

**The effects of environmental enrichment and
environmental stability on the welfare of
laboratory zebrafish**

Submitted by

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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, including fish; and poor welfare may equate to poor science. Laboratory zebrafish, *Danio rerio*, are usually kept in bare aquaria for ease of husbandry and, although it is a well-studied species, little is known about the effects on *D. rerio* of laboratory housing. The first investigation of this thesis shows that environmental enrichment, in the form of gravel and plants, affects survivorship, growth, body condition and behaviour in laboratory-maintained zebrafish. Larvae reared in enriched tanks had significantly higher survivorship than larvae reared in plain tanks. Fish reared in enriched tanks were shorter (20.8 mm) than fish reared in plain tanks (22.7 mm) at 60 days post-fertilisation (pdf) but not at 120 dpf. Females in enriched tanks had higher body condition scores (1.74) than females in plain tanks (1.57) and body condition was more variable in males in plain tanks (1.56 ± 0.14) than in enriched tanks (1.54 ± 0.10). Sex ratio did not differ between treatments. Fish from enriched tanks displayed lower levels of anxiety-like behaviour than fish from plain tanks when acutely transferred to a novel environment. Preference for the enrichment did not differ between treatments but resource monopolisation was higher for enriched fish than for plain fish. Data generated by this study enhance our understanding of what environmental conditions improve housing for laboratory zebrafish.

Although environmental enrichment is often purported as the solution to improving wellbeing in laboratory fish, many enrichments are not compatible with aquaculture or research facilities. The second investigation of this thesis hypothesised that significant welfare benefits may be achievable through simple practical solutions easily adapted to current practices in research laboratories. To investigate these new approaches, this study examined the effects of simple changes in the tank environment on the wellbeing of captive fish, using zebrafish as an experimental model. It was hypothesised that moving fish between tanks of identical status (bare) would provide positive stimulation equating to more complex enriched environments. Groups of zebrafish were housed in 'stable' environments (where groups were maintained in the same tanks throughout the study) or in 'changed' environments (where groups were periodically moved to novel tanks). Comparisons between treatments included effects on morphometry (length, mass and condition), reproductive success (egg output and viability) and aggressive behaviour. For the simple changes adopted—tank and water—no significant effect of environmental stability was found on body condition, reproductive output or aggression. It was concluded from this pilot study that changing the tank did not have any obvious health benefits to the fish, for the periods of time studied.

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Chapter 1 Introduction

This thesis investigates the welfare of laboratory zebrafish. It is a data-driven quantitative inquiry that seeks to measure the effects of laboratory housing conditions on zebrafish. It is a study that attempts to understand how the natural history and ecology of the zebrafish influence its health and wellbeing in the laboratory, an influence that extends also to behaviour and productivity. In this investigation, I studied groups of laboratory zebrafish at various life stages, from embryos to adults, and quantified their survivorship, growth, reproductive output, and behaviour under differing housing conditions. I develop two lines of inquiry that explore the effects that emerged of (1) environmental enrichment and (2) a changed vs stable environment on the wellbeing of laboratory zebrafish. In subsequent chapters I detail these experiments and suggest how their results may be interpreted. In this first chapter I will outline the background to the study, including the biology and behaviour of the species, differences between wild and captive zebrafish, and gaps in the literature that led to the research questions that this thesis addresses.

The laboratory zebrafish

The zebrafish (*Danio rerio*) is a small tropical freshwater teleost fish of the Cyprinidae family. The natural range of the zebrafish is uncertain (Engeszer et

al., 2007) but wild populations have been reported in regions of India, Pakistan, Bangladesh, Nepal and Myanmar (Spence *et al.*, 2006; Engeszer *et al.*, 2007; Arunachalam *et al.*, 2013) where a monsoon climate results in seasonal flooding and wide fluctuation in water spread area (Bassi *et al.*, 2014). The zebrafish is an omnivorous annual species in the wild (Spence *et al.*, 2007). Mature zebrafish are sexually dimorphic with a fusiform body shape and distinctive pattern of alternating dark and light stripes along the flanks and fins. The light stripes of the mature male are yellow or golden and those of the female are silvery. Males are typically slender whereas females have a rounded abdomen and genital papilla that become more pronounced at maturity. The mean standard length (from the tip of the lower jaw to the caudal fin) recorded from a wild population in Bangladesh was 25 mm, with females slightly larger than males (Spence *et al.*, 2008). Surprisingly little is known of the biology and behaviour of wild zebrafish although recent field studies have provided some insights (e.g., Arunachalam *et al.*, 2013; Suriyampola *et al.*, 2015).

The zebrafish was first described by Francis Hamilton in the Kosi river in northern India (Hamilton, 1822). Hamilton's "beautiful fish ... with blue and silver stripes" became a popular aquarium fish following the development of modern air transport that enabled live fish to be moved from source to market (Vitko, 2004). In the late 1960s, George Streisinger, a geneticist and molecular biologist with a passion for tropical fish, brought zebrafish into his laboratory at the University of Oregon and began to use them as a model to study vertebrate development (Stahl, 1995). Streisinger established methods to detect lethal recessive mutations in zebrafish and developed procedures for producing homozygous diploid clones. His pioneering work was published in 1981

(Streisinger *et al.*, 1981), establishing the zebrafish as a promising model organism and leading to the development of new procedures for mutagenesis (Driever *et al.*, 1994). In 1993, work started in Germany and the USA on two large-scale screens for embryonic-lethal mutations, known collectively as 'The Big Screen' (Grunwald & Eisen, 2002). This collaboration recovered and characterized around 4000 mutant phenotypes (Grunwald & Eisen, 2002). The results of The Big Screen were published in 1996 as 37 papers in a special issue of *Development*, confirming the zebrafish as the foremost research model for development biology (Nüsslein-Volhard, 2012). Since then, the zebrafish has also become a prominent model in the fields of toxicology, human diseases, pharmacology, and evolutionary theory (Grunwald & Eisen, 2002).

Importance to science

In 2001, researchers at the Wellcome Trust Sanger Institute began an international project to sequence the entire zebrafish genome (Howe *et al.*, 2013). When the results were compared to the human genome, researchers found that 71% of human genes have a zebrafish orthologue and 82% of genes linked with human disease have a zebrafish equivalent (Howe *et al.*, 2013). These genetic similarities between humans and zebrafish have made the zebrafish a valuable model for studying human development and disease and for discovering and screening new drugs (Santoriello & Zon, 2012). Recently, the development of advanced gene editing techniques such as the Clustered, Regularly Interspaced, Short Palindromic Repeat (CRISPR)/CRISPR-associated 9 (Cas9) system have enabled the zebrafish genome to be

engineered by inducing a double-stranded break at a specific location in the genome and then inserting, removing or changing sections of the DNA sequence (Sertori *et al.*, 2016). The effects of such genetic editing can then be studied in order to better understand the function of affected genes. Zebrafish are amenable to both 'forward' genetics, a process of searching a genome for new gene functions, and 'reverse' genetics, an investigation of the function of a specific known gene, making them particularly useful for modelling human disease and for identifying the genes and pathways underlying diseases (Santoriello & Zon, 2012).

Zebrafish models have been developed for a range of human diseases and disorders, including those affecting the heart, kidneys, brain and central nervous system, muscular system, and behaviour (Santoriello & Zon, 2012). Within cancer research, zebrafish are used to model cancers of organs such as the liver, pancreas and skin; investigate tumour angiogenesis and metastasis; and evaluate new therapies (Zhao *et al.*, 2015).

Zebrafish offer advantages over other model organisms for the study of human development and disease. In contrast to invertebrate models such as the fruit fly (*Drosophila melanogaster*) and nematode (*Caenorhabditis elegans*), zebrafish have a similar body plan and nervous system to humans (Bassett & Currie, 2003). Although zebrafish lack the lungs and mammary glands of mammals, they possess an equivalent to many other human organs.

Compared to mice, zebrafish can be kept in greater numbers in a smaller area. Zebrafish reach sexual maturity at around 3 months of age and can produce

clutches of over 100 eggs several times per week (Markovich *et al.*, 2007). The embryos are transparent and develop externally, with most major organs being fully developed by 24 hours post-fertilisation (Kimmel *et al.*, 1995), making them a powerful model for studying vertebrate growth and development. Recently, researchers have used optical resolution photoacoustic microscopy to observe the formation and development of blood vessels in zebrafish embryos without the use of a fluorescence label or contrast agent, increasing the potential of the zebrafish model for understanding human heart disease, hypertension, stroke, and heart attack (Chen *et al.*, 2017).

Zebrafish have the ability to completely regenerate damaged heart tissue, in contrast to a human heart which, following injury, replaces dead muscle cells with fibrous scar tissue that does not contract and so impairs heart function (Marín-juez *et al.*, 2016). A recent study revealed that a key to the zebrafish's regeneration of heart muscle lies in its ability to quickly revascularise the damaged area (Marín-juez *et al.*, 2016). This finding sheds light on the mechanisms of heart tissue regeneration and paves the way for the development of new therapies to treat human heart disease. Zebrafish can also regenerate damaged fins, making them invaluable for investigations into regeneration and wound healing (Pfefferli & Jazwinska, 2015).

Zebrafish can develop almost all of the cancer types found in humans, with comparable histology and gene expression (Feitsma & Cuppen, 2008). The zebrafish model offers unique experimental advantages over traditional cancer models such as the mouse. These include in vivo visualization of tumour progression in transparent embryos and translucent adults, and transplantation

of cancer cells from humans that could lead to personalised cancer screens (White *et al.*, 2013).

The zebrafish is a powerful model for the study of human muscle disease. The cellular structure of somitic muscle of zebrafish and humans is almost identical (Gibbs *et al.*, 2013) which, coupled with the amenability of zebrafish to large-scale drug screens, makes the zebrafish a powerful model for the study of human muscle disease and the development of therapies. Novel treatments, developed in part using zebrafish models, for Duchenne and other muscular dystrophies have recently entered clinical trials (Gibbs *et al.*, 2013).

Neurodegenerative disorders such as Huntington, and Alzheimer's diseases have been successfully modeled in zebrafish (Santoriello & Zon, 2012) and researchers have recently identified a drug that restored movement in a zebrafish model of Parkinson's disease (Zhang *et al.*, 2017).

Zebrafish are widely used for toxicological studies in both human health and environmental risk assessment. The International Organization for Standardization (ISO) published the first zebrafish toxicity test in 1984 (International Organization for Standardization, 1996). Since then, zebrafish have been used for testing toxicity to vertebrates of a wide range of substances from cigarette smoke (Ellis *et al.*, 2014) to alcohol (Tran *et al.*, 2016), and for monitoring environmental pollutants such as heavy metals (Pawar *et al.*, 2016), endocrine disruptors (Brown *et al.*, 2015), and organic pollutants (Wang *et al.*, 2015). Zebrafish are increasingly used to test chemicals for potential bioactivity. Such predictive-based testing aims to evaluate early biological responses to

chemical exposures, identify potentially hazardous new chemistries, and aid the design of safer chemicals (Noyes *et al.*, 2016).

Why welfare is important

The welfare of zebrafish used in scientific research is important for moral, legal and practical reasons. Morally, we should treat all animals with respect and avoid causing unnecessary suffering or pain (RSPCA, 2014); legally, researchers are obliged to consider the welfare of laboratory animals, including fish (Home Office, 2014a, 2014b); and practically, poor welfare may equate to poor growth rate, poor survival rates and poor science (Weed & Raber, 2005).

In addition to the legal requirements governing the care of research animals, the major funding bodies in the UK have issued guidelines for scientists and animal care staff on the use of animals in research (NC3Rs *et al.*, 2015), the implementation of which is a condition of receiving funds. These guidelines recommend the adoption of a “culture of care” with regard to research animals, which includes ethical review of all experiments involving animals; adoption by researchers and care staff of respectful and careful attitudes and behaviour towards animals in their charge; and standards of animal welfare that exceed the legal minimum (NC3Rs *et al.*, 2015). A culture of care, supported and led by senior management, is one of the guiding principles on good practice for laboratory animal welfare recommended by the RSPCA and LASA (2015).

Three guiding principles form the basis of the humane 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', were developed by the Universities Federation for Animal Welfare (Russell & Burch, 1959) and have since been incorporated into national (Home Office, 2014b) and international (European Union: Council of the European Union, 2010) legislation.

In 2004, the UK government established the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), an independent scientific organisation set up to fund innovation and support technological developments that focus on the 3Rs. Recent NC3Rs-funded projects include the development of 3-D *in vitro* functional assays to replace animals in cancer drug development (Vinci *et al.*, 2012), the development of a new sampling method to reduce the number of fish used in immune studies (Collet *et al.*, 2017), and the assessment of new methods to improve the welfare of laboratory rodents during gas euthanasia (Thomas *et al.*, 2012).

The replacement of animals in research and testing is the ultimate goal of academic and industrial institutions who adopt the principles of the 3Rs and considerable progress has been made in this regard. Animal models in some areas of research have been replaced by the use of computer models, human volunteers, cell lines, or invertebrates such as *Drosophila* and nematode worms (Prescott, 2017). Where there is no viable alternative, refinement of the methods of animal use, housing and husbandry can help to minimise pain,

distress or harm that may be experienced by the animals (Prescott & Lidster, 2017).

Where there are currently no alternatives to the use of animals for research, the refinement of scientific procedures, housing and husbandry can benefit animal welfare and improve the reliability of research data (Prescott & Lidster, 2017). Refinement has the potential to improve the wellbeing and life experience of individual research animals. Even small changes to housing, husbandry or experimental methods can have a measurable effect on welfare. For example, a study of laboratory mice found that mice picked up by the of base of the tail (a traditional handling method) showed high anxiety and poor performance in behavioural tests whereas mice picked up in a handling tube or cupped hand showed lower levels of anxiety and improved performance, indicating that handling methods may influence both the welfare of laboratory mice and experimental results (Gouveia & Hurst, 2017). Another study used a conditioned place avoidance test to compare aversive reactions of zebrafish to three substances commonly used to euthanise fish and found that metomidate hydrochloride and clove oil are less aversive to zebrafish than the more widely-used tricaine methanesulfonate, suggesting that these substances could be used to improve welfare during euthanasia (Wong *et al.*, 2014). Identification and implementation of such refinements to husbandry, housing, or procedures, especially when applied within a culture of care, can create new best practice, deliver higher welfare and improve the quality of science.

Fish are the most diverse group of vertebrates, accounting for half of all described vertebrate species (Fig. 1; IUCN, 2016). Fish species have adapted

to a wide range of environments, from deep seas to anoxic swamps and from polar regions to deserts (Helfman *et al.*, 1997). Fish are found almost

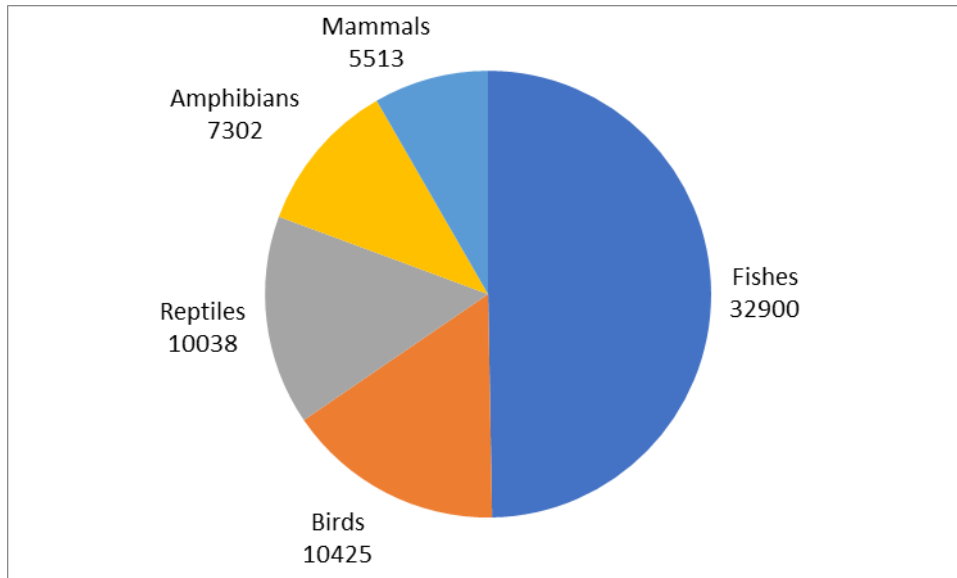


Figure 1. Chart showing the number of described species in each major vertebrate group.

everywhere that there is water. The diversity of aquatic environments is reflected in the vast range of biology, anatomy, physiology and behaviour of fishes. Adult fish body sizes range from the 8-mm-long Indian Ocean goby (*Trimmatom nanus*) to the 12-m-long whale shark (Helfman *et al.*, 1997). Examples of the diverse life histories of fishes include lungfishes which can lie dormant for years when their ponds dry up and revive when immersed in water (Helfman *et al.*, 1997), the killifish (*Nothobranchius kadleci*) which reaches sexual maturity in 17 days and lives in ephemeral muddy puddles (Blažek *et al.*, 2013), and the Greenland shark (*Somniosus microcephalus*), which reaches sexual maturity at 150 years, can live for 400 years, and ranges in Arctic waters from the surface to depths of 1800 m (Nielsen *et al.*, 2016). The pronounced differences among fish species mean that generalised guidelines for their care and welfare are of limited value and species-specific information is essential.

Refinements to housing or husbandry that improve the wellbeing of one species may not work for others. For example, a study of the effects on three species of fish of handling with a scoop versus a dip-net found differences between species (Brydges *et al.*, 2009). Three-spined sticklebacks (*Gasterosteus aculeatus*) and Panamanian bishops (*Brachyrhaphis episcopi*) handled with a scoop had lower operculum beat rates than fish handled with a net, but this effect was not found in rainbow trout (*Oncorhynchus mykiss*) which exhibited a high level of response to both handling methods. Behavioural tests revealed that Panamanian bishops were less motivated to leave a shelter and were more neophobic when handled with a scoop compared to a net, but this effect was not found in sticklebacks and was not measured in trout due to their high level of response to both handling treatments. These findings suggest that using a scoop to move fish between tanks could reduce the negative effects of handling in some species, but not in others (Brydges *et al.*, 2009).

Stocking density, the amount of space provided for each fish, is another husbandry factor that can compromise welfare (Johansen *et al.*, 2006). The effects of stocking density are species-specific. High density causes stress in Senegalese sole (*Solea senegalensis*, Costas *et al.*, 2008) and gilthead seabream (*Sparus auratus*, Montero *et al.*, 1999), low density leads to increased aggression in zebrafish (Paull *et al.*, 2008) and Arctic charr (*Salvelinus alpinus*, Jørgensen *et al.*, 1993), whilst Atlantic salmon (*Salmo salar*) show highest welfare at intermediate stocking densities (Turnbull *et al.*, 2005). In ornamental species, neon tetras (*Paracheirodon innesi*), white cloud mountain minnows (*Tanichthys albonubes*) and tiger barbs (*Barbus tetrazona*) spend more time shoaling and are less aggressive when housed in larger

groups, but this effect is not seen in angelfish (*Pterophyllum scalare*, Saxby *et al.*, 2010). The environmental needs of the species (which may vary throughout its life stages) should be taken into account when designing care protocols and monitoring welfare.

Environmental enrichment

The environmental enrichment of a fish tank by, for example, the addition of physical structures, 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 stereotypies or increased aggression (Näslund & Johnsson, 2016). In many natural aquatic habitats, structures such as rocks, gravel, sand, vegetation and algae create environmental complexity that is useful to most fish species at some life stage (Näslund & Johnsson, 2016). Structurally complex habitats offer shelter from predators, aggressive conspecifics or strong currents (Johansen *et al.*, 2008); additional feeding sites (Thomaz & da Cunha, 2010); or cover from which predators can ambush or stalk prey (Horinouchi *et al.*, 2009). In contrast, most laboratory fish are housed in bare tanks that offer no stimuli. As with other forms of refinement, the design of environmental enrichment should take into account the natural history of the species, including its habitat, behaviour and social structure. 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 (Johnsson *et al.*, 2014).

Environmental enrichment should benefit both fish and research. There is evidence that some forms of enrichment improve welfare for some fish species (reviewed by Näslund & Johnsson, 2016) but every environmental factor, biotic or abiotic, can potentially affect an animal's physiology or behaviour (Killen *et al.*, 2013) and so influence research results. In addition, forms of enrichment such as the addition of plants and gravel are considered impractical or costly by some laboratories (Lidster *et al.*, 2017). The benefits of enrichment, based on evidence, need to be established and weighed against costs and practicalities in order to make a convincing case for the increased use of environmental enrichment for laboratory fish.

Evidence of the effects of environmental enrichment is usually obtained from neurological, physiological or behavioural measurements. Neurological data include comparisons of the size of the brain and its structures, such as the cerebellum, telencephalon, and optic tectum, between fish reared in barren and enriched environments (Näslund *et al.*, 2012). Physiological measures include growth, body condition, sex differentiation, reproductive performance, metabolic rate and hormone levels. Behaviours, such as aggression, stereotypies, resource monopolisation and response to stress and anxiety can be measured, and tests for choice, motivation and aversion used to determine fish preferences, although there is evidence that an animal's choice may not be a reliable indicator of what is best for its wellbeing (Benefiel *et al.*, 2005). While single measurements of the positive or negative effects of enrichment are useful, a combination of different indicators will allow a more comprehensive evaluation of the potential benefits of enrichment (Williams *et al.*, 2009).

The benefits of enrichments such as the provision of cage structures, nesting materials, sensory stimuli, and social partners, have been investigated for a number of laboratory species (Table 1). For all such studies, interpreting results

Table 1. Examples of the benefits of various forms of enrichment for different species of laboratory animals.

Benefit	Enrichment	Species	References
Reduced aggression; reduced monopolization of food	Simulated vegetation	Zebrafish	(Basquill & Grant, 1998)
Increased mass; decreased food consumption	Nesting material	Mice	(Van de Weerd <i>et al.</i> , 1997)
Improved spatial memory	Cage structures	Rats	(Leggio <i>et al.</i> , 2005)
Reduced trichophagia between animals	Dietary (hay)	Guinea pigs	(Gerold <i>et al.</i> , 1997)
Decreased stereotypies (digging, floor chewing, bar biting); increased locomotor activity	Group housing	Rabbits	(Chu <i>et al.</i> , 2004)
Reduced feather pecking	Substrate	Chickens	(Nicol <i>et al.</i> , 2001)
Reduced time spent inactive; reduced chewing of cage furniture	Toys	Dogs	(Hubrecht, 1993)
Reduced aggression and agitation; increased social affiliations	Auditory stimulation (music)	Chimpanzees	(Howell <i>et al.</i> , 2003)

in order to understand the exact cause of improvement in welfare is complex (Young, 2003). For example, Hamilton and Dill (2002) demonstrated that zebrafish in an environmental choice tank prefer to forage in covered habitats

rather than open or vegetated habitats, and that food monopolisation is reduced in covered habitats compared to open or vegetated habitats. These results suggest that (a) cover may be perceived as safer than vegetation and (b) lower food monopolization may result from greater safety or a reduced ability to defend a resource (Hamilton & Dill, 2002). From a welfare perspective, it could be argued that the choice-tank provided fish with the opportunity to express some control over their environment, thus promoting behavioural homeostasis, which is important for good welfare (Garner, 2005). In addition, the reduction of aggression associated with resource defence improved welfare by reducing signs of distress in subordinate fish. Further studies to disentangle the importance of risk, competition and the ability to defend a worthwhile resource could assess the importance of habitat for the welfare of zebrafish. The effects of enrichment can vary with strain (Nevison *et al.*, 1999), sex (Lin *et al.*, 2011) and life-stage (Gerber *et al.*, 2015).

Animal welfare is difficult to define, measure and quantify and is the subject of an ongoing scientific debate based upon arguments for and against evidence that nonhuman animals are sentient and therefore capable of suffering (Weed & Raber, 2005; Duncan, 2006; Volpato *et al.*, 2007).

One of the first definitions of welfare was proposed in 1965 by the Brambell Committee, a body set up by the British government to investigate intensive livestock farming. The committee recommended that 'five freedoms' be adopted as minimum standards for farm animals (Brambell, 1965):

1. Freedom from hunger and thirst
2. Freedom from discomfort

3. Freedom from pain, injury and disease
4. Freedom to express normal behaviour
5. Freedom from fear and distress.

The five freedoms have since been incorporated into the UK legislation (Parliament of the UK, 2006) and widely adopted by veterinarians (British Veterinary Association, 2016) and animal welfare organizations (RSPCA, 2014; Universities Federation for Animal Welfare, 2017). However, disagreement persists regarding the relevance of the freedoms. Some researchers suggest that an animal's affective state as it responds to challenges presented by its environment is a more important indicator of welfare (Broom & Johnson, 1993; Webster, 2005; Mellor & Beausoleil, 2015), while others argue that welfare should be assessed solely in terms of fitness and survival and that welfare is compromised only when an animal's fitness is reduced (Barnard & Hurst, 1996).

Function-based welfare criteria, such as good health and fitness, are relatively easy to measure. In contrast, it is not possible to directly measure an animal's state, i.e. how it feels as it copes with stimuli such as pain or hunger, because feelings are subjective and so known only to the animal experiencing them (Duncan, 2006). However, feelings can be investigated through indirect methods such as preference, avoidance and motivation tests to assess how an animal feels about aspects of its environment, and obstruction and operant response tests to indicate strength of preference and how important a particular choice is for an animal (Duncan, 2006).

Fish welfare is the subject of hot debate centred around differences between nociception (an unconscious response to a noxious stimuli) and pain (a conscious experience), with conflicting views about whether fish have the sense organs and sensory systems required to experience pain, and disagreement over whether fish can be considered sentient animals (Volpato *et al.*, 2007). Sneddon *et al.*, (2003) and Roques *et al.* (2010) provide physiological and behavioural evidence of nociception in fish and conclude that fish also feel pain. Brown (2015) points to evolutionary and comparative neurobiological evidence that nociception and pain perception are ancient adaptive traits that confer fitness benefits to all vertebrates. He argues that, although pain systems may have evolved differently in terrestrial and aquatic vertebrates due to the different demands of their environments, they likely have similar functions. Key (2016) and Rose *et al.* (2014), on the other hand, assert that fish lack the sensory systems for neural processing required for feeling pain and that evidence of consciousness in fishes is inconclusive. Until science can prove or disprove that fish are sentient animals, the ethical position should be to give fish ‘the benefit of the doubt’, treat them with respect, and minimise potential pain and suffering whenever possible.

Knowledge of the effects of environmental enrichment on the welfare of fish lags behind comparative knowledge for mammals (Williams *et al.*, 2009) and many questions remain unanswered about the environmental requirements of different fish species (Williams *et al.*, 2009). There is a need for practical, objective, species-specific welfare indicators for everyday monitoring of research fish. We also need better understanding of fish welfare and how changes to a fish’s environment affect its welfare.

It is generally accepted by researchers that environmental enrichment improves the welfare of research animals and that better welfare leads to better science (Hawkins, 2014). However, concerns persist that enrichment may affect data quality by increasing experimental variability (Hawkins, 2014). Most animal experiments are designed to reduce within-group variability in order to minimize the number of animals used and increase statistical power during hypothesis testing (Griffin, 2012). Enrichment, in the form of gravel and plants, is an experimental variability and its effects on within-group variability can be inconsistent. Some studies report that enrichment does not affect standardization one way or another (Marashi *et al.*, 2004; Wolfer *et al.*, 2004) whereas others show that its effects on variability are parameter-dependent (Mering *et al.*, 2001; Van de Weerd & Aarsen, 2010).

Between-experiment variability is another area of concern. For example, Crabbe and colleagues (1999) reported systematic behavioural differences between mice in different laboratories despite going to “extraordinary lengths to equate test apparatus, testing protocols, and all possible features of animal husbandry.” Interestingly, Richter *et al.* (2009) suggest that reproducibility of animal experiments between laboratories is improved by systematic variation of environmental factors rather than standardization. As support, they present an analysis of data from a multi-laboratory study of behavioural differences between inbred mouse strains that indicates that standardization increases the risk of obtaining idiosyncratic site-specific results that lack external validity (Richter *et al.*, 2009). The authors suggest that reproducibility of experiments

may be improved by including environmental heterogenization in the experimental design.

Environmental enrichment can potentially improve the validity of data. For example, several studies have shown that enrichment affects disease progression in mouse models (Hockly *et al.*, 2002; Glass *et al.*, 2004; Sorrells *et al.*, 2009) suggesting that enriched mice may mimic human disease more accurately. Conversely, a barren environment may produce physiological and behavioural abnormalities in animals that negatively impact the validity of data obtained from them (Bayne & Wurbel, 2014) and could even increase within-group variability through individual variations in the severity of abnormal behaviours such as stereotypies (Garner, 2005). Overall, the provision of appropriate species-specific environmental enrichment that meets the welfare needs of the animal is an ethical imperative (Bayne & Wurbel, 2014) and if enrichment cannot be provided for valid scientific reasons, an explanation should be included in the materials and methods section of the experimental report (Hawkins, 2014).

Environmental enrichment sometimes produces conflicting effects. For example, Collymore and colleagues (2015) found that zebrafish in an environmental-choice tank spent more time associating with conspecifics than swimming near an artificial plant. In contrast, a study by Delaney *et al.* (2002) reported that female zebrafish spent more time swimming near artificial plants than associating with conspecifics. Rainbow trout (*Oncorhynchus mykiss*) fry raised in enriched tanks were reported by Berejikian *et al.* (2000) to be more aggressive and territorial than fry raised in conventional tanks, whereas Tatara

and coworkers (2008) found little difference in aggression between fry raised in the two environments. And in a mouse model of Alzheimer disease, mice that received early-life exposure to environmental enrichment showed reduced behavioural abnormalities, such as hyperactivity, disinhibition and reduced risk-assessment, in a study by Verret *et al.* (2013) but not in a similar study by Hüttenrauch and colleagues (2016).

Conflicting responses to environmental enrichment may result from lack of standardisation between experiments. Even slight differences in laboratory conditions such as lighting levels (Trullas & Skolnick, 1993), noise (Lauer *et al.*, 2009), odours from husbandry procedures (López-Salesansky *et al.*, 2016), and the position of enrichment within the housing unit (Kostomitsopoulos *et al.*, 2007; Riber & Nielson, 2013) can lead to inconsistent test results. Improved descriptions in the literature of housing and husbandry conditions, and increased communication and collaboration between researchers might help to improve standardization and minimize variation of experimental conditions between laboratories.

Results cannot be compared across species. The effects of environmental enrichment for one species of fish may not be applicable to other species, even those with similar ecology. For example, Fischer (2000) found that the respiration rate of burbot (*Lota lota*; a benthic species) was reduced by 30% when cobble substrates were added to their tanks, whereas no significant effect was found when cobbles were added to the tanks of stone loach (*Barbatula barbatula*; another benthic species).

Some studies have shown a negative effect of enrichment on fish welfare. For example, territorial behaviour observed in seven species of fish increased in a diverse environment compared to a plain environment (Nijman & Heuts, 2000) and levels of aggression increased when enrichment was provided to male butterfly splitfins (*Ameca splendens*) (Kelley *et al.*, 2006), and to juvenile perch (*Perca fluviatilis*) (Mikheev *et al.*, 2005).

Enrichment needs to not only benefit the fish, but also be cost effective and practical. Practical issues associated with enrichment include increased labour costs and increased risk of pathogen infections due to the difficulties of cleaning substrates, higher light levels needed by plants encourage the growth of algae (McNabb *et al.*, 2012), and toxicity test regulations that limit the types of enrichment that can be used in toxicity study test areas (Williams *et al.*, 2009). Some aquatic systems are easier than others to incorporate enrichment. For example, the use of enrichment in large recirculating systems with high density racks is impractical and could facilitate pathogen infections which, once established, are difficult to eradicate (Lawrence, 2016). Smaller flow-through or static systems are more amenable to the addition of enrichment.

The natural history of the zebrafish

The life history of the zebrafish comprises four broad stages. The fertilized egg develops rapidly: within 24 hours the transparent embryo forms all of its major organs and tissues and within 48-72 hours hatches and attaches to a hard surface (such as a leaf) using secretory cells in the epidermis of its head (Laale,

1977). Through a succession of attachments at higher levels, the larva moves to the surface where it gulps air to inflate its swim bladder. The newly hatched larva does not have fully formed mouth parts and is reliant on yolk sac nutrients, but within 1-2 days of hatching it begins to feed exogenously and to hunt and capture live prey (Parichy *et al.*, 2009). By 7 days post-fertilization, the yolk sac is completely absorbed and the larva relies on an exogenous food source (Wilson, 2012). From around 14- to 29-dpf, the larva undergoes a metamorphosis to the juvenile form during which the larval fin fold is lost, the gut and nervous system are remodelled, and scales develop (Parichy *et al.*, 2009). At around 3 months, the juvenile becomes sexually mature at which point it enters the adult stage and spawning occurs (Harper & Lawrence, 2012). The duration of each life stage is variable and dependent upon factors such as temperature, rearing density, water quality and food availability (Parichy *et al.*, 2009).

Zebrafish are regarded as a seasonal species. Spence *et al.* (2007) sampled wild fish from a single site in Bangladesh over a 12-month period and determined that the species is relatively short-lived in nature with recruitment linked to the monsoon season and found no evidence of wild zebrafish surviving to breed a second year. In contrast, a lifespan study of laboratory zebrafish by Gerhard *et al.* (2002) reported a mean survival of 42 months, with the oldest individual living for 66 months. The subjects studied by Gerhard and colleagues were outbred wild-type zebrafish whereas a similar study by Herrera & Jagadeeswaran (2004) tested an inbred strain and reported a mean lifespan of 31 months and the oldest fish surviving for 45 months. The variance between

these two studies suggests that life expectancy of laboratory zebrafish may be influenced by strain.

Natural environment

Some field studies report that zebrafish are a floodplain species, found mainly in slow moving or standing water, especially waters associated with paddy fields (Spence *et al.*, 2006, 2007). Other studies found wild populations in mountainous as well as lowland areas; in a wider range of habitats, from stagnant lake-like wetlands to the secondary- and even tertiary-channels of alluvial rivers (McClure *et al.*, 2006; Arunachalam *et al.*, 2013); in waters with pH values from 6.2 to 9.8; and water temperatures from 12.3°C to 28.4°C (Arunachalam *et al.*, 2013). Many of these waters are highly seasonal with monsoon rains causing widespread flooding, increased water levels, and changes to water chemistry and temperature (Suriyampola *et al.*, 2015). During the monsoon, adult zebrafish move from streams and rivers where they spend most of the year, into flooded areas to spawn in paddy fields and other shallow, well vegetated habitats (Engeszer *et al.*, 2007).

The zebrafish's physical environment is varied and complex. Substrates range from silt, sand and gravel to pebbles, boulders and bedrock (Arunachalam *et al.*, 2013). Habitats are typically well vegetated with aquatic plants, riparian vegetation and overhanging canopy, although details of plant species are not reported in the literature (McClure *et al.*, 2006; Spence *et al.*, 2006; Arunachalam *et al.*, 2013; Raja *et al.*, 2016). The social behaviour of wild

zebrafish varies across populations with groups of 4–12 individuals observed in a slow-flowing river compared to shoals of up to 300 in a fast-flowing river (Suriyampola *et al.*, 2015). Zebrafish share their habitat with a variety of other fish, including *Esomus danricus*, *Devario* spp., *Barilius* spp., *Rasbora* spp. and *Chela* spp. (Arunachalam *et al.*, 2013), as well as piscine predators such as *Xenedonton cancila* and *Channa* spp. (Spence *et al.*, 2006).

How zebrafish interact with their environment

Fish obtain information from their environment by using sensory systems that are well developed (Brown, 2014). Fish eyes are similar to those of other vertebrates and are able to detect a wide range of light wavelengths including, in some species and life stages, ultraviolet and polarised light (Helfman *et al.*, 1997). A fish's hearing ability depends on the environment in which it lives. Fish detect sound via particle motion rather than membrane vibration (Brown, 2014). The fish's inner ear receives vibration information from otoliths (ear bones) and from the lateral line, a specialised sense organ that detects water motion and pressure gradients (Brown, 2014). Some species, such as carp, also detect sound through their swim bladder (Brown, 2014). A wide variety of species can detect electric currents or sense the earth's magnetic field and are able to use this information for spatial navigation (Helfman *et al.*, 1997). Chemoreception (taste and smell) in fish is well developed and used to detect chemical cues in the environment. These cues inform a wide variety of behaviours, including prey detection, predator avoidance, mate location, navigation and homing (Brown, 2014). Salmon famously use their sense of smell to detect the particular

combination of chemicals found in their natal stream and guide their return (Helfman *et al.*, 1997).

Effects of environment on zebrafish

The complex, varied and changeable environment across the zebrafish's natural range may account for the wide genetic variation within and among wild populations and the existence of genetically distinct groups corresponding to different geographic locations (Whiteley *et al.*, 2011). Habitat complexity influences survival, reproduction, predation and predator avoidance in fish species (Shumway, 2008), and variation in habitat can change morphological, physiological and behavioural phenotypes (Watters *et al.*, 2003). Although there are no published data on neurology of wild zebrafish, laboratory studies show that environmental change alters brain cell proliferation in laboratory populations (von Krogh *et al.*, 2010) and habitat complexity correlates with brain size in several closely-related species of cichlid fish (Shumway, 2008).

Zebrafish physiology also appears to be affected by habitat. Suriyampola and co-workers (2015) sampled wild zebrafish from four sites that differed in water flow and vegetation, and found that individuals from flowing-water sites were significantly larger than those in still waters, zebrafish from rice paddies were smaller than those from an open channel, and individuals from fast-flowing rivers were smaller than those from slow-moving streams (Suriyampola *et al.*, 2015). These differences among populations were accompanied by differences in social behaviour. In slow-flowing waters, zebrafish occurred in groups of 4-12 fish and were more aggressive than those in faster flowing water, where groups

of up to 300 individuals were found and where very little aggression was observed (Suriyampola *et al.*, 2015). Sex differentiation in zebrafish is also influenced by an environmental factor—temperature—with elevated water temperatures during embryonic development resulting in an increased male proportion of the sex ratio (Abozaid *et al.*, 2011).

Zebrafish in captivity

Wild and captive zebrafish differ in growth rates, age at maturity, reproductive season, lifespan, genetic diversity and behaviour. For example, a growth rate of 183 mm per year during the first 45 dpf was reported for a laboratory population (Eaton & Farley, 1974), whereas a wild population grew at 72 mm per year during the first 60 dpf (Spence *et al.*, 2007), a difference of >250% between the two groups. Reproductive maturity occurred at ~3 months of age in laboratory fish (Eaton & Farley, 1974) compared to ~10 months of age for F₁ wild fish reared in the laboratory (Spence *et al.*, 2007). Laboratory zebrafish breed continuously all year (Nasiadka & Clark, 2012) whereas wild fish generally spawn seasonally, beginning shortly before the start of the monsoon.

Variations in growth, development and behaviour between wild and captive zebrafish are likely due to differences in environmental conditions and selective pressures. Most laboratory zebrafish live in a homogeneous environment with little sensory stimulation, no predators, unnaturally high stocking densities, a homogeneous diet and little opportunity to forage (Johnsson *et al.*, 2014; Lawrence, 2016). Such conditions can lead to changes in levels of boldness

and aggression, changed breeding behaviour, increased growth, early maturity and larger body size (Delaney *et al.*, 2002; Wright *et al.*, 2006; Amaral & Johnston, 2012; Bhat *et al.*, 2015). In addition, although some of the natural selection pressures are removed from laboratory populations, unintentional selection due to captive rearing has been shown to induce substantial changes in gene expression in just nine generations of randomly harvested zebrafish (Uusi-Heikkilä *et al.*, 2017).

Husbandry

The effects on welfare of husbandry practices such as feeding, breeding techniques, stocking densities, handling techniques, tank cleaning and pathogen control are largely unknown. The quantity of food fed to fish and the frequency of feeding varies between laboratories. Some laboratories feed to satiation while others feed only the amount that fish can consume within 5 min; some feed once a day and others feed up to five times a day; some feed live prey, some feed artificial diets, and some feed a combination of live and artificial food (Lawrence, 2007). The effect of feeding regimes on welfare remains to be evaluated. Likewise, breeding techniques, such as the use of small breeding chambers or trays, of various sizes and volumes, placed within home tanks to stimulate spawning, and the use of mass-spawning tanks versus small-group or pair-mating tanks, have not been assessed to determine their effects on behaviour and welfare. Net handling and associated air exposure is known to elicit a cortisol response in zebrafish (Ramsay *et al.*, 2009), and both handling and overcrowding stress increase susceptibility to mycobacterial infection in

zebrafish (Ramsay *et al.*, 2009). Stocking densities affect behaviour such as aggression (Paull *et al.*, 2008). A subordinate fish in a small tank may be unable to escape from an aggressive conspecific and the inability to escape from a situation which affects its survival indicates poor welfare for the subordinate fish (Dawkins, 1990).

Welfare

Any discussion of the effect of housing conditions on fish welfare assumes that we know what welfare is and how to measure it. Studies of fish welfare usually follow one of three approaches. The first, 'feeling-based' approach, sets out to prove or disprove that fish are sentient beings (i.e., that they have the capacity to suffer). Evidence reviewed by Braithwaite and Huntingford (2004) suggests that, despite their relatively simple brain and nervous system, fish do have the capacity to experience pain and fear and, therefore, to suffer. The second approach, 'physiological', attempts to measure pain and stress as indicators of lack of a fish's welfare state (Volpato, 2009). Finally, the 'behavioural' approach uses behavioural analyses to infer learning, preference and choice and to support arguments for fish cognition and emotions (Volpato, 2009; Vila Pouca & Brown, 2017). Fish 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 latter may be discovered through choice/preference tests and operant conditioning techniques (Volpato, 2009).

Welfare is difficult to measure and a combination of different indicators may be needed to produce a comprehensive evaluation of a fish's welfare. The first criteria for good welfare, whether fish are healthy, can be assessed by measuring survivorship, growth, and reproductive performance, and by determining the presence of absence of disease.

Most mortalities in laboratory zebrafish stocks occur between 11 and 16 dpf when larvae first become dependent on exogenous feeding following absorption of the yolk-sac (Wilson, 2012). Such mortalities are likely due to starvation or incorrect nutrition (Wilson, 2012). Mortality in older larvae and juveniles is less common and causes are uncertain but include infectious and non-infectious diseases (Matthews, 2004), and attacks by aggressive conspecifics (Paull *et al.*, 2008). Ideally, evidence of poor health should alert researchers to a welfare problem before mortality occurs, however, mortality can be a welfare indicator to safeguard the surviving fish in the group (Ellis *et al.*, 2012). Uniform growth rates for fish from the same batch of eggs, with fish maturing within 3-4 months, are one sign of positive welfare (Lawrence, 2012) while slow growth can be indicative of chronic stress due to husbandry methods or infectious diseases (Ramsay *et al.*, 2010). In addition to using length and mass measurements as indicators of growth rate, a length-mass relationship, such as Fulton's condition factor (Froese, 2006) can be calculate and used as a proxy for the nutritional state of individual fish.

Reproductive performance may be a welfare indicator as the reproductive performance of teleost fish is known to be affected by nutritional deficiencies (Izquierdo *et al.*, 2001), pathogens (Schreck, 1997), and stress (Billard *et al.*,

1981). Reproductive responses to stress vary depending on the nature of the stressor and, while strong stress has a negative effect on reproduction, mild stress sometimes has a positive effect (Schreck, 2010). For example, a recent study found that increased cortisol levels (induced by feeding fish with cortisol-laced food) increased fecundity in female zebrafish (Faught *et al.*, 2016).

The presence or absence of disease is often used as a measure of health in fish (Segner *et al.*, 2012). However, disease is a poor measure of health and regular screening programmes for early detection of pathogens and the prevention of infection in fish populations, are a preferable indicator and are increasingly used in zebrafish facilities (Lawrence, 2011). Zebrafish are susceptible to a range of pathogens, many of which may be present as subclinical infections for weeks or even months before symptoms are apparent (Lawrence *et al.*, 2012). The most common disease of laboratory zebrafish is microsporidiosis, a parasitic infection that affects the central nervous system and skeletal muscles, causing weight loss, spinal deformity, and lethargy (Ramsay *et al.*, 2010). Other diseases result from mycobacterial infections that cause anorexia, dropsy, skin ulcers and high mortality (Collymore *et al.*, 2016); parasitic and fungal pathogens that damage gills and skin; and viruses that affect the spleen and kidneys (Collymore *et al.*, 2016). Many of these conditions are exacerbated by stress or poor husbandry (Ramsay *et al.*, 2010). Recent studies have found that bacterial infections can alter swimming behaviour (Lee *et al.*, 2015), shoaling behaviour (Spagnoli *et al.*, 2017), and startle response (Spagnoli *et al.*, 2015) in zebrafish and could, therefore, affect the results of experiments that use these behaviours (Spagnoli *et al.*, 2015).

The second criteria for good welfare, whether fish have what they want, can be assessed by measuring stress and behaviour, including preferences and strength of preference. Levels of the stress hormone cortisol in the bloodstream are often used to assess levels of stress in fish, with lower levels of cortisol considered to be a positive welfare indicator, while higher levels are considered to be negative (Ellis *et al.*, 2012). Extracting blood from zebrafish is difficult because of the fish's small size, and stressful for the fish. A non-invasive measurement of cortisol in fish holding water has been validated for zebrafish (Félix *et al.*, 2013) and a method of collecting cortisol from the scales of common carp (*Cyprinus carpio*) allows monitoring of chronic stress and may be applicable to other species, including zebrafish (Aerts *et al.*, 2015). In addition to a rise in cortisol levels, stressed zebrafish show 'emotional fever' (stress-induced hyperthermia) by choosing to spend more time at higher temperatures in response to handling and confinement (Rey *et al.*, 2015).

Observing behaviour is a simple way to assess the welfare of captive fish. The effects of different environmental parameters can be assessed by measuring behaviour of fish in the presence or absence of the variable of interest. Many behavioural assays have been developed for use with zebrafish, including tests for levels of aggression (Way *et al.*, 2015), boldness (Dahlbom *et al.*, 2011), novelty-induced response (Wong *et al.*, 2010), stress (Maximino *et al.*, 2010), social behaviour (Abril-de-Abreu *et al.*, 2015), reproductive behaviour (Henriksen *et al.*, 2016), spatial cognition (Spence *et al.*, 2011), learning (Carrillo & McHenry, 2016), and memory (Gerlai, 2017), some of which involve testing fish individually while others are used with pairs or groups of fish.

Environmental enrichment

The provision of various forms of environmental enrichment for zebrafish have been evaluated by a number of studies, but comparisons among studies are difficult because of differences in the types of enrichment provided, in variables measured, and in confounding variables such as rearing environment, age of experimental fish, and social context before and during the study. For example, one study found that zebrafish spent 99% of their time in areas containing artificial plants (Delaney *et al.*, 2002) whereas another study reported no difference in use of vegetated and bare habitats (Hamilton & Dill, 2002), although the 'vegetation' in this study comprised black plastic strips. The results of a third study (Schroeder *et al.*, 2014) highlight the effect of gender and social structure on the preference of zebrafish for enrichment. The researchers found that males preferred floating vegetation to submerged plants but females had no preference; and group-housed fish preferred an area with submerged plant to a barren area whereas pair-housed fish had no preference for submerged plants versus a barren area (Schroeder *et al.*, 2014).

The provision of enrichment in the form of substrate and plants for laboratory zebrafish was reported by 23 of 95 laboratories that responded to a recent survey on the welfare of zebrafish in research (Lidster *et al.*, 2017). Many respondents expressed concerns about increased labour, risk of disease, inconsistency of scientific results, and financial costs associated with enrichment, and some respondents suggested that evidence of the benefits could help overcome the challenges of providing enrichment (Lidster *et al.*, 2017).

Gaps in the literature

There are many open questions relating to zebrafish welfare. Tank sizes and stocking densities appropriate for welfare need thorough evaluation as reports in the literature show conflicting results. For example, aggression was found to increase at densities ranging from 0.025 fish L⁻¹ (Larson *et al.*, 2006) to 1.4 fish L⁻¹ (Moretz *et al.*, 2007) whereas Paull and colleagues (2008) reported that levels of aggression decreased with increased density. The association between tank size and tank configuration (width, depth, etc.), stocking density and wellbeing remains to be established. Another unknown is whether replicated periods of dawn and dusk benefit laboratory fish (Lidster *et al.*, 2017) and whether light intensity affects welfare. The optimum diet and feeding schedule for laboratory zebrafish is still to be determined (Lawrence, 2016). More knowledge is required about the effects of environmental enrichment, such as substrates and natural or artificial plants, on welfare, including the preferences of zebrafish for individual elements of enrichment, and whether the effects of enrichment or of environmental change differ between life stages.

Many gaps in the literature regarding welfare and husbandry stem from lack of knowledge of zebrafish natural history and behaviour in the wild. There are many questions and few answers. For example, little is known about the social behaviour of wild zebrafish, their shoaling preferences (to shoal with kin or non-kin, and with individuals of uniform or mixed sizes), conditions that affect shoal size, how females choose mates, the fecundity of wild zebrafish, levels of aggression in wild populations, and the natural diet of larval zebrafish. The

typical home range size of zebrafish shoals is also unreported. Knowledge of the behaviour of wild zebrafish is relevant for welfare and husbandry.

The zebrafish research community acknowledges challenges to the implementation of environmental enrichment for laboratory fish due, in part, to the lack of empirical evidence of the value and benefits of such strategies (Lidster *et al.*, 2017). Evidence is needed to inform best choice for enrichment, to identify the best methods to assess the effects of enrichment, and to communicate the value and benefits of enrichment.

Determining which refinement strategies are most effective and result in the highest welfare benefit, and which strategies are incidental, is a challenge for welfare research. Some forms of enrichment, such as the addition of substrates and plants, come at the cost of increased labour requirements, and evidence of the effectiveness of these strategies will allow costs to be weighed against benefits (such as increased reproductive performance or improved survivorship). In addition, evidence of the effectiveness of environmental change as a refinement could inform welfare choices in situations where enrichment is incompatible with research, such as during regulatory based toxicology testing.

To assess the effectiveness of enrichment and environmental change as refinement strategies for laboratory zebrafish, the sensitivity and reliability of welfare measures, such as behavioural tests, need to be assessed and compared and new tests developed in cases where procedures, apparatus, or handling cause stress or affect results. In addition, methods to assess

motivation, such as those developed for Mozambique tilapia (*Oreochromis mossambicus*) (Galhardo *et al.*, 2011) and goldfish (*Carassius auratus*), (Sullivan *et al.*, 2016) could be adapted for zebrafish in order to measure strength of preference for different forms of enrichment.

Once established, the values and benefits of enrichment and/or change need to be communicated, clearly and engagingly, with the research community through the formal publication system, at conferences, and in discussion between facilities. Without clear evidence that enrichment or change can enhance the welfare of zebrafish without affecting experimental results, it is unlikely that researchers who dismiss enrichment as trivial or non-productive (Meredith, 2013) will be persuaded to apply it.

The present study

The effects of laboratory housing on the welfare of zebrafish are poorly understood and many questions remain unanswered about the environmental requirements of this species. Better understanding is needed of zebrafish welfare and how a fish's environment affects its welfare. Laboratory zebrafish are usually kept in bare aquaria in order to reduce variables between experimental groups 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 goals of providing optimal conditions for generating high-quality experimental subjects while creating high welfare standards for laboratory fish.

The present study will address this gap in the literature by investigating the interactions between environment and the welfare of fish used for research.

Aims

The aim of this study was to evaluate the effects of enrichment and of a changed versus stable environment on the welfare of laboratory-held zebrafish. The nature of the topic dictates the use of a combination of different indicators to allow a comprehensive evaluation of the potential benefits of enrichment.

In order to achieve this aim, two separate investigations were undertaken. In the first investigation, groups of zebrafish were raised in plain tanks and in tanks enriched with gravel and plants and the following endpoints were compared between treatments: survivorship; body length and condition; sex ratio; anxiety-like behavior; preference for environment; and tendency to monopolise resources. In the second investigation, groups were housed in 'stable' environments (maintained in the same tanks throughout the study) or in 'changed' environments (were periodically moved to novel tanks with replacement system water) and the following endpoints were compared between treatments: body length, mass and condition; reproductive success (egg output and viability); and aggressive behaviour.

Research questions

The following research questions were addressed:

- Does environmental change affect body condition, reproductive output or levels of aggression?
- Does enrichment confer a fitness benefit to larval fish?
- Does enrichment affect body length, condition or sex ratio?
- Does enrichment affect anxiety-like behavior or resource monopolisation?
- Do zebrafish prefer an enriched or a plain environment?

Thesis outline

The thesis is structured so that the two experimental chapters represent stand-alone pieces of work.

Chapter 2 describes the effects of environmental enrichment on survivorship, growth, development and behaviour of zebrafish. It investigates the relationship between enrichment and survivorship, body length, body condition, sex ratio, anxiety-like behaviour, preference for environment and tendency to monopolise resources. These indicators allow a comprehensive evaluation of the potential benefits of enrichment and the results of this study will advance understanding of what environmental conditions improve welfare for laboratory zebrafish.

Chapter 3 investigates whether welfare benefits are achievable through simple procedures easily adapted to current practices in research laboratories. This study examines the effects of changes in the tank environment on the wellbeing of laboratory zebrafish. Groups of zebrafish were housed in ‘stable’ environments (where groups were maintained in the same tanks throughout the study) or in ‘changed’ environments (where groups were periodically moved to novel tanks with replacement system water). Comparisons between treatments included effects on morphometry (length, mass and condition), reproductive success (egg output and viability) and aggressive behaviour.

Chapter 4 presents a series of conclusions drawn from this work and suggestions for future work.

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Chapter 2 Effects of environmental enrichment on survivorship, growth, sex ratio and behaviour in laboratory-maintained zebrafish

Introduction

The zebrafish *Danio rerio* (Hamilton 1822) is a small tropical freshwater fish of the Cyprinidae family, native to the Indian subcontinent, and typically found in vegetated areas of static or slow moving waters (Spence *et al.*, 2008). *D. rerio* is a prominent research model in a number of fields, including developmental biology, toxicology, human disease, pharmacology and evolutionary theory (Grunwald & Eisen, 2002). Its advantages as a model organism include its small size, robustness, high fecundity, transparent embryos, and its tolerance to a wide range of husbandry conditions. In addition, a fully sequenced genome, transgenic tools and mutant phenotypes make *D. rerio* ideal for research in genetics and embryology (Parichy, 2015)

The welfare of animals used in research is important for moral, legal and practical reasons. Morally, all animals should be treated with respect and with care to avoid causing unnecessary suffering or pain (RSPCA, 2014); legally, researchers are obliged to consider the welfare of laboratory animals, including fish (Home Office, 2014a, 2014b); and practically, poor welfare may equate to poor science (Weed & Raber, 2005).

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, 2014b) and international (European Union: Council of the European Union, 2010) legislation.

Refinement has the potential to improve the wellbeing and life experience of individual research animals (Baumans & Van Loo, 2013). 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, 2016). Structurally complex habitats offer shelter from predators or aggressive conspecifics (Johansen *et al.*, 2008), additional feeding sites (Thomaz & da Cunha, 2010), or cover from which predators can ambush or stalk prey (Horinouchi *et al.*, 2009). In contrast, most laboratory fish are housed in bare tanks that offer 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 (Johnsson *et al.*, 2014).

Animal welfare is difficult to define, measure and quantify. Welfare in fish 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). This definition

avoids the ongoing debate about whether fish have the sense organs and sensory systems required to experience pain, and disagreements over whether fish can be considered sentient animals (Volpato *et al.*, 2007).

Knowledge of the effects of environmental enrichment on the welfare of fish falls behind comparative knowledge for mammals (Williams *et al.*, 2009) and many questions remain unanswered about the environmental requirements of different fish species (Williams *et al.*, 2009). Laboratory *D. rerio* are usually kept in bare aquaria for ease of husbandry 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.

While single measures of the positive or negative effects of enrichment are useful, a combination of different indicators allow a more comprehensive evaluation of the potential benefits of enrichment. This study used seven measures, spanning integrated measures of growth and development to features of behavior, to assess the effects of enrichment on the welfare of laboratory-held *D. rerio*. In particular, it investigated the relationship between environmental enrichment and (1) survivorship from 5–30 dpf, (2) growth, (3) body condition, (4) sex ratio, (5) anxiety-like behavior, (6) preference for environment, and (7) tendency to monopolise resources. The specific hypotheses tested were as follows: environmental enrichment through provision of plants confers a fitness benefit to larval fish by potentially increasing prey diversity; enrichment affects growth and body condition (because fish in enriched tanks may spend more energy on foraging, but not sex ratio; enrichment reduces anxiety-like behavior by improving environmental conditions and reducing aggressive

interactions; fish spend more time in an enriched environment than in a plain environment; and enrichment reduces resource monopolisation because complex habitats are more difficult to defend. Data generated by this study were then applied to enhance our understanding of what environmental conditions improve housing for laboratory fish.

Materials and methods

All experiments were performed in accordance with the guidelines of the animal ethics committee, University of Exeter, and operated under a UK Home Office Project License, 30/2868.

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 Resource Centre at the University of Exeter. Fish were maintained in clear polystyrene tanks (Hagen; West Yorkshire, United Kingdom). Polystyrene tanks were chosen in preference to glass tanks because they are lightweight and manoeuvrable, even when filled with water, and were readily available in the required sizes. 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} $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 9.4 mg l^{-1} NaHCO_3 , 50 mg l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 mg l^{-1} KCl , 50 mg l^{-1} Tropic Marin Sea Salt). The water was

heated to 28°C in a reservoir and 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 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. A laminated sheet of white paper was placed between the tanks to prevent visual interaction between fish in neighbouring tanks. 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 'plain' environments and comprised bare aquaria while others were designed as 'enriched' environments and furnished with 2–5 mm aquarium gravel 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) and obtained from local pet shops. Vallis bunches varied in number of leaves from 2–10 and in length from 50–190 mm. Water trumpet sprigs 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, as experimental endpoints were measured (Fig. 1).





Fish age	5–30 dpf	30–98 dpf	Between 98 and 101 dpf	101-131 dpf
Housing	 Nursery tank	 Rearing tank	 Novel tank	 Choice tank
Endpoints (dpf)	Survivorship, growth (30)	Growth (60)	Anxiety-like behaviour (between 98 and 101 dpf, fish were tested individually for 6 min in a novel tank)	Environmental preference (104–106); resource monopolisation (104–106); growth (120); growth, body condition, sex ratio (131)

Figure 1. Progression of housing conditions and endpoints measured during an experiment to investigate the effects of laboratory housing on *Danio rerio* from 5–131 days post-fertilisation (dpf).

Fish from 5–30 dpf were housed in ‘nursery tanks’. Four nursery tanks were set up, each of 335 x 195 x 170 mm (*L* x *W* x *H*) dimension with a working capacity of 11 l. Two tanks were plain and two were enriched with gravel, 30 bunches of vallis and three sprigs of water trumpet. 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 30–98 dpf were housed in ‘rearing tanks’ of 210 x 130 x 130 mm (*L* x *W* x *H*) dimension, with a working capacity of 2.2 l. Five tanks were plain and

five were enriched with gravel, 10 bunches of vallis and one sprig of water trumpet.

Starting at 98 dpf, fish were removed individually from the rearing tanks and placed into a 'novel tank' 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 visually divided into two horizontal zones marked 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' 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.

Fish from 101–131 dpf were housed in environmental-choice tanks (hereafter 'choice-tanks'). 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 bunches of vallis and one sprig of water trumpet 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 a laminated sheet of

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 with 6-mm plastic hose attached to a hollow glass tube. Tank internal surfaces were cleaned twice weekly by wiping with absorbent, low-linting paper towels.

Table 1. Fish feeding schedule.

<i>Dpf</i>	<i>n</i>	<i>Feeding time and diet</i>				
		<i>0900</i>	<i>1100</i>	<i>1300</i>	<i>1500</i>	<i>1630</i>
5–8	<i>150 per tank</i>	20 mg ZM000 ¹	1 ml artemia ²	20 mg ZM000	1 ml artemia	20 mg ZM000
9–12		20 mg ZM000	2 ml artemia	20 mg ZM000	2 ml artemia	20 mg ZM000
13–16		20 mg ZM000	4 ml artemia	20 mg ZM000	4 ml artemia	20 mg ZM000
17–20		30 mg ZM000	5 ml artemia	30 mg ZM000	5 ml artemia	30 mg ZM000
21–30		30 mg ZM100 ³	8 ml artemia	30 mg ZM100	8 ml artemia	30 mg ZM100
31–40	<i>11 per tank</i>	10 mg ZM100	1 ml artemia	1 ml artemia	-	10 mg ZM100
41–50		10 mg ZM100	2 ml artemia	2 ml artemia	-	10 mg ZM100
51–80		15 mg ZM100	3 ml artemia	3 ml artemia	-	15 mg ZM100
81–120		20 mg ZM100	4 ml artemia	3 ml artemia	-	20 mg ZM100
121–130		20 mg pellets ⁴	4 ml artemia	4 ml artemia	-	20 mg pellets

¹Powdered fry food (ZM-000; ZM, Hampshire, United Kingdom) tapped onto water; ²suspension of freshly hatched artemia (*Artemia salina*) nauplii (ZM Premium Grade Artemia; ZM, Hampshire, United Kingdom) pipetted onto water; ³powdered fry food (ZM-100; ZM, Hampshire, United Kingdom) tapped onto water; ⁴pellets (Gemma Micro 300 Zebrafish Pellets; Skretting, Cheshire, United Kingdom) tapped onto water.

Survivorship from 5–30 dpf

Approximately 650 embryos from mass spawning tanks were collected, cleaned, and placed in Petri dishes containing system water plus methylene blue. Unfertilised eggs were removed. At 2 dpf, 600 embryos were transferred to 60 Petri dishes (10 embryos per dish) and allowed to hatch. Unhatched embryos were replaced from the

original collection so that each Petri dish contained a group of 10 embryos. At 5 dpf, all embryos had hatched and each group was randomly assigned to one of the four nursery tanks (two plain and two enriched). Each nursery tank thus contained 150 larvae. At 30 dpf, survivorship was determined by counting all juveniles in each tank. Prior to counting, plants were removed from enriched tanks to ensure that no fish was overlooked. During counting, a net was used to guide each individual fish into a 400 ml plastic beaker used as a scoop; 55 juveniles were removed from enriched nursery tanks and randomly assigned to five enriched rearing tanks and 55 juveniles were removed from plain nursery tanks and randomly assigned to five plain 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). The remaining juveniles were maintained as laboratory broodstock and took no further part in the study. At 60- and 120-dpf, survivorship was determined by counting fish in each rearing tank.

Growth

Length was used to assess the effects of exposure conditions on growth. For length measurements, a sample of 20 fish from each treatment were individually photographed at 30, 60 and 120 dpf. Fish were 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. To avoid injury, 30 dpf fish were guided into a scoop and then gently poured, in succession,

into a 100 ml beaker, a 10 ml beaker, and finally into the well of the Falcon tissue culture plate. Older fish were gently caught and transferred by net. Overhead photographs were taken 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).

Body condition

At 131 dpf, all fish were sacrificed by anaesthetic overdose (benzocaine; Sigma, Poole, United Kingdom). 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 ($K = \text{mass (mg)}/\text{length (mm)}^3 \times 100$).

Sex ratio

At 131 dpf, fish were sexed based on differences in colouration and body shape between the sexes as described by Paull *et al.* (2008). Male *D. rerio* have dark blue stripes, a golden cast and a streamlined body, whereas females have paler stripes, 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).

Anxiety-like behaviour

The 'novel tank diving 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 diving 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 (5 enriched tanks and 5 plain 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 6 min. At the end of the test, the fish was netted, removed from the novel tank and placed in a choice-tank (see below). 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). Recordings were downloaded onto the laptop computer as AVI files and viewed to analyse behaviour. The following endpoints were measured: latency to reach the upper half of the tank, number of transitions to the upper half (per minute and total number of transitions), time spent in

the upper half (per minute and total time) 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).

Preference for environment

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 feels about 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 and grouped in their original groups 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 fish to the environmental preference tanks was completed by 101 dpf and fish were allowed to acclimate for three days before 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 film 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 analyze 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.

Monopolisation of resources

Increased aggression associated with resource defence may impact welfare by increasing signs of distress in subordinate fish. One way to assess the effects of environmental enrichment on welfare is to compare resource monopolisation between enriched and plain 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 significant differences between treatments and between replicates in the proportion of larvae that survived to 30, 60 and 120 dpf. Mann-Whitney *U*-tests were used to compare standard length between treatments at 30, 60 and 120 dpf, and to compare standard length and body condition at 131 dpf. A chi-square goodness-of-fit test was used to determine whether the sex ratio significantly deviated from the expected 50:50 ratio. Novel tank test data (latency to enter the upper half of the novel tank, total transitions to the upper half, and total time spent in the upper half) were compared between treatments using Mann-Whitney *U*-tests. Environmental preference data were examined by first calculating the daily occupancy count for each group. The occupancy count was converted into a ratio and Jacob's preference index was calculated from the ratio, as in Schroeder *et al.* (2014). For each day of the environmental preference test, an independent samples *t*-test or nonparametric Mann-Whitney *U*-test was used to investigate the effect of rearing environment on occupancy of the bare compartment of the choice-tanks. Within-treatment differences in daily occupancy counts were assessed for groups in enriched tanks by a one-way repeated measures ANOVA and for groups in plain tanks by a nonparametric Friedman test. Data for monopolisation of resources were assessed for each day with a Mann-Whitney *U*-test.

Results

Survivorship from 5–30 dpf

Six hundred larvae were reared in enriched or plain tanks, with 300 in each treatment. At 30 dpf, 248 (83%) of larvae in enriched tanks had survived compared to 161 (54%) of larvae in plain tanks, a difference in proportions of 0.29, $P < 0.001$ (Fig. 2). Survivorship between replicates were not significantly different at 30 dpf for enriched or plain tanks.

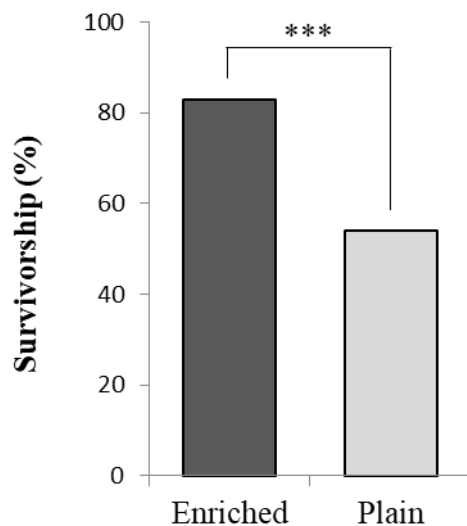


Figure 2. Percentage of *Danio rerio* larvae that survived from 0–30 days post fertilization in enriched tanks and in plain tanks. $N = 2$ tanks per treatment, 150 larvae per tank. Asterisks denote a significant difference between treatments (chi-square test, $P < 0.001$).

Growth

At 30 dpf, fish in enriched and plain tanks were of similar length (9.0 ± 1.3 mm and 8.8 ± 1.4 mm respectively). However, after equal numbers of fish were transferred to the rearing tanks and maintained between 30 dpf and 60 dpf, fish in enriched tanks were shorter (median 20.8 mm) than fish in plain tanks (median 22.7 mm) at 60 dpf and the difference was statistically significant (Mann-Whitney; $U = 282$, $z = 2.22$, $P = 0.026$; Fig. 3). This difference was no longer evident at 120 dpf, when the lengths of fish in enriched and plain tanks were similar (27.4 ± 2.1 mm and 28.6 ± 1.8 mm respectively).

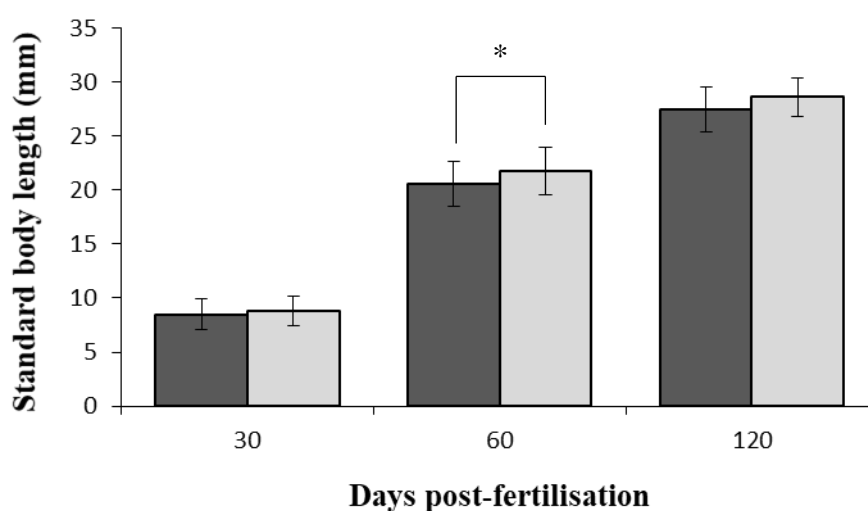
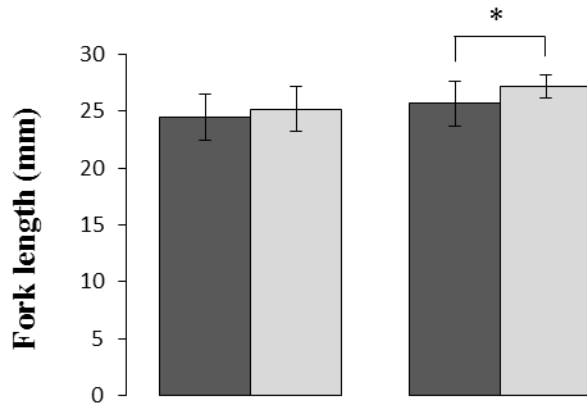


Figure 3. Standard body length at 30, 60 and 60 days post-fertilization of *Danio rerio* reared in enriched tanks (dark bars) and in plain tanks (light bars). Data are presented as medians \pm interquartile ranges; $n = 20$ fish per treatment. An asterisk indicates statistical significance (Mann-Whitney U -test, $P = 0.026$).

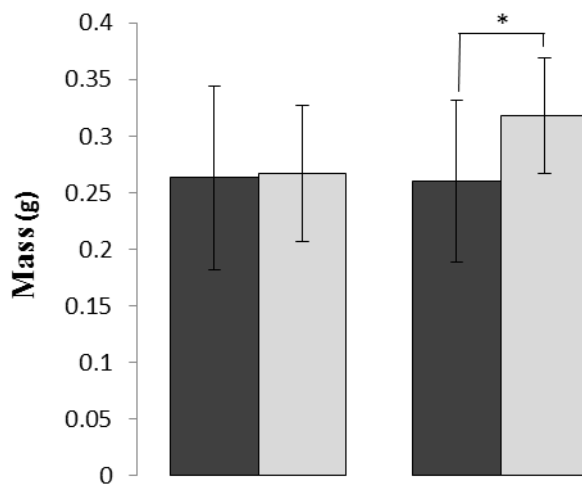
Body condition

At 131 dpf, fork length and mass were used to calculate the body condition of males and females separately. Females in enriched and in plain tanks were of similar length [medians 28.3 mm and 29.5 mm respectively; Fig. 4(a)] and similar 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 plain tanks (1.00) [Mann-Whitney; $U = 44$, $z = -3.86$, $P < 0.001$; Fig. 4(c)]. Males in enriched tanks were significantly smaller in length than males in plain tanks [medians 29.6 mm and 31.5 mm respectively; Mann-Whitney; $U = 231$, $z = 3.18$, $P = 0.001$; Fig. 4(a)] and also smaller in mass [medians 0.26 g and 0.32 g respectively; Mann-Whitney; $U = 227$, $z = 3.03$, $P = 0.002$; Fig. 4(b)] but their body condition scores did not differ [1.00 and 0.99 respectively; Fig. 4(c)].

(a)



(b)



(c)

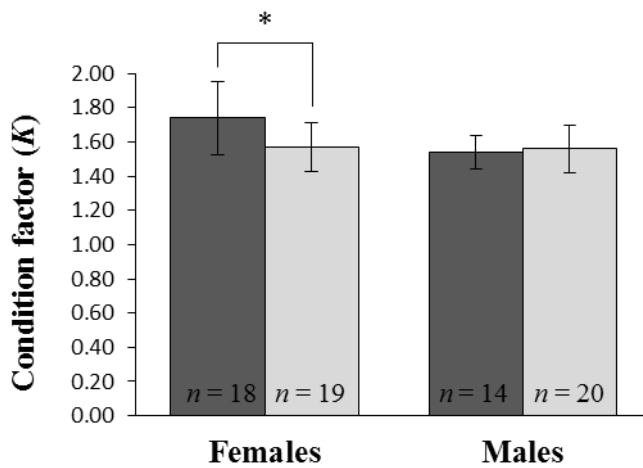


Figure 4. Morphometric measurements at 131 days post-fertilisation of *Danio rerio* females and males reared in enriched tanks (dark bars) and in plain tanks (light bars). Data for fork length (a), mass (b) and condition factor (c) are presented as medians \pm interquartile ranges. Asterisks indicate statistical significance [Mann-Whitney *U*-test, (a) $P = 0.001$; (b) $P = 0.002$; (c) $P < 0.001$].

Sex ratio

There was no significant departure from the expected sex ratio of 50:50 in either treatment group as 52% of fish in enriched tanks were female compared to 49% of fish in plain tanks (chi-square test; $\chi^2_1 = 0.02$, $P = 0.889$).

Anxiety-like behaviour

There was no significant difference between fish in enriched and plain tanks in latency to enter the upper half of the novel tank (Mann-Whitney; $P = 0.142$) or in the total number of transitions to the upper half (Mann-Whitney; $P = 0.242$). However, fish from enriched tanks spent significantly more time than fish from plain tanks in the upper half of the novel tank (Mann-Whitney; $U = 53$, $z = -3.98$, $P < 0.001$; Fig. 5).

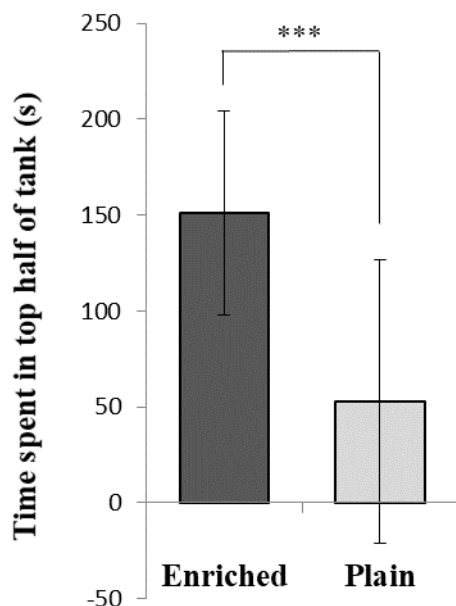


Figure 5. Time spent in the upper half of a novel tank by *Danio rerio* reared in enriched tanks and in plain tanks during a 6-min trial. Data are presented as medians \pm interquartile ranges, $n = 20$ fish per treatment. Asterisks indicates statistical significance (Mann-Whitney U -test, $P < 0.001$).

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

Preference for environment

There was no significant 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: $t_8 = 0.895$, $P = 0.259$; Day 2: $t_8 = -1.627$, $P = 0.142$; Mann-Whitney; Day 3: $U = 17$, $P = 0.421$; Fig. 7). Within-treatment difference in occupancy of the bare compartment over the three test days was not significant for - groups from enriched tanks (ANOVA; $F_{2,8} = 3.001$, $P = 0.107$) or for groups from plain tanks (Friedman test; $\chi^2_2 = 0.947$, $P = 0.623$).

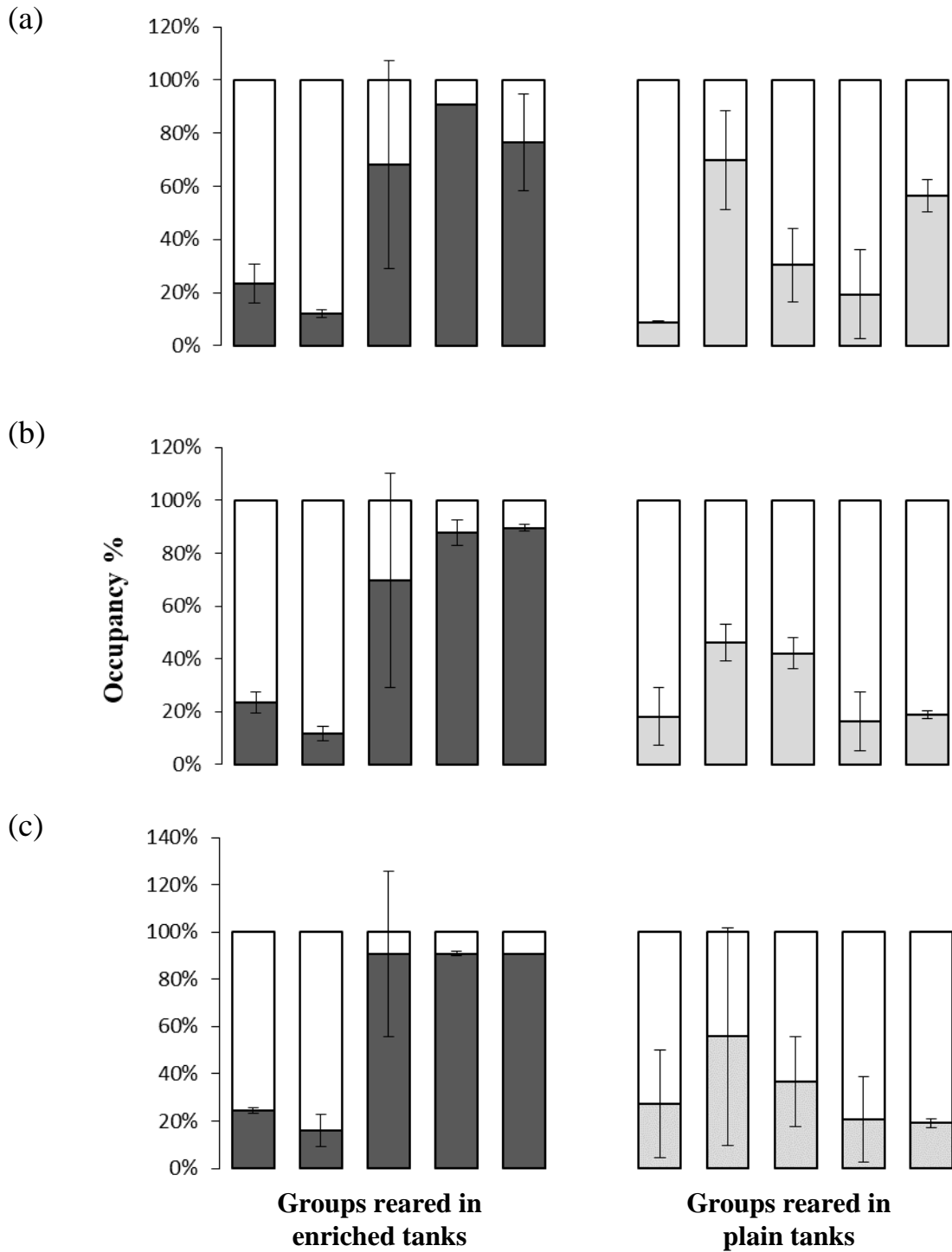


Figure 6. Percentage of occupancy of enriched compartments (grey bars) and plain compartments (white bars) of choice-tanks by *Danio rerio* groups reared in enriched tanks or in plain tanks. No significant difference was found between treatments on (a) Day 1 (t -test, $P = 0.259$), (b) Day 2 (t -test, $P = 0.142$), or (c) Day 3 (Mann-Whitney U -test, $P = 0.421$). Data are presented for Day 1 and Day 2 as means \pm standard deviation, and for Day 3 as medians \pm interquartile range. Each column represents a single group.

Monopolisation of resources

Monopolisation of resources, where a dominant fish excludes subordinate individuals from its preferred compartment, was recorded in $68\% \pm 58\%$ of sampling points for fish reared in enriched tanks compared to $5\% \pm 44\%$ of sampling points for fish reared in plain tanks, a difference that was statistically significant (Mann-Whitney, $U = 40$, $P = 0.002$; Fig 8). In most cases, dominant fish monopolised the compartment of the tank that differed from the environment in which they had been reared, with dominant fish from enriched tanks monopolising the plain compartment in 74% of 530 sampling points, and dominant fish from plain tanks monopolising the enriched compartment in 90% of 213 sampling points.

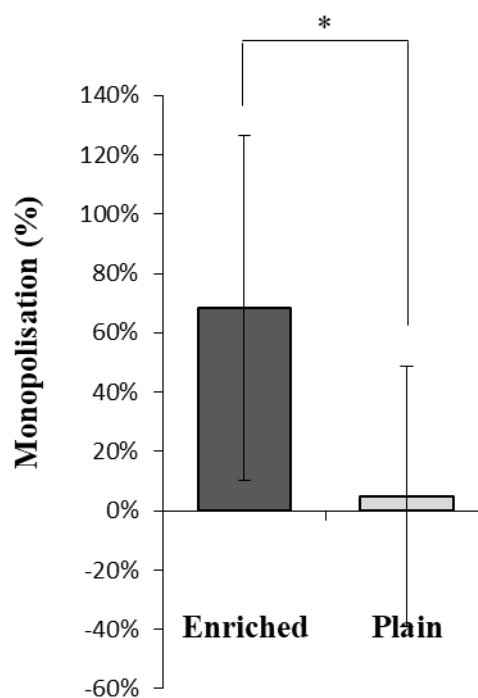


Figure 7. Monopolisation of one half of an environmental choice-tank (where a dominant fish excludes subordinate individuals from its preferred compartment) by *Danio rerio* reared in enriched tanks and in plain tanks during a 3-day trial. Data are presented as medians \pm interquartile range; $n = 5$ tanks per treatment. The asterisk indicates statistical significance (Mann-Whitney U -test, $P = 0.002$).

Discussion

This study set out to assess the effects of environmental enrichment on survivorship, growth, body condition and behaviour of laboratory-held *D. rerio*. In laboratories, *D. rerio* are usually kept in bare aquaria for ease of husbandry, but little is known about the effects of this environment on the fish. Such basic information is of primary importance if optimal conditions are to be provided for the good welfare of laboratory-held fish. The most comprehensive evaluation of the effects of enrichment is obtained from a combination of indicators (Williams *et al.*, 2009) and this study used seven measures (survivorship, body length, body condition factor, sex ratio, anxiety-like behavior, preference for environment, and tendency to monopolise resources) to assess the effects of enrichment on the welfare of *D. rerio*.

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 than larvae reared in plain tanks. Although there are no previous studies for *D. rerio*, 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).

Most *D. rerio* mortalities occur between 11 and 16 dpf when larvae first become dependent on exogenous feeding following absorption of the yolk-sac and are likely due to starvation or incorrect nutrition (Wilson, 2012). Differences in early life survivorship between fish reared in enriched and plain tanks in this study may be linked to three factors: (1) prey diversity, (2) resource availability and (3) the energetic cost of escaping from aggressive conspecifics.

Larvae in enriched tanks likely benefitted from a more varied diet than larvae in plain tanks. From the time that larvae began to free swim, those housed 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. These microfauna were likely present on the leaves and stems of the plants when the plants were brought from local pet shops and survived the surface sterilisation of plants before they were added to the enriched tanks. Such slow-moving organisms on aquatic plants are a potentially important source of food for larval fish as they learn to hunt and develop feeding suction power, and their presence may mimic a contemporary diet for first-feeding larvae which provides live zooplankton, such as paramecia or rotifers, until larvae are able to capture larger and faster prey, such as artemia nauplii (Lawrence *et al.*, 2015). Larvae in planted tanks may also benefit from a continual supply of food items, such as protozoans, algae and detritus. Survival rates of larval *D. rerio* improve when they are fed continually to support their high energy demands (Carvalho *et al.*, 2006; Best *et al.*, 2010). Finally, 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 use less energy for metabolism if they can hide from aggressive larger larvae. Future studies could test whether larvae in enriched tanks benefit from structural complexity or from nutritional diversity. Such an investigation could compare survivorship of larvae in planted tanks with larvae in tanks furnished with inert 'enrichment', such as glass rods provided as potential enrichment for adult zebrafish (Wilkes *et al.*, 2012).

Growth

Fish reared in enriched and in plain tanks were of similar length at 30 dpf, fish in enriched tanks were shorter in length than fish in plain tanks at 60 dpf, but this difference was no longer evident at 120 dpf, suggesting a temporal variation between treatments in energy acquisition, possibly due to differences in food choice or predation success or in age of sexual maturation. Reported lengths of *D. rerio* at given ages vary widely in the literature. For example, Carvallo *et al.* (2006) reported the standard length of larvae at 26 dpf to be 14.3 ± 0.3 mm whereas Singleman and Holtzman (2014) found that standard length at 30 dpf was 8 ± 4 mm. By comparison, the median length of fish at 30 dpf in this study was 8.9 ± 1.3 mm. Differences in growth rates have been reported for different strains (Oswald & Robinson 2008) and diets (Gonzales & Law, 2013), and at different temperatures (Brown *et al.*, 2015) and stocking densities (Ribas *et al.*, 2017), but few studies provide comprehensive information about rearing conditions and the resultant growth curves against which the present results can be compared.

That fish from enriched and plain tanks were of similar length at 30 dpf was contrary to expectations. However, because fewer larvae survived in plain tanks than in enriched, the amount of food provided per fish differed between treatments. For example, at 30 dpf, fish received a daily ration per treatment of 180 mg of processed food and 16 ml of artemia. As a result, the 248 fish in enriched tanks each received, on average, 0.73 mg of processed food and 0.13 ml of artemia, compared to fish in plain tanks who each received, on average, 1.12 mg of processed food and 0.20 ml of artemia. Complex habitats may limit a fish's ability to find and capture prey by reducing visual encounters with prey (Savino & Stein, 1982), affecting the fish's swimming speed (Anderson, 1984) or hunting behaviour (Hovel *et al.*, 2016), or modifying the response of its prey (Anufrieva & Shadrin, 2014). As a result, fish in enriched tanks may spend more energy than fish in plain tanks on foraging and so were expected to grow more slowly. However, larvae in enriched tanks may have compensated for lower predation success by eating a broader, less selective diet, including microorganisms, algae or detritus. Alternatively, the predation success of larvae may not have been affected by habitat complexity. Ryer (1988) reported that prey encounter rates for small (110–130 mm) pipefish *Sygnathus fuscus* Storer 1839 were unaffected by habitat complexity whereas large (180–200 mm) *S. fuscus* showed a significant effect of habitat with higher rates of prey encounter in low complexity habitats. The author attributed this effect to larger fish reacting to prey at a greater distance in low complexity habitats, possibly because larger fish have larger eye size, increased visual acuity, and therefore increased hunting success (Ryer, 1988). If this effect applies also to *D. rerio*, then habitat complexity may not have affected the foraging success of small larvae in this study, resulting in the observed similarity of size between larvae in enriched and in plain tanks at 30 dpf.

The difference in length between fish in enriched and in plain 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 fish in enriched tanks due to the effect of habitat complexity on the rate of prey encounter and resulting in the shorter length of fish in enriched tanks at 60 dpf. Or fish in enriched tanks may have established and defended territories and interfered with the feeding of subordinates, resulting in dominant fish experiencing higher growth rates relative to subordinates, as observed in juvenile steelhead trout *Oncorhynchus mykiss* (Walbaum 1792) (Abbott & Dill, 1989). This theory is supported by data from the present study that indicate that monopolisation of resources occurred more often in enriched groups than in plain groups (Fig. 7).

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 fish in enriched tanks converging with the length of fish in plain tanks by 120 dpf. Further investigation could determine the growth patterns of fish in the two treatments, including the size and age at which segregation into two modal groups starts and ends and whether the convergence observed at 120-dpf is permanent.

Body condition

Females in enriched tanks had higher median condition than females in plain tanks although no significant difference was found between treatments in either mass or length. The reasons for the difference in ratio are unclear but may be related to egg production or energy efficiency. Developing oocytes account for a large part of the body mass of female *D. rerio* and fecundity increases with increased food intake (Forbes *et al.*, 2010). If females in enriched tanks had lower metabolic rates than fish in plain tanks (perhaps due to reduced levels of stress), a lower rate of energy utilisation or greater energetic efficiency, this could explain their increased condition factor. Abbott and Dill (1989) demonstrated that dominant fish grew faster than subordinates. They used pairs of similar-sized *O. mykiss*, comprising one dominant and one subordinate individual, and a feeding regime that ensured that the dominant fish could not receive more food than the subordinate. The authors attributed their results to subordinate growth depression due to higher energetic costs of stress in subordinates. In the present study, males in plain tanks were higher in both length and mass compared to males in enriched tanks and, although median condition scores were similar for both treatments, condition was more variable in males from plain tanks than males from enriched tanks. Further work is needed to determine the causes of differences in body condition between enriched and plain females observed in this study, and the greater variability of body condition among males in plain tanks compared to males in enriched tanks.

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.

Anxiety-like behaviour

To investigate whether enrichment affects levels of anxiety-like behaviour in *D. rerio*, individual fish were placed in a novel tank for 6 min and their behaviour was observed. The 'novel tank diving test' is extensively used to model anxiety in *D. rerio* (Maximino *et al.*, 2010). Fish typically dive to the bottom of the novel tank and stay there for a period of time before beginning to explore their surroundings (Cachat *et al.*, 2010). The time taken to enter the top half of the tank is considered a measure of anxiety, with anxious fish taking longer than other fish to move into the upper half of the tank (Cachat *et al.*, 2010). In this study, fish from enriched and from plain 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 fish from enriched tanks spent significantly more time than fish from plain tanks in the upper half during each minute of the 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 plain fish was similar to that reported for control groups in other studies (e.g. Egan *et al.*, 2009; Wong *et al.*, 2010). Overall, fish from enriched tanks displayed lower levels of anxiety-like behaviour than fish from plain tanks when in a novel environment.

Maximino *et al.* (2010) reported similar results when comparing anxiety-like behaviour of enriched and plain-reared *D. rerio* in a dark/light test.

Preference for environment

Fish preference for an enriched vs plain environment was assessed by housing each group in a choice-tank and measuring the number of fish in the plain 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. In addition, Haynes (2011) warns of the limitations of preference testing. For example, if an animal is given a choice between two options, one cannot know whether it is simply choosing the less unpleasant of two poor choices; and preferences may vary with age, reproductive status, etc. (Haynes, 2011).

Resource monopolisation

Resource monopolisation was significantly higher for fish reared in enriched tanks than for fish reared in plain 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 groups reared in enriched tanks is unclear, as previous studies found that environmental enrichment reduced aggression and resource monopolisation in *D. rerio* (Basquill & Grant, 1998; Hamilton & Dill, 2002), presumably because complex habitats are more difficult to defend. However, Bhat and colleagues (2015) reported the opposite effect—that enrichment increased aggression. Dominant fish in the present study tended to monopolise the compartment of the tank that differed from their rearing environment.

Conclusions and next steps

Overall, the data presented show that 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) are negative. Effects within and between

treatments are sometimes inconsistent and even ambiguous and further investigations will be necessary to understand the specific influence of different elements of enrichment on *D. rerio* at different life stages. Also, the effects of different amounts of enrichment, and of variable vs stable enrichment, remain to be investigated in order to inform what housing conditions promote optimal welfare for *D. rerio* in the laboratory. Interpretation of enrichment effects on both the physiology and behavior of *D. rerio* is complicated. End point measures can be affected by housing conditions which may have indirect as well as direct effects on fish health. For example, algal growth promoted by certain tank conditions may affect food availability which, in turn, can affect growth. The effects of enrichment are likely to differ between life stages, suggesting that no single set of housing conditions is optimal for all life stages. Finally, there is still much to learn about the natural history and normal behavior of *D. rerio*. Such knowledge will aid understanding of which laboratory housing parameters have an impact on wellbeing and how the welfare of fish can be improved without compromising research.

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Chapter 3 Can simple tank changes benefit the welfare of laboratory zebrafish?

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Title

Can Simple Tank Changes Benefit the Welfare of Laboratory Zebrafish?

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Running head

Effects of tank changes on fish welfare

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ABSTRACT

Environmental enrichment is often purported as the solution to improving wellbeing in laboratory fish. However, many enrichments are not compatible with aquaculture or research facilities. It was hypothesised that significant welfare benefits may be achievable through simple practical solutions easily adapted to current practices in research laboratories. To investigate these new approaches, this study examined the effects of simple changes in the tank environment on the wellbeing of captive fish, using zebrafish as an experimental model. It was hypothesised that moving fish between tanks of identical status (bare) and changes in water supply would provide positive stimulation equating to more complex enriched environments. Groups of zebrafish were housed in ‘stable’ environments (where groups maintained in the same tanks throughout the study) or in ‘changed’ environments (where groups were periodically moved to novel tanks with replacement system water). Comparisons between treatments included effects on morphometry (length, mass and condition), reproductive success (egg output and viability) and aggressive behaviour. For the simple changes adopted—tank and water—no significant effect of environmental stability was found on body condition, reproductive output or aggression. It was concluded from this pilot study that changing the tank

and tank water did not have any obvious health benefits to the fish, for the periods of time studied.

KEY WORDS

Zebrafish husbandry; enrichment; environmental change; alternatives to enrichment; welfare.

INTRODUCTION

Fish welfare is of increasing public and regulatory concern, but in the rapid global expansion of aquaculture and in facilities housing fish for research, most attention has focused on facility economics and maximising production, rather than on fish welfare. Most cultured fish are kept in conditions far removed from nature and some of these conditions inevitably result in acute or chronic stress (Braithwaite *et al.*, 2014). Examples include bare tanks used to house laboratory fish (Lawrence, 2012), high stocking densities of ornamental fish in the pet trade (Stevens *et al.*, 2017), and elevated CO₂ rates associated with recirculating aquaculture systems

used in aquaculture (Ellis *et al.*, 2017) . It is generally accepted by researchers that environmental enrichment (increasing the complexity of an animal's environment in order to enhance its wellbeing) improves the welfare of research animals and that better welfare leads to better science (Hawkins, 2014). For example, several studies have shown that enrichment affects disease progression in mouse models (Hockly *et al.*, 2002; Glass *et al.*, 2004; Sorrells *et al.*, 2009) and responses in enriched mice better mimic disease progression in humans. There is evidence that some forms of enrichment improve welfare for some fish species (reviewed by Näslund & Johnsson, 2014). However, many environmental factors, biotic and abiotic, also have the potential to affect an animal's physiology or behaviour (Killen *et al.*, 2013) and so influence research results. In addition, some forms of enrichment such as the addition of plants and gravel are considered impractical, or costly by some laboratories (Lidster *et al.*, 2017) and importantly, there are no studies describing the duration of benefit imparted by the addition of these physical items for fish and how they should be managed day-to-day with other husbandry practices. Therefore, there is a real need for enrichment approaches that provide measurable welfare benefits without compromising research results.

Refinements, versus wholesale changes, in husbandry practices can benefit animal welfare and improve the reliability of research data (Prescott *et al.*,

2017), with even small changes showing a measurable effect. As an example, laboratory mice picked up by the of base of the tail (a traditional handling method) were shown to have high anxiety and poor performance in behavioural tests whereas mice picked up in a handling tube or cupped hand showed lower levels of anxiety and improved performance, indicating that handling methods may influence both the welfare of laboratory mice and experimental results (Gouveia *et al.*, 2017). Another study compared aversive reactions of zebrafish to three substances commonly used to euthanise fish and found that metomidate hydrochloride and clove oil are less aversive to zebrafish than the more widely-used tricaine methanesulfonate, suggesting that these substances could be used to improve welfare during euthanasia (Wong *et al.*, 2014). Housing conditions have also been shown to modulate brain morphology and cognition in some fish species, potentially having a negative impact on the use of these animals for specific research questions. For example, laboratory-reared female guppies *Poecilia reticulata* Peters 1859 had reduced telencephalon and optic tectum size compared to their wild-caught mothers (Burns *et al.*, 2009) and differences in brain morphology were reported between wild-reared Chinook salmon *Oncorhynchus tshawytscha* Walbaum 1792 and hatchery-reared fish spawned from wild-caught adults (Kihslinger *et al.*, 2006), although neither study identified the environmental factors that may have caused these changes. Identification and implementation of

refinements to husbandry practices, including novel approaches, can drive best practice, deliver higher welfare and improve the quality of science.

Anecdotal evidence from researchers and animal care staff (G. Paull, personal observations and discussions with laboratory staff) suggests that laboratory-housed zebrafish *Danio rerio* (Hamilton 1822) that fail to breed (and show reduced activity) may be induced to spawn by changing the tank water or moving the fish to a novel tank. The natural history of *D. rerio* offers clues as to why a changed environment in the laboratory could have such an effect on this species. *D. rerio* is native to the Indian subcontinent, where a monsoon climate creates wide seasonal flooding and variation in water-spread area. Field studies report finding *D. rerio* in a range of habitats, including ponds, stagnant pools, streams, and irrigation ditches associated with rice paddies (Engeszer *et al.*, 2007; Spence *et al.*, 2008; Arunachalam *et al.*, 2013). Many of these waters are highly seasonal and connect to main rivers only during the monsoon rains, when widespread flooding increases water levels, mixing water from different water bodies and changing water chemistry, flow rate, and temperature (Suriyampola *et al.*, 2015). Adult *D. rerio* are thought to spend most of the year in permanent streams and small rivers and to move into flooded areas during the monsoon to spawn in still, shallow, well vegetated areas, such as paddy fields (Engeszer, 2007). It is possible that moving laboratory-housed fish to

a novel tank simulates this movement between water bodies with associated changes in chemical and pheromonal cues. In-house observations by laboratory staff have indicated that fish respond with increased activity throughout the water body, notably in exploratory behaviour, and renewed spawning vigour. Water changes have been reported to stimulate spawning in fish in the aquarist hobby industry also (Ng, 2009) and loss of exploratory activity over time has been well studied in captive zoo animals (Wood-Gush *et al.*, 1989; Morgan *et al.*, 2007) but less so in fish.

This study, with a novel approach to improving the welfare of captive fish, tests the effects of moving groups of *D. rerio* between tanks on body condition, reproductive output and levels of aggression, endpoints commonly used for assessments on welfare of captive fish. Groups of fish were housed in ‘stable’ environments (groups maintained in the same tanks throughout the study) or in ‘changed’ environments (where groups were moved every week or every 3 weeks into novel tanks and tank water) and comparisons made on the above endpoints.

MATERIALS AND METHODS

FISH SOURCE, HOUSING AND HUSBANDRY

Wild Indian Karyotype (WIK) strain zebrafish *Danio rerio* (Hamilton 1822) were bred and housed at the Aquatic Resource Centre, a custom-built zebrafish aquaria facility at the University of Exeter. At the start of the study, 192 fish aged 8 months were sexed and randomly grouped into 12 groups comprising 8 males and 8 females. Each group was housed in a clear polystyrene tank (Hagen; West Yorkshire, United Kingdom) of 300 × 200 × 203 mm ($L \times W \times H$) dimension with a working capacity of 5 l. Tanks were supplied, via a flow-through system from a reservoir, with mains tap water which had been filtered by reverse osmosis (Environmental Water Systems (UK) Ltd), reconstituted with Analar-grade mineral salts to standardized synthetic freshwater (final concentrations to give a conductivity of 300 μ S: 122 mg l⁻¹ CaCl₂·2H₂O, 9.4 mg l⁻¹ NaHCO₃, 50 mg l⁻¹ MgSO₄·7H₂O, 2.5 mg l⁻¹ KCl, 50 mg l⁻¹ Tropic Marin Sea Salt), aerated, and heated to 28°C. Water pH, conductivity, ammonia, nitrate, and nitrite were maintained within U.S. Environmental Protection Agency guidelines (U.S. EPA, 1996). For each tank, the water flow rate was set to 2 l h⁻¹, an air stone was added, an image of gravel placed under the base of

the tank, and laminated black paper placed against 3 tank sides to prevent visual stimulation between groups. Tanks were arranged in a random block design. The photoperiod was set to 12:12 h light:dark with a 30 min artificial dawn to dusk transition. Fish were fed 4 times daily, twice on freshly hatched *Artemia salina* nauplii (4 ml; ZM Premium Grade Artemia; ZM Ltd., Hampshire, UK) and twice on pellets (15 mg; Gemma Micro 300 Zebrafish Pellets; Skretting, Cheshire, United Kingdom). Twice each week, one nauplii meal was replaced with Gamma Slice *Artemia franciscana* brine shrimp (1 ml; Tropical Marine Centre, Chorleywood, Hertfordshire, UK). All experiments were performed in accordance with the guidelines of the animal ethics committee, Department of Biosciences, University of Exeter.

EXPERIMENTAL TREATMENTS

Each group was randomly assigned to one of 3 experimental treatments. Groups in the “1-week change” (1WC) treatment were moved every week into novel tanks and tank water, groups in the “3-week change” (3WC) treatment were moved every 3 weeks into novel tanks and tank water, and groups in the “no change” (NC) treatment were maintained in the same tanks throughout the study. The procedure for moving a group to a novel

tank was to half-fill the novel tank with system water, reduce the water level in the home tank by two-thirds using a siphon fitted with a mesh guard, gently pour fish into the novel tank, and replace the tank in the random block. At the start of the experiment, fish were weighed and photographed as described below, then allowed to acclimate for 2 days before egg collection and behavioural assays began. The first 7 days of assays were considered as Week 0 and groups received their first treatment at the beginning of Week 1.

BODY CONDITION

Fish length and mass were used to determine body condition at the beginning and end of the 9-week study. For this procedure, each fish was gently netted, transferred to a pre-weighed 75 ml crystallising dish containing ~20 mm of system water, and weighed. Overhead photographs were taken 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 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, Rasband, & Eliceiri, 2012). Body condition (K) was

calculated by expressing the cube of fish length as a percentage of fish mass ($K = \text{mass (mg)}/\text{length (mm)}^3 \times 100$).

REPRODUCTIVE OUTPUT

To assess reproductive output between treatments, spawned eggs were collected, counted, and egg viability assessed (at 24-hours post-fertilization) 3 times a week for 9 weeks (weeks 0–8). On evenings prior to egg-collection, 2 spawning trays (Aquatic Habitats, USA) were placed in each tank. Trays were $155 \times 63 \times 40$ mm ($L \times W \times H$) in dimension and had a lattice lid through which eggs could fall into the tray below, thus preventing fish from consuming the eggs. Six plastic *Vallisneria*, each with 3 stems, were threaded through the lattice lid to encourage fish to spawn above the trays. When the 2 trays were placed side-by-side they created a spawning area of 155×126 mm. The next day, 1 h after the artificial dawn, spawned eggs were collected and cleaned, and dead or unfertilized eggs were removed and counted. All egg trays were removed within a 5 min period and the random block arrangement of tanks ensured minimal difference between treatments in the length of time that trays remained in the tanks. Fertilized eggs were transferred into a Petri dish containing system water to which methylene blue had been added as a fungicide (2 ml

of 0.1% methylene blue diluted in 1 l of system water). Eggs were incubated at 28°C for 24 hours after which any dead embryos or infertile eggs were separated from the live embryos. For each tank, the number of live embryos and dead eggs/embryos were counted.

BEHAVIOUR

Fish were filmed on two days of each week for assessment of aggressive behaviour (biting, chasing or sparring). Groups were filmed for 30 min in the afternoon (1400–1430) and 30 min in the evening (1800–1830). This schedule was chosen to avoid the spawning period and feeding times as aggression in zebrafish is known to increase during spawning (Spence *et al.*, 2005) and in the presence of food (Jha, 2010). Two groups from each treatment were simultaneously filmed during one week and the remaining groups were filmed during the following week. Filming was programmed to start and stop automatically and no personnel were present during filming. Each tank was filmed using 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 (Dell Inc., Round Rock, Texas, USA) was used to connect to the NAS via the

network and to record the films. Recordings were downloaded onto the laptop computer as AVI files and viewed to analyse behaviour. The frequency of the most common aggressive behaviours observed in zebrafish (chase, repel, bite, and spar) as defined by Paull et al. (2010) was assessed for each group from the video footage and the rate of aggression per fish per minute was calculated.

DATA ANALYSIS

All data are presented as means \pm standard deviation unless stated otherwise. Statistical analyses were performed using SPSS v. 22 (IBM Inc., USA). Data were first tested for normality using a Shapiro-Wilk test and for equality of variance using Levene's test. Differences among treatments were measured by one-way ANOVA followed by Tukey post-hoc analysis, or a non-parametric Kruskal-Wallis test. Differences among treatments over time were analysed by a non-parametric Friedman test for each individual treatment to determine if data differed across the 9-week study period. This was followed by a Kruskal-Wallis test to look for differences between treatments at each weekly time point. The coefficient of variation (CV; the ratio of standard deviation to the mean) was calculated for

comparisons of variation. All data were considered statistically significant at $P = 0.05$.

RESULTS

BODY CONDITION

At the start of the study, there were no significant differences among treatments in male length (ANOVA; $F_{2,93} = 0.12$, $P > 0.05$) or body condition (Kruskal-Wallis; $H_2 = 1.96$, $P > 0.05$), or in female body condition (Kruskal-Wallis; $H_2 = 0.51$, $P > 0.05$). However, female length differed between 1WC (23.9 ± 1.5 mm) and NC (23.0 ± 1.2 mm) groups, a difference of 0.8 mm (95% CI, 0.0 to 1.6), which was statistically significant (Tukey's *post hoc* test; $P < 0.05$); there was no difference in female length between 3WC groups and other treatments.

At the end of the study, there were no significant differences among treatments in female length (Kruskal-Wallis; $H_2 = 3.34$, $P > 0.05$) or body condition (Kruskal-Wallis; $H_2 = 1.71$, $P > 0.05$), or in male length

(Kruskal-Wallis; $H_2 = 1.25$, $P > 0.05$) or body condition (Kruskal-Wallis; $H_2 = 0.26$, $P > 0.05$).

REPRODUCTIVE OUTPUT

Of the 192 fish used in this study, sex was incorrectly determined in 2 individuals, 1 each in 1WC and NC treatments. Both were initially identified as female and later found to be male. This error impacted on the actual number of females in those fishes' groups, and therefore all spawning data are reported as number of eggs per female rather than number of eggs per group.

All groups in all 3 treatments spawned regularly throughout the study. Over the 9-week assessment period, spawning trays were placed into tanks on 27 days (3 days each week) during which 1WC, 3WC and NC groups spawned on 26.0 ± 0.8 (96%), 25.8 ± 1.9 (95%), and 25.0 ± 1.8 (93%) days, respectively. The total number of eggs produced per female over the study period ranged between 199 and 330 for 1WC groups, 155 and 400 for 3WC groups, and 216 and 267 for NC groups. The mean number of eggs spawned per female per week was 27.6 ± 7.2 for 1WC, 32.0 ± 6.2 for 3WC

and 27.3 ± 4.9 for NC groups. The mean percentage viability of embryos at 24-hpf was $86.0 \pm 3.9\%$ for 1WC, $79.5 \pm 5.8\%$ for 3WC and $78.3 \pm 2.2\%$ for NC groups.

There was no significant difference between the treatments in the total number of eggs spawned per female over the 9-week study period [Fig. 1(a); one-way ANOVA; $P > 0.05$] but egg output was much more variable between 3WC groups (mean CV of 39%) than in 1WC or NC groups (mean CVs of 24% and 10% respectively). Egg viability was less variable between treatments than egg output [mean CVs of 3% for 1WC, 9% for 3WC and 6% for NC groups; Fig. 1(b)]. The patterns of egg output over the 9-week study were similar across treatments (Fig. 2). There was an initial increase in egg output during weeks 1 and 2 followed by a downward trend through week 4. Groups maintained in the same tanks throughout the study had relatively steady egg production from week 4 to the end of the study. Groups that were moved every week or every 3 weeks showed a continued decline in egg output through week 6, after which their production increased slightly. No statistically significant differences were found in egg output over time for fish that were moved every week or that remained in the same tanks (Friedman test; $P > 0.05$ for both treatments). Fish moved every 3 weeks showed a significant difference in egg output between weeks 2 and 6 (Friedman test, $P < 0.05$) but not between other weeks. In general,

however, there did not appear to be any significant time trends in egg output as a function of increasing study time.

BEHAVIOUR

Patterns of aggression over the 9-week study were similar across treatments (Fig. 3). The mean number of aggressive actions per fish per minute over the 9-week study period ranged from 0.69 ± 0.58 to 2.69 ± 1.83 for 1WC groups, 0.34 ± 0.03 to 3.39 ± 2.59 for 3WC groups, and 0.66 ± 0.18 to 2.72 ± 1.47 for NC groups. Data were not normally distributed so a Kruskal-Wallis test was used to measure differences between treatments for each week of the study. No significant difference was found between treatments at any assessment point during the study (Kruskal-Wallis; $H_2 = 1.14, 0.52, 3.43, 3.71, 0.29, 3.43, 1.14, 4.58, \text{ and } 0.86$ for weeks 0–9 respectively, $P > 0.05$ for each week). There was an initial increase in aggression during weeks 1–5, followed by a downward trend in week 6, an upturn in week 7, and a further downward trend in week 8. The increase in aggression in week 7 is noticeable (Fig. 3) but not statistically significant. Lab records show no differences in physical conditions (including water quality) or husbandry practices between week 7 and other weeks and the increased aggression during week 7 is unexplained. Within-treatment aggression did

not significantly differ across time for any of the 3 treatments (Friedman test; $P > 0.05$ for all treatments).

DISCUSSION

Of the growing body of work on *D. rerio* husbandry, this is the first report on the effects of adopting a stable versus changed environment on growth, reproduction and behaviour. The rationale for this approach is to look at alternative methods of enrichment which can be applied easily on a practical basis, incorporating good husbandry practices, rather than the introduction of substrates, refuges or other physical features, that can be more limiting for both system maintenance and research practice. For the simple changes adopted—tank and water—no significant effect of environmental stability was found on body condition, reproductive output, or aggressive behaviour. Levels of aggression were similar across all groups and showed no effect of treatment. It was concluded from this pilot study that changing the tank and tank water did not have any obvious health benefits to the fish, over the 9-week study period. For all tank and water conditions fish health was not impaired, suggesting that the fish were stimulated throughout.

BODY CONDITION

Body condition scores for *D. rerio* did not vary between treatments for either sex in this experiment, suggesting that fish remained healthy and well-stimulated, with no negative effects of the 9-week treatment.

REPRODUCTIVE OUTPUT

Egg production over time was not significantly affected by treatment. The mean estimated number of eggs spawned per female per week was lower than reported by other studies (Spence *et al.*, 2006; Markovich *et al.*, 2007; Paull *et al.*, 2008; Ramsay *et al.*, 2010). This variance may be due to differences between studies in factors known to affect egg output, such as female body size (Uusi-Heikkilä *et al.*, 2010), group size (Paull, 2008) or frequency of egg collection (Nasiadka *et al.*, 2012). However, it is unclear why within-treatment egg output was more variable among 3WC groups than among 1WC or NC groups. High variability of egg output may reflect natural variation in the rate of oviposition among females with some producing a small batch every day while others produce a larger batch

every few days (Paull, 2008). Another possibility is that some females were prevented from accessing the spawning site, or were interrupted during spawning, by aggressive behaviour of dominant males towards rivals (Spence, 2005). However, if changing tanks and tank water resulted in increased territoriality, then this effect should be more pronounced in 1WC groups as they were subjected to more frequent tank changes than 3WC groups. Another possibility is that moving fish to new tanks breaks down social hierarchies and eliminates social dominance such that more fish spawn. However, differences in behaviour were not reflected in the video analyses, although groups were not filmed during the spawning period.

BEHAVIOUR

No significant difference in aggression was found within or between treatments at any point during the 9-week study and patterns of aggression were similar across treatments. For group-living species such as *D. rerio* in which individuals compete for the same resources, research suggests that dominance hierarchies are unstable when environmental conditions change (Sneddon *et al.*, 2006). Further investigations are needed to establish whether changing tanks and tank water affects dominance hierarchies in *D.*

rerio and whether unstable hierarchies affect the wellbeing of individual fish of different ranks.

CONCLUSIONS AND NEXT STEPS

This concept study of relatively short duration found no significant effect of environmental stability on body condition, frequency of spawning, egg output or viability, or aggressive behaviour. In the next phase of this work, the time frame of the study will be extended and effects of a changed environment will be assessed on wider and more subtle aspects of the fish's physiology, including basal metabolic rate, gonadal growth and development and brain morphology and development. Investigations are also planned into other simple changes to the fish's environment, including shading tanks, reducing noise levels, and providing multiple spawning areas, and to husbandry procedures, such as the handling techniques and holding densities used, to assess how these affect fish health and wellbeing. In addition, direct comparisons will be carried out between enriched tanks that are stable or changed and these will also be compared against the equivalent in bare tanks (which are easier to implement in laboratory settings). We believe that welfare in captive fish can be improved through simple refinements to husbandry practices that are practical and,

importantly, likely to be applied across animal facilities. However, more research is needed to identify what these refinements are and the benefits they will impart to welfare and scientific research.

ACKNOWLEDGEMENTS

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Figure 1

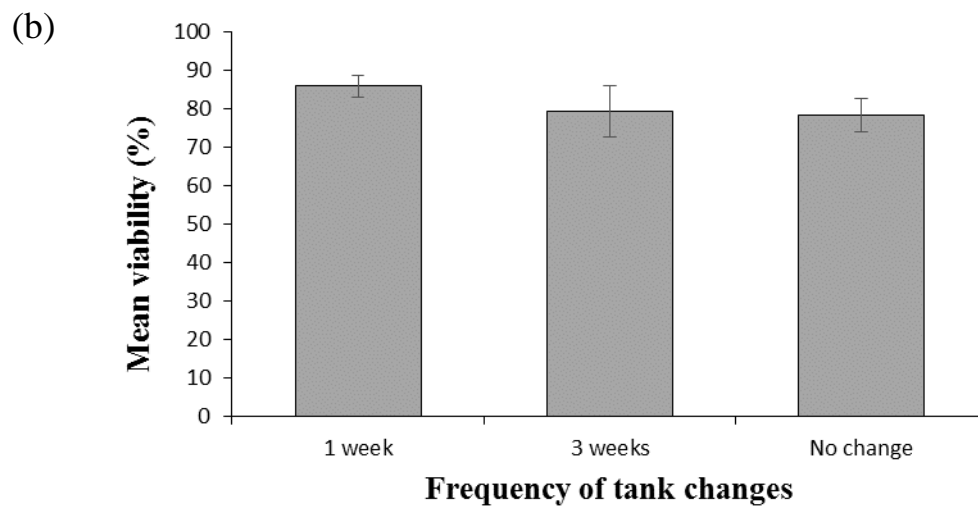
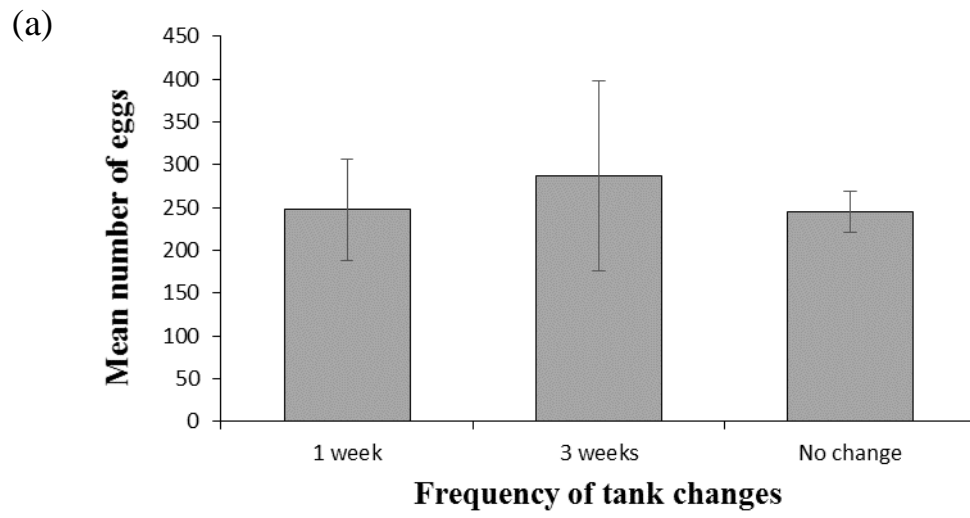


Figure 2

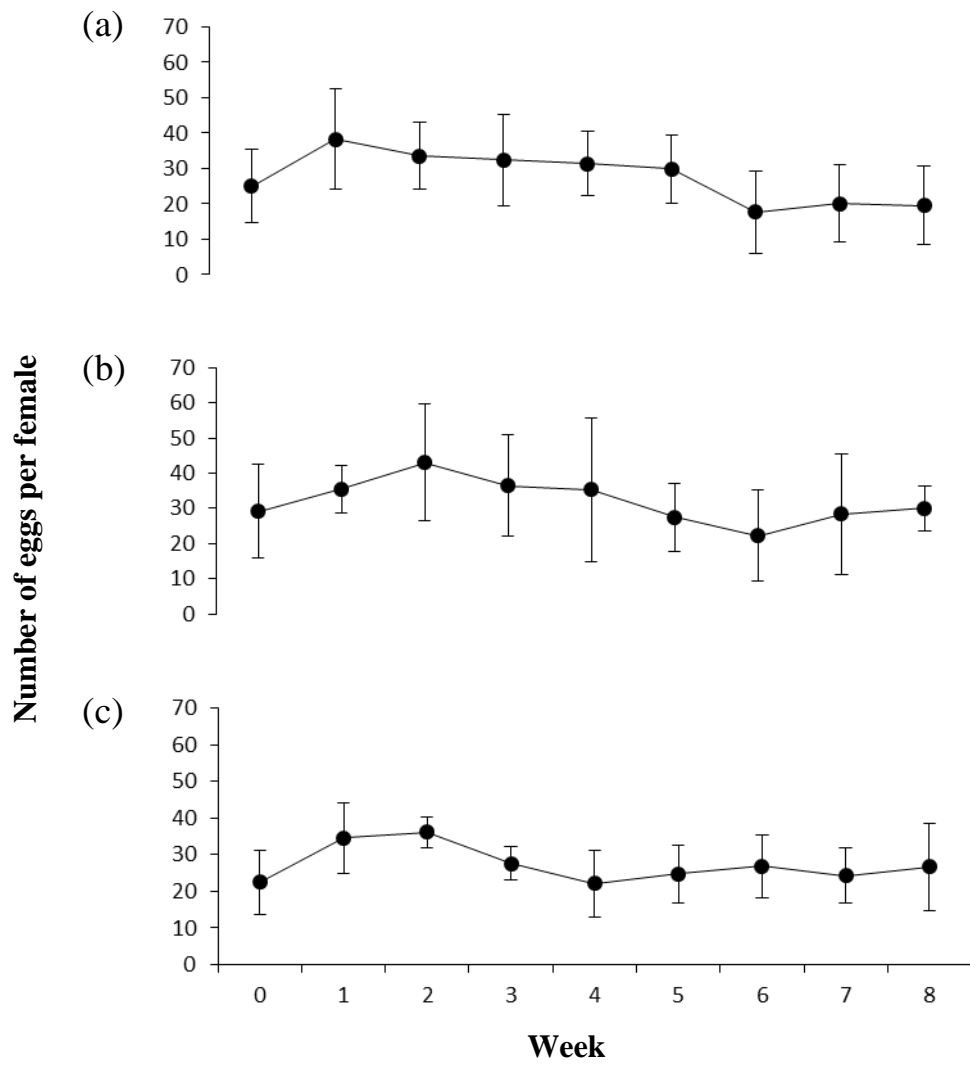


Figure 3

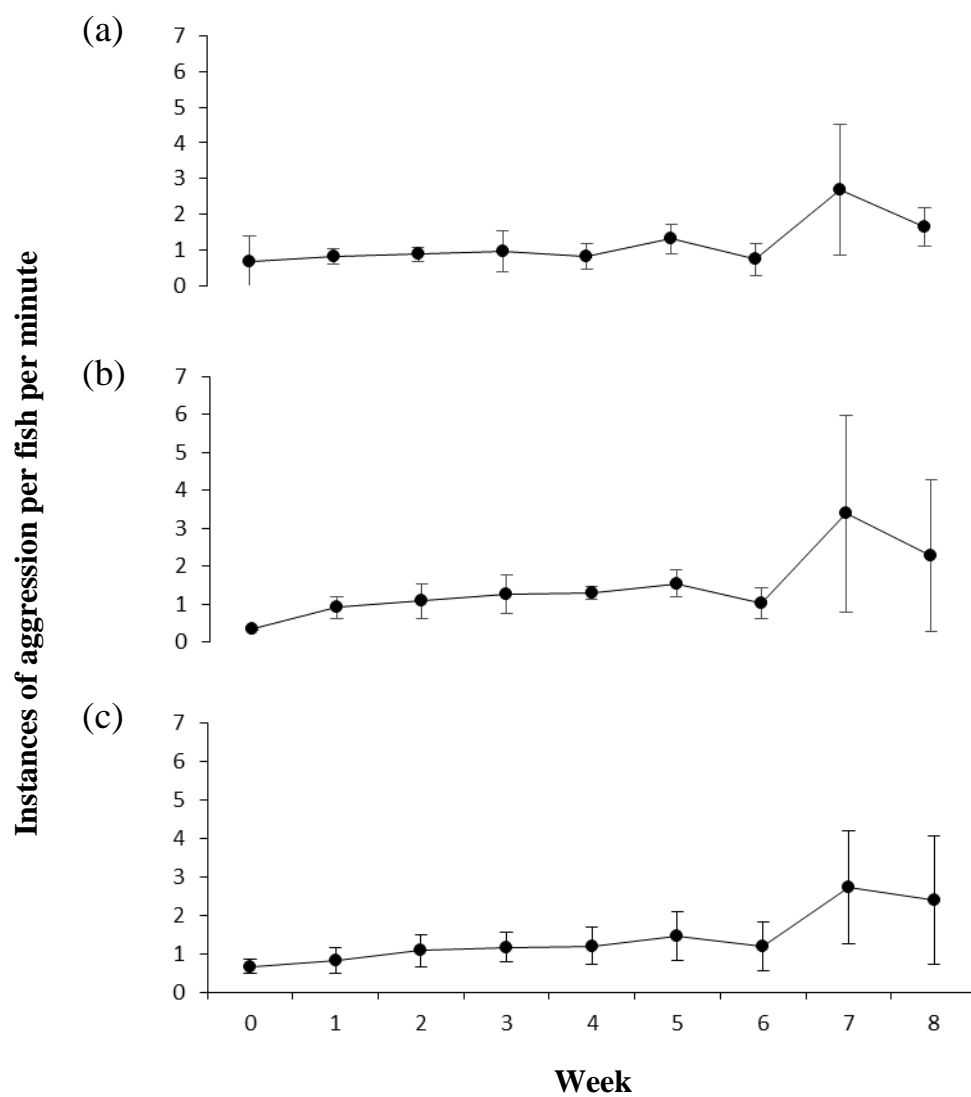


Figure legends

FIG. 1. The effect of periodically moving *Danio rerio* into novel tanks on (a) total number of eggs spawned per female over the 9 week assessment period, and (b) egg viability (percentage of live embryos at 24 h post fertilization). No significant difference was found between treatments (one-way ANOVA; number of eggs: $P > 0.05$; viability: $P > 0.05$). Data are presented as means \pm standard deviation.

FIG. 2. Patterns of egg output across 9 weeks for *Danio rerio* that were moved into novel tanks (a) every week, (b) every 3 weeks, or (c) remained in the same tank for the duration of the study. No significant differences were found in egg output over time for fish that were moved every week or that remained in the same tank (Friedman test; $P > 0.05$ for both treatments). Fish moved every 3 weeks showed a significant difference in egg output between weeks 2 and 6 (Friedman test, $P < 0.05$). Data are presented as means \pm standard deviation.

FIG. 3. Patterns of aggression across 9 weeks for *Danio rerio* that were moved into novel tanks (a) every week, (b) every 3 weeks, or (c) remained in the same tank for the duration of the study. No significant differences were found in aggression over time between treatments (Friedman test, $P > 0.05$ for each treatment). Data are presented as means \pm standard deviation.

Chapter 4 Conclusions

This study set out to provide insights into the provision of optimal conditions for generating high-quality experimental subjects while creating high welfare standards for laboratory zebrafish. I aimed to address the gap in knowledge of the effects of housing conditions on the welfare of zebrafish—a surprising shortfall, considering that over 5 million zebrafish may be used annually in research worldwide (Lidster *et al.*, 2017) and the range of scientific fields in which they are a prominent model. This research investigated the effects of (1) environmental enrichment and (2) a changed vs stable environment on the wellbeing of laboratory zebrafish. In the first study, groups of zebrafish were raised in plain tanks and in tanks enriched with gravel and plants and measures of survivorship, growth, development and behaviour were compared between treatments. In the second study, groups were housed in ‘stable’ environments or in ‘changed’ environments and morphometrics, reproductive success and behaviour were compared between treatments.

Addressing the knowledge gap

This thesis contains one of the first reports on the effects of environmental enrichment on a number of measures. Post-hatch survival of zebrafish larvae reared in enriched tanks was significantly higher than larvae reared in plain

tanks, suggesting that larval zebrafish benefit from the provision of substrate and plants in their rearing tanks, at least in the early stages of their development. This study also revealed that enriched females had improved body condition compared to plain females, and males kept in plain tanks had more variable body condition than males in enriched tanks. No similar studies of *D. rerio* have been published with which these results could be compared and more work is needed to determine the causes of differences in body condition found between treatments in both sexes.

In this study we found that fish kept in enriched environments displayed lower levels of anxiety-like behaviour. This result was similar to that reported for control groups in other studies (e.g. Egan *et al.*, 2009; Wong *et al.*, 2010). Reduced levels of anxiety are likely to be due to plants providing cover and a refuge in which to escape harassment from dominant fish.

The finding that there was no significant departure from the expected sex ratio of 50:50 in either treatment group is a positive result from a welfare perspective, as a skewed sex ratio in zebrafish can imply inappropriate housing conditions or diet. Ultimately, researchers want to avoid skewed sex ratios in their studies.

In fish placed in the choice tanks we found that dominant individuals were able to monopolise the access between the bare and enriched compartments.

Territoriality is a known behavioural trait in zebrafish, especially at low densities.

Despite other studies showing that the addition of planted material reduced monopolisation of resources, we feel that the design of our tanks with a relatively small access between compartments, may have contributed to the

ability of dominant individuals to preclude other fish from entering or moving between compartments. It may be that an alternative tank design or end point is needed to fully elucidate what the fish wants. Overall, the data presented show that enrichment, in the form of gravel and plants, has varied effects on laboratory-maintained *D. rerio*.

The second study investigated the effects of a changed vs stable environment on the wellbeing of zebrafish, an area not previously investigated. This concept study, of relatively short duration, found no significant effect of environmental stability on body condition, frequency of spawning, egg output or viability, or aggressive behaviour and it was concluded that changing the tank and tank water did not have any obvious health benefits to the fish, for the periods of time studied. The rationale for this approach is to look at alternative methods of enrichment which can be applied easily on a practical basis, incorporating good husbandry practices, rather than the introduction of substrates, refuges or other physical features, that can be more limiting for both system maintenance and research practice. However, there is evidence that tank transfers evoke a stress response in zebrafish (Pottinger and Calder, 1995) and that unpredictable stressors may increase anxiety-like behaviours (Fulcher *et al.*, 2017). The temporal basis of a stimulating environment and of the predictability of stressors remain to be determined for zebrafish and should be considered when designing enrichment that aims to improve welfare through environmental change.

Limitations of the study

A factor that needs due consideration here is that the zebrafish used in both investigations here were of the WIK strain. WIK was chosen because it is a popular strain and commonly-used for a wide range of studies. However, there are other well-established laboratory strains and these exhibit between-strain variations in behaviour, growth and stress response. Consequently, the present results may not extrapolate to other strains. It would be useful to test fish from different strains to see whether they differ in response to enrichment and/or a changed environment.

Results of the environmental choice test were confounded by the actions of dominant individuals who monopolised access to tank areas. Choices indicated by the results may not reflect the actual choices of the majority of fish but may more closely represent the choice of the dominant fish in each tank. It would be interesting to test zebrafish at different life stages, including those not influenced by spawning. Also, investigating the endpoints used in this study on different strains of zebrafish.

Future research directions

There is still much to learn about the natural history and ecology of the zebrafish and how these influence its health and wellbeing in the laboratory. Such knowledge will aid understanding of which laboratory housing parameters

promote optimal wellbeing. There is a real need for enrichment approaches that provide measurable welfare benefits without compromising research results.

Opportunities for further study include extending the time frame of the study and assessing the effects of enrichment and of a changed environment on wider and more subtle aspects of the fish's physiology, including basal metabolic rate, gonadal growth and development and brain morphology and development.

Other simple changes to the fish's environment, including shading tanks, reducing noise levels, and providing multiple spawning areas, and to husbandry procedures, such as the handling techniques and holding densities used, could be assessed for effects on fish health and wellbeing. The welfare of captive fish may be improved through simple refinements to husbandry practices that are practical and, importantly, likely to be applied across animal facilities. However, more research is needed to identify what these refinements are and the benefits they will impart to welfare and scientific research. In addition, there are compelling open questions about the effects of enrichment on zebrafish larvae and information on the prey preference of larval *D. rerio*, hitherto unstudied, would be of value in uncovering relationships between environmental enrichment, prey availability and survivorship. Finally, there is a need to develop a model for overall welfare assessment of laboratory zebrafish.

This thesis indicates that there are exciting opportunities to learn more about the effects of laboratory housing on the physiology, behaviour and welfare of laboratory zebrafish. Much awaits discovery, especially in unstudied areas, including multi-generational studies and investigations into maternal effects of environmental enrichment and of a stable versus unstable environment. Such

information will be invaluable for improving housing and husbandry protocols and for promoting the welfare of Hamilton's "beautiful fish".

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