Title

Effects of environmental enrichment on survivorship, growth, sex ratio and behaviour in laboratory maintained zebrafish *Danio rerio*

Authors

C. J. Lee*[1]

G. C. Paull*

C. R. Tyler*

*Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QG, United Kingdom.

Running head

Environmental enrichment for zebrafish

---

[1] Author to whom correspondence should be addressed. Tel.: +44 (0)1647 432732; email: cjl219@exeter.ac.uk

ABSTRACT

Environmental enrichment involves increasing the complexity of a fish's environment in order to improve welfare. Researchers are legally obliged to consider the welfare of laboratory animals and poor welfare may result in less robust data in experimental science. Laboratory zebrafish *Danio rerio* are usually kept in bare aquaria for ease of husbandry and, despite being a well-studied species, little is known about how laboratory housing affects their welfare. This study shows that environmental enrichment, in the form of the addition of gravel substrate and plants into the tank, affects survivorship, growth, and behaviour in laboratory-maintained *D. rerio*. Larvae reared in enriched tanks had significantly higher survivorship compared with larvae reared in bare tanks. Effects of the tank conditions on growth were more variable. Females from enriched tanks had a higher body condition than females maintained in bare tanks, but intriguingly this was not the case for males, where the only difference was a more variable body condition in males maintained in bare tanks. Sex ratio in the rearing tanks did not differ between treatments. Resource monopolisation was higher for fish in enriched tanks than for those in bare tanks. Fish from enriched tanks displayed lower levels of behaviours associated with anxiety compared with fish from bare tanks when placed into a novel environment. This study thus evidences differences in welfare for *D. rerio*

maintained under different environmental conditions with enhancements in welfare more commonly associated with tank enrichment.

KEY WORDS

Laboratory zebrafish, environmental enrichment, fish welfare, survivorship, growth, behaviour

INTRODUCTION

Three guiding principles form the basis of the ethical use of animals in scientific research: (1) the *replacement* of animals in research, (2) the *reduction* in the number of animals used in experiments, and (3) the *refinement* of the care and use of laboratory animals in order to minimise suffering and improve welfare. These principles, known as ‘the 3Rs’, are incorporated into national (Home Office, 2014) and international (European Union: Council of the European Union, 2010) legislation.

Environmental enrichment is a form of refinement that may be appropriate for some laboratory fish. It involves increasing the complexity of the fish's environment in order to improve welfare and minimise maladaptive traits, such as increased aggression (Näslund & Johnsson, 2014). Structurally complex habitats offer shelter from predators or aggressive conspecifics (Johansen *et al.*, 2008), additional feeding sites (Thomaz & da Cunha, 2010) and breeding sites (Beets & Friedlander, 1998). In contrast, most laboratory fish are housed in tanks that offer little, or no, stimuli. The complexities of the natural environment cannot be recreated in the laboratory, so the goal when designing enrichment is to identify elements of the artificial environment that can be modified to provide measurable welfare benefits without compromising research results (Bayne & Wurbel, 2014; Johnsson *et al*., 2014). Welfare is defined here as "the internal state of a fish when it remains under conditions that were freely chosen" as suggested by Volpato (2009) with two criteria for good welfare: whether the fish is healthy and whether it has what it wants (Dawkins, 2017).

The zebrafish *Danio rerio* (Hamilton 1822) is one of the most widely used research models in a number of biological fields, including developmental biology, genetics, toxicology, human disease, pharmacology and evolutionary theory (Grunwald & Eisen, 2002). Laboratory *D. rerio* are usually kept in bare aquaria for ease of maintenance and, although it is a well-studied species, little is known about the effects on *D. rerio* of laboratory housing. This shortfall is a limitation to the dual goals of providing optimal conditions for generating high-quality experimental subjects while fulfilling obligations to consider the welfare of laboratory-held fish.

No single welfare measure is reliable when used in isolation (Ashley, 2007) and therefore this study examined a range of measures in order to gain an overall impression of welfare. It assessed for effects of tank enrichment in laboratory-held *D. rerio* on survivorship, growth (length, mass and body condition), development of sex, and behaviour. The null hypotheses tested were that environmental enrichment through provision of plants and gravel does not affect survivorship, growth, sex ratio or behavior of *D. rerio*.

MATERIALS AND METHODS

FISH SOURCE, HOUSING AND HUSBANDRY

The fish used in this study were Wild Indian Karyotype (WIK) strain *D. rerio*, bred and maintained in-house at the Aquatic Resources Centre at the University of Exeter. Fish were maintained in clear polystyrene tanks (Hagen; West Yorkshire, United Kingdom). Mains tap water was filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts to standardized synthetic freshwater (final concentrations to give a conductivity of 300 µS: 122 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 9.4 mg l^{-1} $NaHCO_3$, 50 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 2.5 mg l^{-1} KCl, 50 mg l^{-1} Tropic Marin Sea Salt), aerated, and heated to 28°C. The water was supplied to each tank via a flow-through system. The pH, conductivity, ammonia, nitrate, and nitrite were maintained within U.S. Environmental Protection Agency guidelines (U.S. EPA, 1996). Each tank (for shapes and sizes, see below) was connected to the system water and the flow rate was set to 1.2 l $h^{–1}$ (slow drip) for larvae from 5–29 days post-fertilisation (dpf), 2.4 l $h^{–1}$ (fast drip) for juveniles from 30–59 dpf, and 6 l $h^{–1}$ (steady stream) for fish from 60 dpf. A filter screen with a 400 µm pore diameter was fitted to the water outflow hole. Laminated white paper was placed between the tanks to prevent visual interaction between fish in neighbouring groups. The photoperiod was set to 12:12 h light:dark with a 30 min artificial dawn to dusk transition.

In each experiment, some tanks were designed as ‘bare’ environments and comprised bare aquaria while others were designed as ‘enriched’ environments and furnished with aquarium gravel (grain size 2–5 mm) to a depth of 3 cm and aquatic plants [vallis (*Vallisneria* spp. including *V. spiralis*, *V. elongata* and *V. tortifolia*) and water trumpet (*Cryptocoryne wendtii*)]. These plant species were chosen for their structural similarity to plants typically found in the natural habitat of *D. rerio* (Spence *et al*., 2006). Vallis plants varied in number of leaves from 2–10 and in length from 50–190 mm. Water trumpet plants varied in number of leaves from 3–5. Plants were washed under running tap water to remove snails and pathogens that may otherwise impact the study, surface-sterilised in 10% commercial bleach for 5 min, rinsed under running de-ionised water for 2 min, blotted on absorbent paper, and planted in an even distribution throughout the enriched tanks.

Fish were housed from 5–131 dpf in a succession of experimental tanks, as described below, and experimental endpoints were measured at various development stages (Fig. 1).

Fish from 5–30 dpf were housed in ‘nursery tanks’ [Fig. 1 (a)]. Four nursery tanks were set up, each of 335 x 195 x 170 mm ($L \times W \times H$) dimension with a working capacity of 11 l. Each tank housed 150 embryos (see below). Two tanks were bare and two were enriched with gravel, 30 vallis plants and three water trumpet plants. For five days prior to the introduction of larvae, nursery tanks were ‘primed’ daily with two drops of liquid fry food (Liquifry; Interpret, Surrey, United Kingdom) to stimulate growth of beneficial microorganisms upon which larvae may feed.

Fish from 31–97 dpf were housed in ‘rearing tanks’ [Fig. 1 (b)] of 210 x 130 x 130 mm ($L \times W \times H$) dimension, with a working capacity of 2.2 l. Each tank housed 11 fish (see below). Five tanks were bare and five were enriched with gravel, 10 vallis plants and one water trumpet plant.

Starting at 98 dpf, fish were removed individually from the rearing tanks and placed into a ‘novel tank’ [Fig. 1 (c)] for assessment of anxiety-like behaviour. The novel tank was trapezoidal and of the following dimensions: 220 mm along the bottom, 261 mm along the top, 95 mm wide at the bottom, 105 mm wide at the top, 150 mm high, with a working capacity of 2.8 l. The tank was divided in half, lengthways, by a PVC plastic sheet which reduced the width of the tank in order to minimise lateral movement but permit easy vertical and horizontal movement (Cachat *et al.*, 2010). The tank was marked into two horizontal zones by a dividing line on the outside wall (Cachat *et al.*, 2010). Each fish remained in the novel tank for 6 min and was then transferred to a ‘choice tank’ [Fig. 1 (d)] where it joined other tested fish from its original group. All fish in any one group were tested and transferred to a choice tank on the same day in order to avoid prior residence affecting the formation of dominance hierarchies. The novel tank tests and transfer of fish to choice tanks were completed by 101 dpf.

Following the novel tank test, fish were housed in choice tanks until 131 dpf. Each tank housed 11 fish (see below). Ten choice tanks were set up, each divided into two equal compartments by a sheet of PVC plastic perforated with 3 mm holes to allow circulation of water. A 40 mm hole in the centre of the sheet allowed fish to swim between compartments. One compartment was furnished with gravel, five vallis plants and one water trumpet plant and the other compartment was bare. To minimize left/right bias, five of the tanks had the bare compartment on the right and five on the left. Tanks were supplied with system water and laminated white paper was placed between tanks to prevent visual interaction between fish in neighbouring groups.

Fish were fed five times a day from 5–30 dpf and four times a day thereafter (Table 1). Mesh filters were cleaned daily and, from 30 dpf, aquaria were cleaned weekly by gently siphoning out detritus. Tank internal surfaces were cleaned twice weekly by wiping with absorbent, low-linting paper towels.

All experiments were performed in accordance with the guidelines of the animal ethics committee, University of Exeter.

SURVIVORSHIP FROM 5–30 DPF

Approximately 650 embryos from mass spawning tanks were collected, cleaned, and placed in Petri dishes (50 embryos per dish) containing system water plus methylene blue as an antifungal agent. Unfertilised eggs were removed. At 2 dpf, 600 embryos were transferred to 60 Petri dishes (10 embryos per dish to facilitate counting) and allowed to hatch. At 5 dpf, all embryos had hatched and each group was randomly assigned to one of the four nursery tanks (two bare and two enriched). Duplicate nursery tanks for each treatment (each containing 150 larva) was adopted to mitigate against tank failure risk. At 30 dpf, survivorship was determined by counting all juveniles in each tank.

GROWTH

At 30-dpf, 55 juveniles were removed from enriched nursery tanks (27 from one tank and 28 from the other) and randomly assigned to five enriched rearing tanks. Similarly, 55 juveniles were removed from bare nursery tanks and assigned to five bare rearing tanks. Each rearing tank thus contained 11 juveniles, representing a shoal size similar to those observed in wild *D. rerio* (2–10 fish; Pritchard *et al*., 2001) and compatible with a recommended stocking density for laboratory *D. rerio* (five fish l^{-1}; Matthews *et al*., 2002).

Body length was used to assess the effects of housing/environmental conditions on growth at 30, 60 and 120 dpf. Body length, mass and body condition were used to assess growth at 131 dpf. For length measurements, a sample of 20 fish from each treatment were individually photographed in reduced-volume containers: 30 dpf larvae in a 12-well Falcon tissue culture plate, well volume 6 ml, half filled with system water; 60 dpf and 120 dpf fish in a 100 ml beaker and 200 ml crystallising dish respectively, each containing ~20 mm of system water. Photographs were taken from an overhead viewpoint with a digital compact camera (Canon PowerShot SX50; Canon, Tokyo, Japan) mounted vertically on a copy stand and lit by a dual fibre optic light source. A ruler for calibration of the measurement was placed next to the container holding the fish and included in the photograph. The distance from the snout to the base of the caudal fin (standard length L_S; ± 1 mm) was determined by image analysis (ImageJ; Schneider *et al*., 2012).

At 131 dpf, all fish were sacrificed by anaesthetic overdose (benzocaine; Sigma, Poole, United Kingdom). Loss of body condition may indicate impaired welfare (Huntingford *et al*., 2006) and to determine whether treatment affected condition, each fish was weighed, measured, and its body condition factor (K) calculated by expressing the cube of fish length as a percentage of fish mass (K = mass (mg)/length (mm)^3 × 100). As body shape/form can differ between the sexes, the results for males and females are presented and discussed separately.

SEX RATI

At 131 dpf, fish were sexed based on differences established in colouration and body shape between the sexes. Male *D. rerio* have a golden cast and a streamlined body, whereas females have a silvery cast and a rounded body shape. The presence of a visible genital papilla in females was also used to help distinguish the sexes (Paull *et al.*, 2008).

BEHAVIOUR

The ‘novel tank test’ is used extensively to model anxiety-like behaviour in *D. rerio*. The test is based on the observation that *D. rerio* display an initial preference for the bottom of a novel tank, and this response slowly diminishes as the fish becomes familiar with the environment (Tran & Gerlai, 2016). The novel tank test was used to assess anxiety-like behaviour in individual fish between the ages of 98 and 101 dpf. Four fish were randomly selected from each rearing tank (five enriched tanks and five bare tanks; $n = 20$ fish per treatment) and transferred individually to a novel tank where their response to the new surroundings was recorded and measured. Laminated sheets of white paper were placed against the back and sides of the tank to prevent visual disturbance during the test. The tank was positioned ~40 cm in front of an AXIS M1054 network camera (Axis Communications, Luton, Bedfordshire, UK) with a video resolution of 1280 × 800 pixels, coupled to a Synology network-attached storage device (NAS) (Synology Inc., Taipei, Taiwan). A laptop computer was used to connect to the NAS, via the network, to view the tank in real time and to record the tests. The video recording was started and a fish was transferred from its rearing tank to the novel tank by gently catching it with a net, placing the net in the novel tank and allowing the fish to swim out. The fish’s behaviour was recorded for a period of 6 min. The water in the novel tank was changed to remove olfactory stimuli before the next fish was tested, as recommended by Cachat *et al*. (2010). The following endpoints were measured: latency to reach the upper half of the tank, number of transitions to the upper half, time spent in the upper half, and freezing behaviour. Freezing was defined as an absence of movement (except for gills and eyes) by the fish while at the bottom of the tank (Kalueff *et al.*, 2013). These endpoints were chosen based on previous studies using the novel tank test to assess anxiety in *D. rerio* (Levin *et al.*, 2007; Egan *et al.*, 2009).

One of the two criteria for good welfare defined in this study is whether fish have what they want, and one way to investigate how a fish responds to aspects of its environment is to measure the amount of time that it spends in one type of environment over another type. This can be done with a simple environmental-preference test. After the novel tank test, fish were transferred to choice tanks together with group-mates that had not been used in the novel tank tests. Each tank was positioned ~40 cm in front of an AXIS M1054 network camera, as described above. During the experiment, equal amounts of food were simultaneously provided to both tank compartments. Transfer of all fish to the choice tanks was completed by 101 dpf. Fish were allowed to acclimate for three days before choice testing began. The occupancy by fish of the enriched and bare compartments of each tank was assessed over three days, from 104–106 dpf, during which the network cameras were set to automatically video the fish for 5 min, three times per day, in the morning, afternoon and evening. Recordings were downloaded onto the laptop computer as AVI files and viewed to analyse behaviour. For each group, data were collected by counting the number of fish occupying the bare compartment at 15 s intervals over the 5 min recording, creating 21 sampling points for each observation period. Occupancy counts for each observation period were totalled and a cumulative count calculated for each day. The daily count was expressed as the percentage of fish occupying the bare compartment.

Increased aggression associated with resource defence may impact welfare by increasing signs of distress in subordinate fish. One way to assess resource defence is to compare resource monopolisation between enriched and bare environments. In this study, resource monopolisation was measured while fish were in the choice tanks. Monopolisation was defined as the occupation of one compartment of a choice tank by a single fish. To investigate monopolisation of resources by *D. rerio,* data were collected for each group by viewing the environmental preference test videos and counting the number of sampling points at which a single fish occupied a certain tank compartment. Counts are expressed as a percentage of total sampling points for each day.

DATA ANALYSIS

Statistical analyses were made using SPSS v. 23 (IBM Inc., USA). All data were tested for normality using a Shapiro-Wilk’s test and for equality of variance using a Levene’s test. When the assumptions for parametric testing were not fulfilled, nonparametric alternative tests were used. Data were considered statistically significant at $P = 0.05$.

Chi-square tests of homogeneity were used to determine whether there were differences between treatments and between replicates in the proportion of larvae that survived from 5 to 30 dpf. Mann-Whitney *U*-tests were used to compare standard length between treatments at 30, 60 and 120 dpf, and to compare fork length, mass and body condition at 131 dpf. A chi-square goodness-of-fit test was used to determine whether the sex ratio deviated from the expected 50:50 ratio. Novel tank test data were compared using Mann-

Whitney *U*-tests. Environmental preference data were examined by converting each group's daily occupancy count into a ratio and calculating Jacob's preference index from the ratio, as in Schroeder *et al*. (2014). For each day of the test, between-treatment differences were assessed by an independent samples *t*-test or Mann-Whitney *U*-test and within-treatment differences were assessed for enriched groups by a one-way repeated measures ANOVA and for groups reared in bare tanks by a nonparametric Friedman test. Data for monopolisation of resources were assessed by Mann-Whitney *U*-tests.

RESULTS

SURVIVORSHIP FROM 5–30 DPF

At 30 dpf, there was a significant difference in survivorship between larvae reared in enriched tanks (248; 83% survivorship) and larvae reared in bare tanks (161, 54%) (chi-square test; $\chi^2 = 58.13$, d.f. = 1, $P = 0.001$; Fig. 2). Survivorship between replicates was not significantly different at 30 dpf for enriched or bare tanks.

GROWTH

At 30 dpf, fish in enriched and bare tanks were of similar length (9.0 ± 1.3 mm and 8.8 ± 1.4 mm respectively). After fish (in equal numbers) were transferred to the rearing tanks and maintained between 30 dpf and 60 dpf, enriched fish were shorter in length (median 20.8 mm) than fish in bare tanks (median 22.7 mm) at 60 dpf (Mann-Whitney; $U = 282$, $z = 2.22$, $P = 0.05$; Fig. 3), however, this difference was no longer evident at 120 dpf, when the lengths of fish reared in enriched and in bare tanks did not differ (27.4 ± 2.1 mm and 28.6 ± 1.8 mm, respectively).

At 131 dpf, females in enriched and in bare tanks were of similar fork length [medians 28.3 mm and 29.5 mm respectively; Fig. 4(a)] and mass [medians 0.26 g and 0.27 g respectively; Fig. 4(b)] but body condition scores were higher for females in enriched tanks (1.12) compared with females in bare tanks (1.00) [Mann-Whitney; $U = 44$, $z = –3.86$, $P = 0.001$; Fig. 4(c)]. Males in enriched tanks were smaller in length than males in bare tanks [medians 29.6 mm and 31.5 mm respectively; Mann-Whitney; $U = 231$, $z = 3.18$, $P = 0.001$; Fig. 4(a)] and smaller in mass [medians 0.26 g and 0.32 g respectively; Mann-Whitney;

$U = 227$, $z = 3.03$, $P = 0.01$; Fig. 4(b)] but their body condition scores did not differ [1.00 and 0.99 respectively; Fig. 4(c)].

SEX RATIO

There was no significant departure from the expected sex ratio of 50:50 in either treatment group as 52% of enriched fish were female compared with 49% of fish in bare tanks (chi-square test; $\chi^2 = 0.02$, d.f. = 1, $P > 0.05$).

BEHAVIOUR

There was no difference between fish reared in enriched and bare tanks in latency to enter the upper half of a novel tank (Mann-Whitney; $U = 254$, $z = 1.48$, $P > 0{\cdot}05$) or in the number of transitions to the upper half (Mann-Whitney; $U = 156$, $z = –1.19$, $P > 0{\cdot}05$). However, enriched fish spent more time than fish from bare tanks in the upper half of a novel tank (Mann-Whitney; $U = 53$, $z = –3.98$, $P = 0{\cdot}001$; Fig. 5).

Freezing behaviour was observed on only one occasion and was not included in the analyses.

There was no difference between treatments in occupancy of the bare compartment of choice tanks on any of the three test days (independent samples *t*-tests; Day 1: $t = 0.90$, d.f. = 8, $P > 0{\cdot}05$; Day 2: $t = –1.63$, d.f. = 8, $P > 0{\cdot}05$; Mann- Whitney; Day 3: $U = 17$, $z = 0.94$, $P > 0{\cdot}05$; Fig. 6). Within-treatment difference in occupancy of the bare compartment over the three test days was not significant for enriched groups (ANOVA; $F_{2,8} = 3.00$, $P > 0{\cdot}05$) or for groups in bare tanks (Friedman test; $\chi^2 = 0.95$, d.f. = 2, $P > 0{\cdot}05$).

Monopolisation of resources, where a dominant fish excludes subordinate individuals from its preferred compartment, was recorded in 68% ± 58% of sampling points for enriched fish compared to 5% ± 44% of sampling points for fish reared in bare tanks, a difference that was significant (Mann-Whitney, $U = 40$, $z = –3.020$, $P = 0{\cdot}05$; Fig 7). In most cases, dominant fish monopolised the compartment of the tank that differed from the environment in which they had been reared; dominant enriched fish monopolised the plain compartment in

74% of 530 sampling points, and dominant plain tank reared fish monopolised the enriched compartment in 90% of 213 sampling points.

DISCUSSION

Comprehensive evaluation of the effects of enrichment requires assessments on a combination of indicators of health and welfare (Williams *et al.*, 2009). In this study measures of survivorship, growth, sex ratio, and behaviour were adopted to assess the effects of environmental enrichment on laboratory-held *D. rerio*. Such basic information is of primary importance if optimal conditions are to be provided for good welfare.

SURVIVORSHIP FROM 5–30 DPF

Of the growing body of work on *D. rerio* husbandry, this is the first report on the effects of enrichment on post-hatch survival. This study found that larvae reared in enriched tanks had significantly higher survivorship from 5–30 dpf compared with larvae reared in bare tanks. These findings support reports of increased survivorship of larvae reared with enrichment in other fish species, including Atlantic salmon *Salmo salar* L. 1758, (Hansen & Moller, 1985), Arctic charr *Salvelinus alpinus* (L. 1758) (Benhaïm *et al.*, 2009) and Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* Mitchill 1815 (Gessner *et al.*, 2009).

Differences in early-life survivorship between fish reared in enriched and bare tanks in this study may be linked to an enhanced prey diversity, greater resource availability and/or the energetic cost of escaping from aggressive conspecifics. Larvae in enriched tanks were frequently seen to pick at plant leaves and stems, and examination of a vallis leaf under a light microscope revealed the presence of various single-celled motile organisms, including ciliated protozoa, on the leaf surface. Availability of slow-moving protozoans on plants during the critical life period of first-feeding may provide a vital source of nutrition while larvae learn to hunt and develop feeding suction power. A diet of zooplankton has been shown to benefit early life survivorship in *D. rerio* (Lawrence *et al.*, 2015) and survival rates improve when larvae are fed continually to support their high energy demands (Carvalho *et al.*, 2006; Best *et al.*, 2010). In addition, larvae in enriched tanks may benefit from hiding places provided by plants and gravel. There is considerable variation in size among larvae (Parichy *et al.*, 2009) and small larvae may expend less energy for metabolism if they can avoid attention from the aggressive larger larvae.

GROWTH

Reported lengths of *D. rerio* at given ages vary widely in the literature and differences in growth rates have been reported for different strains (Oswald & Robison, 2008) and diets (Gonzales & Law, 2013), and at different temperatures (Brown *et al.*, 2015) and stocking densities (Ribas *et al.*, 2017). Few studies however, provide comprehensive information about rearing conditions and the resultant growth curves against which the present results can be compared. Overall, the lengths of fish in this study (in both bare and enriched tanks) were similar to those reported by Eaton and Farley (1974) and by Siccardi *et al*. (2009).

That fish from enriched and bare tanks were of similar length at 30 dpf was contrary to expectations. However, this may be explained by the fact that fewer larvae survived in bare tanks than in enriched, and as an equal overall tank food ration was provided, more resources would have been available per fish for the fish in the bare tanks and stocking density is known to affect growth rate (including length gain) in *D. rerio* (Rabbane *et al.*, 2016).

The difference in length between fish reared in enriched tanks and in bare tanks that occurred between 30 and 60 dpf may have resulted from a variance in the age of puberty, or in the rate of growth after puberty. *D. rerio* are reported to grow rapidly until around 50-dpf, after which their growth rate decreases as energy allocation shifts from growth to sexual maturation (Gómez-Requeni *et al.*, 2010). The timing of this shift in energy budget depends upon feeding history with better fed individuals maturing at a younger age and at a larger size (Parichy *et al.*, 2009; Augustine *et al.*, 2011). Alternatively, differential access to food may have developed as fish grew. Energy spent on foraging may have increased for enriched fish due to the effect of habitat complexity on the rate of prey encounter and resulting in the shorter length of enriched fish at 60 dpf. Growth compensation, defined in the literature as accelerated growth after a period of growth depression (Ali *et al.*, 2003), could account for the length of enriched fish converging with the length of fish in bare tanks by 120 dpf.

Females from enriched tanks had higher body condition scores than females from bare tanks. The reason(s) are unclear but may be related to egg production. Developing oocytes account for a large part of the body weight of female *D. rerio* and fecundity increases with increased food intake (Forbes *et al.*, 2010).

Males reared in bare tanks had greater length and mass than enriched males and, although median conditions scores were similar for both treatments, condition was more variable in males reared in bare tanks than enriched males. Further work is needed to determine the causes of differences in body condition between females reared in enriched and in bare tanks observed in this study, and the greater variability of body condition among males reared in bare tanks compared to enriched males.

SEX RATIO

The observed sex ratio did not deviate from the expected 50:50 ratio. The mode of sex determination in *D. rerio* is uncertain but likely to be controlled by genetic factors that are sensitive to environmental conditions (Wilson *et al.*, 2014) with unfavourable conditions, such as high temperatures (Abozaid *et al.*, 2011), high rearing density (Liew *et al.*, 2012), and poor nutrition (Lawrence *et al.*, 2008), tending to favour male development. In this study, environmental enrichment did not influence sex determination.

BEHAVIOUR

In this study, fish reared in enriched tanks and in bare tanks showed similar latency to enter the upper half of the novel tank and made a similar number of transitions to the upper half, but enriched fish spent significantly more time than fish from bare tanks in the upper half during the 6-min test. Increased time spent in the upper half is considered to indicate lower anxiety levels (Cachat *et al.*, 2010) and the median time spent in the upper half by fish from bare tanks was similar to that reported for control groups in other studies (e.g. Egan *et al.*, 2009; Wong *et al.*, 2010). Overall, enriched fish displayed lower levels of anxiety-like behaviour than fish from bare tanks when in a novel environment. Maximino *et al*. (2010) reported similar results when comparing anxiety-like behaviour of enriched and bare-reared *D. rerio* in a dark/light test. In contrast, Marcon and colleagues (2018) found that fish kept in enriched tanks were more anxious in the novel tank compared to fish kept in standard tanks. Such conflicting results illustrate the risk of relying on a single report when making decisions about fish housing conditions (Bayne, 2005).

Fish preference for an enriched vs bare environment was assessed by housing each group in a choice tank and measuring the number of fish in the bare compartment at various time points. The expectation that fish would prefer an enriched environment was not supported by the data. Preference for the enriched compartment did not differ significantly between or within treatments. These results are similar to those reported by Hamilton & Dill (2002) who found no difference in use by *D. rerio* of (artificially) vegetated and open habitats but differ from those reported by Delaney *et al*. (2002), Kistler *et al*. (2011) and Schroeder *et al*. (2014), who found that *D. rerio* show a clear preference for substrate and plants over a bare tank. Habitat choice in this study may have been confounded by the behaviour of dominant individuals who monopolised access to a preferred compartment. Overall, it is difficult to draw conclusions from this choice study. Further investigation is needed to determine when and why fish gravitate to certain environments within a tank and whether preferences vary with age, reproductive status, social status, group size, or even tank size.

Resource monopolisation was significantly higher for enriched fish than for fish reared in bare tanks. Interference competition among foragers involves aggressive exclusion of competitors by dominant individuals (Godin, 1997) and it seems likely that the design of the choice tanks, with a 40 mm access hole in the divider, allowed dominant fish to defend and exclude subordinates from a compartment. During the experiment, equal quantities of food were provided to each side of the tank, making resource monopolisation an efficient strategy for dominant fish. The reason for resource monopolisation being more prevalent in enriched groups is unclear, but may relate to habitat-linked behavioural plasticity as observed in juvenile Atlantic cod *Gadus morhua* L.1758 (Salvanes *et al.*, 2007) and bluegill sunfish *Lepomis macrochirus* Rafinesque 1819 (Chipps *et al.*, 2004). Bhat *et al*. (2015) observed that certain behavioural responses of *D. rerio* to environmental manipulation varied among populations from different habitats, suggesting that rearing environment may affect behavioural adaptability in this species. The monopolisation of resources by dominant individuals and associated aggression reported in this study have possible negative effects on welfare.

In conclusion, environmental enrichment, in the form of gravel and plants, has varied effects on laboratory-maintained *D. rerio*. Some effects (on survivorship, body condition, and anxiety-like behavior) are positive from the perspective of fish welfare, whereas other effects (such as the tendency to monopolise resources) appear to be negative. Together with the results of previous studies (Basquill & Grant, 1998; Carfagnini *et al.*, 2009; Kistler *et al.*, 2011; Schroeder *et al.*, 2014; Collymore *et al.*, 2015; Keck *et al.*, 2015; Wafer *et al.*, 2016), the findings presented here indicate that (a) multiple welfare indicators are needed in order to make a valid scientific assessment of wellbeing and (b) the effects of enrichment differ between life stages, suggesting that no single set of housing conditions is optimal for all life stages. Future experiments should investigate the effects of different types and amounts of enrichment, and of variable vs stable enrichment, in order to inform what housing conditions promote optimum welfare in *D. rerio*. The challenge is to design enrichment that offers measurable welfare benefits that can be implemented practically without compromising unduly the economics of the housing facility or the protocols applied to address the research questions of interest.

This study was funded by the University of Exeter, including resources provisioned through the Aquatic Resources Centre.

REFERENCES

Abozaid, H., Wessels, S. & Hörstgen-Schwark, G. (2011) Effect of rearing temperatures during embryonic development on the phenotypic sex in zebrafish (Danio rerio). *Sexual Development* **5**, 259–265. doi: 10.1159/000330120.

Ali, M., Nicieza, A. & Wootton, R. J. (2003) Compensatory growth in fishes: a response to growth depression. *Fish and Fisheries* **4**, 147–190. doi:

10.1046/j.1467-2979.2003.00120.x.

Ashley, P. J. (2007) Fish welfare: current issues in aquaculture. *Applied Animal Behaviour Science* **104**, 199–235. doi: 10.1016/j.applanim.2006.09.001.

Augustine, S., Gagnaire, B., Floriani, M., Adam-Guillermin, C. & Kooijman, S. A. L. M. (2011) Developmental energetics of zebrafish, Danio rerio. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **159**, 275–283. doi: 10.1016/j.cbpa.2011.03.016.

Basquill, S. P. & Grant, J. W. (1998) An increase in habitat complexity reduces aggression and monopolization of food by zebra fish (Danio rerio). *Canadian Journal of Zoology* **76**, 770–772. doi: 10.1139/z97-232.

Bayne, K. (2005) Potential for unintended consequences of environmental enrichment for laboratory animals and research results. *Ilar Journal* **46**, 129–139. doi: 10.1093/ilar.46.2.129.

Bayne, K. & Würbel, H. (2014) The impact of environmental enrichment on the outcome variability and scientific validity of laboratory animal studies. *Revue scientifique et technique (International Office of Epizootics)* **33**, 273–80. doi: 10.20506/rst.33.1.2282.

Beets, J. & Friedlander, A. (1998) Evaluation of a conservation strategy: a spawning aggregation closure for red hind, Epinephelus guttatus, in the U.S. Virgin Islands. *Environmental Biology of Fishes* **55**, 91–98.

Benhaïm, D., Leblanc, C. A. & Lucas, G. (2009) Impact of a new artificial shelter on Arctic charr (Salvelinus alpinus, L.) behaviour and culture performance during the endogenous feeding period. *Aquaculture* **295**, 38–43. doi: 10.1016/j.aquaculture.2009.06.024.

Best, J., Adatto, I., Cockington, J., James, A. & Lawrence, C. (2010) A novel method for rearing first-feeding larval zebrafish: polyculture with Type L saltwater rotifers (Brachionus plicatilis). *Zebrafish* **7**, 289–295. doi: 10.1089/zeb.2010.0667.

Bhat, A., Greulich, M. M. & Martins, E. P. (2015) Behavioral plasticity in response to environmental manipulation among zebrafish (Danio rerio) populations. *PLoS ONE* **10**, 1–13. doi: 10.1371/journal.pone.0125097.

Brown, A. R., Owen, S. F., Peters, J., Zhang, Y., Soffker, M., Paull, G. C., Hosken, D. J., Wahab, M. A. & Tyler, C. R. (2015) Climate change and pollution speed declines in zebrafish populations. *Proceedings of the National Academy of Sciences* **112**, E1237–E1246. doi: 10.1073/pnas.1416269112.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., *et al.* (2010) Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols*. Nature Publishing Group **5**, 1786–99. doi: 10.1038/nprot.2010.140.

Carfagnini, A. G., Rodd, F. H., Jeffers, K. B. & Bruce, A. E. E. (2009) The effects of habitat complexity on aggression and fecundity in zebrafish (Danio rerio). *Environmental Biology of Fishes* **86**, 403–409. doi: 10.1007/s10641-009-9539-7.

Carvalho, A. P., Araújo, L. & Santos, M. M. (2006) Rearing zebrafish (Danio rerio) larvae without live food: evaluation of a commercial, a practical and a purified starter diet on larval performance. *Aquaculture Research* **37**, 1107–1111. doi: 10.1111/j.1365-2109.2006.01534.x.

Chipps, S. R., Dunbar, J. A. & Wahl, D. H. (2004) Phenotypic variation and vulnerability to predation in juvenile bluegill sunfish (Lepomis macrochirus). *Oecologia* **138**, 32–38. doi: 10.1007/s00442-003-1396-z.

Collymore, C., Tolwani, R. J. & Rasmussen, S. (2015) The behavioral effects of single housing and environmental enrichment on adult zebrafish (Danio rerio). *Journal of the American Association for Laboratory Animal Science* **54**, 280–285. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26045453.

Dawkins, M. S. (2017) Animal welfare with and without consciousness. *Journal of Zoology* **301**, 1–10. doi: 10.1111/jzo.12434.

Delaney, M., Follet, C., Ryan, N., Hanney, N., Lusk-Yablick, J. & Gerlach, G. (2002) Social interaction and distribution of female zebrafish (Danio rerio) in a large aquarium. *Biological Bulletin* **203**, 240–241.

Eaton, R. C. & Farley, R. D. (1974) Growth and the reduction of depensation of zebrafish, Brachydanio rerio, reared in the laboratory. *Copeia* **1**, 204–209.

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., Elkhayat, S. I., Bartels, B. K., Tien, A. K., Tien, D. H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z. & Kalueff, A. V (2009) Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural brain research* **205**, 38–44. doi: 10.1016/j.bbr.2009.06.022.

European Union: Council of the European Union (2010) Council Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* **L 276**, 33–79. Available at: http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm.

Forbes, E. L., Preston, C. D. & Lokman, P. M. (2010) Zebrafish (Danio rerio) and the egg size versus egg number trade off: Effecte of ration size on fecundity are not mediated by orthologues of the Fec gene. *Reproduction, Fertility and Development* **22**, 1015–1021. doi: 10.1071/RD09257.

Gessner, J., Kamerichs, C. M., Kloas, W. & Wuertz, S. (2009) Behavioural and physiological responses in early life phases of Atlantic sturgeon (Acipenser oxyrinchus Mitchill 1815) towards different substrates. *Journal of Applied Ichthyology* **25**, 83–90. doi: 10.1111/j.1439-0426.2009.01246.x.

Godin, J.-G. J. (ed.) (1997) *Behavioural ecology of teleost fishes*. Oxford: Oxford University Press.

Gómez-Requeni, P., Conceição, L. E. C., Jordal, A. E. O. & Rønnestad, I. (2010) A reference growth curve for nutritional experiments in zebrafish (Danio rerio) and changes in whole body proteome during development. *Fish Physiology and Biochemistry* **36**, 1199–1215. doi: 10.1007/s10695-010-9400-0.

Gonzales, J. M. & Law, S. H. W. (2013) Feed and feeding regime affect growth rate and gonadosomatic index of adult zebrafish (Danio rerio). *Zebrafish* **10**, 532–40. doi: 10.1089/zeb.2013.0891.

Grunwald, D. J. & Eisen, J. S. (2002) Headwaters of the zebrafish — emergence of a new model vertebrate. *Nature Reviews* **3**. doi: 10.1038/nrg891.

Hamilton, I. M. & Dill, L. M. (2002) Monopolization of food by zebrafish (Danio rerio) increases in risky habitats. *Canadian Journal of Zoology* **80**, 2164–2169. doi: 10.1139/z02-199.

Hansen, T. J. & Moller, D. (1985) Yolk absorption, yolk sac constrictions, mortality, and growth during first feeding of Atlantic salmon (Salmo salar) incubated on astro-turf. *Canadian Journal of Fisheries and Aquatic Sciences* **42**, 1073–1078.

Home Office (2014) Guidance on the operation of the Animals (Scientific Procedures) Act 1986. Available at: www.gov.uk/research-and-testing-using-animals.

Huntingford, F. A., Adams, C., Braithwaite, V. A., Kadri, S., Pottinger, T. G., Spandoe, P. & Turnbull, J. F. (2006) Current issues in fish welfare. *Journal of Fish Biology* **68**, 332–372. doi: 10.1111/j.1095-8649.2005.01046.x.

Johansen, J. L., Bellwood, D. R. & Fulton, C. J. (2008) Coral reef fishes exploit flow refuges in high-flow habitats. *Marine Ecology Progress Series* **360**, 219–226. doi: 10.3354/meps07482.

Johnsson, J. I., Brockmark, S. & Näslund, J. (2014) Environmental effects on behavioural development consequences for fitness of captive-reared fishes in the wild 1946–1971. doi: 10.1111/jfb.12547.

Kalueff, A. V, Gebhardt, M., Stewart, A. M., Cachat, J. M., Brimmer, M., Chawla, J. S., Craddock, C., Kyzar, E. J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton, W., Miller, N., Nicolson, T., Braubach, O., *et al.* (2013) Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish* **10**, 70–86. doi: 10.1089/zeb.2012.0861.

Keck, V. A., Edgerton, D. S., Hajizadeh, S., Swift, L. L., Dupont, W. D., Lawrence, C. & Boyd, K. L. (2015) Effects of habitat complexity on pair-housed zebrafish. *Journal of the American Association for Laboratory Animal Science* **54**, 378–383. Available at: http://www.ingentaconnect.com/content/aalas/jaalas/2015/00000054/00000004/art00007.

Kistler, C., Hegglin, D., Würbel, H. & König, B. (2011) Preference for structured environment in zebrafish (Danio rerio) and checker barbs (Puntius oligolepis). *Applied Animal Behaviour Science* **135**, 318–327. doi: 10.1016/j.applanim.2011.10.014.

Lawrence, C., Ebersole, J. P. & Kesseli, R. V. (2008) Rapid growth and out-crossing promote female development in zebrafish (Danio rerio). *Environmental Biology of Fishes* **81**, 239–246. doi: 10.1007/s10641-007-9195-8.

Lawrence, C., James, A. & Mobley, S. (2015) Successful replacement of Artemia salina nauplii with marine rotifers (Brachionus plicatilis) in the diet of preadult zebrafish (Danio rerio). *Zebrafish* **12**, 366–371. doi: 10.1089/zeb.2015.1118.

Levin, E. D., Bencan, Z. & Cerutti, D. T. (2007) Anxiolytic effects of nicotine in zebrafish. *Physiology & behavior* **90**, 54–58. doi: 10.1016/j.physbeh.2006.08.026.

Liew, W. C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K. R. & Orban, L. (2012) Polygenic sex determination system in zebrafish. *PLoS ONE* **7**. doi: 10.1371/journal.pone.0034397.

Marcon, M., Mocelin, R., Benvenutti, R., Costa, T., Herrmann, A. P., de Oliveira, D. L., Koakoski, G., Barcellos, L. J. G. & Piato, A. (2018) Environmental enrichment modulates the response to chronic stress in zebrafish. *Journal of Experimental Biology* **221**. doi: 10.1242/jeb.176735.

Matthews, M., Trevarrow, B. & Matthews, J. (2002) A virtual tour of the Guide for zebrafish users. *Lab animal* **31**, 34–40. doi: 10.1038/5000140.

Maximino, C., Marques de Brito, T., Dias, C. A. G. D. M., Gouveia, A. & Morato, S. (2010) Scototaxis as anxiety-like behavior in fish. *Nature protocols* **5**, 209–16. doi: 10.1038/nprot.2009.225.

Näslund, J. & Johnsson, J. I. (2014) Environmental enrichment for fish in captive environments: effects of physical structures and substrates. *Fish and Fisheries* **17**, 1–30. doi: 10.1111/faf.12088.

Oswald, M. & Robison, B. D. (2008) Strain-specific alteration of zebrafish feeding behavior in response to aversive stimuli. *Canadian Journal of Zoology* **86**, 1085–1094. doi: 10.1139/Z08-085.

Parichy, D. M., Elizondo, M. R., Mills, M. G., Gordon, T. N. & Engeszer, R. E. (2009) Normal table of post-embryonic zebrafish development: staging by externally visible anatomy of the living fish. *Developmental Dynamics* **238**, 2975–3015. doi: 10.1002/dvdy.22113.Normal.

Paull, G. C., Van Look, K. J. W., Santos, E. M., Filby, A. L., Gray, D. M., Nash, J. P. & Tyler, C. R. (2008) Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing population-level effects of environmental chemicals. *Aquatic Toxicology* **87**, 115–26. doi: 10.1016/j.aquatox.2008.01.008.

Pritchard, V. L., Lawrence, J., Butlin, R. K. & Krause, J. (2001) Shoal choice in zebrafish, Danio rerio: the influence of shoal size and activity. *Animal Behaviour* **62**, 1085–1088. doi: 10.1006/anbe.2001.1858.

Rabbane, M. G., Rahman, M. M. & Hossain, M. A. (2016) Effects of stocking density on growth of zebrafish (Danio rerio, Hamilton, 1822). *Bangladesh Journal of Zoology* **44**, 209. doi: 10.3329/bjz.v44i2.32760.

Ribas, L., Valdivieso, A., Díaz, N. & Piferrer, F. (2017) Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response. *Journal of Experimental Biology* **220**, 1056–1064. doi: 10.1242/jeb.144980.

Salvanes, A. G. V., Moberg, O. & Braithwaite, V. A. (2007) Effects of early experience on group behaviour in fish. *Animal Behaviour* **74**, 805–811. doi: 10.1016/j.anbehav.2007.02.007.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**, 671–675. doi: 10.1038/nmeth.2089.

Schroeder, P., Jones, S., Young, I. S. & Sneddon, L. U. (2014) What do zebrafish want? Impact of social grouping, dominance and gender on preference for enrichment. *Laboratory animals* **48**, 328–37. doi: 10.1177/0023677214538239.

Siccardi, A. J., Garris, H. W., Jones, W. T., Moseley, D. B., D'Abramo, L. R. & Watts, S. A. (2009) Growth and survival of zebrafish (Danio rerio) fed different commercial and laboratory diets. *Zebrafish* **6**, 275–280. doi:

10.1089/zeb.2008.0553.

Spence, R., Fatema, M. K., Reichard, M., Huq, K. A., Wahab, M. A., Ahmed, Z. F. & Smith, C. (2006) The distribution and habitat preferences of the zebrafish in Bangladesh. *Journal of Fish Biology* **69**, 1435–1448. doi: 10.1111/j.1095-8649.2006.01206.x.

Thomaz, S. M. & da Cunha, E. R. (2010) The role of macrophytes in habitat structuring in aquatic ecosystems: methods of measurement, causes and consequences on animal assemblages' composition and biodiversity. *Acta Limnologica Brasiliensia* **22**, 218–236. doi: 10.4322/actalb.02202011.

Tran, S. & Gerlai, R. T. (2016) The novel tank test: handling stress and context specific psychopharmacology of anxiety. *Current Psychopharmacology* **5**, 169–179. doi: 10.2174/2211556005666160519144.

Volpato, G. L. (2009) Challenges in assessing fish welfare. *ILAR Journal* **50**, 329–337. doi: 10.1093/ilar.50.4.329.

Wafer, L. N., Jensen, V. B., Whitney, J. C., Gomez, T. H., Flores, R. & Goodwin, B. S. (2016) Effects of evironmental enrichment on the fertility and fecundity of zebrafish (Danio rerio). *Journal of the American Association for Laboratory Animal Science* **55**, 291–294.

Williams, T. D., Readman, G. D. & Owen, S. F. (2009) Key issues concerning environmental enrichment for laboratory-held fish species. *Laboratory animals* **43**, 107–20. doi: 10.1258/la.2007.007023.

Wilson, C. A., High, S. K., McCluskey, B. M., Amores, A., Yan, Y. L., Titus, T. A., Anderson, J. L., Batzel, P., Carvan, M. J., Schartl, M. & Postlethwait, J. H. (2014) Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. *Genetics* **198**, 1291–1308. doi: 10.1534/genetics.114.169284.

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K. M., Stewart, A., Gilder, T., *et al.* (2010) Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research*. Elsevier B.V. **208**, 450–457. doi: 10.1016/j.bbr.2009.12.023.

ELECTRONIC REFERENCES

European Union: Council of the European Union (2010) Council Directive

2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* **L 276**, 33–79. Available at: http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm (last accessed 7 August 2018).

Home Office (2014) Guidance on the operation of the Animals (Scientific Procedures) Act 1986. Available at: www.gov.uk/research-and-testing-using-animals (last accessed 7 August 2018).

U.S. EPA (1996). *Ecological effect test guidelines OPPTS 850.1500 fish life-cycle toxicity*. Washington, D.C.: United States Environmental Protection Agency. Available at https://www.epa.gov/sites/production/files/2015-07/documents/850-1500.pdf (last accessed 28 July 2017).