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RESEARCH

Rapamycin and Post-Deficiency Dietary Recovery Reshape Antioxidant Response and Survival in Offspring of Iron-Deficient Mothers

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Abstract

Maternal iron deficiency (ID) disrupts maternal and offspring health by impairing iron status and antioxidant defenses. Rapamycin is known to promote autophagy, enhance antioxidant activity, and extend lifespan. This study investigates the intergenerational effects of post-deficiency dietary interventions using normal and rapamycin-treated diets on *Drosophila melanogaster*. Female flies (F0) were subjected to an iron-deficient diet for 14 days, followed by a 30-day recovery period on either a normal diet or a rapamycin-supplemented diet. Some F0 females were subsequently mated with normal males to produce F1 offspring. Physiological, biochemical, and gene expression analyses were conducted on F0 flies post-chelation and post-intervention. Post-eclosion evaluations, including a 60-day survival study, were performed on both generations. In F0 females, iron chelation significantly reduced ($p < 0.0001$) body weight, iron levels, and antioxidant enzyme activity, while increasing glutathione (GSH) levels. Gene expression analysis revealed significant changes ($p < 0.05$) in iron storage (Fer1HCH), autophagy (ATG1), and telomere-related genes (dHeT-A, dTahre, dTart). While a normal diet partially restored iron levels and survival, the rapamycin-treated diet improved antioxidant defenses but had mixed effects on survival and gene expression. In the F1 generation, male and female offspring from mothers on a normal diet exhibited reduced and increased iron levels, respectively, alongside improved median survival. Rapamycin increased body weight and iron levels in female offspring but reduced their median survival. Post-deficiency dietary interventions significantly shape antioxidant responses and survival in both iron-deficient mothers and their offspring. While normal diets support recovery of iron status, rapamycin enhances antioxidant defenses but compromises survival, particularly in female offspring.

Keywords Maternal iron deficiency · *Drosophila melanogaster* · Rapamycin · Antioxidants · Autophagy · Telomeres

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Introduction

Iron is a vital element for the survival of most living organisms [30], acting as a cofactor for numerous enzymes and as a component of transcription factors involved in epigenetic modifications [1, 62]. Iron homeostasis is tightly regulated, as both excess and deficiency can lead to significant health consequences. The body relies on complex mechanisms to maintain this balance, with ferritin, a key iron storage protein sequestering excess iron in a soluble and non-toxic form [7]. Ferritin is composed of two subunits: ferritin 1 heavy chain (Fer1HCH) and ferritin 2 light chain (Fer2LCH), which together store iron within cells [16].

Iron deficiency (ID) is a major global health issue that is ranked among the leading causes of disease burden [44]. It is particularly prevalent in developing countries, where"hidden hunger"remains a significant challenge [5]. Children, premenopausal women, adolescents, pregnant women, and women of reproductive age are especially vulnerable due to their increased iron requirements during growth, menstruation, and pregnancy [13]. In these life stages, the demand for iron often exceeds dietary intake, raising the risk of ID [1, 2].

Maternal ID has been linked to poor pregnancy outcomes, including fetal growth restriction and an elevated risk of obesity and hypertension in offspring later in life [4]. These effects can be attributed to mechanisms such as altered placental structure and function, impaired enzyme expression, disrupted nutrient absorption, and abnormal fetal organ development [4].

ID also disrupts antioxidant defenses by impairing iron-dependent antioxidant enzymes, thus promoting oxidative stress [50, 59]. Under normal physiological conditions, a balance exists between reactive oxygen species (ROS) generation and the body's enzymatic and non-enzymatic antioxidant defenses. However, ID tilts this balance toward oxidative stress, with increased ROS production affecting the synthesis of iron-containing enzymes like catalase (CAT) and superoxide dismutase (SOD), further exacerbating oxidative stress [50, 53].

Dietary interventions, like rapamycin, can enhance antioxidant defenses to mitigate these effects. Rapamycin, a potent inhibitor of the mechanistic target of rapamycin (mTOR), modulates nutrient signaling pathways and regulates cellular growth, metabolism, and stress responses [51]. mTOR is composed of two complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Rapamycin has been shown to reduce oxidative stress in yeast cells and rat erythrocytes [35, 54] and also promote the expression of genes involved in free radical scavenging by enhancing antioxidant capacity and inhibiting the mTORC1 [12]. This inhibition improves the body's ability to combat oxidative damage through increased autophagy [41] and enhanced expression of antioxidant enzymes [12]. Autophagy, a cellular process that involves lysosomal degradation of intracellular components through the recycling of damaged organelles and aggregated or misfolded macromolecules, plays a crucial role in maintaining cellular homeostasis, particularly under conditions of stress or nutrient deficiency [21]. Iron is closely linked to autophagy, as it is involved in the function and maintenance of mitochondrial health [18]. Impaired autophagy leads to the accumulation of damaged cellular components, including dysfunctional mitochondria, which increases ROS levels and exacerbates telomere shortening [15]. Telomeres are DNA tandem repeats of the six-nucleotide sequence (TTAGGG) that are situated at the end of chromosomal DNA and are responsible for protecting the chromosomal end from degradation as well as maintaining the overall genomic integrity and stability [32, 52]. Telomere shortening is associated with aging and cellular senescence [52]. Recent studies have highlighted the interplay between telomere maintenance, autophagy, and nutrient availability [26].

Drosophila melanogaster is a well-established model organism widely used for studying genetics, development, and aging due to its genetic similarity to humans. Approximately 75% of human disease-related genes have homologs in *Drosophila* [3], making it a valuable system for investigating molecular processes relevant to human health [8, 31, 38]. Its short lifespan and well-characterized genetics also make it ideal for studying the effects of dietary interventions and other environmental factors across generations. *Drosophila*'s telomeres consist exclusively of head-to-tail arrays, consistently oriented, comprising randomly transposed copies of three specialized non-Long Terminal Repeat (non-LTR) retrotransposons: *Healing Transposon* (d*HeT-A*), *Telomere Associated Retrotransposon* (d*Tart*), and *Telomere Associated and HeT-A Related* (d*Tahre*) [11, 14].

Even though ID is common among women of reproductive age, little is known about its effects on iron and antioxidant status, autophagy, telomere maintenance, and longevity in both the mothers and their offspring. Therefore, the present study aimed to assess the effects of maternal ID and subsequent dietary interventions, including a normal diet and a rapamycin-treated diet on iron and antioxidant status at biochemical and gene expression levels. Additionally, the study assessed the expression of genes related to autophagy and telomere maintenance in *Drosophila melanogaster* and their offspring.

Materials and Methods

Reagents

Bathophenanthroline disulfonic acid disodium salt hydrate, BPS (MW-590.53 g/mol) was purchased from Sigma Aldrich, Switzerland (CAS no. 52746–49-3) while Rapamycin was purchased from Adooq Bioscience (Cat# A10782-5). The designed primers were synthesized by Inqaba Biotech, South Africa.

Fly Stocks and Rearing

Wild-type *Drosophila melanogaster* (*Harwich strain*) flies were obtained from the Fly laboratory at the Centre for Advanced Medical Research and Training (CAMRET), Usmanu Danfodiyo University, Sokoto, Nigeria, and used for this study. Flies were raised on the laboratory standard diet (composed of 10 g Agar Agar, 100 g Corn flour, 10 g Baker's yeast, 1 g methylparaben, and 1.7 l distilled water) under a natural light–dark cycle in an incubator, under a controlled temperature (25 ± 3 ^{0}C) and humidity (55 ± 5%).

Bathophenanthroline Disulfonic Acid/Rapamycin Treatment and Experimental Design

Flies (3-day-old) were separated into males and females. The female flies were transferred onto BPS treated (200 µM BPS) and normal diet (0 µM BPS, to serve as the control group) for 14 days to induce ID, while the males were maintained on a normal diet only (Fig. 1). These flies were designated as the parent (F0) flies. The dose selection was based on a pilot study conducted to determine the dose and duration of time sufficient to induce ID. The iron-chelated diet was prepared by thoroughly mixing a 20 mM stock of BPS with 10 ml of normal diet to make a final concentration of 200 µM BPS. Following 14 days of iron chelation, flies were harvested and divided into three groups. One group was used for biochemical and gene expression analysis the second group was further subjected to either a normal or rapamycin-treated diet along with their control groups for a 60-day survival study, subgroups of these flies were assessed for biochemical and gene expression analysis after 30 days, while the remainder were kept for the full 60-day survival period together with their control groups on the same dietary conditions. The third group was used for crossing to get the first filial generation (F1) on either a normal or rapamycin-treated diet along with their control groups as follows: the F0 ID female flies were crossed with normal male flies and allowed 48 h to lay eggs. The eggs were then allowed to develop on either of the two diets. Rapamycin (200 µM) was dissolved in absolute ethanol (Sigma-Alldrich) and added to the normal diet while for the control group, ethanol alone was added. After successful mating, a subgroup of the emerging F1 offspring from each of the groups was harvested, anesthetized, and crushed in phosphate-buffered saline (PBS) for biochemical analysis and gene expression study. RNA was extracted after crushing the flies with lysis buffer, and the remainder were kept for the full 60-day survival period together with their control groups. To maintain optimal food quality and prevent contamination, fresh media vials were changed once a week for the duration of the experiment.

Survival Analysis

To determine the lifespan of flies in the F0 and F1 generations, 40 flies (both male and female) from each group were placed in fresh media and aged together on either a normal or rapamycin-treated diet under standard conditions [46]. Flies from the untreated group served as controls. The experiment was conducted in duplicate. Fresh media vials were provided every 3–4 days, and dead flies, as well as censored flies (accidental deaths and escapees), were recorded throughout the survival period (60 days).

Determination of Fly Body Weight

The average weight of the flies was determined by gently anaesthetizing groups of ten flies. They were carefully collected and weighed on a sensitive electronic balance (Kern & Sohn Ltd., Balingen, Germany). The weight of each group was recorded in milligrams, allowing for the accurate calculation of the group's average weight.

Sample Preparation for Biochemical Analysis

Flies were anaesthetized on ice and the supernatant was collected by approximately homogenizing 20 flies in 300 µl phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 8000 × g for 10 min at 4 °C. Sixty (60) flies were used per group (3 replicates of 20 flies per group) for biochemical analyses. The supernatant was collected into a sterile and used for biochemical assays.

Assessment of Fly Iron Content

Whole body iron content was determined using a serum iron assay kit (Beijing Solarbio Science & Technology Co., Ltd., China; Cat No BC1735) according to the manufacturer's protocol. The rate of change in sample absorbance at 520 nm was determined spectrophotometrically (using ELISA plate reader S/N: IFTDL231007015, Infitek, Jinan China).

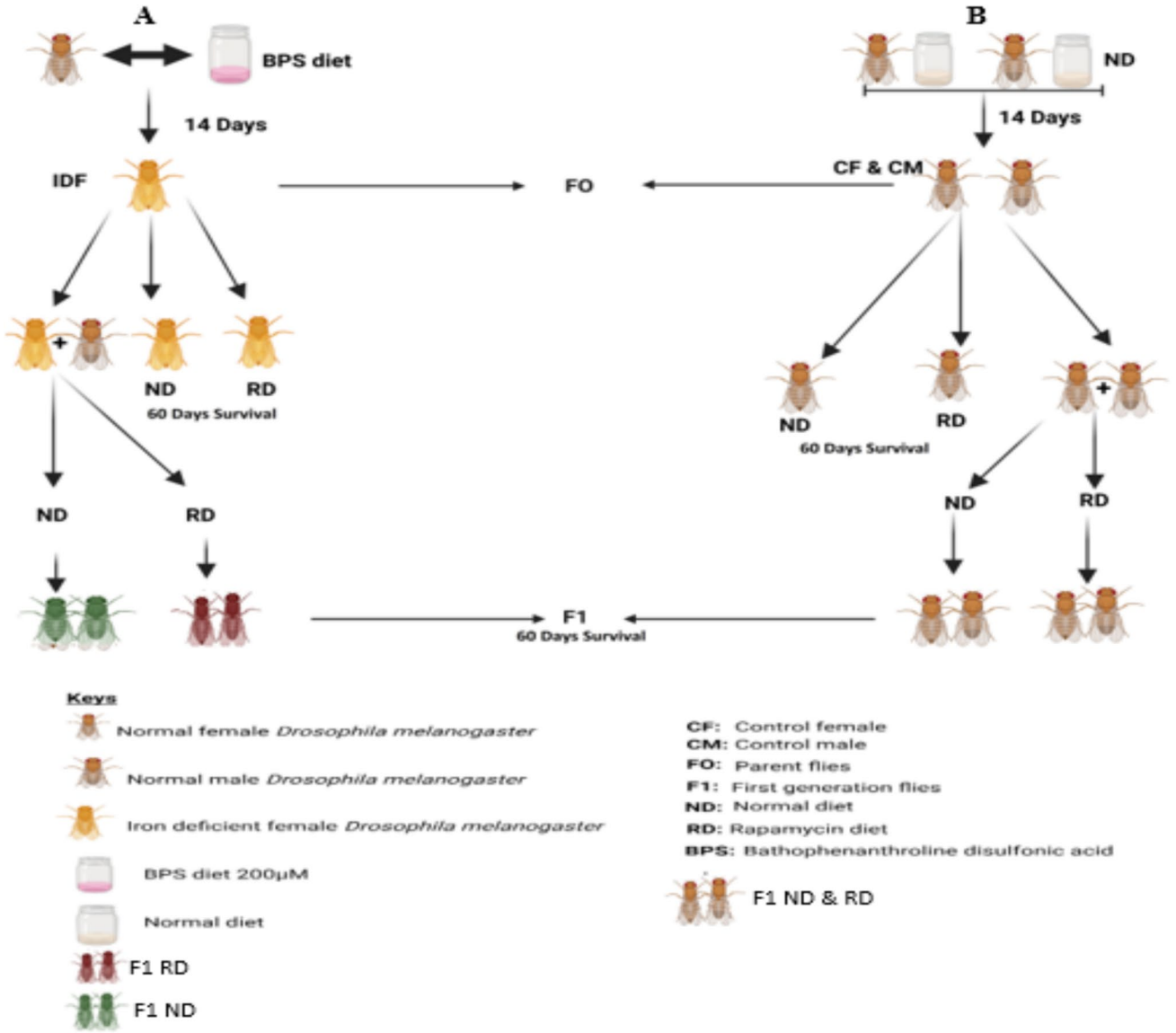

Fig. 1 Experimental design outlining the study protocol. **A** Three-day-old female *Drosophila melanogaster* were placed on a 200 µM bathophenanthroline disulfonic acid (BPS) diet for 14 days to induce iron deficiency (ID). Following ID, a subset of the iron-deficient females (F0) was placed on either a normal diet (ND) containing ethanol only or a rapamycin-treated diet (RD) containing 200 µM rapamycin for a 60-day survival period. Concurrently, iron-deficient females (IDF) were mated with control males (CM), on either a normal or rapamycin diet, and their offspring (F1 generation) were further placed on the same dietary conditions for a 60-day survival study. **B** In parallel, three-day-old control females (CF) and CM *Drosophila melanogaster* (F0) were placed on a normal diet for 14 days. After this period, the CF were switched to either a normal diet containing ethanol alone or RD containing 200 µM rapamycin for a 60-day survival study. Similarly, CF were crossed with CM, and their F1 offspring were placed on the same dietary conditions for a 60-day survival period

Antioxidant Enzyme Assays

GSH levels were determined using a reduced glutathione colorimetric assay kit (Elabscience, Wuhan, China; Catalog No: E-BC-K030-S) in line with the manufacturer's instructions. SOD levels were assessed using the Total Superoxide Dismutase (T-SOD) Activity Assay Kit (Elabscience, Wuhan, China; Catalog No: E-BC-K019-S), adhering to the provided protocol. CAT activity was measured using a Catalase Activity Assay

Kit (Elabscience, Wuhan, China; Catalog No: E-BC-K031-S) as per the manufacturer's instructions. The rate of change in sample absorbance at 405 nm was determined spectrophotometrically (using ELISA plate reader S/N: IFTDL231007015, Infitek, Jinan, China) to calculate the enzyme activity.

Gene Expression Analysis

Ribonucleic Acid (RNA) Extraction

Total RNA was extracted from 20 flies per group (4 replicates of 5 flies per group) using a nucleic acid isolation kit from Hunan Runmei Gene Technology Co., Ltd., Changsha, China, following the manufacturer's protocol. The RNA was quantified using a Nanodrop spectrophotometer (Bioevopeak Nuclei acid analyzer, SP-MUV200 F, Jinan, China). For gene expression analysis, only RNA samples with A260/280 and A260/230 ratios within the range of 1.8–2.2 for high-quality RNA and reliable gene expression analysis were used.

Primer Design

Gene-specific primers to target transcripts for key proteins were designed using the PrimerQuest tool (Table 1).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

For RT-qPCR analysis, the TransScript Green One-Step qRT-PCR SuperMix (AQ211) kit from TransGen Biotech Co., Ltd. (Beijing, China) was used according to the manufacturer's protocol. All the reactions were conducted on a Rotor-Gene Q-5plex HRM platform thermal cycler (Qiagen, Hilden, Germany) and subjected to the cycling conditions listed in the legend of Table 1. After optimization and melt curve analysis, the CT values were normalized to the reference gene (*AcTC5*) and control sample using the delta-delta CT or the Livak method ($2^{-\Delta\Delta ct}$). All experiments were conducted in duplicate.

Data Analysis

Datasets were analyzed using the Statistical Package for Social Sciences SPSS software (version 20). Data were subjected to a normality test (Shapiro–Wilk test) and then expressed as mean ± standard deviation. To analyze the data, a student t-test was conducted for the F0 (parent) generation and a two-way analysis of variance (ANOVA) for the F1 generation of the flies followed by a Tukey post hoc test to compare the differences in the means. The significance level was set at $p < 0.05$. Graphical

Table 1 Primer sequences for target genes

S/N	Gene	Primer	Sequence	Annealing Temperature (°C)
1	*ActC5*	Forward Reverse	GAGCGCGGTTACTCTTTCA TCAAAGTCGAGGGCAACATAG	55
2	*Fer1HCH*	Forward Reverse	CTGGCTGTTCCTGAGATTACC CATGGCCAAGTACTGGTAGG	55
3	*GPx3*	Forward Reverse	GATACCCATGGCAACGATGT CCTTTAGATCCGTCAGCTTCTC	55
4	*SOD3*	Forward Reverse	CTCTGTACCACTTGTGTTCTACC TCTCTGTCATACCTACCCACTC	55
5	*CAT*	Forward Reverse	TGGTCGTCTGTTCTCCTACT CCGCTGGAAGTTCTCAATCT	65
6	*dAtg1*	Forward Reverse	ACAATATGGACGGCGAGTTAG GCCCTGCGTATCAGATCAAT	55
7	*HeT-A*	Forward Reverse	TTGTCTTCTCCTCCGTCCACC GAGCTGAGATTTTTCTCTATGCTACTG	55
8	*Tahre*	Forward Reverse	CTTCCCCTCCGCTCTCATC CCTAGATCTGCATTTGTATTAGTAGCTG	60
9	*Tart*	Forward Reverse	CAAAAAATCCTTTCCGAGATCC GGGCATCAATATTTAGAATGAACAG	55

PCR conditions: reverse transcription at 45 °C for 5 min; pre-denaturation at 94 °C for 30 s; 40 cycles of denaturation at 94 °C for 5 s, annealing at the corresponding temperature for 15 s, and extension at 72 °C for 10 s. *ActC5*; *Actin*, *Fer1HCH*; *ferritin 1 heavy chain*, *GPx3*; *Glutathione peroxidase 3*, *SOD3*; *Superoxide dismutase 3*, *CAT*; *catalase*. *dAtg1*; *Drosophila Autophagy-related gene 1*, *HeT-A*; *Healing Transposon*, *Tahre*; *Telomere associated He-TA related*, *Tart*; *Telomere associated retrotransposon*

illustrations were performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, California).

Results

Maternal ID Alters Body Weight in F_0 Flies and Their F_1 Offspring

The effects of maternal ID on the body weight of F0 flies and their offspring are presented in Figs. 2A-D. A significant ($p < 0.0001$) decrease in body weight of the F0 I.D female was observed compared to the control female (hereafter referred to as F0 control female) following 14 days of iron chelation (Fig. 2A). Upon returning these flies to a normal and rapamycin-treated diet for 30 days, no significant difference was detected ($p > 0.05$) in the body weight of the flies regardless of group and treatment, although it was increased in the normal diet but decreased in the rapamycin-treated diet (Fig. 2B & C). Furthermore, no significant difference ($p > 0.05$) was observed in the body weight of male offspring (hereafter referred to as F1 male offspring) and female offspring (hereafter referred to as F1 female offspring) raised on a normal diet compared to their F1 control male and female offspring (hereafter referred to as control F1 male and F1 female offspring)

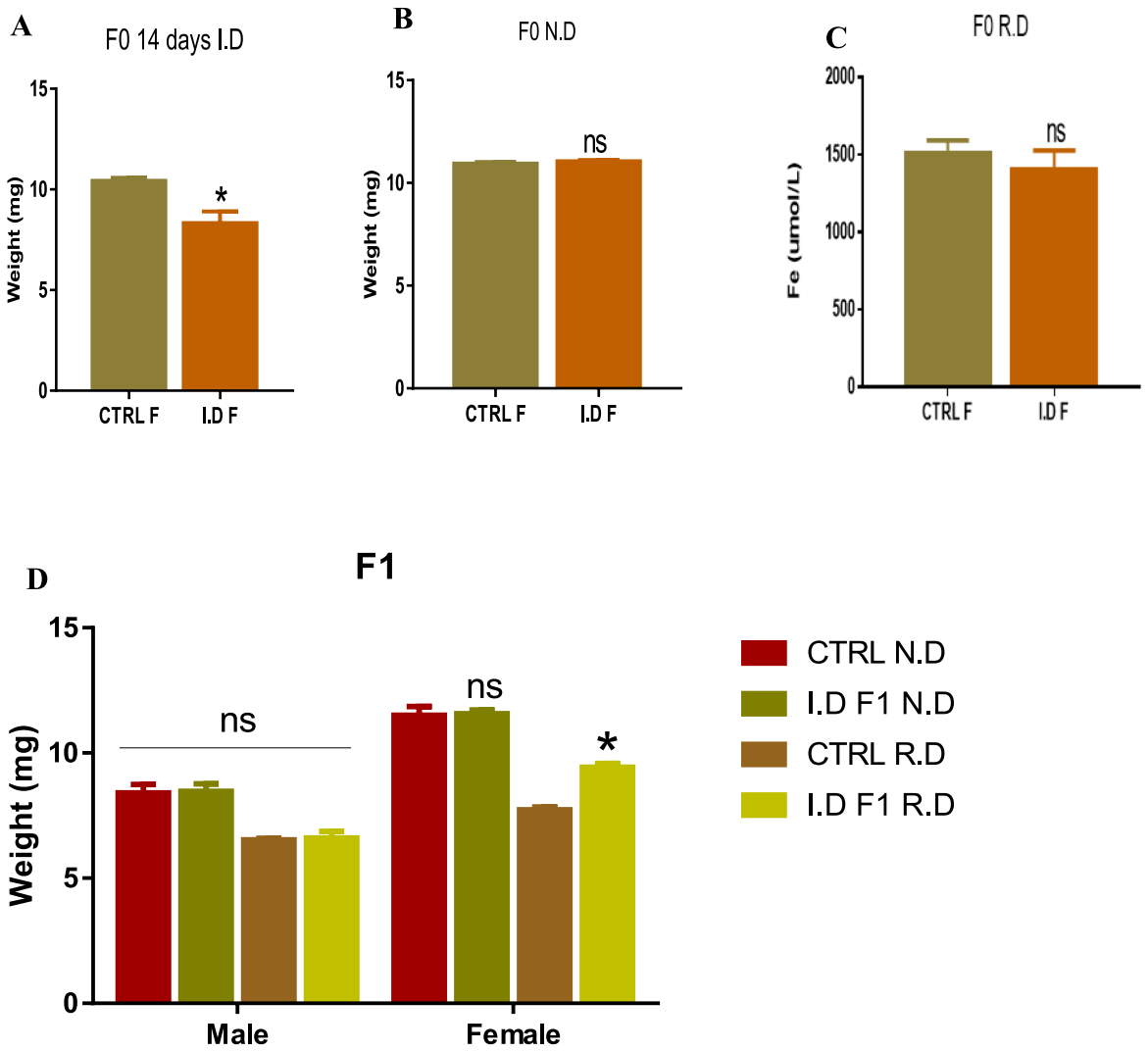

Fig. 2 **A**-**D** Effects of maternal ID on body weight of flies. **A** After 14 days of iron chelation, **B** 30 days on a normal diet, **C** 30 days on a rapamycin-treated diet, **D** F1 offspring on a normal or rapamycin-treated diet. Bars represent mean ± SD. * indicate mean values are significantly different at ($p < 0.05$) compared to the control and treatment groups respectively. ns indicate mean values are not significantly different ($p > 0.05$). Student t-test or two-way analysis of variance (ANOVA) was conducted followed by the Tukey post hoc test. $n = 30$ flies per group (3 replicates of 10 flies per group). CTRL = Control, I.D = Iron Deficiency, N.D = Normal Diet, R.D = Rapamycin Diet, F1 = First Filial Generation

(Fig. 2D). However, when these flies were raised on a rapamycin-treated diet no significant difference ($p > 0.05$) was observed in F1 male offspring while F1 female offspring exhibited a significant increase ($p < 0.0001$) in body weight compared to F1 control females (Fig. 2D).

Maternal ID Alters Iron Homeostasis and Fer1HCH mRNA Expression in F_0 Females and F_1 Offspring in a Sex-Dependent Manner

Maternal ID on iron levels in F_0 flies and their offspring is shown in Figs. 3A-D. After 14 days of iron chelation, F_0 ID females exhibited a significant reduction in iron levels ($p < 0.0001$) compared to F_0 control females (Fig. 3A). This was accompanied by a significant upregulation ($p < 0.020$) of *Fer1HCH* mRNA expression (Fig. 3E), suggesting a compensatory response to iron depletion. When these F_0 ID females were returned to a normal diet for 30 days, their iron levels significantly increased ($p < 0.0001$), surpassing those of the control group (Fig. 3B), while *Fer1HCH* mRNA expression significantly decreased ($p < 0.003$) compared to control (Fig. 3F), indicating restoration of iron homeostasis. Conversely, F_0 ID females maintained on a rapamycin-treated diet showed a non-significant decrease ($p > 0.05$) in iron levels (Fig. 3C), but a significant increase in *Fer1HCH* mRNA expression ($p < 0.050$) (Fig. 3G), suggesting an interaction between rapamycin and iron-regulatory mechanisms.

In the F_1 generation, male offspring raised on a normal diet exhibited significantly lower iron levels ($p < 0.0001$) than F_1 control males, whereas female offspring showed significantly higher iron levels ($p < 0.0001$) than F_1 control females (Fig. 3D). These changes mirrored *Fer1HCH* expression patterns: F_1 males exhibited a significant upregulation ($p < 0.0001$), while F_1 females displayed a non-significant downregulation ($p > 0.05$) of *Fer1HCH* mRNA (Fig. 3H). Interestingly, when raised on a rapamycin-treated diet, both F_1 male and female offspring showed significant downregulation ($p < 0.0001$) of *Fer1HCH* expression, while F_1 males exhibited a significant

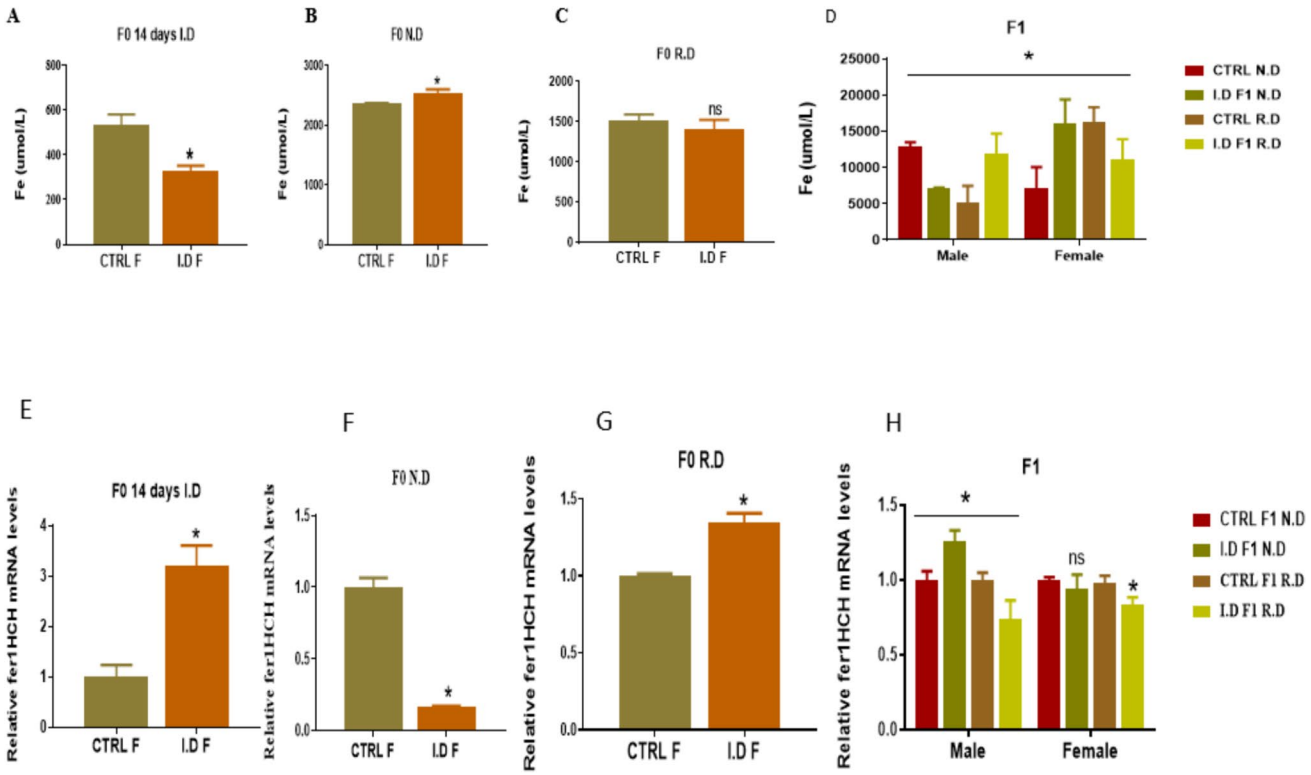

Fig. 3 **A**–**H** Effects of maternal ID on iron levels and Fer1HCH mRNA expression in flies. **A**–**D** Iron levels in flies (**A**) after 14 days of iron chelation, **B** after 30 days on a normal diet, **C** after 30 days on a rapamycin-treated diet, and (**D**) in F1 offspring maintained on a normal or rapamycin-treated diet. E–H: Relative Fer1HCH mRNA expression in flies (**E**) after 14 days of iron chelation, **F** after 30 days on a normal diet, **G** after 30 days on a rapamycin-treated diet, and (**H**) in F1 offspring on a normal or rapamycin-treated diet. Bars represent mean ± SD from 3 biological replicates. * indicates mean values are significantly different ($p < 0.05$), while ns indicates no significant difference ($p > 0.05$) compared to the control and treatment groups. Statistical analyses were conducted using Student's t-test or two-way ANOVA followed by Tukey's post hoc test. $n = 60$ flies per group (3 replicates of 20 flies per group) for iron level analysis (A–D), $n = 20$ flies per group (4 replicates of 5 flies per group) for gene expression analysis (E–H) CTRL = Control, I.D = Iron Deficiency, N.D = Normal Diet, R.D = Rapamycin Diet, F1 = First Filial Generation

increase ($p < 0.0001$) and F_1 females a significant decrease ($p < 0.0001$) in iron levels compared to their respective controls (Figs. 3D and H).

Maternal ID Alters GSH Levels and GPx3 mRNA Expression in F_0 Females and F_1 Offspring

After 14 days of iron chelation, F_0 ID females exhibited a significant increase in GSH levels ($p < 0.0001$) compared to F_0 control females (Fig. 4A). This elevation in GSH persisted after 30 days on a normal diet ($p < 0.0001$) (Fig. 4B), and was also observed in F_0 ID females on a rapamycin-treated diet ($p < 0.0001$) (Fig. 4C). In parallel, GPx mRNA expression in F_0 ID females showed a significant downregulation ($p < 0.003$) compared to F_0 control females (Fig. 4E). This downregulation persisted when these flies were returned to a normal diet ($p < 0.006$) and a rapamycin-treated diet ($p < 0.001$) for 30 days (Figs. 4F and G).

In the F_1 generation, male and female offspring raised on a normal diet showed reduced GSH levels compared to their controls. However, this reduction was statistically significant ($p < 0.0001$) only in F_1 females (Fig. 4D). Likewise, GPx3 mRNA expression in F_1 males exhibited significant downregulation ($p < 0.0001$) compared to F_1 control males (Fig. 4H), while F_1 female offspring showed no significant change ($p > 0.05$). When raised on a rapamycin-treated diet, both male and female F_1 offspring exhibited significantly higher GSH levels ($p < 0.0001$) compared to their respective controls (Fig. 4D). Similarly, both male and female F_1 offspring exhibited significant downregulation ($p < 0.0001$) of GPx3 mRNA expression compared to their control groups (Fig. 4H).

Maternal ID Disrupts SOD in F0 flies and their F1 Offspring

The Impacts of maternal ID on SOD levels AND Relative *SOD mRNA* of F0 flies and their offspring are depicted in Figs. 5A-H. After 14 days of iron chelation, F0 I.D. females exhibited a significant decrease in SOD levels (Fig. 5A, $p < 0.0001$) and SOD3 mRNA expression (Fig. 5E, $p < 0.036$) compared to F0 control females. This trend was maintained after 30 days on a normal diet, with F0 I.D. females showing a persistent decrease in both SOD levels (Fig. 5B, $p < 0.0001$) and SOD3 mRNA expression (Fig. 5F, $p < 0.003$). Upon switching to a rapamycin-treated diet for 30 days, SOD levels were slightly increased (Fig. 5C), but this increase was not statistically significant ($p > 0.05$). Similarly, the downregulation in SOD3 mRNA expression remained significant (Fig. 5G, $p < 0.002$).

In the F1 offspring, regardless of sex or dietary conditions, a similar pattern was observed. Both male and female F1 offspring exhibited significantly lower SOD levels compared

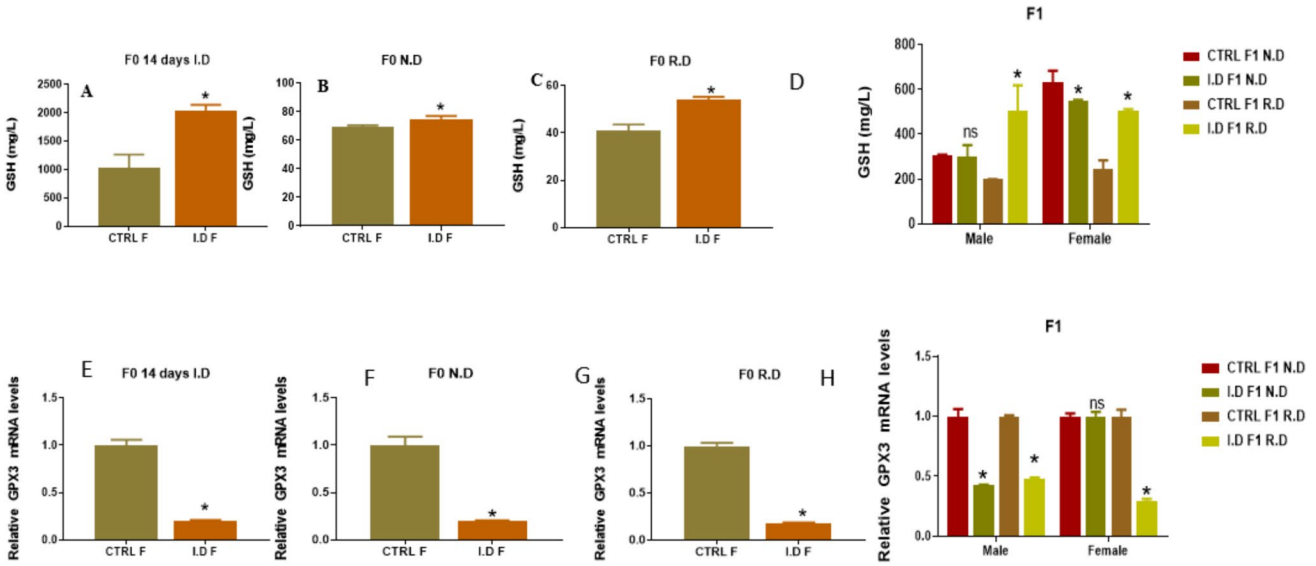

Fig. 4 **A**–**H** Effects of maternal ID on reduced glutathione (GSH) levels and GPx mRNA expression in flies. **A**–**D** GSH levels in flies (**A**) after 14 days of iron chelation, **B** after 30 days on a normal diet, **C** after 30 days on a rapamycin-treated diet, and (**D**) in F1 offspring maintained on a normal or rapamycin-treated diet. **E**–**H** Relative GPx mRNA expression in flies (**E**) after 14 days of iron chelation, **F** after 30 days on a normal diet, **G** after 30 days on a rapamycin-treated diet, and (**H**) in F1 offspring on a normal or rapamycin-treated diet. Bars represent mean ± SD from 3 biological replicates. * indicates mean values are significantly different ($p < 0.05$), while ns indicates no significant difference ($p > 0.05$) compared to the control and treatment groups. Statistical analyses were conducted using Student's t-test or two-way ANOVA followed by Tukey's post hoc test. $n = 60$ flies per group (3 replicates of 20 flies per group) for GSH level analysis (**A**–**D**), $n = 20$ flies per group (4 replicates of 5 flies per group) for GPx gene expression analysis (E–H). CTRL = Control, I.D = Iron Deficiency, N.D = Normal Diet, R.D = Rapamycin Diet, F1 = First Filial Generation

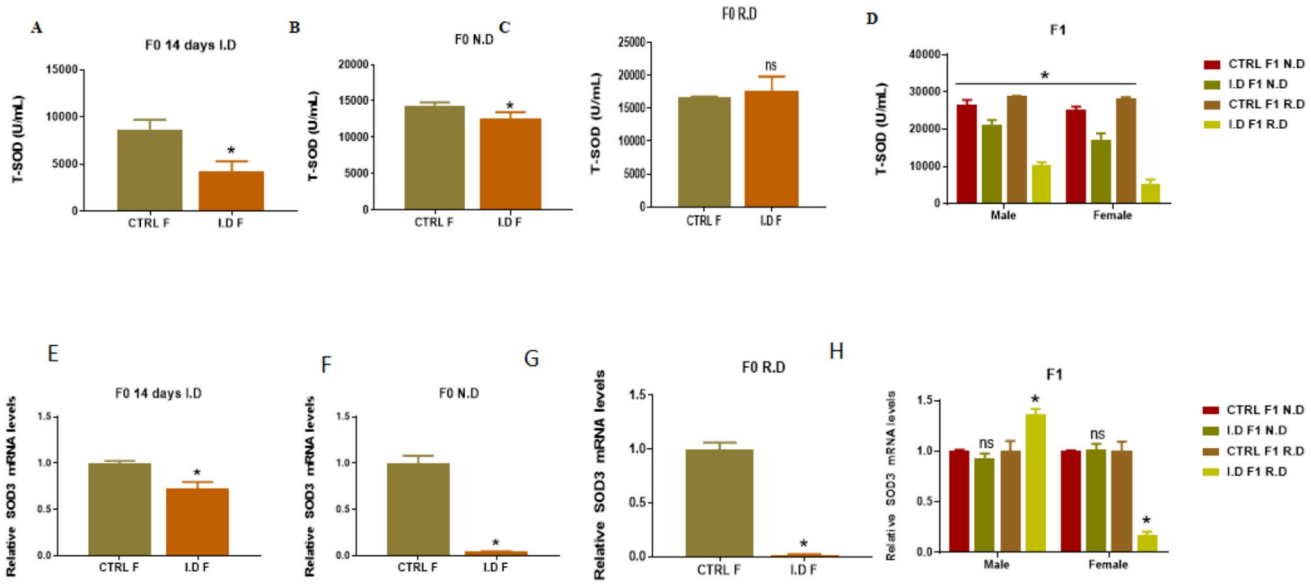

Fig. 5 **A**–**H** Effects of maternal ID on total superoxide dismutase (T-SOD) levels and Relative *SOD mRNA* expression in flies. **A**–**D** T-SOD levels in flies (**A**) after 14 days of iron chelation, **B** after 30 days on a normal diet, **C** after 30 days on a rapamycin-treated diet, and (**D**) in F1 offspring maintained on a normal or rapamycin-treated diet. E–H: Relative Relative SOD mRNA expression in flies (**E**) after 14 days of iron chelation, **F** after 30 days on a normal diet, **G** after 30 days on a rapamycin-treated diet, and (**H**) in F1 offspring on a normal or rapamycin-treated diet. Bars represent mean ± SD from 3 biological replicates. * indicates mean values are significantly different ($p < 0.05$), while ns indicates no significant difference ($p > 0.05$) compared to the control and treatment groups. Statistical analyses were conducted using Student's t-test or two-way ANOVA followed by Tukey's post hoc test. $n = 60$ flies per group (3 replicates of 20 flies per group) for SOD level analysis (A–D), $n = 20$ flies per group (4 replicates of 5 flies per group) for gene expression analysis (E–H) with. CTRL = Control, I.D = Iron Deficiency, N.D = Normal Diet, R.D = Rapamycin Diet, F1 = First Filial Generation

to their control counterparts (Fig. 5D, $p < 0.0001$), and this was consistent with the SOD3 mRNA expression findings. On a normal diet, no significant difference in SOD3 mRNA expression was observed (Fig. 5H, $p > 0.05$), though there was a trend of downregulation in F1 males and upregulation in F1 females. However, when F1 offspring were placed on a rapamycin-treated diet, F1 males showed significant upregulation in SOD3 mRNA expression (Fig. 5H, $p < 0.024$), while F1 females displayed significant downregulation ($p < 0.001$), paralleling the changes in their SOD enzyme levels.

Maternal ID Modulates Catalase in Flies in a Sex-Dependent Manner

The impact of maternal ID on catalase levels and CAT mRNA in F_0 flies and their offspring is illustrated in Figs. 6A–H. After 14 days on an iron-chelated diet F0 ID females exhibited a significant reduction in catalase levels (Fig. 6A, $p < 0.0001$) compared to F0 control females. This reduction persisted even after the flies were returned to a normal diet for 30 days (Fig. 6B, $p < 0.0001$). Interestingly, when these flies were exposed to a rapamycin-treated diet for 30 days (Fig. 6C), catalase levels significantly increased ($p < 0.012$) compared to their control counterparts, suggesting a compensatory effect of rapamycin. Concurrently, F0 ID females exhibited a significant upregulation of CAT mRNA expression after 14 days of iron chelation (Fig. 6E, $p < 0.0001$) compared to F0 control females. Upon returning these flies to either a normal or rapamycin-treated diet for 30 days, CAT mRNA expression in F0 ID females was significantly downregulated (Figs. 6F & G, $p < 0.002$ and $p < 0.007$, respectively) compared to F0 controls.

In F1 offspring, catalase levels did not differ significantly between males and females raised on a normal diet (Fig. 6D, $p > 0.05$), though a trend was observed: F1 male offspring exhibited a slight decrease in catalase levels, while F1 females showed a slight increase. When exposed to a rapamycin-treated diet, both F1 male and female offspring displayed significantly elevated catalase levels (Fig. 6D, $p < 0.0001$), indicating a sex-dependent response to maternal ID and rapamycin intervention. Regarding CAT mRNA expression in F1 offspring, both male and female offspring raised on a normal diet exhibited downregulation compared to their control groups (Fig. 6H). This downregulation was statistically significant in F1 males ($p < 0.003$), but not in F1 females ($p > 0.05$). However, in response to a rapamycin-treated diet, both F1 male and female offspring showed significant upregulation in CAT mRNA expression (Fig. 6H,

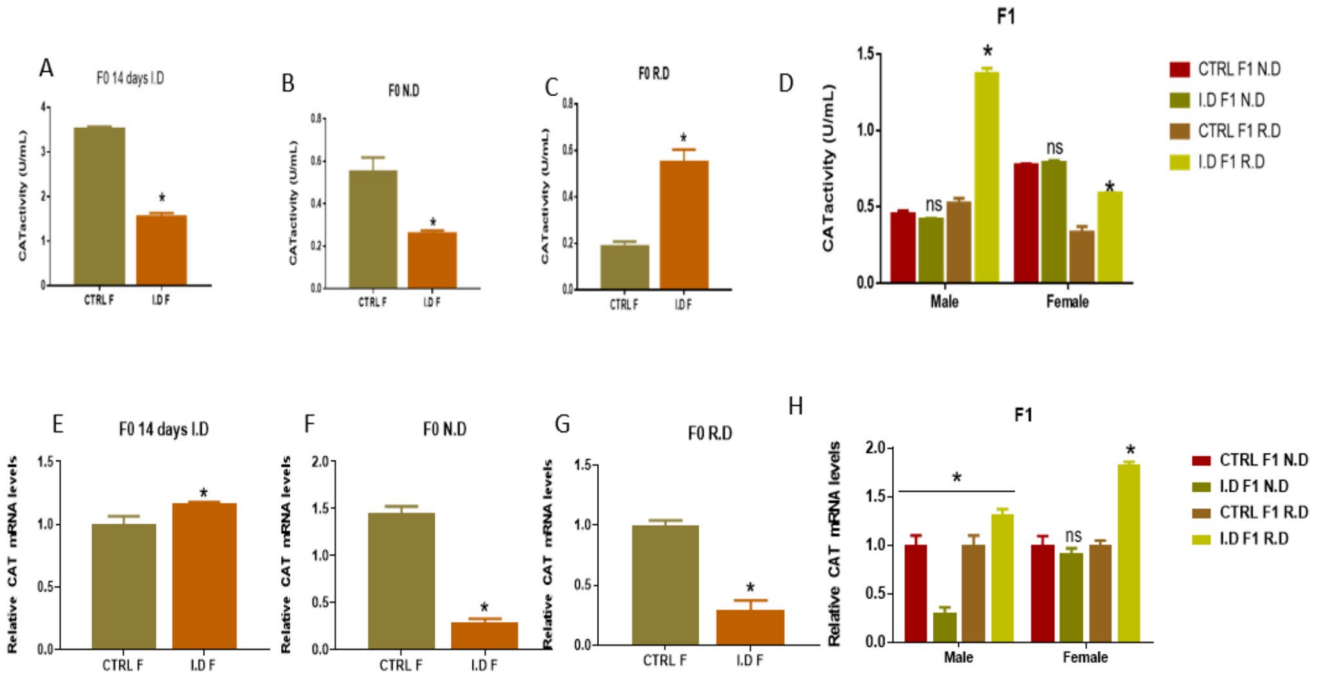

Fig. 6 **A**–**H** Effects of maternal ID on catalase (CAT) levels and Relative *CAT mRNA* expression in flies. **A**–**D** CAT levels in flies (**A**) after 14 days of iron chelation, **B** after 30 days on a normal diet, **C** after 30 days on a rapamycin-treated diet, and (**D**) in F1 offspring maintained on a normal or rapamycin-treated diet. **E**–**H** Relative Relative *CAT mRNA* expression in flies (**E**) after 14 days of iron chelation, **F** after 30 days on a normal diet, **G** after 30 days on a rapamycin-treated diet, and (**H**) in F1 offspring on a normal or rapamycin-treated diet. Bars represent mean ± SD from 3 biological replicates. * indicates mean values are significantly different ($p < 0.05$), while ns indicates no significant difference ($p > 0.05$) compared to the control and treatment groups. Statistical analyses were conducted using Student's t-test or two-way ANOVA followed by Tukey's post hoc test. $n = 60$ flies per group (3 replicates of 20 flies per group) for CAT level analysis (A–D), $n = 20$ flies per group (4 replicates of 5 flies per group) for gene expression analysis (E–H). CTRL = Control, I.D = Iron Deficiency, N.D = Normal Diet, R.D = Rapamycin Diet, F1 = First Filial Generation

$p < 0.023$ and $p < 0.001$, respectively), reflecting a sex-dependent response to maternal ID and rapamycin intervention.

Maternal ID Modulates ATG1 mRNA and dHeT-A mRNA Expression in F_0 Flies and Differentially in Their F_1 Offspring

Figures 7A–D illustrate the effects of maternal ID on the relative expression of dHeT-A Mrna and *ATG1 mRNA* in F_0 flies and their offspring. In F_0 females, relative ATG1 mRNA expression was significantly upregulated (Fig. 7A, $p < 0.005$) in F0 ID females compared to F0 control females following 14 days of iron chelation. This upregulation persisted after returning these flies to a normal diet for 30 days (Fig. 7B, $p < 0.001$), while exposure to a rapamycin-treated diet for 30 days led to a significant downregulation of ATG1 mRNA expression (Fig. 7C, $p < 0.0001$) in F0 ID females compared to F0 control females. In F1 offspring, differential responses were observed: F1 male offspring raised on a normal diet showed significant downregulation of ATG1 mRNA expression (Fig. 7D, $p < 0.001$) compared to F1 control males, whereas F1 female offspring exhibited a significant upregulation (Fig. 7D, $p < 0.0001$) compared to F1 control females. However, under a rapamycin-treated diet, F1 male offspring showed a significant upregulation (Fig. 7D, $p < 0.004$), while F1 female offspring showed significant downregulation ($p < 0.0001$) compared to their control counterparts.

For dHeT-A mRNA, in F0 females, 14 days of iron chelation led to a significant upregulation of dHeT-A mRNA expression (Fig. 7EA, $p < 0.006$) compared to F0 control females. After returning these flies to either a normal or rapamycin-treated diet for 30 days, dHeT-A mRNA expression was significantly downregulated (Figs. 7F and G, $p < 0.0001$) compared to controls. In F1 offspring, male offspring raised on a normal diet exhibited a significant upregulation of dHeT-A mRNA expression (Fig. 7H, $p < 0.0001$) compared to control males, whereas female offspring showed significant downregulation ($p < 0.0001$) compared to control females. However, under a rapamycin-treated diet, both F1 male and female offspring showed significant downregulation of dHeT-A mRNA expression ($p < 0.044$ for males and $p < 0.001$ for females) compared to their respective control groups.

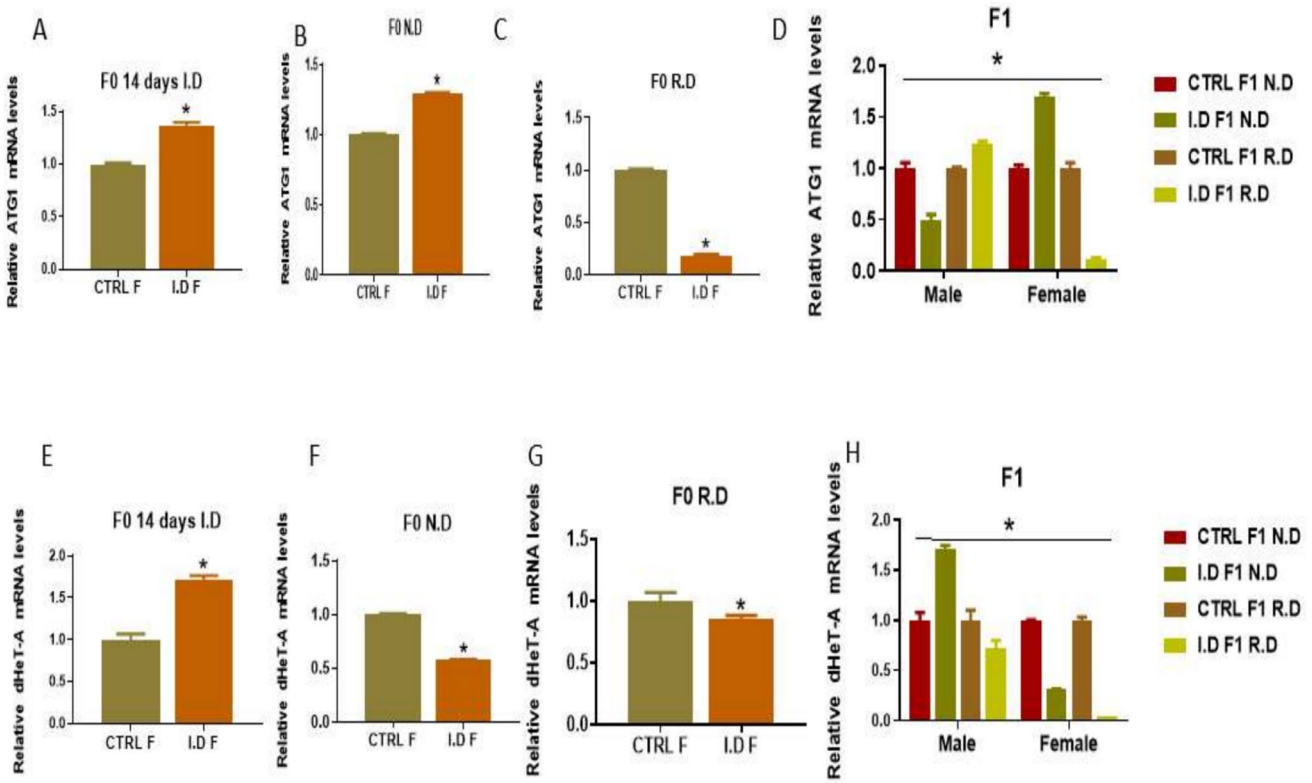

Fig. 7 **A**–**H** Effects of maternal ID on *ATG1 mRNA and dHeT-A mRNA* expression in flies. **A**–**D** *ATG1 mRNA* in flies (**A**) after 14 days of iron chelation, **B** after 30 days on a normal diet, **C** after 30 days on a rapamycin-treated diet, and (**D**) in F1 offspring maintained on a normal or rapamycin-treated diet. **E**–**H** Relative *dHeT-A mRNA* expression in flies (**E**) after 14 days of iron chelation, **F** after 30 days on a normal diet, **G** after 30 days on a rapamycin-treated diet, and (**H**) in F1 offspring on a normal or rapamycin-treated diet. Bars represent mean ± SD. * indicates mean values are significantly different ($p < 0.05$), while ns indicates no significant difference ($p > 0.05$) compared to the control and treatment groups. Statistical analyses were conducted using Student's t-test or two-way ANOVA followed by Tukey's post hoc test = 20 flies per group (4 replicates of 5 flies per group). CTRL = Control, I.D = Iron Deficiency, N.D = Normal Diet, R.D = Rapamycin Diet, F1 = First Filial Generation

Maternal ID Alters dTahre mRNA and dTart mRNA Expression in F_0 Flies and Their Offspring in a Sex-Dependent Manner

The effects of maternal ID on relative *dTahre* and *dTart mRNA* expression of F0 flies and their offspring are shown in Figs. 8A-H. Following 14 days of iron chelation, relative dTahre mRNA expression was significantly upregulated (Fig. 8A, $p < 0.001$) in F_0 ID females compared to F_0 control females. Upon returning these flies to a normal diet for 30 days (Fig. 8B), dTahre mRNA expression was significantly downregulated ($p < 0.005$). In contrast, exposure to a rapamycin-treated diet for 30 days (Fig. 8C) resulted in significant upregulation of *dTahre mRNA* expression ($p < 0.0001$) in F_0 ID females compared to F_0 control females. In the F_1 generation, male offspring raised on a normal diet exhibited a significant downregulation of dTahre mRNA expression (Fig. 8D, $p < 0.0001$) compared to F_1 control males, while female offspring exhibited significant upregulation ($p < 0.025$) compared to F_1 control females. On a rapamycin-treated diet, F_1 male offspring showed a significant downregulation ($p < 0.004$) of dTahre mRNA expression, while no significant difference ($p > 0.05$) was observed in F_1 female offspring compared to their control counterparts.

For *dTart mRNA*, after 14 days of iron chelation, dTart mRNA expression was significantly upregulated (Fig. 8E, $p < 0.028$) in F_0 ID females compared to F_0 control females. Upon returning the flies to a normal diet for 30 days (Fig. 8F) or a rapamycin-treated diet (Fig. 8G), the upregulation persisted and was statistically significant ($p < 0.012$ and $p < 0.013$) compared to F_0 control females. In the F_1 generation, male offspring raised on a normal diet exhibited significant upregulation of dTart mRNA expression (Fig. 8H, $p < 0.0001$) compared to control males, while female offspring showed a significant downregulation ($p < 0.001$) compared to control females. Under the rapamycin-treated diet, both F_1 male and female offspring exhibited significant downregulation of dTart mRNA expression ($p < 0.0001$) compared to their control counterparts.

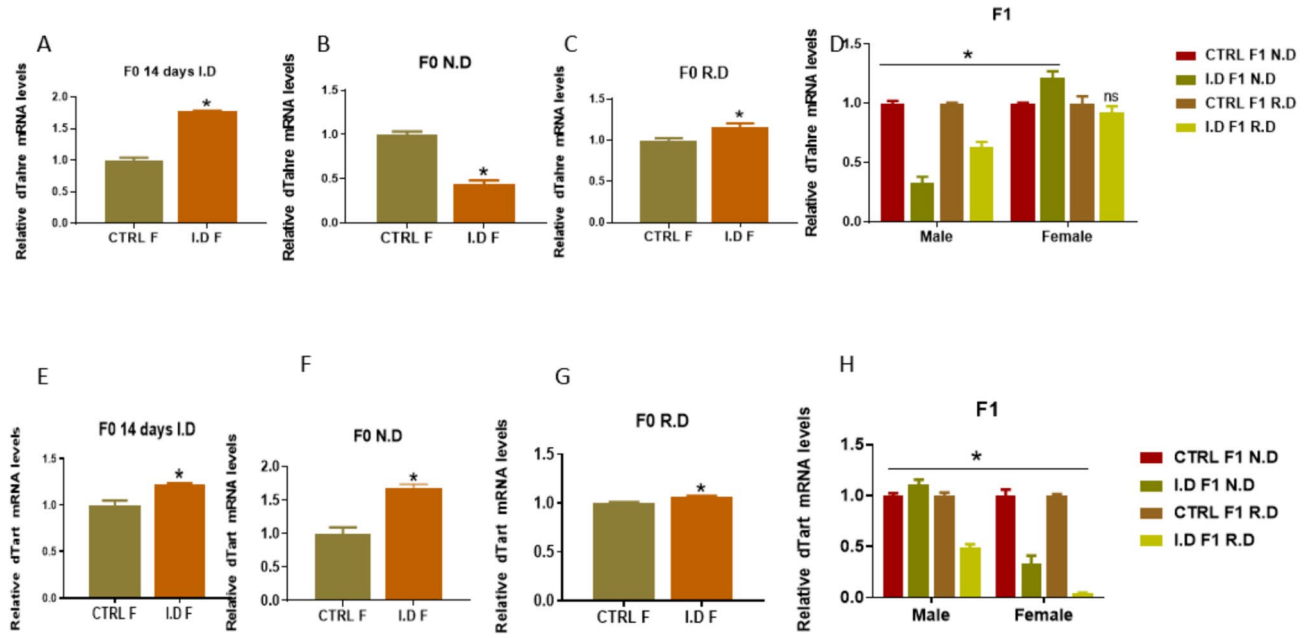

Fig. 8 **A**–**H** Effects of maternal ID on *dTart* mRNA *and dTart mRNA* expression in flies. **A**–**D** *dTart* mRNA in flies (**A**) after 14 days of iron chelation, **B** after 30 days on a normal diet, **C** after 30 days on a rapamycin-treated diet, and (**D**) in F1 offspring maintained on a normal or rapamycin-treated diet. **E**–**H** Relative *dTart mRNA* expression in flies (**E**) after 14 days of iron chelation, **F** after 30 days on a normal diet, **G** after 30 days on a rapamycin-treated diet, and **H** F1 offspring on a normal or rapamycin-treated diet. Bars represent mean ± SD from 3 biological replicates. * indicates mean values are significantly different ($p < 0.05$), while ns indicates no significant difference ($p > 0.05$) compared to the control and treatment groups. Statistical analyses were conducted using Student's t-test or two-way ANOVA followed by Tukey's post hoc test. 20 flies per group (4 replicates of 5 flies per group). CTRL = Control, I.D = Iron Deficiency, N.D = Normal Diet, R.D = Rapamycin Diet, F1 = First Filial Generation

Table 2 Differences in median survival of F0 and F1 flies

Group	Median Survival (80 flies)	
	Normal Diet	Rapamycin Diet
F0 Control female	42.18 ± 3.48	49.24 ± 3.45
F0 I.D female	47.24 ± 4.47[a]	22.86 ± 6.20[a]
F1 Control male	36.48 ± 1.29	43.59 ± 1.74
F1 I.D male	50.17 ± 2.24[a]	42.92 ± 2.08 [ns]
F1 Control female	34.62 ± 1.45	42.28 ± 2.41
F1 I.D female	38.61 ± 1.73[b]	34.62 ± 2.24[b]

a indicate significant differences compared to their respective controls ($p < 0.05$) in the same column, while **ns** indicate mean values are not significantly different ($p > 0.05$). Median survival time refers to the time (days) it takes for 50% of flies to have experienced a terminal event (death). Each fly represents one unit of analysis ($n = 80$), survival time is 60 days

Maternal ID Modulate Lifespan in Drosophila in F0 and F1 Offspring

The effects of maternal ID on the longevity of F0 and F1 flies are summarized in Tables 2. After a 60-day survival study on a normal diet, F0 ID females exhibited a significant increase ($p < 0.05$) in median survival compared to their control group. Conversely, on the rapamycin-treated diet, F0 ID females showed a significant reduction ($p < 0.05$) in median survival relative to controls.

Similarly, F1 male and female offspring raised on a normal diet also demonstrated a significant increase ($p < 0.05$) in median survival compared to their respective control groups. However, under the rapamycin-treated diet, F1 males showed no significant difference ($p > 0.05$) in survival, whereas F1 females exhibited a significant decrease ($p < 0.05$) in median survival compared to their controls.

Discussion

This study examined the effects of maternal ID on physiological, biochemical, and gene expression changes in *Drosophila melanogaster* and their offspring, under both normal and rapamycin-treated diets. Maternal ID led to reduced body weight in F0 ID females after 14 days of iron chelation, but not in F1 offspring raised on a normal diet, regardless of sex. This weight loss may stem from disrupted appetite regulation, as ID has been associated with decreased appetite and altered leptin signaling [20, 23]. While Drosophila lacks leptin, analogous signaling via Upd cytokines and the JAK/

STAT pathway may play a similar role [64]. The rapamycin diet reversed the weight loss seen in F0 ID females on a normal diet, possibly through its effects on lipid and glucose metabolism, as well as appetite regulation [34, 45]. Notably, only F1 female offspring on the rapamycin diet showed increased body weight, suggesting a sex-specific metabolic response to rapamycin [47].

To assess maternal ID effects on iron status, we analyzed iron levels and *Fer1HCH* expression. Iron chelation significantly reduced iron levels in F0 ID females, consistent with the action of chelators [37, 49, 58], and induced a 3.22-fold upregulation of *Fer1HCH*, aligning with earlier findings on ferritin response to ID in *Drosophila* [24, 37]. After 30 days on a normal diet, iron levels rebounded above control levels, while *Fer1HCH* was downregulated, suggesting persistent upregulation of iron uptake pathways and reduced ferritin need.

In F1 offspring raised on a normal diet, gender-specific differences likely reflect epigenetic inheritance from F0. Mechanisms such as DNA methylation and histone modifications may influence gene expression without altering the DNA sequence and can be passed across generations [10, 36]. Sex-specific epigenetic regulation may explain the observed variations in iron metabolism, possibly involving differential gene expression, such as that of hepcidin in mammals [25], or sex hormone influences on iron dynamics. Environmental factors like perinatal lead exposure have also been linked to sex-specific epigenetic changes in metabolism [55].

In contrast, rapamycin treatment in F0 ID females maintained stable iron levels and induced a mild *Fer1HCH* upregulation (1.35-fold), suggesting impaired iron uptake or storage and a cellular stress response [42]. Rapamycin may prevent the rebound effect seen under a normal diet by simulating iron scarcity. F1 offspring of rapamycin-treated F0 flies displayed sex-specific phenotypes: F1 males showed increased iron and reduced *Fer1HCH* expression, indicating enhanced iron uptake or reduced storage, while F1 females showed reduced iron and *Fer1HCH*, pointing to greater iron use or impaired absorption, signifying altered iron homeostasis.

To assess maternal ID's impact on antioxidant status, we measured antioxidant protein levels and gene expression in both F0 and F1 flies. In F0 ID females, elevated GSH levels after 14 days of iron chelation suggest a compensatory response to oxidative stress, as GSH neutralizes radicals and supports detox enzymes [6, 19]. However, *GPx3* expression was downregulated and remained low post-chelation, indicating metabolic reprogramming of antioxidant defense [57, 61].

In F1, male offspring showed stable GSH and reduced *GPx3,* indicating compensation, while females had lower GSH and unchanged *GPx3*, suggesting greater sensitivity to oxidative stress. Rapamycin appeared to enhance antioxidant defense in F0 ID females by increasing GSH and downregulating *GPx3*, possibly shifting to alternative antioxidant pathways. Similar GSH increases and *GPx3* downregulation in F1 offspring suggest transgenerational epigenetic influence [23, 56].

As expected, SOD levels and *SOD3* expression dropped in F0 ID females due to iron dependency, and these reductions persisted post-diet recovery, implying lasting oxidative stress effects. F1 offspring also showed decreased SOD protein despite unchanged *SOD*3, likely due to protein instability inherited from the mother. Under rapamycin, *SOD3* was downregulated in F0 ID females, while in F1 males, it was upregulated (1.37-fold), and in females, downregulated, indicating sex-specific responses potentially linked to mTOR signaling [48].

CAT expression was upregulated in F0 ID females to compensate for low catalase protein but later declined as iron normalized. In F1 males, catalase protein remained stable despite reduced CAT, suggesting alternative regulation. F1 females showed stable catalase levels and expression, implying a more balanced system. Rapamycin improved catalase protein in F0 ID females, possibly by enhancing stability, despite reduced *CAT* expression. In F1 offspring, both *CAT* expression and catalase levels increased, suggesting rapamycin stimulates transcriptional and translational pathways to bolster antioxidant defenses.

To explore the effects of maternal ID on autophagy and telomere maintenance, we assessed *ATG1* and telomere-associated genes (*dHeT-A, dTahre*, *dTart*) expression. *ATG1*, essential for autophagy initiation [21], was upregulated in iron-deficient F0 females, indicating increased autophagic activity likely triggered by oxidative stress. This aligns with studies linking oxidative damage to mitophagy activation [17, 22, 33, 39, 63]. The sustained *ATG1* expression after dietary recovery suggests lingering cellular stress and long-term regulatory changes.

In F1 males raised on a normal diet, *ATG1* downregulation implies reduced autophagy, possibly reflecting cellular normalization due to early exposure to adequate iron. Conversely, F1 females showed *ATG1* upregulation despite elevated iron, suggesting inherited stress or damage driving autophagic activity.

In the rapamycin-treated group, *ATG1* was downregulated in F0 ID females after 30 days, indicating reduced autophagy despite rapamycin's known role as an mTOR inhibitor and autophagy inducer [28]. This suggests that rapamycin's protective effects in this context are likely tied to its antioxidant properties rather than autophagy induction [27]. Interestingly, *ATG1* was upregulated in F1 males from rapamycin-treated mothers, implying elevated autophagic activity for cellular repair. In contrast, F1 females exhibited *ATG1* downregulation, suggesting reduced autophagy,

possibly leading to the buildup of damaged components and highlighting sex-specific regulatory mechanisms.

ID affects telomere length, as shown by increased telomere length after iron chelation in humans [40]. In F0 ID females, upregulation of *dHeT-A, dTahre,* and *dTart* after 14 days of ID suggests a compensatory response to oxidative stress-induced telomere damage. Telomeric retrotransposons help preserve chromosome ends by adding DNA sequences [29, 43], protecting genomic stability [32]. After 30 days on a normal diet, *dHeT-A* and *dTahre* expression dropped, indicating normalization, while *dTart* remained elevated, possibly supporting telomere elongation. The increased lifespan in F0 ID females suggests a hormesis-like effect [9], where initial ID stress may have triggered protective mechanisms that persisted post-recovery.

F1 offspring on a normal diet displayed sex-specific gene expression. F1 males showed upregulated *dHeT-A* and *dTart*, but downregulated *dTahre*, indicating reduced stress and ongoing telomere maintenance. In contrast, F1 females showed the opposite pattern, downregulation of *dHeT*-A and *dTart*, but upregulation of *dTahre*, suggesting selective gene regulation to maintain telomeres and offset inherited stress. The survival benefits observed in both sexes likely result from improved telomere function and autophagic balance.

In the rapamycin-treated group, *dHeT-A* was downregulated in F0 ID females, while *dTahre* and *dTar*t were upregulated. Despite attempts to maintain telomeres, lifespan declined, suggesting rapamycin exacerbated ID effects. Prior studies showed rapamycin could reduce lifespan under nutrient-poor conditions [60], likely due to its dietary restriction mimicry worsening malnutrition. Thus, rapamycin may have disrupted protective pathways like autophagy, leading to shortened lifespan in F0 females.

In F1 males, all telomere-associated genes were downregulated, indicating reduced maintenance activity, possibly due to iron repletion and less cellular stress. Despite molecular changes, no significant survival differences emerged, implying a balanced cellular state. In contrast, F1 females showed reduced *dHeT-A* and *dTart* expression with unchanged *dTahre*, suggesting telomere instability and insufficient compensatory mechanisms, contributing to decreased lifespan. This underscores rapamycin's negative impact under maternal ID conditions, especially in females, where combined effects on iron levels, autophagy, and telomere maintenance severely compromised survival.

Conclusion

This study demonstrates that maternal ID induces long-term alterations in iron homeostasis, antioxidant defenses, autophagy, telomere maintenance, and longevity, with distinct sex-specific responses to post-deficiency dietary interventions. While recovery with a normal diet partially restored iron levels and improved survival, oxidative stress remained elevated, particularly in the F0 generation. Rapamycin enhanced antioxidant responses but produced mixed effects on survival, most notably reducing lifespan in female offspring. Maternal ID also triggered adaptive, sex-specific cellular responses, potentially mediated by epigenetic mechanisms. These findings underscore the complex interplay between iron metabolism, oxidative stress, and lifespan, and highlight the need for deeper investigation into the molecular and epigenetic pathways involved. This should inform future nutritional and therapeutic strategies for populations vulnerable to ID.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval The research is Drosophilla melanogaster based and no ethical approval was required.

Consent to Participate Informed consent was not needed for the study.

Competing interests The authors declare no competing interests.

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