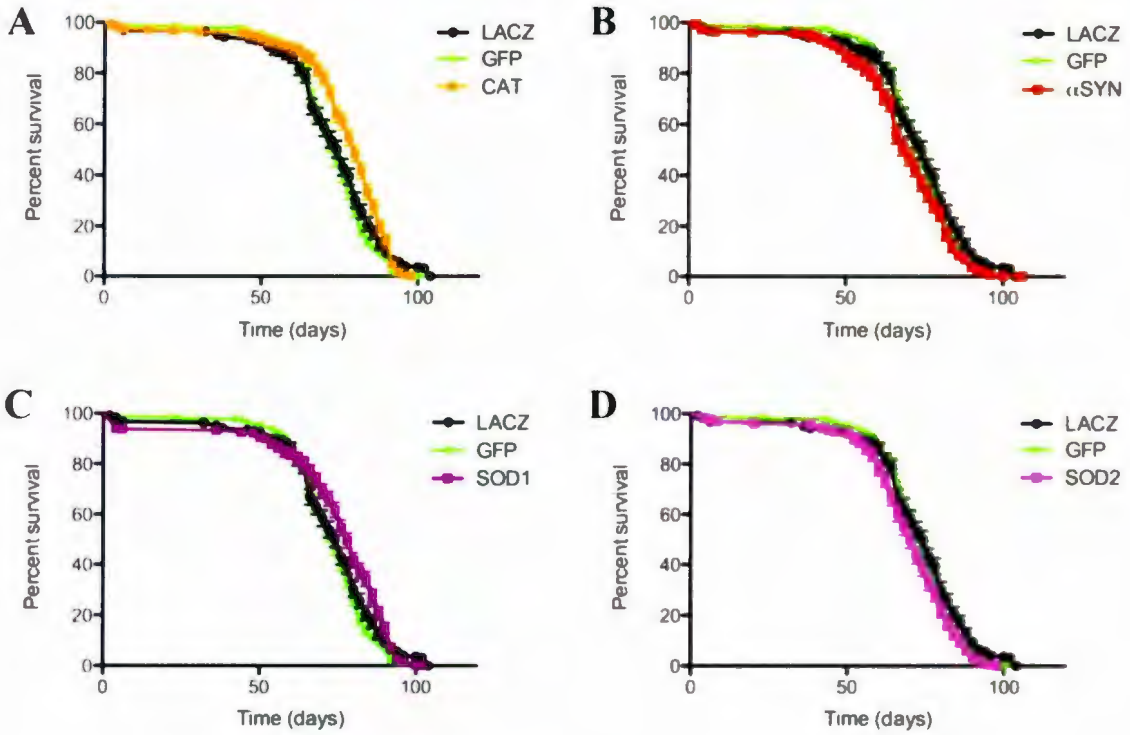

Figure 5 - Directed expression of *catalase* in the dopaminergic neurons slightly extends lifespan in *D. melanogaster*. **A** The longevity of flies expressing *Cat* is significantly longer than both the *lacZ*- and *GFP*-expressing controls ($p < 0.05$). **B - D** Flies expressing *α-synuclein*, *Sod1*, 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*

Genotype	Median survival (days)
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+*	76
w^{1118}; *UAS-GFP/+; Ddc-Gal4/+*	74
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+*	70
w^{1118}; *UAS-Sod1/+; Ddc-Gal4/+*	78
w^{1118}; *UAS-Sod2/+; Ddc-Gal4/+*	70
w^{1118}; *UAS-Cat/+; Ddc-Gal4/+*	80*

* 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 (w^{1118}) 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 (w^{1118} 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.

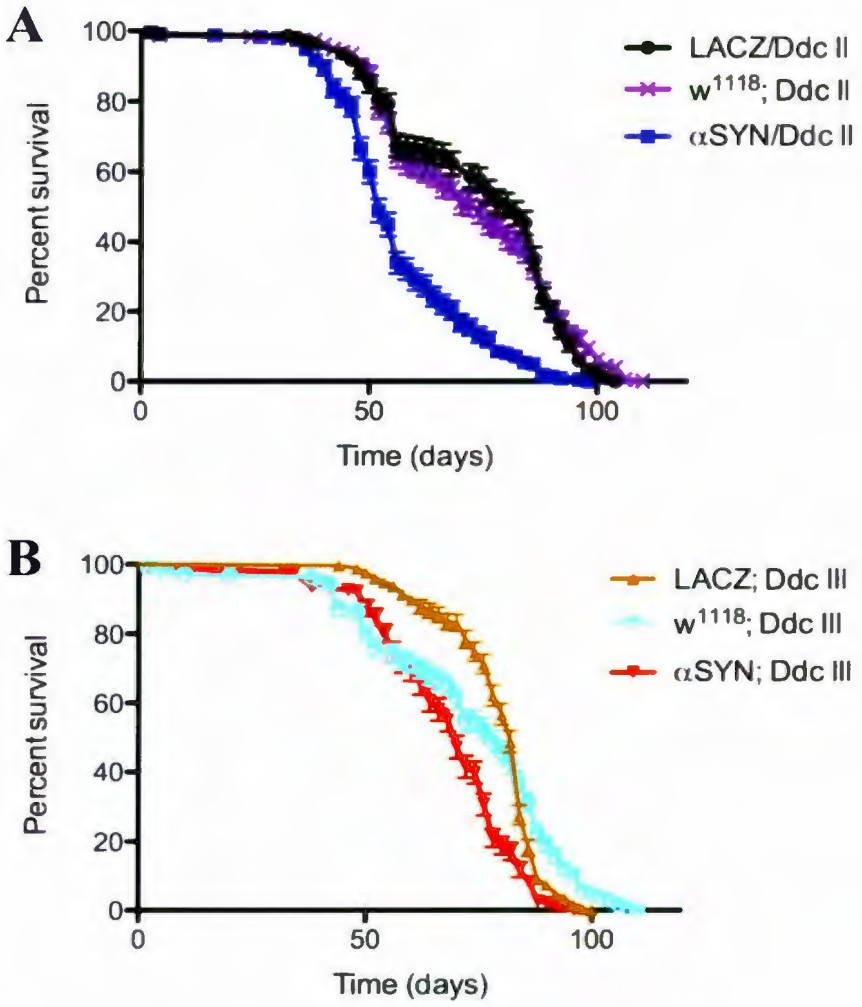

Figure 6 - *α-synuclein* reduces lifespan in *D. melanogaster* when expressed in the dopaminergic neurons. **A** Flies have a severely reduced lifespan when *α-synuclein* expression in the DA neurons is driven by *Ddc-Gal4* II ($p < 0.05$). n = 227, 218, and 229 for αSYN/Ddc II, LACZ/Ddc II and w^{1118}; 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 *α-synuclein* expression directed to their dopaminergic neurons

Genotype	Median survival (days)
w^{1118}; +/*Ddc-Gal4*	74
w^{1118}; *UAS-lacZ/Ddc-Gal4*	82
w^{1118}; *UAS-α-syn/Ddc-Gal4*	52*
w^{1118}; +; *Ddc-Gal4/+*	78
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+*	82
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+*	70*

* 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/Ddc 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/Ddc 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; Ddc III). Neither 1 mg/ml nor 5 mg/ml of BBE extended median survival time in αSYN; Ddc 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.

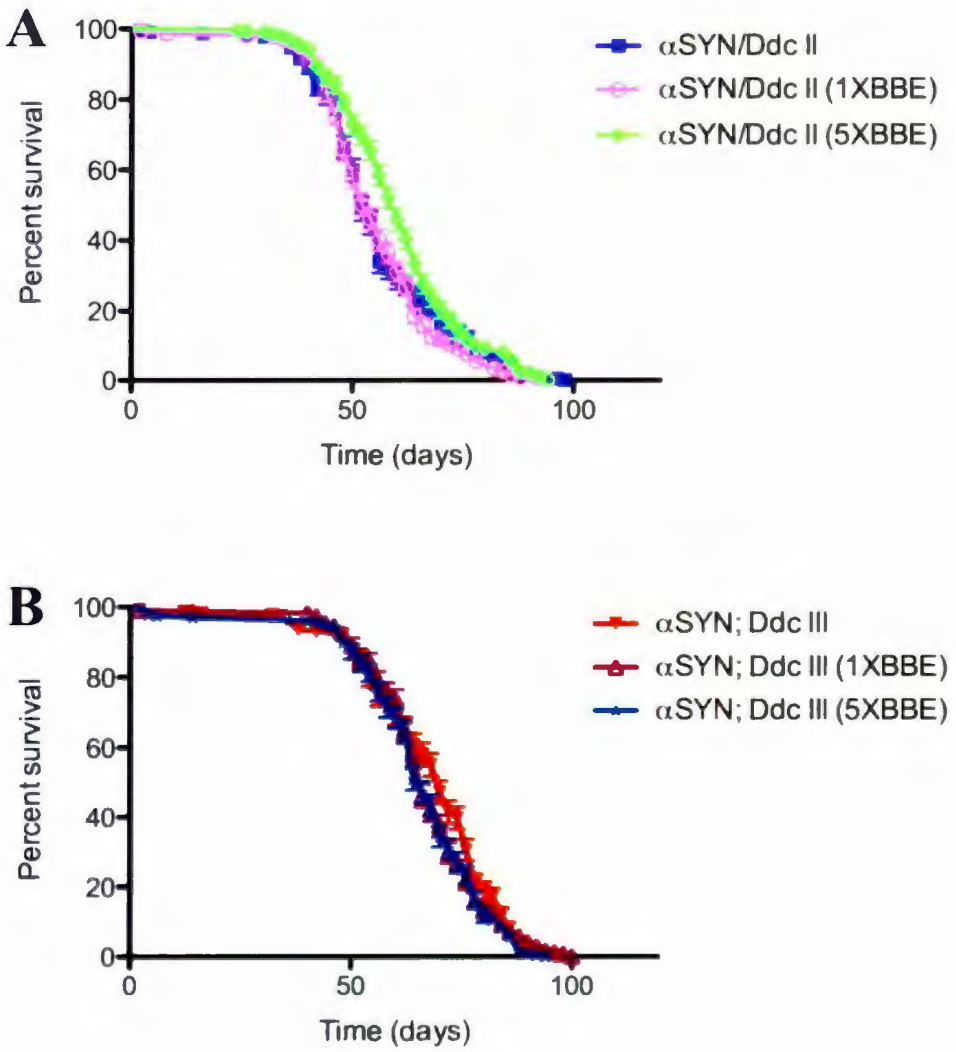

Figure 7 - Pre-eclosion blueberry extract supplementation benefits *α-synuclein*-expressing *D. melanogaster* with severely reduced lifespans. **A** Flies expressing *α-synuclein* in their DA neurons via *Ddc-Gal4 II* that were reared on a diet containing 5 mg/ml BBE survived significantly longer than 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

Genotype (food medium)	Median survival (days)
w^{1118}; *UAS-α-syn/Ddc-Gal4* (control)	52[a]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (1 mg/ml BBE)	54[a]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (5 mg/ml BBE)	60[b]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (control)	70[c]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (1 mg/ml BBE)	66[c]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (5 mg/ml BBE)	66[c]

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* (w^{1118}; 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.

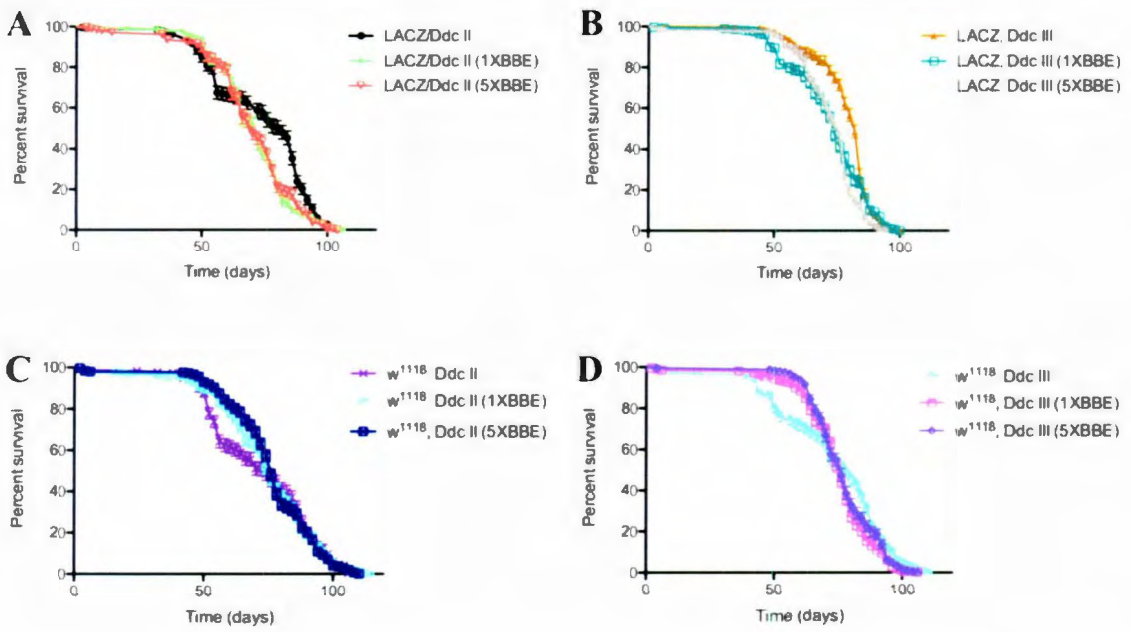

Figure 8 - Pre-eclosion blueberry extract supplementation shortens lifespan in *D. melanogaster* expressing *lacZ* in their dopaminergic neurons. **A, B** Both lines of *lacZ*-expressing flies had shorter median survival times when reared on a diet containing BBE as compared to those reared on a standard 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

Genotype (food medium)	Median survival (days)
w^{1118}; *UAS-lacZ/Ddc-Gal4* (control)	82[a]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (1 mg/ml BBE)	72[b]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (5 mg/ml BBE)	70[b]
w^{1118}; *+/Ddc-Gal4* (control)	74[c]
w^{1118}; *+/Ddc-Gal4* (1 mg/ml BBE)	76[c]
w^{1118}; *+/Ddc-Gal4* (5 mg/ml BBE)	76[c]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (control)	82[d]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (1 mg/ml BBE)	74[e]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (5 mg/ml BBE)	76[e]
w^{1118}; *+; Ddc-Gal4/+* (control)	78[f]
w^{1118}; *+; Ddc-Gal4/+* (1 mg/ml BBE)	76[g]
w^{1118}; *+; Ddc-Gal4/+* (5 mg/ml BBE)	76[fg]

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.

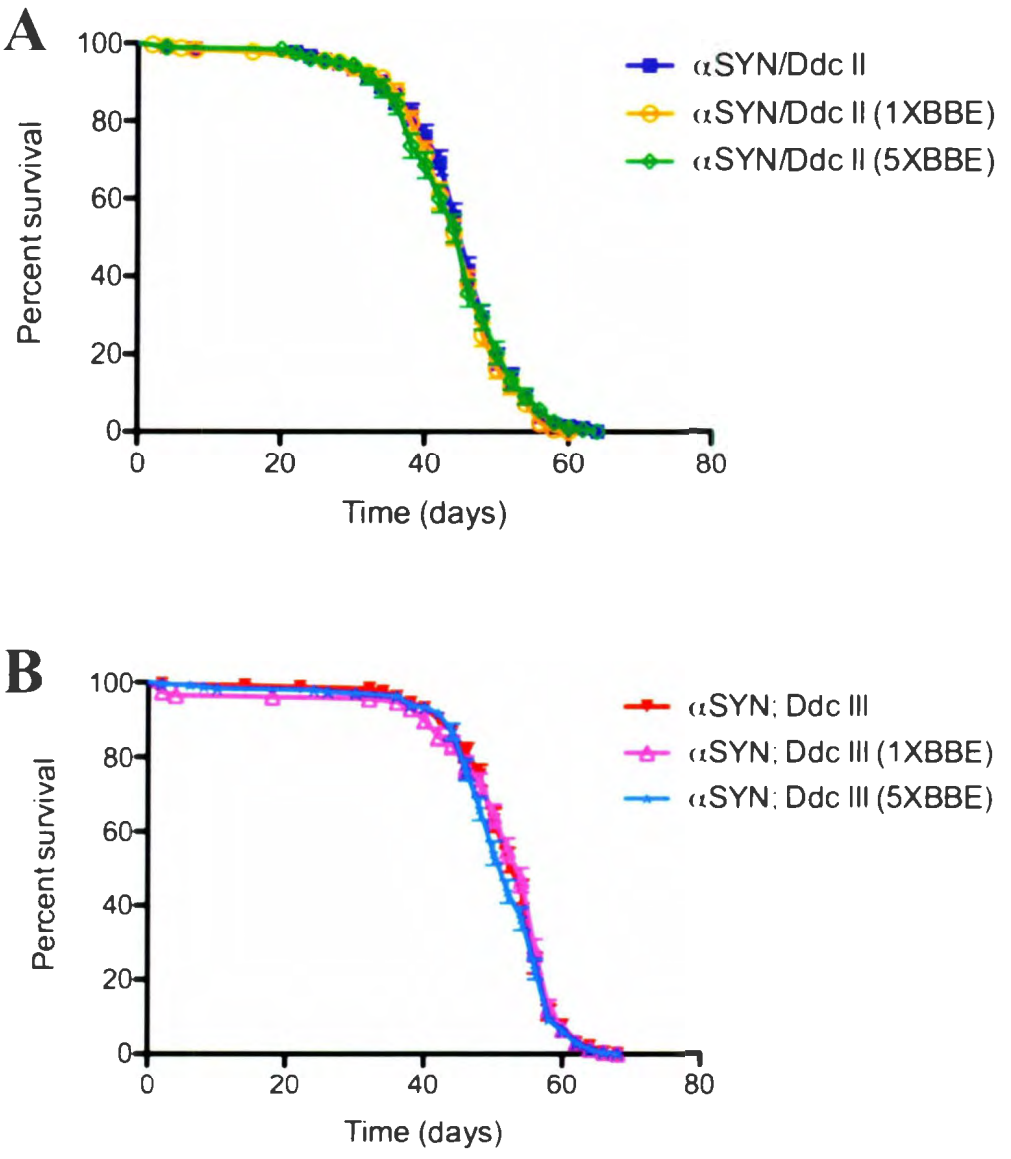

Figure 9 - Post-eclosion blueberry extract supplementation does not affect lifespan in *α-synuclein*-expressing *D. melanogaster*. **A** Longevity curves of flies expressing *α-synuclein* in their DA neurons via *Ddc-Gal4 II* fed control (n = 208), 1 mg/ml BBE (n = 221), or 5 mg/ml BBE (n = 197) medium. **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.

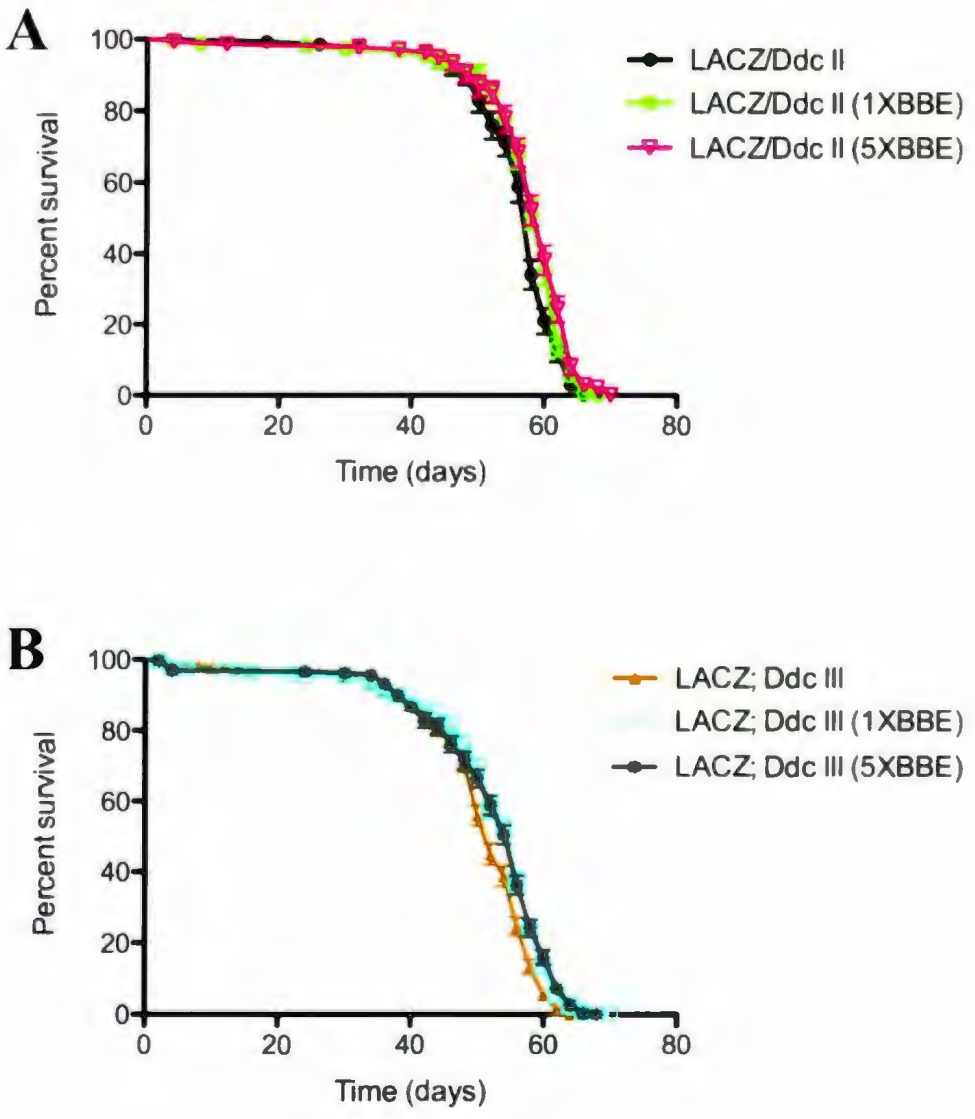

Figure 10 - Blueberry extract fed post-eclosion slightly extends lifespan in *lacZ*-expressing *D. melanogaster*. **A** Flies expressing *lacZ* fed 5 mg/ml BBE (n = 129) survived significantly longer ($p < 0.05$) than those fed either a control diet (n = 145) or one supplemented with 1 mg/ml BBE (n = 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

Genotype (food medium)	Median survival (days)
w^{1118}; *UAS-α-syn/Ddc-Gal4* (control)	46[a]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (1 mg/ml BBE)	46[a]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (5 mg/ml BBE)	46[a]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (control)	54[b]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (1 mg/ml BBE)	54[b]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (5 mg/ml BBE)	52[b]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (control)	58[c]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (1 mg/ml BBE)	60[cd]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (5 mg/ml BBE)	60[d]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (control)	52[e]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (1 mg/ml BBE)	56[f]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (5 mg/ml BBE)	56[f]

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.

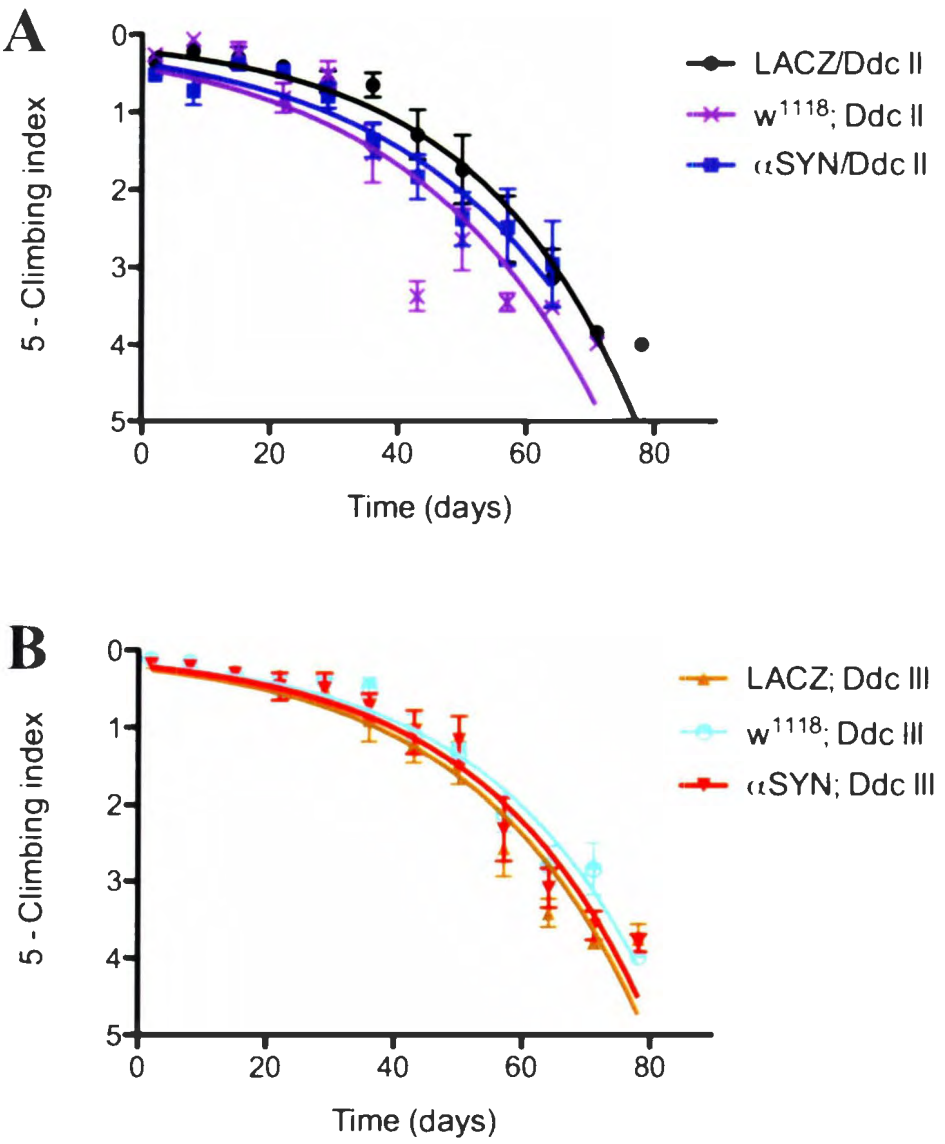

Figure 11 - Targeted *α-synuclein* expression in the dopaminergic neurons of *D. melanogaster* does not affect locomotion. **A** Climbing curves for flies containing *Ddc-Gal4 II*. **B** The climbing ability of flies expressing *α-synuclein* was similar to both the *lacZ* and w^{1118} controls containing *Ddc-Gal4 III*. 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. 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

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

* 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 (Wassef *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.

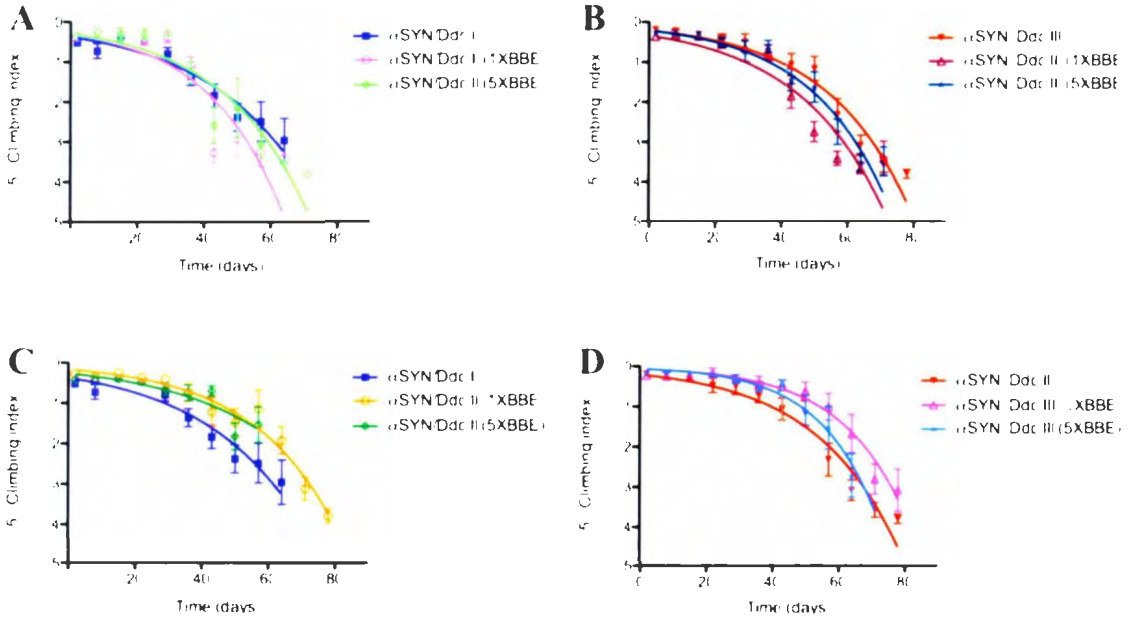

Figure 12 - Blueberry extract supplementation does not affect locomotion in *α-synuclein*-expressing *D. melanogaster*. **A, B** Climbing curves for *α-synuclein*-expressing flies fed BBE-supplemented medium pre-eclosion. **C, D** Climbing curves for *α-synuclein*-expressing flies fed BBE-supplemented 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 *α-synuclein*-expressing *D. melanogaster* fed either control or blueberry extract-supplemented medium

Genotype (food medium)	Slope (k)	Standard error (SE)	95% confidence interval (CI)
Pre-eclosion			
w^{1118}; *UAS-α-syn/Ddc-Gal4* (control)	0.03365	0.003373	0.02704 - 0.04026[a]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (1 mg/ml BBE)	0.04359	0.005264	0.03299 - 0.05418[a]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (5 mg/ml BBE)	0.03902	0.004376	0.03019 - 0.04785[a]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (control)	0.03928	0.002859	0.03362 - 0.04495[b]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (1 mg/ml BBE)	0.03703	0.002955	0.03111 - 0.04294[b]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (5 mg/ml BBE)	0.04209	0.003584	0.03492 - 0.04927[b]
Post-eclosion			
w^{1118}; *UAS-α-syn/Ddc-Gal4* (control)	0.03365	0.003373	0.02704 - 0.04026[c]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (1 mg/ml BBE)	0.03948	0.003183	0.03315 - 0.04580[c]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (5 mg/ml BBE)	0.03199	0.004823	0.02238 - 0.04160[c]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (control)	0.03928	0.002859	0.03362 - 0.04495[d]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (1 mg/ml BBE)	0.05054	0.005597	0.03932 - 0.06176[d]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (5 mg/ml BBE)	0.05742	0.006762	0.04382 - 0.07102[d]

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^{1118} 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.

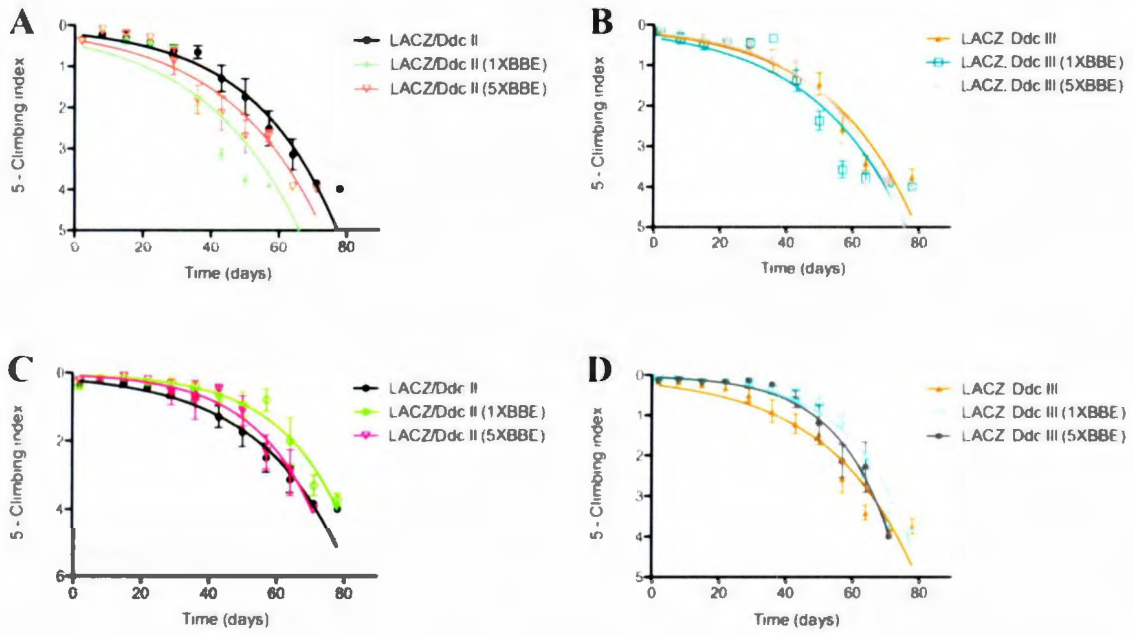

Figure 13 - Post-eclosion blueberry extract supplementation improves mobility in *lacZ*-expressing *D. melanogaster*. **A, B** Climbing curves for *lacZ*-expressing flies fed BBE-supplemented medium pre-eclosion. **C, D** Climbing curves for *lacZ*-expressing flies fed BBE-supplemented medium post-eclosion. **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 *lacZ*-expressing *D. melanogaster* fed either control or blueberry extract-supplemented medium

Genotype (food medium)	Slope (k)	Standard error (SE)	95% confidence interval (CI)
Pre-eclosion			
w^{1118}; *UAS-lacZ/Ddc-Gal4* (control)	0.04015	0.003221	0.03376 - 0.04653[a]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (1 mg/ml BBE)	0.03506	0.003090	0.02885 - 0.04126[a]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (5 mg/ml BBE)	0.03570	0.003278	0.02911 - 0.04228[a]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (control)	0.03797	0.002566	0.03289 - 0.04305[b]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (1 mg/ml BBE)	0.03570	0.002854	0.02999 - 0.04141[b]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (5 mg/ml BBE)	0.04309	0.003401	0.03625 - 0.04993[b]
Post-eclosion			
w^{1118}; *UAS-lacZ/Ddc-Gal4* (control)	0.04015	0.003221	0.03376 - 0.04653[c]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (1 mg/ml BBE)	0.05240	0.005646	0.04107 - 0.06372[c]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (5 mg/ml BBE)	0.05404	0.006958	0.04004 - 0.06804[c]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (control)	0.03797	0.002566	0.03289 - 0.04305[d]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (1 mg/ml BBE)	0.05459	0.003611	0.04736 - 0.06183[e]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (5 mg/ml BBE)	0.06078	0.007640	0.04541 - 0.07615[e]

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

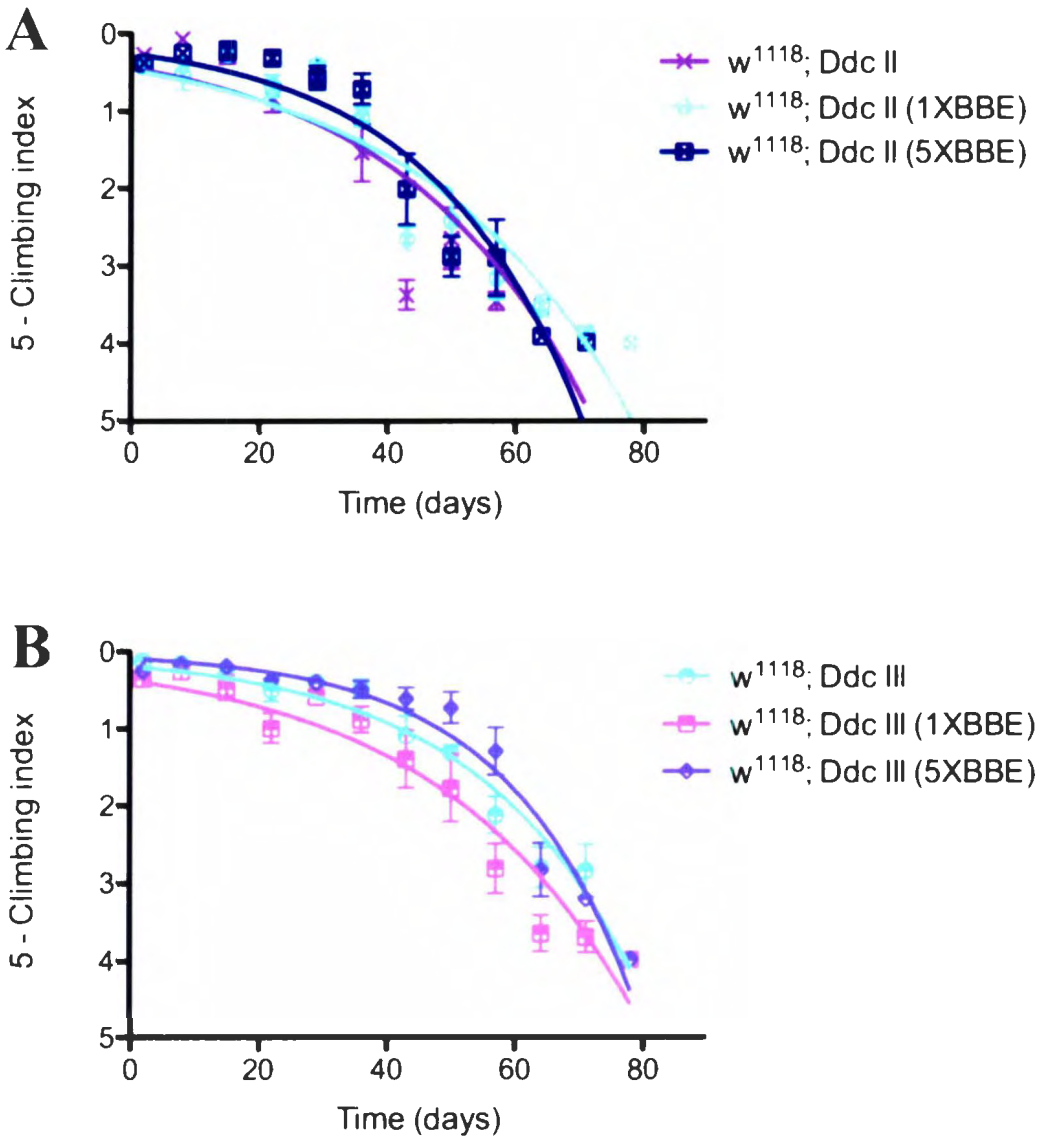

Figure 14 - Blueberry extract supplementation does not affect locomotion in *D. melanogaster* that lack a responsive transgene. **A**, **B** Climbing curves for flies fed a BBE-supplemented diet pre-eclosion. X denotes mg/ml. Genotypes are w^{1118}; *+/Ddc-Gal4* (w^{1118}; Ddc II) and w^{1118}; +; *Ddc-Gal4/+* (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

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

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 multi-cellular 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.

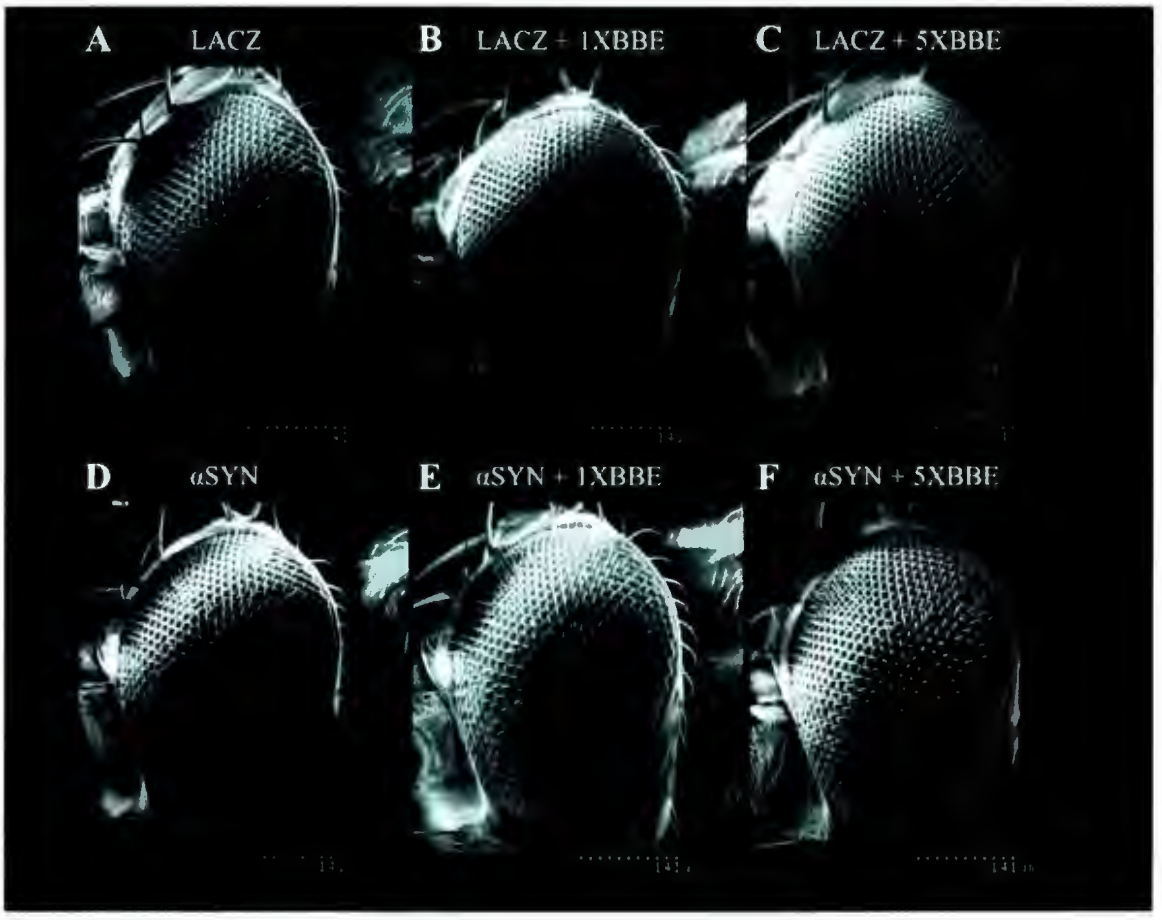

Figure 15 - Expression of *α-synuclein* during eye development does not produce a visible phenotype at 25 °C. **A - F** Scanning electron micrographs of *D. melanogaster* eyes with elevated amounts of either *α-syn* (**D, E, F**) or *lacZ* (**A, B, C**) transcript. Both genotypes were reared on control (**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-α-synuclein/GMR-Gal4* (αSYN) and w^{1118}; *UAS-lacZ/GMR-Gal4* (LACZ).

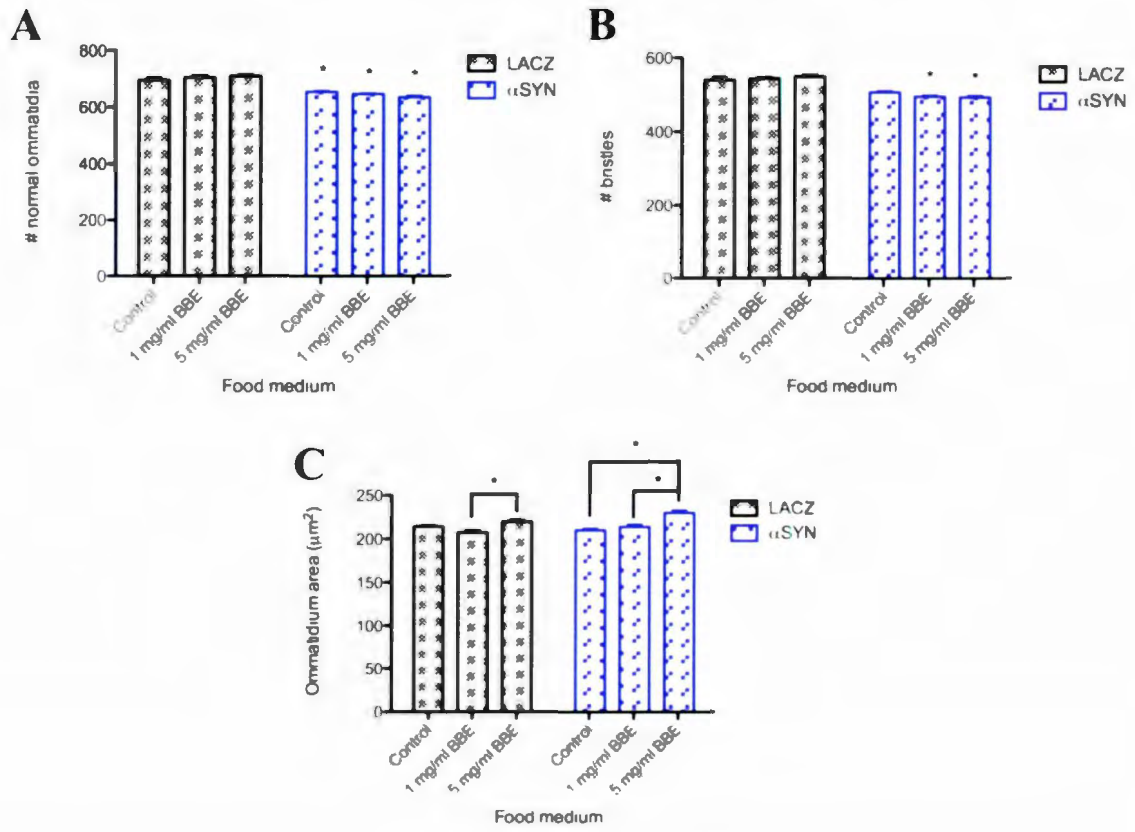

Figure 16 - Blueberry extract supplementation has no effect on moderate *α-synuclein*-induced phenotypes in *D. melanogaster* eyes at 25 °C. **A** Enhanced *α-synuclein* expression significantly reduces the mean number of ommatidia in *D. melanogaster* eyes. **B** Increased levels of *α-synuclein* reduces 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 *α-synuclein* or *lacZ* raised at 25 °C

Genotype (food medium)	# normal ommatidia	# bristles	Ommatidium area (μm^2)
w^{1118}; *UAS-α-syn/GMR-Gal4* (control)	651.8	505.4	208.5
w^{1118}; *UAS-α-syn/GMR-Gal4* (1 mg/ml BBE)	644.0	493.8	212.7
w^{1118}; *UAS-α-syn/GMR-Gal4* (5 mg/ml BBE)	632.8	491.7	228.6
w^{1118}; *UAS-lacZ/GMR-Gal4* (control)	695.3	539.7	214.1
w^{1118}; *UAS-lacZ/GMR-Gal4* (1 mg/ml BBE)	704.4	543.4	207.2
w^{1118}; *UAS-lacZ/GMR-Gal4* (5 mg/ml BBE)	708.9	548.9	219.6

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 ºC (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.

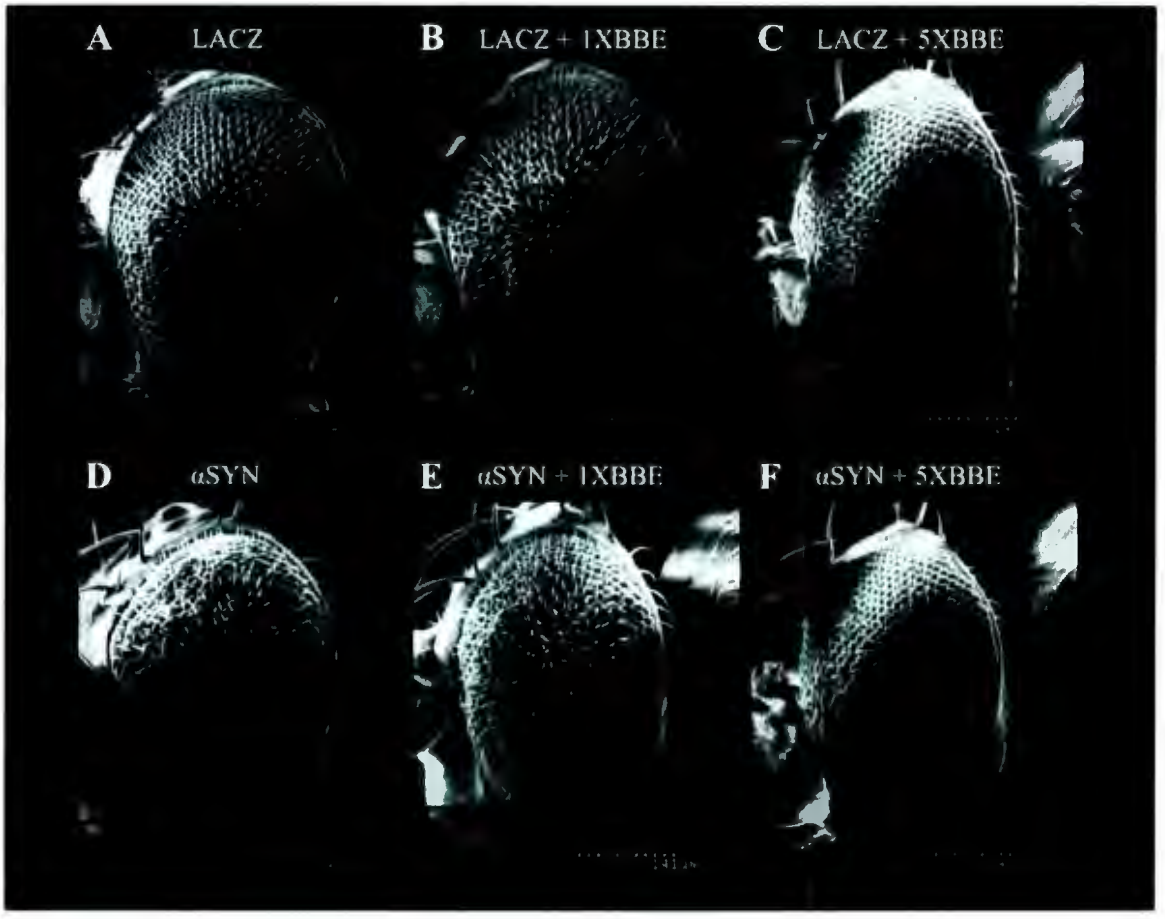

Figure 17 - *α-synuclein* expression during eye development produces a rough external eye morphology at 29 °C. **A - F** Scanning electron micrographs of *D. melanogaster* eyes with elevated amounts of either *α-synuclein* (**D, E, F**) or *lacZ* (**A, B, C**) transcript. Both genotypes were reared on 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^{1118}; *UAS-α-synuclein/GMR-Gal4* (αSYN) and w^{1118}; *UAS-lacZ/GMR-Gal4* (LACZ).

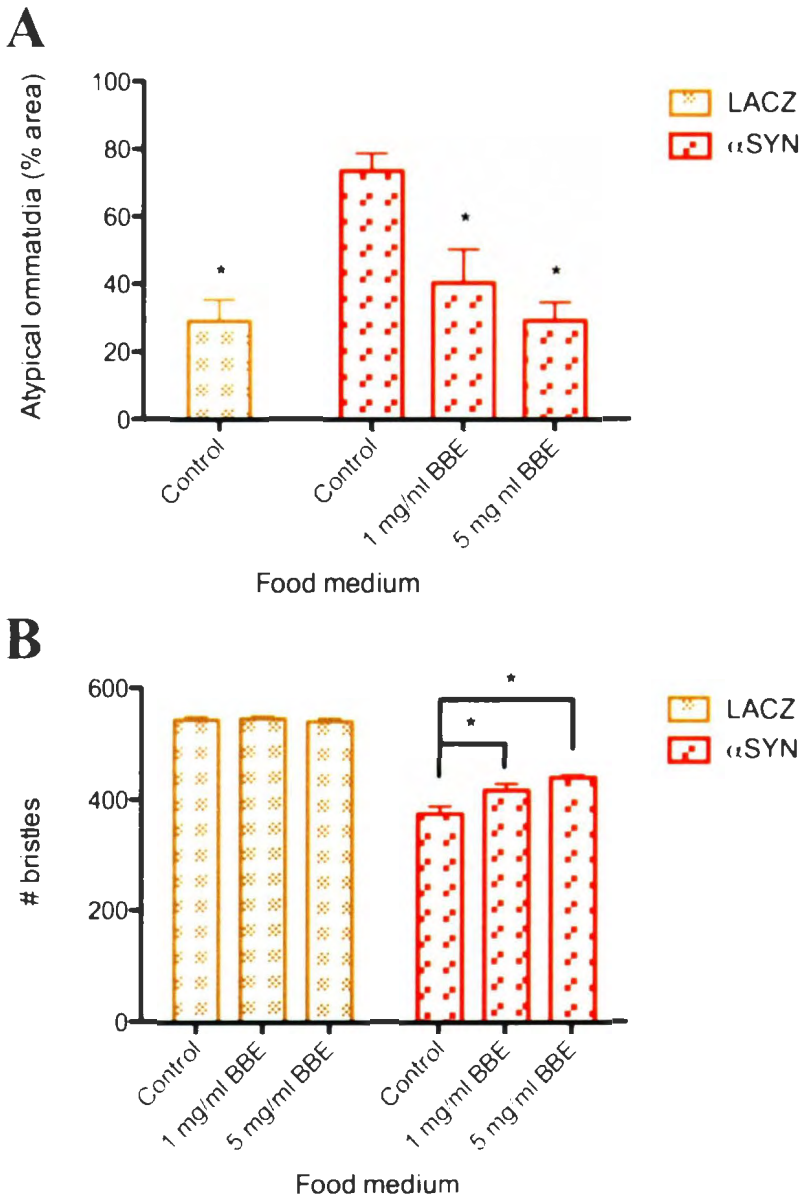

Figure 18 - Severe degenerative phenotypes in *D. melanogaster* eyes caused by *α-synuclein* expression at 29 °C are suppressed by a blueberry extract-supplemented diet. **A** Both concentrations of BBE supplementation completely rescued the amount of atypical ommatidia caused by enhanced *α-synuclein* expression ($p < 0.05$, $n = 10$). **B** BBE supplementation partially rescues the reduced bristle number in *α-synuclein*-expressing eyes ($p < 0.05$, $n = 15$). Genotypes are w^{1118}; *UAS-α-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

Genotype (food medium)	**Atypical ommatidia (% area)**	**# bristles**
w^{1118}; *UAS-α-syn/GMR-Gal4* (control)	73.5	372.9
w^{1118}; *UAS-α-syn/GMR-Gal4* (1 mg/ml BBE)	40.3	414.6
w^{1118}; *UAS-α-syn/GMR-Gal4* (5 mg/ml BBE)	29.2	437.7
w^{1118}; *UAS-lacZ/GMR-Gal4* (control)	28.9	541.3
w^{1118}; *UAS-lacZ/GMR-Gal4* (1 mg/ml BBE)	N/A	543.6
w^{1118}; *UAS-lacZ/GMR-Gal4* (5 mg/ml BBE)	N/A	538.1

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 H_2O_2 (Coyle and Puttfarcken, 1993). Excess CAT may be particularly beneficial to DA neurons since this enzyme converts reactive H_2O_2 into water and could help protect cells against H_2O_2-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 *α-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 *α-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 αSYN 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; Ddc 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-Ga4*. 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 *α-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 *α-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.*,

2006). 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.

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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* (*PARK 1/4*) 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 *α-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 *α-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 *α-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 proprionic 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 CO_2 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 ºC 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 ºC. Surviving flies were preserved at -80 ºC 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 μm^2 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). n

= 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 α-Synuclein levels reduced the mean number of bristles per eye to 373 in flies fed a control diet, whereas *lacZ* flies on the same diet had 541 (Figure 2E). The number of bristles per eye was raised to 415 and 438 when the *α-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 *α-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 *α-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 *Rpn11* messenger RNA, an essential lid component of the 26S proteasome structure (Peng *et al.*, 2012). In humans, the *parkin* gene (PARK2) encodes an E3 ubiquitin ligase and expression of *parkin* in Drosophila eyes suppresses *α-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 *α-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 *α-synuclein*-expressing flies. BBE extract increases the expression of intrinsic antioxidant defense enzymes like *catalase* (*Cat*), *Sod1*, and *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-Synucleinpathic 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.

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TABLES

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

Genotype (food medium)	Median survival (days)
w^{1118}; *UAS-lacZ/Ddc-Gal4* (control)	82[a]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (control)	52[b]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (1 mg/ml BBE)	54[b]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (5 mg/ml BBE)	60[c]

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 *α-synuclein*-induced early mortality. **A** Directed expression of *α-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 α-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-α-synuclein/Ddc-Gal4* (αSYN). 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 w^{1118}; *UAS-lacZ/GMR-Gal4* (control) and w^{1118}; *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

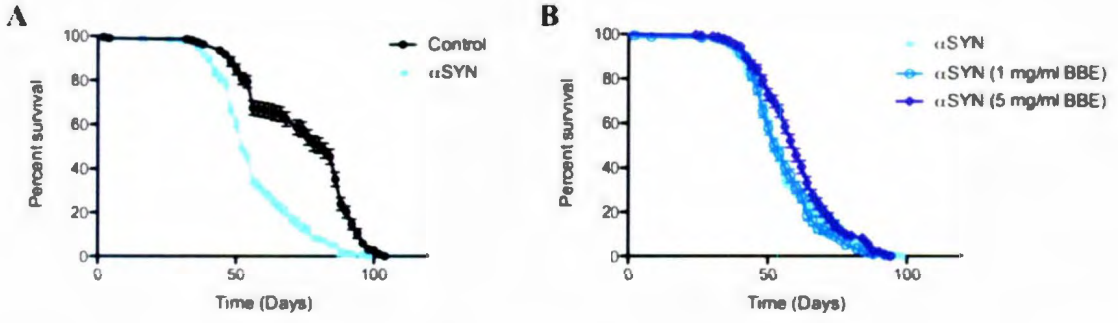

Figure 1 - Blueberry extract (BBE) partially protects *Drosophila melanogaster* against *α-synuclein*-induced early mortality

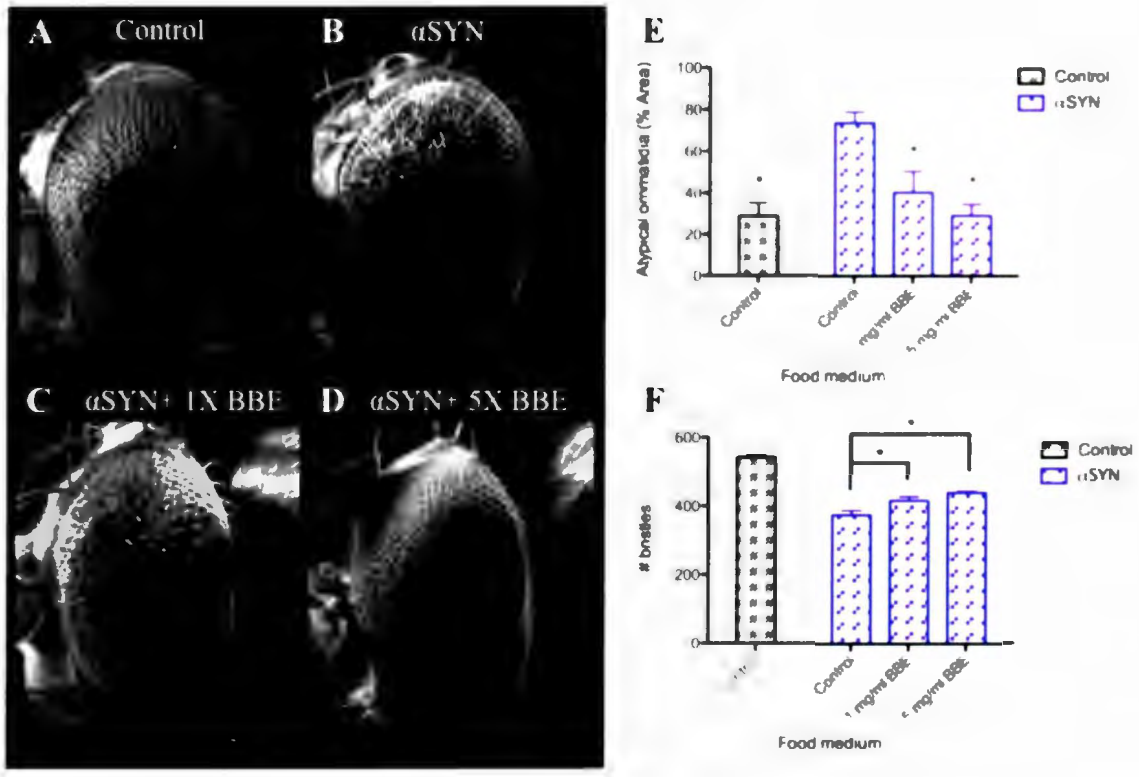

Figure 2 - Blueberry extract (BBE) supplementation counteracts *α-synuclein*-induced developmental defects of the eye

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 *α-syn*-induced phenotypes.

Table A2.1 - Median survival times of *α-synuclein*- and *lacZ*-expressing *D. melanogaster* fed control media from two independent experiments

Genotype	Median survival (days)
w^{1118}; *UAS-α-syn/Ddc-Gal4* (1)	46[a]
w^{1118}; *UAS-α-syn/Ddc-Gal4* (2)	52[b]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (1)	54[c]
w^{1118}; *UAS-α-syn/+; Ddc-Gal4/+* (2)	70[d]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (1)	58[e]
w^{1118}; *UAS-lacZ/Ddc-Gal4* (2)	82[f]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (1)	52[g]
w^{1118}; *UAS-lacZ/+; Ddc-Gal4/+* (2)	82[h]

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