July 2020

Green Coffee Bean Extract Reduces Fat Accumulation in Drosophila melanogaster

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GREEN COFFEE BEAN EXTRACT REDUCES FAT ACCUMULATION IN *DROSOPHILA MELANOGASTER*

A Thesis Presented

by

LYNNEA YOUNG

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

MAY 2020

FOOD SCIENCE

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ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Yeonhwa Park for her guidance and assurance over the past four years of my undergraduate and graduate education. I would not have been able to accomplish this without her. I also want to extend my gratitude to my lab mates, past and present, for their help and friendship over the years. Thank you, Phoebe, Renalison, Yiren, Weipeng, Sida, Zhuojia, Zhenyu, Nick, and everyone else in Dr. Park's Lab. I would also like to thank my committee member, Dr. Lynne McLandsborough for her support.

Thank you to all the faculty in the Food Science department who've taught and mentored me since my freshman year.

I would like to thank the friends I've made through the department—especially Michelle, Christina, and Louisa for their love and encouragement. To my roommates, Emma, Collin, and Taylor, thank you for being my family away from home. Finally, thank you to my family for always being there for me throughout this entire process. You all inspire and motivate me every day.

ABSTRACT

GREEN COFFEE BEAN EXTRACT REDUCES FAT ACCUMULATION IN *DROSOPHILA MELANOGASTER*

MAY 2020

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With obesity on the rise, there has been great interest in identifying functional foods that can alter metabolism to mitigate obesity. One nutraceutical that has gained attention is green coffee bean extract (GCBE), sourced from raw coffee beans. Research has shown that it has a variety of biological effects including antioxidant and anti-inflammatory properties and regulation of lipid and glucose metabolism. It has been reported that the main polyphenolic compound in GCBE, chlorogenic acid (CGA), is likely responsible for these effects. However, GCBE had not yet been studied in *Drosophila melanogaster*, the fruit fly. *Drosophila* are a noted model organism as flies and humans share 60% of disease related genes and similar mechanisms for metabolic regulation. In this study, flies were treated with 0, 1, or 2 mg/mL GCBE and physiological parameters and gene expression were assessed. GCBE significantly reduced fat accumulation in flies, in part by a reduction in food intake. Flies were also treated with an isoform of CGA, 5-caffeoylquinic acid, but this treatment did not change fat accumulation. Overall, this study serves as a foundation for further research on GCBE to determine its potential mechanism of action. This may inform the use of GCBE as a nutraceutical for the treatment of obesity.

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CHAPTER 1

LITERATURE REVIEW: METABOLIC EFFECTS OF GREEN COFFEE BEAN EXTRACT

1.1 Introduction

Current trends in health and wellness show that consumers have an increased interest in living "naturally", with foods and drugs derived from whole, particularly, plant-based sources. In addition to this trend, obesity and its associated metabolic diseases are on the rise (Ogden & Flegal, 2015). In response, researchers have worked to identify therapeutic treatments to mitigate obesity. Nutraceuticals, foods or parts of foods that provide health benefits beyond basic nutrition, may provide a solution that satisfy the desire for natural treatments while helping to decrease levels of obesity in the population. Green coffee bean has been noticed for its ability to modulate metabolism and potentially reduce obesity.

Green coffee bean extract (GCBE) is derived from the unroasted seed of the coffee plant. Without heat processing from roasting, it retains a higher content of polyphenols compared to roasted coffee beans that are typically used to make brewed coffee beans. Therefore, there has been great interest in assessing the bioactive effects GCBE may potentially have. It has been shown that GCBE has beneficial antioxidant and anti-inflammatory effects and is able to regulate lipid and glucose metabolism.

The objective of this review is to summarize the current knowledge on green coffee bean and its main active compound, chlorogenic acid (CGA) and their major biological actions, specifically in anti-inflammatory, antioxidant, and metabolic regulatory effects.

1.2 Green Coffee Bean Extract

1.2.1 Source

Coffee is one of the most widely consumed beverages in the world. It's typically brewed from the dried and roasted seeds, known as coffee beans, of the coffee plant (Farah & Duarte, 2015). The main varieties are *Coffea arabica* and *Coffea canephora* var. *robusta*. However, it is known that the typical method of preparing coffee for consumption, such as roasting at high temperature, causes heat degradation of beneficial polyphenolic compounds (Şemen et al., 2017). Therefore, much attention has been brought to green unroasted coffee beans, because they may serve a better source of polyphenols and have higher antioxidant capacity. Green coffee bean extract (GCBE) is produced by softening raw green coffee beans and using 6# solvent oil, ethanol, and water extraction and filtration to yield an extract powder, with approximately 50% CGA and 12% caffeine content (Cheng et al., 2014). GCBE also contains other bioactive compounds, such as theophylline, theobromine, cafestol, kahweol, tocopherols and trigonelline (Jeszka-Skowron et al., 2016).

1.2.2 Chlorogenic Acid

The main polyphenolic compound in GCBE extract is CGA (Fig. 1). CGA refers to a family of compounds that are esters of cinnamic and quinic acids, including subgroups of mono-, di-, tri-, and tetra-esters of caffeic acid, coumaroylquinic acids and feruloylquinic acids, and mixed di-esters of caffeic and ferulic acid (Clifford, 1999).

In addition, every subgroup of CGA contains several isomeric forms, like 5-O-caffeoylquinic acid (5-CQA), 4-CQA, 3-CQA, and varying residues of caffeic acid or quinic acids generating molecules like caffeoylferuloylquinic acids and caffeoylsinapoylquinic

acids. Thus, a sample of GCBE can contain a diverse and vast set of CGA forms. However, the most common and commercially available isomer of CGA is 5-CQA. It should be noted that after the identity of CGA was determined, the International Union of Pure and Applied Chemistry (IUPAC) reversed the ordering of numbering of atoms on the quinic acid ring, so that what is 5-CQA now, was formerly known as 3-CQA. Though some literature still uses the old convention, this review will refer to the most abundant isomer of CQA as 5-CQA to stay consistent with current IUPAC nomenclature and more recent publications.

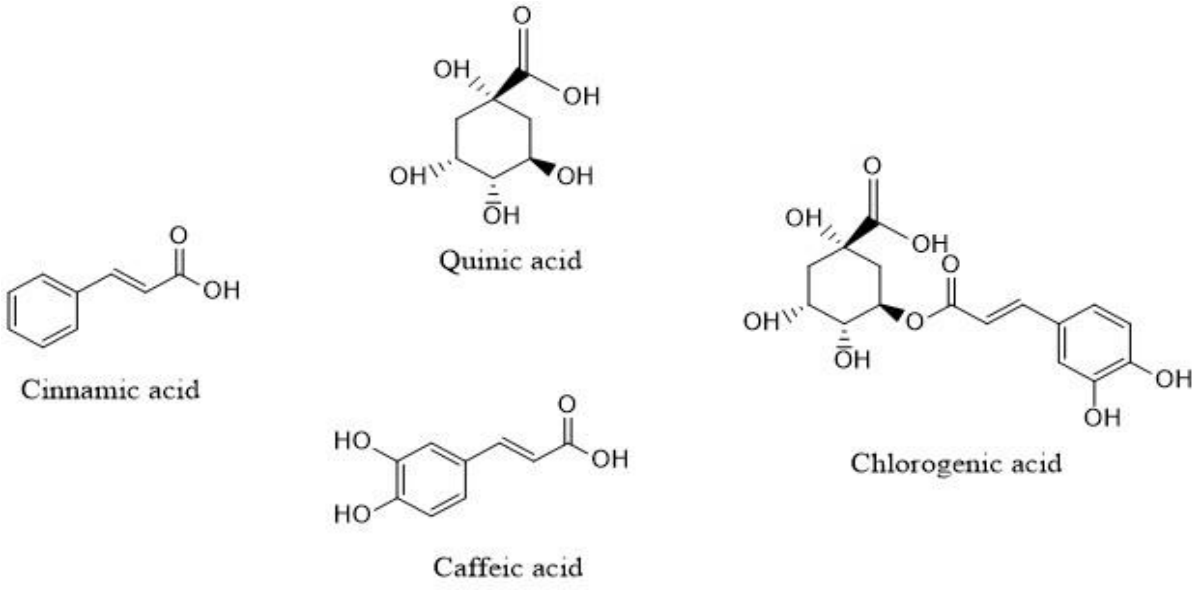


Figure 1.1 Representative structure of Chlorogenic Acid and Constituent Groups.

1.3 Antioxidant and Anti-inflammatory Effects

GCBE has antioxidant and anti-inflammatory properties that are attributed to its polyphenolic compounds (Lee et al., 2019). With CGA as the main fraction of polyphenols, this review will focus primarily on its effects. CGA has antioxidant properties that are protective against toxicities and chronic diseases associated with oxidative stress in various

biological systems (Liang et al., 2016). CGA's mechanisms of action include reducing generation of free radicals and scavenging free radicals (Zang et al., 2002). It was reported that CGA can chelate metals that induce the production of reactive oxygen species (ROS), which are the precursors to free radical species, thus, reducing free radical generation (Wang et al., 2017). It was further reported that CGA can modulate the antioxidant protein transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) to enhance the body's antioxidant and detoxification response, especially in the liver (Wei et al., 2018). In addition, it can improve the activity of antioxidant enzymes like glutathione peroxidase (GSH-Px) and catalase (CAT) that break down cellular ROS (J. Chen et al., 2018). In RAW 264.7 cells, CGA reduced nitric oxide (NO) production and inhibited inducible NO synthase (iNOS) gene expression (Hwang et al., 2014). The same group also showed that CGA reduces the expression of pro-inflammatory cytokines and chemokines, including tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2) by modulating the nuclear factor kappa B (NF-κB) signaling pathway as seen by decreased suppression of I kappa beta (IκB) protein in cells with lipopolysaccharide (LPS)-induced inflammation (Hwang et al., 2014). The proteasome also has a role in regulation of NF-κB and other inflammatory signaling pathways and CGA may modulate inflammation by inhibiting proteasome activity (Francisco et al., 2013). Finally, as an orally delivered nutraceutical, CGA can reach the large intestine and serve as a substrate for microbial fermentation and change the products of the microbiome, including short chain fatty acids (SCFA). It was shown that CGA can reduce hepatic inflammation through modulation of SCFA because of their involvement in anti-inflammatory response pathways (Nishitsuji et al., 2018).

1.4 Effects on Obesity and Metabolic Syndrome

Much research has been conducted regarding the anti-obesity effects of GCBE, with the effects being attributed to its constituent polyphenolic compounds like CGA. GCBE can significantly reduce fat accumulation in animal models (Choi et al., 2016; Farias-Pereira et al., 2018; Li Kwok Cheong et al., 2014; Shimoda et al., 2006). Farias-Pereira et al. investigated GCBE in *Caenorhabditis elegans*, a model organism with conserved fat and energy regulation pathways. The study compared the effects of whole GCBE and 5-CQA to assess the mechanism of action of GCBE. It was suggested that GCBE and 5-CQA were involved in pathways related to lipogenesis, energy homeostasis, and lipid uptake. In mammalian models, GCBE reduced body weight, visceral fat accumulation, insulin resistance, and altered lipid profiles by modulating genes related to fatty acid oxidation, energy homeostasis, adipocyte differentiation, and glucose metabolism (Choi et al., 2016; Li Kwok Cheong et al., 2014; Shimoda et al., 2006). There is a general agreement that polyphenolic compounds like CGA are responsible for the beneficial biological effects of taking GCBE.

1.5 Role of Chlorogenic Acid

The main effect of GCBE is thought to be caused by CGA's modulation of lipid and glucose metabolism. CGA has been shown to decrease triglyceride (TG) and free fatty acid (FFA) levels in the liver and plasma by regulating acetyl CoA carboxylase (ACC) and carnitine palmitoyltransferase 1 (CPT-1), enzymes involved in FFA oxidation and synthesis, respectively (Sudeep et al., 2016). Another critical target for CGA is AMP-activated protein

kinase (AMPK). CGA activates AMPK, which improved lipid and glucose profiles, mitigated hepatic steatosis, and dyslipidemia in $Lepr^{db/db}$ mice model (Ong et al., 2013).

1.5.1 Adiponectin and Leptin Ratio

CGA can moderate adiponectin/leptin ratio in high-fat fed mice by elevating plasma adiponectin levels (Cho et al., 2010). As obesity can be also characterized by increased circulating leptin paired with decrease in adiponectin (Frühbeck et al., 2017), the balance of these 2 adipokines are considered a marker of adipose tissue dysfunction (Vega & Grundy, 2013). More adiponectin can increase activation of peroxisome proliferator-activated receptor (PPAR-α), which may reduce TG by increasing fatty acid oxidation and energy consumption.

1.5.2 Regulation of AMPK

Regulation of AMPK also plays a significant role in CGA's ability to regulate glucose metabolism and mitigate diabetes. First, it can stimulate glucose uptake in skeletal muscle through the activation of AMPK (Ong et al., 2012). The long-term consumption of CGA can affect other metabolic pathways downstream of AMPK phosphorylation. Through activation of AMPK, CGA inhibited hepatic glucose-6-phosphatase expression and activity, attenuated hepatic steatosis, and improved lipid profiles, which improved fasting glucose levels, glucose tolerance, and insulin sensitivity (Ong et al., 2013). These effects on glucose metabolism show that CGA can attenuate the symptoms of type 2 diabetes. However, other research disputes these effects; chlorogenic acid did not reverse glucose intolerance in obese

rats even through CGA was able to reverse physical changes in cardiovascular structure, including reduced systolic blood pressure and diastolic stiffness (Bhandarkar et al., 2019).

1.5.3 Inconsistent Reports

There are inconsistent reports on whether CGA has definitive anti-obesity effects. One study found that supplementation of coffee extract (CE) containing CGA was able to reduce symptoms of metabolic syndrome like glucose intolerance, cardiovascular remodeling, and nonalcoholic liver disease without reducing abdominal obesity (Panchal et al., 2012). Another found that coffee or its components like caffeine and chlorogenic acid did not attenuate body weight or visceral fat in obese mice, but altered the microbiome and reduced liver inflammation (Nishitsuji et al., 2018).

Overall, CGA found in GCBE is a metabolic affecter that can reduce obesity and related disorders caused by metabolic syndrome. Its effects *in vivo* are complicated and need to be studied further to understand its mechanism. CGA is a promising therapy against obesity and metabolic disease.

1.6 Conclusion

This review summarizes the published scientific reports on the effects of GCBE and CGA in antioxidant and anti-inflammatory function and metabolic regulation. Overall, the research suggests that GCBE and CGA exerts these effects through a variety of pathways including ROS scavenging and quenching for antioxidant and anti-inflammatory pathways and modulation of lipid and glucose metabolism (Fig. 2). GCBE and CGA may be useful in the treatment of related disorders and diseases like obesity and metabolic syndrome.

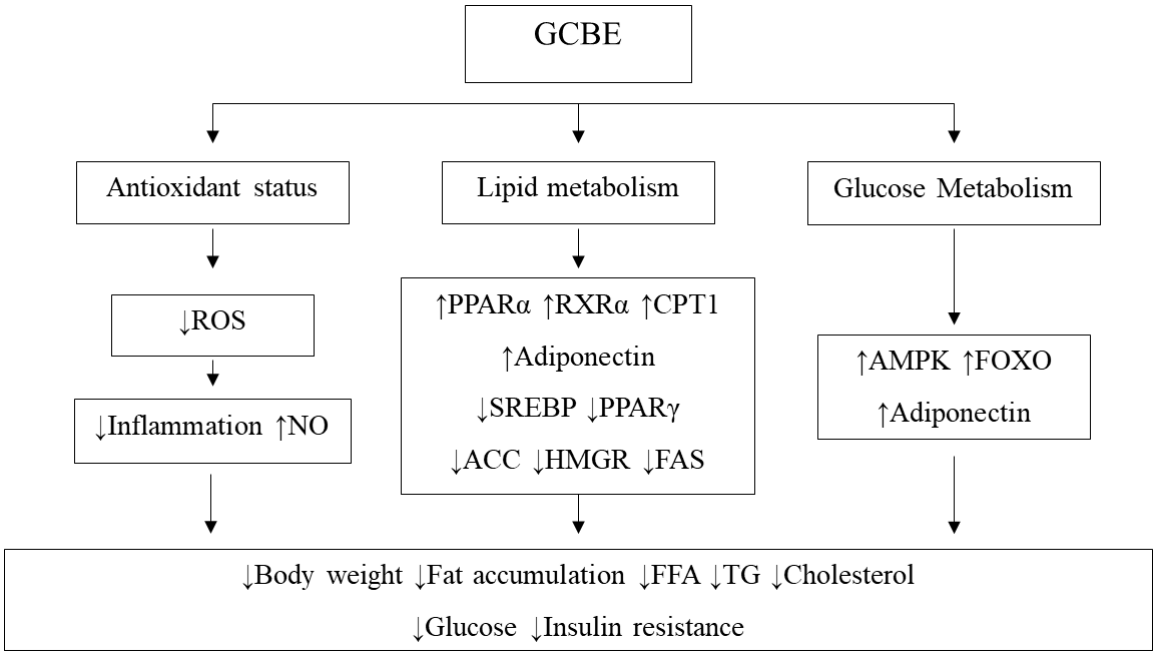


Figure 1.2 Summary of GCBE Biological Effects

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CPT, carnitine palmitoyl transferase; FAS, fatty acid synthase; FFA, free fatty acid; FOXO, forkhead box O; GCBE, green coffee bean extract; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; NO, nitric oxide; PPAR, peroxisome proliferator activated receptor; ROS, reactive oxygen species; RXR, retinoid X receptor; SREBP, sterol regulatory element-binding protein; TG, triglyceride

Table 1.1 Summary of *in vivo* studies of GCBE and CGA on metabolism.

Material	Dosage[a]	Treat-ment Time	Model	Observations[b]	Suggested Mechanisms[c]	Referen-ces
CGA	2.65 mg/mL	3 days	*C. elegans*	↓ TG	↑ ECH4, FOXO; ↓ ACC, ACS2, FAR4, C/EBPα, SREBP	(Farias-Pereira et al., 2018)
CGA	0.02% of diet	7 days	High fat diet fed ICR mice (♂)	↓ BW, visceral fat, plasma leptin, TG, cholesterol; ↑ adiponectin	↑ PPARα; ↓ HMGR, ACAT	(Cho et al., 2010)
CGA	100 mg/kg BW	6-15 weeks	High fat diet fed C57BL/6J mice (♂)	↓BW, cholesterol, FFA, glucose, insulin, TG	↑ ACOX1, CPT1, PPARα; ↓ CD36, FABP4, MGAT, PPARγ	(Ma et al., 2015)
CGA	250 mg/kg BW	2 weeks	$Lepr^{db/db}$ mice (♂)	↓BW, cholesterol, FFA, glucose, insulin, TG; ↑ adiponectin	↑ AMPK, CaMKK; ↓ ACC	(Ong et al., 2012)
CGA	250 mg/kg BW	2 weeks	$Lepr^{db/db}$ mice (♂)	↓BW, cholesterol, FFA, glucose, insulin, TG; ↑ adiponectin	↑ AMPK, CaMKK; ↓ ACC	(Ong et al., 2012)
CGA	90 mg/kg BW	12 weeks	High fat diet fed Sprague–Dawley rats (♂)	↓BW, visceral weight, TG, cholesterol, LDL, FFA	↑ PPARα, RXRα, CPT2; ↓ LXRα, LPL, CD36, ACC, FAS	(K. Huang et al., 2015)
CGA	100 mg/kg BW	8 weeks	High fat/carbohydrate diet fed Wistar rats (♂)	↓ visceral fat, systolic blood pressure, liver inflammation and fat	Modulation of gut microbiota	(Bhandarkar et al., 2019)

CGA	150 mg/kg BW	6 weeks	High fat diet Sprague-Dawley rats (♂)	↓ BW, cholesterol, TG, FFA	↑ AMPK, CPT1; ↓ ACC	(Sudeep et al., 2016)
GCBE (50% CGA, 2% caffeine)	5 mg/mL	3 days	*C.elegans*	↓ TG	↑ ACS2, ECH4, FOXO, HSL; ↓ ACC, FAR4, SREBP	(Farias-Pereira et al., 2018)
GCBE (27% CGA, 10% caffeine)	1 % of diet	2 weeks	ddy mice (♂)	↓BW, visceral fat, TG	↑ CPT	(Shimoda et al., 2006)
GCBE (71% CGA, 0.03% caffeine)	0.5% of diet	12 weeks	High fat diet fed C57BL/6J mice (♂)	No effect	NA	(Li Kwok Cheong et al., 2014)
GCBE (50% CGA)	200 mg/kg BW	6 weeks	C57BL/6J mice (♂)	↓ BW gain, liver weight, WAT weight	↑ PPARα, ATGL, HSL, CPT1 ↓ C/EBPα, SREBP-1c, SREBP-2, PPARγ, FAS	(Choi et al., 2016)

a. BW, body weight

b. BW, body weight; TG, triglyceride; FFA, free fatty acid; LDL, low-density lipoprotein; WAT, white adipose tissue

c. ACAT, acyl-CoA cholesterol acyltransferase; ACC, acetyl-CoA carboxylase; ACOX, acyl-CoA oxidase; ACS, Acetyl-coenzyme A synthetase; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; C/EBP, CCAAT/enhancer binding protein; CaMKK, calcium/calmodulin-dependent protein kinase kinase; CD, cluster of differentiation; CPT, carnitine palmitoyl transferase; ECH, enoyl-CoA hydratase; FABP, fatty acid-binding protein; FAR, fatty acid- and retinoid-binding protein; FAS, fatty acid synthase; FOXO, forkhead box O; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; LXR, liver X receptor; MGAT, monoacylglycerol acyltransferase; PPAR, peroxisome proliferator activated receptor; RXR, retinoid X receptor; SREBP, sterol regulatory element-binding protein

CHAPTER 2

GREEN COFFEE BEAN EXTRACT REDUCES FAT ACCUMULATION IN *DROSOPHILA MELANOGASTER*

2.1 Introduction

Obesity has developed into a global health crisis over the past several decades as rates have tripled since 1975 (Ogden & Flegal, 2015). Obesity is linked to range of conditions including cardiovascular disease, high blood pressure, high blood cholesterol, and type 2 diabetes mellitus (Must et al., 1999). Obese individuals are also at risk of joint and muscular disorders, respiratory problems, and increased cancer mortality (Fruh, 2017). Therefore, it is important to find therapeutic interventions beyond typical diet and lifestyle changes. Nutraceuticals have garnered attention for their ability to affect metabolism, including moderation of obesity (Dulloo, 2011). One nutraceutical that has been identified as having potential anti-obesity effects is green coffee, the unroasted seed of the coffee plant. Though the roasted form is more typical, green coffee contains more bioactive because the phenolic compounds have not been degraded by heat processing (Şemen et al., 2017). The extract of green coffee will be investigated in this study.

Green coffee bean extract (GCBE) and its constituent polyphenolic compound, CGA, are both known for their effects in metabolic regulation. GCBE has been shown to reduce fat accumulation in animals by modulating genes related to lipogenesis, fatty acid oxidation, energy homeostasis, adipocyte differentiation, and glucose metabolism (Choi et al., 2016; Farias-Pereira et al., 2018; Li Kwok Cheong et al., 2014; Shimoda et al., 2006). Though there has been evidence that describes the effects and mechanisms of GCBE and CGA in rodent models, there has not been any investigation of GCBE in *Drosophila melanogaster*.

D. melanogaster, commonly known as fruit flies, are a well-established *in vivo* model for metabolic studies as they have a high degree of conserved biology and physiology with humans including similar lipid and carbohydrate metabolisms (Pandey & Nichols, 2011). Flies also have tissues analogous to mammalian pancreas, liver, and adipose tissues, which all play a role in metabolic regulation (Trinh & Boulianne, 2013). The fat body is the critical organ in maintaining homeostasis in flies—it is equivalent to mammalian liver and adipose tissue for its storage and metabolism of fat and carbohydrates and its ability to act as an endocrine organ (Gutierrez et al., 2007). The fat body is distributed mainly in the abdomen but it also extends into the thorax and head so that it is in contact with the hemolymph for facilitating the exchange of metabolites (Hoshizaki, 2012). In addition, over 60% of all known human disease-causing genes are conserved in flies (Reiter et al. 2001). Therefore, the study of GCBE in *Drosophila melanogaster* is important as it may provide insight into its function that can be applicable to mammalian models and humans.

2.2 Materials and Methods

2.2.1 Materials

GCBE (50% chlorogenic acid and 2% caffeine) was purchased from NuSci Institute & Corp. (Batch No. 201511005, Walnut, CA, USA), the chlorogenic acid isoform 5-O-caffeoylquinic acid (5-CQA, IUPAC numbering, CAS 327-97-9; named previously as chlorogenic acid or 3-O-caffeoylquinic acid, pre-IUPAC numbering; Farias-Pereira et al., 2018) was purchased from Sigma-Aldrich Co. (purity ≥95%, St. Louis, MO, USA), fly stock bottles and vials from Genesee Scientific (San Diego, CA), and trehalase from Megazyme (Chicago, IL). The Jazz-Mix Drosophila food contained 65% brown sugar, 20% cornmeal, 11.5% yeast, 2.5% agar, 1.5% sodium propionate, 0.5% benzoic acid, and 0.5% methyl

paraben (w/v) (ThermoFisher Scientific Inc., Waltham, MA). Liquid food consisted of 5% sucrose and 5% yeast in distilled water (w/v). Capillary tube (5 µl) was from VWR International (Radnor, PA). Infinity triglycerides reagent, the bicinchoninic (BCA) protein assay reagent and Infinity glucose reagent were purchased from ThermoFisher Scientific Inc. (Waltham, MA).

2.2.2 Fly Rearing and GCBE Treatment

Flies were maintained at 25 °C in 12-hour light/dark (L/D) cycle in stock bottles with Jazz-Mix *Drosophila* food as the medium. To prepare the experimental diet, 0, 1, or 2 mg/mL GCBE or 0, 0.5, or 1 mg/mL CGA was directly mixed into the Jazz-Mix food during preparation. For the diet treatment, 2-4-day old flies were fed a control diet (0 mg/mL) or a diet containing the GCBE or CGA for 5 days. 5 days was chosen based on previous work (P. B. Chen et al., 2019).

2.2.3 Food Intake

Capillary feeding (CAFE) assay was performed based on Ja et al., 2007. with minor modifications. Four flies were placed in each vial with 1% agar solution bedding as a water source. After 2 days of habituation with liquid food, experimental and control diets were placed into the capillary tube and inserted into in the vials. Food was changed daily, and daily food intake was determined by volume (µL) with consideration of daily evaporation, measured by volume change in vials without flies.

2.2.4 Triglyceride/Protein Assay

Triglyceride and protein levels were quantified using a triglyceride (TG) and bicinchoninic acid (BCA) assay, respectively. Prior to quantification, *Drosophila* were decapitated and then homogenized in groups of 5 with 500 μL PBS and 0.05% Tween 20. Homogenized samples were used for TG and protein quantifications. TG concentrations were standardized by protein. Instructions were followed as per guidelines from the manufacturers.

2.2.5 Locomotor Activity

Locomotion behavior was monitored using the Drosophila Activity Monitoring System 2 (TriKinetics, Waltham, MA) (Chiu et al., 2010). Flies were briefly anesthetized with carbon dioxide and loaded individually into the monitoring tubes containing control or treatment food containing 1 or 2 mg/mL GCBE. Anesthesia was minimized to less than five minutes to avoid potential impacts on motor behavior (Bartholomew et al., 2015). Flies were habituated for 2 days before the locomotor assay was started. Spontaneous locomotion was measured as total infrared beam block counts for 24 hours. Each interruption of the infrared beams was considered a move.

2.2.6 Trehalose/Glucose Assay

Trehalose, a disaccharide of two glucose monomers, acts as the main circulating sugar in the hemolymph of insects (Yasugi et al., 2017). In this assay, trehalose was extracted from the fly homogenate and converted to glucose using trehalase. Samples of 5 flies were weighed, homogenized in a 0.1 M citrate buffer (pH 5.5), and centrifuged (10,000g for 10 mi). A sample of the supernatant was taken and digested with trehalase in a citrate buffer

with 0.5 mg/ml bovine serum albumin for 2 h at 37°C. The total sugar levels in the flies are expressed as glucose and were normalized by whole body protein levels.

2.2.7 Quantitative Reverse Transcription PCR

Total RNA from flies was extracted with Trizol® according to the manufacturer's instructions. RNA purity was assessed by calculating the 260/280 nm absorption ratio. Samples with values greater than 1.7 were used. Reverse transcription was carried out using a High Capacity cDNA Reverse Transcription Kit, and quantitative reverse transcription PCR (qRT-PCR) was performed on the StepOnePlus Real-Time PCR system (Sun et al. 2016). The following primers were used for qRT-PCR: *actin-42A* (*act42A*; a reference gene; Dm02362162_s1), *adipokinetic hormone* (*akh*; Dm01822073_g1), *brummer* (*bmm*; Dm01805233_m1), *spargel* (*srl*; Dm02136710_g1), *sterol regulatory element-binding protein* (*srebp*; Dm01793855_g1), *target of rapamycin* (*tor*; Dm01843300_g1), *forkhead box sub-group O* (*foxo;* Dm02140207_g1), *lipid storage droplet 2* (*lsd-2*, Dm01838905_m1), *withered* (*whd*, Dm01812891_g1), and *enoyl-CoA hydratase short chain 1* (*echs-1;* Dm01797402_g1). Quantification of the relative transcription level was calculated by the original concept of $2^{-\Delta Ct}$.

2.2.8 Statistical Analysis

GraphPad Prism6 was used for statistical analysis. Data were analyzed by a two-way analysis of variance (two-way ANOVA). The majority of experiments were designed as a 2×3 factorial arrangement with main effects of sex (female vs. male) and diets (Control, 1 mg/mL GCBE, and 2 mg/mL GCBE). Food intake was conducted as a 2×2 factorial

arrangement with effects of sex and diets (Control vs 2 mg/mL GCBE). *P*-values less than 0.05 were considered significant. All data were shown per group for presentation purposes only, regardless of the results of the two-way ANOVA.

2.3 Results

2.3.1 Body Weight

The body weights of flies were significantly different with sex ($P<0.0001$) and GCBE treatment diet ($P=0.0165$) without interaction (Fig. 1). As expected, females were 44% heavier than males. There were significant differences between the control and 1 mg/mL GCBE group ($P= 0.0365$) and between the control and 2 mg/mL GCBE group ($P=0.0261$) (Figure 1). There were no significant differences between the 1 and 2 mg/mL GCBE groups.

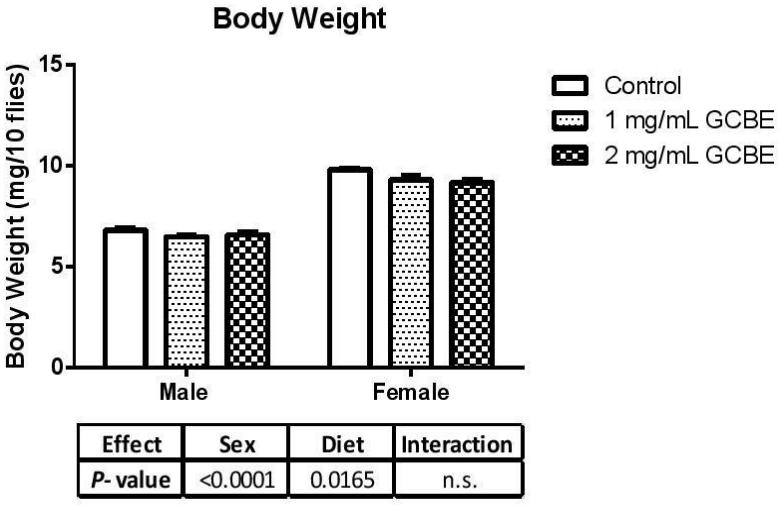
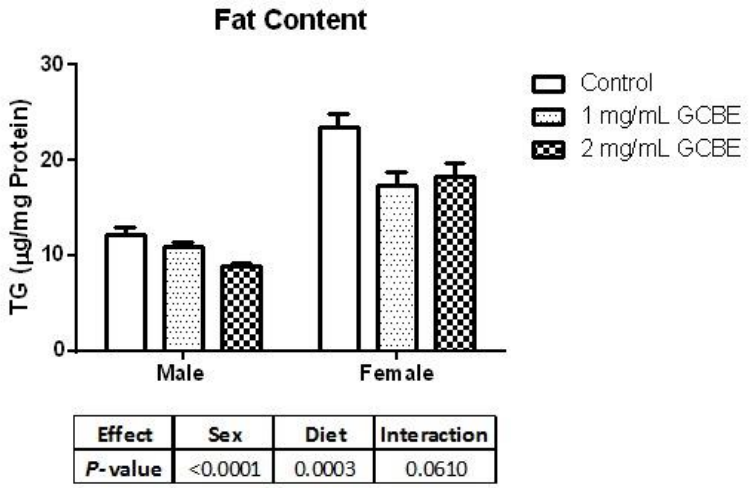


Figure 2.1 Body weight. Body weight of D. melanogaster fed a 0 (control), 1, or 2 mg/mL GCBE diet for 5 days. Values are expressed as mean ± S.E.M. (n=7). GCBE, green coffee bean extract.

2.3.2 Triglyceride Levels

There were significant differences in TG levels between sexes (P<0.0001) and diet groups (P=0.0003) with the interaction reaching significance (P=0.0610, Figure 2). Flies treated with 1 mg/mL and 2 mg/mL GCBE showed a 20% and 24% reduction in TG compared to control groups, respectively.

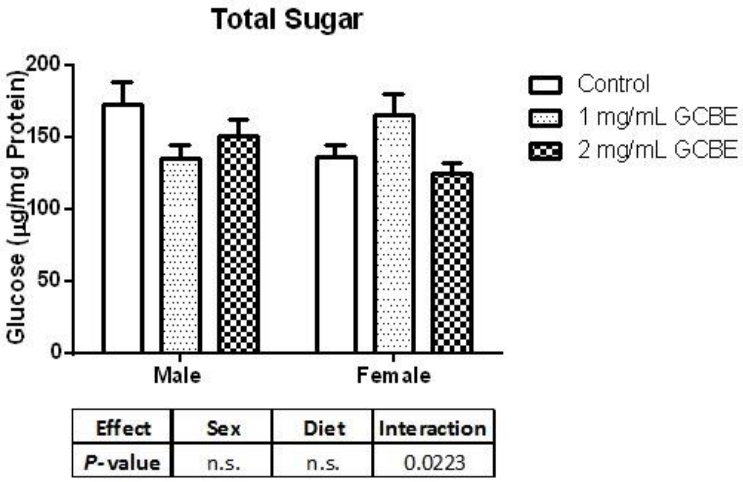


Effect	Sex	Diet	Interaction
P-value	<0.0001	0.0003	0.0610

Figure 2.2 Fat Content. Triglyceride content of *D. melanogaster* fed a 0 (control), 1, or 2 mg/mL GCBE diet for 5 days. Values are expressed as mean ± S.E.M. (n=7). TG, triglyceride; GCBE, green coffee bean extract.

2.3.3 Total Sugar

There were no significant effects of overall sex or diet on total sugar levels, but there was significant sex-diet interaction (P=0.0223, Figure 3). However, there were no significant differences between individual groups.

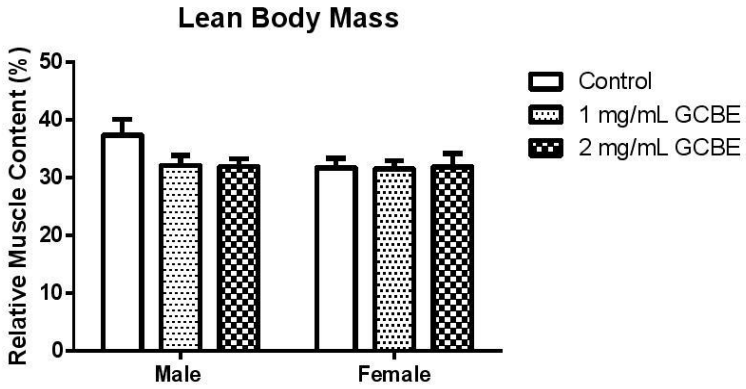


Effect	Sex	Diet	Interaction
P-value	n.s.	n.s.	0.0223

Figure 2.3 Total Sugar. Total sugar content (a summation of trehalose and glucose) of *D. melanogaster* fed a 0 (control), 1, or 2 mg/mL GCBE diet for 5 days. Values are expressed as mean ± S.E.M. (n=4). GCBE, green coffee bean extract.

2.3.4 Lean Body Mass

Green coffee bean extract treatment did not change relative muscle content of flies, nor did relative muscle content vary between males and females (Figure 4).



Effect	Sex	Diet	Interaction
P- value	n.s.	n.s.	n.s.

Figure 2.4 Lean Body Mass. Relative muscle content in *D. melanogaster* fed a 0 (control), 1, or 2 mg/mL GCBE diet for 5 days. After treatment period, thorax weight was standardized by body weight and measured. Values are expressed as mean ± S.E.M. (n=6). GCBE, green coffee bean extract.

2.3.5 Food Intake

There were significant differences in food intake by sex (P<0.0001) and GCBE treatment (P=0.0137) without significant interactions of sex and diet (Fig. 5). Female flies consumed more food than males, which is consistent with their larger body size and greater fat stores. Flies on the 2 mg/mL GCBE diet consumed 8% less food than control groups.

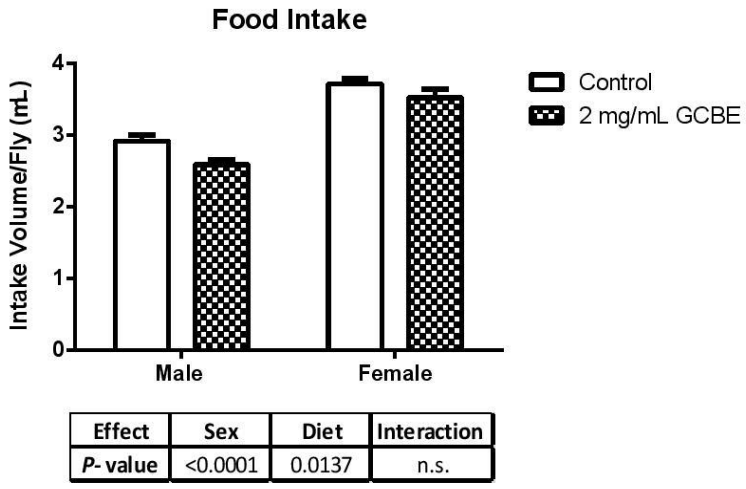


Figure 2.5 Food Intake. Food intake of *D. melanogaster* fed a 0 (control), or 2 mg diet for 5 days. Values are expressed as mean ± S.E.M. (n=4). n.s.: not significant.

2.3.6 Locomotor Activity

Figure 6 depicts the total movement count of flies in a 24-hour period and a movement count over a 24-hour period in 30-minute increments. Flies, like other crepuscular insects, are primarily active at dawn and dusk (Ferguson et al., 2015) and in this experiment, strong peaks of activity were seen when daylight hours began. The total locomotor activities of flies were not different by sex or diet.

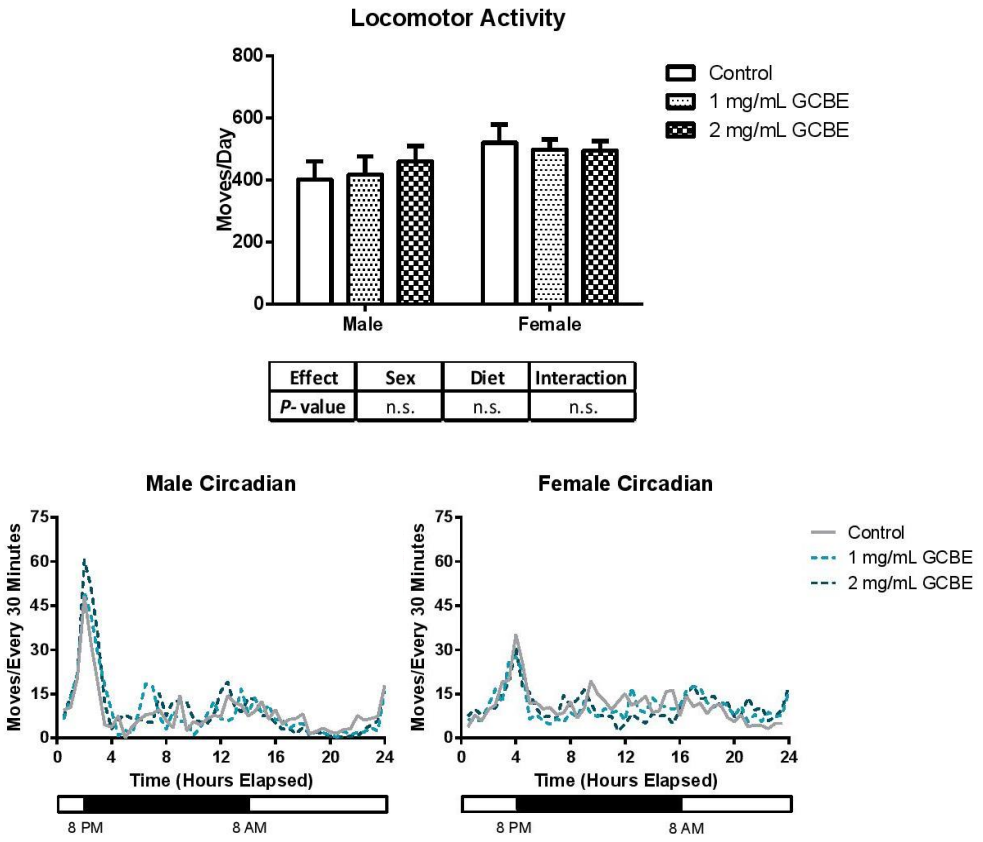


Figure 2.6 Locomotor Activity. Locomotor activity and circadian rhythms of *D. melanogaster* reared 12:12 light/dark cycle at 25°C with diets containing 0 (Control), 1, or 2 mg/mL GCBE. The locomotor activity of each individual fly for 24 h was presented as a 24-hour summation (locomotor activity) and in 30-minute intervals (circadian). Values are expressed as mean ± S.E.M. (n=9-10). GCBE, green coffee bean extract.

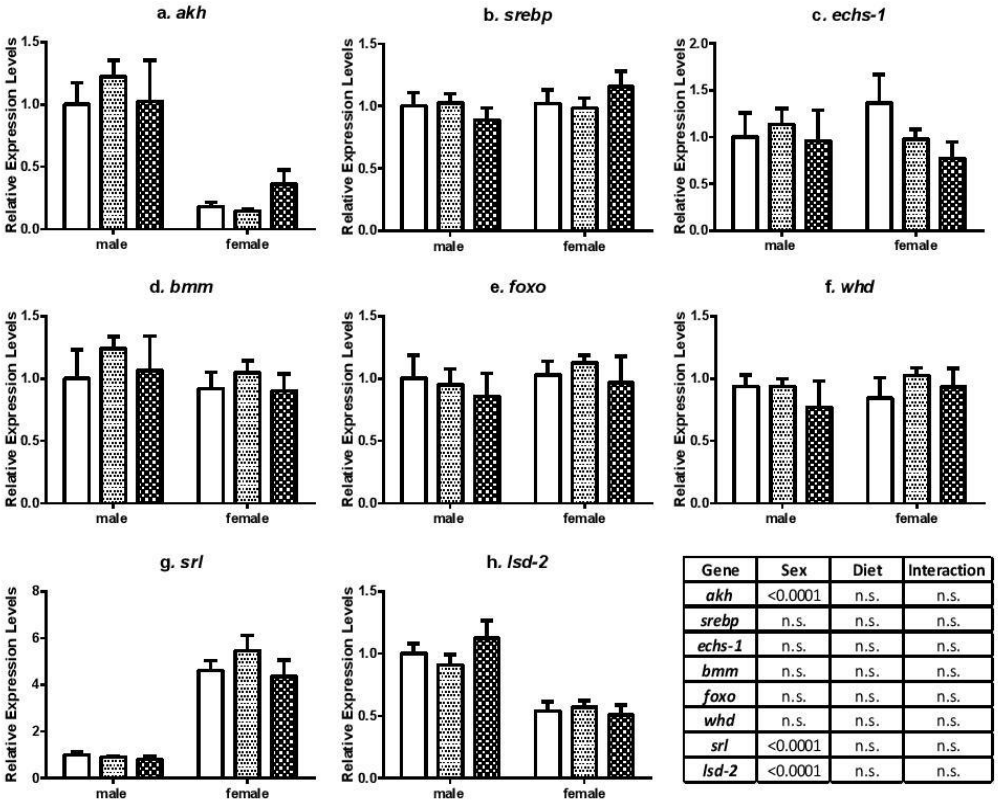
2.3.7 PCR

Akh is the human homolog of glucagon. There was a sex dependent effect on expression of *Akh* ($P<0.0001$) without effects from diet or sex-diet interaction (Fig. 7a). Males had an over 5.5-fold increase in expression compared to female flies.

Srebp is a homolog of human sterol regulatory element-binding protein, a key regulator in lipogenesis. *Echs-1* encodes for enoyl-CoA hydratase, which functions in the second step of mitochondrial fatty acid beta-oxidation. *Bmm* is a homolog of human adipocyte triglyceride lipase, which is a key regulator in lipolysis. *Foxo* is a transcription factor involved in insulin signaling. Its activation can also inhibit growth in response to cellular stresses. *Whd* encodes carnitine palmitoyltransferase I (CPT I), an enzyme required for the import of long-chain fatty acids into the mitochondria for beta-oxidation. There were no effects from sex, diet, or sex-diet interaction for *srebp, echs-1, bmm, foxo,* or *whd* (Fig. 7b-f).

Srl is homolog of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1α), a key regulator in energy homeostasis and mitochondrial biogenesis. There was a sex dependent effect on expression ($P<0.0001$) without effects from diet or sex-diet interaction (Fig. 7g). Females showed 4.6 times greater expression *srl than* male flies.

Lsd-2 prevents the mobilization of lipid stores and is a regulator of lipid storage amount and energy homeostasis. There was a sex dependent effect on expression ($P<0.0001$) without effects from diet or sex-diet interaction (Fig. 7h). Males showed 85.3% greater expression of *Lsd-2* compared to female flies.

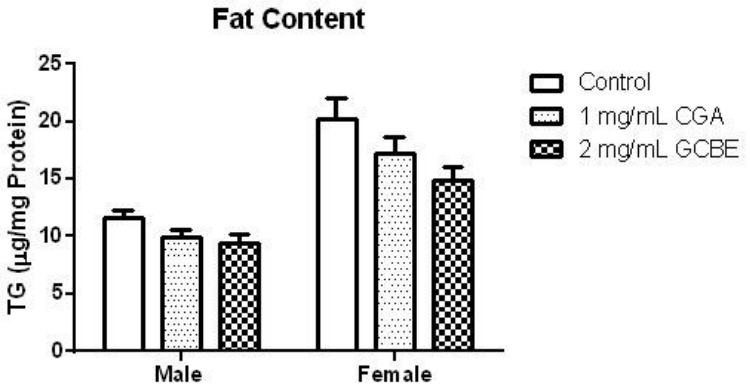


Gene	Sex	Diet	Interaction
akh	<0.0001	n.s.	n.s.
srebp	n.s.	n.s.	n.s.
echs-1	n.s.	n.s.	n.s.
bmm	n.s.	n.s.	n.s.
foxo	n.s.	n.s.	n.s.
whd	n.s.	n.s.	n.s.
srl	<0.0001	n.s.	n.s.
lsd-2	<0.0001	n.s.	n.s.

Figure 2.7 Summary of PCR Results. Expression levels of *adipokinetic hormone (akh*, a*), sterol regulatory element-binding protein (srebp*, b*), enoyl-CoA hydratase short chain 1 (echs-1*, c*), brummer (bmm*, d*), forkhead box sub-group O (foxo*, e*), withered (whd*, f*), spargel (srl*, g*),* and *lipid storage droplet 2 (lsd-2*, h*)* in *D. melanogaster*. Gene expression was determined using whole fly homogenates from flies fed with 0 (Control), 1, or 2 mg expressed /mL GCBE. Data are as the mean ± S.E.M. (n=4). P-value of the gene expressions are shown in the table. GCBE, green coffee bean extract.

2.3.7 The role of Chlorogenic Acid in GCBE

In a comparison of flies fed an equivalent dose of CGA and GCBE, there was a significant difference in triglyceride levels based on sex ($P<0.0001$) and diet ($P=0.0103$) without sex-diet interaction (Figure 8). Flies treated with GCBE groups showed significant decrease from control ($P=0.0080$). Flies treated with chlorogenic acid did not have significant differences compared to the control or GCBE group.



Effect	Sex	Diet	Interaction
P-value	<0.0001	0.0103	n.s.

Figure 2.8 Fat Content. Triglyceride content of *D. melanogaster* fed a 0 (control), 0.5, or 1 mg/mL CGA diet for 5 days. Values are expressed as mean ± S.E.M. (n=6). TG, triglyceride; CGA, chlorogenic acid; GCBE, green coffee bean extract.

2.4 Discussion

This study shows that treatment with GCBE decreases fat accumulation in *Drosophila* without changes in lean body mass or glucose. The current results show that effects of GCBE on fat reduction were sex independent. This is the first investigation to report GCBE reduced fat accumulation, in part through a reduction of food intake in *Drosophila melanogaster*. However, additional research would be needed to clearly elucidate the mechanism of GCBE on body fat reduction.

Previous studies of GCBE in rodent models suggest a wide variety of pathways that are involved in its metabolic effects. GCBE reduced body weight, visceral fat accumulation, insulin resistance, and altered lipid profiles by modulating genes related to fatty acid oxidation, energy homeostasis, adipocyte differentiation, and glucose metabolism (Choi et al., 2016; Li Kwok Cheong et al., 2014; Shimoda et al., 2006). This study did not identify differences in whole-body expression of genes involved in lipogenesis, fatty acid oxidation, energy homeostasis, or glucose regulation. However, whole-body analysis may have obscured the results. Previous work has shown that there are tissue specific differences in gene expression in flies (Girardot et al., 2006). It may be useful to study gene expression in different tissues, like the thorax and fat body of the abdomen to get a clearer view of changes in gene expression.

This study identified decreased food intake by GCBE as a mechanism that contributed to decreased fat in male and female flies. GCBE could have reduced feeding due to its sensory properties. The major flavors associated with polyphenolic compounds, like CGA found in GCBE, are bitter and astringent (Cheynier, 2005), which may be detectable to flies. Fruit flies have the ability to perceive bitter substances through expression of gustatory

receptor (Gr) genes (Weiss et al., 2011) and will show avoidance behaviors toward foods containing bitter substances (French et al., 2015). Therefore, it is possible that flies in this study ate less food simply out of avoidance of GCBE. There was no sex-diet interaction of GCBE on food intake, even though females are typically more sensitive to bitter compounds and show decreased consumption of food containing polyphenols when males show no avoidance (P. B. Chen et al., 2019; Lacaille et al., 2009). This may be caused by sex-based differences in odorant-binding proteins in *Drosophila* (Swarup et al., 2014). One group found that flies entirely did not avoid consuming polyphenolic compounds from tea (Kayashima et al., 2015) and another found that flies can detect and are attracted to hydroxycinnamic acids in their diet (Dweck et al., 2015). Thus, there may be other factors besides taste perception that are responsible for GCBE causing a decrease in food intake. Further study into the effects of GCBE on food intake in *Drosophila* may be needed to determine the cause behind this response.

Factors like the endogenous circadian clock, courtship, and stress behaviors can affect locomotor activity in flies (Manenti et al., 2015). GCBE did not change locomotor activity even though caffeine is expected to increase locomotion. In another study on *Drosophila,* flies showed 25% increase in locomotion with 0.01% caffeine treatment. However, it's likely that the dosage of caffeine in this study, about 0.004%, was not significant enough to cause a physiological change.

In this study, no significant differences in lean body mass or total sugar were found between treatments. Sugars circulate in the *Drosophila* mainly as trehalose with glycogen as the main storage form (Yamada et al., 2018). Trehalose regulation in flies is a contentious topic as there is doubt whether flies hormonally regulate circulating sugars in the body, given

that levels of circulating sugar tend to reflect dietary sugar and metabolic activity (Graham & Pick, 2017). This is supported by the fact that flies require rapid mobilization of energy to power flight (Candy et al., 1997). Therefore, high variation in total sugar between samples may be caused by natural variance in sugar based on diet and metabolism. Further studies should be conducted to evaluate GCBE regulation of sugars in *Drosophila.*

CGA is the main polyphenolic compound present in GCBE, and several studies have shown its efficacy in reducing body weight and TG, improving lipid profiles, and moderating glucose and insulin (Bhandarkar et al., 2019; Cho et al., 2010; Farias-Pereira et al., 2018; Y. Huang et al., 2019; Ma et al., 2015; Ong et al., 2012; Sudeep et al., 2016). Some studies reported that CGA was able to reduce physiological factors like hepatic inflammation and cardiovascular indicators, but not body weight or fat in the models (Nishitsuji et al., 2018; Panchal et al., 2012). In our study, we found that the 5-CQA isomer of CGA had no effect on TG, suggesting that in flies, this may not be the main active compound in GCBE, or that it works in concert with other compounds found in GCBE. The bioavailability of polyphenols is highly variable, and its presence in a food or as an isolated compound can affect its absorption (Manach et al., 2004). It may be useful to assess the absorption of CGA as isolated 5-CQA compared to absorption through GCBE diet before further probing the effects of CGA in fruit flies.

Previous studies evaluating the effects of GCBE on metabolism used dosages of 0.5%-1% diet (Farias-Pereira et al., 2018; Li Kwok Cheong et al., 2014; Shimoda et al., 2006), 2-5 times higher than the dosage used in this study. In clinical trials with overweight subjects, patients used 180-200 mg daily (Onakpoya et al., 2011). There is currently no

knowledge on the relationship of GCBE dosage, oral bioavailability, or metabolism by microbiomes and biological systems in flies and humans, which is a limitation of this study.

2.5 Conclusion

The current study investigated the effects of GCBE on metabolism in the model organism, *Drosophila melanogaster*. GCBE reduced body weight and fat accumulation but did not alter total body sugar or relative lean body mass. Though investigation into locomotor activity or gene expression did not show any changes from GCBE supplementation, the study identified reduced food intake as a mechanism for GCBE's effects. Future investigation into the effects of GCBE should conducted to further elucidate its mechanism. This study provides a foundational basis for understanding of the effects of GCBE in *Drosophila melanogaster*. Overall, this can contribute to the body of knowledge on the use of phenolic compounds as anti-obesity nutraceuticals.

CHAPTER 3

FUTURE DIRECTION

This study provides an introduction to the potential metabolic effects of GCBE in *Drosophila melanogaster*. With reduced body weight, fat accumulation, and food intake, there's evidence that GCBE regulates metabolism in flies. However, no conclusive results were found regarding changes in total body sugar, locomotor activity, or gene expression of the chosen targets. In addition, CGA, the main polyphenolic compound in GCBE, did not reduce fat accumulation in flies. Therefore, the underlying mechanism of GCBE's metabolic effects still need to be further determined.

Though the gene targets chosen in this study were involved in several pathways involved in lipogenesis, fatty acid oxidation, energy metabolism, and insulin signaling, only 8 genes were assessed. There are several other genes that are relevant to these pathways that could be determined. Previous literature has identified genes that showed changes in expression after oral administration of GCBE or CGA. Relevant genes that were not tested in this study include acetyl CoA carboxylase (Sudeep et al., 2016), adiponectin and leptin (Cho et al., 2010), AMP-activated protein kinase, and other downstream targets (Ong et al., 2013). In addition, further literature could be conducted on metabolic regulation by other polyphenolic compounds to identify more potential gene targets.

In this study, RNA was extracted from whole body samples of flies. However, analyzing separate body parts of the flies, like the thorax or abdomen may provide useful results. Different body parts contain different tissues which may have different responses after treatment with GCBE. For example, the fat body contains most of the adipose tissue in the body. In other animal models, adipose tissue can have different expression from other

tissues. So, assessing the expression of genes related to energy metabolism specifically in the fat body could reveal more information on GCBE's potential pathways of action.

A goal for the future of this project is to identify the compounds in GCBE that are responsible for its metabolic effects in flies. In this study, CGA had no significant effect on fat accumulation compared to control groups. This suggests that there may be other compounds in GCBE with bioactive effects. A series of experiments using analytical tools could be used to further investigate. First, the GCBE could be run through a high-performance liquid chromatography (HPLC) column to identify other potential bioactive compounds. Then, flies could be treated with GCBE and isolated compounds, including CGA. The fly samples could be analyzed using HPLC to identify which compounds were absorbed. It is possible that isolated compounds, like the CGA (5-CQA) used in this study, may not have the same bioactivity as in a whole source like GCBE. Also, the compounds in GCBE may have synergistic effects when ingested together. Isolated compounds could be individually administered to flies and the samples could be compared with flies fed a mixture of compounds to assess whether certain compounds in GCBE can exert effects synergistically. This could be the beginnings of an investigation into the source of GCBE's metabolic effects.

Overall, the study of GCBE in *Drosophila* can contribute to the overall knowledge of the mechanism and action of GCBE as a metabolic regulator for its use as an anti-obesity agent. Understanding its role in flies can provide insight into its actions in mammals and eventually, humans.

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