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Green Tea Polyphenols Require the Mitochondrial Iron Transporter, *mitoferrin*, for Lifespan Extension in *Drosophila melanogaster*

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Abstract

Green tea has been found to increase the lifespan of various experimental animal models including the fruit fly, *Drosophila melanogaster*. High in polyphenolic content, green tea has been shown to reduce oxidative stress in part by its ability to bind free iron, a micronutrient that is both essential for and toxic to all living organisms. Due to green tea's iron-binding properties, we questioned whether green tea acts to increase the lifespan of the fruit fly by modulating iron regulators, specifically, *mitoferrin*, a mitochondrial iron transporter, and *transferrin*, found in the hemolymph of flies. Publicly available hypomorph mutants for these iron-regulators were utilized to investigate the effect of green tea on lifespan and fertility. We identified that green tea could not increase the lifespan of *mitoferrin* mutants but did rescue the reduced male fertility phenotype. The effect of green tea on *transferrin* mutant lifespan and fertility were comparable to w^{1118} flies, as observed in our previous studies, in which green tea increased male fly lifespan and reduced male fertility. Expression levels in both w^{1118} flies and mutant flies, supplemented with green tea, showed an up-regulation of *mitoferrin* but not *transferrin*. Total body and mitochondrial iron levels were significantly reduced by green tea supplementation in w^{1118} and *mitoferrin* mutants but not *transferrin* mutant flies. Our results demonstrate that green tea may act to increase the lifespan of *Drosophila* in part by the regulation of *mitoferrin* and reduction of mitochondrial iron.

Keywords

green tea; iron; lifespan; fertility; *mitoferrin*

INTRODUCTION

Green tea polyphenols have been found to increase the lifespan of the fruit fly, *Drosophila melanogaster* (Jimenez-Del-Rio et al., 2010; Li et al., 2007; Lopez et al., 2014; Massie et al., 1993). Despite its well-documented health benefits in humans and among various experimental animal models (i.e., worms, flies and mice), green tea's life-extending mechanisms are not well understood (Abbas and Wink, 2009; Kitani et al., 2007; Li et al., 2007; Strong et al., 2013). Some reports have suggested that green tea's iron-binding activity

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and thus reduction of oxidative stress is the basis of its beneficial health and life-extending effects (Mak, 2012; Massie et al., 1993; Thephinlap et al., 2007; Weinreb et al., 2004). However, the precise relationship between green tea and the modulators of iron metabolism has not been established.

Green tea polyphenols, known as catechins, most notably epigallocatechin gallate (EGCG), have been purported to be responsible for green tea's numerous biological effects (Sinija and Mishra, 2008; Suzuki et al., 2012; Zaveri, 2006). Phenolic compounds have been described as multi-functional antioxidants with metal-chelating abilities, such as iron binding (Khokhar and Apenten, 2003; Perron and Brumaghim, 2009). Iron is a micronutrient that is both essential for and toxic to all living organisms. Free iron can readily catalyze the generation of reactive oxygen intermediates such as hydroxyl radicals which can lead to cell and tissue damage (Papanikolaou and Pantopoulos, 2005). Iron has also been shown to accumulate during aging and a decrease in aging-induced iron accumulation has been found to increase the lifespan of various experimental animal models (Arruda et al., 2013; Massie et al., 1993; Ventura et al., 2005; Xu et al., 2010). For example, fruit flies supplemented with green tea in their diet throughout their life exhibited longer lifespans and reduced total body iron levels further supporting an interaction between green tea's iron-binding and lifespan extension properties (Massie et al., 1993).

In addition to chelating iron, green tea may regulate iron homeostasis in flies through the modulation of essential iron regulators, since the process is partially conserved between flies and humans (Tang and Zhou, 2013). Iron homeostasis involves the action of numerous protein regulators. Among them are *mitoferrin* and *transferrin*, evolutionary conserved proteins involved in important physiological processes in *Drosophila* (Mandilaras et al., 2013; Tang and Zhou, 2013). *Mitoferrin*, located within the inner mitochondrial membrane, is a protein of the mitochondrial solute carrier family known to transport iron into the mitochondria (Mandilaras et al., 2013; Tang and Zhou, 2013). It has previously been reported that a reduction in *mitoferrin* resulted in abnormal development and increased the lifespan of *C. elegans* (Ren et al., 2012). In *Drosophila*, *mitoferrin* mutants display sterility in males (Metzendorf and Lind, 2010). Further mechanistic studies demonstrated that this sterility could be due to the fact that *mitoferrin* plays an important role in spermatogenesis and development (Metzendorf and Lind, 2013). *Transferrin*, found in the plasma of mammals (Gkouvatsos et al., 2012) and hemolymph of fruit flies (Yoshiga et al., 1999), is an endogenous iron-chelator involved in the systemic transport of iron in mammals and plays a protective role in immunity in *Drosophila* (Mandilaras et al., 2013; Yoshiga et al., 1999). While the function of *transferrin* in *Drosophila* may differ from that of mammals, *transferrin* expression in flies has been shown to be influenced by dietary iron availability (Yoshiga et al., 1999). Thus, green tea catechins, which bind non-*transferrin* bound iron (NTBI) (Thephinlap et al., 2007) may modulate the expression of *transferrin* in flies.

Previously, we reported that green tea increased the lifespan of w^{1118} male *Drosophila* by 19% while reducing fertility (Lopez et al., 2014). We further identified negative impairments in the reproductive organs of *Drosophila* treated with green tea, including atrophied testes in male flies (Lopez et al., 2016). In consideration of the inverse relationship between reproduction and lifespan in *Drosophila*, a well-documented occurrence (Flatt, 2011;

Kirkwood and Rose, 1991; Prowse and Partridge, 1997), and the importance of iron for spermatogenesis (Hales, 2010; Metzendorf and Lind, 2010; Tvrda et al., 2015), we questioned whether green tea acts by modulating iron regulation to affect lifespan and fertility. In this study, we investigated the requirement of iron transporters, *mitoferrin* and *transferrin,* for lifespan extension and reproductive function by green tea in *Drosophila*. Using publicly available fruit fly mutants for *mitoferrin* and *transferrin*, we evaluated the effect of green tea on various parameters including lifespan, fertility, and iron levels. We observed that *mitoferrin* and *transferrin* mutant flies exhibited longer lifespans and reduced fertility compared to a standard laboratory strain, w^{1118}. We found that green tea failed to increase the lifespan of *mitoferrin* mutants but increased male fertility. We further tested the effect of green tea on a mutant *Drosophila* strain of *transferrin*. We found that green tea increased the lifespan and reduced fertility of *transferrin* mutants, an effect that parallels the action of green tea in w^{1118} male flies from our previous work (Lopez et al., 2014). In addition, green tea reduced mitochondrial iron levels in all strains suggesting a mitochondria-specific mechanism. Since we observed that green tea increased the lifespan of all strains tested, w^{1118} male flies (Lopez et al., 2014) and *transferrin* mutants, but had an opposing effect in *mitoferrin* mutants, we suggest that the lifespan extension mechanism, as well as reduction in fertility in normal male flies, by green tea is dependent on the mitochondrial iron transporter, *mitoferrin*, and regulation of mitochondrial iron.

MATERIALS AND METHODS

Drosophila stocks

Flies were obtained from the Bloomington Drosophila Stock Center (BDSC) at Indiana University, USA and included w^{1118} (BDSC# 3605), *Drosophila mitoferrin*, $dmfrn^{BG00456}$ (BDSC# 12489), and *transferrin*, $tsf1^{f05108}$ (BDSC# 18838). Mutant flies for *mitoferrin*, $dmfrn^{BG00456}$, and *transferrin*, $tsf1^{f05108}$, were developed with a w^{1118} background genotype.

Fly mutants with reduced expression levels, or hypomorphs, of *mitoferrin* and *transferrin* have a P-element insertion at each respective gene of interest. Hypomorph expression for *Drosophila mitoferrin* (*dmfrn* ^{BG00456}) have previously been characterized (Metzendorf and Lind, 2010). *Drosophila transferrin* ($tsf1^{f05108}$) mutants were previously identified by the Berkeley *Drosophila* Genome Project (BDGP) (Bellen et al., 2004) and mutant stocks are available via BDSC. *Drosophila transferrin* mRNA expression has previously been characterized (Yoshiga et al., 1999).

Treatment diets and experimental conditions

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

the lifespan of male w^{1118} flies (Lopez et al., 2014). To supplement flies with treatment diets, ~3 day old flies were loaded at ratios of 6 males and 6 females per vial. Flies were transferred to freshly treated food vials every day over the course of 10 days prior to measuring iron levels, male fertility, or gene expression.

Mitochondrial isolation and whole fly preparations

Mitochondrial isolation was performed as described by Schriner et al, (2012). Fifty flies per sample were homogenized in 2mL of ice-cold mitochondrial isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.5% fatty acid free BSA, PH 7.2), homogenized using a glass-Teflon dounce homogenizer and filtered through two layers of cotton gauze. A mitochondrial enriched pellet was obtained by centrifugation for 10 min at 6000 x *g* in 4°C. Mitochondrial pellet was re-suspended in 200 μL of lysis buffer (40 mM KCl, 25 mM Tris HCl, pH 7.5 and 1% Triton X-100) and homogenized using a hand pestle.

In preparation for total body iron measurements, whole flies were frozen in groups of 50 in centrifuge tubes. Flies were homogenized in 200 μL lysis buffer using a hand pestle. All samples were centrifuged for 10 mins at 16,000 x *g* in 4°C and supernatant was collected in preparation for iron measurements.

Total body and mitochondrial iron levels

Iron levels were measured using the ferrozine assay as previously described (Missirlis et al., 2006) with the modification of using 50 flies per sample. In brief, the ferrozine assay is a colorimetric assay that utilizes ascorbic acid to reduce ferric ion to the ferrous state. Ferrozine reacts with ferrous ions to form a magenta complex that absorbs at 562 nm. The absorbance is directly related to iron in the fly. Prior to iron measurements, 5 μL of supernatant was collected for protein measurements. Concentrated HCl was added to prepared samples and heated for 20 mins at 95°C. Samples were centrifuged for 2 mins at 16,000 x *g* in 4°C and supernatant collected. Ascorbate (75 mM), ferrozine (10 mM) and saturated ammonium acetate were added and mixed. Absorbance was read at 562nm and measurements were standardized to the amount of protein in each fly sample.

Fertility

Fertility of male flies after iron chelation was evaluated by feeding flies for 10 days varying concentrations of 0 μM, 250 μM, and 500 μM of Ethylenediaminetetraacetic acid (EDTA) (USB Corporation, Cleveland, OH, USA), an iron chelator, mixed in the yeast solution overlay. Fertility of male flies after iron supplementation was evaluated after feeding flies varying concentrations of 0 mM, 5 mM, and 10 mM ferric ammonium citrate (FAC) obtained from Sigma-Aldrich (St. Louis, MO, USA). Flies were fed treatment diets, as described above, or an equivalent control for 10 days and their fertility was measured. Male fertility was assayed by placing one treated male with one untreated virgin female per vial (n=20). Paired flies were allowed to mate for 24 hours and then transferred to new vials each day for the course of 10 days. Eggs were allowed to develop and the offspring was counted 14 days later.

Gene expressions

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

Lifespan

Flies were fed 10 mg/mL of green tea polyphenols as described above throughout their lifespan. A total of 20 vials were loaded with six males and six females in each vial (n = 120 per sex). Flies were transferred every other day to newly yeasted food and deaths were counted after each transfer until all flies died.

Statistical analysis

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

RESULTS

Iron deprivation negatively affects male fly fertility

Iron has been found to be a crucial micronutrient for male *Drosophila* fertility and reproductive systems (Metzendorf and Lind, 2010; Sadraie and Missirlis, 2011). Experiments evaluating the interaction between iron and fertility are typically performed with mutant fly strains that exhibit sensitivity to iron availability. Here we investigated the effect of dietary iron on fly fertility in a standard laboratory strain, w^{1118}, over a 10-day mating period. We observed that iron deprivation by an iron chelator, EDTA, decreased male fly fertility in a dose-dependent manner (P<0.0001, Fig. 1A). Iron supplementation by ferric ammonium citrate (FAC 5μM and 10μM), however, had no effect on the fertility of male flies (P>0.05, Fig. 1B).

Green tea decreases total and mitochondrial iron levels in flies

Green tea has high polyphenolic content with known iron-binding properties (Khokhar and Apenten, 2003; Perron and Brumaghim, 2009). We investigated the ability of a green tea polyphenol extract, consisting of ~80% catechin content (Lopez et al., 2016), to modulate total body iron levels. We found a 39% decrease (245.9±24.9 vs. 403.6±29.4 nmol Fe/mg protein) in total body iron compared to controls (P=0.003, Fig. 2A). Moreover, mitochondrial iron metabolism has been suggested to play a direct role in spermatogenesis of male *Drosophila* (Metzendorf and Lind, 2010). We, therefore, measured the iron levels in mitochondria from flies fed a diet with or without green tea. We found a 36% decrease

(142.3 ±11.9 vs. 223.7±28.6 nmol Fe/mg protein) in mitochondrial iron levels from flies fed green tea versus controls (P=0.04, Fig. 2B).

Hypomorph mutants for mitoferrin and transferrin have increased lifespans and reduced male fly fertility

Using publicly available hypomorphs of *mitoferrin* and *transferrin* (Bellen et al., 2004; Metzendorf and Lind, 2010; Yoshiga et al., 1999), we compared their lifespan and male fertility to a standard w^{1118} fly strain. We first validated the mutation of $dmfrn^{BG00456}$ and $tsf1^{f05108}$ flies by measuring the expression levels of *mitoferrin* and *transferrin*, in each respective mutant. We confirmed a significant reduction in *mitoferrin* (Fig. 3A) and *transferrin* (Fig. 3B) expression in these mutants (P<0.0001). We then identified that *mitoferrin* and *transferrin* hypomorph mutants exhibit longer lifespans compared to w^{1118} (P<0.0001, Fig. 4A). Specifically, *mitoferrin* hypomorphs exhibited the greatest increase in lifespan (42%) followed by *transferrin* (19%). Baseline male fertility levels of all investigated hypomorph mutants were found to be significantly lower than that of w^{1118} flies (P 0.0001, Fig. 4B), 50% in *mitoferrin* and 40% in *transferrin* mutants.

Green tea has no effect on lifespan, but increases male fly fertility of mitoferrin mutants

Previously we reported that green tea increased male *Drosophila* lifespan by 19% in w^{1118} flies while reducing fertility (Lopez et al., 2014). Here we investigated the effect of green tea on the lifespan and male fertility of hypomorph mutants, *mitoferrin and transferrin*. We identified that green tea could no longer increase the lifespan of *mitforerrin* hypomorph mutants (P>0.05, Fig. 5A) but did increase male fertility (P=0.01, Fig. 5B). Interestingly, *transferrin* mutants exhibited an increase in lifespan with supplementation of green tea (P<0.0001, Fig. 5C). However, green tea did have a negative effect on male fly fertility of transferrin hypomorph mutants (P<0.0001, Fig. 5D). The increase in lifespan and decrease in fertility by green tea in *transferrin* mutant flies are similar to those observed in previous reports with w^{1118} [1].

Green tea up-regulates the expression of mitoferrin, but not transferrin, in hypomorph mutants and w^{1118} flies

To determine whether green tea has any effect on the expression levels of iron metabolizing genes, we compared the levels of *mitoferrin* and *transferrin* in a standard laboratory fly strain, w^{1118}. We showed that green tea increased the expression of *mitoferrin* (P=0.04, Fig. 6A) while no difference was detected with *transferrin* expression levels (P>0.05, Fig. 6B). Subsequently, since hypomorph mutants have residual expression for their affected genes (Fig. 3), we questioned whether green tea acts by modulating their expression. We showed that similar to w^{1118} flies green tea up-regulated the expression of *mitoferrin* in *mitoferrin* hypomorph mutants (P=0.04, Fig. 6C). Green tea had no effect on the expression levels of *transferrin* in *transferrin* mutants (P>0.05, Fig. 6D).

Green tea decreases total body and mitochondrial iron levels in mitoferrin but not transferrin hypomorph mutants

Since green tea increased the expression levels of the mitochondrial iron importer, *mitoferrin*, we evaluated the effect of green tea on total body and mitochondrial iron levels in hypomorph mutants. We found that green tea reduced total body and mitochondrial iron levels by 68% and 65%, respectively, in *mitoferrin* hypomorphs, ($P<0.05$, Fig. 7A and $P<0.005$, Fig. 7B). Green tea had no effect on total body iron levels in *transferrin* mutants ($P>0.05$, Fig. 7C) and did not significantly reduce mitochondrial iron ($P>0.05$, Fig. 7D).

DISCUSSION

We and others have previously reported the ability of green tea and its primary active flavonoid, EGCG, to increase the lifespan of different strains of *Drosophila melanogaster* (Jimenez-Del-Rio et al., 2010; Li et al., 2007; Lopez et al., 2014; Massie et al., 1993; Wagner et al., 2015). In this study we found that the mechanism of green tea's action on male *Drosophila* lifespan and fertility involves iron regulators, such as *mitoferrin*. Over the years, the mechanism of action of green tea has been evaluated extensively. Massie et al. (1993) had first suggested that green tea induced lifespan extension in *Drosophila* by its ability to inhibit iron absorption and thus iron accumulation throughout life (Massie et al., 1993). With a number of studies reporting green tea's iron binding activity, some studies have linked the importance of iron to male *Drosophila* spermatogenesis and fertility (Metzendorf and Lind, 2010). Specifically, Metzendorf and Lind (2010) showed that iron chelation in the diet increased sterility in *mitoferrin* hypomorph mutants whereas iron supplementation improved fertility demonstrating the importance of iron for spermatogenesis. To evaluate the interaction between iron and fertility we tested whether iron deprivation in the diet had any effects on male fertility in normal w^{1118} flies. We too identified a reduction in male fertility when iron levels were reduced by an iron chelator, EDTA. Next, we tested whether iron supplementation had any effects on male fertility. Our results did not show any improvements in male fertility with iron supplementation. We surmised that in normal flies, which display typical reproductive abilities and thus functional iron metabolizing pathways, the addition of iron in the diet would have no additive effect on fertility. In healthy flies, excess iron would be stored in ferritin, an iron-storage protein, and iron can be secreted from this storage protein to the gut lumen during iron over-load conditions (Mandilaras et al., 2013). We further explored the levels of iron in whole flies and in fly mitochondria after treatment with green tea polyphenols and identified a significant reduction of iron levels in both homogenates, 39% and 36% reduction, respectively. The reduction in mitochondrial iron is of particular interest since mitochondrial iron involving the iron transporter, *mitoferrin*, has a direct role in the development of sperm. Interestingly, green tea's primary catechin, EGCG, has been reported to accumulate in the mitochondria of neuronal cells (Schroeder et al., 2009). In accordance with this finding, it is thus likely that the presence of green tea catechins in the mitochondria can result in reduced mitochondrial iron levels.

While the reduction in iron levels is a direct consequence of green tea supplementation and hence reduction in male fertility, the reported effects of green tea selectively increasing male

flies' lifespan (Li et al., 2007; Lopez et al., 2014; Massie et al., 1993; Wagner et al., 2015) could be a secondary effect of the treatment. It is well established that impairments in reproductive abilities, such as a decrease in egg laying for females (Flatt, 2011; Kirkwood and Rose, 1991) or decrease in sperm production for males (Prowse and Partridge, 1997), have an inverse relationship with fruit fly lifespan. However, it is likely that the reduction of iron has physiological effects unrelated to reproduction, some of which could result in an increased lifespan. In this study we proposed that the reduction in fertility was caused by the reduction in mitochondrial iron levels, which is critical for spermatogenesis (Metzendorf and Lind, 2010). This decrease in fertility then contributed, at least in part, to an increase in male fly lifespan (Lopez et al., 2014).

To evaluate whether green tea polyphenols act through the regulation of iron metabolism and hence in turn affect fertility and lifespan, we utilized mutant flies with deficiencies in their ability to regulate iron. Flies with defects in *Drosophila mitoferrin* and *transferrin* were specifically chosen as these proteins display a diverse involvement with iron including the transport of iron into the mitochondria, *mitoferrin*, or throughout the fly, *transferrin*. We first established that these mutants exhibit longer lifespans and reduced fertility than normal flies. This supports the notion that these iron-regulators follow an inverted relationship with lifespan and fertility. *Drosophila mitoferrin* mutants, have previously been found to exhibit a reduced male fertility phenotype (Metzendorf and Lind, 2010). However, the lifespan and fertility phenotypes for *transferrin* mutant flies have not yet been characterized. One study, which used *C. elegans* to down-regulate the expression of *mitoferrin*, identified a 50–80% increase in worm lifespan, as well as abnormal developmental phenotypes and reduced production of progeny (Ren et al., 2012). This observation in *C. elegans* is interesting since we previously reported that green tea polyphenols exhibited similar effects on lifespan, development and fertility in *Drosophila* (Lopez et al., 2014; Lopez et al., 2016). It is therefore plausible that green tea polyphenols require *mitoferrin* to affect lifespan. Since *transferrin* hypomorph mutants also exhibited an increase in lifespan and reduced fertility, it was also of interest to determine whether green tea required *transferrin* as well.

The requirement of *transferrin* was evaluated through hypomorph mutant flies with a dysfunction in the *transferrin* gene. We observed that green tea increased the lifespan and reduced male fertility of *transferrin* hypomorph mutants, a result that parallels green tea treatment in normal w^{1118} flies (Lopez et al., 2014). In addition, treatment with green tea showed no change in *transferrin* expression levels. This led us to conclude that green tea polyphenols do not require *transferrin* to increase *Drosophil*a lifespan. This result is not surprising since green tea catechins are known to bind to non-*transferrin* bound iron (NTBI) (Thephinlap et al., 2007) and thus act independently of the action of *transferrin* proteins.

Mitoferrin hypomorph mutants under the treatment of green tea, however, exhibited no increase in lifespan and instead experienced an increase in male fertility. To begin with, *mitoferrin* flies exhibit significantly decreased fertility which may explain their increased lifespans. Green tea polyphenols, which block iron uptake in w^{1118} and *mitoferrin* mutant flies, resulted in an up-regulation of *mitoferrin* expression, likely to compensate for low iron levels. This resulted in a moderate increase in fertility, which was insufficient in magnitude to affect lifespan.

Since green tea did not increase the lifespan of *mitoferrin* hypomorph mutants, blocked iron uptake, and up-regulated the expression levels of *mitoferrin* in both hypomorph mutants and normal flies, green tea polyphenols may require *mitoferrin*, and specifically the reduction of mitochondrial iron, to increase the lifespan of *Drosophila melanogaster*. As for green tea's ability to specifically increase male fly lifespan while negatively affecting the fertility of normal flies, our results suggest that green tea's iron-binding properties are responsible for the unique interplay between the regulation of iron metabolizing proteins, male fly fertility, and lifespan.

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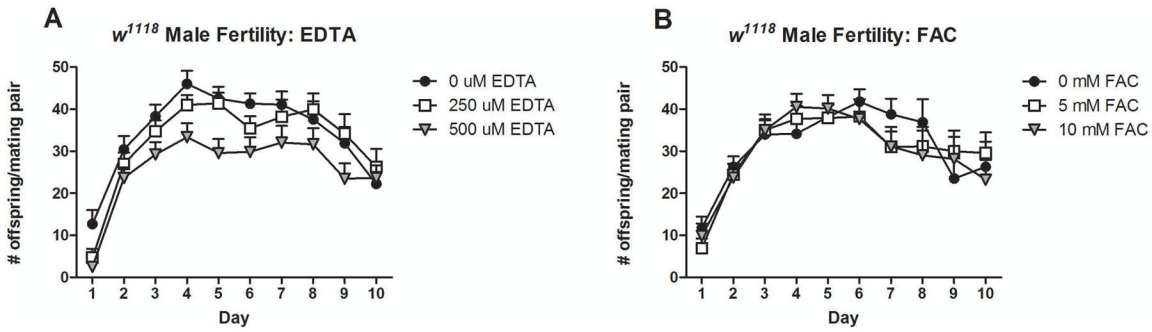

Figure 1.

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

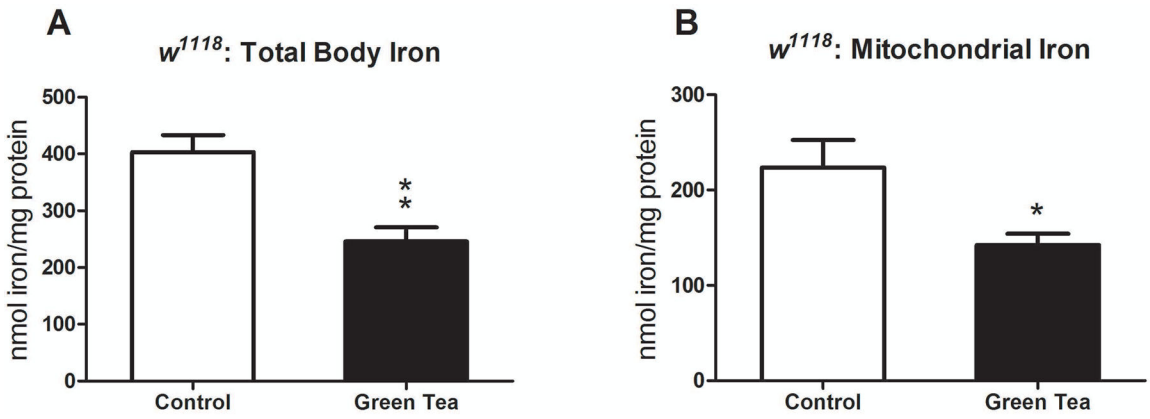

Figure 2.

The impact of green tea on total body and mitochondrial iron levels in *Drosophila*. Green tea decreased total body iron levels in a standard *Drosophila* strain, w^{1118} (P=0.003, n=250) versus controls (**A**). Isolated mitochondria from flies fed green tea had reduced levels of mitochondrial iron (P=0.04, n=200) (**B**). Data are presented as means ± SEM and analyzed by student's t-test.

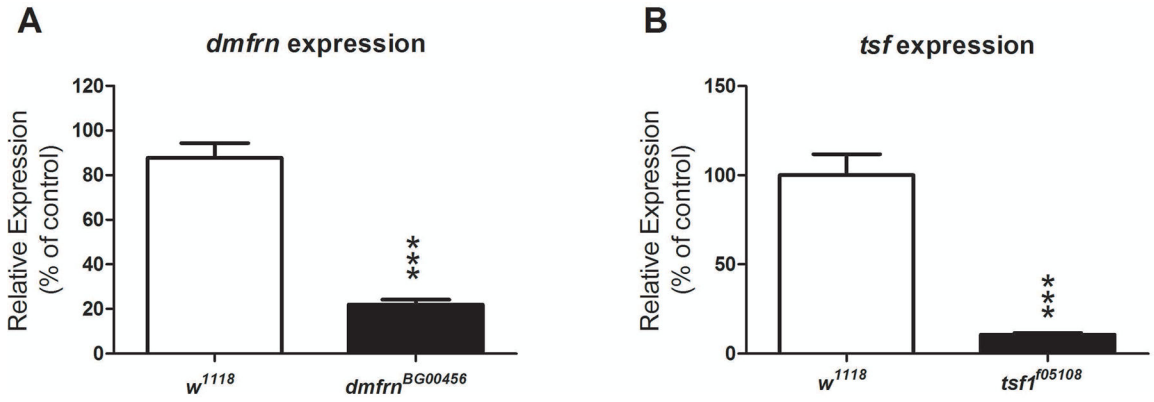

Figure 3.

Background expression levels of *mitoferrin* and *transferrin* in hypomorph mutants. *Mitoferrin* (**A**) and *transferrin* (**B**) expression in $dmfrn^{BG00456}$ and $tsf1^{f05108}$, respectively, was found to be lower than a standard laboratory strain, w^{1118} (***$P<0.0001$). Data are presented as means ± SEM and analyzed by student's t-test. Sample sizes were as follows: for *dmfrn* expression, w^{1118} $n=40$, $dmfrn^{BG00456}$ $n=60$ and for tsf expression, w^{1118} $n=50$, $tsf1^{f05108}$ $n=60$.

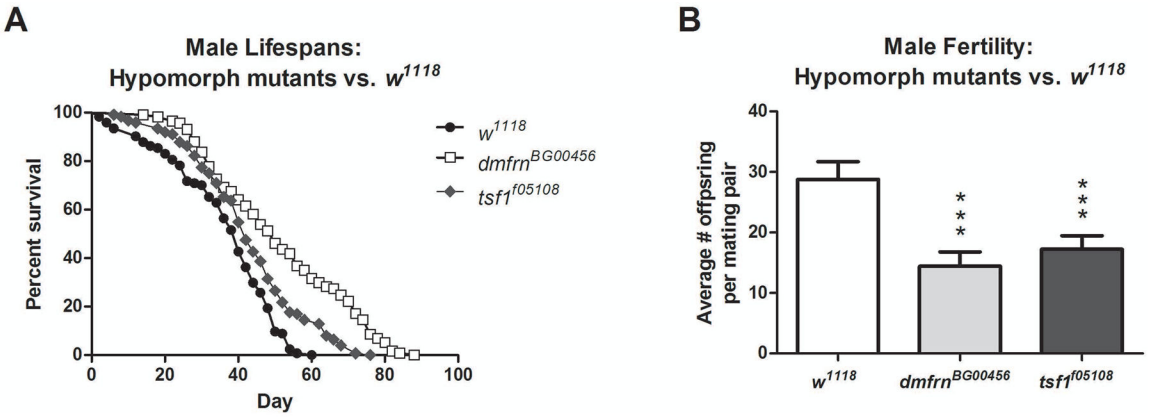

Figure 4.

Lifespan and fertility of *mitoferrin* and *transferrin* mutants compared to a standard laboratory fly strain, w^{1118}. Hypomorph mutants, $dmfrn^{BG00456}$ and $tsf1^{f05108}$ exhibit longer lifespans ($P<0.0001$) than w^{1118} flies (**A**). Hypomorph mutants displayed reduced male fly fertility compared to w^{1118} (**B**). Lifespans were analyzed by Mantel-Cox log-rank test. For each fly strain, lifespan sample sizes, mean lifespan (means ± SEM), maximum lifespan (days) and percent increase compared to w^{1118} were as follows: $dmfrn^{BG00456}$ n=117, 51 ± 1.7 days, 88 days, 42%; $tsf1^{f05108}$ n=124, 43 ± 1.4 days, 76 days, 19%; w^{1118} n=124, 36 ± 1.3 days, 60 days. Fertility experiments were analyzed by student's t-test and data presented as means ± SEM, ***$P\leq0.0001$, n=20 mating pairs.

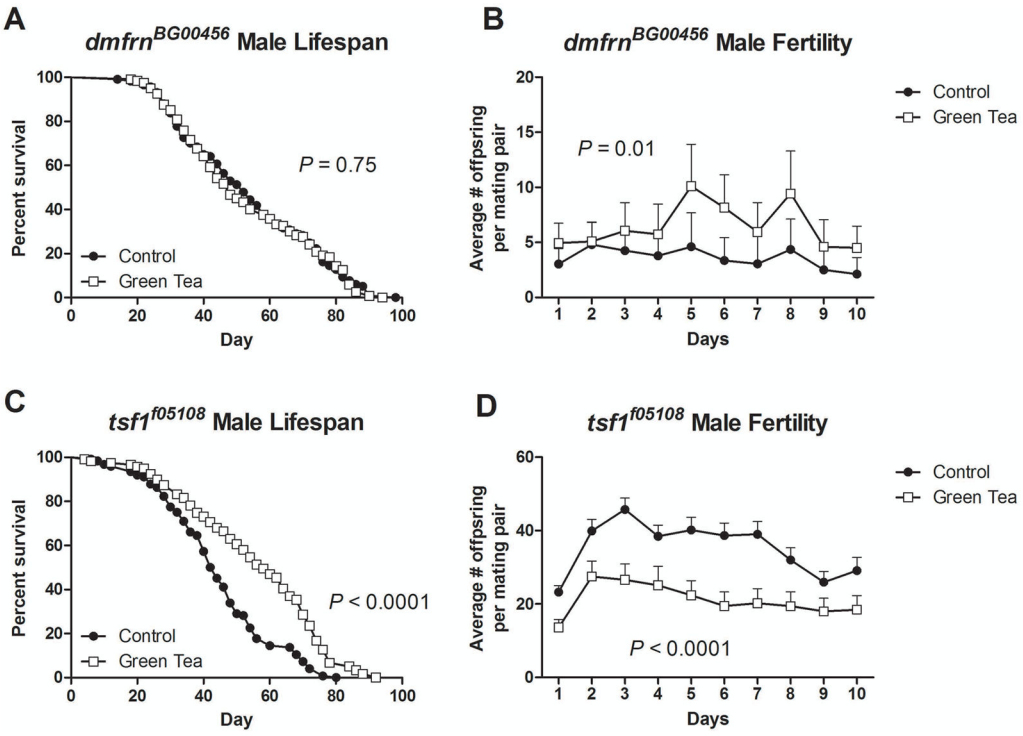

Figure 5.

The impact of green tea on lifespan and fertility of *mitoferrin* and *transferrin* mutants. Green tea had no effect on *dmfrn*^{BG00456} hypomorph mutant lifespan (**A**) but increased male fly fertility (**B**). Green tea increased the lifespan of *tsf1*^{f05108} mutants (**C**) however exhibited a decline in male fertility (**D**). Lifespans were analyzed by Mantel-Cox log-rank test. Sample sizes for lifespans, control and green tea respectively, were as follows: *dmfrn*^{BG00456} n=117,120; *tsf1*^{f05108} n=124,119. Fertility experiments were analyzed by Two-way ANOVA and data are presented as means ± SEM per day, n=20 mating pairs.

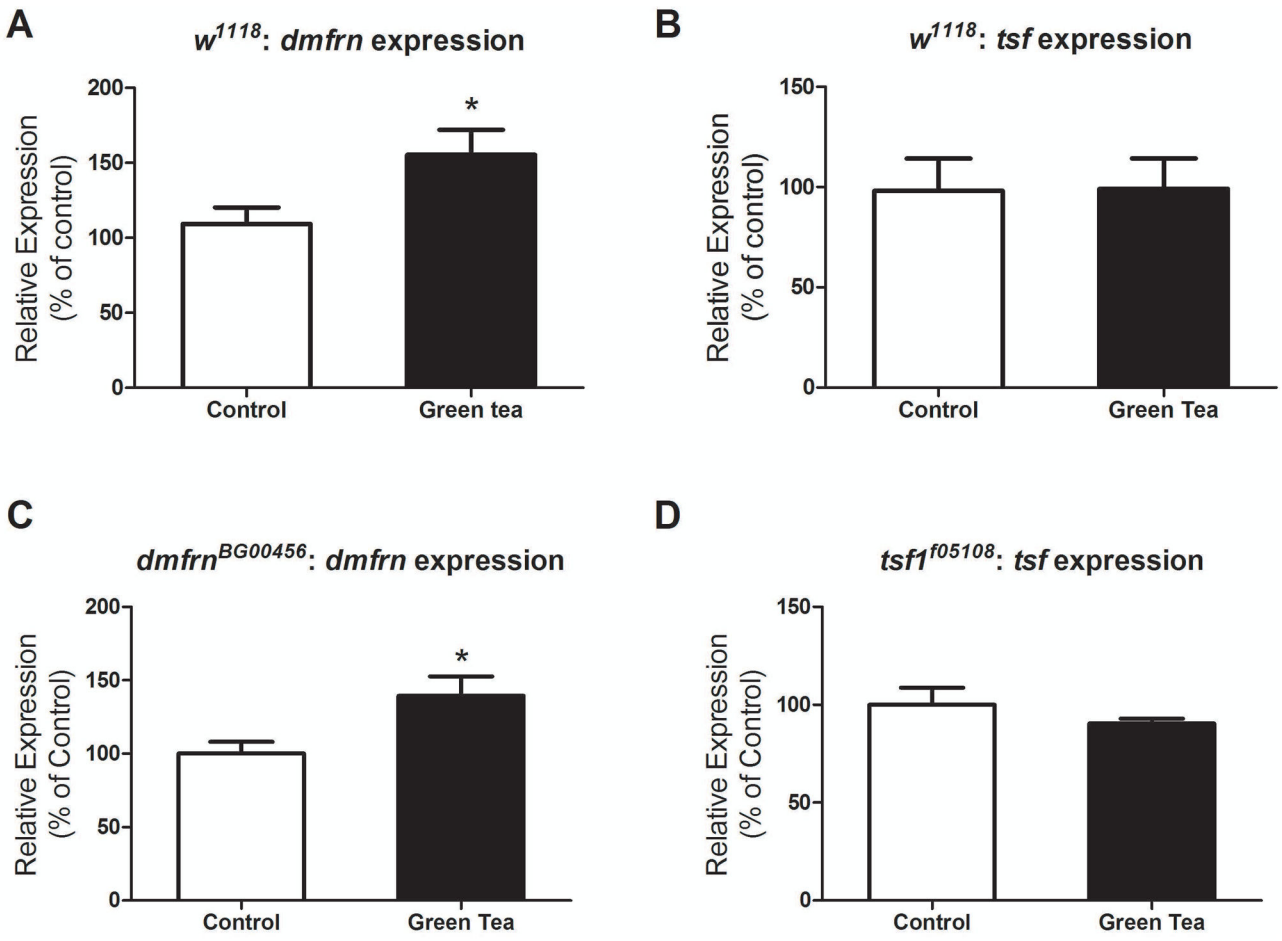

Figure 6.

The effect of green tea on *mitoferrin* and *transferrin* expression in w^{1118} and hypomorph mutants. Green tea up-regulated expression of *mitoferrin* (**A**) (P=0.04, n=50 per treatment) in w^{1118} flies but had no effect on the expression levels of *transferrin* (**B**) (P>0.05, control n=60, green tea n=50). Green tea up-regulated *mitoferrin* in $dmfrn^{BG00456}$ hypomorph mutants (**C**) (P=0.04, n=50 per treatment). Green tea did not affect *transferrin* expression levels in $tsf1^{f05108}$ flies (**D**) (P>0.05, control n=60, green tea n=50). Data are represented as means ± SEM and analyzed by student's t-test.

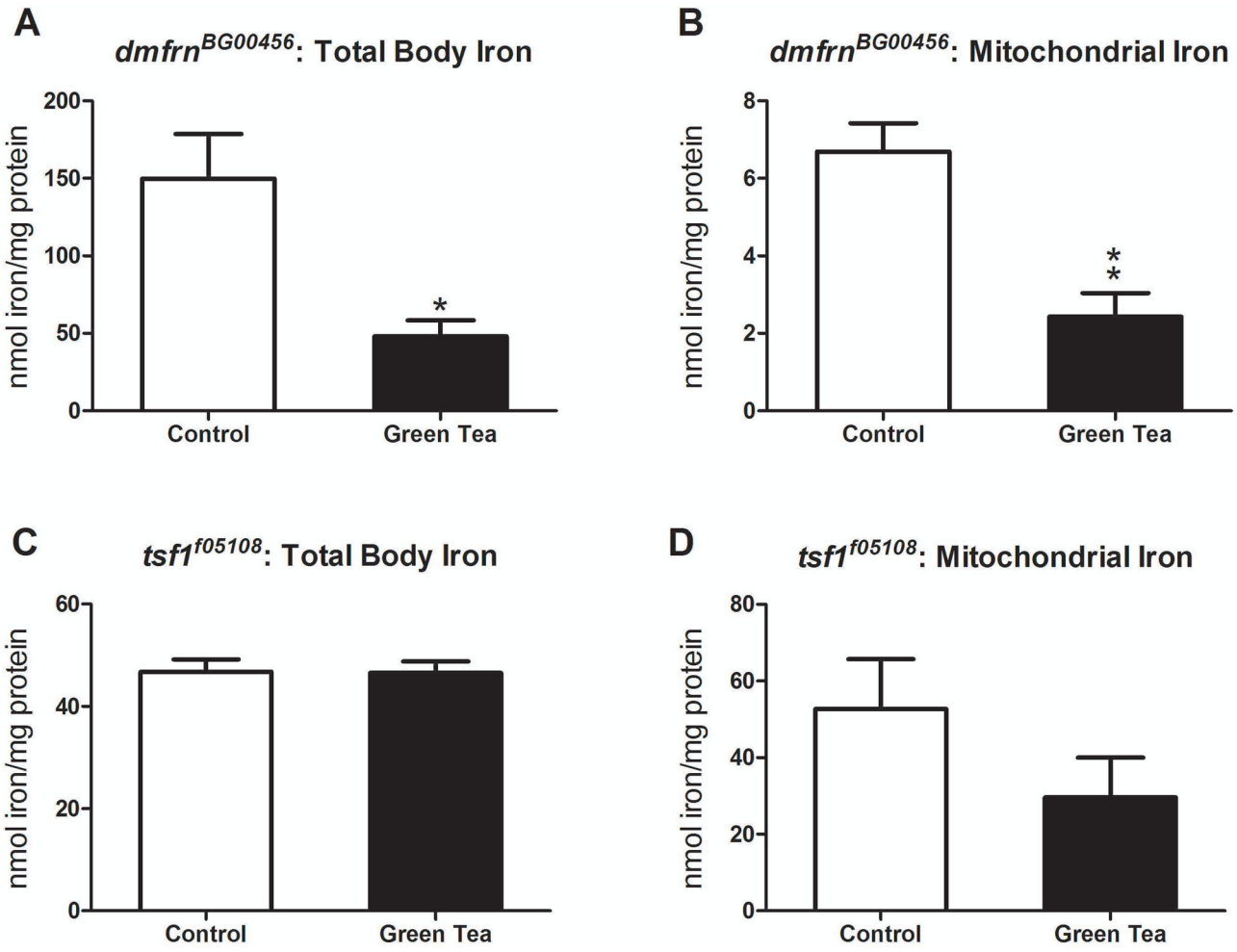

Figure 7.

The effect of green tea on total body and mitochondrial iron levels in *mitoferrin* and *transferrin* mutants. Green tea significantly reduced total body (**A**) and mitochondrial iron levels (**B**) in $dmfrn^{BG00456}$ hypomorph mutants (*P<0.05 and **P<0.005, respectively, n=50 per treatment). Green tea did not affect total body iron levels (**C**) nor mitochondrial iron levels (**D**) of *transferrin* mutants (P>0.05, n=40 per treatment). Data are represented as means ± SEM and analyzed by student's t-test.

Table 1

Gene	Primer Sequence (5′→ 3′)	Source
*GAPDH	F-GTTGCGGCTGAGGGCGGATT R-AGTTGATGTTGGCCGGGTCGC	Primers were designed by NCBI/Primer-BLAST
dmfrn	F-TTTGCCGCCTACGAGATG R-TAGAAATGGCGTCGTGTATG	Navarro *et al*, 2015 (Navarro et al., 2015) Metzendorf *et al*, 2009 (Metzendorf et al., 2009)
tsf1	F-AAGTACTTTGGTCTGCCGGG R-GTGCCATCCTCGCACAGATA	Primers were designed by NCBI/Primer-BLAST

Abbreviations: F, forward; R, reverse

*Reference gene