


## RESEARCH ARTICLE

# Interacting effects of environmental enrichment across multiple generations on early life phenotypes in zebrafish

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**Abstract**

The environment plays an important role in an individual's development during early life, however, parents may also influence offspring development through so called "parental effects." We examined the effects of environmental enrichment in zebrafish (*Danio rerio*) across two generations through the paternal lineage. Fathers and grandfathers were exposed to either standard or high levels of housing enrichment for 4-weeks during adulthood. First-generation (F1) and second-generation (F2) offspring were obtained from controlled breeding and tested as larvae for changes in morphology at hatching stage (72hpf), and in locomotor activity at larval stage (120hpf) in both generations. We found paternal experience of enrichment resulted in changes in trunk length of F1 offspring and changes in spine curvature and dorsal length of F2 offspring, while changes in snout morphology of F2 offspring seemed to be driven by whether grandpaternal and paternal experience of the environment was matched or not. We found that while paternal enrichment increased the frequency of spontaneous movement in F1 and F2 offspring, interacting effects of paternal and grandpaternal enrichment on movement distance were seen in F2 offspring, and that spontaneous movement and the distance that larvae swam are thus distinct phenotypes that were differentially affected by the experiences of previous paternal generations. Taken together, these findings suggest that the parental and grandparental environment influence zebrafish behavior and morphology. The nature of these effects and the design of this study mean that these phenotypes were likely the result of nongenetic transmission through the paternal germline.

**KEYWORDS**

environmental enrichment, locomotion, morphology, paternal effects, transgenerational effects, zebrafish

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## 1 | INTRODUCTION

Environmental enrichment through greater structural complexity is often used with captive animals to replicate natural environments and is generally considered a positive intervention for animals housed in captivity (Newberry, 1995; Young, 2003). In rodents for example, structural enrichment has been shown to improve well-being, offset neuronal ageing, and modulate social cognition and plasticity (Bayne, 2018; Gubert & Hannan, 2019; Speisman et al., 2013). For fish in captivity, enrichment may come in the form of gravel or sand substrate, artificial vegetation, shelters, tunnels, and novel objects (Näslund & Johnsson, 2016). These have been shown to affect behaviors such as exploration, swimming performance, and agility (Ahlbeck Bergendahl et al., 2017; Arechavala-Lopez et al., 2019), and to have positive effects on neural plasticity, learning ability, and spatial cognition (Salvanes et al., 2013). Zebrafish (*Danio rerio*) have been shown to make decisions with regard to their physical environment, and to favor structural enrichment over barren housing conditions (Schroeder et al., 2014). Enrichment can also have effects when provided in early life, suggesting that developmental trajectories can be influenced by the provision of structural complexity in captivity (Hegde et al., 2020; Zocher et al., 2020) and that phenotype can be influenced by the early physical environment through developmental plasticity (Gilbert, 2001).

As well as an individual's own early life environment, the personal experience of mothers and fathers can also influence offspring development through so-called "parental effects" (Badyaev & Uller, 2009; Uller, 2008). For example, rat mothers that experience environmental enrichment subsequently have offspring with enhanced cognition and neuronal plasticity (Cutuli et al., 2015), effects thought to result from altered maternal care. Variation in maternal care in rodents has effects on multiple offspring phenotypes (Caldji et al., 2000; Champagne et al., 2003), and this phenomenon may represent maternal programming of offspring for the predicted future environment (Bateson et al., 2014). Paternal effects have also been documented in response to different paternal experience, however, as fathers in most species contribute little more to offspring than sperm and do not typically play a role in rearing, their influence is limited to epigenetic marks in the male germline, including DNA methylation patterns and cytoplasmic RNAs (Curley et al., 2011). For example, offspring of male mice fed a low protein diet exhibit metabolic changes in lipid and cholesterol biosynthesis, and elevated expression of genes involved in lipid and cholesterol biosynthesis, changes that are accompanied by differences in cytosine methylation despite no contact between offspring and fathers (Carone et al., 2010). Evidence for the involvement of cytoplasmic RNA in such paternal effects can be seen again in mice, where early life traumatic stress has been found to alter microRNA (miRNA) expression in both fathers and their offspring (Gapp et al., 2014).

Investigation of such paternal effects in many vertebrates is complicated by other sources of influence on offspring, such as maternal effects (pregnancy, egg constituents, hormone allocation (Mashoodh et al., 2018)) or parental care. Investigations in other

vertebrate species, such as the three-spined stickleback, have also shown that effects of paternal experience, for example, due to predation exposure, can be transmitted via sperm to offspring and can affect offspring survival (Hellmann, Bukhari, et al., 2020). Consequently, externally fertilizing species with no paternal or maternal care offer great potential for understanding such effects. The common laboratory model the zebrafish is one such species where these attributes mean that observed paternally inherited effects are likely the result of nongenetic germline inheritance. For example, levels of sperm competition between male zebrafish affect both offspring and sperm traits: high sperm competition resulted in fathers that produced faster and more motile sperm, and whose offspring hatched faster but had lower survival (Zajitschek et al., 2014). Variation in paternal social status has also been shown to not only influence sperm traits, but also affect offspring behavior, with activity levels elevated among offspring of dominant males (Zajitschek et al., 2017). Sperm from male zebrafish exposed to paternal stress exhibit alterations in levels of small noncoding RNA and are accompanied by changes in offspring stress response (Ord et al., 2020), suggesting that such paternal experiences are transmissible via sperm contents.

The aim of this study was to examine the multigenerational effects of different levels of structural enrichment experienced by fathers and grandfathers, in first-generation (F1) and second-generation (F2) zebrafish larvae. We used externally fertilizing zebrafish and a split clutch design to investigate interactions between parental and grandparental effects, and focused specifically on paternal inheritance by only manipulating the fathers and by controlling for maternal effects. We first compared F1 offspring whose fathers experienced standard (STD) or enriched (ENR) housing environments. Then, a full factorial  $2 \times 2$  design was used to measure the effects on F2 offspring of exposing both preceding generations to standard or enriched housing, resulting in four treatment groups based on grandpaternal-paternal experience (STD-STD, STD-ENR, ENR-STD, and ENR-ENR). Larvae were examined using two approaches: 1) examination of body morphology after hatching at 72 hours post fertilisation (hpf), and 2) quantification of locomotor behavior at 120hpf. These traits were selected as physical enrichment has been shown to influence swimming behavior in fish (Ahlbeck Bergendahl et al., 2017; Jones et al., 2021), effects which we predicted might be inherited given that larval zebrafish hatch into the same physical environment as their parents. As larval swimming is affected by body shape (McHenry & Lauder, 2006) and timing of developmental events, for example, swim bladder inflation (Lindsey et al., 2010), we also measured changes in morphology.

If transgenerational effects occurred as a result of paternal enrichment alone, morphology or locomotion differences would be detectable between F1 offspring groups, with no effect of grandpaternal enrichment in F2 offspring groups. If, however, phenotypes were influenced by both paternal and grandpaternal enrichment, we would also expect to see multi-generational effects, which could be either cumulative (Lock, 2012) or interacting (Shama & Wegner, 2014). Cumulative effects could for example lead to phenotypes of the F2 larvae being most different between

STD-STD larvae and ENR-ENR larvae, with ENR-STD and STD-ENR larvae intermediate between them. Interactions between grandpaternal and paternal experience could occur in a number of ways, for example different paternal experiences might mask the effects of grandpaternal experience, or even reverse it (Bell & Hellmann, 2019).

## 2 | RESULTS

### 2.1 | F1 morphology

The main sources of body shape variation in F1 offspring were spine curvature (F1-PW1), snout elongation (F1-PW2) and trunk length (F1-PW3), and F1 offspring of enriched fathers had longer trunks than offspring of control fathers.

For F1 offspring, images of 95 standard and 94 enriched larvae were analyzed. Eleven images were excluded due to larvae being misoriented. The first three partial warps cumulatively accounted for 65.34% of total F1 body shape variation (F1-PW1 = 31.20%, F1-PW2 = 17.86%, F1-PW3 = 16.32%). F1-PW1 explained the curvature of the spine in the sample images; positive scores described arching of the spine, and negative scores inverted arching. F1-PW2 explained elongation of the snout; positive scores described protrusion and negative scores compression of the snout. F1-PW3 explained the length of the trunk, as indicated by both ventral and dorsal length. Positive scores described a shorter trunk and negative scores a longer trunk.

The mixed-effects models fitted to the partial warp scores indicated that there was no effect of paternal enrichment on F1-PW1 (spine

curvature;  $\chi^2 = 1.143$ ,  $DF = 1$ ,  $p = 0.285$ ). Similarly, there also appeared to be no significant effect of paternal enrichment on F1-PW2 (snout protrusion;  $\chi^2 = 2.96$ ,  $DF = 1$ ,  $p = 0.052$ ). However, there was a significant effect of paternal enrichment on F1-PW3 (trunk length;  $\chi^2 = 11.849$ ,  $DF = 1$ ,  $p < 0.001$ ): offspring from enriched fathers had significantly longer trunks than those from standard fathers (Figure 1a).

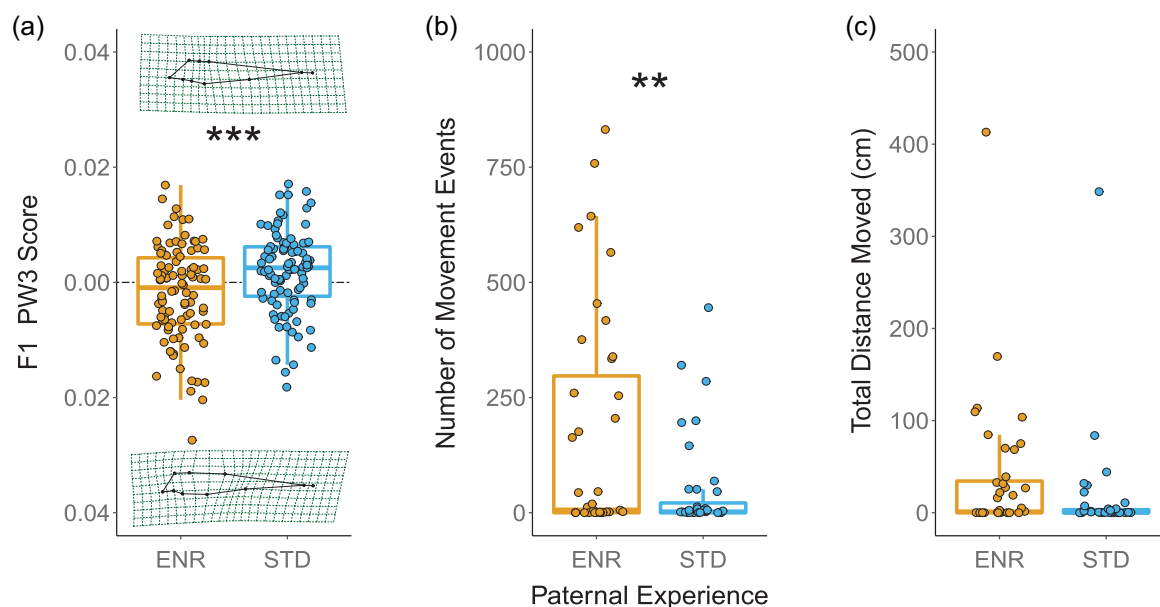
### 2.2 | F1 movement

Paternal enrichment affected locomotor activity as F1 offspring from enriched fathers initiated movement more frequently than F1 offspring from control fathers.

The movement of 40 standard and 40 enriched F1 offspring was analyzed, showing that there was a significant effect of paternal enrichment on the number of movement events ( $\chi^2 = 6.633$ ,  $DF = 1$ ,  $p = 0.010$ , Figure 1b): offspring of enriched fathers exposed to enriched housing conditions moved significantly more frequently than those from standard fathers. However, there was not a significant effect of paternal enrichment on the total distance moved ( $\chi^2 = 2.932$ ,  $DF = 1$ ,  $p = 0.071$ ; Figure 1c).

### 2.3 | F2 morphology

The main sources of body shape variation in F2 offspring were snout protrusion (F2-PW1), curvature of the spine (F2-PW2), and dorsal length (F2-PW3). F2 offspring with matching paternal and grandpaternal



**FIGURE 1** Effects of paternal enrichment on F1 larvae morphology and locomotion. (a) Offspring of enriched (ENR) fathers ( $n = 94$ ) displayed increased trunk length in partial warp F1-PW3 compared to those from standard (STD) fathers ( $n = 95$ ). Trunk length is defined as the distance between landmarks 8 and 9 and between landmarks 3 and 4. Illustrative shape deformations of the most positive and negative observed scores for F1-PW3 are shown. (b) Offspring from ENR fathers ( $n = 40$ ) also moved more frequently than offspring from STD fathers ( $n = 40$ ). (c) Total distance moved was not different between the two conditions. Box-and-whisker plots of median, IQR and 1.5X IQR are shown, with overlaid raw data.

backgrounds displayed increased snout protrusion compared to those with mismatched paternal and grandpaternal backgrounds. F2 offspring from enriched fathers also exhibited reduced dorsal length and increased upward arching of the spine relative to those from standard-housed fathers, however, grandpaternal experience did not affect these partial warps.

Images of 37 STD-STD, 40 STD-ENR, 39 ENR-STD, and 40 ENR-ENR F2 offspring were analyzed. Four images were excluded due to misorientation of the larvae. The first three partial warps cumulatively accounted for 67.51% of total F2 body shape variation (F2-PW1 = 38.89%, F2-PW2 = 17.55%, F2-PW3 = 11.07%). F2-PW1 explained elongation of the snout: positive scores described compression and negative scores protrusion of the snout. F2-PW2 described curvature of the spine: positive scores indicated inverted arching and negative scores an arched spine. F2-PW3 was associated with dorsal length: positive scores indicated longer dorsal length and negative scores shorter dorsal length.

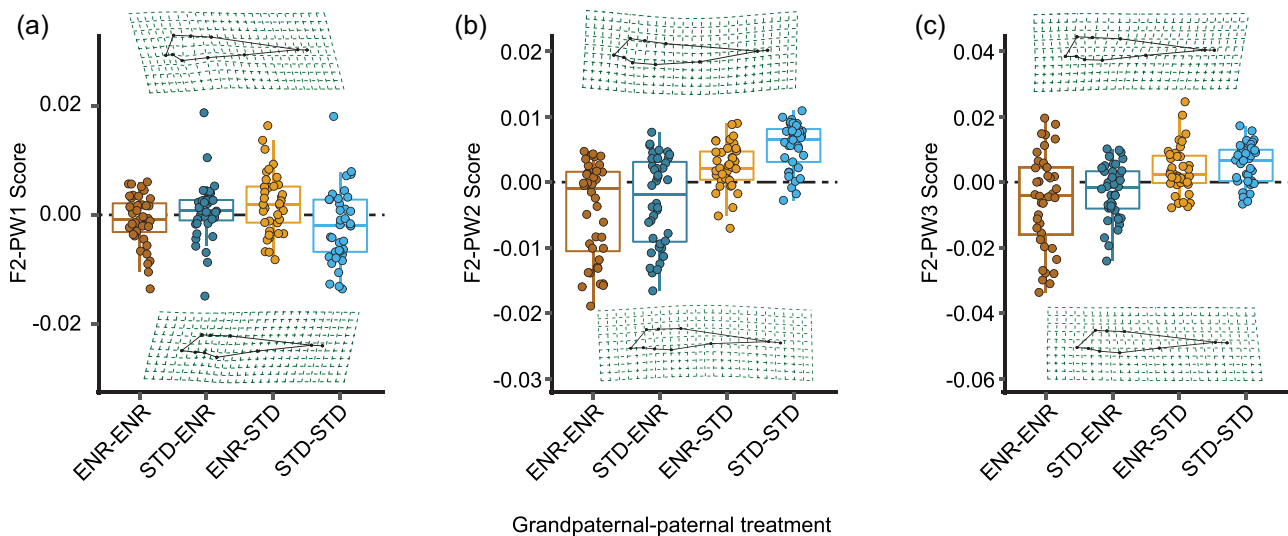
In F2 larvae, although there were no main effects of grandpaternal ( $\chi^2 = 1.86$ , DF = 1,  $p = 0.173$ ) or paternal ( $\chi^2 = 0.044$ , DF = 1,  $p = 0.833$ ) enrichment on F2-PW1 (snout protrusion), there was a significant interaction effect ( $\chi^2 = 11.544$ , DF = 1,  $p < 0.001$ ) between these two factors (Figure 2a). When grandpaternal and paternal environments matched, larvae displayed increased snout protrusion. Conversely, when there was mismatch between grandpaternal and paternal environment, larvae displayed reduced snout protrusion. There was a significant main effect of paternal environment on F2-PW2 (arching of the spine) in F2 larvae ( $\chi^2 = 34.54$ , DF = 1,  $p < 0.001$ ): F2 larvae from enriched F1 fathers had more arched spines, whereas F2 offspring from standard F1 fathers had inverted arching of the spine (Figure 2b). There was no main effect of grandpaternal

environment ( $\chi^2 = 3.281$ , DF = 1,  $p = 0.701$ ) and no interaction effect (Chisq = 0.45, DF = 1,  $p = 0.504$ ) on F2-PW2. There was also a significant main effect of paternal environment on F2-PW3 (dorsal length) in F2 offspring ( $\chi^2 = 15.58$ , DF = 1,  $p < 0.001$ ). Offspring from standard fathers exhibited increased dorsal length than offspring from enriched fathers (Figure 2c). There was no effect of grandpaternal environment ( $\chi^2 = 1.060$ , DF = 1,  $p = 0.303$ ) and no interaction ( $\chi^2 = 0.039$ , DF = 1,  $p = 0.842$ ).

## 2.4 | F2 movement

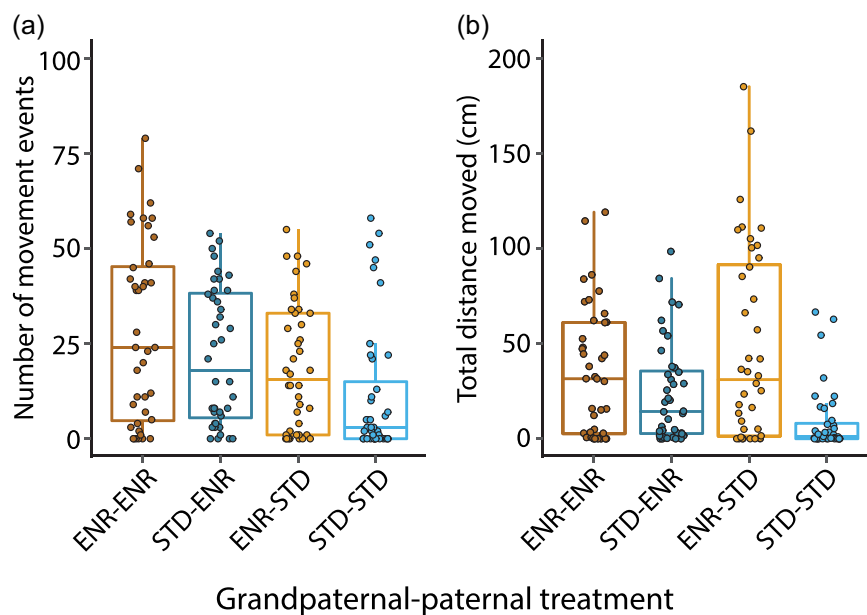
Total distance moved by F2 larvae was affected by an interaction between paternal and grandpaternal experience as grandpaternal enrichment increased movement distance in the offspring of standard fathers, but not in the offspring of enriched fathers. F2 larvae moved more frequently if their father experienced enrichment rather than standard housing.

Forty F2 offspring from each of the four experimental treatment groups were tested for locomotor activity. We found a significant interaction between grandpaternal and paternal environment with regard to total distance traveled ( $\chi^2 = 6.28$ , DF = 1,  $p = 0.012$ ). Among the F2 offspring of standard-housed F1 fathers, larvae whose grandfathers experienced enrichment (ENR-STD) swam further than those whose grandfathers experienced standard housing (STD-STD). In contrast, there was a far smaller difference between F2 offspring of enriched fathers whose grandfathers experienced enriched (ENR-ENR) versus standard (STD-ENR) housing (Figure 3a). There was a significant effect of grandpaternal environment ( $\chi^2 = 7.00$ , DF = 1,  $p = 0.010$ ) but no effect of paternal environment on total distance



**FIGURE 2** Effects of paternal and grandpaternal enrichment on F2 larval morphology in partial warps F2-PW1 (a), F2-PW2 (b) and F2-PW3 (c). F2 offspring from matching ancestral conditions exhibited reduced snout protrusion compared with those from mismatched backgrounds (a). F2 offspring from enriched (ENR) fathers had arched spines (b) and shorter dorsal length (c) relative to those from standard (STD) fathers. Images of  $n = 37$  STD-STD,  $n = 40$  STD-ENR,  $n = 39$  ENR-STD, and  $n = 40$  ENR-ENR F2 offspring were analyzed. Box-and-whisker plots of median, IQR, and 1.5x IQR are shown, with overlaid raw data. Illustrative shape deformations of the most positive and negative scores for each partial warp are shown.

**FIGURE 3** Effects of paternal and grandpaternal enrichment on number of movement events (a) and distance moved (b) in F2 larvae. (a) F2 offspring of enriched (ENR) fathers moved more frequently than those from standard (STD) fathers. (b) F2 offspring from STD fathers differed in total distance moved according to their grandfathers' experience, while total distance moved by F2 offspring from ENR fathers was less affected by grandfathers' experience. Movement was analyzed in  $n = 40$  larvae for each F2 condition (STD-STD, STD-ENR, ENR-STD, and ENR-ENR). Box-and-whisker plots of median, IQR, and 1.5x IQR are shown, with overlaid raw data.



traveled ( $\chi^2 = 3.35$ ,  $DF = 1$ ,  $p = 0.067$ ). We also found a significant effect of paternal environment on the number of movement events ( $\chi^2 = 7.60$ ,  $DF = 1$ ,  $p = 0.010$ ): offspring of enriched fathers moved more frequently than those of standard fathers (Figure 3b). There was not a significant effect of grandpaternal environment ( $\chi^2 = 2.41$ ,  $DF = 1$ ,  $p = 0.121$ ) or interaction effect ( $\chi^2 = 0.68$ ,  $DF = 1$ ,  $p = 0.410$ ).

### 3 | DISCUSSION

Our results suggest that variation in the physical environment experienced by both fathers and grandfathers can influence early life phenotypes in zebrafish larvae. This manifested as changes in locomotor activity at 5dpf and changes in body shape at 72hpf in both F1 and F2 offspring. Moreover, we found significant interactions between grandpaternal and paternal experience affecting both locomotion and morphology in F2 larvae. For morphology, these seem to be driven by whether grandpaternal and paternal enrichment regimes were matched or not: F2 offspring whose grandfathers and fathers experienced the same enrichment regime, whether enriched or standard, exhibited increased snout protrusion compared to offspring from mismatched grandpaternal-paternal backgrounds, as indicated by the results obtained for F2-PW1. For movement behavior of F2 larvae, grandpaternal enrichment was associated with longer movement duration in offspring whose fathers experienced standard housing, however paternal experience of enrichment appeared to mask this grandpaternal effect.

Previous theoretical work has suggested that transgenerational effects have the potential to be detrimental to offspring when parental conditions are mismatched to their own (Dewitt et al., 1998; Uller et al., 2013) and some empirical work supports this. In guppies (*Poecilia reticulata*), offspring whose temperature regime was either matched or mismatched to that of their parents exhibited maximum swimming

performance when parental and offspring acclimation temperatures matched (Le Roy & Seebacher, 2018). In sticklebacks (*Gasterosteus aculeatus*), male mating success is higher when paternal and offspring thermal experience is matched, although this is also modulated by developmental temperature (Fuxjäger et al., 2019). Other empirical evidence has suggested that matches or mismatches between paternal and maternal environments are also important for offspring development (Hellmann, Bukhari, et al., 2020). For example, in the mosquito (*Aedes aegypti*), parents who were exposed to similar nutritional conditions sired offspring who developed more quickly than those exposed to dissimilar nutrient conditions (Zirbel & Alto, 2018). In sticklebacks, exposure of a single parent to a model predator-induced changes in sexual selection via mate preference (Lehto & Tinghitella, 2020), changes which were reversed when both parents were exposed, suggesting that paternal and maternal effects may not necessarily combine additively to produce parental effects. We were interested in the effects of contrasting environments in two ancestral generations, rather than between offspring and their parents, or between parents, and our results indicate that offspring phenotypes are indeed sensitive to whether the experiences of preceding generations are consistent or not. We did not assay larval fitness directly, so we cannot determine whether the changes in larval development and movement we observed as a result of mismatched paternal and grandpaternal experience are maladaptive. However growth and swimming are critical phenotypes for larval zebrafish, and both are sensitive to the physical environments experienced by the previous two male generations. As zebrafish are an externally fertilizing species with no parental care (unlike guppies and sticklebacks respectively), the phenotypes we observed cannot be the result of parental influences arising from gestation or rearing, but instead are likely to result from nongenetic inheritance via the male germline (Curley et al., 2011).

The main areas of morphological variation were the shape of the snout, curvature of the spine, and trunk and dorsal length. The



associated partial warps were found to be the largest sources of body shape variation in both F1 and F2 offspring, although the sensitivity of these to ancestral enrichment varied between generations. Among F1 larvae, paternal enrichment affected only the partial warp for trunk length (F1-PW3), which accounted for the least variation in body shape of the three partial warps we examined. In contrast, all three partial warps were affected by ancestral experience in F2 larvae, with the experience of both preceding generations influencing snout shape (F2-PW1), which accounted for the greatest proportion of variation in body shape of the three F2 partial warps. Variation in this dimension is interesting as during zebrafish embryonic development, a prominent morphological feature at the end of the 72hpf hatching period is the protruding snout, which must rapidly elongate to form the mouth (Kimmel et al., 1995). This key developmental step permits independent feeding to commence as soon as the yolk is exhausted and involves a series of morphological changes as the cranial architecture forms (Hernández et al., 2002), and this observed interaction between grandpaternal and paternal enrichment may represent a subtle but potentially significant shift in development, as mouth gape size and pharyngeal shape are known limiting factors for food selection and feeding in fish larvae (Rønnestad et al., 2013). Parentally programmed changes in development have previously been observed in response to varying thermal regimes in sheepshead minnows (Salinas & Munch, 2012): maximal offspring growth was seen when parents had previously experienced temperatures matching those of the offspring. As both parents in this study were exposed to the thermal regimes however, inheritance could have occurred through either the paternal or the maternal lineage, for example as a result of changes during egg formation (Fellous et al., 2022), while the effects we see can only result from paternal effects.

We also saw that F1 offspring from enriched paternal backgrounds displayed a high activity phenotype, transitioning from stationary to active significantly more often than offspring from standard paternal backgrounds. Experience of enrichment improves adult swimming performance (Ahlbeck Bergendahl et al., 2017), but these results suggest adult enrichment also influences offspring locomotion. While we cannot rule out the possibility that these changes in locomotor activity are a consequence of changes in stress responsiveness due to the enrichment treatments, in a separate study we found no effects of enrichment on adult stress responses in open tank trials (unpublished data), and the larval swimming phenotypes we report are consistent with effects of enrichment on adult swimming behavior. A similar result was found in F2 offspring, where movement events were more frequent in offspring of enriched fathers. Interestingly, there may have been a cumulative effect here, as the highest frequency of movement was in subjects whose fathers and grandfathers both experienced enrichment, and the lowest in those whose fathers and grandfathers both experienced standard housing. These findings contrast with the swimming distance results, indicating clear differences between our two measures of behavior in larval responses to paternal and grandpaternal experience. F1 subjects did not differ in swimming distance as a result of paternal experience, however, in F2 offspring there was a significant

interaction between paternal and grandpaternal experience. F2 offspring whose fathers experienced standard housing swam much further if their grandfather experienced enrichment instead of standard housing, while this grandpaternal effect was absent in subjects whose fathers experienced enriched housing. This suggests that while structural complexity of the paternal and grandpaternal environment influence swimming behavior in zebrafish larvae, the frequency, and distance of swimming are distinct phenotypes that are differentially sensitive to ancestral experiences. Grandpaternal and paternal experience has also recently been reported to affect activity in sticklebacks. Sons and granddaughters of predator-exposed males exhibit changes in activity phenotypes (Hellmann, Carlson, et al., 2020), indicating that these effects can also vary depending on an individual's sex. It is not clear whether grandpaternal and paternal experience interact in sticklebacks, however, findings such as these and our own suggest that different aspects of paternal and grandpaternal experience can influence locomotion and activity.

While the effects of ancestral enrichment on swimming behavior were consistent in both F1 and F2 offspring, morphological responses to enrichment were seemingly less so. For example, F1 larvae whose fathers experienced enrichment had increased trunk length (F1-PW3), while F2 larvae whose fathers experienced enrichment had decreased dorsal length (F2-PW3). This is somewhat surprising, however, it is important to note that the partial warps for each generation cannot be directly compared across generations. The partial warps were computed independently within each generation from the variation among all sampled individuals with these separate generations. Although F1-PW3 and F2-PW3 both included changes in length, they are different measures of shape change with distinct and separate characteristics, for example, they differ considerably in how much variation in ventral length and in rostral-caudal body angle they each involve.

We deliberately employed a relatively simple environmental manipulation to try to determine how ecologically relevant experience, such as variation in the structural complexity of the environment, might impact phenotypes across generations. In sticklebacks, paternal effects have been recently shown to mediate responses to potential predators, with stronger responses seen when fathers experience more ecologically relevant predator stimuli (Chen et al., 2021). In the mangrove killifish (*Kryptolebias marmoratus*), manipulation of ecologically relevant conditions in parents has also been shown to exert transgenerational effects, as offspring from parents reared in enriched environments displayed higher activity levels regardless of their own environment (Berbel-Filho et al., 2020). While transgenerational effects on larval locomotor activity and behavior in zebrafish have been reported, these have often been the result of aggressive manipulations, such as parental exposure to toxins or ionizing radiation (Huang et al., 2020; Hurem et al., 2018; Lombó et al., 2015). It is interesting then that despite using a much less aggressive experimental manipulation, we still detected clear transgenerational effects on locomotor activity. This fits with other work in zebrafish that has demonstrated that naturalistic manipulations, such as social status or sexual competition, can induce

transgenerational effects during early development (Zajitschek et al., 2014; Zajitschek et al., 2017). However, our results show that effects are not limited to the experience of fathers but also those of grandfathers, and that information from both these sources can be integrated into the phenotypes of zebrafish larvae.

While the importance of enrichment for mammals in captivity has been understood for some time (Young, 2003), it is increasingly clear that for other vertebrates, including fish, enrichment is important not just for animal welfare (Schroeder et al., 2014; Stevens et al., 2021), but also for improving the reliability of research findings (Jones et al., 2021). The experience of animal research subjects before study has become a topic of recent discussion, and the importance of considering sources of potential sampling bias and thus improving research is increasingly understood (Webster & Rutz, 2020). Our findings show that animals' phenotypes are not only influenced by their own experiences of the physical environment, but also by that experienced across multiple previous generations. As such, researchers may need to consider the ancestral history, as well as the direct experiences, of their subjects to improve study reliability and minimize sampling bias. Enrichment is a positive measure that enhances animal welfare in captivity, and our findings suggest that it should be implemented not just for study subjects but also the stock populations they are drawn from to avoid unwanted effects arising from the experiences of preceding generations.

## 4 | CONCLUSION

Our results indicate that the experiences of male zebrafish can influence phenotypes of offspring and grand-offspring in response to relatively subtle variations in environmental enrichment. This is particularly interesting as males in this externally fertilizing, non-parental species contribute little more than genetic material and its accompanying epigenetic patterns to the next generation, thus the inheritance effects we saw likely resulted from nongenetic transmission via the male germline. These results indicate that both behavior and morphology are labile traits that vary according to the environment experienced by fathers and grandfathers and that matches or mismatches between these ancestral experiences can drive variation in these phenotypes.

## 5 | MATERIALS AND METHODS

### 5.1 | Animals and husbandry

Wildtype zebrafish used to start this study were taken from our stock population at Liverpool John Moores University, originally established with larvae obtained from the University of Manchester. Fish were housed in 5 L polycarbonate tanks (dimensions: 176(W) × 325(L) × 150(H) mm) arranged on racks with constant recirculation of water via a sump, with both biological and particulate filtration. Standard housing density was 10 fish per tank and the

facilities featured a 14 h/10 h light/dark cycle and a constant water temperature of 28°C. All fish were fed once per day with ground-dried flake food and once with live *Artemia* nauplii. All experimental procedures had received approval from the Liverpool John Moores University Animal Welfare and Ethics Review Board.

### 5.2 | Experimental design

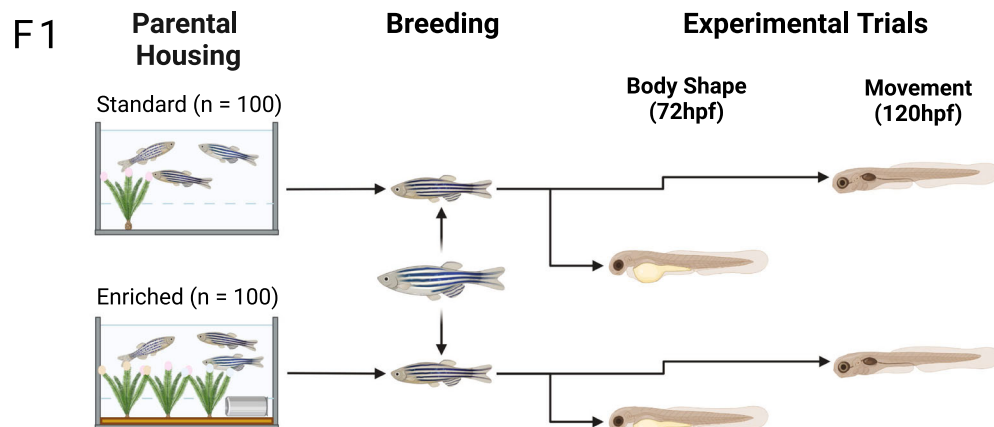
Adult male zebrafish at 8 months of age were randomly selected from our stock population and allocated to either standard or enriched housing for 4 weeks. Stock fish were housed in the same conditions as for our standard housing treatment, which consisted of a 5 L polycarbonate tank with a single artificial plant (green with orange flowers) as per UK Home Office minimum enrichment requirements for laboratory zebrafish. Ten standard tanks of 10 adult fish were set up, each containing one plastic plant (green with orange flowers). Ten enriched tanks of 10 fish were set up, each containing gravel substrate, three plastic plants (green with orange, pink and blue flowers), and a gray PVC tunnel shelter. After 4 weeks of experimental housing, a single male from each tank was randomly selected for breeding and paired with a single female for breeding of F1 offspring. These 8-month-old females were randomly drawn from our stock population for use in breeding. Each female was paired up in separate matings with a male from each housing condition, so that the female background was controlled across both treatments.

The F1 offspring from these matings were phenotyped as larvae for body shape and movement and then males of each clutch were reared to adulthood. At 4 months of age, these F1 males from standard (STD) and enriched (ENR) paternal backgrounds were then split and allocated to standard or enriched housing for 4 weeks. They were then paired up for breeding with 4-month-old females in the same way as described above. The resulting F2 offspring thus came from four different possible paternal-grandpaternal backgrounds: STD-STD, STD-ENR, ENR-STD, and ENR-ENR. These F2 offspring were then also phenotyped as larvae for body shape and movement in the same way as F1 offspring (see Figure 4 for an experimental overview).

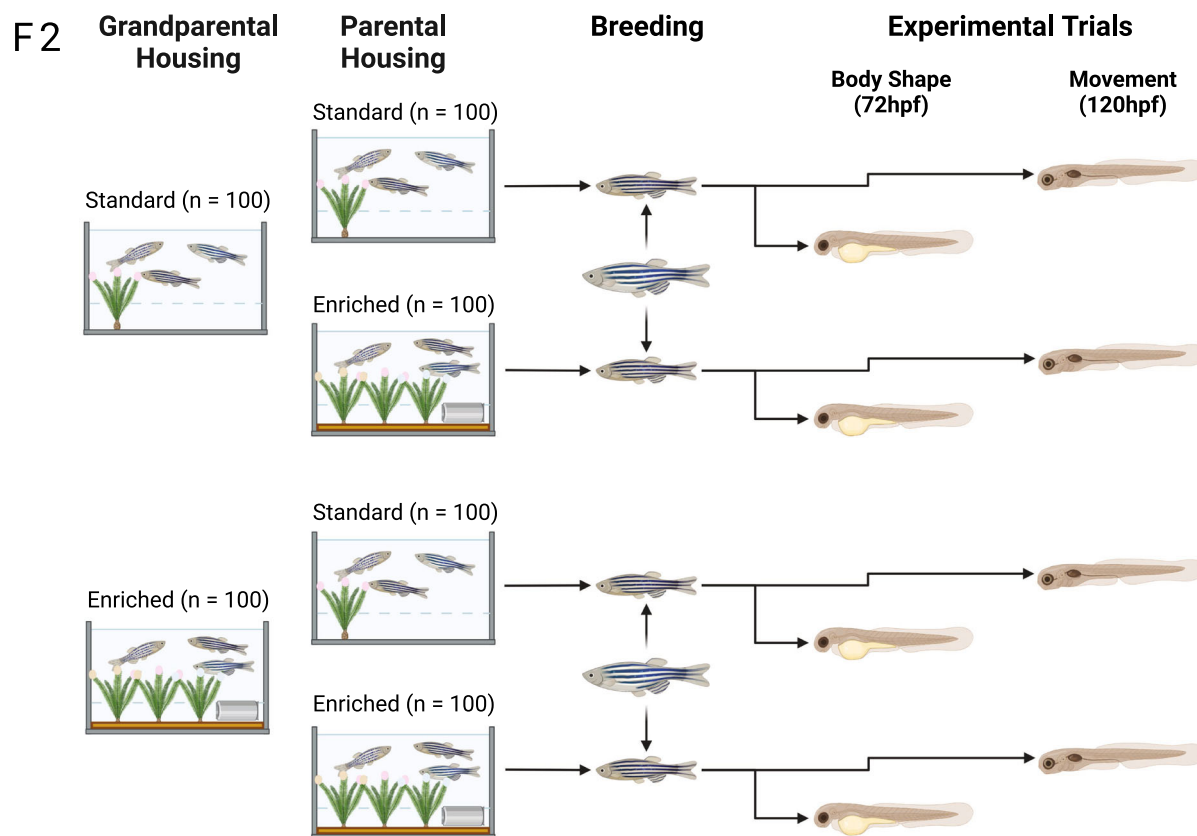
### 5.3 | Breeding

All matings were performed with a single experimental male and a single non-experimental female, referred to here as "mating pairs." This was used control for mate choice and dominance effects that are present when using mass mating techniques. A single father from each experimental tank was randomly chosen for mating. Mating pairs were moved at 8 am (30 min before lights came on) to an empty 5 L breeding tank with suspended netting through which eggs could fall. Pairs were left undisturbed for two hours and then checked every hour for the presence of eggs at the base of the tank. When eggs were observed, the mating pair was placed back into their respective housing tanks. Eggs were collected and transferred into a

(a)



(b)



**FIGURE 4** Experimental overview for F1 offspring (a) and F2 offspring (b). Fathers and grandfathers were exposed to either enriched (ENR) or standard housing (STD) for 4 weeks. After controlled breeding with a separate cohort of females, offspring were obtained and used in morphometric analysis at 72hpf and locomotion trials at 120hpf. Created with [BioRender.com](https://BioRender.com).

petri dish containing 0.0001% methylene blue in conditioned water. Embryos were incubated in separate dishes per clutch at 28°C on a 14 h/10 h light/dark cycle at a maximum density of 100 eggs per dish. Once larvae had hatched at approximately 3 days post-fertilization (dpf) they were moved to fresh conditioned water in Petri dishes until 5dpf. F1 larvae that were not

used for body shape measurement were transferred at 5dpf to standard 5 L tanks, fed daily with zebrafish fry food (ZM Systems), and reared to adulthood. Both F1 and F2 larvae were housed in rearing tanks until adulthood, these were identical to our standard housing condition, with the exception of the flow rate of water into the tank being greatly reduced.



## 5.4 | Imaging of larval body shape

Whole-body imaging was performed at 72hpf. A total of 20 F1 clutches were obtained, 10 from F0 standard fathers and 10 from F0 enriched fathers, and 10 larva were randomly selected from each clutch and imaged. A total of 36 F2 clutches were obtained from F1 fathers, eight clutches from each of the four experimental groups (STD-STD, STD-ENR, ENR-STD, and ENR-ENR) and five larvae were taken from each clutch and imaged.

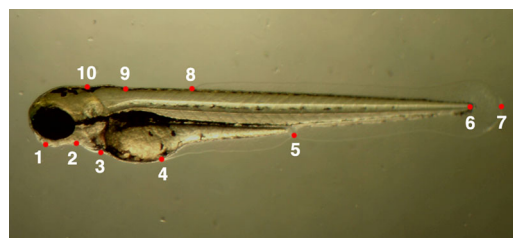
Imaging was carried out with a Leica M50 dissection microscope (Leica Microsystems) fitted with a GXCAM-EYE-5 eyepiece camera (GT Vision). Each larva was moved into a petri dish containing ice water for 5 min before imaging, then moved onto a clean slide and excess water removed. A single drop of 3% methylcellulose solution was placed directly on top of each larva. Larvae were oriented so that they were always imaged on the lateral side, in full extension and with the body straight. Images were captured in 1920 × 2560 pixel resolution using GXCapture imaging software (GT Vision). Larvae used for body shape measurement were euthanized immediately following imaging.

## 5.5 | Movement trials

Movement trials were performed at 120hpf. In F1 offspring, five larvae were taken from each of eight standard and eight enriched clutches. In F2 offspring, five larvae were taken from each of 32 clutches (eight each for STD-STD, STD-ENR, ENR-STD, and ENR-ENR). Movement was recorded using a 24-well assay plate with opaque black sides and transparent flat bases in each circular well. Individual larvae were placed in the center five wells, the assay plate was placed on top of a MiniSun A3 light pad (Minisun) and the behavior of the larvae was recorded for 15 min using a Sony PJ410 camcorder (Sony Europe B.V.) suspended directly above. The first five minutes of each trial were treated as habituation and ignored, movement was measured in the following 10 min. Larvae were rehoused with their clutches after each trial. Movement of individual larvae was tracked using the idTracker application (Pérez-Escudero et al., 2014). A custom MATLAB (MathWorks) script was written to extract x-y coordinates from every frame for each larva, and from this the total distance moved by each larva and the number of transitions from stationary to active was calculated. Scripts were written to ensure that position jumps (periods where a larvae would drift and then start moving again) were corrected for and all larval path detections were in the correct wells.

## 5.6 | Data analysis: Morphology

Morphology was examined using geometric morphometric analysis of the images of larvae. Digitization of 10 pre-defined landmarks (Figure 5) was performed using tpsDig232 ver. 2.31 (Rohlf, 2016a). These landmarks were chosen to capture key morphological features



**FIGURE 5** Landmark configuration used for geometric morphometric analysis. Ten landmarks were placed on images of 72hpf larvae and used for geometric morphometric analysis of body shape: 1) tip of the snout; 2) rear of the jaw; 3) ventral junction of heart/yolk sac; 4) junction of ventral fin/yolk sac; 5) urinary tract opening; 6) caudal tip of the tail; 7) tip of caudal fin; 8) dorsal fin insertion; 9) caudal point of dorsal hump; 10) nape.

in development and to retain the whole body shape. The consensus configuration of landmarks across all samples was calculated using the generalized orthogonal least-squares Procrustes (GPA) method of superimposition. A relative warp analysis was then performed on aligned landmark coordinates using tpsRelw32 ver.1.69 (Rohlf, 2016b). A matrix of partial warp (PW) scores was extracted and used in further statistical tests to examine differences in shape deformations between experimental groups (Rohlf et al., 1996). Principle warps were generated separately for F1 and F2 offspring using all sampled individuals from all experimental groups, and so within each generation, the principle warp loadings between groups were the same. Shape deformation grids were plotted in R (R Core Team, 2022) using the “geomorph” package (Adams & Otárola-Castillo, 2013).

Separate linear mixed-effects models were fitted to the scores for the first three partial warps for the F1 and the F2 larvae using the “lme4” package (Bates et al., 2015) in R. The first three partial warps were chosen as they cumulatively accounted for over 65% of total shape variation and individually accounted for at least 10% of the total shape variation at each larval generation. In F1 offspring, paternal experience was included in the models as a single fixed factor, paternal and maternal identity were included as random effects to control for similarities between offspring from the same lineages. In F2 offspring, paternal experience and grandpaternal experience were included as separate fixed factors and an interaction term was included to examine the relationship between these factors. Paternal, maternal, and grandpaternal identity were included as random effects. Type III Wald chi square tests were performed to determine the significance of model terms.

## 5.7 | Data analysis: Movement

Two behavioral measures were obtained for each subject, total distance moved (cm) and movement events (number of transitions from stationary to active). Some larvae did not move at all and so data were not normally distributed. Both measures were therefore

analyzed using generalized linear mixed-effects models with a negative binomial distribution to account for the overdispersion present in the data. These models were fitted using the “lme4” (Bates et al., 2015) and “MASS” (Venables & Ripley, 2002) packages in R. In F1 offspring, paternal experience was included as a single fixed factor, paternal and maternal identity were included as random effects to account for any similarities between subjects from the same lineage. In F2 offspring two fixed factors were included, paternal and grandpaternal experience, as well as paternal, maternal and grandpaternal identity as random factors. Type III Wald chi square tests were performed to assess the significance of all model terms. All movement and morphology models were assessed using the “performance” package (Lüdecke et al., 2021) in R to check that model assumptions were met. Overdispersion, zero inflation, variance and normality of residuals, and normality of random effects were assessed as appropriate using both visual methods and formal tests and found to be well within tolerances of the chosen models.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data and analysis code for this manuscript are available at <https://github.com/greenm91/zf-larva-data>.

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