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Green Tea Polyphenols Extend the Lifespan of Male *Drosophila melanogaster* While Impairing Reproductive Fitness

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ABSTRACT Green tea is a popular beverage believed to have many health benefits, including a reduction in the risks of heart disease and cancer. Rich in polyphenolic compounds known as catechins, green tea and its components have been shown to increase the lifespan of various animal models, including *Drosophila melanogaster*. Here, we investigated the gender-specific effects of green tea on the lifespan of fruit flies and observed that green tea 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 an inverse correlation between lifespan and reproduction, the impact of green tea on male reproductive fitness was also investigated. We found that green tea negatively impacted male fertility as shown by a reduced number of offspring produced and increased mating latency. We further identified that the lifespan extension properties of green tea was only observed in the presence of females which alludes to a reproductive (or mating) dependent mechanism. Our findings suggest that green tea extends the lifespan of male flies by inhibiting reproductive potential, possibly by limiting iron uptake. To our knowledge, our study is the first to report the negative impact of green tea on *Drosophila* male reproduction. Our results also support previous studies that suggest that green tea might have a negative effect on reproductive fitness in humans.

KEY WORDS: • ***aging*** • ***green tea*** • ***iron*** • ***reactive oxygen species***

INTRODUCTION

TEA IS THE MOST CONSUMED beverage in the world after water. Green tea, a nonfermented product of the plant *Camillia sinensis*, has been popularized as an excellent source of health modulating dietary antioxidants.[1–3] Rich in flavonoids known as catechins (epigallocatechin gallate [EGCG], epicatechin gallate [ECG], epigallocatechin [EGC], epicatechin [EC], and catechin),[4,5] green tea has been reported to improve blood lipid profiles, reduce the risks of coronary heart disease, control body weight, significantly increase plasma antioxidant capacity in humans, and reduce the risks of some cancers.[1–4] Furthermore, green tea polyphenols (GTPs) have been implicated as neuroprotective agents in diseases such as Alzheimer's and Parkinson's through the reduction of iron accumulation, reactive oxygen species (ROS), and inflammation.[3,6] Being regarded as a functional food and broad-spectrum botanical, green tea appears to impact various biological processes that may contribute to a reduction in the rates of aging and age-associated diseases.[3,6]

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Previous reports have found green tea to extend the lifespan of various animal models. In *Caenorhabditis elegans*, daily administration of 220 μM of EGCG, its most prevalent and active flavonoid, increased the mean lifespan up to 14%.[7] This same study showed that under lethal oxidative stress, survival rates increased by 65%.[7] 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.[8] In *Drosophila melanogaster*, flies fed on a diet of green tea extract had an increased mean lifespan of 16% in male flies with a corresponding upregulation of superoxide dismutase (SOD) and catalase.[9] Another study reported a 21% increase in male fly lifespan by inhibition of age-related iron accumulation.[10] 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, $P < .05$).[11] However, reports on heterogeneous mice revealed no differences in lifespan.[12,13] A more recent report in Wistar rats revealed that EGCG significantly increased median lifespan to 105 weeks compared with controls at 92.5 weeks.[14] These authors found that EGCG delayed death of healthy rats by $\sim$8–12 weeks and attributed the effect to a reduction in age-associated inflammation, oxidative stress, liver and kidney damage.[14]

In this study, we report that GTPs extend the lifespan of male fruit flies whereas simultaneously reducing male fertility. A number of compounds that had negatively impacted reproduction had also resulted in an increase in *Drosophila* lifespan.[15,16] 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.[17,18] Female fruit flies exhaust their energy resources in the production of eggs.[18] Male fruit flies, however, require an exertion of energy during mating, which involves a complex ritual of courtship behaviors.[19,20] 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.[15,16]

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

MATERIALS AND METHODS

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 through 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 before use. Treatment diets consisted of 10 mg/mL GTPs 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°C ± 1°C under 12-h light–12-h 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.

DR lifespans

A total of 120 flies per treatment group per sex were fed either control or GTP diets 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.

Mitochondrial assays and enzyme activities

Mitochondrial isolation, respiration, and fumarase activity were performed as detailed in Schriner *et al.*[21] Rates of mitochondrial superoxide production were measured with the fluorescent dye MitoSOX (Invitrogen, Carlsbad, CA, USA) based on a protocol by Robinson *et al.*[22] SOD activities were determined as described in Winterbourn *et al.*[23] Catalase activity was measured by direct decomposition of H_2O_2 as detailed by Beers and Sizer.[24]

Environmental and oxidative challenges

All environmental and oxidative challenges were performed as outlined in Schriner *et al.*[21] Death counts were recorded as follows: every hour under heat stress, every 2 h under desiccation, and every 4 h for oxidative challenges. Water and lipid content in flies were also determined using methods described in Schriner *et al.*[25]

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 nontreated food every day at the same time for 10 days. Offspring were allowed to develop and counted 2 weeks later.

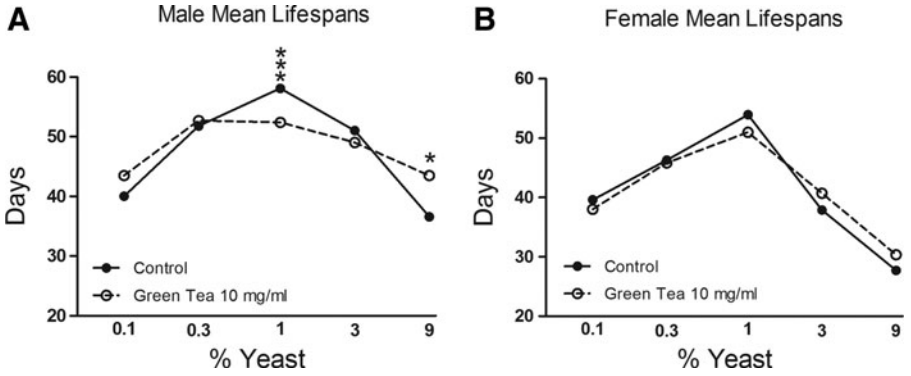

FIG. 1. The impact of dietary yeast manipulations on the lifespan of male and female fruit flies supplemented with green tea polyphenols (GTPs). Male fruit flies **(A)** supplemented with GTP exhibited a 19% increase in mean lifespan at the 9% dietary yeast content, $*P = .01$ and a 10% decrease in mean lifespan at the 1% dietary yeast content, $***P = .0006$. There was no effect of GTP on the lifespan of female flies at all yeast levels, P values $< .05$ **(B)**. Survival curves were analyzed by Mantel–Cox log-rank test. Sample sizes for control and treated groups were as follows: Males: 0.1%: 121, 116; 0.3%: 116, 119; 1%:115, 116; 3%: 107, 100; 9%: 123, 129; Females: 0.1%: 109, 109; 0.3%: 114, 102; 1%: 104, 111; 3%: 116, 113; 9%: 107, 98.

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.

Statistical analysis

All statistical analysis was performed using Prism (GraphPad Software, San Diego, CA, USA). 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 Student's *t*-test. Fisher's exact test was used to validate mating proportions for the virility assay. *P* values less than .05 were considered statistically significant.

RESULTS

GTPs extend the lifespan of male flies only at the highest dietary yeast content

DR, defined as decreased caloric intake without malnutrition, is the most notable intervention for lifespan extension in model organisms.[26–28] In flies, DR is performed by decreasing dietary yeast content. This increases the lifespan up to a point at which a further reduction will shorten lifespan. A compound or extract acting as a DR mimetic will increase the 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.[26–29] We observed that GTP significantly extended mean lifespan of male w^{1118} flies by 19% at the 9% yeast content (Fig. 1A and Table 1). Surprisingly, GTP severely compromised male survival at the 1% yeast content ($P<.05$). There were no differences in female mean lifespan at all yeast levels (Fig. 1B). Since 9% dietary yeast content resulted in the highest lifespan, for all subsequent experiments, we chose to use this diet.

Table 1. Mean Lifespan Values for Green Tea-Fed Versus Control-Fed Flies Under Varying Yeast Content

% Yeast content	Sex	Dose (mg/mL)	Mean	P value	Maximum
0.1	♂	0	40.0 ± 1.9	.13	86
	♂	10	43.5 ± 1.9		84
	♀	0	39.6 ± 1.7	.41	82
	♀	10	38.0 ± 1.8		86
0.3	♂	0	51.8 ± 2.0	.95	84
	♂	10	52.7 ± 1.8		82
	♀	0	46.3 ± 2.1	.58	88
	♀	10	45.8 ± 1.8		88
1	♂	0	58.1 ± 2.0	.03*	100
	♂	10	52.4 ± 1.9		78
	♀	0	53.9 ± 2.0	.22	88
	♀	10	51.0 ± 1.9		90
3	♂	0	51.5 ± 2.3	.47	88
	♂	10	49.3 ± 2.3		84
	♀	0	37.9 ± 1.5	.15	74
	♀	10	40.7 ± 1.3		84
9	♂	0	36.6 ± 1.7	.007*	82
	♂	10	43.5 ± 1.8		80
	♀	0	27.7 ± 1.4	.30	64
	♀	10	30.4 ± 1.7		78
9 (males only)[a]	♂	0	39.7 ± 1.9	.92	72
	♂	10	41.2 ± 1.8		72

Values are means ± SEM. Units are days. *P* values were calculated by Mann–Whitney analysis, control versus green tea polyphenols (GTP).

[a]Lifespan performed in the absence of females.

*P values < .05 are statistically significant.

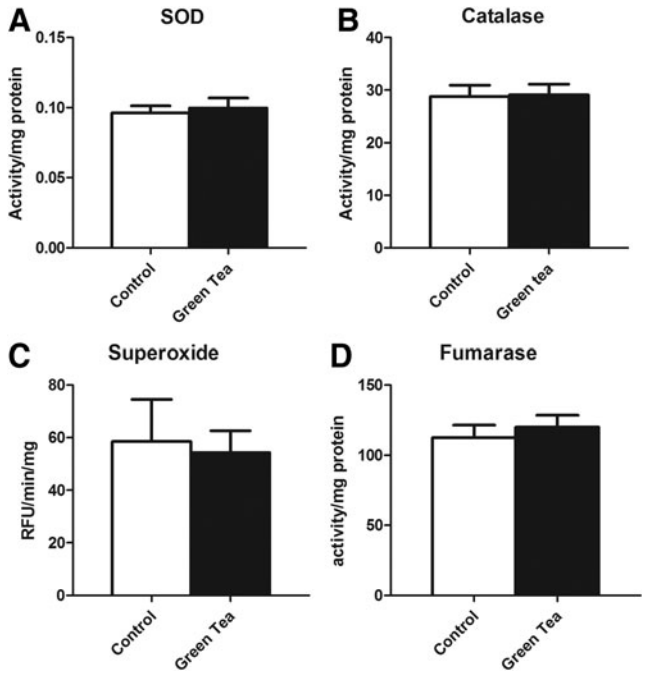

FIG. 2. Effect of GTPs on antioxidant defenses, mitochondrial superoxide and fumarase levels. GTPs did not alter superoxide dismutase (**A**) or catalase (**B**) levels. Furthermore, GTP had no effect on superoxide production (**C**) or fumarase activities (**D**). Data are presented as means ± SEM, $n=6$ (50 flies per sample). Unpaired *t*-test, *P* values > .05. SOD, superoxide dismutase.

GTPs do not alter oxidative energy metabolism

Normal aerobic metabolism, which uses oxygen as an electron acceptor resulting in the production of ROS is

Table 2. The Effect of Green Tea Polyphenols on Mitochondrial Respiration Rates

Diet	State 3	State 4	RCR	Uncoupled
Control	203 ± 16	53 ± 3	3.8 ± 0.2	422 ± 25
Green tea	216 ± 11	57 ± 1	3.8 ± 0.2	442 ± 13
P values	.53	.23	.82	.48

Rates are mean ± SEM, $n=6$ (50 flies per sample). Units are nmol O_2/min/mg protein except for the respiratory control ratio (RCR), which is a ratio of state 3/state 4.

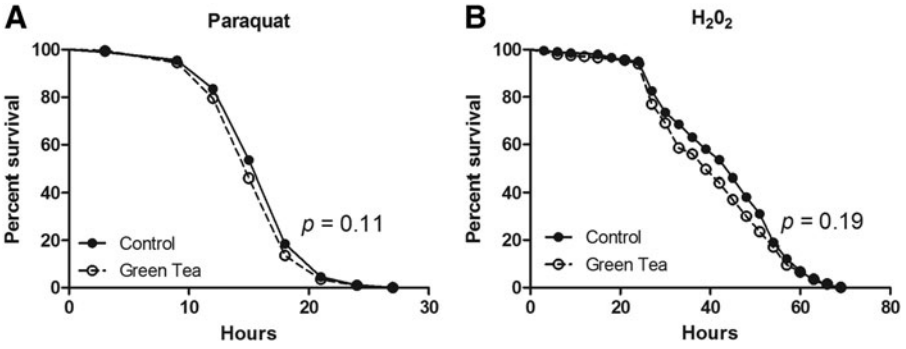

FIG. 3. Impact of GTPs on the tolerance to oxidative challenges. Flies fed with GTP conferred no protection against oxidative stressors paraquat (**A**) or H_2O_2 (**B**). *P* values were calculated by Mantel–Cox log-rank test, $n = 200$ for all groups.

suggested to play a role in aging and age-related disorders.[30–33] It is, therefore, purported that the enhancement of antioxidant enzymes such as SOD, which converts superoxide to H_2O_2, and catalase, which converts H_2O_2 to water and oxygen, may attenuate age-related oxidative damage, thus resulting in the extension of life span.[34,35] 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 (Fig. 2A, B). Furthermore, GTP did not alter mitochondrial superoxide production in flies (Fig. 2C) and had no effect on fumarase activity levels, an integral enzyme in the TCA cycle and indicator of mitochondrial content (Fig. 2D). We further showed that GTP had no impact on mitochondrial respiratory activity (Table 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 (Fig. 3A) or H_2O_2 (Fig. 3B).

GTPs 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.[36,37] Furthermore, this increased resistance to stress is positively correlated with lipid and water content.[38,39] We, therefore, tested green tea's ability to increase stress resistance. Although water levels were not impacted, GTP significantly increased the lipid content (Fig. 4; *P* value $< .05$). Under conditions of desiccation and starvation, GTP afforded no protection to flies (Fig. 5A, B). Moreover, GTP sensitized flies to heat stress (Fig. 5C; $P < .05$). The ability of GTP to protect against iron stress was also evaluated. Flies fed with GTP exhibited a modest protection against iron toxicity versus controls (Fig. 5D; *P* value $< .0001$). GTPs appear to offer some protection against environmental stressors particularly iron toxicity.

GTPs 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.[18–20] We found that GTP significantly decreased the number of viable offspring produced from male flies at doses ≥ 10 mg/mL versus controls (Fig. 6). We further investigated whether GTP impacted the mating behavior by monitoring mounting time (copulation) and mating latency (time for mating). No effect on copulation was observed, however, GTP significantly increased mating latency (Fig. 7; $P < .05$). To ensure that unsuccessful mating events did not affect experimental results, we evaluated mating and observed that both treatment groups had successful mating (Table 3).

GTPs 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 (Fig. 8).

DISCUSSION

We report that GTPs extend lifespan in male *Drosophila melanogaster*, impairs reproductive fitness in male flies, and

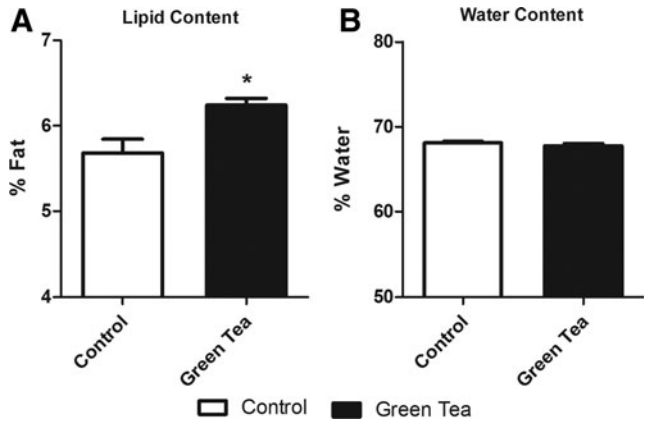

FIG. 4. Effect of GTPs on lipid and water content. Flies supplemented with GTP in their diet significantly increased lipid (**A**), but not water content (**B**). Data are presented as means ± SEM, $n = 6$ (10 flies per sample). Data analyzed by unpaired *t*-test $*P = .01$ ($P = .01$ for lipid content and $P > .05$ for water content).

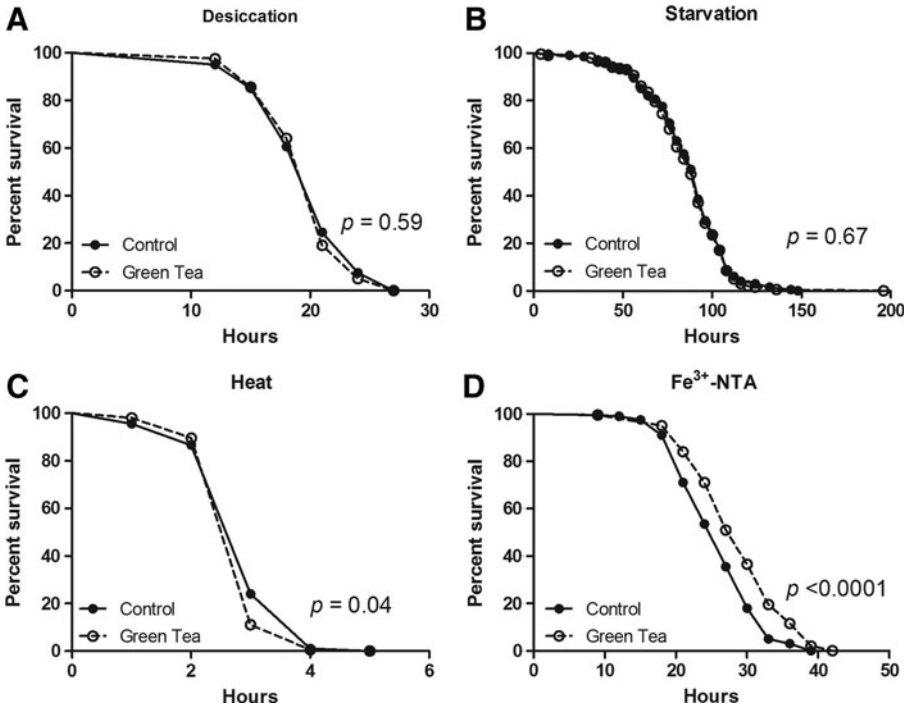

FIG. 5. Impact of GTPs on the tolerance to environmental stress. Flies fed with GTP conferred no protection against desiccation (**A**) or starvation (**B**). GTP slightly sensitized flies to heat stress (**C**) and afforded modest enhanced survival against Fe^{3+}-NTA (**D**). *P* values were calculated by Mantel–Cox log-rank test, $n = 200$ for all groups.

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.*[9] They reported that Oregon-R flies supplemented with 10 mg/mL green tea had prolonged survival with a corresponding upregulation of endogenous antioxidant enzymes SOD and catalase. This group also reported that green tea protected against paraquat and H_2O_2.[9] 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 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.[1,40] Most likely, the Chinese

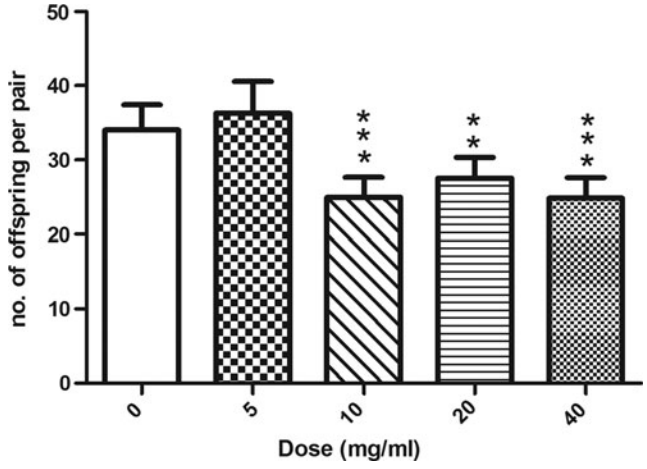

FIG. 6. Dose-dependent effect of GTPs on male fertility. GTP significantly reduced mean male fertility at 10, 20, and 40 mg/mL doses over a 10-day period. Data are presented as means ± SEM, $n = 20$ per treatment group. Data points were removed where female deaths occurred. Data were analyzed by paired *t*-test ($P > .05$ for 5 mg/mL, $^{**}P = 0.002$, $^{***}P < .001$).

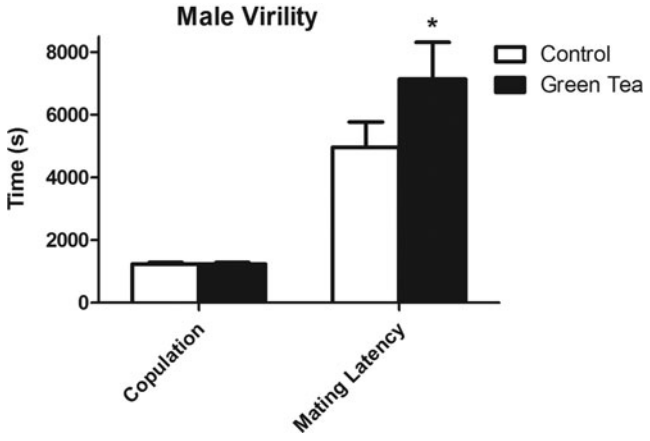

FIG. 7. The effect of GTPs on the mating behavior. GTPs significantly increased mating latency versus controls. The proportion of mating was equal in both treatment groups. Male virility data are presented as means ± SEM and analyzed by Mann–Whitney test (*P* value > .05 for copulation; $^{*}P < .05$ for mating latency).

Table 3. Mating Proportions in Virility Experiment

Mating proportions	*Control*	*Green tea*	*Total*
Mated	25	24	49
Did not mate	11	12	23
Total	36	36	72
Fisher's exact test		P value = 1.0	

Longjing green tea contains other components contributing to an increase in SOD and catalase. Despite the reported upregulation of antioxidant enzymes by Li *et al.*, we report that green tea extends the lifespan of *Drosophila* 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,[41] and the inhibition of iron accumulation has been shown to increase the lifespan of *Drosophila*.[10] The protection of GTP against iron, however, is not surprising as polyphenols are known for their metal chelating activities.[42] Furthermore, clinical reports have shown that green tea inhibits dietary iron absorption.[43] Since green tea has consistently shown a protective effect against the sources of iron, presumably the mechanism associated with an extension in lifespan could be iron related.

The action of GTP through DR was also investigated since this practice is known to increase the lifespan and improve the health of all laboratory species examined,[29] with the possible exception of rhesus monkeys.[44] In the case of GTP, an extension in lifespan was noteworthy only at the highest dietary yeast content. Whereas this would imply that GTP is acting by restricting the caloric intake, no DR effects were observed in female flies (Fig. 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 sublethal 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.[45–48] 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.[49,50] In the case of green tea, its polyphenols have been shown to have pro-oxidant activities[46] 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*.[18–20] 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 the total mounting time. Surprisingly, however, GTP significantly increased mating latency. It remains unclear as to why GTP is affecting the mating time, however, we speculate that GTP may be impairing the central nervous system function since successful mating in *Drosophila* requires complex and precise movements.[51] Lastly, GTP increased the lifespan of male flies only when housed with females for the course of their life. GTPs 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.

Although the research underlying the 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.[52] 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.[52] The authors further emphasized that the negative effects of EGCG is dose dependent since low doses improved the overall sperm motility and function. Other reports in rats showed that green tea has negative effects on male reproductive biology and endocrinology.[53,54] Furthermore, male rats supplemented with green tea showed altered morphology and histology of testis and accessory sex organs. Additionally, a significant

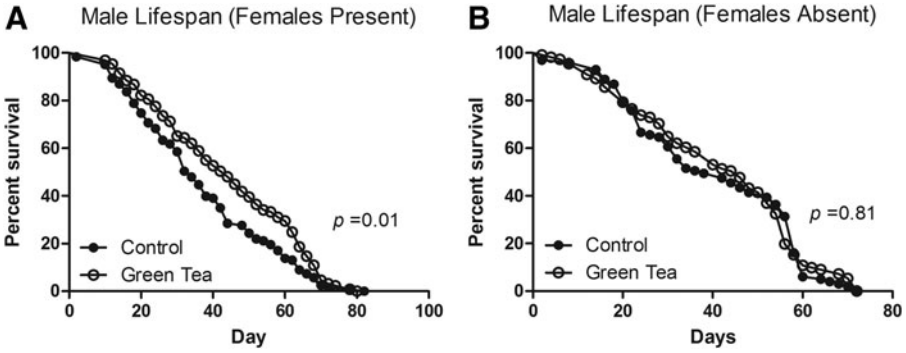

FIG. 8. The requirement of females in the action of green tea. Green tea supplementation significantly extended the lifespan of male flies housed with females (**A**), however had no effect on the lifespan of males in the absence of females (**B**). P values were calculated by Mantel–Cox log-rank test. The sample sizes are as follows and listed as control and green tea, respectively: females present, $n = 123$ and $n = 129$ and females absent, $n = 99$ and $n = 111$.

dose-dependent decrease in sperm counts, serum testosterone levels, luteinizing hormone, and inhibition of spermatogenesis was observed.[54] 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.[55,56]

Based on our observations, we conclude that GTP is increasing the lifespan of male *Drosophila* 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.[57] 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.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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