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Research Article

Born in complexity: How the early life environment shapes zebrafish larvae phenotype

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ABSTRACT

The biotic and abiotic environments are potent drivers of individual plasticity. Zebrafish, a popular research model around the world, has been extensively used for understanding the proximate cause and mechanisms of phenotypic plasticity to environmental enrichment. Despite awareness of providing high-standard rearing condition for increasing fish welfare, rearing of zebrafish larvae involves little more than stable temperature and daily water changes. We asked whether including Lego bricks as additional visual and mechanosensory stimuli during early rearing would induce developmental plasticity across morphological (growth, forebrain cell proliferation), physiological (stress response), and behavioural (anxiety, habituation) endpoints. We raised zebrafish in Petri dishes with complexity (Lego bricks) from fertilization to 5 days post-fertilization (dpf) and then compared phenotypes to those of traditionally reared larvae (barren). Relative to barren-reared larvae, those raised with complexity were shorter and did not increase whole body cortisol in response to an acute agitation stressor. Forebrain cell proliferation was not different between treatments, however, larvae raised with complexity habituated sooner to an adverse stimulus and showed a tendency towards reduced anxiety-like behaviour, suggesting the anxiolytic effects of environmental enrichment may be initiated at early life stages. Taken together, our results invite consideration into how current practices in raising zebrafish may influence neurodevelopment and behavioural research findings, and may be applied to improve outcomes in fish breeding programs for conservation initiatives.

1. Introduction

Developmental plasticity defines the cause-and-effect relationship between an animal's early life environment and its realized phenotype, and includes morphological, physiological, and behavioural changes induced by biotic and abiotic cues. For example, the suite of anti-predator responses in aquatic invertebrates (e.g., *Daphnia*) and vertebrates (e.g., tadpoles) are classic models of developmental plasticity explored across the spectrum from molecular mechanisms to fitness implications (Miner et al., 2005; Mitchell et al., 2017). For experimentalists using captive-reared animal models, this topic is of particular import as rearing efficiency may be prioritized without full consideration of the phenotypic consequences. Zebrafish (*Danio rerio*) are an increasingly popular vertebrate species used in research (Stewart et al., 2014; Tsang and Gerlai, 2024). One advantage of this model species that has catalyzed its popularity is the convenience of early life stage rearing. Thousands of zebrafish can be simultaneously raised from fertilization through to yolk sac resorption in Petri dishes with no more than a stable thermal environment and daily water changes.

Zebrafish develop rapidly in a transparent chorion, hatching around 55 h post fertilization (hpf) and becoming a free-swimming juvenile ready to consume exogenous food after 5 d (Kimmel et al., 1995; Westerfield, 2000). During this period, the neural ectoderm transforms into a recognizable brain complete with ventricles and regional subdivisions (Mueller and Wullimann, 2016), and a peripheral nervous system capable of receiving, transducing and transmitting sensory information to the brain. Formation of the visual system is initiated at 11 hpf with the appearance of the optic vesicles, followed by innervation of the optic tectum around 40 hpf and a quantifiable behavioural startle response to visual stimuli by 70 hpf (Neuhauss, 2010). Concurrently, the lateral line system—which integrates mechanosensory information derived from water motion for object avoidance, schooling, and many other behaviours—is initiated in embryogenesis with appearance of the posterior lateral line primordium at 24 hpf and maturation of neuromasts with functional hair cells by 72 hpf (Ma and Raible, 2009). The endocrine stress axis also develops early in zebrafish (Alderman, 2024),

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and larvae are capable of increasing cortisol levels in response to an acute stressor as early as 3 d post-fertilization (dpf) (Alderman and Bernier, 2009). Thus, larvae have systems in place to detect and respond to external environmental cues during the first days of life.

Zebrafish originate from tributarian habitats in Southeast Asia, but most laboratory fish have been maintained for dozens of generations under highly stable and controlled conditions. A recent comparison between fully domesticated zebrafish (150 generations in captivity) and first-generation captive-reared offspring of wild-caught zebrafish highlighted that long-term stable rearing significantly reduces thermal plasticity across molecular, physiological, and behavioural endpoints (Morgan et al., 2022). This encourages reflection on how past and present laboratory conditions influence zebrafish phenotypes and, by extension, experimental results (Tsang and Gerlai, 2024). Indeed, relatively simple modifications to standard housing, such as plastic plants and substrate impart quantifiable phenotypic responses in adult zebrafish (Gallas-Lopes et al., 2023; Stevens et al., 2021; Volgin et al., 2018). For example, zebrafish from enriched aquaria have reduced anxiety-like behaviours and live longer (De Pasquale et al., 2016; Lee et al., 2019), invest less in somatic growth and display reduced within-shoal aggression (Lavery and Mason, 2023), and are less responsive to acute stress (Giacomini et al., 2016). At the same time, enhanced sensory input (e.g., olfactory cues) promotes neurogenesis in brain regions linked to the specific sensory modalities activated by the stimuli (Lindsey et al., 2014). A behavioural effect of adding environmental complexity to zebrafish habitat is evident even in juveniles. Gatto et al. (2022) added multicoloured Lego bricks to arenas containing zebrafish starting at 4 dpf and found reduced anxiety-like behaviours in these fish at 14 and 21 dpf compared to fish reared in barren arenas (Gatto et al., 2022). Although the authors did not observe an anxiolytic response at 7 dpf, a second experiment confirmed that environmental complexity experienced between 4 and 6 dpf alone was sufficient to induce the observed behavioural plasticity at 14 dpf (Gatto et al., 2022). This suggests that a critical window for complexity-induced behavioural plasticity occurs during the larval stage. A wider scope of developmental plasticity was not addressed by Gatto and colleagues (2022) and it remains uncertain whether introducing environmental complexity prior to 4 dpf can influence zebrafish phenotype.

We tested the hypothesis that environmental complexity experienced from fertilization to the end of the larval period will induce broad phenotypic responses in zebrafish. Since neural tissue is expensive (Mink et al., 1981) and zebrafish have a finite energy source (i.e., yolk) prior to exogenous feeding, the hypothesis predicts a trade-off between neural development and other energy consuming processes, a concept consistent with the expensive tissue hypothesis (Aiello and Wheeler, 1995). Therefore, we assessed forebrain cell proliferation and behavioural endpoints as indicators of morphological and functional neural investment, and used somatic growth and stress-induced cortisol release to gauge morphological and energy-intensive functional trade-offs, respectively. Lego bricks were selected to introduce complexity as these are universally available, cost-effect, sterilizable, and stable, thus ensuring complexity without compromising the ease of high-throughput care enjoyed by zebrafish researchers. In addition, Legos have been used by others to enrich juvenile zebrafish housing (Gatto et al., 2022) and in adult behavioural assays (Hamilton et al., 2013), highlighting that zebrafish readily sense and respond to these structures in their environments.

2. Materials and methods

2.1. Zebrafish rearing

Zebrafish embryos were obtained from an in-house breeding colony of TU wild-type adults (Hagen Aqualab, University of Guelph) group-housed with tank enrichment (marbles, plastic plants). Eggs collected from at least five breeding tanks were pooled and cleaned, and then randomly distributed into sterile polystyrene plastic Petri dishes with white opaque bottoms at a maximum density of 3 embryos/mL E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgCl_2$). Embryos were maintained at constant temperature 28.5 °C under a 12 L:12 D photoperiod with daily media changes.

2.2. Experimental design

Immediately upon sorting, eggs were allocated into one of two treatment groups, barren or complex. Embryos in the barren treatment were reared exactly as described above. For embryos in the complex treatment, a random assortment of small Lego pieces was included in the Petri dish (standard colours: blue, red, yellow, and green; shapes: rectangular 1 × 1 or 2 × 1, cylindrical, or irregular; coverage: ~12 % surface area; Fig. 1). After 24 hpf, stocking density in each dish was standardized to ~1.5 embryos/mL E3 by removing any nonviable eggs and, if necessary, culling at random to achieve the desired density. After 5 d of rearing in these conditions, larvae were used for behavioural assays, an acute stress test, or mitotic cell labeling as described below. At the end of all experiments, larvae were rapidly euthanized by direct transfer to ice-cold E3 medium. A total of six replicate Petri dishes were used for each treatment, and each endpoint was measured in larvae collected from at least 3 independent dishes.

2.3. Behavioural testing

At 5 dpf, individual larvae were transferred to one of six 24-well plates containing 1 mL E3 medium (1 larvae/well; $N = 12$ each barren and complex larvae per plate; $N = 6$–11 plates per test from at least 3 experimental replicates). Larval behaviour was assessed using a DanioVision observation chamber (Noldus Information Technology, Wageningen, The Netherlands) equipped with an external temperature control unit held at 28.5 °C, and an infrared Basler Ace video camera with Gigabit Ethernet interface (1280 × 1024 resolution). All behavioural testing was conducted between 12:00 h and 19:00 h, when locomotor activity tends to be more stable (MacPhail et al., 2009). To minimize any potential confounding effects of diurnal variation in behaviour, consistent start times were used across days for assay replicates, and a maximum of two tests per day. Larvae were acclimated in the observation chamber for 30 min in the dark, and then video recording commenced for future analysis of larval movements in Ethovision Software (background sensitivity = 113, object size = 5 pixels, tracking rate = 5/s, minimum displacement threshold = 0.5 mm). As described below, the light-dark assay was carried out on separate cohorts of larvae from the thigmotaxis and vibrational startle response

Fig. 1. Representative rearing environments. Zebrafish were housed from fertilization to 5 dpf in either barren (left) or complex (right) Petri dishes, with the base and sides obscured by white tape. Increased environmental complexity was achieved by including a random set of small Legos to the rearing dish to occupy ~12 % of the surface area.

assay, which were conducted in series.

2.3.1. Light-dark assay

A 20 min baseline recording in the dark was followed by 3 replications of 10 min light and 10 min dark as previously described (Best and Vijayan, 2017; MacPhail et al., 2009), using maximum illumination for the light phase (~4400 lx) and infrared only for the dark phase (0 lx). The distance moved during one-minute intervals throughout each light and dark period was measured for each larva. This test exploits the natural tendency for dark-acclimated zebrafish larvae to freeze under sudden bright light, with lower distance moved in the light interpreted as anxiety-like behaviour. This assay was repeated across a total of 11 plates ($N = 132$ larvae per treatment).

2.3.2. Thigmotaxis

Following the initial dark acclimation, larval movement was recorded for an additional 30 min of free swimming in the dark as previously described (Best and Vijayan, 2017). The area of each well was divided into an outer and inner zone (outer diameter = 16 mm, inner diameter = 8 mm), creating a 4 mm thigmotaxis zone along the circumference of the well roughly equivalent to one larval body length from the outside wall (Schnörr et al., 2012). For each larva, thigmotaxis behaviour was quantified as the percent time it occupied the outer zone of the arena, with increased tendency towards wall-hugging behaviour used as an indicator of anxiety-like behaviour. This assay was repeated across a total of 6 plates ($N = 72$ larvae per treatment).

2.3.3. Vibrational startle response assay (VSRA)

Immediately following the thigmotaxis assay, larvae were acclimated for 30 min in the light. The VSRA was then carried out in the light as previously described (Faria et al., 2019). This test uses the aversive vibrational stimulus (tap) feature of the DanioVision to elicit a startle response, and habituation of the startle response over successive stimulations is used as an indicator of short-term associative learning. The total distance moved (TDM) was quantified prior to and following 20 repeated taps at 1 s interstimulus intervals. This assay was repeated across a total of 6 plates (N = 72 larvae per treatment).

2.4. Acute stress response and cortisol measurement

After 5 d of rearing, a subset of 10 larvae per dish were either immediately euthanized (time 0) or were transferred to a 20 mL glass scintillation vial containing 10 mL E3 medium. An acute agitation stressor was applied by gently swirling for 2 min using a motorized rotor plate (Roto Mix 50,800, Fisher) on the lowest setting as previously described (Alsop and Vijayan, 2008), and then larvae were allowed to rest undisturbed for 10 min or 15 min before euthanasia as described above ($N = 4$–6 pools of 10 larvae per treatment per time point). Samples were snap frozen on dry ice and stored at −80 °C prior to analysis of cortisol content. Frozen larvae were partially thawed on ice, then briefly sonicated on ice in 120 mL of 1× extraction buffer (Neogen, Lexington, U.S.A.) and clarified by brief centrifugation at 4 °C. Cortisol content of homogenates was quantified in duplicate using a commercial enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions (Neogen, Lexington, U.S.A.). A dilution series of a representative homogenate was used to confirm the accuracy of cortisol measurements from non-extracted larval samples, as has been reported by others (Faught et al., 2016).

2.5. Morphometrics

To evaluate the effect of rearing environment on growth, digital images of larvae were collected after euthanasia at 5 dpf using a Nikon Stereomicroscope SMZ1500 under constant magnification and lighting (~50 individuals per replicate; $N = 325$ and 300 larvae for barren and complex, respectively). Body length was measured as the greatest linear distance between the rostral and caudal end of an individual using the line tool in ImageJ, following calibration to a micrometer imaged under identical settings. All measurements were taken blind to treatment.

2.6. Mitotic cell labeling and forebrain cell proliferation

The rate of forebrain cell proliferation was determined using a short, non-saturating pulsed exposure to a thymidine analogue, 5-ethynyl-2′-deoxyuridine (EdU). Briefly, a subset of 5 dpf larvae from each treatment ($N = 7$) were transferred to a 6-well plate containing 2.5 mM EdU (ThermoFisher) prepared in E3 medium. Larvae were incubated in the dark for 1 h at 28.5 °C and then euthanized as described above. Larvae were then transferred to 4 % paraformaldehyde (prepared in phosphate buffered saline, PBS; pH 7.4) and fixed for 90 min at room temperature, rinsed 3 × 15 min in PBS, and then embedded in Cryomatrix (Fisher). Serial 20 μm transverse sections through larval heads were collected on SuperfrostPlus slides (Fisher), dried, and stored at −20 °C until staining. Thawed sections were rehydrated in PBS with 0.1 % Tween-20 (PBT), and then mitotic cells were visualized via "Click-iT" chemistry as previously described (Lindsey et al., 2019). Briefly, sections were incubated in a dark humidified chamber for 30 min with reaction cocktail (100 mM L-ascorbic acid, 100 mM Tris buffer at pH 8.5, 1 mM copper II sulphate) containing 1 mM 488 Azide Fluor (Invitrogen A10266) and DAPI in PBS. Sections were rinsed in PBT and washed extensively in PBS before aqueous mounting. Digital 10-step *Z*-stacked images of all sections were collected using a Nikon Ti2 fluorescent microscope to visualize $DAPI^+$ and EdU^+ cells throughout each section. For each larva, EdU^+ cells were manually tallied in the pallium and subpallium across 4–7 serial sections (i.e., rostral to the habenula and anterior commissure) (Mueller and Wullimann, 2016), and the number of mitotic cells per 20 μm was estimated by dividing the total EdU cell counts by the number of sections examined.

2.7. Statistical analysis

The effect of rearing environment on total distance moved (TDM) during the Light-Dark assay was compared using separate nested *t*-tests for each illumination phase (light, dark), and differences among the 3 interval repetitions (nested factor) were assessed with Chi-square. The analysis was repeated using a pared dataset that included only the first 5 min of each light interval ($n = 132$ larvae per treatment), as shorter cycle lengths are also used for this assay (Hillman et al., 2024). The effect of rearing environment on thigmotaxis behaviour was assessed using an unpaired *t*-test ($n = 72$ larvae per treatment). A mixed-effects model (REML) and Tukey's multiple comparisons test were used to detect and compare habituation of the startle response between rearing environments, considering *treatment* (barren, complex) and *tap number* (Pre, 1–20) as main factors and allowing for their interaction. Habituation was taken as the lowest tap number at which TDM no longer differed from pre-stimulus TDM for all subsequent taps in the VSR assay (n = 72 larvae per treatment). An area under the curve (AUC) analysis was used to calculate the mean distance moved during the VSR assay for each treatment and then compared via *t*-test. Differences in the acute stress response were determined by two-way ANOVA, using *treatment* (barren, complex) and *time* (0-, 10-, and 15-min post-stress) as main factors and allowing for their interaction. Where significant differences were detected, post hoc pairwise comparisons were performed using Tukey's tests ($N = 4$–6 per treatment per time). Differences in body lengths of larvae raised in barren or complex environments were assessed using a nested *t*-test ($n = 23$–93 larvae per dish, totaling 325 and 300 individuals in barren and complex environments, respectively), and differences among the 6 Petri dishes (nested factor) were assessed with Chi-square. The effect of rearing environment on forebrain cell proliferation was assessed by two-way ANOVA, using *treatment* (barren, complex) and *brain region* (pallium, subpallium) as main factors and allowing for their interaction. For all analyses, differences were considered significant at p

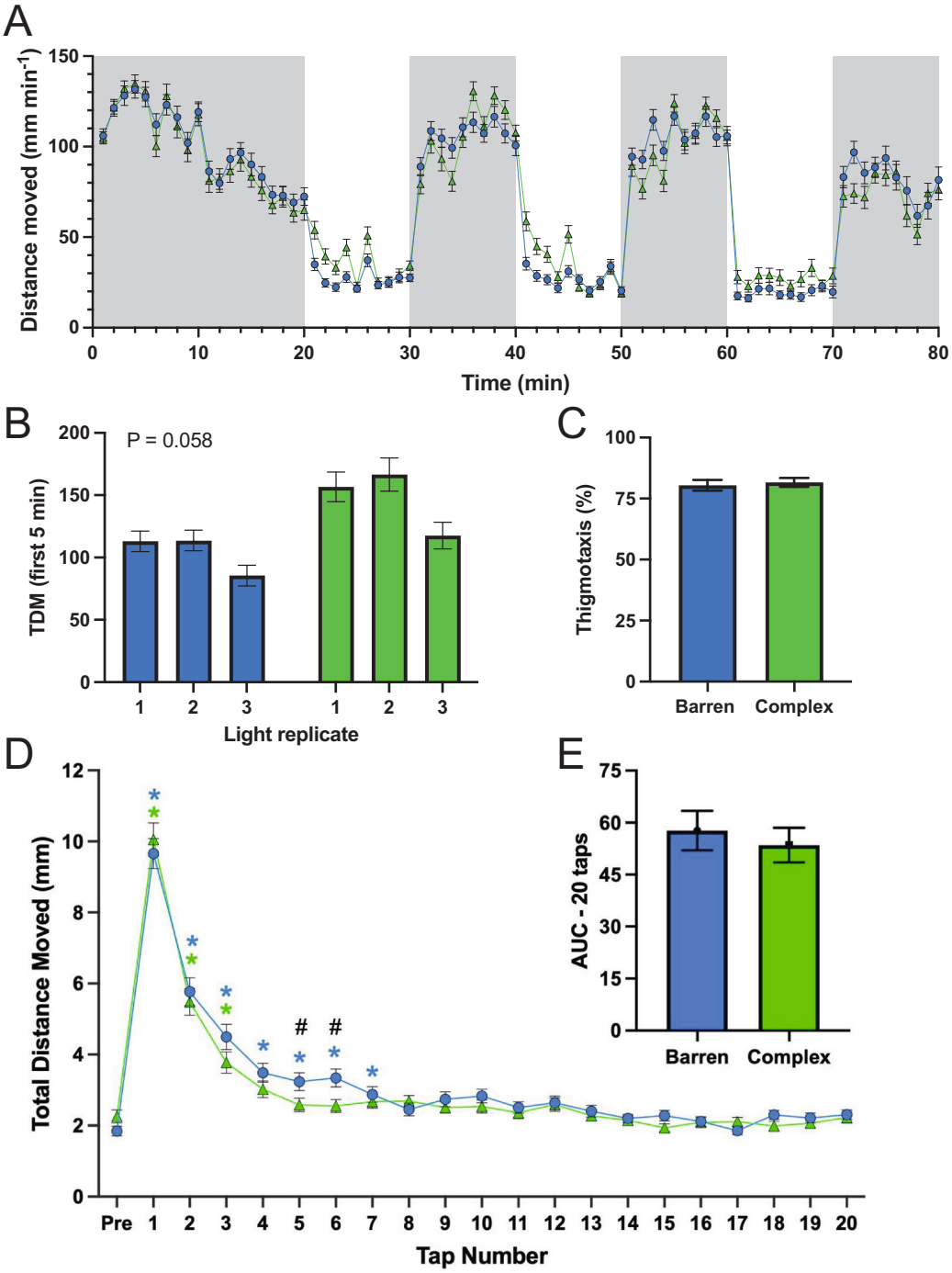

Fig. 2. Effect of barren (blue) and complex (green) rearing environments on larval behaviours. (A) Movement summary of larvae during a Light-Dark assay, including a 20-min baseline followed by 3 × 10 min intervals of alternating light and dark illumination (indicated by white and grey shading, respectively). A nested *t*-test was used to detect differences in total distance moved (TDM) between barren and complex reared larvae during each replicate light interval ($n = 132$ larvae per treatment; $P_{light} = 0.081$; $P_{dark} = 0.782$). (B) Mean TDM during the first 5 min of the replicate light intervals for larvae reared in barren (blue) and complex (green) Petri dishes (n = 132 per treatment; nested t-test, $P = 0.058$). (C) Thigmotaxis behaviour, expressed as distance moved in the outer zone relative to total distance moved ($n = 72$ larvae per treatment; unpaired t-test, $P = 0.676$). (D) Movement summary of larvae during the Vibrational Startle Response assay, expressed as total distance moved (mm s^{-1}) following each of 20 taps. Blue (barren) and green (complex) asterisks denote significant differences from pre-stimulus movement (Pre) in the same treatment; hashtags (#) indicate significant differences between barren and complex at a given tap number (n = 72 larvae per treatment; mixed-effects REML and Tukey's test, $P_{interaction} = 0.049$). (E) Area under the curve (AUC) for the data in D (t-test; $p > 0.05$). Data are mean ± S.E.M. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

< 0.05.

3. Results

3.1. Behavioural assessments

The effect of rearing environment on larval behaviour was assessed using three discrete behavioural assays. In the Light-Dark assay, the total distance moved during dark intervals was similar for larvae in both treatments (nested *t*-test; $P = 0.782$; Fig. 2A). There was a tendency for larvae reared in complex Petri dishes to exhibit a dampened freeze response in the light relative to larvae reared in barren dishes (nested t-test; $P = 0.081$; Fig. 2A). This was especially evident in the first 5 min of the light intervals (nested t-test; $P = 0.058$; Fig. 2B). Of note, larval movement in both treatments was reduced in the third light and dark intervals relative to the first two intervals (Chi-square tests; $P = 0.074$ and $P < 0.0001$, respectively). Thigmotaxis was similar for larvae reared in barren or complex Petri dishes (unpaired t-test; $P = 0.676$; Fig. 2C). Rearing environment affected time to habituation, assessed in the VSR assay. While pre-stimulus movement and initial startle response at tap 1 were similar for both treatments, larvae reared in barren Petri dishes required double the number of stimuli to habituate compared with larvae reared in complex dishes (tap 8 vs tap 4, respectively; mixed-effects model REML; $P_{interaction} = 0.049$; Fig. 2D). Despite these differences, average movement across the entirety of the VSR assay was similar in both treatments (AUC = 57.7 ± 5.7 and 53.5 ± 5.0 for barren and complex, respectively; $P = 0.580$; Fig. 2E).

3.2. Acute stress response

Rearing environment had a significant effect on the acute stress response of 5 dpf larvae (two-way ANOVA; $P_{interaction} = 0.023$; Fig. 3). In larvae raised in barren Petri dishes, whole body cortisol increased from 0.65 pg/embryo to 2.17 pg/embryo 10 min after the swirling stress ($P = 0.0006$) and remained elevated above baseline for at least 15 min ($P = 0.020$). In contrast, whole body cortisol levels were stable across all three time points in larvae raised in complex dishes, with values ranging from 1.31 pg/embryo to 1.94 pg/embryo ($p > 0.05$). Baseline cortisol was 2-fold higher in larvae raised in complex dishes relative to larvae from barren dishes, however this did not reach statistical significance ($P = 0.07$). At 10 min post-stress, cortisol was significantly higher in the barren larvae compared to complex larvae ($P = 0.033$), but larval cortisol content was similar in both groups at 15 min ($P = 0.693$).

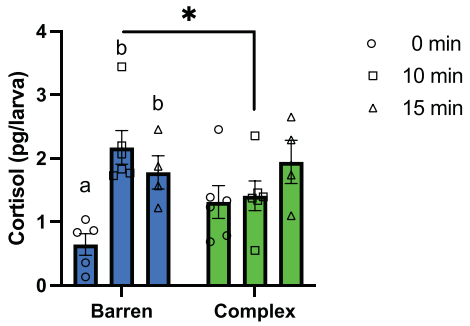

Fig. 3. Acute stress response in 5 dpf zebrafish larvae raised under barren or complex conditions. Cortisol was quantified in replicate pools of 10 larvae prior to stress (0 min) or after recovering from an acute 2 min agitation stressor for 10 min or 15 min, and values compared using a two-way ANOVA and Tukey's tests ($N = 4$–6; $P_{interaction} = 0.023$). Values are means ± S.E.M. Within a treatment, bars that do not share a letter are significantly different. An asterisk denotes a significant difference between barren and complex at a specific time point.

3.3. Morphometrics

Zebrafish larvae reared in complex dishes were ~ 2 % smaller at 5 dpf relative to larvae raised in barren dishes (3.84 ± 0.01 mm vs 3.91 ± 0.01 mm, respectively; $P = 0.017$; Fig. 4A). Mean body length did not differ across replicates within each treatment (Chi-square = 1.23, $P = 0.267$; Fig. 4B).

3.4. Forebrain cell proliferation

A one-hour incubation in the presence of the thymidine analogue, EdU, resulted in consistent and unambiguous marking of S-phase cells in 5 dpf larvae in the forebrain (Fig. 5), as well as in other tissues not assessed in this study (e.g., caudal brain regions, the olfactory epithelium, retina, pharynx). An average of 176.5 ± 19.3 mitotic cells were counted in each larval forebrain, equating to ~15–18 mitotic cells per section in both subregions of the forebrain. The number of mitotic cells was similar in all larvae, independent of rearing environment ($P_{environment} = 0.389$) and forebrain region ($P_{region} = 0.857$), nor did these variables have an interactive effect ($P_{interaction} = 0.974$; two-way ANOVA; Fig. 5B).

4. Discussion

The present study demonstrates that introducing a modest level of environmental complexity to the early rearing environment of zebrafish induces a phenotypic response consistent with changes incurred in adult zebrafish housed with tank enrichments. Specifically, zebrafish raised with environmental complexity from fertilization to 5 dpf were shorter and less responsive to an acute stressor. Although forebrain cell proliferation was unaffected by rearing environment, larvae from complex dishes habituated faster to an aversive stimulus and displayed a tendency towards reduced anxiety-like behaviour relative to larvae raised in barren dishes. This suggests that environmental complexity can drive plastic responses in zebrafish from very early in development, at a time when most laboratories maintain embryos in simple, unadorned Petri dishes.

4.1. Morphological responses to complexity

Two morphological endpoints were evaluated in this study: body length and forebrain cell proliferation. Consistent with the effects of environmental enrichment in adult zebrafish (Lavery and Mason, 2023), larvae raised with complexity were smaller at 5 dpf compared to larvae raised in barren dishes. This is an intriguing result, considering initial yolk quantity and quality should be similar between the treatments as they were established from a common pool of eggs. Zebrafish rely exclusively on this finite energy resource for growth and development during the first 5 d of life (Kimmel et al., 1995; Westerfield, 2000), thus the reduced length of larvae raised in complexity indicates underlying differences in energy utilization. One possibility is that the activity of complexity-reared larvae was greater than that of barren-reared larvae, mandating enhanced catabolism of yolk reserves to fuel ATP synthesis for muscle contraction and leaving less resources for somatic growth. Although we did not monitor larval activity outside of behavioural assays, the activity patterns of 5 dpf larvae during the three behavioural assays do not support this explanation. Larvae from both treatments moved a similar distance during the acclimation and dark phases of the light-dark assay, throughout the thigmotaxis assay, and following habituation in the VSR assay, suggesting that increased activity is not a general characteristic of complexity-reared larvae, and therefore unlikely to account for the observed growth restriction. Quantifying oxygen consumption and estimating remaining yolk reserves at 5 dpf would help rule out differences in the rate of yolk sac utilization between the two treatments.

Another possible explanation for the observed difference in length is

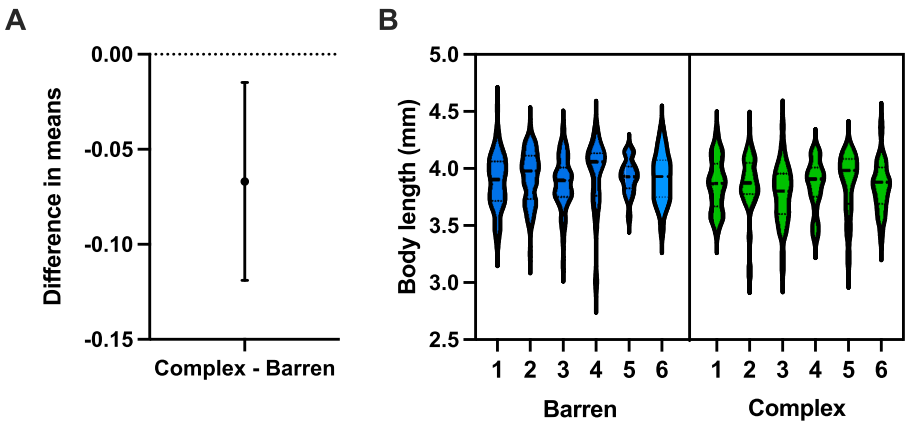

Fig. 4. Effect of rearing environment on zebrafish growth. Embryos were reared at equal densities from fertilization to 5 dpf in either barren Petri dishes or dishes containing small Lego blocks of various colours and dimensions (complex). The body lengths of individual larvae from 6 replicate treatments ($N = 325$ and 320 per treatment, respectively, from 23 to 93 individuals per replicate) were measured in ImageJ, blind to treatment. A significant difference in body length was determined using a nested t-test and visualized as (A) mean difference ± 95 % confidence intervals alongside (B) violin plots for each of six replications of barren and complex rearing environments.

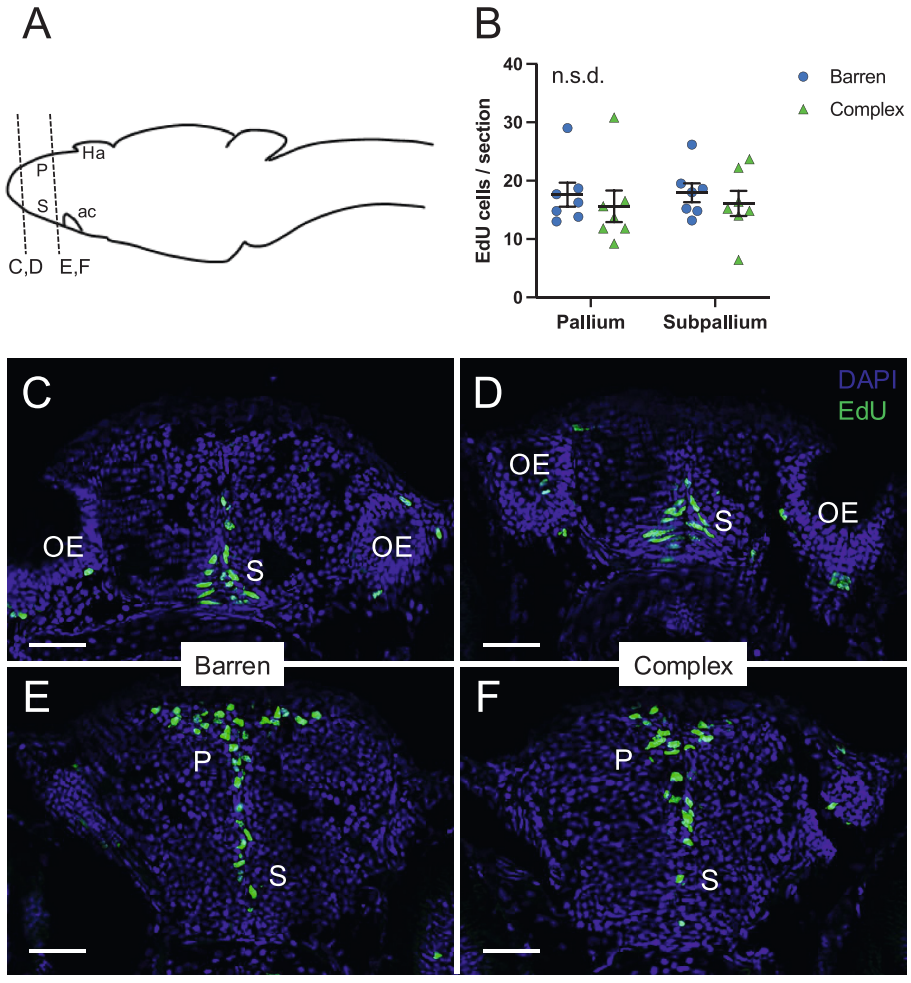

Fig. 5. Effect of rearing environment on forebrain cell proliferation in 5 dpf zebrafish larvae. (A) Schematic of a 5 dpf brain indicating regions of interest and approximate plane of section for representative images (dashed lines). Image is in sagittal orientation, left facing, modified from Mueller and Wullimann (2005). (B) Mean number of mitotic cells (EdU) per 20 mm section in the pallial and subpallial regions of the forebrain (n = 7 larvae/treatment; see text for analysis details) for larvae raised in barren (blue circles) or complex (green triangles) Petri dishes. (C-F) Representative images indicating EdU^+ cells (green) in two planes of forebrain sections corresponding to approximately panel 5 A (C,D) and 10 A (E,F) of Mueller and Wullimann (2005). Scale bar is 50 mm in each. ac, anterior commissure; Ha, habenula; n.s.d., not statistically different; OE, olfactory epithelium; P, pallium; S, subpallium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that larvae from the complex dishes are partitioning the available energy differently from larvae raised in barren dishes. Examples of such trade-offs are numerous, including the relatively larger tail and smaller body of tadpoles raised with predator cues relative to tadpoles raised without these cues (Van Buskirk and Mccollum, 2000), and the inverse relationship between gut and brain mass in guppies (*Poecilia reticulata*) selected for brain size over two generations (Kotrschal et al., 2013). The latter example conforms to the expensive tissue hypothesis (Aiello and Wheeler, 1995), which posits an obligate trade-off between investment in the central nervous system and investment in other energetically expensive tissues or processes. Given that environmental enrichment stimulates neurogenesis (Lindsey and Tropepe, 2014), increases relative brain size (De Pasquale et al., 2016; Edmunds et al., 2016) and alters behaviour (De Pasquale et al., 2016; Lavery and Mason, 2023) in adult zebrafish, the addition of Lego bricks to the early rearing environment in the present study may have enhanced investment in central nervous system development at the expense of somatic growth. Yet, forebrain cell proliferation rate was comparable at 5 dpf in complex- and barren-reared larvae, results inconsistent with a simple structural trade-off. Although the forebrain was selected because it is highly proliferative (Zupanc et al., 2005) and functionally linked to the behavioural outputs quantified in this study (Wullimann et al., 1996), it is possible that the effects of environmental complexity on the rate of brain cell proliferation are restricted to other brain regions such as the optic tectum, which processes visual stimuli. Alternatively, other morphological metrics (e.g., neuronal density) or activity indicators (e.g., functional imaging) may better evaluate whether rearing environment alters investment in neural systems. Indeed, the observed behavioural changes discussed below offer some support for differential investment in the functionality of the brain, which may contribute to the observed changes in growth.

4.2. Physiological responses to complexity

In the present study, a brief, standardized, agitation stressor followed by 10- and 15-min rest periods was used to assess the effects of rearing environment on the acute stress response, based on previous studies (Alsop and Vijayan, 2008; Theodoridi et al., 2021). Whereas traditionally reared larvae had significantly elevated whole-body cortisol within 10 min of experiencing an acute agitation stressor, cortisol did not increase above baseline levels in larvae raised with environmental complexity. Adult zebrafish are capable of increasing cortisol levels within 5 min of an acute stressor (Fuzzen et al., 2010), thus it is possible that the complexity-reared larvae experienced a rapid rise and recovery in cortisol that was not captured in this sampling protocol. A more likely explanation is that environmental complexity blunts responsivity to acute stress. In rodents, for example, positive early life experiences like maternal care reduce the sensitivity of the endocrine stress response (Franklin et al., 2012; Romeo, 2015).

4.3. Behavioural responses to complexity

In the present study, the effect of rearing environment on behaviour was assessed across three common assays for zebrafish larvae. The clearest indication that early exposure to complexity affects behavioural phenotype was the rapid habituation in the VSR assay. Larvae reared in complex dishes stopped responding to the vibrational stimulus in half the time as their barren-reared counterparts. Importantly, the initial startle response was identical in both treatments, ruling out neural circuitry differences from mechanosensory sensitivity to neuromuscular output as explanations for the earlier habituation in complexity-reared larvae. Our findings are consistent with recent work showing earlier habituation time in 16 dpf zebrafish raised with similar environmental complexity as the present study beginning at hatch (Gatto et al., 2024). Habituation is a simple form of learning achieved through presynaptic (short-term) and postsynaptic (long-term) modifications (Glanzman, 2009), allowing animals to reject predictable, non-noxious stimuli based on prior experience. Ultimately, the earlier an individual habituates, the less energy it expends on non-productive activity.

The anxiolytic effects of environmental enrichment are broadly accepted in mammalian literature and are readily observed (e.g., reduction in stereotypic behaviours like obsessive grooming and pacing in captive animals; (Clark et al., 2023)). In zebrafish, too, inclusion of environmental enrichment reduces anxiety-like behaviours (De Pasquale et al., 2016; Lee et al., 2019), among other benefits (Gallas-Lopes et al., 2023; Stevens et al., 2021; Volgin et al., 2018). The present study offers new evidence that environmental complexity experienced by embryonic and larval zebrafish may also reduce anxiety-like behaviour, as the light-induced freezing response was less pronounced in larvae raised with Legos relative to barren-raised larvae. We present this interpretation with caution, as differences in larval movement were most distinct during the first 5-min of the light phase, and thigmotaxis was not reduced by complexity. Gatto and colleagues (Gatto et al., 2022) suggest that anxiety-like behaviours may be easier to distinguish at later life stages. In their study, the novel object and novel odor tests failed to detect behavioural differences at 7 dpf despite experimental evidence that the 4–6 dpf rearing window was sufficient to induce behavioural plasticity at 14 dpf (Gatto et al., 2022). Thus, investigating latent behavioural differences between the two treatments may provide stronger support for anxiolytic effects of early complexity. At the same time, more nuanced analysis of larval behaviour during the light phase may better detect behavioural differences between the two treatments. For example, advances in tracking software now enable refined measures of larval activity, including directional movements and velocity that were not considered in the present study.

The fact that any behavioural differences were observed in this study is, in itself, a remarkable finding as it suggests that plasticity in zebrafish behavioural phenotypes can be experimentally induced from very early in development. It may be fruitful, therefore, for laboratories invested in zebrafish models for neurodevelopment and behavioural research to augment Petri dish rearing with some sensory stimuli.

4.4. Considerations and conclusions

Adding Lego bricks to Petri dishes changed zebrafish phenotype when measured at 5 dpf. Although the environmental complexity was introduced during initial sorting of the fertilized eggs, adding complexity at hatch is likely sufficient to induce such phenotypic responses assuming the chorion acts as a significant barrier to external visual and mechanosensory stimuli. We are not advocating for universal change in embryonic rearing practices for laboratory zebrafish, but suggest that the addition of complexity, like Lego bricks, may be worthwhile for some research endeavours, particularly if these early phenotypic responses persist and/or magnify into later life stages. In rodents, for example, early exposure to environmental enrichment improves visual acuity (Cancedda et al., 2004). If the same holds true for zebrafish, early complexity may improve live prey capture, and, by extension, survival, as larvae transition to exogenous feeding. The results of this study may also warrant consideration in applications beyond laboratory zebrafish. Captive breeding programs are widely used for species conservation but with varying success, potentially due in part to reduced scope and/or alteration of neural phenotypes (Clark et al., 2023; Rabin, 2003). For example, many fish populations are managed through re-introduction programs, but hatchery-reared individuals may be disadvantaged relative to wild fish (Milot et al., 2013). Adding enrichment to juvenile tanks of cod (Braithwaite and Salvanes, 2005; Salvanes et al., 2007), Atlantic salmon (Brown et al., 2003; Salvanes et al., 2013), and steelhead trout (Berejikian et al., 2001; Lee and Berejikian, 2008) has been shown to support behavioural development in ways expected to benefit individuals upon reintroduction. The present study adds to this literature by emphasizing that supplying enrichment as early as logistically feasible may augment these benefits.

CRediT authorship contribution statement

Julia A. Bourdeau: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Sarah L. Alderman:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use any generative AI or other AI-assisted technologies for the scientific writing process of this work.

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Declaration of competing interest

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

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Data availability

Data will be made available on request.

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