Paternal Zinc Deficiency and Its Transgenerational Effects on Zinc Transporters in *Drosophila*

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Summary The nutritional status of fathers plays a significant role in influencing the growth, metabolism, and susceptibility to diseases in their offspring. Paternal zinc deficiency can lead to developmental programming effects on the offspring's zinc homeostasis. This study investigated the effects of paternal zinc deficiency on the zinc homeostasis of offspring in a *Drosophila melanogaster* (fruit fly) model. Male flies were reared on a diet supplemented with a zinc-chelator, *N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), from the egg stage until adulthood, and their offspring were subsequently reared on a normal diet for 7 d. Body zinc status and zinc transporters were assessed afterwards. The results indicated that the prenatal zinc deficiency significantly lowered total body zinc levels ($p<0.05$) compared to the controls. Additionally, the mRNA levels of zinc transporters, dZip42C.1, dZnT63C, and dZnT35C, were lower in the zinc-deficient male parents ($p<0.05$) and their male offspring ($p<0.05$). These findings suggested that paternal zinc deficiency can alter offspring zinc homeostasis, even when the offspring was fed a zinc-sufficient diet. This is an important finding, as zinc is an essential nutrient that is required for a variety of bodily functions. Further research is needed to better understand the mechanisms by which zinc deficiency in the male parent affects the health of the offspring and to develop strategies to prevent this from happening.

Key Words paternal, zinc deficiency, zinc transporters, *Drosophila melanogaster*, transgenerational

Zinc is an essential nutrient that is required for a variety of bodily functions, including growth, development, reproduction and immunity (*1*). However, zinc deficiency is a significant global health issue affecting billions of people worldwide (*2*), with particular consequences during pregnancy and early childhood (*3*).

In recent years, emerging evidence suggests that paternal factors, including environmental exposures and dietary deficiencies, can have transgenerational effects on offspring health. This is a new paradigm based on the concept of the paternal origin of health and disease (POHaD) (*4, 5*). It centers on the scientific evidence that environmental factors impacting the father can play a role in reprogramming the health of their offspring throughout their life span. These effects are believed to be mediated through epigenetic modifications and alterations in germ cells, leading to changes in gene expression patterns in subsequent generations (*6*).

Epigenetic factors, influenced by parental lifestyle and exposure to various dietary and environmental substances, can have a profound impact on offspring health. Sperm, previously considered a carrier of paternal DNA only, has been discovered to carry epigenetic information, including non-coding RNAs, which are involved in embryonic development and adaptation to stress across generations (*7*). Evidence suggests that a man's diet can affect sperm quality through epigenetic mechanisms, resulting in transgenerational effects on offspring health (*8*). For example, paternal diets before conception impact male germline programming and offspring breast cancer risk (*9*). In fact, adverse paternal environmental exposure has been shown to cause congenital disorders including ventricular septal defects in newborn, increased susceptibility to *Pseudomonas* in-

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fection, impaired cognitive behavior and motor ability, among others (*10*).

The study by Croxford and colleagues focused on the role of zinc deficiency in compromising sperm production and function (*11*). They investigated the expression of zinc transport proteins during spermatogenesis in mice and observed alterations in zinc transporters involved in various stages of sperm development (*11*). Other previous studies in animals have also demonstrated that paternal zinc deficiency can disrupt offspring health, affecting fasting blood glucose levels, insulin sensitivity, and insulin secretion (*12, 13*), although the mechanisms are still not fully understood. However, it is believed to involve the modulation of gene expression related to development, metabolism, and immunity in the sperm (*14*).

To further understand the transmission of health-related traits and the role of zinc in this context, this study aimed to investigate paternal line effects on offspring health, focusing on body zinc levels and the expression of zinc transporters involved in zinc absorption and excretion. *Drosophila melanogaster* (fruit fly) is an ideal model organism for studying such genetic and molecular phenomena due to its short lifespan, rapid reproductive cycle, and well-characterized genetic and physiological traits (*15, 16*).

Like mammals, *Drosophila* obtains zinc from its diet, mainly from plant-based and microbial sources. Zinc transporters, including the ZnT and ZIP families, facilitate zinc movement across cell membranes, aiding its uptake into tissues. While mammals have at least fourteen ZIPs and ten ZnTs, *Drosophila* possesses ten ZIPs and seven ZnTs, which are vital for maintaining zinc homeostasis (*17*). In *Drosophila melanogaster*, zinc absorption mainly occurs in the midgut epithelium through ZIP transporters such as dZip42C.1 and dZnT63C. These transporters facilitate zinc uptake from the diet, with their expression and activity regulated by dietary zinc levels. When zinc levels are low, ZIP transporter expression increases, enhancing zinc absorption to maintain zinc balance (*18*).

Zinc excretion in *Drosophila* involves two pairs of Malpighian tubules, similar to mammalian kidneys, which play roles in osmoregulation and detoxification. These tubules excrete zinc primarily through the faeces (*19*). Zinc excretion is regulated by specific transporters in the tubules. dZnT35C acts as a zinc exporter, localizing to the tubule cell's apical membrane to pump zinc out (*19, 20*). Meanwhile, dZip71B facilitates zinc import into the tubules from the body (*20*). These transporters, along with dZnT63C involved in zinc reabsorption, work together to regulate zinc excretion effectively.

By utilizing the *Drosophila* model, this study examined the impact of paternal zinc deficiency on the total body zinc levels of male parents and their F1 to F3 generations of the male and female offspring. Moreover, we investigated the expression levels of key transporters involved in zinc absorption and excretion in both the parent and offspring groups. We hypothesized that insights into the mechanisms underlying paternal zinc deficiency effects on offspring health could shed light on the molecular basis of zinc transport regulation in this context.

Table 1. Dietary composition.

Component	Normal diet	Zinc-chelated diet
Corn flour	100 g	100 g
Agar-agar	12 g	12 g
Baker's yeast	20 g	20 g
Distilled water	1.5 L	1.5 L
Methyl paraben	1 g	1 g
Others		100 μM TPEN

TPEN: *N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine.

MATERIALS AND METHODS

Materials. Corn flour was obtained from Faso Farine Mais Blanc (Ouagadougou, Burkina Faso). Nitric acid (169215) was purchased from CDH Ltd. (New Delhi, India). *N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) was purchased from Sigma-Aldrich, while Baker's yeast was from STK ROYAL Ltd. (Lagos, Nigeria). The bacteriological grade agar-agar powder was obtained from Titan Biotech Ltd. (Rajasthan, India), while methyl paraben (16260) was a product of Molychem (Mumbai, India).

Fruit flies. The adult W1118 strain of *Drosophila melanogaster* aged 7 to 10 d were acquired from the fly lab of the Centre for Advanced Medical Research and Training (CAMRET), Usmanu Danfodiyo University, Sokoto, Nigeria. They were cultured and maintained at fly optimum temperature (22–25°C) with access to standard cornmeal diet (Table 1) and a natural light-dark cycle.

Experimental design. To study the effects of zinc deficiency through the paternal line, 100 μM of TPEN, a zinc chelator, was added to the diets of the fruit flies to induce zinc deficiency (*21*). Accordingly, adult flies aged 7 to 10 d were transferred to the zinc-chelated diet, and allowed 24 h to lay eggs. The eggs laid were allowed to develop on the zinc-chelated diet, and the emerging adult males were termed F0. Some of the male flies were collected at about 7 d after eclosion and assessed for zinc content. Another section of the male flies aged 7 to 10 d was mated with age-matched virgin control female flies to generate F1. The male offspring (F1) of 7 to 10 d old were in turn mated with age-matched virgin control females to generate F2, while the male offspring (F2) aged 7 to 10 d were also mated with age-matched virgin control females to generate F3 (Fig. 1). The offspring from F1 to F3 were maintained on normal diet for 7 d and analyzed for body zinc status and the expression of the mRNA levels of zinc transporters (dZip42C.1, dZnT63C, dZip71B, and dZnT35C).

Assessment of body zinc status. Ten flies of three replicates per group which were aged 7 to 10 d were anaesthetized, weighed and rinsed in distilled water. The flies

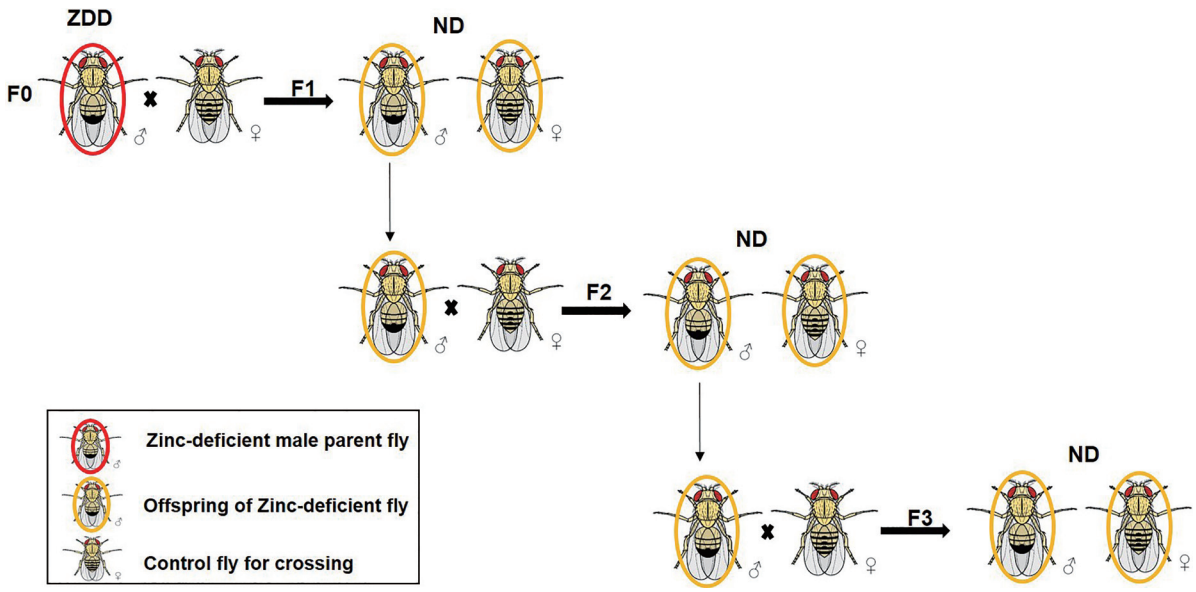

Fig. 1. Fly crossing of parent and offspring generation. F0–F2 adult flies were 7 to 10-d old at the time of mating. F0 flies were cultured on zinc-deficient diet, while F1–F3 flies were maintained on normal standard diet. ZDD: zinc-deficient dietary status, ND: normal dietary status, F0: parent generation, F1: first generation, F2: second generation, F3: third generation.

Table 2. Gene name, accession number and primer sequences used for qPCR.

Gene	Accession	Primer	Sequence[1]	Annealing temperature (˚C)[1]
dZnT63C	NM_168015.2	Forward	CACCATTCAGCCAGAGTTCA	60
		Reverse	CTTCTTCCGTGGTAGGACAATC	
dZip42C.1	NM_143006.2	Forward	AGGCTCAACAACCCTACTTTC	60
		Reverse	TTACCACCCTTGTGTGTTTCT	
dZip71B	NM_001316435.1	Forward	CCCAGTAGCCTTCATGGTAATC	62
		Reverse	GCAAAGGCGGTAGCAAATC	
dZnT35C	NM_135897.5	Forward	GTGTTGTAACGTGTGGTGTTAG	62
		Reverse	CGTTTGGCAATCGGTGTATC	
RpL32	NM_170460.2	Forward	GGATCGATTCCTGTGAGAGTTC	60
		Reverse	TGGGCAGTATCCATTGAGTTT	

[1] 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. dZnT63C: *Drosophila* Zinc Transporter 63C; dZip42C.1: *Drosophila* Zrt-, Irt-like Protein 42C; dZip71B: *Drosophila* Zrt-, Irt-like Protein 71B; dZnT35C: *Drosophila* Zinc Transporter 35C; RPL-32-60S: ribosomal protein large subnunit-32.

were digested in 1 mL of concentrated (65%) metal-free nitric acid at 100˚C for 10 min in closed tubes using a thermoshaker. Samples were diluted with distilled water to 5 mL. Zinc concentration was measured against calibration curves and a digestion blank in an Agilent microwave plasma atomic emission spectrometers (MP-AES, MY19479002). Calibration curve was performed between 0.00 to 6.00 ppm.

Gene expression analysis.

Extraction of RNA. From each group, RNA was extracted from 45 flies aged between 7 and 10 d. The nucleic acid isolation kit (Hunan Runmei Gene Technology Co., Ltd., Hunan, China) was used according to the manufacturer's instructions. The extracted RNA was considered acceptable when it had purity readings for A260/230 and A260/280 between 1.8 and 2.2 using a Bioevopeak Nucleic Acid Analyzer (SP-MUV2000F, Jinan, Shandong, China).

Primer design. The PrimerQuest tool software of Integrated DNA Technologies was used to design primers using *Drosophila melanogaster* gene sequences obtained from the National Centre for Biotechnology Information GenBank Database (Table 2). The genes of interest were dZip42C.1, dZnT63C, dZip71B, and dZnT35C. RpL32 was used as the housekeeping gene.

Quantitative real-time polymerase chain reaction (RT-qPCR) analysis. RT-qPCR of RNA samples was carried out using TransScript Green One-Step qRT-PCR SuperMix (AQ211) (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. Briefly,

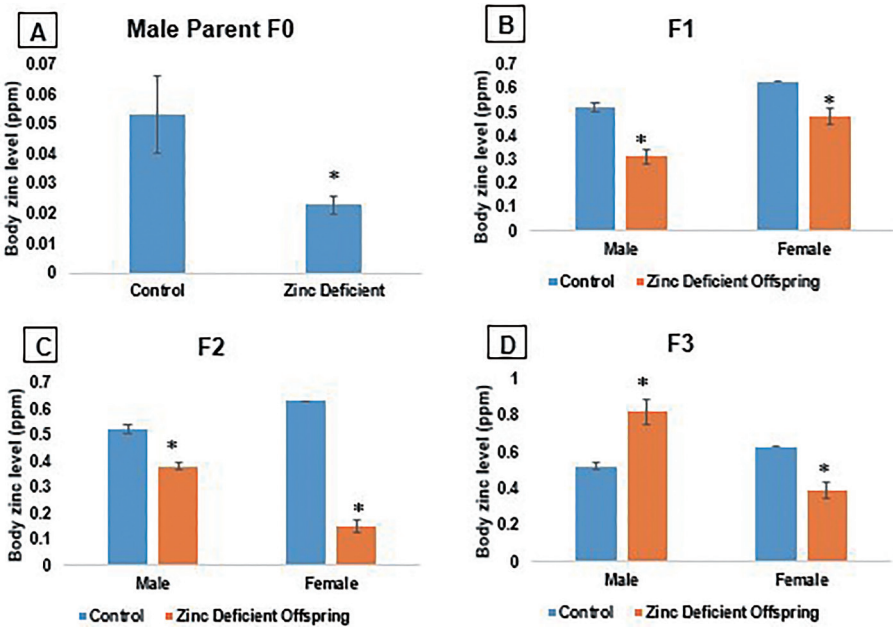

Fig. 2. Body zinc levels in male parent flies (F0) reared on zinc deficient diet and their male and female offspring (F1–F3) reared on normal diet. Three technical and biological replicates (10 flies per replicate) were done using MPAES (microwave plasma atomic emission spectrometry). Bars represent mean±SD. Bars with asterisks are significantly different from control at $p<0.05$.

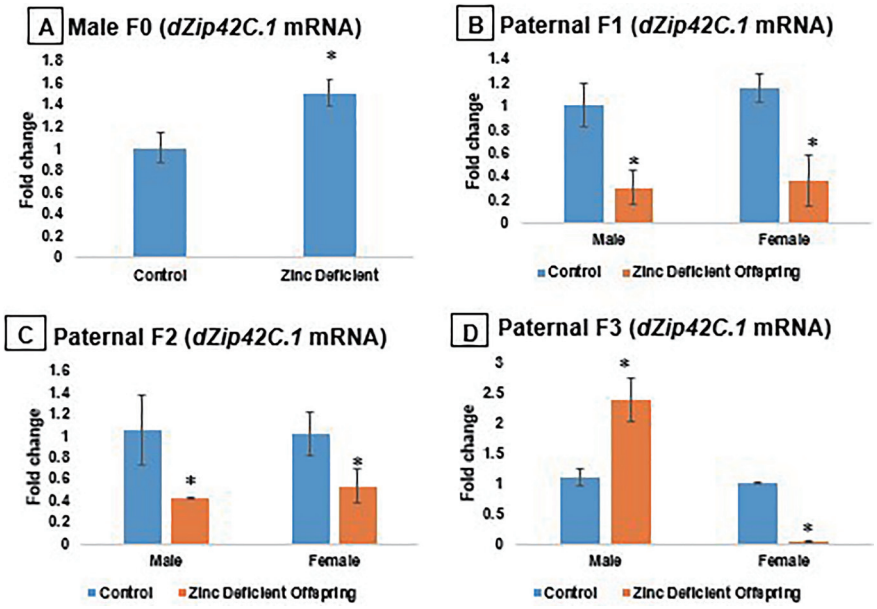

Fig. 3. Expression of dZip42C.1 mRNA in male parent flies (F0) reared on zinc deficient diet and their male and female offspring (F1–F3) reared on normal diet. qPCR was done in duplicates (45 flies per replicate). Fold change was calculated using Livak method ($2^{-\Delta\Delta CT}$). Bars represent mean±SD. Bars with asterisks are significantly different from control ($p<0.05$).

the reaction mixture was prepared by mixing RNA template (200 ng/μL), forward primer (0.4 μL), reverse primer (0.4 μL), SuperMix (10 μL), enzyme mix (0.4 μL) and RNase-free water added to a final volume of 20 μL. The mixture was loaded on a Rotor-Gene Q-5plex HRM platform thermal cycler (Qiagen, Hilden, Germany) set with the cycling conditions on Table 2. The fold change was determined using $2^{-\Delta\Delta CT}$.

Data analysis. Data were analyzed using the statistical software package IBM SPSS (Version 20.0., IBM Corp., Armonk, NY, USA), while graphs were plotted using Microsoft excel. Student's *t* test was used for data from F0 while two-way ANOVA was used for data from F1 to F3, followed by Bonferroni's multiple comparison post hoc test. Data were expressed as mean±standard deviation. The results were considered statistically significant at $p<0.05$.

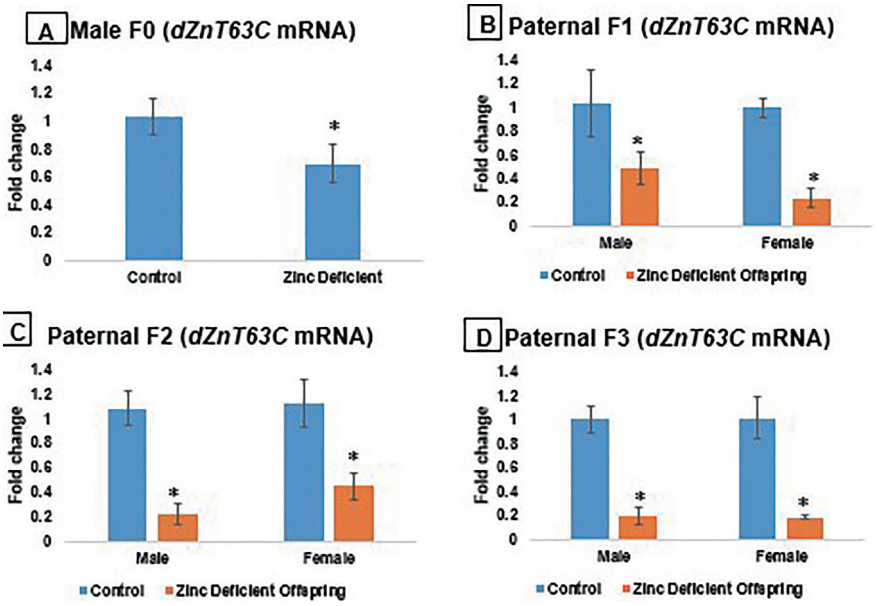

Fig. 4. Expression of dZnT63C mRNA in male parent flies (F0) reared on zinc deficient diet and their male and female offspring (F1–F3) reared on normal diet. qPCR was done in duplicates (45 flies per replicate). Fold change was calculated using Livak method ($2^{-\Delta\Delta CT}$). Bars represent mean±SD. Bars with asterisks are significantly different from control ($p<0.05$).

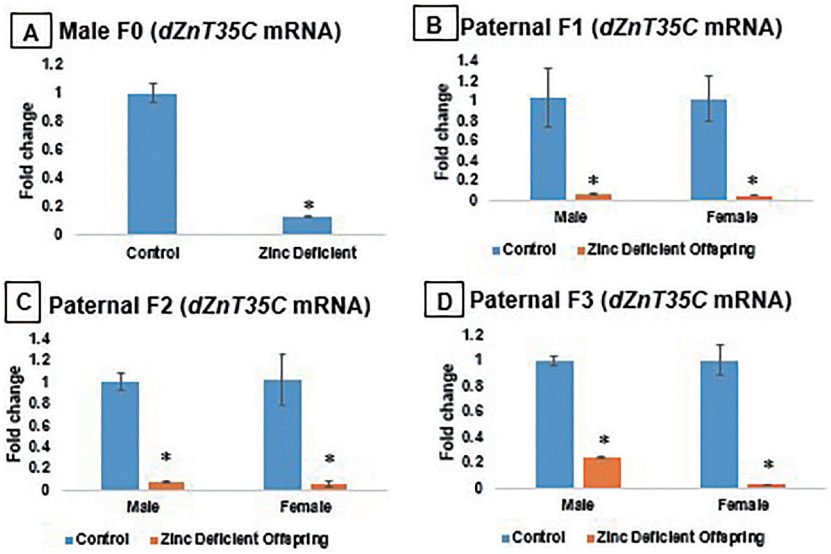

Fig. 5. Expression of dZnT35C mRNA in male parent flies (F0) reared on zinc deficient diet and their male and female offspring (F1–F3) reared on normal diet. qPCR was done in duplicates (45 flies per replicate). Fold change was calculated using Livak method ($2^{-\Delta\Delta CT}$). Bars represent mean±SD. Bars with asterisks are significantly different from control ($p<0.05$).

RESULTS

Effect of paternal zinc deficiency on offspring body zinc level

We found a significant ($p<0.05$) reduction in total body zinc levels in the male parent flies that were reared on a zinc-deficient diet compared to the control group (Fig. 2). Similarly, the male and female offspring (F1 to F3) exhibited significantly ($p<0.05$) lower body zinc levels, except for the F3 male offspring, which showed higher levels ($p<0.05$) (Fig. 2D). Thus, we felt this exception needed to be further explored.

Expression of dZip42C.1 in male parent flies reared on a zinc-deficient diet and their offspring

Our findings revealed a significant ($p<0.05$) increase in the expression of dZip42C.1 mRNA in male parent flies (F0) reared on a zinc-deficient diet (Fig. 3A). In contrast, male and female offspring from F1 to F3 exhibited a reduction ($p<0.05$) in dZip42C.1 expression (Fig. 3B–D). However, the male offspring at F3 showed similar dZip42C.1 expression levels to the male parent flies (Fig. 3D).

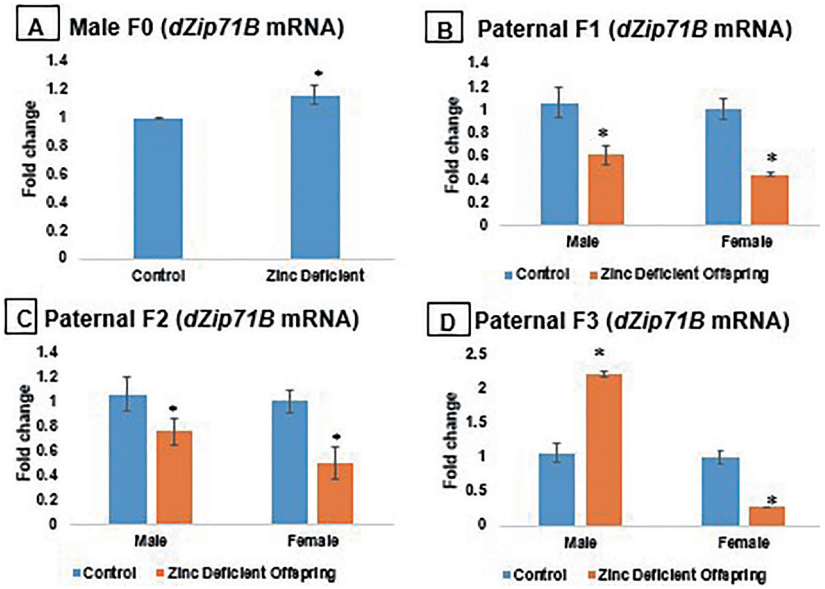

Fig. 6. Expression of dZip71B mRNA in male parent flies (F0) reared on zinc deficient diet and their male and female offspring (F1–F3) reared on normal diet. qPCR was done in duplicates (45 flies per replicate). Fold change was calculated using Livak method ($2^{-\Delta\Delta CT}$). Bars represent mean±SD. Bars with asterisks are significantly different from control ($p<0.05$).

Expression of dZnT63C in male parent flies reared on a zinc-deficient diet and their offspring

We observed a significant ($p<0.05$) reduction in the expression of dZnT63C mRNA in both the male parent flies (F0) and the male and female offspring from F1 to F3 (Fig. 4).

Expression of dZnT35C in male parent flies reared on a zinc-deficient diet and their offspring

We also found a significant ($p<0.05$) reduction in the expression of dZnT35C mRNA, in the male parent flies (F0) and the male and female offspring from F1 to F3 (Fig. 5).

Expression of dZip71B in male parent flies reared on a zinc-deficient diet and their offspring

We observed a significant ($p<0.05$) increase in dZip71B expression in the male parent flies (F0), but a significant ($p<0.05$) reduction in the male and female offspring from F1 to F3 (Fig. 6), except for the male offspring at F3 (Fig. 6D), which also increased.

DISCUSSION

Despite the increasing focus on maternal dietary imbalances, understanding the relationship between paternal factors, zinc levels, and zinc transporters has implications for human health. It can help develop strategies to optimize zinc status and address risks associated with zinc deficiencies in populations affected by paternal dietary imbalances or environmental exposures. Additionally, studying the molecular mechanisms involved in intergenerational zinc regulation contributes to understanding transgenerational inheritance of micronutrient status and its impact on health outcomes.

The nutritional status of fathers plays a significant role in influencing the growth, metabolism, and susceptibility to diseases in their offspring (*22*). These effects are believed to be mediated, at least in part, by epigenetic changes (*23*). Although research on the impact of paternal zinc deficiency on offspring health is limited, studies conducted in mice have revealed disrupted energy metabolism in male offspring born to fathers with low zinc levels (*12*). These findings highlight the importance of considering paternal factors in understanding the long-term health implications for the next generation.

The reduction observed in the body zinc level of the male parent corresponds to the reduced zinc bioavailability as a result of dietary zinc chelation. Interestingly, even when the offspring were consuming a normal diet, they still exhibited reduced body zinc levels. This led us to explore the molecular basis of this phenotype by assessing the expression levels of zinc transporters involved in zinc absorption.

Previous research has indicated the importance of *Drosophila melanogaster* zinc transporters, such as dZip42C.1, dZip71B, dZnT63C, and dZnT35C (human homologue of ZIP1, ZIP5, ZnT1, and ZnT2/3/8 respectively) in regulating zinc homeostasis and influencing systemic zinc levels. (*19, 20, 24–28*). However, their specific roles in the context of paternal line effects and transgenerational inheritance have not been fully elucidated. In the context of *Drosophila*, our study adds to the growing body of evidence regarding the importance of zinc and zinc transporters in development and metabolism.

The dZnT63C and dZip42C.1 are known to play a crucial role in zinc absorption in *Drosophila melanogaster*, and previous studies have demonstrated that their expression is transcriptionally regulated in response to dietary zinc levels (*26–28*). In line with these findings,

our study observed an increase in dZip42C.1 expression, which aligns with the findings of the study conducted by Qin et al., where dZip42C.1 was found to be upregulated under conditions of dietary zinc deficiency (*28*). The increased expression of dZip42C.1 in the male parent flies can be considered an expected homeostatic response to enhance zinc absorption in response to the reduced zinc bioavailability. However, the reduction in dZip42C.1 expression in male and female offspring from F1 to F3 could probably be due to normal diet intake. On the other hand, it could explain the reduced body zinc levels observed in these generations. More so, the male offspring at F3 which showed similar dZip42C.1 expression levels to the male parent flies, could be indicating a possible inheritance pattern. Other potential factors for these differences could be sex-specific gene regulation or variations in zinc metabolism between male and female offspring. Moreover, further studies could investigate the role of sex hormones or epigenetic modifications in mediating the transgenerational effects of paternal zinc deficiency.

In addition, the reduction we observed in dZnT63C, a zinc exporter responsible for releasing zinc into the haemolymph during absorption, correlated with the reduced body zinc levels observed in the parent and offspring groups. However, the reduction in dZnT63C expression in the male offspring at F3, despite the increased total body zinc levels, suggests the involvement of other zinc transporters associated with excretory functions. This led us to further assess other important zinc transporters such as dZnT35C and dZip71B localized in the Malpighian tubules.

Furthermore, dZnT35C and dZip71B are respective exporter and importer proteins that act as counterparts for zinc excretion. This reduction observed in dZnT35C expression in the parent flies may contribute to the reduced zinc excretion observed when zinc bioavailability is limited due to zinc deficiency. Interestingly, this reduction in dZnT35C expression in the offspring might be developmentally programmed, despite their exposure to a normal diet.

It is suggested that dZip71B functions upstream of dZnT35C to transport zinc from the haemolymph into the enterocyte (*20*). In turn, dZnT35C, which is apically localized in the enterocyte, acts to pump zinc out of the body through the tubular lumen (*29*). Thus, the reduction in the offspring dZip71B in our study is consistent with the reduction in dZnT35C in order to reduce zinc excretion. In addition, the increase in dZip71B in the male F3 offspring is consistent with its increase in dZip42C.1, which could be attributed to the corresponding increase in body zinc level in this group of offspring. This also aligns with the hypothesis of probable developmental programming, POHaD, since the male parent also exhibits an increase in the expression of dZip71B. Moreover, this could point to a new role of dZip71B in the absorption and accumulation of zinc.

In essence, the correlation we observed between decreased body zinc levels and reduced zinc bioavailability in our study is in line with previous research conducted in other models (*18, 30*). This relationship has been examined in various studies investigating the interplay between dietary zinc intake, zinc transporters, and zinc status in human intestinal cells and cell lines (*30*). Consistent with findings in different models (*18*), our results suggest that reduced zinc availability triggers the upregulation of zinc transporters to facilitate increased zinc absorption and ensure the maintenance of zinc homeostasis.

In conclusion, our study provides insights into the effects of paternal zinc deficiency on offspring health, focusing on body zinc levels and the expression of zinc transporters. The observed reductions in body zinc levels in offspring, even with a normal diet, suggest possible developmental programming effects. The coordinated regulation of zinc transporters indicates complex mechanisms underlying zinc homeostasis and their potential roles in zinc absorption, accumulation, and excretion. Our findings on the interplay between paternal zinc deficiency, zinc transporters, and offspring health in *Drosophila* offer intriguing possibilities for future human health research. It sheds light on the role of zinc in developmental programming and disease susceptibility. Further research can build upon these findings to unravel the molecular mechanisms involved and explore interventions to optimize zinc status and improve offspring health.

Limitations and future directions

Although our study provides valuable insights, it however has some limitations. The study's design used a single food source, time of year, and fly generation, potentially limiting its generalizability. Also the use of the w1118 mutant strain and whole-body analysis approach may introduce background effects and restrict our understanding of specific mechanisms, respectively. Additionally, the absence of a TPEN-Zn complex control group, focus on mRNA expression levels only, and lack of exploration into metallothionein roles further highlight areas for improvement. Future research should incorporate biological replicates, tissue-specific analyses, and explore protein-level and epigenetic changes to build upon these preliminary findings and enhance our understanding of paternal zinc deficiency's transgenerational impact on zinc homeostasis in *Drosophila*.

Authorship

Conceptualization: M.U.I.; data curation: M.B.A., K.G.I., and M.U.I.; formal analysis: K.O.S.; funding acquisition: M.U.I.; investigation, methodology, project administration: K.O.S., M.B.A., K.G.I., and M.U.I.; resources, supervision and validation: M.B.A., K.G.I., and M.U.I.; writing–original draft: K.O.S.; and writing–review & editing: K.O.S., M.B.A., K.G.I., and M.U.I.

Disclosure of state of COI

No conflicts of interest to be declared.

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