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Paternal zinc deficiency alters offspring metabolic status in *Drosophila melanogaster*

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ABSTRACT

Background: This study delves into the understudied yet potentially crucial role of paternal zinc deficiency in programming offspring metabolic outcomes. By examining paternal zinc deficiency, we aim to shed light on a previously unexplored avenue with the potential to significantly impact future generations. We investigated the intergenerational effects of paternal zinc deficiency on metabolic parameters in *Drosophila melanogaster*.
Methods: Dietary zinc deficiency was induced by supplementing the diet of Drosophila F0 male flies with TPEN (N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine) from egg stage. The F0 male flies after eclosion were mated with age-matched virgin female flies from the control group, resulting in the F1 offspring generation. The F1 generation were then cultured on a standard diet for subsequent metabolic analyses, including assessments of body weight, locomotion, and levels of glucose, trehalose, glycogen, and triglycerides as well as the expression of related genes.
Results: We observed an increase ($p<0.05$) in body weight in male parent flies and female offspring. Negative geotaxis performance was also impaired in the female offspring. Paternal zinc deficiency exerted distinct effects on carbohydrate and lipid metabolism, as evidenced by a significant ($p<0.05$) increase in trehalose and triglyceride levels in both parent and offspring. Additionally, zinc deficiency led to alterations in the expression of key metabolic genes, including significant ($p<0.05$) increase in *DILP2* mRNA levels, highlighting potential links to insulin signaling. Also, there were reduced mRNA levels of *SOD1* and *CAT* in both parental and offspring generations. Parental zinc deficiency also increased the expression of *Eiger* and *UPD2* mRNA in the offspring, suggesting potential perturbations in the immune response system.
Conclusion: These findings underscore the link between zinc status and various physiological and molecular processes, revealing both immediate and intergenerational impacts on metabolic, antioxidant, and inflammatory pathways and providing valuable insights on the implications of paternal zinc deficiency in *Drosophila melanogaster*.

1. Introduction

While it is well known that the maternal diet during the periconceptional period affects the health of the offspring, there is growing evidence that the paternal diet also plays a role in disease onset in their offspring. It is now understood that sperm not only contributes half of the genetic material to the offspring, but also has an influence on an offspring's adult life health and disease risks [1]. The nutritional status and environmental exposure of fathers during their childhood and/or the periconceptional period have significant transgenerational consequences. In fact, a Drosophila model of paternal-diet-induced intergenerational metabolic reprogramming showed that as little as 2 days of

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dietary intervention in fathers led to obesity in offspring [2].

Furthermore, research suggests that environmental and lifestyle factors experienced by fathers, including zinc deficiency, can leave an epigenetic mark on their sperm, potentially influencing the health and development of their offspring across generations [3]. Moreover, zinc is an essential micronutrient that is involved in a myriad of physiological processes critical to the maintenance of overall organismal health. From metabolic regulation to antioxidant defense and inflammatory responses, the role of zinc in cellular homeostasis is increasingly recognized [4–7]. Despite substantial research on the consequences of maternal zinc deficiency on offspring health [8–12], the interplay between a spectrum of metabolic and molecular parameters and their intergenerational implications remains a focal point to be explored through the paternal lineage.

Linking these metabolic parameters is crucial for understanding health and disease. For instance, the impact of zinc deficiency on body weight might be intimately linked with altered carbohydrate metabolism [13], reflected in changes in glucose, trehalose, and glycogen levels [14]. Oxidative stress markers are tied to the broader antioxidant defense system, which itself is closely related to metabolic processes [15, 16]. Moreover, the potential modulation of inflammation by zinc status introduces another layer of complexity in this network [17,18].

Furthermore, gender-specific responses to zinc deficiency add another dimension to this investigation, acknowledging potential divergences in male and female offspring across these parameters. Thus, in this study we aimed to unravel the relationships between paternal zinc deficiency and various physiological parameters in *Drosophila melanogaster* offspring. By studying the link between paternal zinc deficiency and offspring metabolic health, we can gain insights into how early life programming can affect susceptibility to conditions like obesity, diabetes, and even certain types of cancers. Bridging the gap between model organism studies and clinical relevance, the findings may pave the way for a deeper understanding of the metabolic effects of zinc deficiency and inform targeted strategies to mitigate its adverse impacts on health.

2. Materials and methods

2.1. Fruit fly (Drosophila melanogaster) husbandry

The W^{1118} strain of *Drosophila melanogaster* was acquired from the Fly Laboratory of the Centre for Advanced Medical Research and Training (CAMRET) at Usmanu Danfodiyo University, Sokoto, Nigeria. These flies were cultured and maintained under controlled conditions: a temperature range of 22–25°C, 50–60 % relative humidity, and a natural light-dark cycle. Their diet consisted of a standard cornmeal mixture containing corn flour, agar, yeast, methyl paraben, and distilled water. To induce zinc deficiency in the parent generation (F0), a zinc-chelator, TPEN (N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine) was added to their diet at a concentration of 100 μM [19]. The diets were replaced weekly to ensure optimal food quality and prevent contamination.

2.2. Experimental design

Gravid adult flies aged seven to ten days were transferred to the zinc-chelated diet and given a 24-hour period to lay eggs. The eggs were allowed to develop on the zinc-chelated diet, and the resulting adult males were referred to as the F0 generation. The F0 male flies were collected within four to six hours after eclosion and mated with age-matched virgin female flies from the control group at post-eclosion day seven to ten, giving rise to the F1 generation. The offspring (F1) were maintained on a normal diet for seven days and subsequently analysed for physical variables, biochemical variables and gene expression.

2.3. Assessment of body zinc

The total body zinc levels of the parent flies raised on zinc deficient diet were measured using an Agilent Microwave Plasma Atomic Emission Spectrometer (MP-AES, MY19479002, Santa Clara, United States). Three groups of ten adult flies, aged seven to ten days, were collected and anesthetized by ice immobilization. Each group was then rinsed with distilled water. For analysis, the flies were individually digested in 1 mL of 65 % nitric acid (HNO3) within sterile microcentrifuge tubes. These tubes were heated on a block at 100°C for ten minutes, cooled, and diluted to 5 mL with distilled water. Finally, the total body zinc concentration was measured for each group against calibration curves ranging from 0.00 to 1.00 ppm established on the MP-AES (see supplementary information).

2.4. Body weight measurement

To measure the average weight, groups of ten flies were first gently anesthetized by chilling them on ice. Three separate groups of flies were collected from each experimental group and then weighed on a sensitive electronic balance (Kern & Sohn Ltd Balingen, Germany). The weight was recorded in milligrams for each group.

2.5. Locomotor performance (negative geotaxis assay)

Flies were placed in an empty 50 mL measuring cylinder with a line drawn 6 cm from the bottom. They were gently tapped to the bottom of the vial and the number of flies able to cross the line within 10 s were recorded. This was repeated thrice for each vial, and the percentage of flies climbing above the line was averaged for a given group [20].

3. Biochemical analysis

3.1. Sample preparation

The gut contents of the flies were cleared prior to the biochemical analysis. The flies were first transferred to empty vials and held for an hour. Then they were anesthetized on ice and rinsed with phosphate buffered saline (PBS) at a pH of 7.4 and a 1:5 ratio (w/v). Next, the flies were homogenized and centrifuged at 3000×g for 6 minutes at 4°C in a refrigerated centrifuge (MX-301 Highspeed, Tomy Kogyo Co., Ltd., Tagara, Japan). The upper layer was thereafter collected and served as the haemolymph for the biochemical assay [21].

3.2. Glucose assay

The haemolymph glucose level was determined using the Spinreact™ kit (Girona, Spain) according to the manufacturer's protocol. The absorbance of the samples and standard was read against the blank at 505 nm. The concentration was calculated using the following formula.

Glucose concentration (mg/dl) = Absorbance of the sample / Absorbance of the standard X 100

3.3. Trehalose assay

The haemolymph level of trehalose was quantified using the Trehalose Content Assay Kit (Solarbio Life Science, Beijing, China) according to the manufacturer's protocol. The absorbance of samples was read at 620 nm. The final concentration was calculated using the following formula:

Trehalose (mg/g sample) = Concentrations from y-axis / Fresh weight of the sample

3.4. Glycogen assay

The glycogen level in the haemolymph was determined using the Glycogen Content Assay kit (Solarbio Life Science, Beijing, China) according to the manufacturer's protocol. The absorbance was detected at 620 nm. The final concentration was calculated using the following formula.

Glycogen (mg/g fresh weight) = (Cs x V 1) x (A3 – A1) ÷ (A2 – A1) ÷ (W x V1 ÷V2) ÷ 1.11

Where 1.11 = is a constant that glucose content converted to glycogen content; Cs = the concentration of the standard, 0.1 mg/mL; V1 = sample volume, 25 μl; V2 = extraction volume, 1 mL; W = sample weight; A1 = absorbance of blank; A2 = absorbance of standard; A3 = absorbance of sample

3.5. Triglyceride assay

The haemolymph triglyceride level was quantified using the Spinreact kit (Girona, Spain) according to the manufacturer's protocol. The absorbance (A) of the samples and standard were read against the blank at 505 nm. The triglyceride concentration was calculated as follows.

Triglycerides (mg/dl) = A (Sample) – A (Blank) / A (Standard) – A (Blank) X 100

3.6. Catalase (CAT) assay

The CAT activity in the haemolymph was detected using the Catalase Activity Assay Kit (Solarbio Life Science, Beijing, China) according to the manufacturer's protocol. The principle is based on its action in decomposing H_2O_2 into H_2O and O_2 and absorbance detected at 240 nm. The final concentration was calculated using the following formula.

CAT activity (U/mL) = (ΔA x Extraction volume ÷ (Ɛ x d) x 109) ÷ Sample volume÷ Reaction time.

Where Ɛ= molar coefficient and d= light path

3.7. Total antioxidant capacity (TAOC) assay

To measure the total antioxidant capacity of the samples, the Total Antioxidant Capacity Assay Kit (Solarbio Life Science, Beijing, China) was used, following the manufacturer's instructions. The kit utilizes a specific chemical called Fe3+-TPTZ. In the presence of antioxidants, this compound gets transformed into Fe2+-TPTZ, which has a distinct blue color. The intensity of this blue color directly reflects the total antioxidant capacity of the sample. This intensity is measured using a spectrophotometer, which reads the absorbance of the solution at 505 nm. The final concentration was obtained from the following formula.

Total antioxidant capacity (μmol/ml) =x× Vrv÷ Vs =34×x

Where Vrv: total reaction volume, 1.02 mL; Vs: sample volume, 0.03 mL; x: concentration from the standard curve.

3.8. Malondialdehyde (MDA) assay

The haemolymph MDA level was determined by lipid peroxidation using the Malondialdehyde Content Assay Kit (Solarbio Life Science, Beijing, China) according to the manufacturer's protocol. The final concentration was calculated using the following formula.

MDA (nmol/g) = 5 (6.45 x (ΔA532-ΔA600) – 1.29 x ΔA450) ÷ sample weight

4. Gene expression analysis

4.1. Extraction of RNA

Ribonucleic acid (RNA) was extracted from 45 flies per group. This was done using a nucleic acid isolation kit (Hunan Runmei Gene Technology Co., Ltd) following the manufacturer's protocol. The purity of the extracted RNA was then assessed using the Bioevopeak Nucleic Acid Analyzer (SP-MUV2000F, Jinan, Shandong, China). Only RNA samples with A260/230 and A260/280 ratios between 1.8 and 2.2 were considered acceptable for further analysis.

4.2. Primer design

Using the sequences obtained from the GenBank Database of the National Centre for Biotechnology Information (NCBI), primers for the genes of interest were designed using the PrimerQuest software (https://www.idtdna.com/PrimerQuest/Home/Index) (Table 1). RPL32 was used as the housekeeping gene.

4.3. 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 following the manufacturer's instructions. The reaction mixture comprised RNA template (200 ng/μl each), forward primer (0.4 μl of 100 μM), reverse primer (0.4 μl of 100 μM), SuperMix (10 μl), Enzyme mix (0.4 μl), and RNase-free water, resulting in a final volume of 20 μL. The prepared mixture was loaded onto a Rotor-Gene Q-5plex HRM platform thermal cycler (Qiagen, Hilden, Germany) and subjected to the cycling conditions on Table 1. Calculation of fold change was performed using the comparative CT method with the formula $2^{-\Delta\Delta CT}$.

4.4. Data analysis

GraphPad Prism 9.5.1.733 (GraphPad Software Inc, San Diego, CA) was used for statistical analysis. For data derived from F0, Student's t-test was used, while data obtained from F1 were subjected to a two-way

Table 1
List of genes and primer sequences.

S/N	Gene	Primer	Sequence	Annealing Temperature
1	*DILP2*	Forward	GTACTCAATTCCCTGGCTGAA	55
		Reverse	CGCAGAGCCTTCATATCACA	
2	*PEPCK*	Forward	TCAATGGCGAATCCTGCTAC	60
		Reverse	CTTCACGTCCACCTTATCCTTC	
3	*SOD1*	Forward	CGGTCACACCATAGAAGATACC	65
		Reverse	CAGACAGCTTTAACCACCATTTC	
4	*CAT*	Forward	TGGTCGTCTGTTCTCCTACT	65
		Reverse	CCGCTGGAAGTTCTCAATCT	
5	*UPD2*	Forward	TTGACCATAAACGCCTCCTATC	60
		Reverse	GTGAAAGTTGAGACGCTCCT	
6	*EGR*	Forward	TGAGGCAACTTCCAAAGAGAG	60
		Reverse	CGGATCTGGCTGAAAGAAGAG	
7	*RPL32*	Forward	GGATCGATTCCTGTGAGAGTTC	60
		Reverse	TGGGCAGTATCCATTGAGTTT	

PCR conditions used were 5 mins at 45 °C for reverse transcription; 30 sec at 94 °C for pre-denaturation; 40 cycles of 5 sec at 94 °C for denaturation, 15 sec at the corresponding temperature above for annealing, and 10 sec at 72 °C for extension. *CAT*: Catalase; *dILP2*: Drosophila Insulin like peptide-2; *EGR*: Eiger; *PEPCK:* Phosphoenolpyruvate carboxykinase; *RPL-32*: 60S ribosomal protein large subnunit-32; *SOD1*: Superoxide dismutase 1; *UPD2*: Unpaired 2.

analysis of variance (ANOVA), followed by Bonferroni's multiple comparison *post hoc* test. The results were expressed as mean ± standard deviation, and statistical significance was established at a $p<0.05$.

5. Results

5.1. Paternal zinc deficiency and offspring's zinc status, body weight and locomotor performance

Dietary zinc chelation significantly ($p<0.05$) reduced body zinc levels in the parent flies and the offspring (Fig. 1a and b). Moreover, there was a significant ($p<0.05$) increase in body weight in the male parent (Fig. 1c) and the female offspring (Fig. 1d). Notably, the body weight was significantly ($p<0.05$) higher in the female offspring compared to the male counterpart (Fig. 1d). On negative geotaxis performance, no significant difference was observed in the zinc-deficient male parent flies compared to the control (Fig. 1e). However, a significant ($p<0.05$) reduction in performance was observed in the female offspring (F1) of zinc deficient group compared to their male counterparts or the control flies (Fig. 1f).

5.2. Effects of paternal zinc deficiency on glucose and trehalose levels

No significant differences ($p>0.05$) were observed in the haemolymph glucose levels of the zinc-deficient parent as well as the male and female offspring compared to the controls (Fig. 2a and b). However, zinc deficiency significantly ($p<0.05$) increased trehalose levels in the male parent (F0) compared to the control (Fig. 2c). F1 male offspring of zinc deficient parents had significant ($p<0.05$) increase in the trehalose levels, although it was significantly reduced ($p<0.05$) in the female (Fig. 2d).

5.3. Effects of paternal zinc deficiency on glycogen and triglyceride levels

Zinc deficiency caused a significant ($p<0.05$) reduction in glycogen levels in the male parent flies (F0) compared to the control (Fig. 3a). However, F1 males and females from the zinc deficiency group showed significant increases ($p<0.05$) in glycogen levels compared to controls (Fig. 3b). Zinc deficiency also caused significant increases ($p<0.05$) in triglycerides levels in the male parent flies and both male and female offspring compared to their respective controls (Fig. 3c-d).

5.4. Effects of paternal zinc deficiency on catalase activity, total antioxidant capacity, and malondialdehyde level

Zinc-deficient male parents showed a significant ($p<0.05$) reduction in catalase activity compared to controls (Fig. 4a). However, the F1 male and female showed significant ($p<0.05$) increases in activities compared to the control (Fig. 4b). Total antioxidant capacity in the male parent (F0) flies as well as the male and female offspring showed a significant ($p<0.05$) reduction compared to the control (Fig. 4c and d). There was a significant ($p<0.05$) increase in the malondialdehyde level in the male parent (Fig. 4e) as well as in the male and female F1 compared to the control (Fig. 4f).

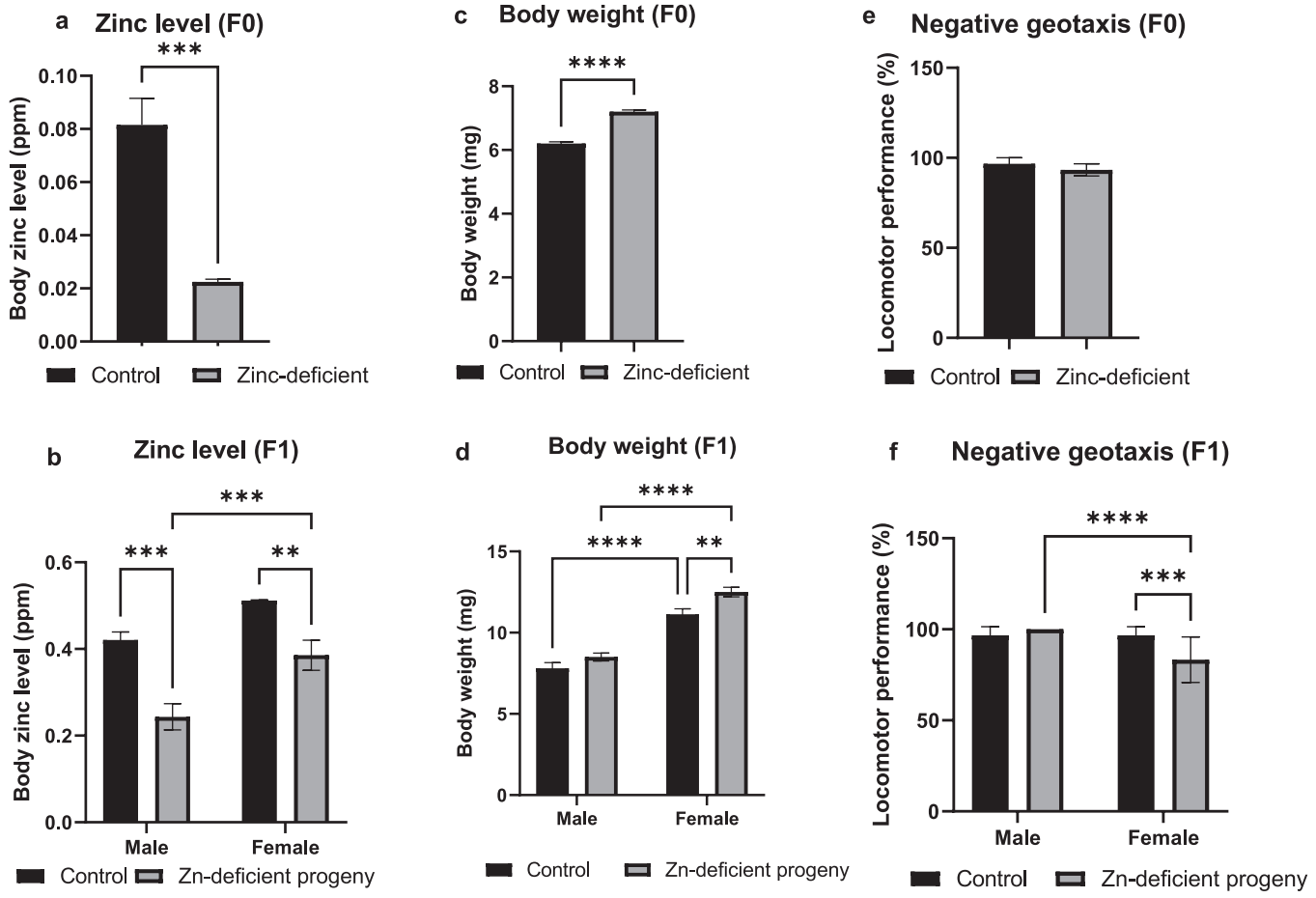

Fig. 1. Effects of paternal zinc deficiency on offspring's zinc status, body weight and locomotor performance. Bars represent mean ± SD. F0 data were analysed using Student's t-test while F1 data were analysed using two-way ANOVA followed by Bonferroni's *post hoc* test. Asterisks represent significant difference at varying p value (*: 0.0332, **: 0.0021, ***: 0.0002, ****: <0.0001), n = 30 per group.

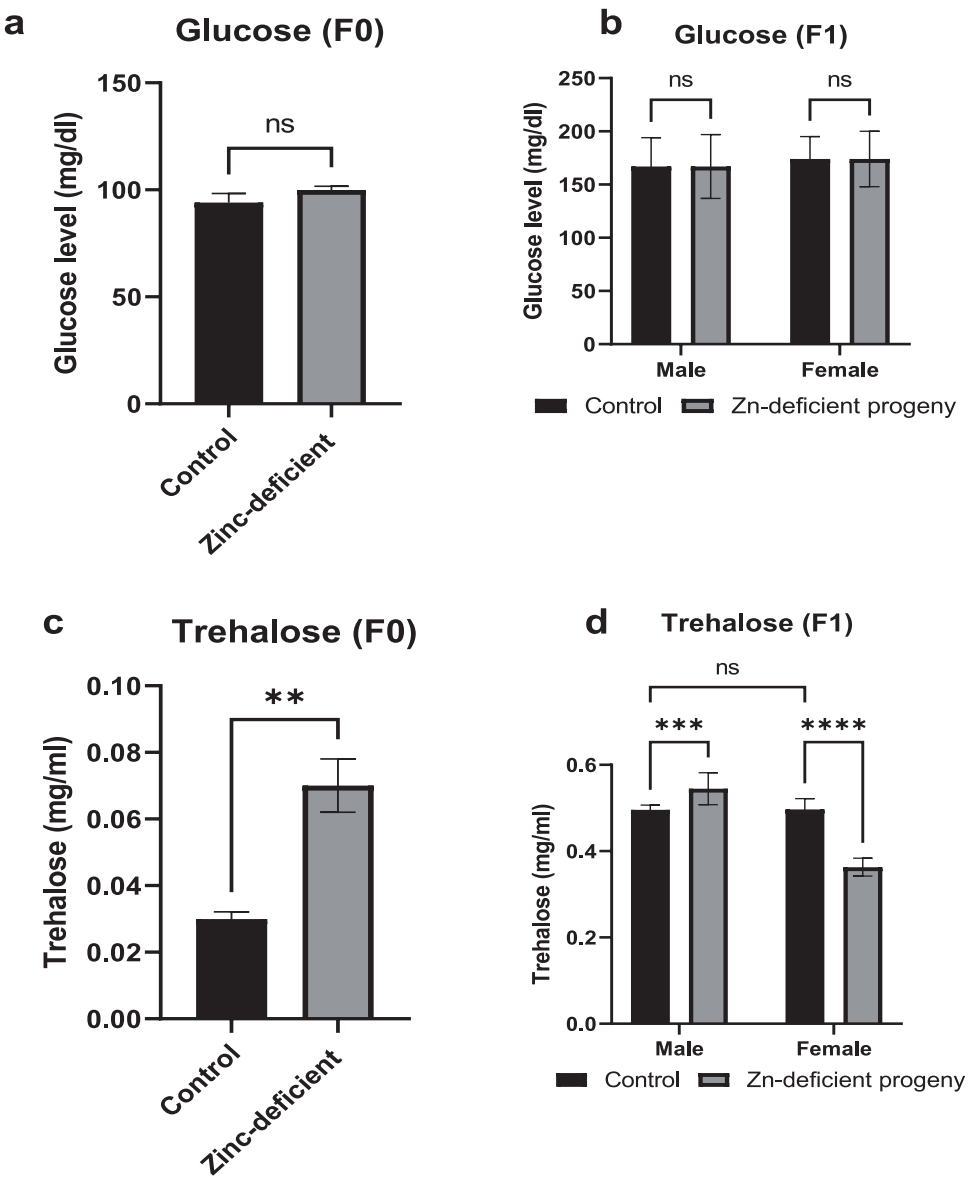

Fig. 2. Effects of paternal zinc deficiency on glucose and trehalose levels of flies. Bars represent mean ± SD. F0 data were analysed using Student's t-test while F1 data were analysed using two-way ANOVA followed by Bonferroni's *post hoc* test. ns: not significant. Asterisks represent significant difference at varying p value (*: 0.0332, **: 0.0021, ***: 0.0002, ****: <0.0001). n = 30 per group.

5.5. Effects of paternal zinc deficiency on the expression of DILP2 and PEPCK mRNA

Zinc deficiency significantly ($p<0.05$) increased the expression of *DILP2* mRNA in the male parent (Fig. 5a) as well as both male and female offspring compared to the control (Fig. 5b). Similarly, there was significant ($p<0.05$) upregulation in expression of For *PEPCK* mRNA in the zinc deficient male parent (Fig. 5c), although the expression was significantly ($p<0.05$) reduced in both the male and female offspring compared to the control (Fig. 5d).

5.6. Effects of paternal zinc deficiency on the expression of SOD1 and CAT mRNA

There were significant ($p<0.05$) decreases in the expression of *SOD1* and CAT mRNA in the zinc-deficient male parent compared to the control (Fig. 6a and c). Similarly, male and female offspring in the zinc deficiency group showed significant ($p<0.05$) decreases in the expression of *SOD1* and CAT mRNA compared to the control (Fig. 6b and d).

5.7. Effects of paternal zinc deficiency on the expression of EGR and UPD2 mRNA

There was significant ($p<0.05$) increase in the expression of *EGR* mRNA in the zinc deficient male parent and both male and female offspring compared to the control (Fig. 7a-b). In addition, the expression of *UPD2* mRNA was significantly ($p<0.05$) decreased in the zinc deficient male parent flies (Fig. 7c), but significantly ($p<0.05$) increased in the male and female offspring compared to controls (Fig. 7d).

6. Discussion

Metabolic programming, shaped by early life exposures such as paternal zinc deficiency, can profoundly influence metabolic parameters like body weight, carbohydrate metabolism, and lipid profiles, predisposing offspring to later-life metabolic disorders [22,23]. Indeed, zinc deficiency is a significant factor in altering insulin signalling and thereby impacting body weight [24,25]. Thus, the observed increase in body weight among male parents and female offspring suggests a

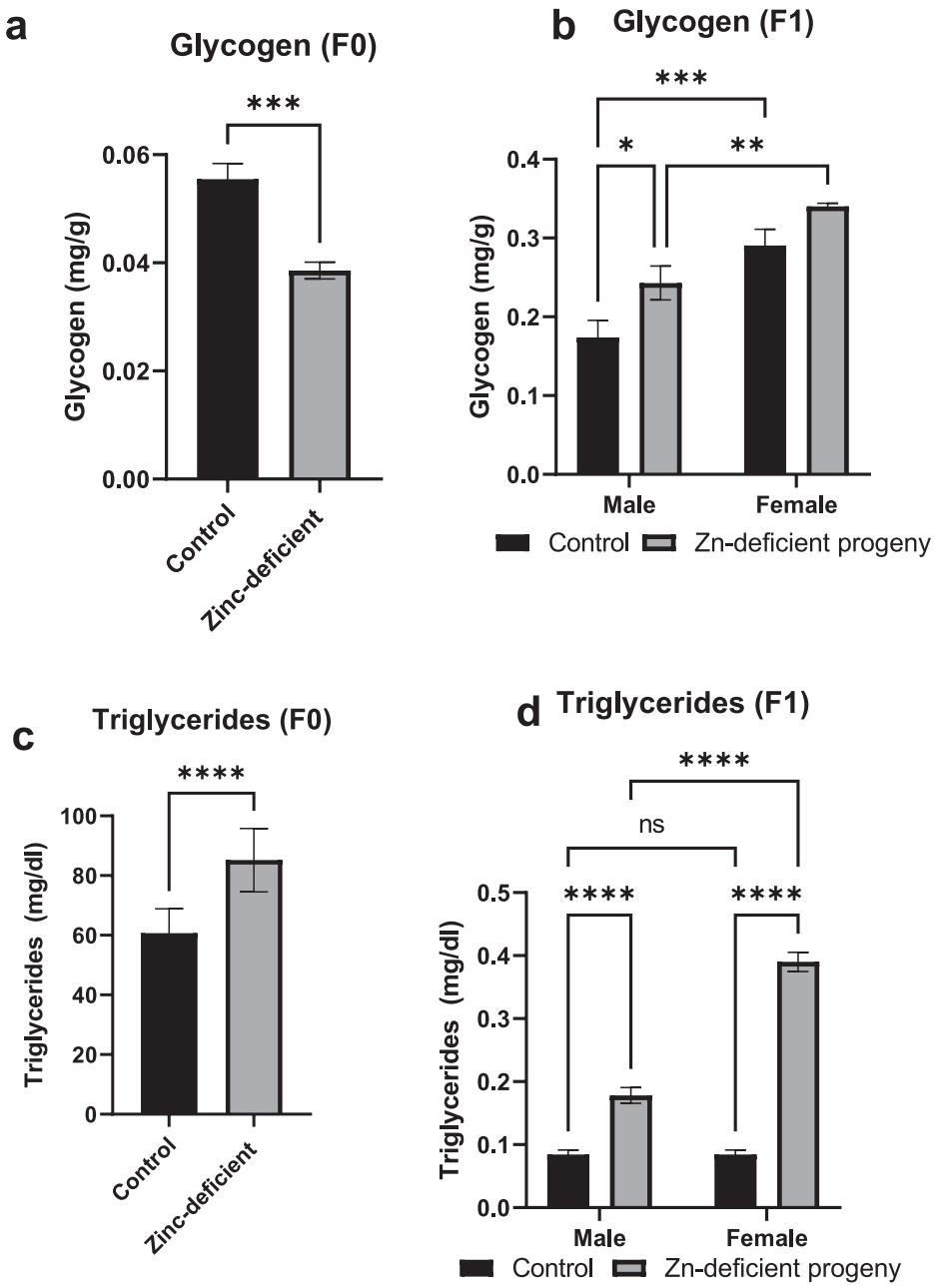

Fig. 3. Effects of paternal zinc deficiency on glycogen and triglyceride levels of flies. Bars represent mean ± SD. F0 data were analysed using Student's t-test while F1 data were analysed using two-way ANOVA followed by Bonferroni's *post hoc* test. ns: not significant. Asterisks represent significant difference at varying p value (*: 0.0332, **: 0.0021, ***: 0.0002, ****: <0.0001). n = 30 per group.

programming effect of zinc deficiency on body weight regulation.

Expanding beyond body weight, our study delved into the effects of paternal zinc deficiency on motor function, revealing a notable reduction in negative geotaxis performance among female offspring. Negative geotaxis, a well-established assay in Drosophila, measures the ability to climb against gravity and offers insights into motor function [26]. These findings not only highlight the susceptibility of female progeny to motor dysfunction but also prompt further investigation into the relationship between paternal zinc levels and offspring health outcomes. Additionally, research suggesting a link between zinc deficiency and alterations in neurotransmitter pathways further elucidates the mechanisms underlying observed motor deficits [7,27].

Furthermore, our study examined the impact of zinc deficiency on carbohydrate metabolism in *Drosophila melanogaster*, revealing intriguing gender-specific variations in trehalose levels. While zinc deficiency did not directly influence glucose levels, a significant increase in trehalose levels was noted in the male parent, indicating potential alterations in carbohydrate storage or utilization. Notably, gender-specific responses were observed, with males showing increased trehalose levels while females exhibited a reduction. These variations likely stem from hormonal differences, with females having higher levels of insulin-like peptide 2 secretion, leading to increased insulin signalling and potentially lower glucose and trehalose levels [28]. Such gender-specific effects on trehalose levels align with the growing recognition of sex-dependent responses in metabolic processes, reflecting broader trends observed in both humans and experimental animals, including Drosophila [29,30]. Interestingly, previous studies have shown that male offspring of zinc deficient female rat had liver damage, altered pancreatic beta cell activation and as well as zinc transporters [11,12]. These findings collectively contribute to our understanding of

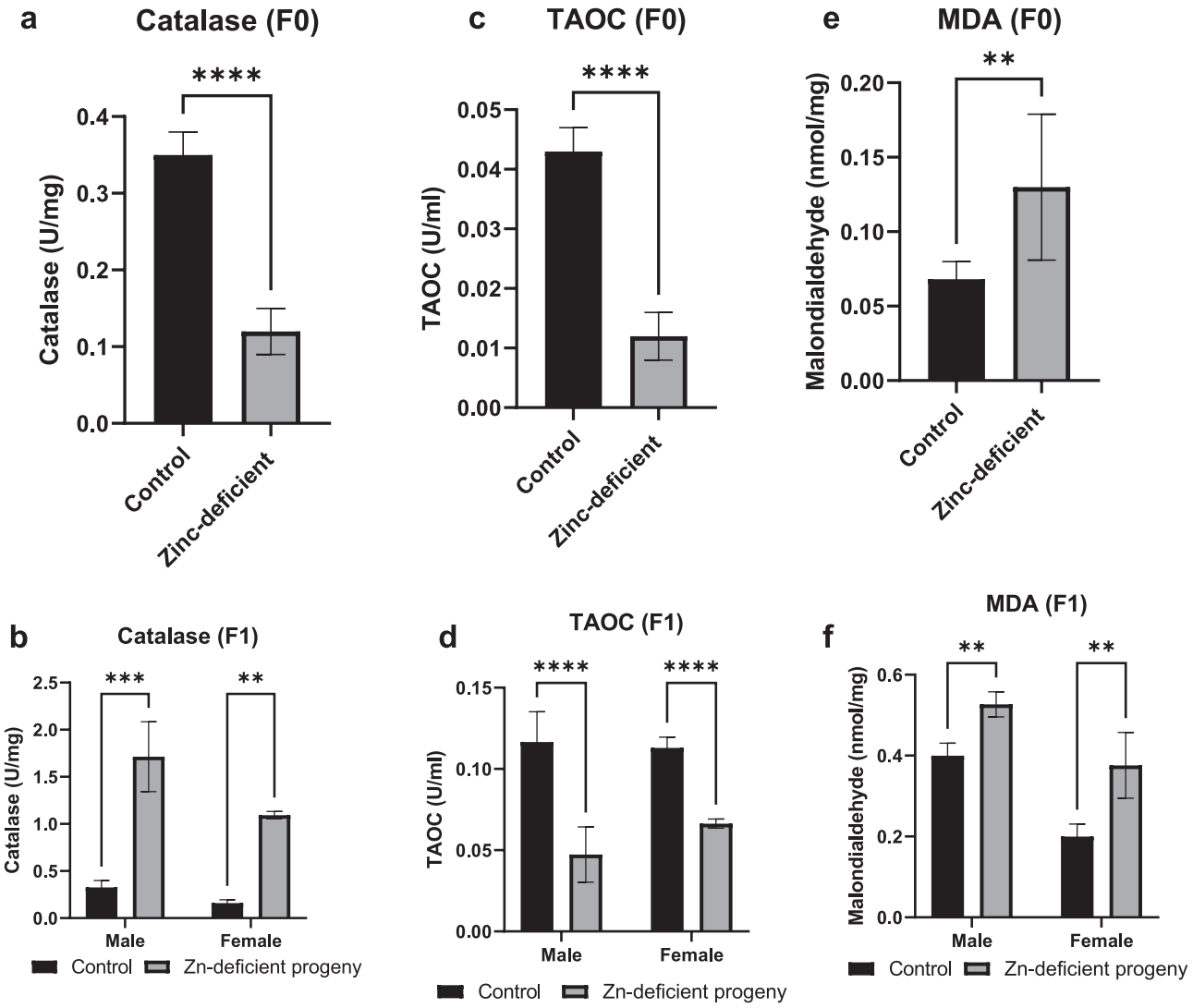

Fig. 4. Effects of paternal zinc deficiency on catalase activity, total antioxidant capacity (TAOC), and malondialdehyde (MDA) level in flies. Bars represent mean ± SD. F0 data were analysed using Student's t-test while F1 data were analysed using two-way ANOVA followed by Bonferroni's *post hoc* test. Asterisks represent significant difference at varying p value (*: 0.0332, **: 0.0021, ***: 0.0002, ****: <0.0001). n = 30 per group.

how paternal zinc status influences offspring health outcomes, shedding light on the link between environmental factors and metabolic programming.

Zinc also plays a crucial role in both energy storage and lipid metabolism. It regulates glycogen synthesis by inhibiting the activation of glycogen synthase kinase-3beta, ultimately leading to increased glycogen storage [31]. This supports the observed significant reduction in glycogen levels in the offspring of zinc-deficient parent flies, highlighting how zinc deficiency disrupts glycogen metabolism. The reduction indicate alterations in glycogen synthesis or breakdown processes, implicating zinc deficiency in modulating energy storage pathways in male parents. In addition, the stored glycogen might have been depleted and converted to glucose and in turn trehalose to increase the circulating sugar due to the perceived insulin resistance. However, the increase in glycogen levels in the offspring might be an adaptive response to restore normalcy.

In the regulation of lipid metabolism, zinc acts as a cofactor for enzymes like lipoprotein lipase (LPL), responsible for breaking down triglycerides into fatty acids for cellular uptake and energy [32]. Adequate zinc levels might enhance LPL activity, leading to reduced triglyceride storage. The significant increase in triglyceride levels observed in the zinc-deficient male flies is consistent with research indicating a potential link between zinc deficiency and altered lipid metabolism [5]. This finding suggests that zinc deficiency may contribute to an increase in triglyceride storage. Similarly, the significant increase in triglyceride levels in the offspring further emphasizes the intergenerational effects of paternal zinc deficiency on lipid metabolism.

As a cofactor for antioxidant enzymes, zinc directly neutralizes harmful reactive oxygen species (ROS) [33]. Additionally, it supports the immune system, which helps fight off infections and the associated oxidative stress they create [34]. Furthermore, zinc might offer cellular protection by stabilizing cell membranes from damage caused by ROS. Essentially, our findings contribute to the broader understanding of the role of zinc in maintaining redox homeostasis. The observed significant reduction in catalase activities in zinc-deficient male parents linked zinc deficiency to decreased antioxidant enzyme activities. Catalase is a crucial enzyme involved in the breakdown of hydrogen peroxide [35], and the reduction in its activity suggests a compromised antioxidant defense system in male parents under zinc deficiency. However, the unexpected significant increase in catalase activities in the offspring may indicate a compensatory response or a differential regulation of antioxidant enzymes in subsequent generations. Moreover, the significant reduction in total antioxidant capacity observed in both the zinc-deficient male parent flies and the male and female offspring

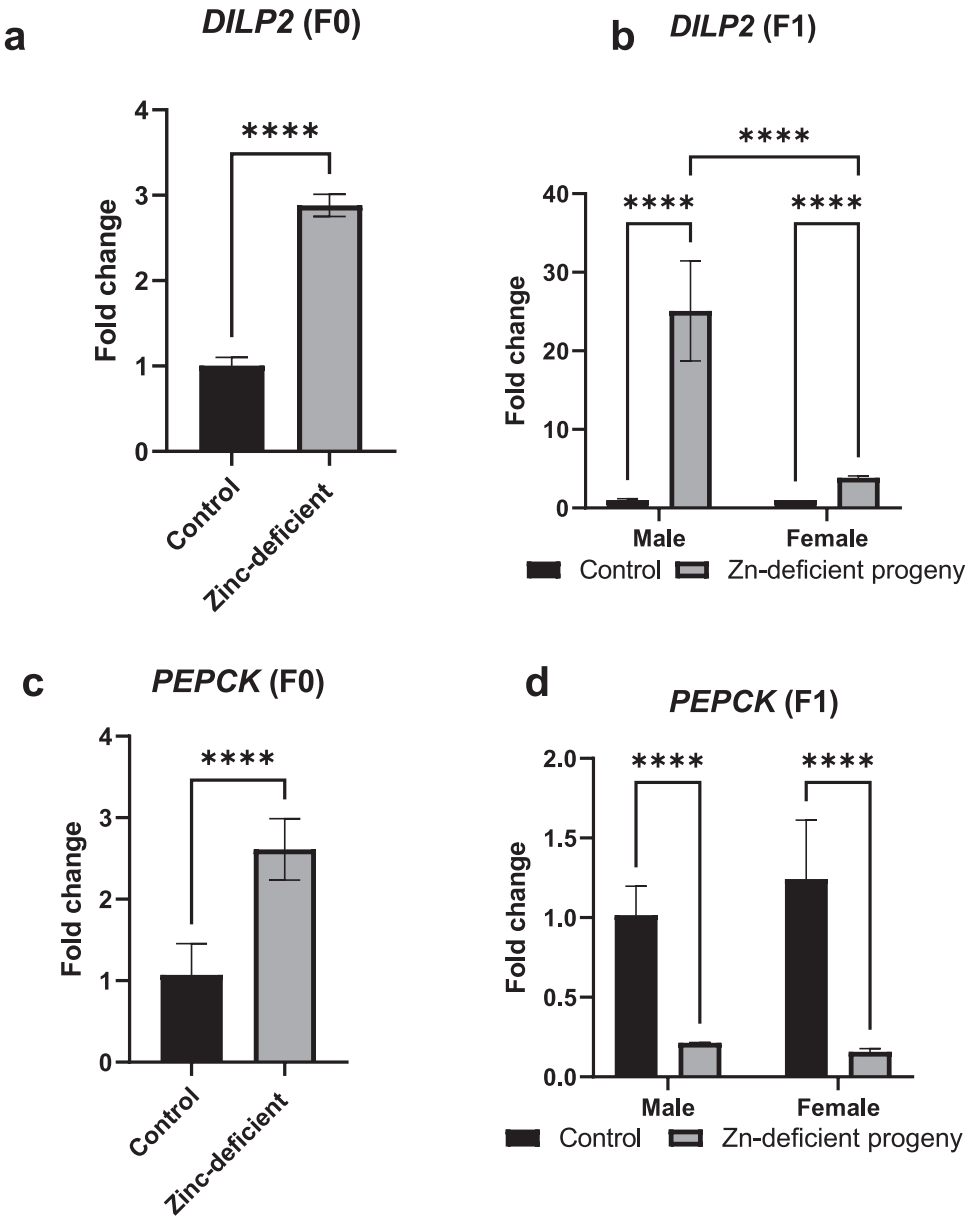

Fig. 5. Effects of paternal zinc deficiency on the expression of *DILP2* and *PEPCK* mRNA in flies. Bars represent mean ± SD. F0 data were analysed using Student's t-test while F1 data were analysed using two-way ANOVA followed by Bonferroni's *post hoc* test. Asterisks represent significant difference at varying p value (*: 0.0332, **: 0.0021, ***: 0.0002, ****: <0.0001). n = 45 per group.

suggests the overall reduction in the activity of the antioxidant system, since zinc is a cofactor for various antioxidant enzymes. This reduction implies a compromised ability to neutralize ROS, leading to increased oxidative stress in zinc-deficient flies and their offspring. Furthermore, the significant increase in MDA levels, a marker of lipid peroxidation and oxidative stress, in zinc-deficient male parents and both F1 male and female offspring aligns with the observed reductions in total antioxidant capacity. This elevation in MDA levels suggests an imbalance between ROS production and antioxidant defense, leading to increased oxidative damage.

Gene expression patterns offer valuable insights into how zinc deficiency may influence key regulators of metabolism, providing a window into potential molecular mechanisms associated with observed metabolic changes. The significant increase in *DILP2* mRNA expression in the zinc-deficient parent highlights the role of zinc in regulating insulin-like peptides crucial for growth and metabolism in Drosophila [36,37]. This upregulation of *DILP2* mRNA expression in both male and female offspring suggests a programming impact on the insulin signaling pathway. Similarly, alterations in *PEPCK* mRNA expression reflect the dynamic response to zinc deficiency. While the significant increase in *PEPCK* mRNA expression in the male parent aligns with its role in gluconeogenesis related to energy homeostasis [38], the reduction in offspring suggests a possible compensatory mechanism, indicating the regulatory feedback in gene expression across generations.

Moreover, the decreases in *SOD1* and *CAT* mRNA expression offer molecular insights into how zinc status influences the antioxidant defense system. The reduced expression of *SOD1* mRNA indicates compromised capacity to neutralize superoxide radicals, while the decrease in *CAT* mRNA expression suggests impaired catalase activity, collectively indicating an impaired ability to cope with oxidative stress in response to zinc deficiency across generations.

Furthermore, the increase in *EGR* mRNA expression highlights a potential proinflammatory response associated with paternal zinc deficiency in Drosophila. EGR (eiger), a tissue necrotic factor, is involved in

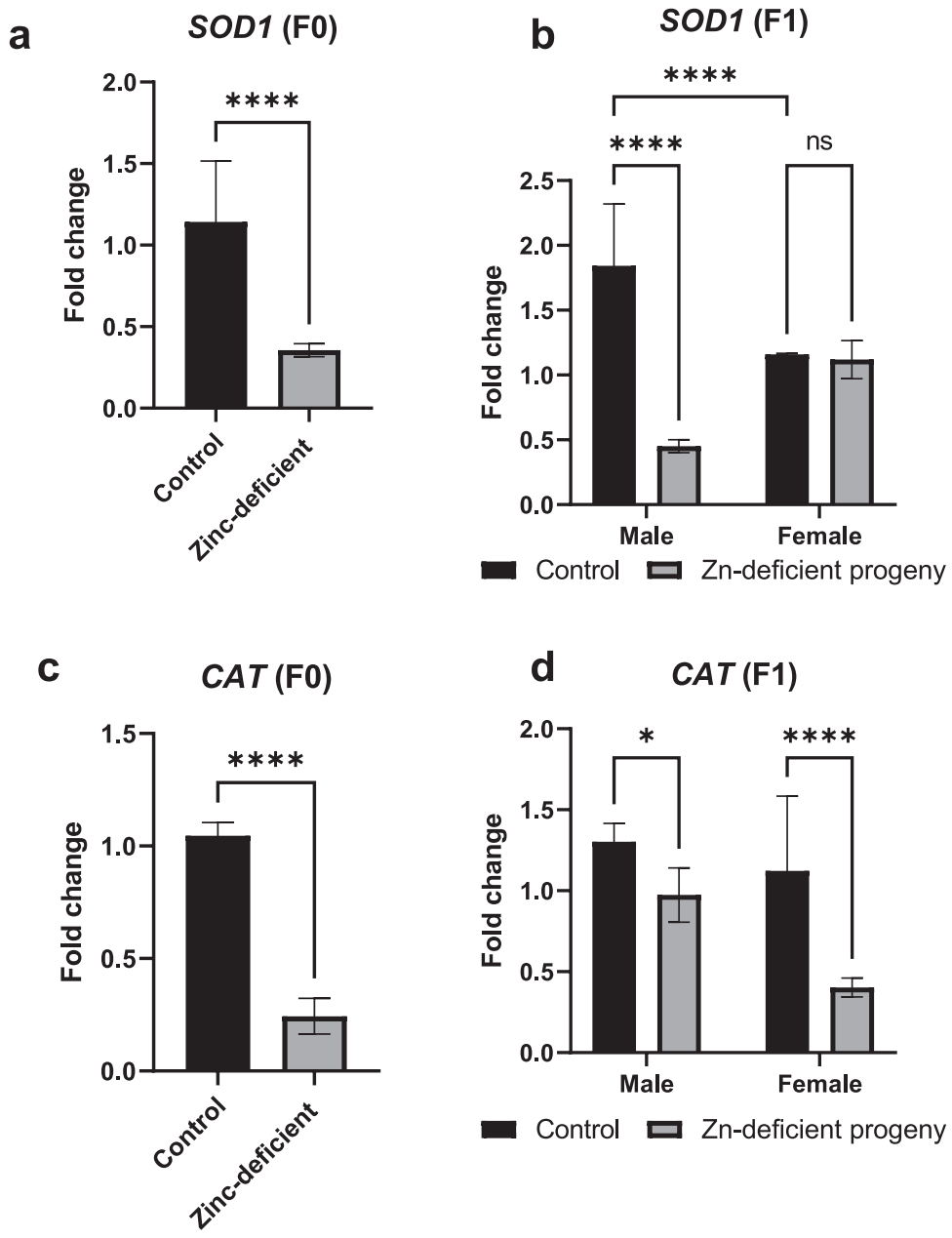

Fig. 6. Effects of paternal zinc deficiency on the expression of *SOD1* and *CAT* mRNA in flies. Bars represent mean ± SD. F0 data were analysed using Student's t-test while F1 data were analysed using two-way ANOVA followed by Bonferroni's *post hoc* test. ns: not significant. Asterisks represent significant difference at varying p value (*: 0.0332, **: 0.0021, ***: 0.0002, ****: <0.0001). n = 45 per group.

immune responses and inflammation [39], suggesting an activation of inflammatory pathways in response to cellular stress induced by zinc deficiency.

Interestingly, the unexpected downregulation of *UPD2* mRNA expression (an IL-6-like pro-inflammatory cytokine in Drosophila) in the zinc-deficient parent may signify complex regulatory mechanisms, potentially involving compensatory responses, requiring further investigation. However, the upregulation of *UPD2* expression in offspring suggests a potentially increased proinflammatory response, indicating that the impact of paternal zinc deficiency on immune-related pathways may manifest differently across generations. These findings collectively shed light on the molecular mechanisms underlying metabolic programming and its intergenerational effects.

7. Conclusion

Our study showed the multi-faceted consequences of paternal zinc deficiency on both immediate and intergenerational physiological and transcriptional profiles in *Drosophila melanogaster*. The observed alterations in body weight, carbohydrate metabolism, antioxidant defense mechanisms, and gene expression patterns underscore the critical role of zinc in maintaining homeostasis and regulating key metabolic pathways. The gender-specific responses and transgenerational effects highlight the complexity of the impact of zinc on health and inheritance. The findings emphasize the significance of zinc in modulating insulin signaling, carbohydrate metabolism, inflammation, and oxidative stress responses. The identified changes in gene expression, including upregulation of *DILP2* and *EGR* and downregulation of *SOD1* and *CAT*, point towards potential regulatory mechanisms that warrant further exploration.

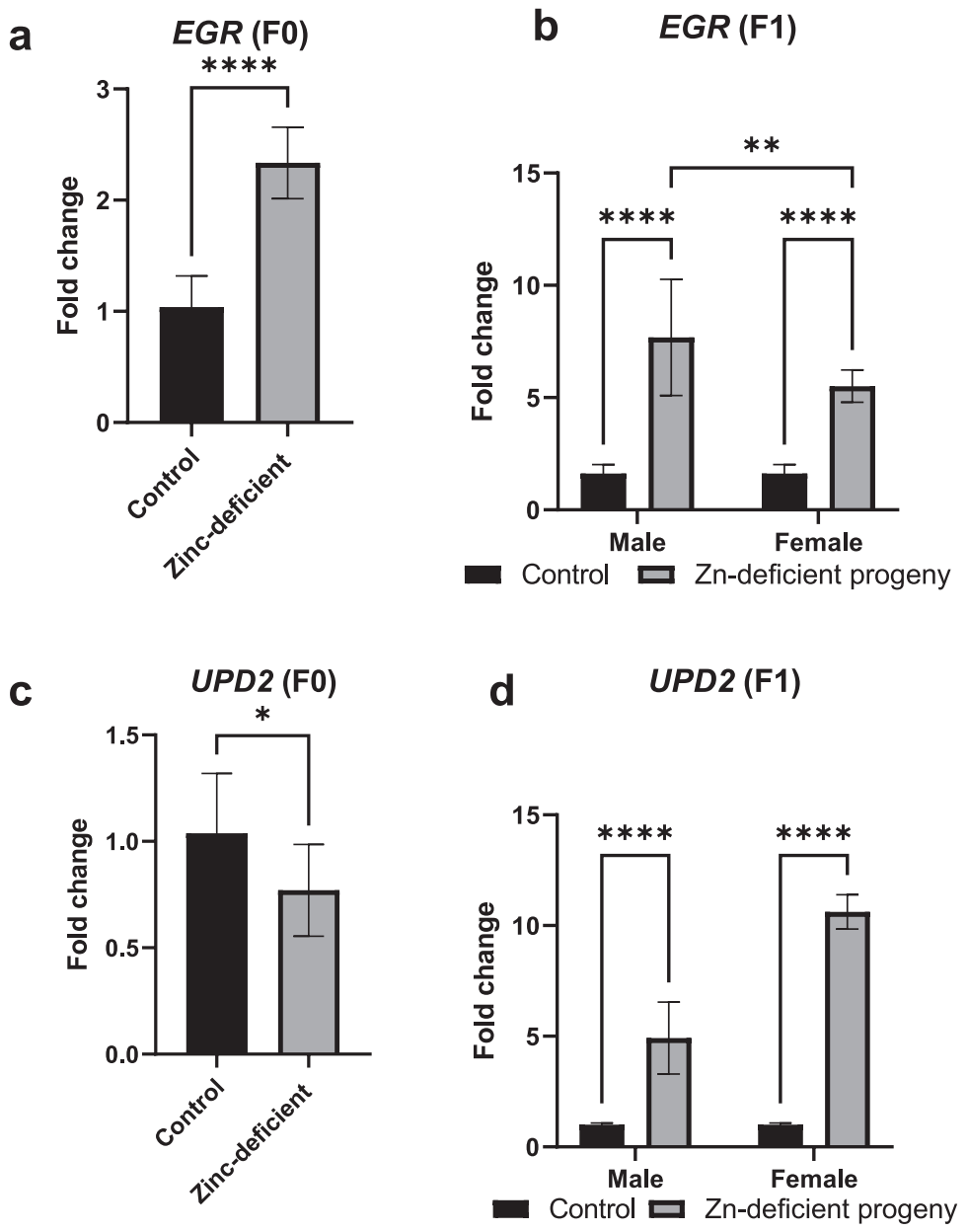

Fig. 7. Effects of paternal zinc deficiency on the expression of *EGR* and *UPD2* mRNA in flies. Bars represent mean ± SD. F0 data were analysed using Student's t-test while F1 data were analysed using two-way ANOVA followed by Bonferroni's *post hoc* test. Asterisks represent significant difference at varying p value (*: 0.0332, **: 0.0021, ***: 0.0002, ****: <0.0001). n = 45 per group.

8. Limitations and recommendations

While our study provides valuable insights into the effects of zinc deficiency on metabolic parameters in Drosophila melanogaster and their offspring, to definitively attribute the observed effects solely to zinc deficiency, a future study incorporating a zinc-deficient diet control group devoid of TPEN would be beneficial to eliminate any background effect. This would provide a more robust comparison and strengthen the cause-and-effect relationship between zinc deficiency and the observed outcomes.

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CRediT authorship contribution statement

Mustapha Umar Imam: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Murtala Bello Abubakar:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology. **kasimu Ghandi Ibrahim:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Data curation. **Kamaldeen Olalekan Sanusi:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtemb.2024.127519.

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