Title
The effect of green tea polyphenols on Drosophila melanogaster lifespan and healthspan

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The effect of green tea polyphenols on *Drosophila melanogaster* lifespan and healthspan

DISSErTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Pharmacological Sciences

by

Terry Enriquez Lopez

Dissertation Committee:
Associate Professor Mahtab Jafari, Chair
Professor Richard Chamberlin
Professor Laurence D. Mueller

2016
DEDICATION

To my mom and dad,
for their love and support

And to my sisters,
for always believing in me
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>xii</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>xiii</td>
</tr>
<tr>
<td>ABSTRACT OF THE DISSERTATION</td>
<td>xiv</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Drosophila melanogaster as a Model Organism for Aging Studies</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Screening Algorithm and Candidate Compounds</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Green tea: History, Cultivation, Preparation and Health Benefits</td>
<td>4</td>
</tr>
<tr>
<td>1.4 Green tea Phytochemistry and Metabolism</td>
<td>5</td>
</tr>
<tr>
<td>1.5 Green tea’s Antioxidant/Pro-oxidant and Hormetic Mechanisms</td>
<td>7</td>
</tr>
<tr>
<td>1.6 Lifespan Eextension by Green Tea in Humans and Experimental Animal Models</td>
<td>9</td>
</tr>
<tr>
<td>1.7 Lifespan Extension by Iron-based Mechanisms of Green Tea Polyphenols</td>
<td>10</td>
</tr>
<tr>
<td>1.8 Iron in Physiological Processes in Male Drosophila Fertility</td>
<td>12</td>
</tr>
<tr>
<td>1.9 The Impact of Reproduction on Lifespan</td>
<td>13</td>
</tr>
<tr>
<td>1.10 Hypothesis and Scope of Study</td>
<td>14</td>
</tr>
<tr>
<td>1.11 References</td>
<td>17</td>
</tr>
<tr>
<td>CHAPTER 2: GREEN TEA POLYPHENOLS EXTEND THE LIFESPAN OF MALE DROSOPHILA MELANOGASTER WHILE IMPAIRING REPRODUCTIVE FITNESS</td>
<td>26</td>
</tr>
</tbody>
</table>
# 2.1 Introduction

# 2.2 Materials and Methods

## 2.2.1 *Drosophila* Strains and Experimental Conditions

## 2.2.2 Dietary Restriction Lifespans

## 2.2.3 Mitochondrial Assays and Enzymatic Activities

## 2.2.4 Environmental and Oxidative Challenges

## 2.2.5 Male Fertility

## 2.2.6 Male Virility

## 2.2.7 Statistical Analysis

# 2.3 Results

## 2.3.1 Green Tea Polyphenols Extend the Lifespan of Male Flies Only at the Highest Dietary Yeast Content

## 2.3.2 Green Tea Polyphenols Do Not Alter Oxidative Energy Metabolism

## 2.3.3 Green Tea Polyphenols Protect Against Iron Toxicity

## 2.3.4 Green Tea Polyphenols Compromise Male Reproductive Fitness

## 2.3.5 Green Tea Polyphenols Have No Effect on the Lifespan of Male Flies Housed in the Absence of Females

# 2.4 Discussion

# 2.5 References
3.1 Introduction

3.2 Materials and Methods

3.2.1 Green Tea

3.2.2 Fruit Fly Strains and Experimental Conditions

3.2.3 Toxicity and Developmental Assay

3.2.4 Size and Weight of Emerged Offspring

3.2.5 Measurement of DNA Content

3.2.6 Fertility Assay

3.2.7 Dissections of Testes and Ovaries

3.2.8 Measurement of Water, Lipid and Protein Contents

3.2.9 Stress Assays

3.2.10 Gene Expression Assay

3.2.11 Lifespan Assay

3.2.12 Statistical Analysis

3.3 Results

3.3.1 Composition of Green Tea Polyphenols

3.3.2 Green Tea Polyphenols Impair Development and Reduce Offspring Size and Weight at a High dose

3.3.3 Green Tea Polyphenols Reduce Cells Numbers

3.3.4 Green Tea Polyphenols Fed During Larval Stages Impact Subsequent Reproductive Output in Females

3.3.5 Green Tea Polyphenols Cause Morphological Defects in Reproductive Organs
3.3.6 Green Tea Polyphenols Increase Water Content and Decrease Lipid Levels Without Effecting Protein Levels 57

3.3.7 Green Tea Polyphenols Confer a Modest Protection Against Desiccation but Sensitize Flies to Starvation, Heat and Oxidative Stress 58

3.3.8 Green Tea Polyphenols Reduce Adult Fly Survival in Females but Has No Effect on Male Lifespan 60

3.4 Discussion 60

3.5 References 66

CHAPTER 4: GREEN TEA POLYPHENOLS REQUIRE MITOFERRIN AND THE DIVALENT-METAL TRANSPORTER-1 HOMOLOG, MALVOLIO, FOR LIFESPAN EXTENSION IN DROSOPHILA MELANOGASTER 72

4.1 Introduction 72

4.2 Materials and Methods 74

4.2.1 Fly Strains and Experimental Conditions 74

4.2.2 Total Body and Mitochondrial Iron Levels 75

4.2.3 Fertility 76

4.2.4 Gene Expression 76

4.2.5 Lifespan 77

4.2.6 Statistical Analysis 77

4.3 Results 78

4.3.1 Iron Deprivation Negatively Affects Male Fly Fertility 78
4.3.2 Green Tea Decreases Total and Mitochondrial Iron Levels in Flies 78
4.3.3 Hypomorph Mutants for Mitoferrin, Transferrin and Mvl Have
    Increased Lifespans and Reduced Male Fly Fertility 79
4.3.4 Green Tea Has No Effect on Lifespan, but Increases Male Fly
    Fertility of Mitoferrin and Mvl but Not Transferrin Hypomorphs 80
4.3.5 Green Tea Up-regulates the Expression of Mitoferrin and Malvolio,
    but not Transferrin in Hypomorph Mutants and w^{118} Flies 83
4.4 Discussion 84
4.5 References 90

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS 95
5.1 Summary of Dissertation 95
5.2 Future Directions 99
    5.2.1 Green Tea Consumption and Metabolism in Drosophila –
        Extrapolation to Humans 99
    5.2.2 Evaluation of Reproductive Effects Pertaining to Sperm
        Physiology and Requirements 101
    5.2.3 Modulation of Iron Regulators and Their Role in
        Lifespan Extension 102
5.3 References 104

APPENDIX A: Capillary Feeding of Green Tea Polyphenols and Measurements 109
APPENDIX B: Verification of Green Tea Consumption in Flies Using Food Dyes 110
<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 1.1.</strong> Effect of green tea extract on the lifespan of male and female JIV flies.</td>
<td>3</td>
</tr>
<tr>
<td><strong>Figure 1.2.</strong> Green tea catechins.</td>
<td>6</td>
</tr>
<tr>
<td><strong>Figure 1.3.</strong> Antioxidant activities of tea polyphenols.</td>
<td>7</td>
</tr>
<tr>
<td><strong>Figure 1.4.</strong> Fenton reaction.</td>
<td>8</td>
</tr>
<tr>
<td><strong>Figure 1.5.</strong> Iron chelation mechanism of tea polyphenols.</td>
<td>11</td>
</tr>
<tr>
<td><strong>Figure 1.6.</strong> Comparison of iron metabolism in mammals and <em>Drosophila melanogaster</em>.</td>
<td>13</td>
</tr>
<tr>
<td><strong>Figure 1.7.</strong> Schematic of hypothesis – Proposed mechanism of green tea polyphenols (GTP) on the fertility and lifespan of male <em>Drosophila melanogaster</em>.</td>
<td>15</td>
</tr>
<tr>
<td><strong>Figure 2.1.</strong> The impact of dietary yeast manipulations on the lifespan of male and female fruit flies supplemented with green tea polyphenols.</td>
<td>30</td>
</tr>
<tr>
<td><strong>Figure 2.2.</strong> Effect of green tea polyphenols on antioxidant defenses, mitochondrial superoxide and fumurase levels.</td>
<td>32</td>
</tr>
<tr>
<td><strong>Figure 2.3.</strong> Impact of green tea polyphenols on the tolerance to oxidative challenges.</td>
<td>33</td>
</tr>
<tr>
<td><strong>Figure 2.4.</strong> Effect of green tea polyphenols on lipid and water content.</td>
<td>34</td>
</tr>
<tr>
<td><strong>Figure 2.5.</strong> Impact of green tea polyphenols on the tolerance to environmental stress.</td>
<td>34</td>
</tr>
<tr>
<td><strong>Figure 2.6.</strong> Dose dependent effect of green tea polyphenols on male fertility</td>
<td>35</td>
</tr>
<tr>
<td><strong>Figure 2.7.</strong> The effect of green tea polyphenols on mating behavior.</td>
<td>36</td>
</tr>
<tr>
<td><strong>Figure 2.8.</strong> The requirement of females in the action of green tea.</td>
<td>37</td>
</tr>
<tr>
<td><strong>Figure 3.1.</strong> The effect of green tea polyphenols on development</td>
<td>53</td>
</tr>
<tr>
<td><strong>Figure 3.2.</strong> Phenotypic effects of emerged offspring from food treated with green tea polyphenols</td>
<td>54</td>
</tr>
<tr>
<td><strong>Figure 3.3.</strong> DNA content in flies treated with green tea polyphenols.</td>
<td>55</td>
</tr>
</tbody>
</table>
Figure 3.4. Reproductive output of adult Drosophila melanogaster fed green tea polyphenols during larval stages.

Figure 3.5. The effect of green tea polyphenols on reproductive organs.

Figure 3.6. The effect of green tea polyphenols on water, lipid and protein content.

Figure 3.7. The effect of green tea polyphenols on the tolerance towards desiccation, starvation, heat and paraquat.

Figure 3.8. The effect of green tea polyphenols on heat shock proteins

Figure 3.9. Drosophila male and female lifespan after treatment with green tea polyphenols during development

Figure 4.1. Effects of iron availability on male Drosophila fertility.

Figure 4.2. The impact of green tea on total and mitochondrial iron levels in Drosophila

Figure 4.3. Background expression levels of mitoferrin and transferrin in hypomorph mutants.

Figure 4.4. Lifespan and fertility of mitoferrin, transferrin and malvolio mutants compared to a standard laboratory fly strain, w^{1118}.

Figure 4.5. The impact of green tea on lifespan and fertility of mitoferrin (dmfrn), transferrin (tsf1) and Malvolio(Mvl) mutants.

Figure 4.6. The effect of green tea on mitoferrin, Malvolio and tsf expression in w^{1118} and hypomorph mutants.

Figure A1. Capillary feeder (CAFE) feeder apparatus.

Figure B1. Verification of consumption in flies using colored dyes.

Figure C1. HPLC chromatogram of flies fed green tea polyphenols versus controls.
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 2.1.</strong></td>
<td>Mean lifespan values for green tea-fed versus control-fed flies under varying yeast content.</td>
<td>31</td>
</tr>
<tr>
<td><strong>Table 2.2.</strong></td>
<td>The effect of green tea polyphenols on mitochondrial respiration rates.</td>
<td>32</td>
</tr>
<tr>
<td><strong>Table 2.3.</strong></td>
<td>Mating proportions in virility experiment.</td>
<td>36</td>
</tr>
<tr>
<td><strong>Table 3.1.</strong></td>
<td>Composition of green tea polyphenols.</td>
<td>52</td>
</tr>
<tr>
<td><strong>Table 4.1.</strong></td>
<td>List of primer sequences.</td>
<td>77</td>
</tr>
<tr>
<td><strong>Table A1.</strong></td>
<td>Measurements of green tea consumption by capillary feeding.</td>
<td>109</td>
</tr>
</tbody>
</table>
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Associate Professor Mahtab Jafari, Chair

Green tea is a popular beverage believed to have many health benefits including reducing the risks of neurodegenerative disorders, cardiovascular disease, and some cancers. As such, it has been suggested as a candidate life-extending natural product. Rich in polyphenolic compounds known as catechins, green tea has been shown to increase the lifespan of various animal models including the fruit fly, Drosophila melanogaster. Despite this, inconsistencies in reported data have been cited. In this dissertation, green tea’s life extension mechanisms were explored in a standard laboratory Drosophila strain, w1118.

Green tea was found to increase male Drosophila lifespan by 19%, but had no effect on female lifespan. Interestingly, this effect was associated with a reduction in male fly fertility. This is important as impairments in reproduction can modulate fruit fly lifespan. Moreover, green tea showed a modest protection against iron-induced oxidative stress, likely by its ability to bind iron. Since iron has been evidenced to play an essential role in male fly fertility, this dissertation explored the unique interplay of green tea on iron homeostasis, fertility and Drosophila lifespan. Processes that can modulate fly healthspan,
such as development and reproduction, were evaluated. Green tea was found to delay larval development, reduce offspring sizes, and atrophied reproductive organs. From this it was suggested that green tea is toxic to *Drosophila*’s physiological processes. Green tea’s ability to chelate iron and modulate iron metabolism was evaluated using hypomorph mutants of iron-regulating proteins. Specifically, the lifespan and fertility of mitoferrin, a mitochondrial iron transporter, transferrin, an extracellular iron binding protein, and *Malvolio*, the fly homolog of the divalent-metal transporter-1, were characterized. Hypomorph flies displayed increased lifespans and reduced fertility than normal flies. Interestingly, green tea could no longer increase the lifespan of mitoferrin and *Malvolio* flies but did rescue the reduced male fertility phenotype. This was associated with an up-regulation of mitoferrin and *Malvolio* expression. Combined, these results support the possibility that green tea increases male *Drosophila* lifespan in part by the modulation of iron homeostasis that is essential for male fly fertility.
CHAPTER 1: INTRODUCTION

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Aging is the single greatest risk factor for chronic disorders and disease [1, 2]. This includes cancer, Alzheimer’s disease, and cardiovascular disease. Simultaneously, as individuals advance in age, the incidence of mortality increases. Although, aging is defined as physiological and metabolic disorders, no single biological mechanism is specific to the progression of aging [3]. Therefore, rather than targeting any specific disease or underlying biological abnormalities, slowing aging as a whole can help prevent the onset of chronic conditions and improve the quality of life.

While it would be ideal to evaluate the process of aging in human populations, lifespan studies in humans pose multiple logistical difficulties and ethical concerns. Aside from the varying genetic, environmental and cultural factors associated with each unique individual, detailed and reliable human studies are not feasible due to the average human lifespan being approximately 78 years. To study aging, an ideal model organism must be utilized that is relevant to human aging [4, 5]. A good model system is one which displays high genetic and physiological conservation with humans, is easy to manipulate genetically, has short generational turn-arounds, and generally has a short enough lifespan to monitor the aging process over a reasonable period of time [6]. The fruit fly, Drosophila melanogaster,
fits such needs for aging research and is considered an excellent model system to study aging.

1.1 *Drosophila melanogaster* as a Model Organism for Aging Studies

*Drosophila melanogaster* is one of the most commonly used and extensively studied organisms in biology. The fly has been used to investigate a number of developmental and cellular processes common to those of higher eukaryotes [6, 7]. Since the full genome sequencing of *D. melanogaster* in March 2000, it has been affirmed that flies are a suitable model to study human diseases, drugs, and toxins [8]. Moreover, known genetic signaling pathways involved in the process of aging are conserved between flies and humans. These include the target of rapamycin (TOR) and insulin/insulin-like growth factor signaling (IIS), two nutrient-sensing pathways that are known to modulate aging in both flies and humans [9]. Under normal conditions the TOR pathway regulates translation and growth through phosphorylation of downstream effectors. Inhibition of this pathway, however, is associated with lifespan extension. TOR inhibition can occur under unfavorable conditions, such as nutrient deprivation or stress, in which a switch to the synthesis of other proteins responsible for cell survival occurs. Similarly, IIS responds to circulating signaling molecules and regulates the synthesis of proteins, fats, and glycogen. Inhibition of IIS initiates a similar response as the inhibition of TOR which results in an overall increase in cell protection and defense systems thereby increasing lifespan. With a short lifespan of approximately 80-90 days in a standard *Drosophila* laboratory strain, rapid developmental cycle of 12-14 days at room temperature, powerful genetics, and conserved genetic pathways of aging, the fruit fly makes an excellent model for aging research.
1.2 Screening Algorithm and Candidate Compounds

Plant derived products are the foundation of modern medicine today. Plants have been used for thousands of years for their medicinal properties to relieve a vast array of ailments including but not limited to, anxiety, cardiovascular function, inflammation, neurological disorders and some cancers [10]. As such, they make excellent candidates to slow the aging process, prevent the onset of chronic disorders and increase lifespan [6]. In our lab, we utilized a pharmacological approach by subjecting numerous products to a screening algorithm that evaluates whether a candidate product with known medicinal properties demonstrates an ability to increase the lifespan and improve the healthspan of our model system, the fruit fly – Drosophila melanogaster [6]. In a preliminary screen of pharmaceutical and botanical agents, green tea was found to increase the lifespan of an outbred population, derived by PT Ives, in South Amherst, MA [11, 12], of male Drosophila melanogaster by 22% (Figure 1.1). No effect on female lifespan was observed. Further

![Figure 1.1. Effect of green tea extract on the lifespan of male and female JIV flies. GTP significantly increased survival in male flies (A), however had no effect on the survival of female flies (B). P-values were calculated by Mantel-Cox log rank test. The sample sizes are as follows and listed as control and green tea, respectively: males, n=88 and n=90 and females, n=103 and n=108.](image-url)
investigation into the mechanism underlying male fly lifespan extension by green tea was warranted including the evaluation of healthspan parameters [4, 13].

1.3 Green tea: History, Cultivation, Preparation and Health Benefits

The entire world drinks tea. Tea comes second only to water as the most consumed beverage worldwide. Categorized into three types, black, oolong and green tea, annual production and consumption of each is approximately 78%, 2% and 20%, respectively [14]. The discovery and drinking of tea likely originated during the Shen-Nong era in ancient China approximately 5000 years ago [15]. Used first as a medicinal for various illnesses, tea has since evolved as a widely accepted beverage. The commonly consumed tea today is typically derived from the plant *Camellia sinensis*, which is native to East Asia and the Indian Subcontinent but is cultivated worldwide in tropical and subtropical regions [15, 16].

*Camellia sinensis* is a perennial evergreen that usually grows as a small shrub and has characteristically murky green colored leaves with notched edges [16]. Tea is made by infusion of the dried leaves in hot water. While black, oolong and green tea are derived from the same plant, the process in which they are manufactured differentiates the three tea types. Unlike black and oolong tea, which are subjected to complete or partial fermentation after harvesting, green tea is the non-fermented product of *Camellia sinensis* and is prepared by drying and steaming the fresh leaves [17]. The preparation process of green tea allows the tea leaves to retain non-oxidized polyphenolic compounds which have been purported to elicit numerous beneficial health effects [17].

Often regarded as a broad-spectrum botanical and functional food, green tea has been extensively studied for its numerous health benefits [14, 17]. Among various investigations,
green tea has been reported to improve lipid profiles[18, 19], reduce the risks of coronary heart disease [20], control body weight [21, 22], increase plasma antioxidant capacity [16] and reduce the incidence of some cancers [23-25]. In addition, green tea’s chemical compounds have been implicated as neuroprotective agents in diseases such as Alzheimer’s disease and Parkinson’s disease [26] through the reduction of iron accumulation, reactive oxygen species (ROS) and inflammation.

1.4 Green tea Ptochemistry and Metabolism

Green tea has a complex composition with the dry weight of leaves consisting of approximately 15-20% proteins, 1-4% amino acids, 5-7% carbohydrates and 5% of various minerals and trace elements [17]. In addition, green tea is rich in polyphenols, the most widely distributed class of plant secondary metabolites [27]. Polyphenols are chemically defined by the presence of one or more aromatic rings bearing hydroxyl groups. In plant biology, polyphenols including flavonoids, play a role in leaf structure, UV protection, herbivore defense, nutritional allocation, wound sealing and programmed cell death [27, 28]. Tea polyphenols are predominantly catechins namely (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) (Figure 1.2). After ingestion, most tea constituents will be absorbed by the small intestine followed by a series of metabolic processes in the liver. Catechin metabolism involves glucuronidation or sulfation of the parent compounds followed by methylation. According to a clinical study in which healthy volunteers were given a 500 ml bottle of green tea infusion consisting of approximately, 648 μmol of flavan-3-ols, principally 230 μmol of EGCG, peak plasma concentrations, C_{max}, ranged from 25-126 nmol/L by 1.6 to 2.3 hours, T_{max} [29].
Interestingly, EGCG and ECG appeared unchanged chemically in plasma. It has been suggested that the galloyl group of these compounds inhibit phase II metabolism thereby maintaining the original structure. Furthermore, of the total ingested flavan-3-ols, 28% of epicatechin metabolites were recovered in urine, whereas only 11% of epicatechingallate metabolites were found. It has been speculated that EGCG and ECG are excreted from the body by other means, such as biliary excretion or uptaken by foam cells. These observations of the metabolic fate of green tea and its constituents are consistent with other investigations [30-32].

**Figure 1.2.** Green tea catechins. Tea polyphenols are predominantly catechins namely (-)-epigallocatechin-3-gallate (EGCG) (A), (-)-epicatechin-3-gallate (ECG) (B), (-)-epigallocatechin (EGC) (C) and (-)-epicatechin (EC) (D). Polyphenols are chemically defined by the presence of one or more aromatic rings bearing hydroxyl groups as depicted in the catechin structure (E).
1.5 Green tea’s Antioxidant/Pro-oxidant and Hormetic Mechanisms

Of all the tea types, green tea has a significantly higher polyphenolic content than black or oolong tea and its most copious and potent catechin, EGCG, has been attributed to green tea’s beneficial effects [16, 17]. Some of green tea’s beneficial effects can be owed to the antioxidant properties of its plant polyphenolic compounds [33]. The antioxidant capacity of tea catechins have been well-documented in several in vitro and chemical assays [34, 35]. Free radical species, or molecules with a lone pair of electrons, which are known to induce cell and tissue damage, are present in an array of chronic diseases. Green tea’s

![Antioxidant activities of tea polyphenols. Gallate and hydroxyl groups in tea polyphenols, such as in (-)-epigallocatechin-3-gallate (EGCG) (shown), eliminate free radical species by the transfer of a hydrogen atom to stabilize free radical molecules (A). Resulting superoxide products are then subjected to further stabilization by antioxidant enzymes, such as superoxide dismutase (SOD) which converts superoxide to H₂O₂ (B), and catalase, which converts H₂O₂ to water and oxygen (C). Tea polyphenols may also be involved in the enhancement of these antioxidant enzymes.](image)
antioxidant activities are due to its gallate and hydroxyl groups, which eliminate free radical species by the transfer of a hydrogen atom to stabilize free radical molecules (Figure 1.3A) [36]. Moreover, by a similar action, green tea has been shown to protect against lipid peroxidation, a radical reaction in which hydrogen atoms are taken from unsaturated fatty acids resulting in alkyl radicals that react with molecular oxygen to yield lipid hydroperoxyl radicals [16, 36]. Furthermore, as an anti-oxidant, green tea has been shown to improve mitochondrial function and up-regulate anti-oxidant enzymes such as superoxide dismutase (SOD) (Figure 1.3B), glutathione peroxidase, and catalase (Figure 1.3C).

However, as a potential chemopreventive agent, green tea may depend more on its pro-oxidant properties. In this manner, green tea may promote cytotoxicity of cancer cells by either directly producing hydrogen peroxide or in the presence of transition metals, reduce Fe (III) to Fe (II) which triggers the Fenton reaction to create more potent reactive oxygen species (ROS) such as hydroxyl radicals (Figure 1.4). As a result of green tea’s pro-oxidant actions green tea is considered a hormetric compound [37-39]. Hormetic compounds follow a bi-phasic dose response in which a low dose results in beneficial effects and a high dose results in toxicity [40, 41]. The low dose benefit is dependent on mild toxicity caused by the treatment and results in the induction of a number of defense systems such as antioxidant defenses, heat shock proteins or repair enzymes.

\[
\text{Fenton Reaction:}\ \\
(1) \ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \\
(2) \ \text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \cdot\text{OOH} + \text{H}^+
\]

**Figure 1.4.** Fenton reaction. Iron can readily catalyze the generation of reactive oxygen intermediates including hydroxyl radicals, superoxide and hydrogen peroxide based on the Fenton reaction.
1.6 Lifespan Extension by Green Tea in Humans and Experimental Animal Models

As a result of green tea’s array of health benefits, green tea has been suggested as a candidate life-extending natural product. Some meta-analysis investigations have found that green tea consumption is associated with a positive impact on mortality rates. In one study in which Chinese individuals aged 80 years and older were surveyed, found that tea consumption was associated with a lower risk of mortality by 10% than those who reported infrequent tea drinking [42]. In a similar study by the Chinese Longitudinal Healthy Longevity Survey (CLHLS), only men who drank tea almost everyday had a 10-20% lower risk of death [43]. Aside from mortality rates, older individuals who drink green tea displayed a better overall health status than non-tea drinkers. For instance, the Ohsaki Cohort 2006 Study reported that the incidence of functional disability declined in individuals who drank more than 1 cup of green tea per day [44]. Improved cognitive function [45, 46] and reduced incidence of neurodegenerative disorders [26, 47] have also consistently been reported with consumption of green tea.

There have been numerous studies evaluating the ability of green tea to increase of the lifespan of experimental animal models, including worms, flies and mice. In C. elegans, daily administration of 220 µM of EGCG, its most prevalent and active flavonoid, increased mean lifespan up to 14% [48]. This same study showed that under lethal oxidative stress, survival rates increased by 65%. However, other groups have determined that despite improved mean longevity under heat and oxidative challenges, EGCG could not extend the lifespan of C. elegans under normal culture conditions [49]. In Drosophila melanogaster, a diet of green tea extract increased male mean lifespan by 16%, with a corresponding up-regulation of superoxide dismutase (SOD) and catalase [50]. Another study reported a 21%
increase in male fly lifespan by inhibition of age-related iron accumulation [51]. In male C57BL/6 mice, one study reported a longer lifespan in green tea fed mice versus controls (801 ± 121.5 vs. 852.7 ± 88.2 days, p < 0.05) [52]. However, reports on heterogenous mice revealed no differences in lifespan [53, 54]. Another report in Wistar rats revealed that EGCG significantly increased median lifespan to 105 weeks compared to controls at 92.5 weeks [55]. This increase in lifespan was attributed with a reduction in age-associated inflammation, oxidative stress, liver and kidney damage [55].

1.7 Lifespan Extension by Iron-Based Mechanisms of Green Tea Polyphenols

A number of mechanisms have been investigated to describe green tea’s ability to increase lifespan in experimental animal models. One proposed mechanism centered on the concept of iron homeostasis during aging. Iron accumulation during aging is a common finding observed in various aging research studies including humans [56]. Mechanistically, if iron homeostasis is not maintained, iron can readily catalyze the generation of reactive oxygen intermediates including hydroxyl radicals, superoxide and hydrogen peroxide based on the Fenton reaction (Figure 1.4). The presence of hydroxyl radicals in immediate proximity to DNA can result in the abstraction of hydrogen atoms found on the deoxyribose sugar backbone leaving a DNA radical adduct ultimately resulting in strand scission [33]. Alternatively, hydroxyl radicals may damage nucleotide bases. In either case, DNA damage of this manner ultimately leads to genetic mutations, cell and tissue damage, or cell death. Similar to increased serum ferritin levels in aged individuals [57], Massie et al. [51] had observed that older fruit flies had increased levels of total body iron than young flies. Massie and colleagues further observed that flies supplemented with green tea in their diet
maintained lower levels of iron in their body throughout their life and lived longer than flies fed control diets. Other investigations have identified similar correlations between iron levels, aging, and associated diseases [58-61] revealing a strong interaction between iron and lifespan.

It is well known that many functional derivatives of polyphenol compounds are effective metal chelators. Structurally, the presence of catechol and gallate groups on green tea polyphenols is responsible for the binding and stabilization of iron [33]. In the presence of O₂, catechol and gallate groups can undergo an auto-oxidation reaction with ferrous, Fe²⁺, to yield a Fe³⁺-polyphenol complex (Figure 1.5). In this manner green tea acts to protect the

**Figure 1.5.** Iron chelation mechanism of tea polyphenols. The presence of catechol and gallate groups on green tea polyphenols, such as in (-)-epigallocatechin-3-gallate (EGCG) (shown), are responsible for the binding and stabilization of iron (A). In the presence of O₂, catechol and gallate groups can undergo an auto-oxidation reaction with ferrous, Fe²⁺, to yield a Fe³⁺-polyphenol complex (B).
cell by binding free iron that may cause cell damage. Tea polyphenols have also been found to inhibit the absorption of dietary iron at the gut intestinal monolayer by up to 83% [62-65].

1.8 Iron in Physiological Processes and in Male Drosophila Fertility

Despite the known toxicities associated with excess and unregulated iron, iron plays a crucial role in vital biochemical activities of all living organisms. In humans, iron is distributed widely throughout the body and is responsible for essential functions such as heme biosynthesis, oxygen transport, energy production, cellular proliferation, and neuron development [66]. In flies, iron acts as an essential mitogen for cell proliferation during larval growth and development [67, 68]. In addition, the fruit fly makes an excellent model to study the impact of various compounds on iron metabolizing pathways as many iron proteins are shared between flies and humans (Figure 1.6). Increasing evidence has also revealed the importance of iron in male Drosophila spermatogenesis [69, 70] after the characterization of mitochondrial ferritin sequences were identified to be predominantly expressed in testis [71].

Further studies on the role of iron and Drosophila fertility revealed that the mitochondrial iron transporter, mitoferrin, is required for normal reproductive functions [69, 72]. Hypomorph mutant flies, with reduced expression levels of mitoferrin, exhibited complete sterility, spermatid defects, and abnormal testis structures when subjected to low dietary iron conditions [69]. Male fly fertility was increased when iron was supplemented in the diet of these mitoferrin mutants [69]. From this study, a strong association between iron availability and male Drosophila fertility was established.
Figure 1.6. Comparison of iron metabolism in mammals and Drosophila melanogaster. Dietary iron, generally obtained in the ferric (Fe3+) form, is reduced to ferrous (Fe2+) and absorbed by intestinal epithelia in both mammals and Drosophila. Iron levels then post-transcriptionally regulate the expression of iron-binding proteins via the iron-responsive element (IRE) system where iron-regulatory proteins (IRP1 or IRP2) bind to mRNAs. Intracellular iron levels (ICIL) can then be stored in ferritin complexes, exported via transferrin to non-intestinal tissues or transported via mitoferrin into mitochondria for storage by mitoferritin.

1.9 The Impact of Reproduction on Lifespan

Candidate life-extending products must also be evaluated for their ability to improve healthspan [13]. Pharmacological interventions which cause negative impacts on healthspan are not appropriate candidates for lifespan extension [4]. The reasoning behind this is that negative impacts on healthspan may be indicators of unanticipated side-effects [4, 5, 7, 13]. In addition, interventions which impair essential processes may consequently affect lifespan extension [4, 11]. One established biomarker of evaluating healthspan in fruit flies is
reproduction. The inverse relationship between reproduction and lifespan is a widely recognized and established concept that has been demonstrated in various organisms [73]. Interventions, whether physical such as castration or pharmacological such as drugs, which limit reproduction can significantly increase lifespan in *Drosophila* [4, 74-76]. For example, female fruit flies that experienced high-mating events had a significantly shorter lifespan than females with incidences of low-mating [77]. Similarly, in males, male fruit flies never housed with females had a significantly longer lifespan than those that had females present [74]. Therefore, it is critical to evaluate whether the increase in lifespan by pharmacological agents is due to impaired reproduction. In female *D. melanogaster*, reproductive costs have been attributed to egg production, exposure to males and mating itself [77]. Whereas, in male flies, although reproductive costs are less understood, increased courtship rate, mating rate and production of accessory fluid and sperm have been reported to reduce lifespan [74, 76].

**1.10 Hypothesis and Scope of Study**

As described above, in a preliminary screening of pharmaceutical and botanical agents, green tea increased the lifespan of an outbred population of male *Drosophila melanogaster* by 22%, but had no effect on female lifespan. In Chapter 2 of this dissertation, I describe the initial work performed in a standard laboratory strain of male fruit flies, *w*^1118^. Within this work, I investigated the mechanism of lifespan extension of green tea polyphenols in *Drosophila* with respect to dietary restriction (DR) or reduced caloric intake without malnutrition [78, 79], mitochondrial energy metabolism [80], environmental [81] and oxidative stress resistance [82], and reproductive fitness [73] all of which are known to modulate fly lifespan. I observed that green tea elicited its life-extending benefit in a sex
specific manner, once again extending the lifespan of male flies only. Interestingly, the life-extending properties of green tea were not associated with alterations in antioxidant enzymes, modulating mitochondrial activities, or protection against oxidative or environmental insults, with the exception of iron. The sex-specific increase in male fly lifespan justified the need to evaluate the impact of green tea on reproductive fitness further.

**Figure 1.7.** Schematic of hypothesis – Proposed mechanism of green tea polyphenols (GTP) and effects on iron levels, fertility and lifespan of male *Drosophila melanogaster*. This schematic outlines that fruit fly lifespan is inversely related to fertility. High fertility is known to negatively impact *Drosophila* lifespan. Iron homeostasis has been demonstrated to play a role in *Drosophila* fertility. This hypothesis suggests that green tea polyphenols may modulate iron homeostasis which will in turn affect fertility, and ultimately the lifespan of male *Drosophila*. 
The established evidence of green tea’s ability to chelate iron, and increasing evidence for the role of iron in male *Drosophila melanogaster* reproduction and development, led me to question whether there is an interaction between the protective effects of green tea against iron toxicity and reduced reproductive potential by green tea, as described in **Chapter 2**. As mentioned above, iron metabolism has been evidenced to be essential for male *Drosophila* spermatogenesis, development and fertility [69, 72, 83]. Since green tea is a known metal chelator and inhibitor of dietary iron absorption, *I hypothesized that green tea polyphenols increase male Drosophila lifespan by modulating iron homeostasis essential for male reproductive systems* (Figure 1.7).

I first explored the effects of green tea on *Drosophila* healthspan. This included an in-depth evaluation of green tea’s effect on development, and reproduction which are described in **Chapter 3**. Since toxicity was apparent in developing offspring exposed to green tea, toxicity parameters were evaluated which included an examination of reproductive organs, ability to protect against stress, and measurement of the levels of heat shock proteins. I then examined the role of key iron metabolizing proteins on *Drosophila* lifespan and male reproduction in **Chapter 4**. For this, I utilized publicly available hypomorph mutants fly strains with reduced expression for iron metabolizing proteins and evaluated their lifespan and fertility with and without green tea supplementation. In addition I measured expression levels of iron-related genes in normal and mutant strains also in the presence or absence of green tea supplementation. **Chapter 5** summarizes key findings, provides a speculation of the relationship between iron metabolism, green tea, and lifespan extension in male *Drosophila*, and promotes future work.


1.11 References


CHAPTER 2: GREEN TEA POLYPHENOLS EXTEND THE LIFESPAN OF MALE DROSOPHILA MELANOGASTER WHILE IMPAIRING REPRODUCTIVE FITNESS

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2.1 Introduction

Here, we report that green tea polyphenols (GTP) extend the lifespan of male fruit flies while simultaneously reducing male fertility. A number of compounds that had negatively impacted reproduction had also resulted in an increase in *Drosophila* lifespan [1, 2]. This inverse relationship is due to the fact that reproduction is a high energy and resource dependent expenditure in which fruit flies trade late life survival for increased reproduction [3, 4]. Female fruit flies exhaust their energy resources in the production of eggs [4]. Male fruit flies, however, require an exertion of energy during mating which involves a complex ritual of courtship behaviors [5, 6]. Furthermore, negative impacts on reproductive fitness not only have the ability to increase lifespan but, if a treatment is involved, it can also reveal a potential adverse effect associated with the treatment [1, 2].

In this work, we investigated the gender specific effects of green tea polyphenols (GTPs) on *Drosophila* lifespan and evaluated the impact of GTP’s on dietary restriction, oxidative energy metabolism, stress resistance and reproductive fitness. We concluded that
green tea polyphenols extend male fly lifespan by protecting against iron toxicity and negatively impairing reproductive fitness.

## 2.2 Materials and Methods

### 2.2.1 Drosophila Strains and Experimental Conditions

All assays were performed using w^1118^ flies (FBID #3605) obtained from the Bloomington Drosophila Stock Center (BDSC) at Indiana University, USA via FlyBase.

Standard *Drosophila* banana-molasses food was used in all feeding assays. Control diets included a 75 µL yeast solution (2 g yeast/52 mL 1% acetic acid) overlay on food, which was allowed to dry and refrigerated for at least 24 h prior to use. Treatment diets consisted of 10 mg/mL green tea polyphenols (GTP) consisting of 47% EGCG, purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), which was added to the yeast solution.

Flies were maintained 12 to a vial (6 males and 6 females) at 22 ± 1°C under 12 h light:dark cycles. Flies were fed either control or treatment diets for the duration of the experiment. Every 2 days, flies were transferred to new vials containing fresh food.

### 2.2.2 Dietary Restriction Lifespans

A total of 120 flies per treatment group per sex were fed either control or GTP throughout their life. Yeast concentrations, both in food and yeast solution, were varied at 0.1%, 0.3%, 1%, 3%, and 9%. Every 2 days, flies were transferred to fresh food and deaths were recorded.

### 2.2.3 Mitochondrial Assays and Enzyme Activities

Mitochondrial isolation, respiration and fumurase activity were performed as detailed in Schriner et al., 2012 [7]. Rates of mitochondrial superoxide production were
measured with the fluorescent dye MitoSOX (Invitrogen, Carlsbad, CA) based on a protocol by Robinson et al., 2006 [8]. SOD activities were determined as described in Winterbourn et al., 1975 [9]. Catalase activity was measured by direct decomposition of H$_2$O$_2$ as detailed by Beers and Sizer 1952 [10].

2.2.4 Environmental and Oxidative Challenges

All environmental and oxidative challenges were performed as outlined in Schriner et al., 2012 [7]. Death counts were recorded as follows: every hour under heat stress, every two hours under desiccation and every four hours for oxidative challenges. Water and lipid content in flies were also determined using methods described in Schriner et al., 2013 [11].

2.2.5 Male Fertility

Flies were fed a diet of 0, 5, 10, 20 or 40 mg/mL GTP. Following feeding, a single control or treated male was placed in a food vial along with a virgin female (n = 20 per treatment group). Mating pairs were transferred to fresh non-treated food everyday at the same time for 10 days. Offspring were allowed to develop and counted 2 weeks later.

2.2.6 Male Virility

A single virgin female was paired with either a control or GTP-fed male in an individual well of a 24-well plate (12 wells per treatment, n = 3). Mating behaviors were recorded using a webcam connected to a laptop. Male virility was assessed by two different measurements: mating latency and copulations. Mating latency was recorded as the time required for a male to initially mount a female. Copulation was recorded as the total time a male was in a mounted position.

2.2.7 Statistical Analysis
All statistical analysis was performed using Prism (GraphPad Software, San Diego, CA). The Mantel-Cox log rank test was used to evaluate fly survival. Mean lifespans were evaluated using the Mann-Whitney nonparametric test. Statistical evaluation of differences between treatment groups was analyzed by students t-test. Fisher’s exact test was used to validate mating proportions for the virility assay. P-values less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 Green Tea Polyphenols Extend the Lifespan of Male Flies Only at the Highest Dietary Yeast Content

Dietary restriction (DR), defined as decreased caloric intake without malnutrition, is the most notable intervention for lifespan extension in model organisms [12-14]. In flies, DR is performed by decreasing dietary yeast content. This increases lifespan up to a point at which a further reduction will shorten lifespan. A compound or extract acting as a DR mimetic will increase lifespan at the highest dietary yeast content and compromises survival at the lowest dietary yeast content due to a deprivation of nutrients of reduced yeast and the added effect of the compound or extract [12-15]. We observed that GTP significantly extended mean lifespan of male w1118 flies by 19% at the 9% yeast content (Figure 2.1A; Table 2.1). Surprisingly, GTP severely compromised male survival at the 1% yeast content (p < 0.05). There were no differences in female mean lifespan at all yeast levels (Figure 2.1B). Since 9% dietary yeast content resulted in the highest lifespan, for all subsequent experiments, we chose to use this diet.
2.3.2 Green Tea Polyphenols Do Not Alter Oxidative Energy Metabolism

Normal aerobic metabolism, which uses oxygen as an electron acceptor resulting in the production of reactive oxygen species (ROS) is suggested to play a role in aging and age-related disorders [16-19]. It is therefore purported that enhancement of antioxidant enzymes such as SOD, which converts superoxide to H$_2$O$_2$, and catalase, which converts H$_2$O$_2$ to water and oxygen, may attenuate age-related oxidative damage, thus resulting in the extension of lifespan [20, 21]. We investigated the ability of GTP to enhance these antioxidant enzymes. We found that GTP-fed flies showed no alteration in SOD or catalase levels (Figure 2.2A and 2.2B). Furthermore, GTP did not alter mitochondrial superoxide production in flies (Figure 2.2C) and had no effect on fumurase activity levels, an integral enzyme in the TCA cycle and indicator of mitochondrial content (Figure 2.2D). We further show that GTP had no impact on...
on mitochondrial respiratory activity (Table 2.2). In addition to enzymatic assays, SOD and catalase, other forms of oxidative stress were used to test green tea’s protective effects against ROS. Consistent with the results from enzymatic assays, GTP did not confer protection against paraquat, a superoxide generator (Figure 2.3A) or H2O2 (Figure 2.3B).

Table 2.1. Mean lifespan values for green tea-fed versus control-fed flies under varying yeast content.

<table>
<thead>
<tr>
<th>% Yeast Content</th>
<th>Sex</th>
<th>Dose (mg/ml)</th>
<th>Mean</th>
<th>P-value</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>♂</td>
<td>0</td>
<td>40.0 ± 1.9</td>
<td>0.13</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>43.5 ± 1.9</td>
<td>0.13</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>0</td>
<td>39.6 ± 1.7</td>
<td>0.41</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>38.0 ± 1.8</td>
<td>0.41</td>
<td>86</td>
</tr>
<tr>
<td>0.3</td>
<td>♂</td>
<td>0</td>
<td>51.8 ± 2.0</td>
<td>0.95</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>52.7 ± 1.8</td>
<td>0.95</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>0</td>
<td>46.3 ± 2.1</td>
<td>0.58</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>45.8 ± 1.8</td>
<td>0.58</td>
<td>88</td>
</tr>
<tr>
<td>1</td>
<td>♂</td>
<td>0</td>
<td>58.1 ± 2.0</td>
<td>0.03*</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>52.4 ± 1.9</td>
<td>0.03*</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>0</td>
<td>53.9 ± 2.0</td>
<td>0.22</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>51.0 ± 1.9</td>
<td>0.22</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>♂</td>
<td>0</td>
<td>51.5 ± 2.3</td>
<td>0.47</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>49.3 ± 2.3</td>
<td>0.47</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>0</td>
<td>37.9 ± 1.5</td>
<td>0.15</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>40.7 ± 1.3</td>
<td>0.15</td>
<td>84</td>
</tr>
<tr>
<td>9</td>
<td>♂</td>
<td>0</td>
<td>36.6 ± 1.7</td>
<td>0.007*</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>43.5 ± 1.8</td>
<td>0.007*</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>0</td>
<td>27.7 ± 1.4</td>
<td>0.30</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>30.4 ± 1.7</td>
<td>0.30</td>
<td>78</td>
</tr>
<tr>
<td><em>(males only)</em></td>
<td>♂</td>
<td>0</td>
<td>39.7 ± 1.9</td>
<td>0.92</td>
<td>72</td>
</tr>
</tbody>
</table>

Values are means ± sem. Units are days. P-values were calculated by Mann-Whitney analysis, control vs. green tea polyphenols (GTP). †Lifespan performed in the absence of females. *P-values < 0.05 are statistically significant.
Table 2.2. The effect of green tea polyphenols on mitochondrial respiration rates.

<table>
<thead>
<tr>
<th>Diet</th>
<th>State 3</th>
<th>State 4</th>
<th>RCR</th>
<th>Uncoupled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>203 ± 16</td>
<td>53 ± 3</td>
<td>3.8 ± 0.2</td>
<td>422 ± 25</td>
</tr>
<tr>
<td>Green Tea</td>
<td>216 ± 11</td>
<td>57 ± 1</td>
<td>3.8 ± 0.2</td>
<td>442 ± 13</td>
</tr>
<tr>
<td><em>p</em>-values</td>
<td>0.53</td>
<td>0.23</td>
<td>0.82</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Rates are mean ± sem, n = 6 (50 flies per sample). Units are nmol O₂/min/mg protein except for RCR, which is a ratio of state 3/state 4.

Figure 2.2. Effect of green tea polyphenols on antioxidant defenses, mitochondrial superoxide and fumurase levels. Green tea polyphenols did not alter superoxide dismutase (A) or catalase (B) levels. Furthermore, GTP had no effect on superoxide production (C) or fumurase activities (D). Data are presented as means ± sem, n= 6 (50 flies per sample). Unpaired t-test, *p*-values > 0.05.
2.3.3 Green Tea Polyphenols Protect Against Iron Toxicity

The action of GTP on environmental stressors was also evaluated. Previous studies in *Drosophila* have shown that flies selected for increased stress resistance have longer lifespans [22, 23]. Furthermore, this increased resistance to stress is positively correlated with lipid and water content [24, 25]. We therefore tested green tea’s ability to increase stress resistance. Although water levels were not impacted, GTP significantly increased lipid content (Figure 2.4; *p*-value < 0.05). Under conditions of desiccation and starvation, GTP afforded no protection to flies (Figure 2.5A and 2.5B). Moreover, GTP sensitized flies to heat stress (Figure 2.5C; *p* < 0.05). The ability of GTP to protect against iron stress was also evaluated. Flies fed GTP exhibited a modest protection against iron toxicity versus controls (Figure 2.5D; *p*-value < 0.0001). Green tea polyphenols appear to offer some protection against environmental stressors particularly iron toxicity.
Figure 2.4. Effect of green tea polyphenols on lipid and water content. Flies supplemented with GTP in their diet significantly increased lipid (A) but not water content (B). Data are presented as means ± sem, n = 6 (10 flies per sample). Data analyzed by unpaired t-test (p = 0.01 for lipid content and p > 0.05 for water content).

Figure 2.5. Impact of green tea polyphenols on the tolerance to environmental stress. Flies fed GTP conferred no protection against desiccation (A) or starvation (B). GTP slightly sensitized flies to heat stress (C) and afforded modest enhanced survival against Fe$^{3+}$-NTA (D). P-values were calculated by Mantel-Cox log rank test, n = 200 for all groups.
2.3.4 Green Tea Polyphenols Compromise Male Reproductive Fitness

In the evaluation of compounds and their effect on lifespan, it is critical to evaluate their impact on reproductive fitness as it is known that reduced reproduction will increase lifespan.\textsuperscript{18-20} We found that GTP significantly decreased the number of viable offspring produced from male flies at doses $\geq 10$mg/mL versus controls (Figure 2.6). We further investigated whether GTP impacted mating behavior by monitoring mounting time (copulation) and mating latency (time to mating). No effect on copulation was observed, however, GTP significantly increased mating latency (Figure 2.7; $p < 0.05$). To ensure that unsuccessful mating events did not affect experimental results, we evaluated mating and observed that both treatment groups had successful mating (Table 2.3).

![Figure 2.6](image)

**Figure 2.6.** Dose dependent effect of green tea polyphenols on male fertility. GTP significantly reduced mean male fertility at 10, 20 and 40 mg/mL doses over a 10-day period. Data are presented as means $\pm$ sem, $n=20$ per treatment group. Data points were removed where female deaths occurred. Data analyzed by paired t-test ($p > 0.05$ for 5 mg/mL, $p < 0.0001$ for 10 mg/mL, $p = 0.002$ for 20 mg/mL and $p < 0.0001$ for 40 mg/mL).
2.3.5 *Green Tea Polyphenols Have No Effect on the Lifespan of Male Flies Housed in the Absence of Female Flies*

Since GTP extends male lifespan with a corresponding decrease in fertility, we tested whether GTP acts through a negative impairment on reproduction. There are many ways to decrease reproductive potential. The simplest is to remove females. In the absence of females, we found that GTP could no longer extend male lifespan (Figure 2.8).

![Figure 2.7](image)

**Figure 2.7.** The effect of green tea polyphenols on mating behavior. Green tea polyphenols significantly increased mating latency versus controls. The proportion mating was equal in both treatment groups. Male virility data are presented as means ± sem and analyzed by Mann-Whitney test (p-value > 0.05 for copulation; p-value = 0.04 for mating latency).

<table>
<thead>
<tr>
<th>Mating Proportions</th>
<th>Control</th>
<th>Green Tea</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mated</td>
<td>25</td>
<td>24</td>
<td>49</td>
</tr>
<tr>
<td>Did not mate</td>
<td>11</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>36</td>
<td>72</td>
</tr>
</tbody>
</table>

Table 2.3. *Mating proportions in virility experiment.*

Fisher’s Exact Test

\[ p\text{-value} = 1.0 \]
2.4 Discussion

We report that green tea polyphenols (GTP) extend lifespan in male *Drosophila melanogaster*, impairs reproductive fitness in male flies and protect against iron toxicity. Lifespan extension properties of GTP were not associated with alterations in antioxidant enzymes, protection against oxidative or environmental insults, with the exception of iron, or altering mitochondrial energy metabolism.

Our results are somewhat contrary to that reported by Li et al. [26] They reported that Oregon-R (OR) flies supplemented with 10 mg/mL green tea had prolonged survival with a corresponding up-regulation of endogenous antioxidant enzymes SOD and catalase. This group also reported that green tea protected against paraquat and H$_2$O$_2$ [26]. The cause of this discrepancy in results could be due to numerous factors such as using different fly strains, type of green tea formulation used, feeding duration or that only males were used in

![Figure 2.8](image-url)
their study. Li et al extracted tea catechins from dry leaves of Chinese Longjing green tea with a 62% EGCG content. We utilized a primarily polyphenolic extract consisting of 47% EGCG. It has previously been shown that the extent of green tea’s beneficial health effects is highly dependent on its preparation method and dose [27, 28]. Most likely, the Chinese Longjing green tea contains other components contributing to an increase in SOD and catalase. Despite the reported up-regulation of antioxidant enzymes by Li et al, we report that green tea extends Drosophila lifespan without the modulation of these defense systems.

A notable effect of GTP is its ability to protect against exogenous sources of iron. Iron is known to accumulate in flies as they age, and the inhibition of iron accumulation has been shown to increase Drosophila lifespan [29]. The protection of GTP against iron, however, is not surprising as polyphenols are known for their metal chelating activities [30]. Furthermore, clinical reports have shown that green tea inhibits dietary iron absorption [31]. Since green tea has consistently shown a protective effect against sources of iron, presumably the mechanism associated with an extension in lifespan could be iron-related.

The action of GTP through dietary restriction (DR) was also investigated since this practice is known to increase the lifespan and improve the health of all laboratory species examined [15], with the possible exception of rhesus monkeys [32]. In the case of GTP, an extension in lifespan was noteworthy only at the highest dietary yeast content. While this would imply that GTP is acting by restricting caloric intake, no DR effects were observed in female flies (Figure 2.1B). Hence, if GTP is acting by restricting caloric intake, the effects should be observable in both sexes. Despite these observations, it is unclear as to why GTP extended the lifespan of males only and under specific dietary conditions (9% yeast content).
Another possible mode of action of green tea could be through hormesis. Hormesis occurs when a sub lethal dose of a compound or treatment (e.g., exercise, radiation, heat, or substance) is administered to an animal, which then confers an enhanced protection against further insults [33-36]. This secondary benefit is dependent on the mild toxicity caused by the treatment and is thought to be the result of the induction of antioxidant defenses, heat shock proteins, repair enzymes, etc. [37, 38]. In the case of green tea, its polyphenols have been shown to have pro-oxidant activities [34] and thus may actually be mildly toxic, consistent with hormesis as a plausible mechanism of action.

It is known that decreased reproduction is inversely related to lifespan in Drosophila [4-6]. Since GTP exhibited a gender specific effect by increasing the lifespan of male flies only, we investigated GTP’s impact on male reproductive fitness. We first investigated the effect of GTP on male fertility and observed that GTP significantly reduced male fertility at doses ≥ 10 mg/mL. This suggests that GTP may be negatively impacting sperm viability. We further questioned whether GTP was affecting mating behaviors. We observed that GTP did not affect total mounting time. Surprisingly, however, GTP significantly increased mating latency. It remains unclear as to why GTP is affecting time to mating, however, we speculate that GTP may be impairing central nervous system function since successful mating in Drosophila requires complex and precise movements [39]. Lastly, GTP increased the lifespan of male flies only when housed with females for the course of their life. Green tea polyphenols had no effect on the lifespan of males housed alone. Since courtship and reproduction has a high cost in energy expenditure, we presume that GTP is protecting male flies from the negative impacts of reproduction possibly by allocating energy for mating that can be used throughout the course of the male lifespan.
While the research underlying adverse impacts of GTP on male fertility is limited, some studies did identify significant negative effects of green tea on the reproductive system. An in vitro study investigating the impact of EGCG on human normozoospermic samples showed that EGCG significantly reduced sperm physiology and function at high doses [40]. The authors stated that the impact on sperm function is due to EGCG’s antiestrogenic and cytotoxic capabilities resulting in oxidative cellular damage of human sperm [40]. The authors further emphasized that the negative effects of EGCG is dose-dependent since low doses improved overall sperm motility and function. Other reports in rats showed that green tea has negative effects on male reproductive biology and endocrinology [41, 42]. Furthermore, male rats supplemented with green tea showed altered morphology and histology of testis and accessory sex organs. Additionally, a significant dose dependent decrease in sperm counts, serum testosterone levels, luteinizing hormone and inhibition of spermatogenesis was observed [42]. Our results are in line with these observations as there was a dose-dependent effect on male fertility. Despite this, no genotoxic or negative impacts on male reproductive systems have been associated with green tea consumption in humans [43, 44].

Based on our observations, we conclude that GTP is increasing male Drosophila lifespan by protecting against iron toxicity and impairing reproductive fitness. The link between iron and reproductive fitness has yet to be determined. However, recent research has revealed that iron may play a role in Drosophila spermatogenesis [45]. Presumably GTP’s metal chelating activities is preventing the uptake of iron which is essential for sperm function. This can then negatively impact male fertility and hence increase the lifespan of male flies.
2.5 References


CHAPTER 3: THE IMPACT OF GREEN TEA POLYPHENOLS ON DEVELOPMENT AND REPRODUCTION IN DROSOPHILA MELANOGASTER

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3.1 Introduction

Green tea, derived from the plant Camellia sinensis, is not only a popular drink worldwide but it is also considered a nutraceutical [1] as a neuroprotective [2], cardioprotective [3, 4] and anti-carcinogenic agent [5]. In spite of reported health benefits with green tea, there are some reports on possible adverse effects with its excessive consumption. In 2008, the US Pharmacoepia identified 34 cases of cytolytic and cholestatic liver damage associated with long term use or high usage of green tea products [6, 7]. Furthermore, green tea components, particularly its primary active flavonoid, epigallocatechin gallate (EGCG), has been shown to interact with and influence metabolism of a number of drugs [8, 9] and cytochrome P450 substrates [7, 10], which could result in therapeutic failure or toxic drug levels. During investigations of green tea’s actions on different organ systems, unwarranted effects have been cited at high doses suggesting limitations to its use and questioning its safety. Such effects include liver [11], gastrointestinal [7], hematological and renal toxicities [12], as well as decreased hormonal levels [13, 14], sperm counts [13, 15] and atrophy of reproductive organs [12, 16]. While
such toxicities have been cited in a number of reports, they are often over-looked, and their mechanisms are not fully understood. In line with some of these observations, we have previously reported that while green tea polyphenols (GTP) increased the lifespan of *Drosophila melanogaster*, it resulted in a reduction in male fertility [17].

The fruit fly, *Drosophila melanogaster*, with its evolutionary conserved biological pathways, is a commonly used model organism in biomedical research [18] and an emerging model to screen for adverse drug reactions [19]. Evaluation of drug induced developmental and reproductive effects is commonly used to assess adverse drug reactions [20, 21].

The *Drosophila* life cycle consists of distinct developmental stages that include embryogenesis, 1st, 2nd and 3rd larval instars, pupae and adults [22]. Each stage is highly regulated by transcriptional control in response to nutritional, environmental and hormonal cues [22]. Considering the highly conserved pathways between fruit fly and mammalian reproductive systems, the fruit fly is considered an excellent model system for the evaluation of drug toxicities [19]. Reproductive phenotypes, including egg production, mating behavior and fertility, are notable measurable characteristics. Female fecundity, defined as egg production, is a complex yet established phenotype to evaluate toxicity and has been used to evaluate the reproductive adverse effects of chemotherapeutic agents such as methotrexate [23, 24]. In addition, *Drosophila* male fertility, defined as production of viable offspring, is also considered a phenotype to evaluate drug induced toxicity since it has been shown to be influenced by a number of factors such as mating behavior, hormones, testes development, reproductive morphology, and spermatogenesis [25].

In this study, we observed that GTP at a high dose of 10 mg/mL resulted in delayed emergence, smaller offspring, morphological abnormalities in reproductive organs and
reduced reproductive output. Collectively, our findings indicate that green tea at high doses can negatively impact development and reproductive physiology.

3.2 Materials and Methods

3.2.1 Green Tea

Green tea polyphenols (GTP) were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). HPLC grade standards, individual catechins, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and included epigallocatechin gallate (98% EGCG), epicatechin gallate (98% ECG), epigallocatechin (95% EGC), epicatechin (98% EC) and internal standard ethylgallate (≥96% EG). Standards were dissolved in water/methanol (1:1, v/v) solution and quantified by ESI-LC/MS/MS using a Micromass Waters Quattro Premier XE (Waters Corp., Milford, MA, USA) coupled with an Acquity UPLC BEH C18 Column (Waters Corp.). The injection volume was 20 μL with an eluent flow rate of 0.3 mL/min. Gradient elution solvent A consisted of a mixture of water with 2% acetonitrile (ACN) and 0.2% ammonium acetate (5mM AA). Solvent B was ACN with 0.2% AA. The eluent gradient was ramped from 10% to 90% B in one minute. All acquisitions were performed in negative ion mode. Data acquisition and processing was performed using Waters MassLynx 4.1 (Waters Corp.).

3.2.2 Fruit Fly Strains and Experimental Conditions

All assays were performed using w^{1118} flies (FBID #3605) obtained from the Bloomington Drosophila Stock Center (BDSC) at Indiana University, Bloomington, IN, USA through FlyBase. Flies were maintained at 23°C ± 1°C and 60-70% humidity under 12-h light–12-h dark cycles. Standard banana–molasses food, composed of 9% carbohydrate content and 3.6% yeast content, was used in all feeding assays. For developmental feedings,
which included the exposure of embryos and larvae to GTP, food was prepared by mixing the
treatment within banana-molasses food and refrigerated for 24h. Unless stated, the GTP
dose that was used for all the experiments was 10 mg/ml in fly food. In our work, this dose
resulted in lifespan extension.

3.2.3 Toxicity and Developmental Assay

Toxicity assays were performed by preparing larval food as described above at 0, 2.5,
5, and 10 mg/mL concentrations of GTP. For each concentration, 6 flies per sex were placed
in each vial (n=120 per treatment) for egg laying. After 24 h, flies were removed and the
number of eggs laid was recorded. Larval development was checked every 24 h, and the
number of pupae and emerged offspring, including dates of occurrence, were recorded.

3.2.4 Size and Weight of Emerged Offspring

For size measurements, 6 flies per treatment (control or 10 mg/mL GTP) per sex were
randomly selected from the population of emerged offspring. Flies were imaged using a ruler
for scale, and pictures were analyzed using the open access Image Processing and Analysis
in Java program (Image J, NIH, Bethesda, MD, USA). Fly length was estimated with one line
from the top of the head to the end of the abdomen. Scaling in image J was set to
pixels/centimeter. To determine weight, flies were sedated on a CO₂ plate and weighed using
a Sartorius SE2 Ultra Micro Balance (Bradford, MA, USA) in groups of 10 (n=60 per
treatment, per sex).

3.2.5 Measurement of DNA Content

A total of 5 flies per sex, per treatment, were weighed and flash frozen. Flies were
homogenized in DNA isolation buffer (50 mM Tris-HCL, pH 8.0, 5 mM
ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 0.5% SDS) and supplemented with
proteinase K (0.5 mg/mL final concentration) and digested overnight at 55°C with mild shaking. The samples were then extracted by standard phenol-chloroform procedures and precipitated by ethanol. DNA content was quantified and normalized to fly weight.

3.2.6 Fertility Assay

Male fertility was performed as outlined in our previous work, Lopez et al. (2014) [17], with the following modifications: 6 male and 6 female mating pairs were used per vial instead of 1 mating pair. Embryos and larvae were exposed to GTP or control throughout their development. Offspring were collected for fertility assays.

3.2.7 Dissections of Testes and Ovaries

Six flies per sex from each treatment (control or GTP) were randomly selected for dissection. Using fine forceps, the internal reproductive organs were teased out of the abdomen into a drop of phosphate buffer saline (PBS). Samples were stained with DAPI nuclei stain (Sigma-Aldrich, St. Louis, MO, USA) and were visualized under fluorescence using a Zeiss Axio Scope.A1. (Carl Zeiss Industrial Metrology LLC., Maple Grove, MN, USA)

3.2.8 Measurement of Water, Lipid and Protein Contents

Water, lipid and soluble protein levels were measured as previously described in Schriner et al., 2013 [26]. Flies were collected and weighed as described above. Water content was determined after drying flies for 48 h at 70°C, re-weighing them and taking the difference in weights. Lipid content was determined after lipid extraction with diethyl ether. Samples were dried and re-weighed. Fat content was determined by taking the difference in the weights before and after diethyl ether extraction divided by the initial weight. Soluble protein was determined from the supernatant of homogenized flies and measured by reaction with Coomassie Brilliant Blue normalized to fly weight.
3.2.9 Stress Assays

A total of 120 flies per sex per treatment were used for each stress assay. For desiccation, flies were housed in empty vials and deaths were recorded every 2 hours. For starvation, flies were housed in vials containing 2% agarose to provide moisture without any food and deaths were recorded every 4 hours. To evaluate heat tolerance, flies were housed at 37°C and deaths recorded every hour. To evaluate the protection against superoxide, flies were exposed to 12.5 mM of paraquat (98% methyl viologen dichloride hydrate, Sigma-Aldrich, St. Louis, MO, USA) mixed in standard banana molasses food and deaths were recorded every 4 h. Survival analysis for all stress assays was determined by log-rank Mantel-Cox test.

3.2.10 Gene Expression Assay

Heat shock protein expressions were performed as described in Schriner et al. 2013 [26]. In brief, flies were frozen in groups of 10 and RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Samples were treated with DNase (New England Biolabs, Ipswich, MA, USA) and converted to cDNA by the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative PCR was performed on a MiniOpticon realtime PCR system with SYBR green dye (Bio-Rad). Relative amplification was calculated by the threshold cycle of each respective gene divided by the threshold cycle of the reference gene, RNA polymerase II. Primer sequences and product sizes were as follows. RNA pol II forward: AGGGCGGCGAGGACATGGAT, and reverse: CGACGCTGTAAGTGGACC. HSP70 forward: ACCAAGGGTGTTGCCCCAAGA, and reverse: CTGGCTTTGCCCCTGCTCA. HSP22 forward: TTGCGGATGCGCCGAGGAGA, and reverse: AGCGCCACTCCAACGGG. Primers were designed by NCBI/Primer-BLAST.
3.2.11 Lifespan Assay

A total of 120 emerged offspring from control and GTP were collected and utilized for lifespan assays. For this particular assay, the flies were no longer exposed to GTP-treated food throughout their life; the only exposure to GTP was during development as described above. Flies were transferred every 2 days to fresh food and deaths were recorded until all flies died.

3.2.12 Statistical Analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical evaluation of differences between treatment groups was analyzed by Student’s t-test. Data analysis of green tea and control groups separated by sex was analyzed by two-way ANOVA. The Mantel–Cox log-rank test was used to evaluate fly survival in stress and lifespan assays. *P* values < 0.05 were considered statistically significant.

### Table 3.1. Composition of green tea polyphenols

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% in GTP</th>
<th>RT (mins)</th>
<th>Transitions [M-H]-</th>
<th>Mean ± SD (µg/mL)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epigallocatechin gallate (EGCG)</td>
<td>54</td>
<td>0.83</td>
<td>458 &gt; 169</td>
<td>5.38 ± 0.16</td>
<td>3.0</td>
</tr>
<tr>
<td>Epicatechin gallate (ECG)</td>
<td>11</td>
<td>0.90</td>
<td>441 &gt; 289</td>
<td>2.18 ± 0.093</td>
<td>4.2</td>
</tr>
<tr>
<td>Epigallocatechin (EGC)</td>
<td>6</td>
<td>0.68</td>
<td>305 &gt; 125</td>
<td>2.46 ± 0.071</td>
<td>2.9</td>
</tr>
<tr>
<td>Epicatechin (EC)</td>
<td>8</td>
<td>1.45</td>
<td>289 &gt; 245</td>
<td>1.90 ± 0.18</td>
<td>9.8</td>
</tr>
</tbody>
</table>

GTP, green tea polyphenols; RT, retention time; SD, standard deviation; RSD, relative standard deviation

3.3 Results

3.3.1 Composition of Green Tea Polyphenols

Analysis of the GTP by ESI-MS revealed 54% EGCG, 11% ECG, 8% EC, 6% EGC and <0.1% caffeine as summarized in Table 3.1. The composition of the green tea that we used in
this study was consistent with the composition of standardized green tea extracts used in scientific studies [27].

3.3.2 Green Tea Polyphenols Impair Development and Reduce Offspring Size and Weight at a High Dose

The effect of green tea on *Drosophila* development was evaluated by exposing embryonic and larval stages to a mixture of GTP at varying doses. The number of eggs laid by adults exposed to GTP was normal at all doses (Figure 3.1A). No effects on development involving pupation and emergence were observed at the 0, 2.5 or 5 mg/mL doses but GTP at 10 mg/mL significantly inhibited the formation of pupae and subsequent offspring emergence (Figure 3.1A). A surprising finding was the extended delay in pupation and emergence of offspring as doses of GTP increased (Figure 3.1B).

Male and females from the GTP treatment group emerged as smaller adult flies compared to controls (Figure 3.2A and 2D). Recorded lengths from a random sample from each treatment showed a significant reduction in fly length by GTP for both males (Figure

---

**Figure 3.1.** The effect of green tea polyphenols on development. Green tea polyphenols fed to larvae during development had no effect on adult emergence at 0, 2.5, and 5 mg/mL doses (*P*>0.05). However, a significant reduction in pupae and adults was observed at the 10 mg/mL dose (*P*<0.0001) (A). Time to development was significantly increased as doses of GTP were increased (*P*<0.05) (B). Data are presented as means ± SEM, *n*=10 vials with 6 mating pairs (A) and *n*=3 experimental trials (B) and analyzed by two-way ANOVA.
3.2B) and females (Figure 3.2E). This observation was further confirmed by measuring weights from each group. A dose-dependent reduction in weight was observed in both sexes with the greatest reduction occurring at 10 mg/mL of GTP (Figure 3.2C and 2F).

3.3.3 Green Tea Polyphenols Reduce Cell Numbers

To evaluate whether the emergence of smaller offspring by GTP is the result of reduced cell size or reduction of cell numbers, we measured the total DNA content relative to fruit fly weights. We found no significant increase in the total DNA content/body weight ratio in either sex (Figure 3.3) which is consistent with fewer cells in GTP treated flies.

**Figure 3.2.** Phenotypic effects of emerged offspring from food treated with green tea polyphenols. Emerged offspring from the 10 mg/mL dose of green tea polyphenols were noticeably smaller in size than controls (A, D). Lengths of both males (B) and females (E) were significantly smaller after being exposed to 10 mg/mL GTP during development (n=6 per treatment per sex, ***P=0.0005, student’s t-test). Weight of emerged offspring also decreased in a dose dependent manner for both males (C) and females (F) with the greatest reduction occurring at the 10 mg/mL dose (n=60 per treatment per sex, *P=0.01, **P=0.001 and ***P<0.001).
3.3.4 Green Tea Polyphenols Fed During Larval Stages Impact Subsequent Reproductive Output of Adult Females

It is well established that reproductive output in flies, egg production from females or viable offspring from males, is a marker of toxicity in Drosophila [19, 25]. We evaluated the reproductive output of emerged offspring after being treated with GTP during development. Female offspring exposed to GTP exhibited a significant reduction in reproduction as adults compared to control (Figure 3.4A, 4B, 4C). The fertility of male flies, or number of eggs laid by treated males upon mating with females, was not affected by the treatment (Figure 3.4D, 4E). Interestingly, an increase in offspring numbers was observed (Figure 3.4F).

3.3.5 Green Tea Polyphenols Cause Morphological Defects of Reproductive Organs

Reduced fly fertility can be associated with a number of factors including morphological defects in reproductive organs [20, 28]. Male offspring emerged from control food had normal reproductive structures such as full testes, seminal vesicles and accessory
glands (Figure 3.5A). However, male offspring from the GTP group exhibited atrophy of reproductive organs and a dramatic reduction of the number of nuclei in testes (Figure 3.5B). Similarly, control females had normal ovaries and recognizable structures including ovarioles and mature eggs (Figure 3.5C), whereas emerged females from GTP group revealed smaller ovarian structures and absence of mature eggs (Figure 3.5D).

**Figure 3.4.** Reproductive output of adult *Drosophila melanogaster* fed green tea polyphenols during larval stages. The fertility of emerged offspring from GTP-treated food was determined by measuring the number of eggs, pupae and adults formed from each sex over a 10-day mating period. Female flies exhibited a significant reduction in overall reproductive output including eggs laid (A), pupae formed (B) and emerged offspring (C) (*P*<0.0001). Male flies showed no difference in fertility by the number of eggs laid (D) or pupae (E) formed (*P*>0.05) however, an increase in the number of emerged flies was observed (*P*=0.01) (F). Data are presented as means ± SEM (*n*=60 per treatment per sex) and analyzed by two-way ANOVA. *P*>0.05 are not significant.
3.3.6 Green Tea Polyphenols Increase Water Content and Decrease Lipid Levels Without Effecting Protein Levels

Increased water, lipid and protein levels are often associated with enhanced stress resistance and increased survival [29]. Offspring emerged from GTP food had increased water (Figure 3.6A) but reduced lipid (Figure 3.6B) contents in both sexes. No changes in soluble protein levels were observed (Figure 3.6C).
3.3.7 Green Tea Polyphenols Confer a Modest Protection Against Desiccation but Sensitize Flies to Starvation, Heat and Oxidative Stress

Flies emerged from GTP food were evaluated for their ability to confer protection against stress, a marker of health and survival in *Drosophila melanogaster* [30]. Flies emerged from GTP were resistant to desiccation (Figure 3.7A and 7B) but sensitized to starvation (Figure 3.7C and 7D) and heat stress (Figure 3.7E and 7F).

To evaluate the protective ability of GTP against oxidative stress, we subjected flies to paraquat, a superoxide generator. While we observed no significant differences in survival for males from paraquat exposure (Figure 3.7G), female survival was significantly reduced (Figure 3.7H). The expression levels of heat shock proteins (HSPs), specifically HSP70 and HSP22, were measured as a marker of cellular damage under a stress-induced environment, in this case caused by GTP. We observed that GTP caused a significant up-regulation in the expression levels of HSP70 (Figure 3.8A) and HSP22 (Figure 3.8B) in male flies. While an
increase in HSP70 and HSP22 may be present in females as well, the effect was not statistically significant.

Figure 3.7. The effect of green tea polyphenols on the tolerance towards desiccation, starvation, heat and paraquat. Offspring emerged from GTP-treated food exhibited a modest protection against desiccation in male (A) and female (B) flies. However, GTP sensitized offspring to starvation (C, D), and heat stress (E, F). Male offspring from GTP-treated food did not exhibit any significant reduction in survival with exposure to paraquat (G). Female offspring from GTP-treated food exhibited a significant reduction in survival when exposed to paraquat (H). The sample sizes were n=120 per sex and per treatment. P values were calculated by Mantel-Cox log rank test.

Figure 3.8. The effect of green tea polyphenols on heat shock proteins. Male offspring emerged from GTP-treated food exhibited a significant upregulation in the expression levels of HSP70 (P=0.002) (A) and HSP22 (P=0.004) (B). Although an increase in the expression levels of HSP70 and HSP22 was observed in females as well, the effect was not significant (P>0.05). Data are presented as means ± SEM (n=60 per treatment per sex) and analyzed by two-way ANOVA with Bonferroni post-test.
3.3.8 Green Tea Polyphenols Reduce Adult Fly Survival in Females but Has No Effect on Male Lifespan

Flies were fed GTP only during early development and the lifespan of emerged offspring was examined. Female flies exhibited a 17% reduction in overall lifespan (Figure 3.9A), whereas males were unaffected (Figure 3.9B).

3.4 Discussion

Previously, we reported that green tea polyphenols (GTP) fed to adult *Drosophila melanogaster* increased male mean lifespan by up to 19% but resulted in a decrease in male fertility at doses ≥10 mg/mL [17]. This adverse effect on male fertility was surprising and warranted further investigation on the extent of toxicity caused by green tea. In this work, we explored the impact of GTP on health by exposing *Drosophila melanogaster* to GTP during...
early stages of development. We surmised that since developing embryos and larvae are highly susceptible to environmental factors, exposing flies to GTP during early development would amplify subsequent negative phenotypic effects in emerged adults. Our results showed a number of phenotypic effects by GTP, most notably a delay in emergence, smaller offspring, reduced reproductive output by females and abnormalities of reproductive organs in both sexes. Moreover, we evaluated whether GTP provided any protection against stress and found that emerged GTP-treated offspring were resistant to desiccation but were sensitized to starvation and heat stress, phenotypes consistent with water and lipid changes in flies. Lastly, female flies emerged from GTP-treated food exhibited a significant decrease in survival whereas male fly lifespan was unaffected.

Numerous studies, particularly those investigating the effects of environmental pollutants on *Drosophila* development, have revealed that flies exposed to adverse food conditions emerge as smaller adults and require longer time periods for development [20, 31, 32]. It has been suggested that these observations are the result of somatic effects caused by the relevant treatment [20]. However, studies evaluating adverse effects of green tea on organismal development are scarce. Epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), which make up more than half of green tea’s polyphenolic composition, have been shown to induce embryonic toxicity and impair development in mouse blastocysts. These compounds also induced apoptosis with a corresponding decrease in cell numbers, decreased implantation rates, and resulted in embryos with lower fetal weight [33, 34]. These observations are consistent with our results showing that *Drosophila* embryos subjected to green tea-treated food had a remarkable reduction in offspring numbers, and the larvae that successfully emerged into adults were dramatically smaller in body size than
respective controls. We questioned whether the small body size was the result of a decrease in individual cell sizes or a decrease in cell numbers. If there was a decrease in cell size, the GTP treated flies would have the same number of cells, and hence total DNA content, as controls. Since GTP-treated flies are smaller compared to controls, this would result in an increased DNA content/body weight ratio, which we did not see. Therefore, our results are consistent with either elevated apoptosis or decreased cell division or both. The striking developmental defects by green tea can be attributed to green tea’s pro-oxidant and pro-apoptotic activity. In fact, green tea’s ability to induce apoptosis is the basis of its reported classification as an anti-carcinogenic agent by initiating cell-cycle arrest and growth inhibition in carcinoma cells [35-37]. This mechanism is likely the cause of green tea’s impairment of fly development since Drosophila development is highly dependent on cell cycle progression and cell survival, events which are influenced in part by environmental factors [31].

Hormetic effects of GTP may provide an explanation about these observed adverse effects. Hormesis is a phenomenon in which low dose exposure to a potential toxic compound is beneficial but higher doses of the same compound become harmful [38, 39]. In flies, beneficial effects after exposure to hormetic compounds can lead to increased resistance against further stressors such as environmental and oxidative stress [40, 41]. With the exception of desiccation, emerged offspring from GTP were more susceptible to heat, starvation and oxidative stress. The protective effect exhibited by GTP against desiccation and sensitization to starvation could be explained by the increased levels in water and decreased levels in lipid content, respectfully, since such phenotypes are known to be positively correlated with one another [29]. Green tea’s inability to protect against
superoxide free radicals could be due to a higher dose of GTP resulting in green tea’s pro-
oxidant toxic effect [42]. Some of our results may appear to disagree with our previous work
where we did not observe protective effects against desiccation, and GTP did not increase
water content [17]. The reason for this discrepancy is likely due to feeding GTP to adult flies
in our previous work versus exposing embryos to GTP in this work. Presumably, green tea
could act through different mechanisms at different stages of fly development. Early
embryonic development is a phase dependent on cell cycle progression and larval transitions
[22, 43] but in adult stages, flies are primarily post-mitotic [43]. Studies have shown that
green tea constituents can bind to DNA and RNA, inhibit replication, increase topoisomerase
II-mediated DNA cleavage, induce apoptosis and attenuate cell cycle progression at the
G1/G0 phase [36, 44-46]. It is plausible that GTP is acting in this manner to inhibit growth
and development of Drosophila melanogaster.

We observed remarkable abnormalities in the reproductive organs of both male and
female flies. While male testes showed fewer nuclei and were smaller in size, female ovaries
appeared to be almost entirely absent of mature eggs indicating that females emerged from
GTP-treated food were more susceptible to the effects of GTP than males. The toxic effects of
GTP on the female reproductive system were further evident by the reduced number of eggs
laid and viable offspring produced. Male fertility did not appear to be largely affected. These
sex-specific effects on male and female reproduction could be due to the difference in the
numbers and sizes of the male and female gametes. Since sperm are so small and numerous,
a significant reduction in gamete production could result in a nearly undetectable effect on
the fertility and organ morphology. Whereas in females, eggs make up a large proportion of
the female reproductive system, and similar degree of impairment in gamete production could result in marked morphological and reproductive effects.

Hormetic compounds, such as those found in green tea, are known to induce heat shock proteins (HSPs), stress proteins that function during harmful conditions to aid cell survival by binding and refolding damaged proteins [41]. Studies evaluating the toxic effects of chemical pollutants on Drosophila utilize the expression levels of HSPs, particularly the induction of HSP70, as a marker of stress response and cellular damage [20]. The small mitochondrial heat shock protein, HSP22, is essential in the maintenance of protein homeostasis and up-regulated in response to heat and oxidative stress [47, 48]. Additionally, both HSP70 and HSP22 are up-regulated during Drosophila aging [49]. We observed a sex-specific effect of GTP on HSP70 and HSP22. Emerged male offspring from GTP-treated food exhibited an up-regulation in HSP70 and HSP22. The effect was not observed in females.

Previous studies have shown that green tea and its primary active flavonoid, EGCG, induce HSPs as a protective mechanism to combat stress-induced environments and increase survival [50, 51]. In our study, HSPs were up-regulated by GTP treatment alone which is indicative of stress caused by the treatment itself.

Additionally, we explored the effect of GTP on HSP22 as it has been suggested as a predictive biomarker for Drosophila survival [52]. Yang and Tower (2009) [52] identified that flies robustly expressing HSP22 at a younger age tended to die sooner. Additionally, over-expression of HSP22 made flies more sensitive to heat and oxidative stress and reduced fly lifespan [47]. Similar to our results, flies exposed to GTP exhibited higher levels of HSP22 which sensitized flies to stress and compromised survival. Female flies, in particular, were more susceptible to oxidative stress by paraquat than male flies. In addition to sex-specific
differences that affect fly lifespan [53, 54] and those described above, we speculate the sensitivity to oxidative stress induced by GTP contributed to an overall shorter lifespan for females. Lastly, the observed difference in HSP expression between control and GTP-treated groups suggested that at high doses, GTP is detrimental and leads to cell loss and subsequent morphological changes.

In summary, our results demonstrate that high concentrations of green tea impair development and reproduction of Drosophila melanogaster, as demonstrated by delayed emergence, small offspring sizes, reduced offspring numbers and atrophy of reproductive organs. The conserved biological pathways, including similarities between reproductive and developmental genes and hormones in flies and humans, make flies a valid model organism to evaluate drug induced adverse effects [19, 24]. Our results indicate that when consumed at high doses, green tea could potentially be detrimental to human physiological processes such as development and reproduction. However, it is difficult to draw direct conclusions on human implications since the mechanisms of bioavailability of polyphenols in both mammals and Drosophila remain poorly understood.
3.5References


46. Saiko, P., et al., *Epigallocatechin gallate, ellagic acid, and rosmarinic acid perturb dNTP pools and inhibit de novo DNA synthesis and proliferation of human HL-60*


CHAPTER 4: GREEN TEA POLYPHENOLS REQUIRE MITOFERRIN AND THE DIVALENT-METAL TRANSPORTER-1 HOMOLOG, MALVOLIO, FOR LIFESPAN EXTENSION IN DROSOPHILA MELANOGASTER

4.1 Introduction

Green tea polyphenols (GTP) have been found to increase the lifespan of the fruit fly, Drosophila melanogaster [1-4]. Despite its well-documented health benefits in humans and among various experimental animal models (i.e., worms, flies and mice), green tea’s life-extending mechanisms are not well understood [2, 5-7]. Some reports have suggested that green tea’s iron-binding activity and thus reduction of oxidative stress is the basis of its beneficial health and life-extending effects [4, 8-10]. However, the precise relationship between green tea and the modulators of iron metabolism has not been established.

Green tea polyphenols, known as catechins, most notably epigallocatechin gallate (EGCG), have been purported to be responsible for green tea’s numerous biological effects [11-13]. Phenolic compounds have been described as multi-functional antioxidants with metal-chelating abilities, such as iron binding [14, 15]. Iron is a micronutrient that is both essential for and toxic to all living organisms. Free iron can readily catalyze the generation of reactive oxygen intermediates such as hydroxyl radicals which can lead to cell and tissue damage [16]. Iron has also been shown to accumulate during aging and a decrease in aging-induced iron accumulation has been found to increase the lifespan of various experimental animal models [4, 17-19]. For example, fruit flies supplemented green tea in their diet throughout their life exhibited longer lifespans and reduced total body iron levels further
supporting an interaction between green tea's iron-binding and lifespan extension properties [4].

In addition to chelating iron, green tea may regulate iron homeostasis in flies through the modulation of essential iron regulators, since the process is partially conserved between flies and humans [20]. Iron homeostasis involves the action of numerous protein regulators. Among them are mitoferrin, transferrin and the divalent-metal transporter-1 (DMT1), evolutionary conserved proteins involved in important physiological processes in *Drosophila* [20, 21]. Mitoferrin, located within the inner mitochondrial membrane, is a protein of the mitochondrial solute carrier family known to transport iron into the mitochondria [20, 21]. It has previously been reported that a reduction in mitoferrin resulted in abnormal development and increased the lifespan of *C. elegans* [22]. In *Drosophila*, mitoferrin mutants resulted in male sterility [23] and additional studies demonstrated that mitoferrin played an important role in spermatogenesis and development [24]. Transferrin, found in the plasma of mammals [25] and hemolymph of fruit flies [26], is an endogenous iron-chelator involved in the systemic transport of iron in mammals and plays a protective role in immunity in *Drosophila* [21, 26]. While the function of transferrin in *Drosophila* may differ from that of mammals, transferrin expression in flies has been shown to be influenced by dietary iron availability [26]. Thus, green tea catechins, which bind non-transferrin bound iron (NTBI) [9] may modulate the expression of transferrin in flies. DMT1, most notably located in the gut lumen in mammals, including humans, is an essential iron transporter for the cellular uptake of non-heme iron [21]. In *Drosophila*, mutants of the protein homolog of
DMT1, Malvolio (Mvl), have depleted intestinal iron stores which makes Mvl flies are a good model system to study iron metabolism [27, 28].

In this study, we investigated the requirement of mitoferrin, transferrin and Mvl for lifespan extension and reproductive function by GTP in Drosophila. Using publicly available fruit fly mutants for the aforementioned iron transporters, we evaluated the effect of GTP on lifespan and fertility. We observed that mitoferrin, transferrin and Mvl mutant flies have longer lifespans and reduced fertility compared to a standard laboratory strain, w1118. Previously, we reported that GTP increased the lifespan of male Drosophila by 19% while reducing fertility [1]. Here, we found that GTP failed to increase the lifespan of Mvl and mitoferrin mutants but increased male fertility in these mutant strains. We further tested the effect of GTP on a mutant Drosophila strain of transferrin. We found that GTP increased the lifespan and reduced fertility of transferrin mutants, a similar observation in w1118 controls. Since we observed that green tea increased the lifespan of all strains tested except mitoferrin and Mvl mutants, we suggest that the lifespan extension mechanism, as well as reduction in fertility in normal male flies, by green tea is dependent on these endogenous iron transporters.

4.2 Materials and methods

4.2.1 Fly Strains and Experimental Conditions

Flies were obtained from the Bloomington Drosophila Stock Center (BDSC) at Indiana University, USA and included w1118 (BDSC# 3605), Drosophila mitoferrin, dmfrnEY01302
Fly mutants with reduced expression levels of mitoferrin, transferrin and the divalent metal transporter-1 (DMT) have a P-element insertion at each respective gene of interest. Hypomorph expression for *Drosophila* mitoferrin (dmfrn) and *Malvolio* (*Mvl*) have previously been characterized by Metzendorf and Lind, 2010 [23], Rodrigues *et al.*, 1995 [28] and Bettedi *et al.*, 2010 [27]. *Drosophila* tsf1 mutants were previously identified by the Berkeley *Drosophila* Genome Project (BDGP) [29] and mutant stocks are available via BDSC. *Drosophila* transferrin mRNA expression has previously been characterized by Yoshiga *et al.*, 1999 [26].

All flies used for experimental assays were fed a standard *Drosophila* banana–molasses food diet composed of 9% carbohydrate content and 9% yeast. Control diets included a 75 µL of 9% yeast solution overlay on food, which was allowed to dry and refrigerated for at least 24 h before use. Treatment diets consisted of 10 mg/mL GTP, purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), which was added to the yeast solution. Flies were maintained at 22 ± 1 °C under a 12 h light:12 h dark cycle for all experiments. The 10 mg/mL dose was chosen based on our previous work in which a 10 mg/mL dose increased the lifespan of male *w^1118* flies [1].

### 4.2.2 Total Body And Mitochondrial Iron Levels

Iron levels were measured using the ferrozine assay as previously described by Missirilis *et al.*, 2006 [30] with the modification of using 50 flies per sample. In brief, the ferrozine assay is a colormetric assay that utilizes ascorbic acid to reduce ferric ion to the
ferrous state. Ferrozine reacts with ferrous ions to form a magenta complex that absorbs at 562 nm. The absorbance is directly related to iron in the fly. Fifty male flies per sample ($n=300$, per treatment) were homogenized in lysis buffer and centrifuged. Supernatant was then collected at which point 5 µL was collected for protein measurements. Ferrozine assay absorbance measurements were standardized to the amount of protein in each fly sample.

4.2.3 Fertility

Fertility of male flies after iron chelation was evaluated by feeding flies varying concentrations of 0 µM, 250 µM, and 500 µM of Ethylenediaminetetraacetic acid (EDTA) (USB Corporation, Cleveland, OH, USA), an iron chelator, that was mixed in the yeast solution overlay. Fertility of male flies after iron supplementation was performed by feeding flies varying concentrations of 0 mM, 5 mM, and 10 mM ferric ammonium citrate (FAC) obtained from Sigma-Aldrich (St. Louis, MO, USA).

Flies were fed treatment diets, as described above, or an equivalent control for 10 days and their fertility was measured. Male fertility was performed by placing one treated male with untreated virgin female per vial. Paired flies were allowed to mate for 24 hours and then transferred to new vials each day for the course of 10 days. Remaining eggs were allowed to develop and the offspring was counted 14 days later.

4.2.4 Gene Expressions

Gene expressions were performed as described in Schriner et al, 2012 [31]. In brief, flies were frozen in groups of 10 and RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Samples were treated with DNase (New England Biolabs, Ipswich, MA, USA) and converted to cDNA by the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA).
Quantitative PCR was performed on a MiniOpticon real-time PCR system with SYBR green dye (Bio-Rad, Hercules, CA, USA). Relative amplification was calculated by the threshold cycle of each respective gene divided by the threshold cycle of the reference gene. Primer sequences are summarized in Table 1.

**Table 4.1 List of primer sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’→ 3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>*GAPDH</td>
<td>F-GTTGCCGGCTAGGGCCGATT</td>
<td>Primers were designed by NCBI/Primer-BLAST</td>
</tr>
<tr>
<td></td>
<td>R-AGTTGATGTGGGCCCCGTCGC</td>
<td></td>
</tr>
<tr>
<td>*Rp 49</td>
<td>F-CGGCTTCAAGGGACAGTACTTG</td>
<td>Metzendorf <em>et al</em>, 2009</td>
</tr>
<tr>
<td></td>
<td>R-CAGTTTGTGCCACGAACTTT</td>
<td></td>
</tr>
<tr>
<td><em>Mvl</em></td>
<td>F-GGGCTGATTGCATTATTC</td>
<td>Tang <em>et al</em>, 2013 [33]</td>
</tr>
<tr>
<td></td>
<td>R-CTGCGCTACGGTCTTTG</td>
<td></td>
</tr>
<tr>
<td>dmfnn</td>
<td>F-CTTGCCGCCTACGAGATG</td>
<td>Navarro <em>et al</em>, 2015 [34]</td>
</tr>
<tr>
<td></td>
<td>R-TAGGATGCGCTCGATG</td>
<td>Metzendorf <em>et al</em>, 2009 [32]</td>
</tr>
<tr>
<td><em>Tsf1</em></td>
<td>F-AAGTACTTTTGTCTGCGG</td>
<td>Primers were designed by NCBI/Primer-BLAST</td>
</tr>
<tr>
<td></td>
<td>R-GTGCCATCTCGCAGAGATA</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: F, forward; R, reverse

*Reference gene

4.2.5 Lifespan

Flies were fed as described above throughout their life. A total of 20 vials were loaded with six males and six females in each vial (*n* = 120 per sex). Flies were transferred every other day to newly yeasted food and deaths were counted after each transfer until all flies died.

4.2.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Fertility experiments were analyzed by Two-way ANOVA and data was presented as means ± SEM. Lifespans were analyzed by Mann-Whitney log-rank test. All other experimental data were analyzed by student t-test.
4.3 Results

4.3.1 Iron Deprivation Negatively Affects Male Fly Fertility

Iron has been found to be a crucial micronutrient for male *Drosophila* fertility and reproductive systems [23, 35]. Such experiments are typically performed with mutant fly strains that exhibit sensitivity to iron availability. Here we investigated the effect of dietary iron on fly fertility in a standard laboratory strain, *w*^1118^, over a 10-day mating period. We observed that iron deprivation by an iron chelator, EDTA, decreased male fly fertility in a dose-dependent manner (*P*<0.0001, Figure 4.1a). Iron supplementation by ferric ammonium citrate (FAC 5µM and 10µM), however, had no effect on the fertility of male flies (*P*>0.05, Figure 4.1b).

![Figure 4.1](image-url)

**Figure 4.1.** Effects of iron availability on male *Drosophila* fertility. Iron chelation by EDTA decreased the fertility of a standard *Drosophila* strain, *w*^1118^ (*P*< 0.0001) over a 10-day mating period (A). Iron supplementation with ferric ammonium citrate (FAC) had no effect on male fly fertility (*P*> 0.05) (B). Data are presented as means ± SEM per day and analyzed by Two-way ANOVA. Sample sizes for each treatment were as follows: n=18, 15, 19 for 0µM, 250µM and 500µM EDTA, respectively, and n=12, 15, 16 for 0mM, 5mM and 10mM FAC, respectively.
4.3.2 Green Tea Decreases Total and Mitochondrial Iron Levels in Flies

Green tea has high polyphenolic content with known iron-binding properties [14, 15]. We investigated the ability of a green tea polyphenol extract, consisting of ~80% catechin content [36], on total body iron levels. We found a 39% decrease (245.9±24.9 vs. 403.6±29.4 nmol Fe/mg protein) in total body iron compared to controls (P=0.003, Figure 4.2a). Moreover, mitochondrial iron metabolism has been suggested to play a direct role in spermatogenesis of male Drosophila [23]. We, therefore, measured the iron levels in mitochondria from flies fed with or without green tea. We found a 36% decrease (142.3 ±11.9 vs. 223.7±28.6 nmol Fe/mg protein) in mitochondrial iron levels from flies fed green tea versus controls (P=0.04, Figure 4.2b).

4.3.3 Hypomorph Mutants for Mitoferrin, Transferrin and Mvl Have Increased Lifespans and Reduced Male Fly Fertility

Using publicly available hypomorphs of mitoferrin, transferrin and Mvl [23, 26-29] we compared their lifespan and male fertility to standard w1118 flies. We first validated dmfrn

![Figure 4.2](image-url). The impact of green tea on total and mitochondrial iron levels in Drosophila. Green tea decreased total body iron levels in a standard Drosophila strain, w1118 (P=0.003, n=250) versus controls (A). Isolated mitochondria from flies fed green tea had reduced levels of mitochondrial iron (P=0.04, n=200) (B). Data are presented as means ± SEM and analyzed by unpaired t-test.
and tsf1 hypomorph flies by measuring their expression levels and confirmed a significant reduction in mitoferrin (Figure 4.3a) and transferrin (Figure 4.3b) expression in these mutants ($P<0.0001$). We then identified that mitoferrin, transferrin and Mvl hypomorph mutants exhibit longer lifespans compared to $w^{1118}$ ($P<0.0001$, Figure 4.4a). Specifically, mitoferrin hypomorphs exhibited the greatest increase in lifespan (42%) followed by transferrin (19%) and Mvl (11%). Baseline male fertility levels of all investigated hypomorph mutants were found to be significantly lower than that of $w^{1118}$ flies ($P\leq 0.0001$, Figure 4.4b) by 50% in mitoferrin, 40% in transferrin, and 95% in Malvolio mutants.

**4.3.4 Green Tea Has No Effect on Lifespan, but Increases Male Fly Fertility of Mitoferrin and Mvl but Not Transferrin Hypomorphs**

Previously we reported that GTP increased male Drosophila lifespan by 19% in $w^{1118}$ flies while reducing fertility [1]. Here we investigated the effect of GTP on the lifespan and
male fertility of hypomorph mutants dmfrn, Mvl and tsf1. We identified that green tea could no longer increase the lifespan of dmfrn flies ($P>0.05$, Figure 4.5a) but did increase male fertility ($P=0.01$, Figure 4.5b). Similarly, green tea did not affect the lifespan of Mvl flies ($P>0.05$, Figure 4.5c) but rescued the reduced fertility phenotype ($P<0.0001$, Figure 4.5d). Interestingly, tsf1 flies exhibited an increase in lifespan with supplementation of green tea ($P<0.0001$, Figure 4.5e). However, green tea did have a negative effect on tsf1 male fly
Figure 4.5. The impact of green tea on lifespan and fertility of mitoferrin (dmfrn), transferrin (tsf1) and Malvolio (Mvl) mutants. Green tea had no effect on dmfrn\(^{BG00456}\) hypomorph mutant lifespan (A) but increased male fly fertility (B). Green tea had no effect on Mvl mutant lifespan (C) but had a pronounced increase in Mvl male fertility (D). Green tea increased the lifespan of tsf mutants (E) however exhibited a decline in male fertility (F). Lifespans were analyzed by Mann-Whitney log-rank test. Sample sizes for lifespans, control and green tea respectively, were as follows: dmfrn\(^{BG00456}\) \(n=117,120\); Mvl \(n=116,118\); and Tsf1 \(n=124,119\). Fertility experiments were analyzed by Two-way ANOVA and data are presented as means ± SEM per day, \(n=20\) mating pairs.
fertility ($P<0.0001$, Figure 4.5f). The increase in lifespan and decrease in fertility by green tea in tsf1 flies are similar to those observed in previous reports with $w^{1118}$ [1].

### 4.3.5 Green Tea Up-regulates the Expression of Mitoferrin and Malvolio, but not Transferrin in Hypomorph Mutants and $w^{1118}$ Flies.

To determine whether green tea has any effect on the expression levels of iron metabolizing genes we compared the levels of mitoferrin, transferrin and $Mvl$ in a standard laboratory fly strain, $w^{1118}$. We showed that green tea increased the expression of mitoferrin

![Figure 4.6.](image)

**Figure 4.6.** The effect of green tea on mitoferrin, *Malvolio* and tsf expression in $w^{1118}$ and hypomorph mutants. Green tea up-regulated expression of mitoferrin (A) ($P=0.04$, $n=50$ per treatment) and $Mvl$ (B) ($P=0.04$, control $n=30$, green tea $n=40$) in $w^{1118}$ flies. Green tea did not impact the expression levels of transferrin (C) ($P>0.05$, control $n=60$, green tea $n=50$). Green tea up-regulated mitoferrin in mfrn$^{BG00456}$ hypomorph mutants (D) ($P=0.04$, $n=50$ per treatment) and increased the expression of *Malvolio* in $Mvl$ mutant flies (E) ($P=0.0008$, $n=50$ per treatment). Green tea did not affect transferrin expression levels in transferrin hypomorph mutants (F) ($P>0.05$, control $n=60$, green tea $n=50$). Data are represented as means ± SEM and analyzed by student’s t-test.
(P=0.04, Figure 4.6a) and Mvl (P=0.04, Figure 4.6b) while no difference was detected with transferrin expression levels (P>0.05, Figure 4.6c). Subsequently, since hypomorph mutants have residual expression for their affected genes (Figure 4.3), we questioned whether green tea may act by modulating their expression. We showed that similar to w1118 flies green tea up-regulated the expression of mitoferrin in dmfrn flies (P=0.04, Figure 4.6d) and Malvolio in Mvl hypomorph mutants (P=0.0008, Figure 4.6e). Green tea had no effect on the expression levels of transferrin in tsf1 mutants (P>0.05, Figure 4.6f).

4.4 Discussion

We and others have previously reported the ability of green tea and its primary active flavonoid, EGCG, to increase the lifespan of different strains of Drosophila melanogaster [1-4, 37]. In this study we found that the mechanism of green tea’s action on male Drosophila lifespan and fertility involves iron regulators, such as mitoferrin and Malvolio (Mvl). Over the years, the mechanism of action of green tea has been evaluated extensively. Massie et al, 1993 [4] had first suggested that green tea induced lifespan extension in Drosophila by its ability to inhibit iron absorption and thus iron accumulation throughout life [4]. With a number of studies reporting green tea’s iron binding activity, more recent reports have linked the importance of iron to male Drosophila spermatogenesis and fertility [23]. Specifically, Metzendorf and Lind 2010 [23] showed that iron chelation in the diet increased sterility in dmfrn hypomorph mutants whereas iron supplementation improved fertility demonstrating the importance of iron for spermatogenesis. To evaluate the interaction between iron and fertility we tested whether iron deprivation in the diet had any effects on
male fertility in normal w^{118} flies. We too identified a reduction in male fertility when iron levels were reduced by an iron chelator, EDTA. Next, we tested whether iron supplementation had any effects on male fertility. Our results did not show any improvements in male fertility with iron supplementation. We surmised that in normal flies, which display typical reproductive abilities and thus functional iron metabolizing pathways, the addition of iron in the diet would have no additive effect on fertility. In healthy flies, excess iron would be stored in ferritin, an iron-storage protein, and iron can be secreted from this storage protein to the gut lumen during iron over-load conditions [21]. We further explored the levels of iron in whole flies and in fly mitochondria after treatment with green tea polyphenols and identified a significant reduction of iron levels in both homogenates, 39% and 36% reduction, respectively. The reduction in mitochondrial iron is of particular interest since mitochondrial iron involving the iron transporter, mitoferrin, has a direct role in the development of sperm. Interestingly, green tea’s primary catechin, EGCG, has been reported to accumulate in the mitochondria of neuronal cells [38]. In accordance with this finding, it is thus likely that the presence of green tea catechins in the mitochondria can result in reduced mitochondrial iron levels.

While the reduction in iron levels is a direct consequence of green tea supplementation and hence reduction in male fertility, the reported effects of green tea selectively increasing male flies’ lifespan [1, 2, 4, 37] is likely a secondary effect of the treatment. It is well established that impairments in reproductive abilities, such as a decrease in egg laying for females [39, 40] or decrease in sperm production for males [41], have an inverse relationship with fruit fly lifespan. In this study we proposed that the
reduction in fertility was caused by the reduction in mitochondrial iron levels, which is critical for spermatogenesis [23]. Thus the decrease in fertility resulted in an increase in male fly lifespan [1].

To evaluate whether green tea polyphenols do act through the regulation of iron metabolism and hence in turn affect fertility and lifespan, we utilized mutant flies with deficiencies in their ability to regulate iron. Flies with defects in Drosophila mitoferrin, Mvl and transferrin were specifically chosen as these proteins display a diverse involvement of iron including the transport of iron into the mitochondria (dmfrn), into the cell (Mvl) or throughout the fly (tsf). We first established that these mutants exhibit longer lifespans and reduced fertility than normal flies. This supports the notion that these iron-regulators follow an inverted relationship with lifespan and fertility. With the exception of Drosophila dmfrn mutants, which previously was reported to exhibit reduced male fertility [23], Mvl and tsf1 mutant lifespan and fertility phenotypes (e.g., decrease in the production of offspring) have not yet been characterized. One study, which used C. elegans to down-regulate the expression of mfrn, identified a 50-80% increase in worm lifespan, as well as abnormal developmental phenotypes and reduced production of progeny [22]. This is an interesting observation since we previously reported that green tea polyphenols exhibited similar effects on lifespan, development and fertility [1, 36]. It is plausible that green tea polyphenols require mitoferrin to increase lifespan. Since Mvl and tsf1 hypomorph mutants also exhibited an increase in lifespan and reduced fertility, it was also of interest to determine whether green tea requires these proteins as well.
Typically, if a treatment requires a specific gene in order to elicit its effect, that treatment will no longer work in the event of a mutation or dysfunction of that gene. In this case, we utilized hypomorph mutants due to resulting sterility and lack of feasibility in maintaining complete knock-outs [23]. As previously discussed, green tea increased the lifespan and reduced male fertility of tsf1 flies. In addition, treatment with green tea polyphenols showed no change in tsf expression levels. This leads us to conclude that since tsf1 flies exhibit the same effects under green tea treatment as normal w1118 flies [1], green tea polyphenols do not require transferrin to increase Drosophila lifespan. This result is not surprising since green tea catechins are known to bind to non-transferrin bound iron (NTBI) [9] and thus act independently of the action of transferrin proteins. Dmfrn and Mvl mutants under the treatment of green tea, however, exhibited no increase in lifespan and instead experienced an increase in male fertility. In mitoferrin mutants, since the expression of the mitochondrial iron transporter is reduced, it is plausible that green tea’s polyphenolic compounds are no longer able to accumulate in the mitochondria as previously suggested [38], thus preventing green tea polyphenols from binding to iron inside the mitochondria. This in turn would normalize the levels of iron inside the mitochondria. Due to the presence of iron, no benefits in lifespan would be observed, and instead male fertility would be improved as a result of a supply of iron for spermatogenesis. Similar to the effects of dmfrn mutants in the presence of green tea, we speculate that Mvl mutants are exhibiting a similar effect at the site of transport. Further studies are needed to confirm the action of green tea on mitoferrin and Malvolio iron-transporters.
In the evaluation of the effect of green tea on the aforementioned iron-metabolizing proteins we found an increase in the expression levels of mitoferrin and *Mvl* in hypomorph mutants but no effect on the expression levels of transferrin in tsf1 hypomorphs. We speculate that the residual presence of mitoferrin and *Mvl* in hypomorph mutants was sufficient for green tea to modulate their expression levels. Similarly, green tea increased the expression of mfrn and *Mvl* in normal flies as well. This is somewhat contrary to the results identified in *C. elegans* where an increase in the lifespan of worms and reduced progeny numbers were observed by a reduction in mitoferrin expression [22]. However, the reduction in mitoferrin expression was caused by genetic manipulation not by a dietary treatment as presented in our study. Typically homeostatic responses to iron supply, which are mediated by iron regulatory proteins (IRPs), can normalize cellular iron levels by either increasing or repressing synthesis of iron transport proteins [16]. It has been suggested that during increased iron level conditions, mRNA translation of iron transport proteins are inhibited whereas low iron level conditions would signal increased expression and synthesis of iron importers [21]. Therefore, it is plausible that green tea, which reduces intracellular iron levels, causes an increase in the expression of mitoferrin and *Mvl* in order to compensate and allow a greater uptake of iron into the cell and mitochondria.

Since green tea could no longer increase the lifespan of dmfzn and *Mvl*, rescued the impaired fertility phenotype and up-regulated the expression levels of mfrn and *Mvl* in both hypomorph mutants and normal flies, green tea polyphenols may require these proteins to increase the lifespan of *Drosophila melanogaster*. As for green tea’s ability to specifically increase male fly lifespan while negatively affecting the fertility of normal flies, our results
suggest that green tea’s iron-binding properties are responsible for the unique interplay between the regulation of iron metabolizing proteins, male fly fertility and lifespan.
4.5 References


CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

5.1 Summary of Dissertation

Chapter 1 served as an introduction to the dissertation which outlined sequential events leading up to the scope of the research. The complexity of aging, lifespan, and healthspan studies were introduced with an emphasis on the interaction between lifespan extension and reproductive changes. In this chapter, utilization of Drosophila melanogaster as an appropriate model system to investigate lifespan extension mechanisms was presented. It was concluded that the fruit fly makes an excellent model for aging research due to its conserved metabolic and genetic pathways, and relatively short lifespan. Preliminary screenings of products with known medicinal properties, primarily plant components, were examined for their ability to increase Drosophila lifespan and improve healthspan using a well-established algorithm. Of those tested, green tea polyphenols increased the lifespan of male Drosophila by 22%. Green tea’s health benefits and mechanistic properties as an antioxidant, prooxidant and hormetic were outlined. Some meta-analysis investigations in older individuals who drank green tea revealed a lower risk in mortality, particularly in men. Green tea’s ability to increase lifespan has been observed in various model species including worms, flies, and rodents. Suggested mechanisms of lifespan extension have included increases in antioxidant defense enzymes, such as superoxide dismutase and catalase, increased ability to withstand stressful environments, decrease in oxidative damage, and reduction in iron accumulation. The role of excess iron and its dysregulation in chronic diseases, and aging was also presented in this chapter. Massie et al, purported that limiting iron absorption was the underlying mechanism of
lifespan extension by green tea in *Drosophila melanogaster*. Green tea’s iron chelation abilities were also summarized in this chapter. Studies in which iron levels were measured after consumption of green tea found that iron absorption from dietary sources was inhibited by up to 83%. The importance of iron as an essential micronutrient for physiological processes was also presented. In flies, iron is required for various processes including development and male *Drosophila* spermatogenesis. Hence, limiting iron absorption impairs developmental and reproductive processes in *Drosophila*. It has been well established that impairments in reproductive systems affects fruit fly lifespan. A discussion of this phenomenon was presented in this chapter. Chapter 1 also introduced selected preliminary data including the observation that green tea increased the lifespan of a standard laboratory *Drosophila* strain while reducing fertility. Chapter 1 presented the foundation and the rationale behind the hypothesis with a focus on green tea’s effect on the interaction between lifespan extension, reproduction, and iron metabolism.

Chapter 2 explored potential mechanisms responsible for the increase in lifespan by green tea polyphenols in *Drosophila*. For this investigation, a standard inbred laboratory *Drosophila* strain, *w*¹¹¹⁸, was selected. Interestingly, green tea once again extended the lifespan of male flies only. This effect was found to be independent of typical aging interventions such as dietary restriction, modulation of oxidative energy metabolism, and improved tolerance to environmental stresses. The one exception was that green tea did protect male flies against iron toxicity. Since there is a known inverse correlation between lifespan and reproduction, the impact of green tea on male reproductive fitness was also investigated. Green tea was found to have a negative effect on male fertility as shown by a reduction in offspring produced and increased mating latency. Another interesting finding
was that the lifespan extension properties of green tea were only observed in the presence of females which alludes to a reproductive (or mating) dependent mechanism. These findings suggested that green tea extends male fly lifespan by inhibiting reproductive potential.

**Chapter 3** evaluated green tea’s effects on healthspan, including development and reproduction, which are known to modulate *Drosophila* lifespan. Since toxic effects associated with green tea in *Drosophila* were observed, the introduction of this chapter summarizes the literature on green tea’s reported toxicities. Chapter 2 evidenced a decrease in male *Drosophila* fertility, here, the extent of green tea toxicity on development and reproduction was investigated. *Drosophila melanogaster* embryos and larvae were exposed to various doses of green tea polyphenols (GTP). Larvae exposed to 10 mg/mL GTP were slower to develop, emerged smaller, and exhibited a dramatic decline in the number of emerged offspring. When subjected to stress-induced environments, green tea polyphenols protected flies against desiccation but sensitized them to starvation and heat stress. Female offspring exhibited a decline in reproductive output and decreased survival while males were unaffected. Green tea had a negative impact on reproductive organs in both males and females (e.g., atrophic testes in males, absence of mature eggs in females). Collectively, the data show that high doses of GTP adversely affect development and reproduction of *Drosophila melanogaster*.

**Chapter 4** investigated a potential mechanism of action of green tea related to iron homeostasis in *Drosophila*. The introduction of this chapter revisited green tea’s high polyphenolic content, and ability to decrease oxidative stress in part by its iron binding properties. The role of iron as both an essential micronutrient and its toxic capabilities was
once again discussed. Due to green tea’s iron-binding activities, it was questioned whether green tea acts to increase the lifespan of the fruit fly by modulating iron regulators, specifically, mitoferrin, transferrin and Malvolio (Mvl, the Drosophila homolog of the divalent metal transporter-1). Publicly available hypomorph mutants for these iron-regulators were utilized to investigate the effect of green tea on lifespan and male fly fertility. This work identified that green tea could not increase the lifespan of mitoferrin and Mvl flies but did rescue the reduced male fertility phenotype. Green tea had no effect on transferrin hypomorph flies. Expression levels in w^{1118} flies supplemented with green tea showed an up-regulation of mitoferrin and Mvl but not transferrin. These results demonstrated that green tea may act to increase the lifespan of Drosophila in part by the regulation of mitoferrin and Mvl.

Chapter 5 provides a summary of the dissertation and future directions of the study. Overall, green tea was found to increase male Drosophila lifespan with no effect on female lifespan. Interestingly, this effect was associated with a reduction in male fly fertility. Moreover, green tea showed a modest protection against iron-induced oxidative stress, likely by its ability to bind iron. Since iron has been evidenced to play an essential role in male fly fertility, this dissertation explored the unique interplay of green tea on iron homeostasis, fertility and Drosophila lifespan. Processes that can modulate fly healthspan, such as development and reproduction, were evaluated. Green tea was found to delay larval development, reduce offspring sizes, and atrophied reproductive organs. From this it was suggested that green tea is toxic to Drosophila’s physiological processes. Green tea’s ability to chelate iron and modulate iron homeostasis was evaluated using hypomorph mutants of iron-regulating proteins. Specifically, the lifespan of hypomorph mutants of
mitoferrin and Malvolio were unaffected by green tea in the diet. However, green tea did rescue the reduced male fertility phenotype exhibited by these hypomorph mutant flies. This was associated with an up-regulation of mitoferrin and Malvolio expression. Combined, these results support a mechanism by which green tea increases male Drosophila lifespan in part by the modulation of iron homeostasis that is essential for fertility.

5.2 Future Directions

Green tea’s vastly explored health benefits have been well studied in experimental animal models and cannot be undermined [1]. Green tea shows great promise in the prevention of chronic conditions particularly neurodegenerative disorders [2], diabetes [3] and some cancers[4-6]. As a candidate life-extending natural product, it is critical to evaluate all factors associated with the aging process as well as those which modulate lifespan extension in experimental animal models [7]. In addition, aging research which limits the outcome to a long lifespan without the evaluation of health-related parameters poses issues with the benefits associated with a life-extending intervention. In this dissertation, green tea was evaluated for its ability to modulate lifespan while taking into account associated health factors, specifically reproduction, effect on iron pathways, and the combined relationship of the two as it affects lifespan. Future efforts include 1) those which address limitations to the use of Drosophila in drug studies, 2) an evaluation of reproductive effects pertaining to sperm physiology and requirements, and 3) investigation of green tea’s modulation of other iron regulators and their role in lifespan extension.

5.2.1 Green Tea Consumption and Metabolism in Drosophila – Extrapolations to Humans
Aside from the common genetic and metabolic similarities between fruit flies and humans [8, 9], one of the major concerns with the use of Drosophila in drug studies includes the verification of consumption and metabolic fate of drugs [10, 11]. Methods used to address this have included addition of colored dyes to the food/drug mixture, administration of radiolabeled drugs, and measurement of drug concentrations in fruit flies using mass spectrometry techniques [10]. It is critical to monitor consumption since food aversion could lead to reduced caloric intake, inadvertently curtail fertility, and ultimately impact lifespan [12]. For the purpose of this dissertation, green tea consumption was verified in fruit flies by utilization of various techniques including 1) capillary feeding and measurement as described by William et al, 2007 [11] using the CAFÉ method (Appendix A) 2) use of colored dyes in Drosophila food containing the green tea polyphenol mixture and the observation of colored dye in flies (Appendix B), and 3) verification of polyphenol compounds in flies, specifically EGCG, using HPLC methods (Appendix C). Collectively, these methods confirmed the consumption of green tea polyphenols by flies in all experimental investigations throughout this dissertation.

Despite assuring the uptake of green tea by flies, the metabolic fate of green tea catechins have not been documented and are not well-understood in Drosophila. As outlined in Chapter 1, tea catechins undergo various metabolic processes in humans including glucuronidation, sulfation, and methylation [13]. While flies are known to share similar metabolic processes, and possess various cytochrome P450s [9, 14, 15], drug-metabolizing enzymes in humans, their functions may differ from those of mammalian systems. In addition, several pharmacokinetic investigations have also reported variability in excreted metabolites [16] and limited bioavailability of tea catechins [17-19]. This poses
issues in pharmacokinetic studies of not only *Drosophila* but of all experimental animal models.

Finally, pharmacological studies in *Drosophila* may not be directly extrapolated to human studies. For instance, in *Drosophila* studies, drug concentrations are often much higher than those used in humans which presents a major challenge for extrapolations. The most common reason for this is that drug concentrations reported in drug studies are the concentration mixed in the food [20]. Although food uptake is measurable, physiological concentrations of the drug in the fly are typically unknown and difficult to quantify for the reasons mentioned above. Other factors for the requirement of high treatment concentrations may be due to the ingredients in *Drosophila* food in which the treatment is mixed, feeding behaviors, and metabolism [10, 11, 15]. It is important to note that although flies may consume higher concentrations of drug treatments, toxicity effects are dose-dependent [10, 20] and dose response studies need to be performed to identify the effective and toxic doses [8, 21]. Future efforts into the thorough understanding of fruit fly drug metabolism, prandiology behaviors, and extrapolation of *Drosophila* doses to humans would present greater possibilities of utilizing the fly for pharmacokinetic studies.

5.2.2 Evaluation of Reproductive Effects Pertaining to Sperm Physiology and Requirements

Within this dissertation, the effects of green tea on reproduction and reproductive organs were explored. While it can be concluded that green tea does have a dramatic impact on reproductive systems in the fly, an evaluation of reproductive effects pertaining to sperm physiology would provide a greater understanding of the molecular targets green tea is acting on. Specifically, within the research of this dissertation, male *Drosophila* fertility was defined as the number of viable offspring produced by a green tea-fed male
fruit fly after mating with a non-mated control female. Since a reduction in the number of viable offspring was observed in green tea-fed male flies, green tea’s action on sperm physiology was questioned. The possibility that green tea arrests cell-cycle progression [22] or causes iron deprivation [23] inhibiting spermatogenesis [24, 25] was suggested from experimental data performed in this dissertation and supported by prior studies. Further evaluation of green tea on spermatogenesis would provide further insight to green tea's mechanism of action as it relates to Drosophila male fertility [26]. The reproductive systems of the male fruit fly are complex. Sperm development in male flies involves several rounds of mitotic and meiotic events resulting in elongated spermatids and eventually mature sperm [27]. Drosophila spermatogenesis is regulated by several key regulators, such as the meiotic regulator of spermatogenesis, boule [28], the regulator of cell proliferation, merlin [29] and accessory gland proteins (Acps) [30], essential for the storage, transfer and competition of sperm. These genes are of interest as they encode reproductive proteins in mammals as well.

5.2.3 Modulation of Iron regulators and Their Role in Lifespan Extension

The fruit fly shares a number of similar iron metabolizing proteins with humans, as outlined in Chapter 1 and Chapter 4 of this dissertation. These proteins have been used in a number of studies investigating iron homeostasis [31-34]. As described in Chapter 4, one study in C. elegans reported that reduced expression of mfrn increased lifespan by 50% to 80% [35]. This lifespan increase was associated with a number of phenotypic abnormalities including small body size, reduced fecundity, slow movement and increased sensitivity to superoxide generator, paraquat. In Drosophila, mitoferrin mutants, as those used in Chapter 4 of this dissertation, exhibited altered iron metabolism and reduced
fertility [25]. These adverse phenotypes further support the notion that iron-binding proteins are essential for lifespan and development and iron chelators such as green tea can adversely affect important functions. Although iron metabolizing pathways are conserved between flies and mammals, the functionality of most iron regulators in dipterans have not been thoroughly characterized [34, 36]. Moreover, information on their importance in aging and lifespan with modulations of iron availability is lacking. Exploring the role of other proteins involved in iron metabolism [36], including iron-regulatory proteins (IRPs), ferritin, and mitoferritin, as they relate to lifespan and fertility of Drosophila, could reveal potential new aging pathways or therapeutic targets. The study of green tea in the modulation of these iron proteins would also be of interest as green tea has been shown to influence many physiological functions.
5.3 References


APPENDIX A: Capillary Feeding of Green Tea Polyphenols and Measurements

Figure A1. Capillary feeder (CAFE) apparatus. Blue dye was added to the treatment mixture to allow easier measurement of meniscus descent and verification of consumption in Drosophila.

Table A1. Measurements of green tea consumption by capillary feeding.

<table>
<thead>
<tr>
<th></th>
<th>Length of feeding: 20 h</th>
<th>Blank vials (no flies)</th>
<th>Control (0 mg/mL)</th>
<th>Green tea (10 mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg. descent of meniscus (mm) (Equivalent volume - µL)</td>
<td>19.0 mm (1.72 µL)</td>
<td>32.8 mm (2.98 µL)</td>
<td>37.5 mm (3.41 µL)</td>
<td></td>
</tr>
<tr>
<td>Amount consumed (mm) (Equivalent volume - µL)</td>
<td>N/A</td>
<td>13.8 mm (1.25 µL)</td>
<td>18.5 mm (1.68 µL)</td>
<td></td>
</tr>
<tr>
<td>[C] of EGCG recovered</td>
<td>N/A</td>
<td>0 mg/mL</td>
<td>0.014 mg/ml</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX B: Verification of Green Tea Consumption in Flies Using Food Dyes

Figure B1. Verification of green tea consumption in flies using colored food dyes. Food dye was added to Drosophila food which contained a 10 mg/mL mixture of green tea polyphenols. Food consumption was confirmed by the presence of blue dye in fruit flies.
APPENDIX C: HPLC Chromatogram of Flies Fed Green Tea Versus Controls

Figure C1. HPLC chromatogram of flies fed green tea polyphenols versus controls. Control and green tea fed flies (10 mg/mL) were collected, homogenized and injected into HPLC to validate the presence of tea catechins versus controls. The resulting chromatogram shows peaks in green tea flies that are absent in control fed flies.