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The impact of green tea polyphenols on development and reproduction in *Drosophila melanogaster*

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Abstract

Although, green tea has numerous health benefits, adverse effects with excessive consumption have been reported. Using *Drosophila melanogaster*, a decrease in male fertility with green tea was evidenced. 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. GTP 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. GTP 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*.

Keywords

development; *Drosophila*; green tea; reproduction; toxicity

1. Introduction

Green tea, derived from the plant *Camellia sinensis*, is not only a popular drink worldwide but it is also considered a nutraceutical (Mak, 2012) as a neuroprotective (Weinreb, Mandel, Amit, & Youdim, 2004), cardioprotective (Basu & Lucas, 2007; Zhong, Huan, Cao, & Yang, 2015) and anti-carcinogenic agent (Fujiki, Sueoka, Watanabe, & Suganuma, 2015). 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 (Sarma, Ko, & Dog, 2008; Schonthal, 2011). Furthermore, green tea

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest

components, particularly its primary active flavonoid, epigallocatechin gallate (EGCG), has been shown to interact with and influence metabolism of a number of drugs (Andersen et al., 2013; Cuzzolin, Zaffani, & Benoni, 2006) and cytochrome P450 substrates (Schonthal, 2011; Yang & Pan, 2012), 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 (Mazzanti, Di Sotto, & Vitalone, 2015), gastrointestinal (Schonthal, 2011), hematological and renal toxicities (Wu, Yao, & Boring, 2011), as well as decreased hormonal levels (Chandra, Choudhury, De, & Sarkar, 2011; Kao, Hiipakka, & Liao, 2000), sperm counts (Chandra et al., 2011; De Amicis, Santoro, Guido, Russo, & Aquila, 2012) and atrophy of reproductive organs (Kapetanovic et al., 2009; Wu et al., 2011). 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 (Lopez et al., 2014).

The fruit fly, *Drosophila melanogaster*, with its evolutionary conserved biological pathways, is a commonly used model organism in biomedical research (Jafari, Long, Mueller, & Rose, 2006) and an emerging model to screen for adverse drug reactions (Avanesian, Semnani, & Jafari, 2009). Evaluation of drug induced developmental and reproductive effects is commonly used to assess adverse drug reactions (Siddique et al., 2009; Weisbrot, Lin, Ye, Blank, & Goodman, 2003).

The *Drosophila* life cycle consists of distinct developmental stages that include embryogenesis, 1st, 2nd and 3rd larval instars, pupae and adults (Kozlova & Thummel, 2000). Each stage is highly regulated by transcriptional control in response to nutritional, environmental and hormonal cues (Kozlova & Thummel, 2000). 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 (Avanesian et al., 2009). 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 (Affleck, Neumann, Wong, & Walker, 2006; Kislukhin, King, Walters, Macdonald, & Long, 2013). 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 (Tiwari, Pragya, Ram, & Chowdhuri, 2011).

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.

2. Materials and Methods

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.).

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.

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.

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 CO_2 plate and weighed using a Sartorius SE2 Ultra Micro Balance (Bradford, MA, USA) in groups of 10 (n=60 per treatment, per sex).

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.

2.6 Fertility assay

Male fertility was performed as outlined in our previous work, Lopez *et al*. (2014), 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.

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)

2.8 Measurement of water, lipid and protein contents

Water, lipid and soluble protein levels were measured as previously described in Schriner *et al.*, (Schriner et al., 2013). 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.

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.

2.10 Gene expression assay

Heat shock protein expressions were performed as described in Schriner *et al*. (Schriner et al., 2013). 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: CGACGGCTGGTAGTGACCGC. HSP70 forward: ACCAAGGGGTGTGCCCCAGA, and reverse: CTTGGCCTTGCCCGTGCTCA. HSP22 forward: TTGGCGGATGGCCGAGGAGA, and reverse: AGCGCCACACTCCAAACGGG. Primers were designed by NCBI/Primer-BLAST.

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.

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.

3. RESULTS

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 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 (Svoboda, Vlčková, & Nováková, 2015).

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 (Fig. 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 (Fig. 1A). A surprising finding was the extended delay in pupation and emergence of offspring as doses of GTP increased (Fig. 1B).

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

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 (Fig. 3) which is consistent with fewer cells in GTP treated flies.

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* (Avanesian et al., 2009; Tiwari et al., 2011). 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 (Fig. 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 (Fig. 4D, 4E). Interestingly, an increase in offspring numbers was observed (Fig. 4F).

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 (Anderson, 1945; Siddique et al., 2009). Male offspring emerged from control food had normal reproductive structures such as full testes, seminal vesicles and accessory glands (Fig. 5A). However, male offspring from the GTP group exhibited atrophy of reproductive organs and a dramatic reduction of the number of nuclei in testes (Fig. 5B). Similarly, control females had normal ovaries and recognizable structures including ovarioles and mature eggs (Fig. 5C), whereas emerged females from GTP group revealed smaller ovarian structures and absence of mature eggs (Fig. 5D).

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 (Chippindalet, 1998). Offspring emerged from GTP food had increased water (Fig. 6A) but reduced lipid (Fig. 6B) contents in both sexes. No changes in soluble protein levels were observed (Fig. 6C).

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* (Rose, Vu, Park, & Graves, 1992). Flies emerged from GTP were resistant to desiccation (Fig. 7A and 7B) but sensitized to starvation (Fig. 7C and 7D) and heat stress (Fig. 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 (Fig. 7G), female survival was significantly reduced (Fig. 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 (Fig. 8A) and HSP22 (Fig. 8B) in male flies. While an increase in HSP70 and HSP22 may be present in females as well, the effect was not statistically significant.

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 (Fig. 9A), whereas males were unaffected (Fig. 9B).

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 (Lopez et al., 2014). 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 (Böhni et al., 1999; Gupta et al., 2007; Siddique et al., 2009). It has been suggested that these observations

are the result of somatic effects caused by the relevant treatment (Siddique et al., 2009). 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 (Fan & Chan, 2014; Tu, Chen, & Chan, 2010). 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 (Ghasemi-Pirbaluti et al., 2015; Gupta, Ahmad, Nieminen, & Mukhtar, 2000; Lambert & Elias, 2010). 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 (Böhni et al., 1999).

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 (Murakami, 2014; Son, Camandola, & Mattson, 2008). In flies, beneficial effects after exposure to hormetic compounds can lead to increased resistance against further stressors such as environmental and oxidative stress (Birringer, 2011; Mattson, 2008). 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 (Chippindalet, 1998). 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 (Forester & Lambert, 2011). 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 (Lopez et al., 2014). 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 (Kozlova & Thummel, 2000; Lehner & O'Farrell, 1989) but in adult stages, flies are primarily post-mitotic (Lehner & O'Farrell, 1989). 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 (Bandele & Osheroff, 2008; Gupta et al., 2000; Kuzuhara, Sei, Yamaguchi, Suganuma, & Fujiki, 2006; Saiko et al., 2015). 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 (Mattson, 2008). 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 (Siddique et al., 2009). 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 (Bhole, Allikian, & Tower, 2004; Morrow & Tanguay, 2015). Additionally, both HSP70 and HSP22 are up-regulated during *Drosophila* aging (Tower, 2011). 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 (Abbas & Wink, 2009; Zhang, Jie, Zhang, & Zhao, 2009). 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 (Yang & Tower, 2009). Yang and Tower (2009) 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 (Bhole et al., 2004). 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 (Adler, Cassidy, Fricke, & Bonduriansky, 2013; Maklakov et al., 2008) 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 (Avanesian et al., 2009; Kislukhin et al., 2013; Kozlova & Thummel, 2000). 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.

Acknowledgments

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Abbreviations

GTP	green tea polyphenols
EGCG	epigallocatechin gallate
ECG	epicatechin gallate
EGC	epigallocatechin
EC	epicatechin
EG	ethylgallate
HSP	heat shock protein

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HIGHLIGHTS

- Fruit fly larvae were exposed to green tea polyphenols (GTP) only during development
- A high dose delayed development, reduced offspring sizes and impaired reproduction
- Morphological abnormalities of reproductive organs were observed
- High doses of GTP may cause developmental adverse effects in humans

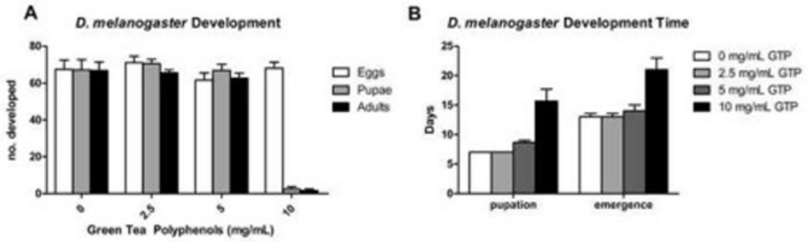

Fig. 1. The effect of green tea polyphenols on development

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.

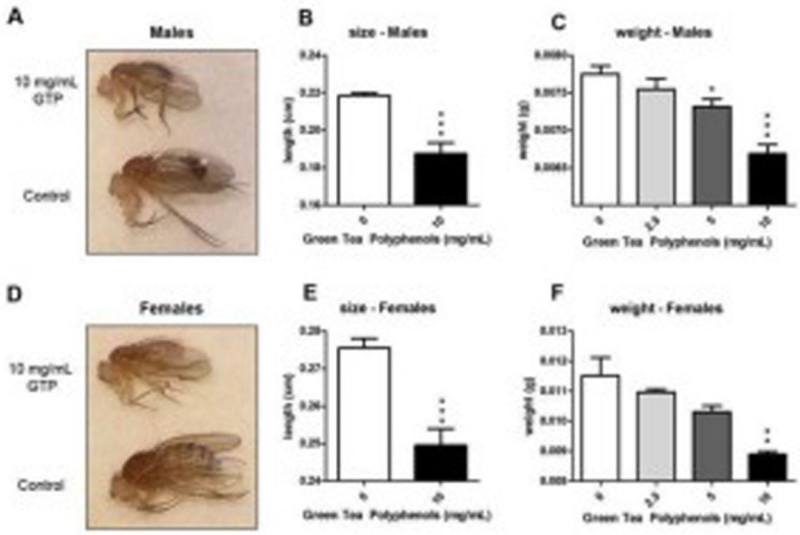

Fig. 2. Phenotypic effects of emerged offspring from food treated with green tea polyphenols

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).

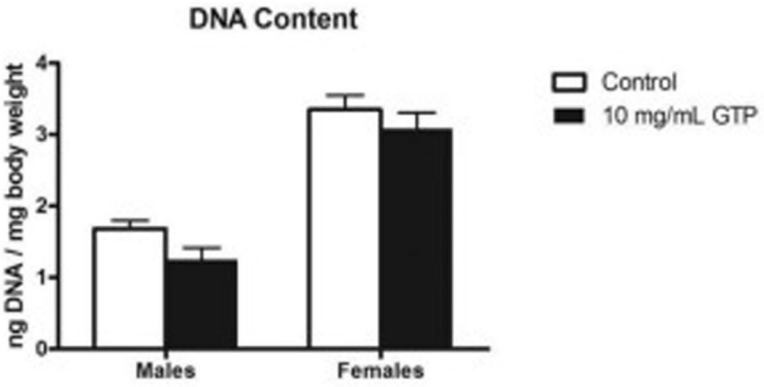

Fig. 3. DNA content in flies treated with green tea polyphenols

DNA content isolated from treated flies normalized to body weight was used to determine whether cell numbers were affected by GTP treatment. Flies treated with GTP did not exhibit an increase in DNA content normalized to body weight. Data are presented as means ± SEM, *n*=30 per treatment, per sex and analyzed by two-way ANOVA.

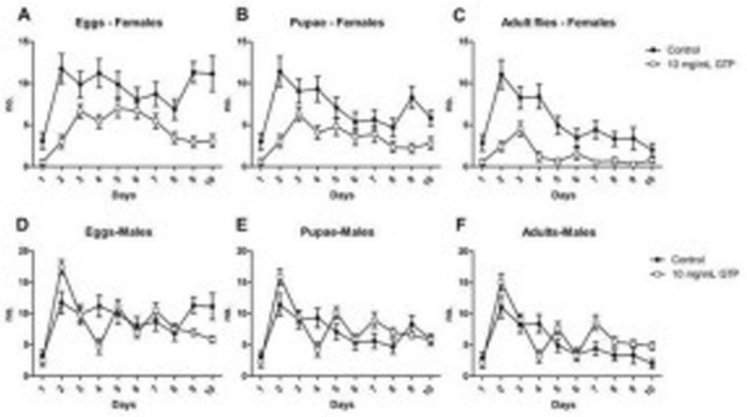

Fig. 4. Reproductive output of adult *Drosophila melanogaster* fed green tea polyphenols during larval stages

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.

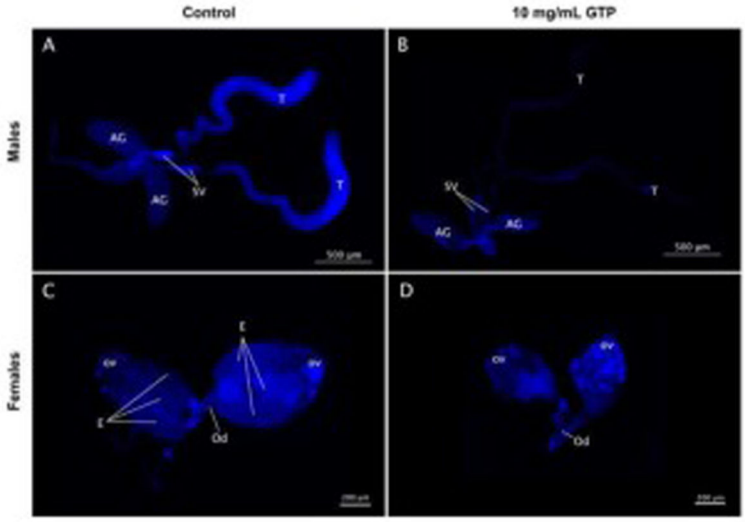

Fig. 5. The effect of green tea polyphenols on reproductive organs

Reproductive organs of emerged offspring from GTP-treated food evaluated by DAPI nuclei stain revealed morphological abnormalities. Male reproductive organs from control males (A) showed normal and full structures of accessory glands (*AG*), seminal vesicles (*SV*) and testes (*T*). Males emerged from GTP food exhibited an overall reduction in organ size, atrophy of testes and reduced number of nuclei (B). Ovaries from control females (C) revealed normal organ structures including the presence of ovarioles (*ov*), mature eggs (*E*) and ovarian duct (*Od*). Females exposed to GTP exhibited a dramatic reduction in ovary size and absence of mature eggs (D).

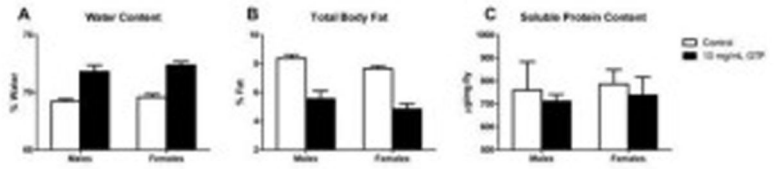

Fig. 6. The effect of green tea polyphenols on water, lipid and protein content

Larval fed GTP offspring exhibited higher levels of water (A) and lower total body fat content (B) than controls ($P<0.0001$). Offspring did not exhibit significant changes in soluble protein content ($P>0.05$) (C). Data are presented as means ± SEM ($n=60$ per treatment per sex) and analyzed by two-way ANOVA with Bonferroni post-test. $P>0.05$ are not significant.

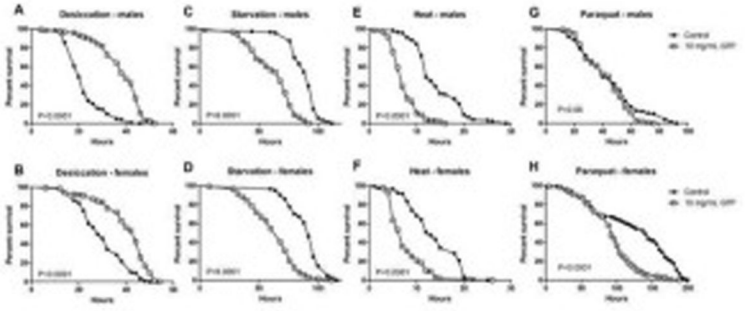

Fig. 7. The effect of green tea polyphenols on the tolerance towards desiccation, starvation, heat and paraquat

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.

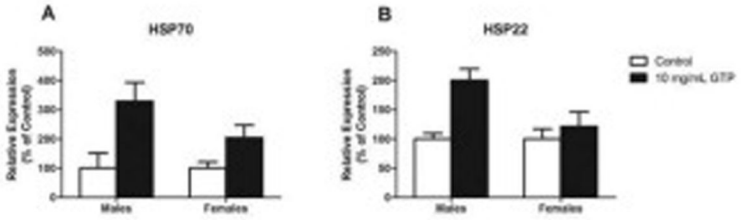

Fig. 8. The effect of green tea polyphenols on heat shock proteins

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.

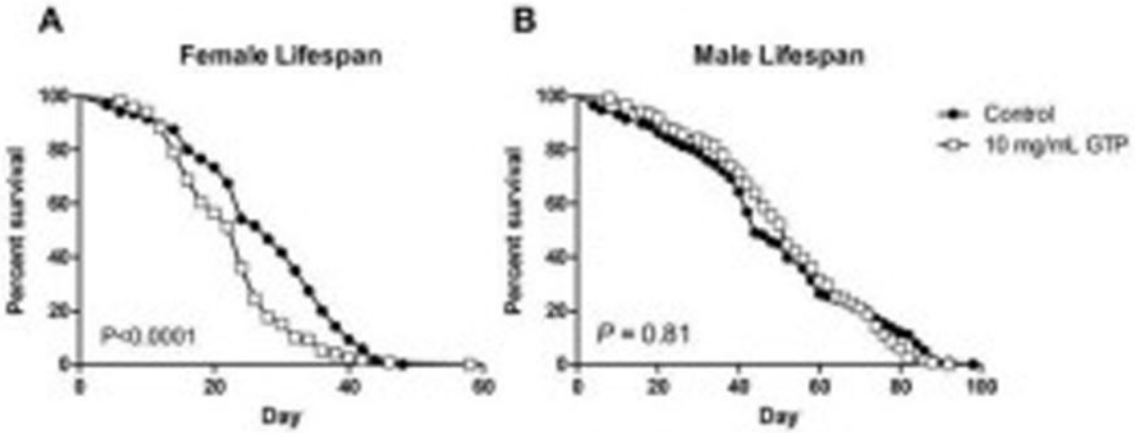

Fig. 9. *Drosophila* male and female lifespan after treatment with green tea polyphenols during development

Female *Drosophila* exposed to a 10 mg/mL treatment of GTP during larval stages subsequently exhibited a 17% decrease in adult lifespan (A). Emerged male offspring from treated food were not affected from the treatment (B). The sample sizes were n=120 per sex and per treatment. P values were calculated by Mantel-Cox log rank test.

Table 1

Composition of green tea polyphenols

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

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