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Adverse effects of parental zinc deficiency on metal homeostasis and embryonic development in a zebrafish model

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Abstract

The high prevalence of zinc deficiency is a global public health concern, and suboptimal maternal zinc consumption has been associated with adverse effects ranging from impaired glucose tolerance to low birthweights. The mechanisms that contribute to altered development and poor health in zinc deficient offspring are not completely understood. To address this gap, we utilized the *Danio rerio* model and investigated the impact of dietary zinc deficiency on adults and their developing progeny. Zinc deficient adult fish were significantly smaller in size, and had decreases in learning and fitness. We hypothesized that parental zinc deficiency would have an impact on their offspring's mineral homeostasis and embryonic development. Results from mineral analysis showed that parental zinc deficiency caused their progeny to be zinc deficient. Furthermore, parental dietary zinc deficiency had adverse consequences for their offspring including a significant increase in mortality and decreased physical activity. Zinc deficient embryos had

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altered expression of genes that regulate metal homeostasis including several zinc transporters (ZnT8, ZnT9) and the metal-regulatory transcription factor 1 (MTF-1). Zinc deficiency was also associated with decreased expression of genes related to diabetes and pancreatic development in the embryo (Insa, Pax4, Pdx1). Decreased expression of DNA methyltransferases (Dnmt4, Dnmt6) was also found in zinc deficient offspring, which suggests that zinc deficiency in parents may cause altered epigenetic profiles for their progeny. These data should inform future studies regarding zinc deficiency and pregnancy and suggest that supplementation of zinc deficient mothers prior to pregnancy may be beneficial.

Keywords

Zebrafish; zinc deficiency; epigenetics; zinc homeostasis; fitness; learning

1 Introduction

Zinc is an essential micronutrient that is required for many biological processes including enzyme activity, immune function, neurological function, and reproduction [1, 2–6]. While severe zinc deficiency is rare, mild-to-moderate zinc deficiency is quite common, with about one-third of the world's population estimated to have a zinc deficiency [7]. Zinc deficiency in pregnant women is a public health concern because it has been estimated that up to 82% of pregnant women worldwide have inadequate zinc uptake [8]. Zinc supports normal growth and development from pregnancy through childhood [1]. In humans, suboptimal zinc consumption has been associated with increased premature birth, low birthweights, and increased congenital malformations [9–16]. Marginal maternal zinc status has also been associated with impaired glucose tolerance, increased susceptibility to diabetic stress, and impaired learning and memory in the offspring [5, 17, 18]. Overall, this evidence supports that maternal zinc deficiency has adverse effects for their offspring, and suggests that zinc supplementation of pregnant women might be beneficial. However, the evidence that zinc supplementation in pregnant women is beneficial has been limited [19]. This issue is complicated by the fact that there are limited human biomarkers for zinc deficiency. There is also limited information regarding the mechanisms that contribute to altered embryonic development and poor health in zinc deficient offspring. These disconnects in our knowledge suggests there is still much to learn about the impact of zinc during development, and new approaches are warranted.

Zebrafish *(Danio rerio)* are a premier model to address developmental questions. Zebrafish have been utilized for decades to understand the developmental and mechanistic consequences of environmental toxicant exposures [20], but have not been used extensively to understand the developmental consequences of nutrients or nutrient deficiencies. Zebrafish are a small, complex vertebrate organism that have a short generation time, rapid development, and a short life cycle [21]. Female zebrafish are capable of producing hundreds of embryos per spawn, thereby providing high statistical power for analysis [22]. The embryos develop externally and are optically clear to allow for non-invasive assessments. The genetic homology between human and zebrafish is approximately 70%, and ~84% of human genes known to be associated with human diseases are also present in

zebrafish. It is estimated that 99% of embryonic-essential fish genes are homologues in human embryonic development [23, 24]. Importantly, like humans, zebrafish require dietary zinc intake and have similar regulatory mechanisms that maintain zinc homeostasis. Because zinc plays a fundamental role in many cellular processes, like insulin storage, secretion, and signaling, its uptake and intracellular distribution is tightly regulated by zinc importers (ZIPs/SLC39) and zinc exporters (ZnT/SLC30), and these genes are conserved between zebrafish and humans [25].

Herein we developed a defined zinc deficient diet for zebrafish that resulted in zinc deficient adult fish. We evaluated the effect of zinc deficiency in the adults and then used these zinc deficient fish to test the hypothesis that parental zinc deficiency has an impact on their offspring's mineral homeostasis and embryonic development. We also evaluated whether the offspring had increased mortality, altered development, and changes in expression of genes that regulate zinc homeostasis, DNA methylation, and pancreatic development.

2 Materials and Methods

2.1 Fish Husbandry and Diet Preparation

Wild type zebrafish (5D) were raised and maintained at the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University, in accordance with protocols approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC). All adult animals were fed standard lab diet (Gemma Micro. Skretting, Tooele, France) until 8 weeks post fertilization when the fish were moved to clean enclosures and housed at densities of ~6 fish/liter. Adult fish were then randomized to either zinc deficient or control defined diets. The defined diet was based on the previously published diet for zebrafish, with minor modifications as detailed [26]. (Supplemental Table 1 and 2). Each diet was prepared by mixing dry ingredients and incorporating reverse osmosis (RO) water and wet ingredients until a homogenous mixture was achieved. Batter was spread onto large baking sheets and then oven dried at ~212 °F until dry (~1.5 hours). The diet was then cooled to room temperature, crushed into chips and ground to appropriate life stage micron size using a bur grinder and stored frozen at −20 °C until fed to the zebrafish. Diets were used within 4–6 months of preparation. Feeding volumes for all feeds were ~5% body weight/day, or until satiation, given over 2–3 feedings in a day depending on life stage. Fish were maintained on either control or zinc deficient diet for a minimum of 8 weeks prior to the first spawn. In order to obtain the zinc deficient embryos from the adults, the same control and zinc deficient fish were subsequently spawned every 2–3 weeks in small group crosses. All embryos were screened for viability and weighed to estimate total yield (1 embryo equals ~0.8mg).

2.2 Metal analysis

Zinc, copper, iron, selenium, calcium and magnesium, essential metals in human health were evaluated in diets, adult fish, and embryos with the Prodigy High Dispersion Inductively Coupled Plasma-Optical Emissions Spectroscopy (ICP-OES) (Teledyne Leeman Labs, Hudson, NH, USA) and a previously described method [27]. Starting materials were homogenized adult fish (100 µl from a 2ml homogenized volume), 30 fish embryos, or ~50

mg of diet. Samples were digested overnight in 70% ultrapure nitric acid and then diluted 10-fold with Chelex-treated nanopure water. Additionally, fish water was tested periodically using the same ICP-OES technique and shown to have no detectable zinc where the detection limit was 0.5 ng/mL. Samples were analyzed against known metal standards (Ultra Scientific).

2.3 Adult Oxygen Consumption Rates

An AutoResp swim tunnel, a Fibox fiber optic oxygen probe, and an oxy4 sensor (Loligo Systems, Denmark) were used to measure the oxygen consumption rate in groups of 8 adult zebrafish as previously described with small modifications [28]. Briefly eight animals per group were weighed prior to the experiment and then placed in the large swim tunnel in a 2.5 L tank with an oxygen sensor connected to the AutoResp software. Oxygen consumption rate measurements (O_2 mg/kg/hr) were taken every 10 min, consisting of a 240s flush period, in which water was renewed in the swim tunnel with a flush pump, then the pump was turned off to create a closed system during a 60s wait period, and a 300s measure period, as described in [28]. The zebrafish were given a 30 min acclimation period followed by a 30 min free swim at a water flow of 5 cm/sec (108 rpm) before swimming against a water current of 25 and 45 cm/sec (504 and 901 rpm respectively). The AutoResp software was used to calculate oxygen consumption rates from the slope of oxygen depletion. Three measurements were taken at each swimming speed; 5, 25, and 45 cm/sec, with the mean value used in statistical analysis. Three to four experiments were run for each diet group, with all data points included in analysis having an R^2 value of 0.8 or higher. [29–31].

2.4 Adult Learning and Memory Assay

To test learning and memory in adult zebrafish, custom built shuttleboxes were used with a modified protocol as described in Truong *et al.* [32, 33]. The protocol programmed into the shuttlebox was to condition the zebrafish to leave the compartment with green light (“reject side”) and swim to the unlit dark side (“accept side”). At the beginning, there was a 10 minute acclimation period (in the dark) prior to beginning the trials which consisted of giving the zebrafish 4 secs to “seek” the condition side to avoid a moderate shock, and if it did not move to the accept side, the 20 s shock period was initiated. A moderate pulse of 5 V was delivered at 1 s intervals, for a duration of 500 ms. There were two sets (a train and test session) of 50 trials with a 1 h rest in between, where the fish were kept in the dark. Fault outs occurred when fish did not swim to the “accept side” at all during 8 consecutive trials. Data is analyzed with and without the fault out fish as appropriate. The statistical method remained the same as previously described, with the data fit using linear regression models and intercept and slopes were calculated for each recorded parameter [32]. An analysis of variance (AOV) followed by a Tukey’s statistical difference was used to calculate statistical significance amongst the groups.

2.5 Developmental Malformation Evaluation

Embryo chorions were enzymatically removed using pronase (83 μL of 25.3 U/mg; Roche, Indianapolis, IN, USA) at 4 hours post fertilization (hpf) using a custom automated dechorionator and protocol described in Mandrell *et al.* [34]. Dechorionated embryos were placed in individual wells of a 96-well plate (Falcon U-bottom; supplier number: 353227)

with 100 μL of E2 embryo media using automated embryo placement system [35]. At 24 and 120 hpf, embryos were assessed for developmental toxicity endpoints as described in Truong *et al.* [36]. Briefly, 4 endpoints were observed at 24 hpf, and 18 separate morphological evaluations were conducted at 120 hpf. Each endpoint was scored in a binary fashion (present/absent) and collected using a custom zebrafish laboratory integration system (Zebrafish Acquisition and Analysis Program; ZAAP). Statistical analysis was performed using R code from the methodology described in Truong *et al.*, which is a binomial test that calculates lowest effect levels (LELs) for each endpoint to identify incidences that exceed a significance threshold above that endpoints control [37, 38].

2.6 Photomotor Response Assay

At 120 hpf, embryos were tested in the 96-well exposure plates by placing them into the Viewpoint Zebrabox HD (software version 3.2, Viewpoint Life Sciences, Lyon, France) and measuring locomotor activity using the tracking setting during 3 minute periods of alternating light and dark for a total of 24 minutes. The integration time was set to 6 seconds to increase statistical power. The total movement (swim distance) in response to the multiple light-dark transition was tracked by an HD camera at 30 frames/s [38]. Any dead or malformed animals at 120 hpf (41 control, and 67 zinc deficient embryos) were excluded from the data analysis and 247–278 embryos were analyzed. Raw data files were processed using custom R scripts to average the total distance traveled for each integration time point, and then the area under the curve was computed [38]. The overall area under the curve was compared to the control (zinc sufficient) using a Kolmogorov-Smirnov test ($p<0.05$).

2.7 Metal Homeostasis, Pancreatic Development and DNA methyltransferase gene expression

Expression of genes related to zinc transport, metal homeostasis, pancreatic development, insulin and DNA methyltransferases were evaluated by quantitative real-time PCR. Total RNA was collected from homogenized embryos at 6, 24 and 48 hpf using a standard Trizol extraction method (Life Technologies). cDNA was synthesized using 1 μg of total RNA and SuperScript III First-Strand Synthesis SuperMix (Life Technologies). Real time PCR was done using the following zebrafish-specific primers: Odc1, (forward) 5′-GTGGGCGACTGGCTGCTGTT-3′ and (reverse) 5′-CCGCAGTGGGATGGCACGTT-3′, ZIP1 (forward) 5′-GGTGAGAGTTGGAGCTCTGG-3′ and (reverse) 5′-AGTGGGAAGCCATCATCAAG-3′; ZnT7 (forward) 5′-CCCTTCCTGAATGCTACCAA-3′ and (reverse) 5′-CACCGACCTGTGTGAAGATG-3′; ZnT8 (forward) 5′-ATCGTCTTGATGGAAGGCAC-3′ and (reverse) 5′-TTTCTCGAAGCACCTCCTGT-3′; ZnT9 (forward) 5′-CCTGTTTTGGTTGGCAAAGT-3′ and (reverse) 5′-GAATGCTCTCTGCCTTCGTC-3′; metal-regulatory transcription factor 1 (MTF1) (forward) 5′-CGCTGTGCCTCAGTGATTTA-3′ and (reverse) 5′-TCTGTAGGTTTGGGGTCGTC-3′; Dnmt4 (forward) 5′-ACTGCCACGGCTGCCATGAA-3′ and (reverse) 5′-AGTCCTGGGGCGAGGCCTTT-3′, Dnmt6 (forward) 5′-TGGGCATCCAGGTGGAGCGT-3′ and (reverse) 5′-CCGACCGGTGCCCTCGTAGA-3′; Pax4 (forward) 5′-AGAGTTTGATGCCGATGTGTCACC-3′ and (reverse) 5′-TGCAATTTCTTTCCTTTGCCGCCC-3′; Pdx1 (forward) 5′-

AATCTCACACGCACGCATGGAAAG-3′ and (reverse) 5′-ATTCATCCTTAAGATCCCCGGACGTG-3′; Insa (forward) 5′-TAAGCACTAACCCAGGCACA-3′ and (reverse) 5′-GATTTAGGAGGAAGGAAACC-3′; Insb (forward) 5′-ACTCTTCACAGACTCTGCTC-3′ and (reverse) 5′-ACAGATGCTGGGATGGAGAA-3′. Fourteen additional zinc transporters and metallothionein (MTII) were screened in embryos of control or zinc deficient fish but were not pursued further because no changes in gene expression between the two groups were found. Reactions were performed using Fast SYBR Green Mastermix (Life Technologies) on 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). PCR conditions were programmed as follows: 95°C for 20 s, followed by 40 cycles of denaturing at 95°C for 1 s, annealing and extension at 58°C for 20 s, followed by a dissociation curve at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. A dilution series of 10^3, 10^4, 10^5, 10^6, and 10^7 copies of template DNA served as internal standard for quantification [39]. Data in graphs represent the copy number of the gene of interest, divided by the copy number of the housekeeping gene *Odc1* to normalize the genes expression relative to input of cDNA.

3 Results

3.1 Effects of Zinc Deficiency on Adult Zebrafish

In order to create zinc deficiency in zebrafish, we developed a defined fish diet that was moderately deficient in zinc. The diet was designed to mimic nutrient levels similar to commercially available diets that are typically used in zebrafish research, and utilized a similar approach to a Vitamin E deficient defined diet that has been previously published (Figure 1A, Supplemental Table 2) [26]. The control and zinc deficient diets contained the same ingredients with the exception of custom designed mineral mixes that contained zinc (control diet), or did not contain zinc (zinc deficient diet). The zinc deficient diet was significantly lower in zinc content (12 μg of zinc/g of diet) than the control diet (33 μg of zinc/g of body weight), but zinc was not completely absent primarily due to zinc present in provided casein protein. Adult fish were tested for zinc content and the control fish had 74 μg zinc/g of body weight while the fish on the zinc deficient diet had significantly less zinc at 45.59 μg zinc/g of body weight (Figure 1B). There was no difference between the control and zinc deficient adults in the amount of other essential metals including calcium, iron, magnesium and selenium (Supplemental Table 2).

Next we characterized the effects of zinc deficiency on growth, fertility, fitness, learning and memory, in adult zebrafish. Zinc deficient adult fish were significantly smaller in size than the controls (Figure 1C). The numbers of eggs produced every two weeks was recorded at the time of spawning and zinc deficient fish produced fewer eggs than control fish but this was not statistically significant (Figure 1D). These eggs were subsequently used for evaluation of the effect of parental zinc deficiency on their offspring (discussed below). We did note a trend that zinc deficient fish had a higher percentage of eggs that were non-viable (Figure 1E). To evaluate physical fitness, oxygen consumption was measured. Initially there were no differences between the control and zinc deficient fish during the acclimation period (Figure 1F). As the speed of the current increased, the fish had to swim more vigorously, resulting in a significant increase in oxygen consumption in the zinc deficient fish ($p < 0.001$,

by two-way ANOVA). Importantly, there was also a significant diet effect (p=0.0001, two-way ANOVA) where the zinc deficient fish consumed more oxygen than the controls. At the highest speed (901 rpm) the zinc deficient fish consumed 28% more oxygen than control fish (Figure 1G, Bonferroni post-test).

The learning and memory capacity of adult zinc deficient fish were evaluated with the shuttlebox assay (Figure 2). During the training period there was no difference between the two diets in the number of fish that learned (Figure 2A). However, out of the fish who completed the test, the zinc deficient fish were initially shocked more than the control fish during the training period (Figure 2B and C, Intercept). This indicates a negative impact on learning and behavior among the zinc deficient fish. The number of control fish who learned in this assay significantly improved from the training period (40%) to the test period (58.1%) (Figure 2A). In contrast, the percent of fish that learned in the zinc deficient group did not improve, indicating an impairment in learning and memory in zinc deficient fish (Figure 2A). The decreasing slope in both the training and testing periods, among the fish that did learn, indicates that the fish were shocked less over time in both diet conditions (Figure 2B and C, slope). By the test period, the zinc deficient fish learned at the same rate as the control fish (Figure 2B and C). Taken together, this data shows that zinc deficient fish have learning and memory deficits, but they are not completely deficient in these processes.

3.2 Effects of Parental Zinc Deficiency on Embryonic Zinc Status and Developmental Outcomes

The control and zinc deficient adult fish were allowed to mate to understand the effect of parental zinc deficiency on the offspring. Zinc levels were significantly lower in embryos of zinc deficient parents at all time points examined (Figure 3A). The progeny had ~23% less zinc when born from parents that consumed a zinc deficient diet. We also examined if parental zinc consumption had an impact on developmental malformations and mortality in their offspring. A very modest but significant increase in malformations of the snout and eye were found in offspring of zinc deficient parents (Figure 3B). A significant 2-fold increase in the mortality of the offspring was observed both at 24 hours and 5 days post fertilization (Figure 3C). Since we saw a difference in the zinc deficient parents' oxygen consumption in a movement based assay, we then investigated whether the progeny born of zinc deficient parents had altered movement. Assessment of locomotor activity was done in larval fish (120 hpf) exposed to repeated cycles of light and dark stimulus. A typical control response is little movement while in the light and increased movement in the dark (Figure 3D). Offspring of zinc deficient fish moved during the dark phases but their total movement was 27% less, as shown by the decreased height of the three peaks (Figure 3D and E). The hypoactivity of zinc deficient progeny is apparent when comparing the total movement of the fish over time, where the area under the curve is significantly different between the two groups (Figure 3E and F).

3.3 Zinc homeostasis genes are altered in zebrafish embryos

The uptake of zinc and its intracellular distribution is tightly regulated by zinc importers (ZIPs/SLC39) and zinc exporters (ZnT/SLC30). In order to understand if zinc deficient embryos had altered expression of these genes, we examined the expression of multiple zinc

transporters. An initial screen was done for multiple times post fertilization for the expression of 10 zinc importers and 8 exporters (ZIP 1,3, 4, 6–11 13 and ZnT 1, 2, 4–9) with 3 samples of embryos that were from control and zinc deficient parents (Supplemental Table 3). We then expanded the number of samples we tested but focused on ZIP1, ZnT7, ZnT8 and ZnT9 as these transporters appeared the most different between the two diet groups in the screen (Figure 4). ZnT7 mRNA levels were not significantly changed in zinc deficient embryos, but the expression of ZnT8 mRNA levels were significantly down regulated in zinc deficient progeny by 2-fold at 24 and 48 hpf. ZnT9 mRNA expression levels were also significantly reduced in the zinc deficient progeny at 48 hpf. ZIP1 mRNA levels were also reduced in offspring of zinc deficient parents at 24 and 48 hpf but this was not significant when tested by a two-way ANOVA. We also examined the expression of metal-regulatory transcription factor 1 (MTF-1) which regulates expression of genes involved in metal homeostasis in response to exposure to zinc, cadmium, copper, and silver. MTF-1 was significantly decreased, by 2-fold, in zinc deficient embryos at 48 hpf.

3.4 Expression of genes related to insulin, pancreatic development, and DNA methylation are altered in zinc deficient embryos

Zinc is known to play important functions in insulin storage, secretion, and signaling [25]. Also marginal maternal zinc status has also been associated with impaired glucose tolerance, increased susceptibility to diabetic stress [17, 18]. For these reasons, we wanted to evaluate if parental consumption of a diet low in zinc would influence the expression of genes related to pancreatic development and insulin production in the offspring (Figure 5). Paired box 4 (Pax4) is a transcriptional regulator that is important in pancreatic islet beta cell differentiation and development [40]. An additional transcription factor that regulates pancreas development and particularly endocrine function and beta cell formation is Pancreatic and Duodenal Homeobox 1 (Pdx1) [41, 42]. Pdx1 is also a key player in glucose-dependent regulation of insulin gene expression. Both Pax4 and Pdx1 mRNA levels are suppressed in the offspring of zinc deficient parents at 48 hpf (Figure 5). We next examined the expression of the two insulin genes that are found in zebrafish (insulin-a (Insa) and insulin-b (Insb)). As expected, Insa expression significantly increased with time while Insb decreased over time in both control embryos [43]. Zinc deficient offspring had a significant decrease in Insa expression at 48 hpf (Figure 5). Insb mRNA levels were also lower in progeny of zinc deficient parents but this was not significant by 2-way ANOVA. It is worth noting that a pair wise comparisons showed a significant decrease in Insb in the embryos at 48 hpf with zinc deficiency ($p=0.0086$).

DNA methyltransferases (Dnmts) play a critical role in regulating gene expression and development through a heritable epigenetic mechanism that regulates DNA methylation. We next sought to evaluate if the expression of Dnmts were altered in the offspring of zinc deficient parents. We focused on Dnmt4 and Dnmt6 because they fall into the mammalian DNMT3 family and regulate *de novo* DNA methylation in embryos. The expression levels of both Dnmt4 and Dnmt6 mRNA were significantly reduced in the progeny of zinc deficient parents at 48 hpf (Figure 5).

4 Discussion

In order to address how zinc deficiency alters development, we established a new dietary model utilizing zebrafish that were fed a defined zinc diet. Through this model we effectively characterize the effects of zinc deficiency in both parental adult fish and consequences to their offspring. Zinc deficient adult fish were significantly smaller in size, had an impairment in learning and memory function, and had increased consumption of oxygen during exercise. Parental zinc deficiency had significant acute effects on their offspring including a two-fold increase in mortality, decreased physical activity, and embryonic zinc deficiency. Zinc deficient embryos also had altered expression of genes that regulate metal homeostasis (ZnT8, ZnT9, MTF-1), pancreatic development and glucose regulation (Insa, Pax4, Pdx1). Offspring of zinc deficient parents also had decreased expression of epigenetic regulators (Dnmt4, Dnmt6). Given the important role these genes play in the developmental origins of disease, our data suggest that parental zinc deficiency may also have long term health consequences for their offspring.

In the past, zinc deficiency has been associated with reduced working memory, reduced neurogenesis, and increased apoptosis in the hippocampus of mice. Zinc is known to modulate synaptic activity and neuronal plasticity which are directly related to cognitive function [5, 44, 45]. Studies on zinc intake and cognitive function in humans have mixed results. Yasuda et al. have eluded to an association with infantile dietary zinc deficiency and an increased susceptibility to autism spectral disorders [46]. Independent from this, a recent meta-analysis concluded there was no significant overall effect of zinc intake on cognitive function in children but noted some small indicators of improved executive function and motor development with zinc supplementation [47]. Zinc supplementation studies in children may have mostly failed because the zinc deficient mothers should have been supplemented to gain the most benefit. Unfortunately, because of limitations in growing larval fish on the defined diets, and the lack of a learning and memory test in embryos, we were not able to directly address the effect of parental zinc deficiency on learning and memory. This is an important avenue of future research. Our data in adult zebrafish does support a role for zinc in cognitive function as adult zinc deficient zebrafish had deficits in learning and memory. Our results demonstrate that zinc deficient fish were smaller in size and had a small decrease in reproductive capacity, which is consistent with previously documented functions of zinc [1, 6]. We also found zinc deficiency in adults was associated with decreased fitness, as measured by a significant increase in oxygen consumption during exercise. Oxygen consumption is related to mitochondrial function and there is a large body of evidence where zinc deficiency is also regularly associated with increased oxidative stress [48–51] but this is the first report where we are aware of an association between zinc deficiency and increased oxygen consumption during exercise. In a zinc deficiency study focused on metabolic rate, Evans *et al.* found no significant changes in oxygen consumption of rats on a zinc deficient diet as compared to zinc adequate pair-fed controls [52]. There are some major differences in methodology between this paper and our study, including the period of zinc deficiency and length of oxygen measurement, which likely contribute to the different results. Nevertheless, the effect of zinc deficiency on exercise is of concern among athletes and worthy of further investigation [53, 54].

Zheng *et al*. has also completed a zinc study in adult zebrafish with a focus on zinc uptake through the gills, movement through the body, and effect on other elements [55]. They showed zinc deficiency was associated with a significant decrease in zinc in the whole body, but it did not produce a decrease in the gills or intestine. Interestingly they found that zinc deficiency was associated with a significant decrease in calcium, potassium, and sodium in the whole body of zebrafish following 3 weeks of low zinc diet and water. This result is different from ours as we did not see changes in the calcium, iron, magnesium or selenium in the whole body after months of zinc deficiency. The difference between these two results is probably related to differences in diet formulations, as our water also had low zinc content, or the length of time the fish were zinc deficient before analysis.

There is intense interest and controversy in the literature regarding if parental zinc deficiency has adverse outcomes for their progeny. For example, sub-optimal zinc consumption has been associated with increased premature birth, congenital malformations, and decreases in learning and memory, but there is also literature showing little effect of zinc deficiency on outcomes in rhesus monkeys and little benefit from zinc supplementation during pregnancy [19, 56–59]. Here, we observed that parental zinc deficiency was associated with a two-fold increase in embryo mortality, a slight but significant increase in malformations of the snout and eye, and larval hypoactivity in their progeny. Our observations show that parental zinc deficiency does have adverse consequences for their progeny. This is similar to the congenital defects seen in rats and is likely associated with cleft palate in humans [60, 61]. Given the significant increase in mortality, and changes in gene expression at early time points in development, it suggests that parental zinc supplementation, especially in populations at risk for zinc deficiency, may need to occur prior to conception in order to get full benefits to the developing embryo.

We also investigated how parental zinc deficiency altered metal homeostasis in the developing embryo. Our results show that parental zinc deficiency causes their progeny to be compromised in zinc status and have alternations in expression of MTF-1 and specific zinc transporters. This is consistent with results in other model organisms where maternal zinc deficiency was associated with lower amounts of zinc in their offspring’s liver or plasma [57, 62, 63]. Also, given MTF-1’s important role in regulating metallothioneins and metal homeostasis in relationship to heavy metal exposure, it is possible that zinc deficiency, either in the parent or the embryo, may cause embryos to be more sensitive to toxic metals. In support of this possibility, zinc deficient cells in culture are more sensitive to hexavalent chromium exposure [64]. An important area of future research would be to examine subcellular localization of MTF-1 or altered metal-response-element binding in response to zinc deficiency to understand the functional perturbations in MTF-1. With respect to zinc transporters, in particular, ZnT8 and ZnT9, were identified as transporters that were decreased in embryos spawned from zinc deficient parents. ZnT8 is predominantly expressed in the secretory granules of pancreatic β-cells and functions to deliver zinc into granules for insulin maturation and secretion [65]. Defects in these zinc transporters have been associated with abnormalities in insulin synthesis, maturation, and secretion, subsequent glucose metabolism, and may alter risk for diabetes [66]. ZnT9 is a ubiquitous zinc transport, with unidentified function, however decreases in ZnT9 have also been apparent in obese women [67]. Together this data suggests that parental zinc deficiency has

impact on zinc levels, and zinc transport that may have implications in future health of the offspring.

There is a wide body of evidence that parental nutritional deficits are contributors to adult onset diseases, including obesity and diabetes. There is a growing amount of literature linking zinc deficiency, and maternal zinc deficiency, to alterations in body composition, glucose tolerance, and/or insulin response [17, 62, 63, 68]. As with much of our data, we are not currently capable of teasing out if the changes in embryonic gene expression we observed are caused directly by low levels of zinc in the embryos, or by some epigenetic change that has been passed from the zinc deficient parent to their offspring. Currently there are only a few studies examining the intersection of zinc deficiency and epigenetics [2, 3, 69–74]. DNA methyltransferases are key players in regulating epigenetics by methylating the DNA. Here we show that zinc deficiency is associated with lowered expression of DNA methyltransferases at the mRNA level in the zinc deficient offspring. This is an interesting but limited finding as we were not able to explore if it was associated with suppressed DNMT protein expression, or changes in global methylation patterns, or changes in DNA methylation patterns in key genes. This will be an important direction of our future research. Interestingly, ZnT8 has recently been shown to have increased DNA methylation in type 2 diabetes patients [71, 72]. In another report, parental zinc deficiency altered the epigenetic status of the metallothionein gene MT2 in the liver of one day old pups but did not have a significant effect on adults [73]. Also, acute maternal zinc deficiency in mice for 3–5 days post ovulation caused large decreases in global DNA methylation and histone H3K4 trimethylation in the oocyte and this was associated with decreased fertilization [74]. Given this interesting literature, and our own findings, it is possible that dysregulated epigenetic mechanisms that occur with zinc deficiency may play a key role in producing adverse effects associated with zinc deficiency in the embryo, and this is an important area of future research.

Here we show that parental zinc deficiency causes a significant decline in ZnT8 mRNA expression at 24 and 48 hpf when ZnT8 is expressed in the developing pancreas [39, 75]. ZnT8 has been shown to be involved in insulin processing and dysregulated zinc transporters may lead to glucose tolerance and diabetes [75, 76]. Interestingly, we also found that zinc deficiency is associated with decreased expression of Insa mRNA, and to a lesser degree Insb mRNA levels, primarily at the 48 hour time point. Insa is known to be expressed in the pancreas at 48 hpf and Insb is expressed in the pancreas and the brain [43]. There are limitations to interpreting the effect of these changes in gene expression because suppression of mRNA expression does not always align with a decline in protein expression or function. Never the less, future detailed molecular analysis of zinc deficiencies effects on the development of the pancreas and effects on insulin production is warranted because the decrease in Ins expression that we observe, taken together with the literature, and the decline in the mRNA levels of transcription factors that regulate pancreatic development (Pdx1 and Pax4 also at 48 hour time point), suggest that parental and/or embryonic zinc deficiency may dysregulate normal pancreatic development. This in turn could affect insulin production and glucose metabolism in the zinc deficient embryo which may contribute to diabetes development.

Zinc deficiency is highly prevalent in pregnant women and thus an important public health concern. Overall, we illustrate zinc deficiency in adult zebrafish decreases learning, memory, and fitness. We also show zinc deficiency in adults has both acute and possible long term health consequences for their offspring, like potential increased susceptibility to chronic diseases including diabetes. Taken together our data suggest that supplementation of zinc deficient mothers may be beneficial.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Zinc deficient adults had decreased size, learning and fitness.
- Parental zinc deficiency increased mortality and decreased activity in offspring.
- Zinc deficient embryos had altered expression of genes that regulate metal homeostasis.
- Zinc deficient embryos had decreased expression of genes related to diabetes.
- Zinc deficient embryos had decreased expression of DNA methyltransferases genes.

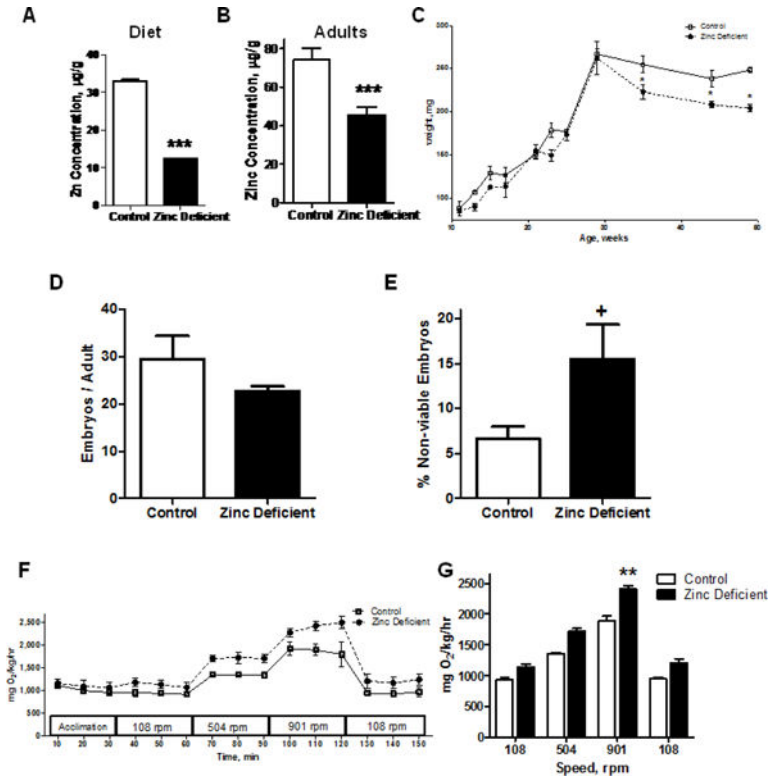


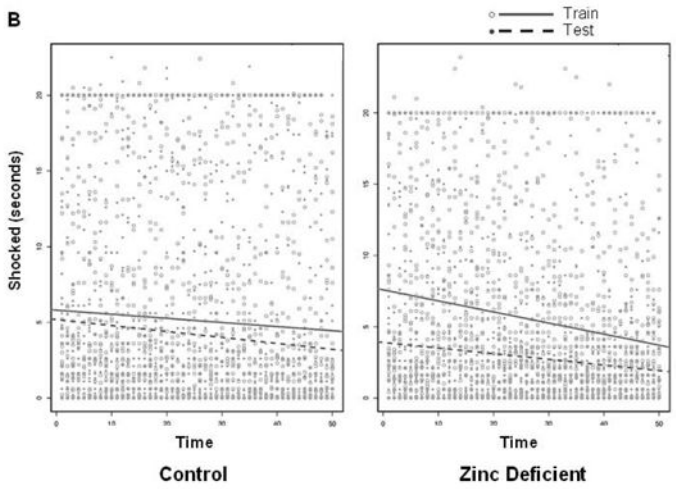
Figure 1. Zinc deficient adult zebrafish are smaller in size and have decreased fitness

A–B) Bars indicate the average amount of zinc (µg) per g of diet (A, n=5) or µg of zinc/g of body weight of fish (B, n=12) as measured by ICP-OES. B) Data was obtained on fish that were 6 months old and had been on the diet for 4 months. C) The average weight of zebrafish was measured over time (n=3–6). D–E) Bars indicate the mean number of embryos/adult fish (D) and % of non-viable embryos at 8 hpf, relative to total eggs produced (E). D–E) Data are from three sequential spawns. F–G) Oxygen consumption was measured using the AutoResp swim tunnel as a measure of fitness in control and zinc deficient adults that were on the diets for 7–8 months. Data is representative of three independent experiments, where n=3–9. F) Data are an average of the three time points for each rpm shown in graph G. A–G) Significant differences between the control and zinc deficient samples were calculated using t-tests (A, B, D, E) or two-way ANOVAs with Bonferroni post-tests (C, G). *, **, and *** indicate significant differences between the groups where $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively, and + indicates p=0.0929.

A

Treatment	Period	Total fish	% Learned	% Failed	% Faulted Out	% Learned - Fault Out	p value
Control	Train	35	34.3	65.7	14.3	40.0	
Zinc Deficient	Train	36	30.6	69.4	11.1	34.4	0.13492
Control	Test	35	51.4	48.6	11.4	58.1	
Zinc Deficient	Test	36	30.6	69.4	11.1	34.4	< 0.0001

B

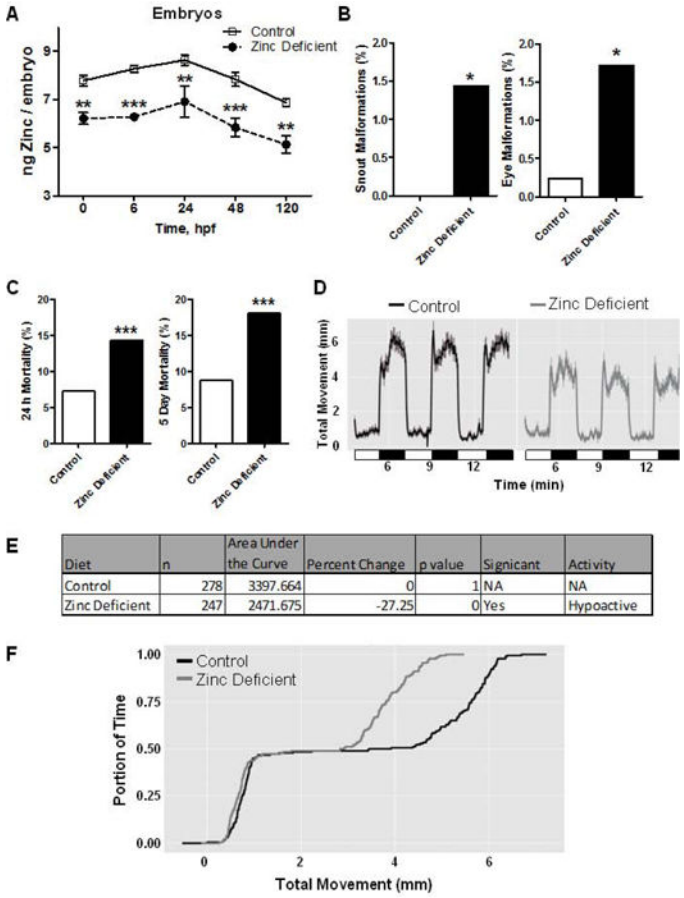


C

Diet	Period	Intercept	Slope
Control	Train	5.807	-0.027
Zinc Deficient	Train	7.589	-0.078
Control	Test	5.181	-0.039
Zinc Deficient	Test	3.902	-0.040

Figure 2. Zinc deficiency results in decreased learning and memory in adult fish

A–C) Zinc deficient and control fish were tested for learning and memory using the shuttle box assay after 7–8 months of being on the control or zinc deficient diet. Fish were evaluated during a training and then testing period, and 50 sequential trials were completed for each period. For humane purposes, a fault out parameter was in place, for animals that did not move into the lighted side over 8 sequential trials. B) Each plot represents the control or zinc deficient diet fed fish that underwent the shuttlebox assay. Data are obtained from 3 independent experiments where n=35–36. A linear regression model was computed for each diet and illustrated on the plot in either a solid or dashed line (the train and test period, respectively). C) For each period, the model parameters are listed where the intercept corresponds to the initial shock time (s) and the slope, representing the learning rate. B–C) Analysis was completed without the animals that faulted out.



Diet	n	Area Under the Curve	Percent Change	p value	Signicant	Activity
Control	278	3397.664	0	1	NA	NA
Zinc Deficient	247	2471.675	-27.25	0	Yes	Hypoactive

Figure 3. Parental zinc deficiency results in zinc deficiency in their offspring, and is associated with increased mortality and hypoactivity

Embryos were collected from control or zinc deficient fish and analyzed for zinc content (A), developmental malformations and mortality (B–C), and larval activity (D–F). A) Zinc was measured in the embryos, at the indicated time points, and points on the graph represent the mean zinc level (± SEM, n=6–9). Significant differences between the control and zinc deficient group was calculated by a two-way ANOVA with Bonferroni post-tests. B–C) Bars represent mean developmental malformations and mortality for 348–456 embryos analyzed over at least 3 independent experiments. Significant differences between groups were calculated with a binomial test that calculates lowest effect levels (LELs) for each endpoint. D–F) Locomotor activity of 5-day old embryos were measured by larval photomotor response assay and the overall area under the curve for movement was compared using a Kolmogorov-Smirnov test. D) The light conditions during the assay are indicated over time by white (light) or black (dark) bars on the x-axis. F) A cumulative distribution plot illustrating the total distance moved overtime. A–F) All data come from at least three independent experiments. *, **, and *** indicates $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively.

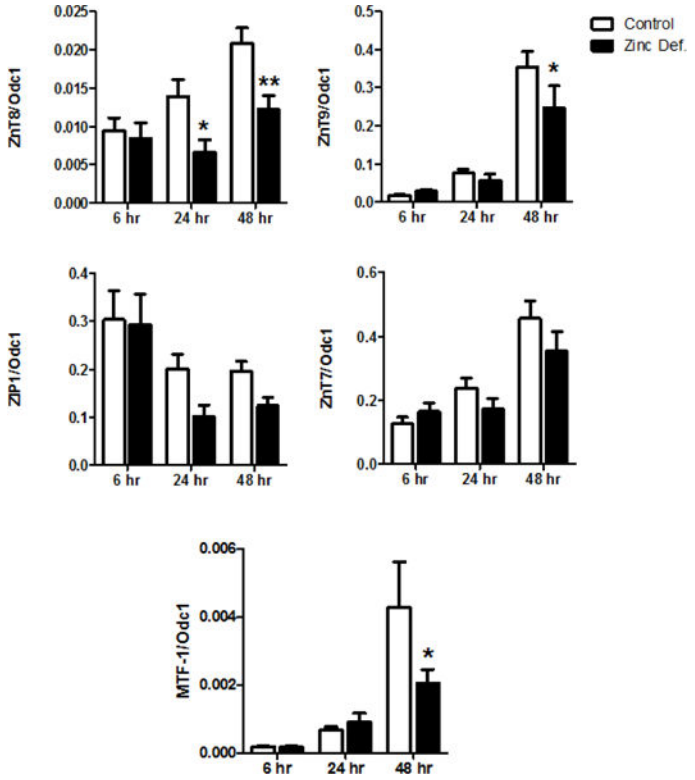


Figure 4. Zinc deficiency decreases the expression of genes that regulate metal homeostasis in embryos

Embryos were collected from control (white bars) or zinc deficient (black bars) fish at 6, 24 and 48 hpf. mRNA expression levels were determined using qPCR for zinc importer (ZIP1), zinc exporters (ZnT8, ZnT9, ZnT7) and metal-regulatory transcription factor 1 (MTF-1). Data represent an average of 9 replicates per treatment group and were obtained from 3 independent experiments. Bars are indicative of the mean ± SEM and significant differences between the control and zinc deficient group were calculated by a two-way ANOVA with Bonferroni post-tests where * indicates $p < 0.05$, and ** indicates $p < 0.01$. Because ZIP1 approached statistical significance with the ANOVA, we performed t-tests for the 24 and 48 hour time points and found significantly lower levels of ZIP1 mRNA with zinc deficiency where p=0.0184 for 24 hpf, and p=0.0136 for 48 hpf.

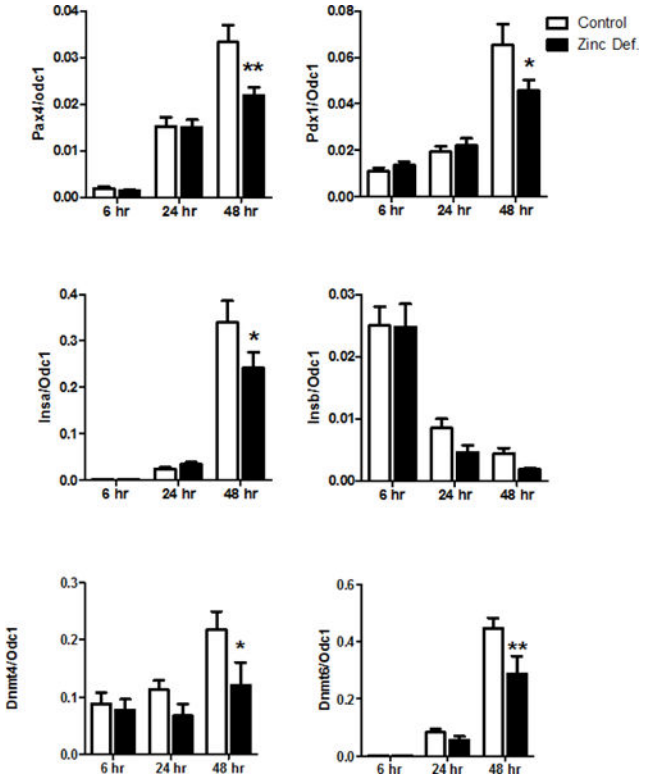


Figure 5. Zinc deficiency decreases the expression of genes that regulate pancreas development, insulin production, and DNA methyltransferases in embryos

Embryos were collected from control (white bars) or zinc deficient (black bars) fish at 6, 24 and 48 hpf. mRNA expression levels were determined using qPCR for Paired box 4 (Pax4), Pancreatic and Duodenal Homeobox 1 (Pdx1) insulin genes (Insa and Insb) and DNA methyltransferases (Dnmt4 and Dnmt6). Data represent an average of 9 replicates per treatment group and were obtained from 3 independent experiments. Bars are indicative of the mean ± SEM and significant differences between the control and zinc deficient group were calculated by a two-way ANOVA with Bonferroni post-tests where * indicates $p < 0.05$, and ** indicates $p < 0.01$. Because Insb approached statistical significance with the ANOVA we performed t-tests for the 24 and 48 hour time points and found significantly lower levels of Insb mRNA with zinc deficiency at the 48 hpf time point where p=0.0086.