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Impact of Intermittent Fasting on Survival and Gene Expression Profiles Associated with Autophagy, Metabolism, and Antioxidant in *Drosophila melanogaster*

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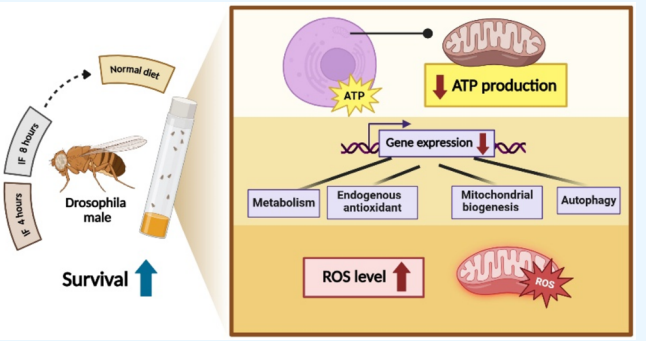
ABSTRACT: Intermittent fasting (IF) has attracted significant interest as a potential approach with promising health benefits, including lifespan extension and metabolic improvement. Previous studies have shown that IF influences autophagy, metabolism, and oxidative stress across various organisms. *Drosophila melanogaster* was used in this study due to its genetic structure and metabolic responses that closely resemble those of humans, making it a valuable model for studying the effects of IF on cellular and molecular mechanisms. This study investigates the effects of IF on survival and the expression of genes related to autophagy, metabolic, and endogenous antioxidants in *D. melanogaster* to elucidate the molecular mechanisms underlying these effects. Male w^{1118} flies were subjected to either a 4-h or 8-h fasting protocol, three times per week. The results revealed that both fasting protocols improved the survival of flies, with the 8-h IF group showing the most significant benefit (over 60% survival at day 10 compared to ∼50% in the control). Gene expression analysis demonstrated a significant downregulation in the fasting groups, with *dilp2* decreasing by 45%, *tom40* by 40%, *cat* by 50%, *srl* by 35%, and *atg8a* by 48%, indicating reduced insulin signaling, altered mitochondrial function, decreased antioxidant defense, and suppressed autophagy-related pathways. Notably, these findings contrast with previous studies reporting upregulation of autophagy and antioxidant responses during fasting, suggesting context-dependent regulatory mechanisms. Given the genetic and physiological similarities between *D. melanogaster* and mammals, these results provide insights into IF-induced metabolic adaptations and their potential implications for longevity and cellular homeostasis. Further research is warranted to clarify the molecular pathways involved.



1. INTRODUCTION

Intermittent fasting (IF) is a dietary regimen that alternates between periods of fasting and eating within specific time frames, with growing evidence supporting its role in promoting metabolic health, extending lifespan, and influencing key cellular processes such as autophagy and oxidative stress regulation.[1] In mammalian models, IF has been shown to enhance metabolic efficiency, reduce insulin signaling, and activate autophagy to maintain cellular homeostasis. These mechanisms are closely linked to longevity and protection against age-related diseases, including neurodegeneration, cardiovascular disorders, and metabolic syndromes.[2−4] Given these well-documented benefits in mammals, it is essential to explore whether similar mechanisms operate in simpler and genetically tractable model organisms such as *Drosophila melanogaster*.

The choice of *D. melanogaster* as a model organism is well-supported due to its short lifespan, ease of genetic manipulation, and high degree of evolutionary conservation with humans.[5,6] Approximately 75% of human disease-associated genes have homologues in *Drosophila*, making it a highly relevant model for studying conserved molecular mechanisms.[7] Past studies using *D. melanogaster* have significantly advanced our understanding of insulin signaling, mitochondrial function, and oxidative stress responses.[8] For instance, mutations in *Drosophila insulin-like peptide* (*dilp*) genes have been shown to extend lifespan and enhance stress resistance, paralleling findings in mammalian systems. Additionally, *D. melanogaster* models have contributed to

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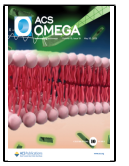

Table 1. Sequences of Primers Used in RT-qPCR Analysis

genes	forward primers	reverse primers
dilp2	TCTGCAGTGAAAAGCTCAACGA	CAACTGCAGGGGATTGAGG
tom40	TGCACGTGTGCTACTACCAG	ATTCCGCCTCTGAAGACCAG
cat	TTCCTGGATGAGATGTCGCACT	TTCTGGGTGTGAATGAAGCTGG
srl	CTCTTGGAGTCCGAGATCCGCAA	GGGACCGCGAGCTGATGGTT
atg5	GCACTACATGTCCTGCCTGA	AGATTCGCAGGGGAATGTTT
atg8a	TACCAGGAACATCACGAGGA	AAACGCGGTTCTGCATGAG

elucidating mechanisms of dietary restriction and lifespan extension via FOXO and TOR signaling pathways.[9,10]

Despite the growing body of research on IF, the molecular mechanisms underlying its effects on autophagy, metabolism, and oxidative stress in *D. melanogaster* remain underexplored. These pathways were selected for investigation due to their central roles in cellular maintenance, energy homeostasis, and longevity.[11] Autophagy is critical for the degradation of damaged organelles and proteins and has been directly linked to lifespan extension across species.[12,13] Metabolic regulation, especially through insulin signaling and mitochondrial function, influences not only energy balance but also stress resistance.[14,15] Oxidative stress, a key contributor to aging, is modulated by fasting,[16,17] but the specific gene expression responses in *D. melanogaster* have yet to be fully characterized. Moreover, previous findings on IF-related gene regulation in *D. melanogaster* have been inconsistent, with some studies reporting upregulation of autophagy and antioxidant defenses, while others show suppression,[16,18,19] highlighting the need for further investigation.

A notable advantage of using *D. melanogaster* in IF research is the ability to bypass ethical concerns associated with mammalian studies while maintaining strong translational relevance.[20] Experiments in *D. melanogaster* are also cost-effective and scalable and allow for rapid screening of genetic and environmental factors affecting physiology. These advantages underscore its utility in dissecting the long-term impact of IF on gene expression and survival.[21,22]

This study investigates how IF influences survival and gene expression in *D. melanogaster*, with a specific focus on genes involved in autophagy (*atg5*, *atg8a*), metabolism (*dilp2*, *tom40*), and oxidative stress (*cat*, *srl*). By addressing existing gaps in nonmammalian IF research and offering a more comprehensive gene expression analysis, this work aims to refine our understanding of the molecular adaptations induced by IF and their implications for aging and metabolic regulation.

2. MATERIALS AND METHODS

2.1. *Drosophila* Stock. *D. melanogaster* (strain w^{1118}) was used in this study. This strain is widely utilized in genetic and metabolic research due to its well-characterized genome and sensitivity to dietary interventions such as intermittent fasting. The flies were obtained from the Laboratory of Host Defense and Responses at Kanazawa University, Japan, and were maintained at 25 °C under a 12-h light/dark cycle with controlled humidity. Only male flies (1−2 days old) were used for all experiments to minimize sex-dependent variability in metabolic and survival responses.

2.2. Fly Feed Preparation. Two types of feed were prepared: normal feed and fasting feed. For the normal feed, 7.5 g of cornmeal, which serves as a primary source of complex carbohydrates and dietary fiber to provide sustained energy and bulk, was combined with 2.5 g of yeast, a rich source of protein, amino acids, and B-complex vitamins essential for development and metabolic function. Additionally, 0.9 g of agar was added as a gelling agent to solidify the medium, allowing flies to move and feed without the risk of drowning. To support fly activity, 4.5 g of granulated sugar was included as a readily accessible energy source. These ingredients were combined in a beaker, and the final volume was adjusted to 100 mL using mineral water. The mixture was then heated on an electric stove, stirring continuously with a rod until it thickened. Once the desired consistency was achieved, 400 μL of propionic acid and 450 μL of methylparaben were added as preservatives. The feed was allowed to cool before being poured into vials for use. The fasting feed consisted of 1% agar in water, without nutrients, to prevent starvation-induced dehydration while eliminating caloric intake. This formulation has been previously validated as a fasting medium in *D. melanogaster* studies.

2.3. Intermittent Fasting Protocol. Flies were divided into three groups: Control group − Fed *ad libitum* on the control diet, 4-h IF group − Placed on 1% agar for 4 h, three times per week (Monday, Wednesday, Friday), and 8-h IF group − Placed on 1% agar for 8 h, three times per week (Monday, Wednesday, Friday). After fasting, flies were returned to normal feeding.[19] The feeding schedule was designed to mimic IF regimens used in mammalian studies while ensuring minimal stress.

2.4. Survival Assay and Reactive Oxygen Species Measurement. To evaluate survival, flies were maintained in groups of 10 per vial and monitored daily. Mortality was determined by the absence of movement and lack of response to gentle tapping. Environmental conditions (temperature, humidity, and light/dark cycle) were kept constant throughout the experiment. Survival data were recorded until all flies had died. Reactive Oxygen Species (ROS) levels were determined using a modified nitroblue tetrazolium (NBT) reduction assay on whole-body homogenates. For each group (UC and IF), 100 adult male flies were collected and rinsed with phosphate-buffered saline (PBS) to eliminate any food residue. Hemolymph was extracted on ice to minimize melanization. A 300 μL hemolymph solution was prepared by combining 100 μL of hemolymph with 200 μL of 1× PBS, then mixed with an equal volume of NBT solution. The mixture was incubated at room temperature in the dark for 1 h. The reaction was halted by the addition of 300 μL of glacial acetic acid. Following centrifugation at maximum speed for 1 min, absorbance was recorded at 595 nm spectrophotometer (Shimadzu UV-vis 1800, Kyoto, Japan). The intensity of the blue formazan product indicated the relative ROS concentration.

2.5. Gene Expression Analysis. For gene expression analysis, five male flies per group were used. Total RNA was extracted using the Pure Link RNA Mini Kit (Invitrogen, Thermo Fisher Scientific Inc., Massachusetts, U.S.) according

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to the manufacturer's protocol. RNA purity and concentration were assessed with a NanoDrop spectrophotometer, using the A260/A280 ratio, with only samples showing ratios between 1.8−2.1 included in further analysis. Gene expression was quantified using reverse transcription-quantitative PCR (RT-qPCR) with the Luna Universal One-Step RT-qPCR Kit (New England Biolabs, Massachusetts, U.S.) on a Rotor-Gene Q system (Qiagen, Germany). Reactions were carried out in 10 μL volumes. The thermal profile included a 10 min RT activation at 50 °C, polymerase initiation at 95 °C for 2 min, followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. A melt curve analysis was performed to ensure product specificity. The housekeeping gene *rp49*, was used as the internal control for normalization. All primers were validated for specificity, and no-template controls (NTCs) were included to detect potential contamination or nonspecific amplification. Primer sequences used in the study are listed in Table 1.

2.6. Data Analysis. All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, MA). Survival data were analyzed using Kaplan−Meier survival curves, a statistical method used to estimate the probability of survival. Differences in survival between groups were assessed using the log-rank test to determine statistical significance. Gene expression data were analyzed using *t* tests. Three biological replicates were included for each experimental condition to ensure reproducibility.

2.7. Experimental Design and Workflow. The overall experimental design is summarized in Figure 1.

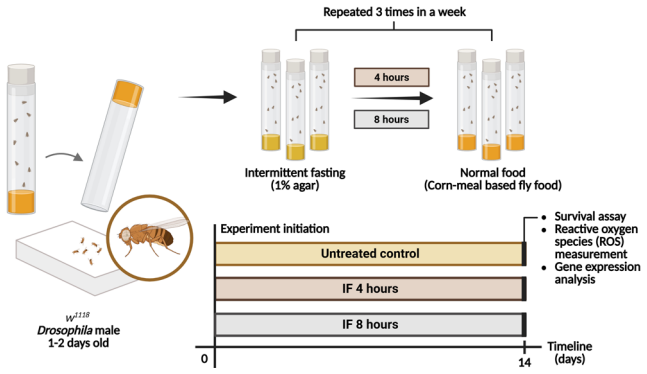

Figure 1. *D. melanogaster* males were allocated into three groups: untreated control (fed *ad libitum*), 4-h intermittent fasting (IF) group (exposed to 1% agar for 4 h, 3 times per week), and 8-h IF group (exposed to 1% agar for 8 h, 3 times per week). Following treatment, flies were subjected to survival assay, reactive oxygen species measurement, and gene expression analysis.

2.8. Ethical Considerations. Although *D. melanogaster* studies do not require formal ethical approval, all experiments were conducted following established guidelines for humane handling and minimal stress exposure.

3. RESULT AND DISCUSSION

3.1. Effect of Intermittent Fasting on the Survival of *Drosophila melanogaster*. This study investigates the impact of intermittent fasting (IF) on the survival of *D. melanogaster*. Survival analysis revealed that both 4-h and 8-h IF protocols significantly prolonged lifespan compared to the *ad libitum*-fed control group. As shown in Figure 2A, 60% of flies in the 8 h IF group survived beyond day 10, compared to 50% in the control group, indicating a notable survival advantage. Similarly, 60% of flies in the 4 h IF group remained alive on day 10 (Figure 2B), although the effect was slightly less pronounced than that observed with 8 h IF.

The lifespan extension observed in the 8-h IF group may be attributed to a more robust physiological adaptation to fasting. Previous research has shown that extended fasting can activate protective mechanisms such as increased mitochondrial efficiency, improved stress resistance, and enhanced autophagic clearance. A longer fasting duration can trigger greater metabolic reprogramming, potentially optimizing energy utilization and stress responses. However, excessive fasting durations may result in diminishing returns or induce cellular stress due to increased oxidative burden or protein degradation.[23−25] Thus, future studies should assess whether durations longer than 8 h preserve benefits or elicit metabolic dysfunction.

3.2. Effect of Intermittent Fasting on the Expression of Metabolism-Related Genes. The present research examined how IF influences the expression of genes associated with metabolic pathways, particularly *dilp2* and *tom40*, in *D. melanogaster*. Flies were divided into two IF groups with fasting durations of either 4 or 8 h, while the control group had constant food availability (*ad libitum*). The gene expression analysis revealed significant downregulation of *dilp2* and *tom40* in both fasting groups (Figure 3A−3D).

Gene expression analysis revealed a 45% reduction in *dilp2* ($p < 0.01$) and a 40% decrease in *tom40* ($p < 0.05$) in the fasting groups compared to the control (Figure 3A−D). These findings suggest a suppression of insulin signaling and mitochondrial transport activity, which are central to metabolic adaptation during fasting. The downregulation of *dilp2* aligns with previous studies showing that reduced insulin/IGF-like signaling (IIS) contributes to lifespan extension in *Drosophila*.[26] Lower IIS activity has been linked to enhanced stress resistance and metabolic flexibility, allowing flies to better endure fasting periods.[27] However, the decrease in *tom40* expression contrasts with earlier studies that reported increased mitochondrial biogenesis under fasting conditions.[28] One possible explanation is the timing of sample collection, if flies were sampled after refeeding rather than during fasting, mitochondrial gene expression patterns may have differed.[29] This highlights the need for time-course studies to capture the dynamic regulation of mitochondrial function during IF.

3.3. Effect of Intermittent Fasting on the Expression of Endogenous Antioxidant and Mitochondrial Biogenesis-Related Genes. The influence of IF on the expression of genes involved in endogenous antioxidant defense and mitochondrial biogenesis, such as *cat* (encoding Catalase), and *srl* (encoding Spargel), was examined in w^{1118} *D. melanogaster* subjected to 8-h and 4-h fasting durations, with a control group, fed *ad libitum*, as shown in Figure 4.

Contrary to expectations, both *cat* and *srl* showed significant downregulation under fasting conditions, with *cat* expression reduced by 50% ($p < 0.01$) and *srl* by 35% ($p < 0.05$) (Figure 4A−D). This is surprising, as previous studies often report increased antioxidant gene expression as a compensatory response to fasting-induced oxidative stress.[30,31] A potential explanation for this discrepancy is the accumulation of reactive oxygen species (ROS) beyond a certain threshold, which might inhibit antioxidant gene transcription. Mitochondrial dysfunction and ROS accumulation have been reported in prolonged fasting conditions, potentially leading to oxidative stress

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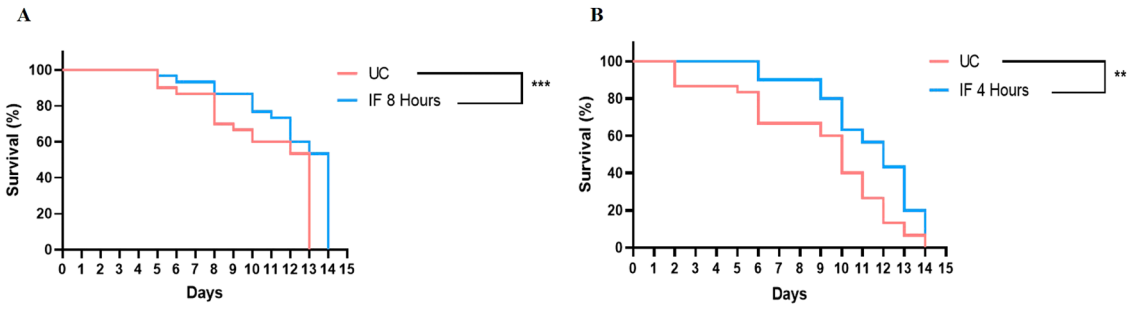

Figure 2. Effect of 8-h (A) and 4-h (B) Intermittent Fasting Protocols on Survival Rates of w^{1118} *D. melanogaster*. Survival was monitored daily using the Kaplan–Meier method. Data are presented as survival percentages, and statistical analysis was performed using the log-rank test. IF, intermittent fasting; UC, untreated control; **, $p < 0.01$; ***, $p < 0.001$. Sample size ($n = 30$ flies/group).

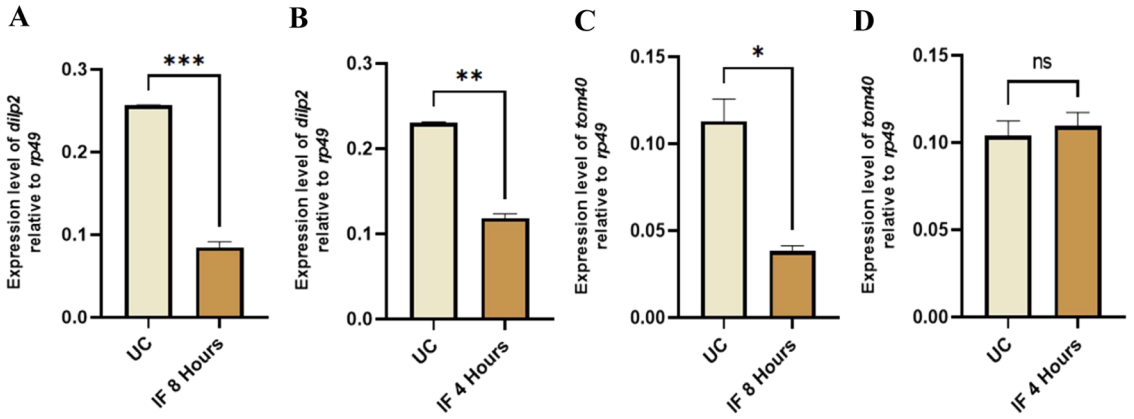

Figure 3. Expression of metabolism-related genes in w^{1118} *D. melanogaster* after 8-h (A, C) and 4-h (B, D) IF. Bars represent mean ± SEM. Error bars denote standard error. IF, intermittent fasting; UC, untreated control; ns, nonsignificant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Expression of target genes was normalized to *rp49*.

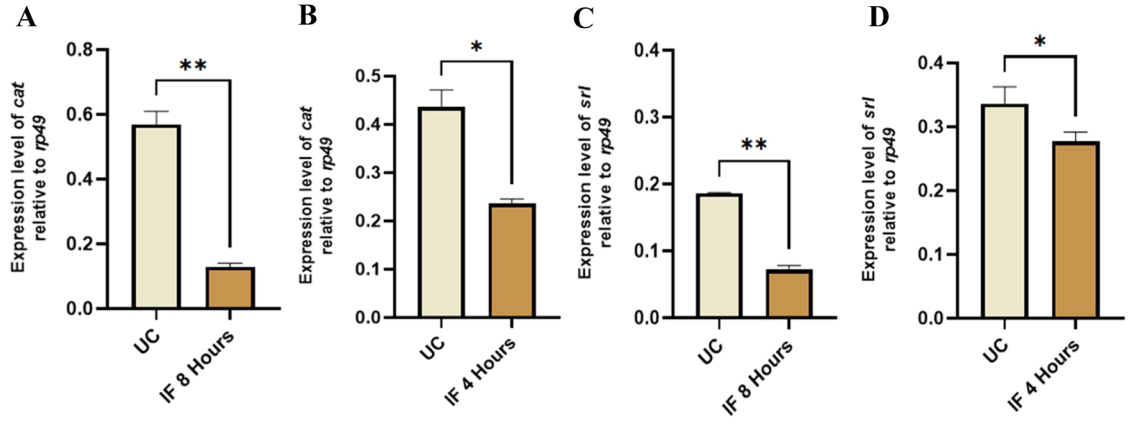

Figure 4. Expression of endogenous antioxidant (*cat*) (A, B) and mitochondrial biogenesis (*srl*) genes (C, D) in *D. melanogaster* subjected to IF. Data presented as fold change relative to control. Bars indicate mean ± SEM. IF, intermittent fasting; UC, untreated control; *, $p < 0.05$; **, $p < 0.01$.

responses that differ from acute fasting effects.[32] Additionally, *srl*, which encodes Spargel (a PGC-1α homologue), plays a crucial role in mitochondrial biogenesis.[33] Its downregulation suggests that IF may reduce mitochondrial turnover in certain contexts, possibly as an energy conservation strategy.[34] Further studies are needed to determine whether these effects are transient or sustained.

3.4. Effect of Intermittent Fasting on the Expression of Autophagy-Related Genes. This study examined how IF influences the expression of *atg5* and *atg8a*, essential genes that play a role in autophagy and cellular upkeep in *D. melanogaster*. The flies were separated into two groups: an IF treatment group subjected to fasting durations of 8 and 4 h, and a control group maintained on *ad libitum* feeding, as illustrated in Figure 5.

Autophagy is a critical process for cellular maintenance during nutrient deprivation,[35] yet *atg8a* was significantly downregulated in fasting groups (55% and 48% reduction, respectively; $p < 0.05$) (Figure 4A–D). This finding contrasts with previous studies that report increased autophagic activity

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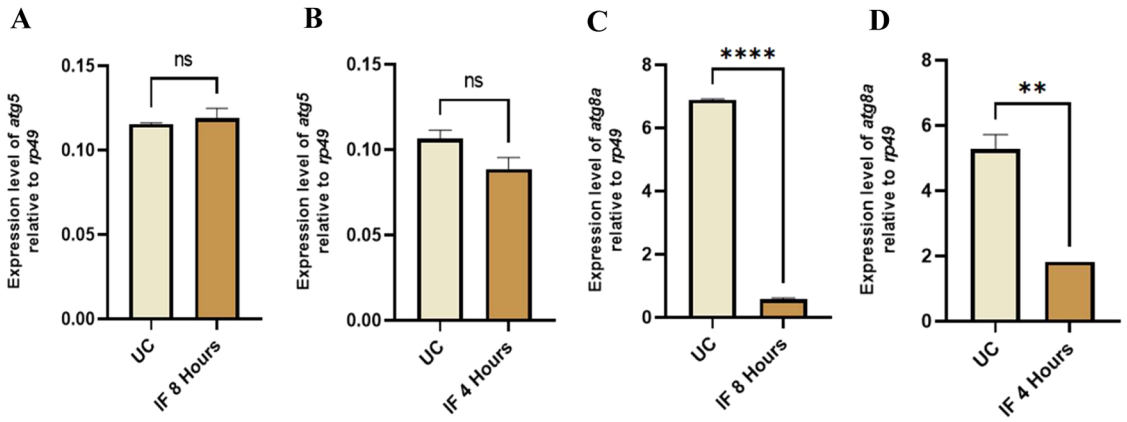

Figure 5. Expression levels of autophagy-related genes (*atg5* and *atg8a*) after 8-h (A, C) and 4-h (B, D) fasting protocols. Expression normalized to *rp49*. Data represent mean ± SEM. IF, intermittent fasting; UC, untreated control; ns, not significant; **, $p < 0.01$; ****, $p < 0.0001$.

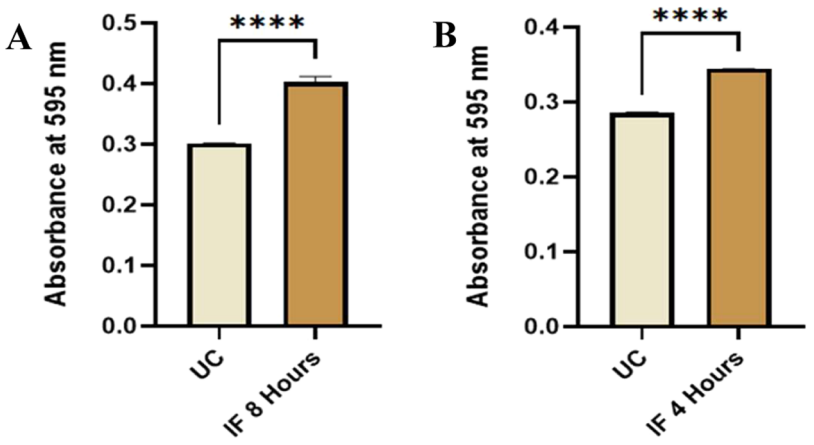

Figure 6. Effect of intermittent fasting on ROS levels in *D. melanogaster*. Flies subjected to both 8-h (A) and 4-h (B) intermittent fasting exhibited significantly elevated ROS levels compared to the control group, with the 8-h IF group showing the highest ROS accumulation. Data are shown as mean ± SEM. IF, intermittent fasting; ROS, reactive oxygen species; UC, untreated control; ****, $p < 0.0001$.

under fasting conditions.[35,36] One possible explanation is that autophagy induction follows a dynamic pattern, where gene expression peaks at the onset of fasting but declines after prolonged fasting or refeeding. The timing of sample collection is crucial, as autophagy-related gene expression might be transiently suppressed once cells have adapted to fasting-induced stress. Additionally, prior studies showing upregulation of *atg* genes often focus on different fasting regimens or stress intensities, which may account for the observed discrepancies.[29] Future research should examine autophagy dynamics over multiple time points to clarify these regulatory patterns.

3.5. Effect of Intermittent Fasting on Reactive Oxygen Species (ROS) Levels. To complement the analysis of antioxidant gene expression, we assessed the intracellular ROS levels in *D. melanogaster* to determine the oxidative stress status following IF interventions. ROS measurements showed a significant increase in ROS accumulation in both the 4-h and 8-h IF groups compared to the control group. Specifically, flies subjected to 8-h IF exhibited the highest ROS levels ($p < 0.01$), while those in the 4-h IF group also showed elevated ROS levels relative to control ($p < 0.05$), as shown in Figure 6. These results are consistent with the observed downregulation of *cat* expression in fasting groups, suggesting a reduced capacity to detoxify hydrogen peroxide and other ROS.

Elevated ROS levels under IF may reflect a fasting-induced oxidative shift due to metabolic reprogramming and increased mitochondrial activity. However, the sustained ROS increase observed here could surpass the compensatory capacity of the antioxidant system, particularly when *cat* expression is concurrently suppressed. This imbalance may contribute to mild oxidative stress, which in some models is linked to beneficial hormetic responses, yet chronic exposure could impair cellular homeostasis.[11,17,37]

These findings indicate that IF in *D. melanogaster*, particularly at longer durations, can increase oxidative load despite concurrent reductions in antioxidant gene expression. This discrepancy supports the hypothesis that IF triggers complex stress responses that may be beneficial in moderation but potentially detrimental if antioxidant defenses are overwhelmed. Further investigation into downstream ROS-sensitive signaling pathways and time-course changes in oxidative balance is needed to fully understand the adaptive versus maladaptive roles of ROS during IF.

4. CONCLUSIONS

This study demonstrates that intermittent fasting (IF) significantly extends lifespan and alters gene expression in *D. melanogaster*, with the 8-h IF protocol providing the most pronounced survival benefit. IF-induced changes included the

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downregulation of genes associated with metabolism (*dilp2*, *tom40*), oxidative stress response (*cat*, *srl*), and autophagy (*atg8a*), suggesting a complex adaptive response to fasting. A key novel finding of this study is the unexpected suppression of autophagy-related gene under IF condition, contrasting with previous studies that report autophagy activation during fasting. Additionally, the downregulation of antioxidant genes despite the known link between fasting and oxidative stress resistance raises important questions about the role of ROS accumulation and mitochondrial function in fasting adaptations. Overall, this study provides valuable insights into the molecular mechanisms underlying fasting-induced longevity and lays the foundation for future research into dietary interventions aimed at promoting healthspan and lifespan in higher organisms.

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Author Contributions

F.D.I.N., Ar.Ar., and F.N. were responsible for conceptualization, F.D.I.N., Ar.Ar., and F.N. were responsible for methodology, F.D.I.N., Ar.Ar., Am.Am., An.Ar., As.As., N.P.L., N.R.R., and F.N. were responsible for data curation and formal analysis, F.D.I.N., Ar.Ar., N.P.L., and F.N. were responsible for writing the original draft preparation, F.D.I.N., Ar.Ar., Am.Am., An.Ar., As.As., N.P.L., N.R.R., and F.N. were responsible for writing, review, and editing, F.D.I.N. was responsible for visualization, and Ar.Ar. and F.N. were responsible for supervision. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

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