

Improving zebrafish laboratory welfare and scientific research through understanding their natural history

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ABSTRACT

Globally, millions of zebrafish (*Danio rerio*) are used for scientific laboratory experiments for which researchers have a duty of care, with legal obligations to consider their welfare. Considering the growing use of the zebrafish as a vertebrate model for addressing a diverse range of scientific questions, optimising their laboratory conditions is of major importance for both welfare and improving scientific research. However, most guidelines for the care and breeding of zebrafish for research are concerned primarily with maximising production and minimising costs and pay little attention to the effects on welfare of the environments in which the fish are maintained, or how those conditions affect their scientific research. Here we review the physical and social conditions in which laboratory zebrafish are kept, identifying and drawing attention to factors likely to affect their welfare and experimental science. We also identify a fundamental lack knowledge of how zebrafish interact with many biotic and abiotic features in their natural environment to support ways to optimise zebrafish health and well-being in the laboratory, and in turn the quality of scientific data produced. We advocate that the conditions under which zebrafish are maintained need to become a more integral part of research and that we understand more fully how they influence experimental outcome and in turn interpretations of the data generated.

Key words: zebrafish, *Danio rerio*, welfare, physical environment, social environment, natural habitats, laboratory conditions, data quality

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I INTRODUCTION

There are several reasons why it is important to review the welfare of laboratory zebrafish (*Danio rerio* Hamilton). First, researchers have a duty of care for laboratory animals and in many countries are legally obliged to consider their welfare when designing experiments (e.g. UK: Home Office, 2014a; European Union: Council of the European Union, 2010; USA: National Research Council, 2011; Australia: National Health and Medical Research Council, 2013). Second, awareness and support for better welfare of research animals is increasing in the public domain and research funders are now requesting high standards of welfare as a condition of receiving research funds (NC3Rs *et al.*, 2015). A survey by Ipsos MORI in 2018 found that 59% of the public want to know more about work to improve the welfare of research animals, a rise of 5% since 2016 (Clemence, 2018). Thirdly, good animal welfare is linked to improved quality of science (Prescott & Lidster, 2017).

The zebrafish is a key experimental model and worldwide millions are used for studies spanning toxicology, drug discovery, and the study of human diseases, but a lack of defined conditions and standards for zebrafish care have resulted in the protocols for their housing and maintenance varying among laboratories (Alestrom *et al.*, 2019). Advances in the science of zebrafish husbandry and management and more detailed reporting of maintenance conditions of experimental fish are needed to address this issue (reviewed by Lieggi *et al.* 2020). Most guidelines are concerned primarily with maximising production and minimising costs and pay less attention to how aspects of the laboratory environment affect welfare or the science that they support.

We identify the science behind the conditions in which laboratory zebrafish are often kept and aim to identify and draw attention to factors likely to affect their welfare. Understanding a species' natural biology can help to guide good welfare practice (Howell & Cheyne, 2019) and here we draw on field observations and from experiments in mesocosms and laboratory tanks, to compare and contrast the physical and social environment experienced by wild zebrafish with those of laboratory-maintained fish. A variety of physiological and behavioural changes, including increased growth rate and reduced startle response, have been documented in zebrafish raised in a captive environment compared to fish collected from their natural habitat in India (Robison & Rowland, 2005). Captivity creates different fitness pressures compared to the fish's natural environment, potentially leading to differences in selection on various traits (Robison & Rowland, 2005). We use measures of physiology and behaviour to indicate factors likely to affect zebrafish welfare. We also identify gaps in knowledge and discrepancies among current regulatory and laboratory guidelines and illustrate some of the challenges and opportunities that

confront researchers and facility managers for providing best welfare for zebrafish. We furthermore consider how these factors can affect the many fields of science that employ zebrafish as study models, including areas such as neuroscience where the recent discovery of astrocytes in zebrafish is predicted to open new research avenues into brain development, function and disease (Chen *et al.*, 2020). Such research will likely be influenced by the environment in which the experimental fish are produced and tested. For example, temperature can affect the zebrafish nervous system, as evidenced by changes in the brain proteome and behaviour (Nonnis *et al.*, 2021), and behavioural testing of isolated fish modifies their physiological, neuroendocrine and behavioural responses (Giacomini *et al.*, 2015).

II WELFARE

Optimising welfare, based on scientific evidence, is not always compatible with research methods or purpose. For example, some forms of environmental enrichment can improve welfare for some fish species (Näslund & Johnsson, 2014) but can potentially also alter features of an animal's physiology or behaviour (Killen *et al.*, 2013), influencing research results. The benefits of refinements to housing or husbandry need to be measured against their costs and practicalities in order to make a convincing case for improvements that deliver higher welfare. This requires that welfare is quantified. Measures of fish welfare include survivorship, growth, health, reproductive performance, levels of blood/body cortisol, behaviour and affective states. These indicators of fish welfare (Table 1) along with how they are measured, examples of their use, and their advantages and disadvantages, are reviewed extensively elsewhere (Huntingford *et al.*, 2006; Sadoul & Geffroy, 2019; Toni *et al.*, 2019). Each of these methods reflects a particular aspect of welfare but none provide a complete evaluation, so multiple methods are often used (Huntingford & Kadri, 2008).

Studies of fish welfare usually follow one of several approaches. The first, a 'feeling-based' approach, sets out to prove or disprove that fish are sentient beings, i.e. that they have the capacity to suffer (Vettese, Franks & Jacquet, 2020). A second approach, 'physiological', attempts to measure pain and stress as negative indicators of a fish's welfare state (Daskalova, 2019). A third approach uses behavioural analyses to infer learning, preference and choice and to support arguments for fish cognition and emotions (Vila Pouca & Brown, 2017). In a fourth approach, a fish's welfare is considered good if it is able to lead a natural life and express the behaviours that it would in the wild (Huntingford & Kadri, 2009). In our opinion, all of these approaches are

Table 1. Commonly used measures of fish welfare

Welfare indicator	Metric	Examples of use	Advantages	Disadvantages
Survivorship	Simple counts	Evaluate effects of processed diets (Goolish, Okutake & Lesure, 1999) and environmental enrichment (Lee, Paull & Tyler, 2019a)	Easily understood	Difficult to differentiate between underlying and immediate causes (Ellis <i>et al.</i> , 2012a)
Growth	Body length, mass, body condition	Evaluate effects of social isolation (Forsatkar, Safari & Boiti, 2017), stocking density (Rabbane, Rahman & Hossain, 2016), and the use of body condition scoring to assess health and welfare (Clark <i>et al.</i> , 2018)	Straightforward to measure	Depends upon many factors, including temperature, photoperiod, strain, diet, life stage (MacIntyre <i>et al.</i> , 2008); optimal growth for zebrafish has yet to be established (Siccardi <i>et al.</i> , 2009)
Health	Fish appearance (skin, fin, eye and gill integrity and colour) and behaviour (feeding, air-gasping, balance, activity) (Segner <i>et al.</i> , 2012); regular health surveillance (Harper & Lawrence, 2012); body condition (Wilson <i>et al.</i> , 2013)	Evaluate husbandry stress on mycobacterial infections (Ramsay <i>et al.</i> , 2009) and compare success of pathogen detection during health inspections (Marancik <i>et al.</i> , 2019)	Assessing is pragmatic; incidence of disease relatively easy to recognise and measure	High cost of full health evaluations; retrospective results; high number of sampled fish needed to detect pathogens (Collimore, Crim & Lieggi, 2016). The link between health and welfare is complex and can be hard to interpret – although a diseased fish is in a poor state of welfare, a healthy fish is not necessarily in a good state of welfare (Segner <i>et al.</i> , 2012)
Reproductive performance	Clutch size, egg viability, spawning frequency	Evaluate effects of stocking density (Castranova <i>et al.</i> , 2011), stress (Abdollahpour <i>et al.</i> , 2020a), and simple tank changes (Lee, Tyler & Paull, 2018)	Straightforward to measure	Counting or sizing eggs or embryos is tedious and time-consuming. High variability among individuals and populations so high replication needed (Paull <i>et al.</i> , 2008).
Cortisol levels	Whole-body cortisol; cortisol in blood, mucus, faeces, water, scales or fins	Evaluate auditory enrichment as method of reducing stress (Barcellos <i>et al.</i> , 2018), compare effects of handling fish with a scoop <i>versus</i> with a net (Brydges <i>et al.</i> , 2009), and assess transport stress (Dhanasiri, Fernandes & Kiron, 2013)	Measuring cortisol in blood is relatively simple (Ellis <i>et al.</i> , 2012b); non-invasive measurements of cortisol in fish holding water is suitable for small species, such as zebrafish (Stevens <i>et al.</i> , 2017)	May be difficult to interpret for welfare. Cortisol release is a natural reaction to challenges (Huntingford <i>et al.</i> , 2006) but is not synonymous with suffering (Dawkins, 1998). Short-term stress may be beneficial, chronic stress may compromise welfare (Huntingford <i>et al.</i> , 2006)
Behaviour	Observation of latency, frequency or duration of behaviours such as	Impact of invasive procedures on swimming behaviour (Deakin <i>et al.</i> , 2019;	Easy to observe, with training; ideal for daily use; can be early sign of welfare problems	Often variable over time or in response to husbandry events such as anticipation of feeding

(Continues)

Table 1. (Cont.)

Welfare indicator	Metric	Examples of use	Advantages	Disadvantages
	aggression, foraging, shoaling or breeding	Thomson <i>et al.</i> , 2019), and shoal cohesion as an indicator of positive welfare (Franks, Graham & von Keyserlingk, 2018)		(Martins <i>et al.</i> , 2012); may be affected by social dynamics when removing fish from social groups for individual testing (Kleinhappel, Pike & Burman, 2019); individual variance within a group; may be manipulated by parasites (Poulin, 2013); may be difficult to measure and interpret for welfare
Affective state	Cognitive bias, judgement bias, decision-making, preference	Assess decision-making after losing aggressive encounter (Rogers <i>et al.</i> , 2020), assess preference for a range of tank enrichments (Schroeder <i>et al.</i> , 2014), assess sensitivity to reward shifts (Tan <i>et al.</i> , 2020)	Indicates an animal's subjective perception of internal or external stimuli; can identify positive as well as negative welfare (Jirkof, Rudeck & Lewejohann, 2019)	May be difficult to quantify; lack of established measures; judgement bias tests require training of fish but some fail to learn task so tests may systematically exclude some individuals (Rogers <i>et al.</i> , 2020); interpretation of preference tests requires caution as choices restricted to resources provided in the test, fish preferences may change over time, or they may choose the lesser of two non-preferred items (Maia & Volpato, 2016)

needed to define the welfare status of a fish most reliably. Optimising welfare is critical if researchers are to avoid variation in research results caused by changes in the physiology and behaviour of experimental animals due to compromised welfare (Prescott & Lidster, 2017).

III PHYSICAL ENVIRONMENT

The optimum range of conditions for zebrafish, and the husbandry parameters that are most important for welfare, and therefore good science, are largely unknown (Tsang *et al.*, 2017) with many laboratories modifying environmental conditions for zebrafish based on their own opinions, experience, and/or old literature rather than from any systematic analyses (Tsang *et al.*, 2017). In their natural environment in India, Bangladesh and Nepal, zebrafish occupy a variety of habitats, including rice paddies, ponds, ditches, and streams, and tolerate a wide range of fluctuating temperatures and water conditions (Lee, Tyler & Paull, 2020). This

adaptability accounts for the species' ability to tolerate the wide-ranging conditions operating among laboratories, and why ease of care is often listed as a zebrafish attribute. However, there is a need for empirical studies to evaluate the effects of environmental parameters on the health and welfare of zebrafish in order to understand which environmental factors may affect research results (Tsang *et al.*, 2017). For example, a recent study found that temperature affects the nervous system and cognitive abilities of adult zebrafish and even short-term variations can alter brain protein expression and behaviour (Nonnis *et al.*, 2021). This, in turn, suggests that temperature is an important environmental factor influencing zebrafish physiology and behaviour and that the duration of thermal change may induce different responses in laboratory fish.

(1) Water quality

Water physicochemistry is hugely important for the welfare of fish but is often overlooked as a variable in zebrafish research (Hammer, 2020). Fish are sensitive to poor water

quality and to contaminants (Huntingford *et al.*, 2006) and provision of appropriate water conditions for fish maintenance does not necessarily align with the needs of the research. For example, fish used to investigate the relationship between gene function and disease may be housed in static containers during genotyping and experience a rapid decrease in water quality (Goodwin *et al.*, 2016).

The UK Government's code of practice for the housing and care of fish used for scientific purposes (Home Office, 2014b, p. 37) states that “adequate water supply of suitable quality shall be provided” and that “water quality parameters shall be within the acceptable range that sustains normal activity and physiology for a given species”, but does not state the specific requirements for zebrafish. European legislation specifies water parameters for zebrafish embryos and larvae used for ecotoxicology studies (OECD, 2013) but recommendations for adults are vague (European Commission, 2019). United States guidelines for the care of research animals acknowledge that water quality affects aquatic animal wellbeing and that requirements vary with species, but do not define the specific needs of zebrafish (National Research Council, 2011). Given that poor water quality can directly affect fish growth and reproduction (Hammer, 2020) this lack of prescribed water parameters for breeding stock represents a significant shortfall in official guidance.

Zebrafish are widely used in (eco)toxicology (Tal, Yaghoobi & Lein, 2020), where water quality is likely to affect study outcomes through effects, for example, on the fate and behaviour of the exposure chemical (Hamm *et al.*, 2019). It also has been shown that, during the process of fish domestication (Teletchea & Fontaine, 2014), water physicochemistry in which embryos are incubated can affect their ability to cope with laboratory conditions and such information may be passed on to future generations *via* epigenetic memory in the gametes (Labbé, Robles & Herraes, 2017).

In their natural environment, water conditions experienced by zebrafish are highly dynamic seasonally. By contrast, water conditions in laboratories are controlled and monitored to reduce fluctuations and maintain stability throughout the year (Fig. 1; Table 2 provides tolerance ranges for water chemistry parameters in wild zebrafish, recommendations for laboratory-maintained zebrafish, and implications for welfare). How the lack of variation in water physicochemistry and seasonality affects zebrafish is unknown, but further insight could help inform both the management of stock animals and certain areas of research, for example, how climate affects disease. As a case example, a direct link has been established between temperature during zebrafish development and cardiac anatomy (Dimitriadi *et al.*, 2018).

(2) Water flow

Zebrafish have been used to model the effects of exercise on growth (Palstra *et al.*, 2010), ageing (Gilbert, Zerulla & Tierney, 2014), heart regeneration (Rovira *et al.*, 2018) and

anxiety (DePasquale & Leri, 2018). In these experiments, swim tunnels with adjustable flow are used to induce exercise. As repeated exercise improves the overall condition of fish (Palstra & Planas, 2011), there may be differences in the pre-study condition of fish reared in tanks with differing flow rates, from static to high flow depending on tank system design and fish life stage. It may therefore benefit the comparison of results if flow rates in rearing tanks are standardised, or at least reported. However, none of the above-referenced studies report rearing tank flow rates.

Wild zebrafish are found in still waters and waters that flow up to 18 cm s⁻¹ (Spence *et al.*, 2006, 2008; Suriyampola *et al.*, 2015). Complex, fragmented flow patterns in natural streams and rivers are created by structures such as boulders (Branco *et al.*, 2013), woody debris (Manga & Kirchner, 2000) and vegetation (Sukhodolov & Sukhodolova, 2010). Wild fish can move between these microhabitats as conditions or needs change. By contrast, water flows in laboratory tanks are homogeneous, with recommended flow rates of 3–5 tank volume exchanges per hour for adult zebrafish (Brand, Granato & Nüsslein-Volhard, 2002; Varga, 2016). The flow of water through laboratory tanks varies among laboratories, and for fish at different life stages.

Water flow may benefit the welfare of zebrafish by helping to minimise mycobacteriosis (Murray *et al.*, 2011), promote movement (Gilbert *et al.*, 2014), and possibly stimulate the immune system (Palstra *et al.*, 2010), but little is known about the effects of flow on other measures of welfare. Water flow may also be negative for welfare, especially in small tanks, such as the 1–10 l tanks used in commercial rack units (Lawrence & Mason, 2012), where the inability to move away from flow may increase stress and energetic costs for fish (Suriyampola *et al.*, 2017).

Interestingly, captive zebrafish have been shown to be more active, more aggressive, and to form less-cohesive groups in flowing, rather than still, water conditions (Suriyampola *et al.*, 2017) but only when the areas were furnished with plants and substrate (DePasquale *et al.*, 2019). Similarly, zebrafish in the wild are more aggressive and form less-stable groups in flowing, rather than still, waters but, when compared to captive fish, their social behaviour is less affected by vegetation (Suriyampola *et al.*, 2015). These studies suggest that water flow influences the social behaviour of wild and captive zebrafish and may be an important parameter to be considered in experimental/housing design.

(3) Light

Zebrafish are increasingly used as models to study light-induced eye diseases and retina regeneration (Wan & Goldman, 2016), and light itself can act as a stressor for zebrafish in biomedical tests (Lee *et al.*, 2019b). Despite the importance of light in the zebrafish rearing environment, in a sample of 10 published studies on eye disorders in adult zebrafish, none stated the type of light used or the light intensity (search conducted in *Web of Science* Core Collection, using “eye disorders adult zebrafish” and dates 2010-01-01 to

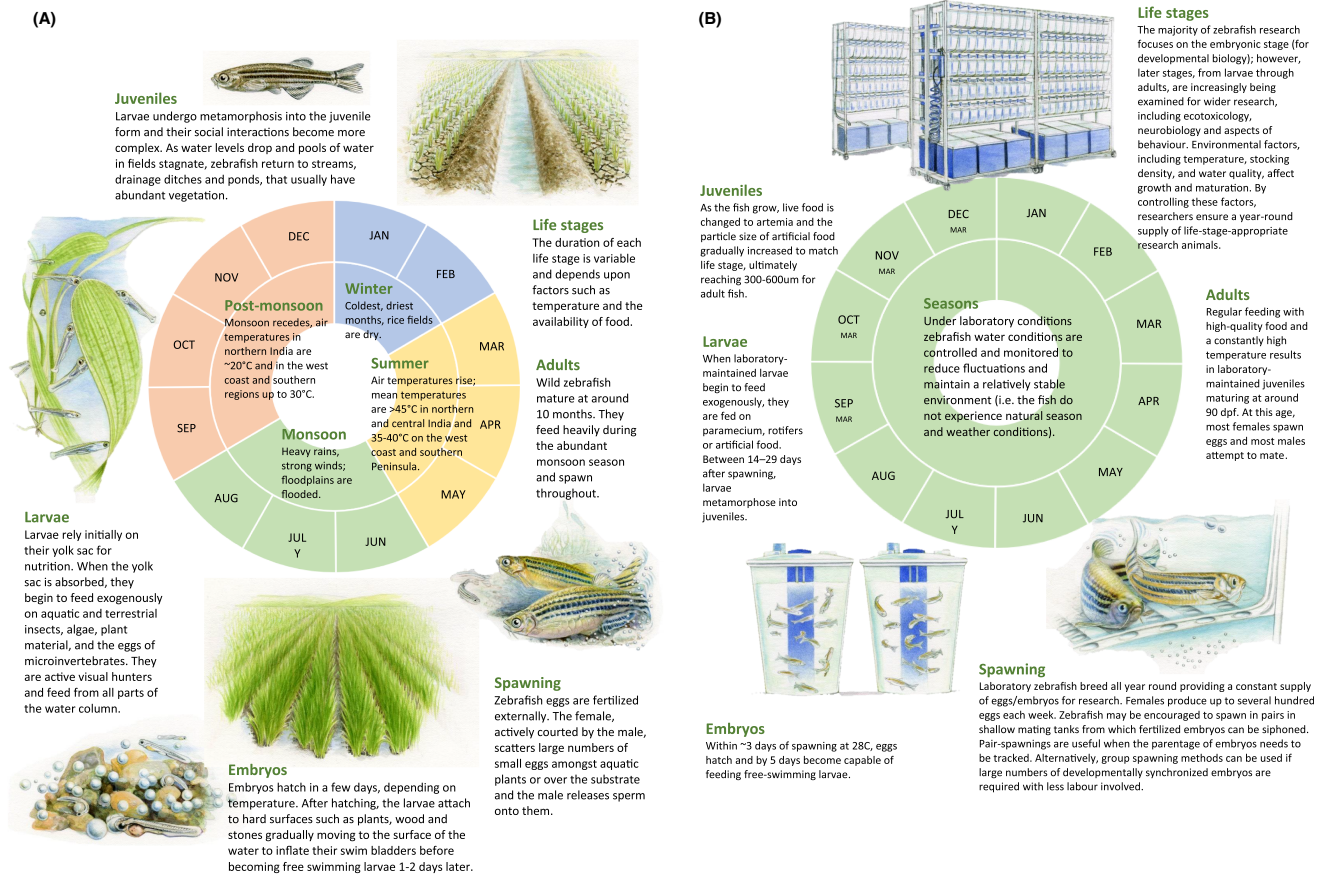


Fig. 1. A schematic representing the life cycle and living conditions experienced by (A) wild zebrafish and (B) laboratory maintained zebrafish. Credit: Sandra Doyle.

2020-12-01 as search criteria). Light is therefore a possible confounding factor among these experiments due to inconsistencies among laboratories and lack of similarity between lighting conditions in the laboratory and in the natural environment.

Light affects fish behaviour in many ways. Diel cycles of day and night shape the times at which fish feed, breed, and rest (Helfman, 1993) and seasonal light rhythms influence reproduction and spawning (Migaud, Davie & Taylor, 2010). A rhythm of activity and rest occurs in zebrafish from the first day post-fertilisation (Dekens & Whitmore, 2008; Krylov *et al.*, 2021), reflecting the light–dark cycle in nature. In India and Bangladesh, hours of daylight range from 10 h in December to 13 h in June (www.timebic.com/sun) with the longest day lengths coinciding with the start of the monsoon, when wild zebrafish are thought to breed (Spence *et al.*, 2007). The sun provides up to 100000 lux of light at the Earth’s surface at midday (Thorington, 1985) and sunlight covers the full spectrum from infrared to ultraviolet as it gradually changes from dawn to dusk.

In the laboratory, artificial light is provided through incandescent, fluorescent or light-emitting diode (LED) lamps, each of which produces a different spectrum of light per

wavelength. Light intensity may vary depending on the position of tanks in the rack or room, or may be reduced to inhibit algal growth (Bhargava, 2018). In addition, most artificial light fluctuates with low intensity (incandescent bulbs) or with a pronounced flicker (fluorescent and LED bulbs) (Inger *et al.*, 2014) that can cause headaches and eye fatigue in humans (Wilkins *et al.*, 1989), affect mate choice in captive birds (Evans, Cuthill & Bennett, 2006) and induce myopia in mice (Yu *et al.*, 2011). Despite the potential for these factors to affect behaviour, experimental results, and reproducibility (Adatto, Krug & Zon, 2016; Sabet, van Dooren & Slabbekoorn, 2016a; Gerlai, 2018), light source and intensity are rarely reported in the literature. A cycle of 14 h of light and 10 h of darkness with phased transitions has been suggested as appropriate for laboratory zebrafish (Reed & Jennings, 2011; Tsang *et al.*, 2017) but, across the year, this adds 1–4 h of light per day compared to the natural environment. Such an extension of day length increases growth and reproductive performance (Abdollahpour, Falahatkar & Lawrence, 2020b), but its long-term effects on welfare have not been evaluated. In addition, biological rhythms may be disrupted by light pollution or leakage during the hours of darkness from extraneous light sources such as hallway lights

Table 2. Summary of water chemistry parameters reported in wild zebrafish (*Danio rerio*) habitats and in zebrafish facilities and their implications for welfare

Parameter	Wild habitats	Laboratory conditions for adult zebrafish			Implications for welfare
		Tolerance limits	Optimal range	Recommendations	
Dissolved oxygen	Unknown	Levels of 0.8 mg l ⁻¹ are lethal within 2 days and levels of 0.4 mg l ⁻¹ are lethal within 12 h (Rees, Sudradjat & Love, 2001)	Unknown	Range from 6 mg l ⁻¹ (Matthews, Trevarrow & Matthews, 2002) to 7.8 mg l ⁻¹ (Harper & Lawrence, 2012); with a suggested minimum of 4 mg l ⁻¹ (Lawrence & Mason, 2012)	Uneaten food, decaying solids and high fish densities can reduce levels (Hammer, 2020). Warning signs include hyperventilation, surface swimming and gulping air (Kramer, 1987); extreme depletion can damage gills, impair growth, cause immunosuppression, and lead to death.
Ammonia	Unknown	Levels >1.0 mg l ⁻¹ are lethal to many fish (Murray, Lains & Spagnoli, 2020) but the specific tolerance limits of zebrafish are unknown	Unknown	As close to 0 mg l ⁻¹ as possible (Hammer, 2020; Murray <i>et al.</i> , 2020)	Highly toxic. Chronic exposure to non-lethal levels can result in immunosuppression and reduced growth (Murray <i>et al.</i> , 2020). Acute exposure can cause hyperexcitability, anorexia, and death (Murray <i>et al.</i> , 2020).
Nitrite	Unknown	Levels of 386 mg l ⁻¹ are lethal within 4 days (Voslářová <i>et al.</i> , 2008)	Unknown	As close to 0 mg l ⁻¹ as possible: <0.5 mg l ⁻¹ (Hammer, 2020); <1.0 mg l ⁻¹ (Murray <i>et al.</i> , 2020)	Warning signs in fish include lethargy, remaining near water inlet, hyperventilating; chronic exposure impairs growth (Murray <i>et al.</i> , 2020).
Nitrate	Unknown	Unknown	Unknown	<100 mg l ⁻¹ (Pereira <i>et al.</i> , 2017); <50 mg l ⁻¹ (Hammer, 2020); <25 mg l ⁻¹ (Alestrom <i>et al.</i> , 2019)	Less toxic than nitrite but may accumulate over time in recirculating systems with high fish densities (Learmonth & Carvalho, 2015). Chronic exposure can damage gills, skin, kidneys, liver and intestines (Pereira <i>et al.</i> , 2017).
pH	Varies from 5.9 (Engeszer <i>et al.</i> , 2007) to 9.8 (Arunachalam <i>et al.</i> , 2013)	Lower and upper lethal limits: 3.0 and 12.0 respectively (Zahangir <i>et al.</i> , 2015)	7.4–7.5 for reproduction (Alestrom <i>et al.</i> , 2019)	7–8 (Hammer, 2020); 6.5–8 (Alestrom <i>et al.</i> , 2019)	Exposure to pH near the lower or upper limits damages skin and gills, leads to loss of balance, convulsions and death (Zahangir <i>et al.</i> , 2015).

(Continues)

Table 2. (Cont.)

Parameter	Wild habitats	Laboratory conditions for adult zebrafish			Implications for welfare
		Tolerance limits	Optimal range	Recommendations	
Salinity	From 0.01 to 0.6 g l ⁻¹ (Spence <i>et al.</i> , 2006)	Unknown	Unknown	0.5–2 g l ⁻¹ (Harper & Lawrence, 2012); 0.06–0.17 g l ⁻¹ (Tsang <i>et al.</i> , 2017); 0.08–0.93 g l ⁻¹ (Alestrom <i>et al.</i> , 2019)	Long-term exposure to low or high salinity may negatively affect energy expenditure and fecundity (Boisen <i>et al.</i> , 2003).

and exit signs (Adatto *et al.*, 2016). Lighting type and light cycle regime may even vary between rooms within a facility, which needs to be considered in facility design and reporting of results.

(4) Temperature

Zebrafish are increasingly used to model population sex ratios and investigate how epigenetic inheritance contributes to adaptation to new environmental conditions such as temperature exposure (Valdivieso *et al.*, 2020). They are also used to assess the effect of temperature on cardiovascular function (Rayani *et al.*, 2018), and to study fish diseases and infections at different temperatures (Jorgensen, 2020).

Temperature exerts a fundamental impact on all aspects of development, growth, feeding, metabolism, reproduction and behaviour in fish (Coutant, 2006). For laboratory zebrafish, rearing temperature and daily temperature cycles influence larval survival, growth, phenotype and sex determination (Schaefer & Ryan, 2006; Sfakianakis *et al.*, 2011). However, effects of temperature on development and growth are complex.

Villamizar *et al.* (2014) found that a constant temperature of 24°C or 28°C resulted in male-biased sex ratios whereas a daily thermocycle resulted in a higher proportion of females. Adults kept at 34°C were bolder and less anxious than fish kept at 26°C (Angiulli *et al.*, 2020), a finding that could impact results and reproducibility, especially for studies of neuroscience or behaviour. Generally, the highest survival and growth rates are reported in fish maintained at a constant temperature of 28°C (Schaefer & Ryan, 2006; Sfakianakis *et al.*, 2011), the most commonly used set point for laboratory zebrafish, or when thermocycles are set so that the transition to higher temperature coincides with dawn and the transition to lower temperature coincides with dusk (Villamizar *et al.*, 2014).

Zebrafish are cold-blooded (poikilothermic) and eurythermal (Nonnis *et al.*, 2021), reflecting wide seasonal and diurnal temperature ranges reported in their natural environment, from 12°C in Arunachal Pradesh to 39°C in Orissa (Arunachalam *et al.*, 2013). Air temperature, water depth and vegetation combine to create fine-scale microclimates

throughout a fish's home range (Welch, Jacoby & May, 1998). Riparian vegetation provides shade along the banks of streams and lakes, creating refuges that are cooler in summer and warmer in winter than similar habitats with no vegetation (Welch *et al.*, 1998).

Zebrafish are used to investigate the effects of thermal tolerance on aquatic ectotherms (Schaefer & Ryan, 2006), however, evidence of differences in thermal tolerance between wild and laboratory-reared zebrafish (Morgan *et al.*, 2019) suggests that the tolerance of wild populations could be overestimated if reliance is placed solely on the results of laboratory experiments. Understanding species' thermal limits is central to understanding how they will respond to climate change (Bennett *et al.*, 2021).

Temperature is a potentially useful tool for the husbandry and health management of zebrafish. When allowed to move freely across a gradient of 18–35°C, fish infected with a virus spent most of their time at a temperature of 29°C and showed temperature-dependent changes in the brain transcriptome higher than those of fish with limited thermal choices (Boltaña *et al.*, 2013). A comparable experiment with larval zebrafish produced similar results (Rey *et al.*, 2017). Thermal choice and, conversely, thermal restriction may therefore impact upon a fish's health and welfare.

Little is known about the temperature preferences of zebrafish. When allowed to choose between water temperatures of 20°C and 24°C, zebrafish showed a consistent preference for the higher temperature (López-Olmeda & Sánchez-Vázquez, 2009). In addition, there is evidence that fish personality may affect temperature preference, with bold, aggressive zebrafish preferring warmer waters than shy, passive fish (Rey, Digka & Mackenzie, 2015). More research is needed to define the preferences of unstressed fish, increase understanding of the effects of temperature, diurnal cycles, and thermal gradients on zebrafish, and evaluate the potential benefits of temperature choice on welfare and fitness.

(5) Physical space

Tank size is a potential confounding factor for studies of fish cognition and spatial learning (Salena *et al.*, 2021). Like rodents and birds, fish use geometry to solve spatial tasks

and differences in the size and shape of the tanks in which they are reared can affect their subsequent performance in spatial tests (Brown, Spetch & Hurd, 2007; Carbia & Brown, 2019; Salena *et al.*, 2021). Tank size can also affect studies of the growth rates of Atlantic salmon (*Salmo salar*; Espmark *et al.*, 2017) and aggression and welfare in the Midas cichlid (*Amphilophus citrinellus*; Oldfield, 2011). However, few experiments have measured zebrafish responses to different tank sizes and little attention has been paid to the small amount of space a laboratory zebrafish has compared to what it may use as a free-living animal.

Laboratory housing often involves confining fish at high densities, with no opportunity for dispersal or exploration of novel space, and in groups differing in size and social structure from those found in nature, where shoals are dynamic and individuals interact in an ever-changing social network (Krause *et al.*, 2000). One consequence is an effect on mating strategy. Group spawning accounted for 72% of spawning events when fish were held at high densities in 17 l tanks, whereas most fish spawned in pairs when housed at low densities in 450–1000 l tanks, suggesting that group spawning may result from mating pairs being unable to escape from conspecifics (Hutter *et al.*, 2010). Undoubtedly, the movement and behaviour of laboratory zebrafish is greatly restricted compared to that of wild fish, with consequences for research into genetic architecture and the evolution of behavioural traits (Wright *et al.*, 2006a). Fish may be routinely and repeatedly moved from their home tanks to smaller spawning chambers with the potential for additional stress due to handling and confinement, and to changes in water conditions and flow, social structure and density, room position, lighting, etc. In addition, visual health checks are more challenging in small tanks as sick fish cannot easily isolate from the group and behavioural signs of disease, such as changes in swimming activity or social behaviour (Kirsten *et al.*, 2018), are more easily missed.

There are no data-driven standards for the size and shape of tanks used to house zebrafish. Neither the UK Home Office Code of Practice for the housing and care of animals used for scientific purposes (Home Office, 2014a) nor the European Directive on the protection of animals used for scientific purposes (Council of the European Union, 2010) give guidance on tank dimensions. RSPCA guidelines state simply that “tanks need to be of sufficient size to accommodate the physical and behavioural needs of zebrafish and to allow appropriate social interactions. The necessary dimensions depend on the size and age of the fish” (Reed & Jennings, 2011). For this advice to be of practical use, the physical and behavioural preferences of zebrafish at different life stages need to be determined and defined and the number of fish in the tank taken into account. Unsurprisingly, given the lack of published standards, tank sizes and shapes and stocking densities vary considerably among facilities. Tanks reportedly used for rearing larvae range in volume from 1.3 l housing 50 larvae (38 larvae l⁻¹) to 0.75 l for 100 larvae (133 larvae l⁻¹) while tanks for adult fish range from 1.8 l for 18 fish (10 fish l⁻¹) to 75 l for 300 fish (4 fish l⁻¹)

(Harper & Lawrence, 2012; Lawrence & Mason, 2012; Varga, 2016). Commercially available rack units that hold tanks, typically from 1 to 10 l, are used by many research establishments to house zebrafish (Lawrence & Mason, 2012) as they enable a large number of fish to be held in a relatively small space.

The size and depth of water bodies change drastically between wet and dry seasons in ponds, rice fields and streams throughout zebrafish natural habitats and are linked to different life-history stages (Lee *et al.*, 2020). This seismic shift in water volume is often mimicked for captive zebrafish during their first few days of life when they are kept in shallow water until swim bladder inflation, at which point they are transferred to shallow tanks which are filled gradually, over a period of a few hours to a day, often to the depth at which they will remain for their adult lives. The size of water bodies in which wild zebrafish have been found range from puddles of 1 m² to lakes of 3.4 ha, with depths from 5 cm in irrigation channels to 1 m in rivers and lakes (Spence *et al.*, 2007; Arunachalam *et al.*, 2013; Suriyampola *et al.*, 2015; Sundin *et al.*, 2019). Even the smallest water body in nature offers more physical space than typical laboratory conditions, which may impact the responses that zebrafish give to cognition, learning and other tests. Careful reporting of tank size and shape during rearing and testing is therefore recommended.

(6) Environmental enrichment

Despite growing interest among neuroscientists in how environmental enrichment influences brain processes and behaviours, its role in zebrafish models is poorly understood (Volgin *et al.*, 2018). Enrichment improves reproducibility of rodent studies by increasing animal well-being and positively influencing brain physiology and behaviour (Volgin *et al.*, 2018). It stimulates several brain regions, promotes neuroprotection and neurogenesis, and protects against the effects of unpredictable chronic stress (Marcon *et al.*, 2018). In fish species, enrichment has been found to affect several aspects of biology, from aggression and stress to disease susceptibility, but these differ depending upon the species and life stage (reviewed by Näslund & Johnsson, 2014). In zebrafish, enrichment appears to reduce anxiety and provide positive neurological stimulation, but more studies are needed to determine its influence fully (Volgin *et al.*, 2018).

Zebrafish live in diverse environments within their natural range, where substrates and vegetation are varied and structurally complex. By contrast, laboratory fish are almost always housed in bare tanks with few stimuli. Such barren housing may be associated with poor welfare even when animals are otherwise well provisioned (Schroeder & Mocho, 2014).

Environmental enrichment for laboratory fish involves increasing the complexity of the tank environment, usually by the addition of substrates or plants, in order to improve welfare and minimise unwanted behaviour such as aggression (Näslund & Johnsson, 2014). The goal when designing

enrichment is to identify elements of the natural environment that can be modified to provide measurable welfare benefits without compromising research (Johnsson, Brockmark & Näslund, 2014), and can be accommodated in small laboratory tanks and practically maintained by animal care staff. Preference tests can be informative since they allow fish to choose what they want. However, the interpretation of such tests requires caution as choices are restricted to the resources provided in the test, fish preferences may change over time, or they may choose the lesser of two non-preferred items that are presented to them (Maia & Volpato, 2016).

Physical items placed in the tank, such as gravel, plants and other objects, are often seen as desirable enrichment features, however they may not be compatible with research studies. For example in (eco)toxicology, these objects add surfaces for adherence of the test chemical and establishment of microbe biofilms that may increase the rate of degradation of the test chemical (Wilkes *et al.*, 2012). Such inclusions can also serve to harbour pathogens as well as restrict viewing of the fish, affecting the likelihood of early detection of disease and/or other health-related problems (Williams, Readman & Owen, 2009). In some cases the provision of these enrichment features is not feasible, and even for laboratories where it is, the ambiguity surrounding their benefits, the cost of enrichment objects and the time required for their day-to-day management are concerns (Lidster *et al.*, 2017).

Despite the importance of the sensory system for the zebrafish's view of the world (Moorman, 2001), few studies have examined the effects of sensory enrichment for zebrafish. Tank background colour affects anxiety-like behaviour and cortisol stress response but effects change with age (de Abreu *et al.*, 2020). Facilities' tanks are usually blue, green or clear depending on the manufacturer, but little work has been done on the importance of tank colour to help define the most suitable tank design for welfare and to guide commercial companies as to what to manufacture based on scientific evidence. Zebrafish display preference for images of gravel beneath their tanks and this preference is almost as high as for the actual substrate (Schroeder *et al.*, 2014). By contrast, visual enrichment in the form of seascape backing paper, along with an artificial plant and upturned pot, increased aggression, but the effect, if any, of the backing paper is unknown (Woodward, Winder & Watt, 2019). Classical music as auditory enrichment resulted in zebrafish appearing less anxious in behavioural tests and had a positive effect on immune gene expression (Barcellos *et al.*, 2018). Together, these results suggest that auditory and visual stimulation may have potential as enrichment, although further research is needed to determine their merits and long-term impacts. There is a growing body of work comparing soundscapes in zebrafish natural habitats and the laboratory and the possible impact that sound could have on research outcomes and reproducibility (Sabet *et al.*, 2016b; Lara & Vasconcelos, 2019, 2021).

Environmental enrichment in various forms has varied effects on zebrafish. Some effects are positive from the perspective of welfare (Basquill & Grant, 1998), some are

negative (Hamilton & Dill, 2002), while others show inconsistent responses (Kistler *et al.*, 2011). Understanding and comparing study results is hampered by confounding variables such as the age and provenance of experimental fish, the environment in which they were reared, the social context before and during the experiment, tank size and shape, and different types of enrichment offered. Measures used to assess the effects of enrichment, and whether time points used for behavioural assessments were appropriate to avoid confounding by other husbandry practices, may also affect comparison of results (see Table 3 for examples illustrating differences in study design that hinder comparison of the effects of various forms of environmental enrichment). A more systematic approach is needed to tease apart the influence of different elements of enrichment on zebrafish at different life stages, and the effects on welfare of variable, stable and different amounts of enrichment (Stevens, Reed & Hawkins, 2021).

IV SOCIAL ENVIRONMENT

The social environment is an important consideration when designing and maintaining housing and husbandry that best suit zebrafish. Zebrafish show a strong preference to associate with conspecifics, experience isolation stress when separated from the group, and recover better from an aversive event when housed in a group rather than as pairs or individuals (White *et al.*, 2017). Zebrafish pay attention to social interactions between conspecifics. They learn to assess risk by observing the behaviour of others, and use social information to adjust their behaviour (Nunes *et al.*, 2017). Adults exhibit a rich repertoire of social behaviour from shoaling and courtship to aggression. They react to social stimuli, such as the sight and smell of conspecifics, and consistently recognise familiar conspecifics (Spence & Smith, 2006; Oliveira, 2013; Madeira & Oliveira, 2017) while larvae as young as 6 days post-fertilisation (dpf) can discriminate between kin and non-kin (Hinz *et al.*, 2013).

(1) Sociodynamics

The zebrafish's sociality, capacity for cognitive processing and decision making, and similar brain architecture to that of humans, makes them a powerful model for neuroscience (Kalueff, Echevarria & Stewart, 2014), psychiatric disorders such as schizophrenia (Gawel *et al.*, 2019) and autism (Meshalkina *et al.*, 2018), and the impact of social interaction and isolation on the young, developing brain (Tunbak *et al.*, 2020). But zebrafish social behaviour is plastic and dependent upon their surroundings and the presence, number, age, sex ratio, and relatedness of conspecifics. Even a short period of isolation evokes changes in behaviour and brain activity (Tunbak *et al.*, 2020) yet most behavioural tests are conducted on isolated fish (Pagnussat *et al.*, 2013), in contrast to how zebrafish live in nature. This discrepancy could

Table 3. A representative sample of reports investigating the effects on zebrafish (*Danio rerio*) of various forms of environmental enrichment (EE) to illustrate differences in study design that hinder comparison of results and replication of studies

Enrichment	Rearing environment	Social context		Age of fish	Measures	Results	Study
		Before study	During study				
Gravel, sand, image of gravel or sand, artificial plants (floating and submerged), air stone	Barren tanks	Mixed-sex groups of 10	Pairs and groups of 8	9 months	Preference for enrichments and combinations of enrichments	Pairs preferred substrate over barren tanks. Groups preferred substrates and plants over barren areas; strong preference for gravel and images of gravel; males preferred floating plants to submerged plants; air stones not preferred	Schroeder <i>et al.</i> (2014)
Plastic plants, water flow	3 populations: wild fish from still waters; wild fish from flowing waters; laboratory-reared in bare tanks	Groups of 15–20	Groups of 6	Unreported	Latency to feed, aggression, shoal distance	Plastic plants increased aggression; water flow decreased latency to feed	Bhat, Greulich & Martins (2015)
Plastic plants	Bare tanks	10 fish per 2.5 l tank	Single fish or groups of 5 fish per 2.5 l tank	6 months	Novel-tank, light-dark, and place-preference tests	Single housing in barren tank increased anxiety. Single-housed in barren tanks and group-housed with or without EE spent more time with conspecifics than with artificial plants; single-housed fish with EE showed no preference for conspecifics or artificial plant	Collymore, Tolwani & Rasmussen (2015)
Plastic plants	Bare tanks	5 fish l ⁻¹	Single males; mixed sex pairs	5 months	Survival; cortisol levels	EE prevented deaths from fighting; higher cortisol levels among pairs on day 5 but lower levels by day 10 so no overall reduction in cortisol	Keck <i>et al.</i> (2015)
Sand, artificial plant, artificial rock formation	Barren or enriched housing from hatching	20 fish in bare tank; 24 fish in enriched tank	Single fish	6, 12, and 24 months	Black and white preference test; inhibitory avoidance test; expression of neuroplasticity; gene expression	EE decreased anxiety-like behaviour and increased exploration; reduced inhibitory avoidance in 6- and 12-month-old fish, but not in 24-month-old fish; differences in gene expression at 6 months but not at 24 months; delayed inhibitory avoidance at 24 months compared with 6 months.	Manuel <i>et al.</i> (2015)

(Continues)

Table 3. (Cont.)

Enrichment	Rearing environment	Social context		Age of fish	Measures	Results	Study
		Before study	During study				
Plastic grass, plastic leaves	Bare tanks	Groups of 30	Pairs	90–180 dpf; 0–6 dpf	Fertility, fecundity, survivorship	More eggs spawned on plastic grass than on plastic leaves; no effect on fry survivorship	Wafer <i>et al.</i> (2016)
Flowing water, plastic plants	Wild-caught fish held in bare tanks for 3 months prior to study	Groups of 6 (3 males, 3 females) for 1 month prior to study	Groups of 6 (3 males, 3 females)	Unreported	Shoal cohesion, aggression, activity level	Flowing water resulted in less cohesive, more aggressive, more active groups. Effect not exaggerated by turbulence. Fish more active and aggressive in complex tank.	Suriyampola <i>et al.</i> (2017)
Auditory enrichment (classical music)	Unreported	Unreported	Groups of 3 in 3 l tanks	1 year	Novel tank and light–dark tests; whole-body cortisol; gene expression	Fish with EE were less anxious, less active; decreased levels of pro-inflammatory cytokines and increased activity of some CNS genes; no effect on whole-body cortisol	Barcellos <i>et al.</i> (2018)
Gravel, plastic object, plastic plants	Unknown; fish obtained from pet shop	Group of 96 in 16 l tank for 15 days prior to study	Single fish	Adults	Effects of unpredictable chronic stress	EE reduced effects of stress on behaviour and cortisol, and prevented effects on reactive oxygen species levels	Marcon <i>et al.</i> (2018)
Seascape backing paper, artificial plant, plant pot	Bare tanks	Not reported	Groups of 10 (5 males, 5 females)	Adults	Aggression, fertilisation success, growth	EE increased aggression over time; EE fish were shorter; no effect on mass or fertilisation success	Woodward <i>et al.</i> (2019)
Above-tank shade; plastic plants	Tanks enriched with gravel and plants	Mixed-sex groups of 36 in 54 l tanks	Unsexed groups of 3 from same housing tank	Adults	Preference	No preference for shade or plants	Jones <i>et al.</i> (2019)
Plastic plants, sand substrate, flowing water	Unknown; fish obtained from pet shop	Groups of 8 (4 males, 4 females) for 2 weeks prior to study	Groups of 8 (4 males, 4 females)	12 months	Preference	Fish preferred EE plus flowing water, avoided flowing water only and plain zones, EE plus exercise more important than either factor alone	DePasquale <i>et al.</i> (2019)
Plastic plants, marbles, mesh strips, PVC pipe, various images, mirrored paper, sight of conspecifics	Not reported	Group housed at density of 5 fish l ⁻¹	Single fish	6–12 months	Preference	Fish preferred mirrored paper and sight of conspecifics	Krueger <i>et al.</i> (2020)

CNS, central nervous system; dpf, days post-fertilisation; PVC, polyvinyl chloride.

affect experimental results and lead to flawed conclusions or reproducibility problems.

The limited space in laboratory tanks restricts behaviours such as courtship, aggression and territoriality (Hutter *et al.*, 2010), and prevents the fission and fusion dynamics seen in wild shoals. A typical laboratory tank lacks spawning sites and places to hide from aggressive conspecifics. Lack of variability in the age and size of fish in the tank restricts an individual's choice of shoal mates (Wright *et al.*, 2006b). In addition, inadvertent and natural selection on laboratory fish may change their social behaviour compared to wild fish, decreasing the stimulus required to provoke agonistic responses and so increasing aggression (Ruzzante, 1994). Whether such changes matter to science depends on the type of research the fish are used for. An ecologist, for example, might study the richness of natural behaviour and how a species adapts to diverse environments, whereas a neuroscientist may concentrate on a single aspect of behaviour under artificial conditions. In both cases, the brain evolved to generate behaviour in complex natural environments, and behaviours of interest may not be captured under reduced conditions (Orger & de Polavieja, 2017).

In the wild, zebrafish aggregate in shoals, compete for food and spawning sites, cooperate in predator inspection, and reproduce with each other in a dynamic network of social interactions (Nunes *et al.*, 2017; Fig. 1). In natural systems, zebrafish have been recorded in groups of up to 300 individuals (Suriyampola *et al.*, 2015). It is likely that individuals transfer between shoals frequently and that shoal sizes change rapidly, as in other freshwater species (Krause *et al.*, 2000). Simple social decisions drive the movements of individuals and determine which conspecifics an individual encounters (He, Maldonado-Chaparro & Farine, 2019). Factors affecting such decisions include habitat structure and the spatial distribution of resources, predators, mates, and competitors (He *et al.*, 2019). These factors are dynamic and create temporal variation in the social structure of wild populations (He *et al.*, 2019).

Zebrafish are usually reared in social groups, but may then be housed in pairs for breeding or individually for behavioural tests or for procedures such as genotyping. Stress levels, as evidenced by water-borne and whole body cortisol levels, are higher in fish housed singly compared to fish housed in groups (White *et al.*, 2017), with isolated individuals taking longer to recover from stressful events, probably due to increased base-level stress caused by social isolation (White *et al.*, 2017). Pair-housed fish show an even greater response to stressors, possibly because of the additional strain imposed by dominance or subservience in the housing situation (White *et al.*, 2017). Overall, zebrafish are less stressed when housed in groups, suggesting that social housing may hasten recovery from stressors and enhance welfare and should be considered in experimental and housing design.

(2) Sex

Sex ratio in experimental zebrafish is often unreported, possibly because its ascertainment from external morphology is

subjective and requires expertise. But there is growing evidence that zebrafish behaviour and responses to pharmacological treatments and pollutants are influenced by sex (Genario *et al.*, 2020a). For example, when a novel tank test was used to measure behavioural reactions to two common anxiolytic drugs, both sexes responded to melatonin, but only males responded to diazepam (Genario *et al.*, 2020b). Sex is therefore an important variable. Improved data reporting, including methods used to determine the sex of fish, could increase the replicability and reproducibility of experiments performed with zebrafish. The traditional method of determining the sex of zebrafish relies on visually identifying differences in colour, shape and behaviour between the sexes (Paull *et al.*, 2008) and is highly subjective. Other methods include examination of the pectoral fins to reveal the presence or absence of breeding tubercle clusters in male fish (Dai *et al.*, 2021); microscopic examination of dissected gonad tissue (Abozaid, Wessels & Hörstgen-Schwark, 2011), which requires the fish to be killed; and machine learning strategies to identify the sex of an individual accurately based on body colour and pattern and caudal fin colour (Hosseini *et al.*, 2019). Recent studies have looked for sex-specific genetic markers in zebrafish, similar to those found in roach (*Rutilus rutilus*) (Lange *et al.*, 2020), that will allow non-destructive polymerase chain reaction (PCR) determination of sex from skin swabs or fin clips and improve sex determination in juvenile zebrafish (King, Gut & Zenker, 2020). However, given that genetic sex determination in zebrafish is dependent upon the fish line, and some lines have lost the sex chromosome, it is possible that a sex-specific marker may not be attainable for some zebrafish lines.

Male and female zebrafish differ in their motivation, preferences and behaviour, driven by the potential fitness consequences of being male or female (Magurran & Garcia, 2000). Studies investigating female and male preferences for shoal size and for members of the opposite sex have not provided any consensus in outcomes (Delaney *et al.*, 2002; Pylon, 2003; Ruhl & McRobert, 2005; Spence & Smith, 2006; Hutter *et al.*, 2010; Paull *et al.*, 2010), likely due to differences in study design. For example, experimental tank sizes ranged from 17 l to 1100 l; some tanks were furnished with plastic plants, others were not; study length varied from 10 min to 5 days; and focal fish were tagged in one experiment.

Sex ratios are highly variable in zebrafish (Wilson *et al.*, 2014). The ratio in wild populations appears to be 1:1 (Spence *et al.*, 2007), but stressful environmental conditions, increased temperature and hypoxia may alter the ratio in favour of males (Shang, Yu & Wu, 2006). In research facilities, there is evidence that rearing zebrafish at high densities increases the number of males in the population (Ribas *et al.*, 2017) and that male-bias increases aggression (Spence & Smith, 2005) with associated welfare implications, so most laboratories aim for a 1:1 ratio for long-term maintenance and for practical reasons such as spawning requirements. Female bias can result in females becoming egg-bound as the oviduct becomes blocked with degenerating eggs (Stevens *et al.*, 2021), and females held in single-sex

groups prior to spawning have been found to produce fewer eggs (Osborne *et al.*, 2016). For this information to be of practical use, further studies are needed to determine (i) how sex ratio mediates breeding strategy, reproductive output and social hierarchies, (ii) how these factors are affected by laboratory housing and experimental design, and (iii) how they interact to influence welfare and affect research results.

(3) Kinship

There is growing interest in using zebrafish to study a diverse range of social interactions, including altruism, group-living, and self-organisation (Robinson *et al.*, 2019). The concept of kinship is central to understanding the evolution of social behaviours and new ways to estimate kinship in natural and domestic animal populations (Goudet, Kay & Weir, 2018) will be useful for understanding how zebrafish compete with conspecifics for access to resources.

Shoaling with kin can reduce competition among relatives, increase stability of dominance hierarchies, and improve foraging performance (Frommen & Bakker, 2004). In addition, females may use kin recognition to avoid mating with related males (Gerlach & Lysiak, 2006). Little is known about the social structure or genetic relatedness of wild zebrafish, due to the practical difficulties of such studies, and so kin-biased behaviour of wild fish is inferred from laboratory studies. Zebrafish represent one of the best-documented examples of kin recognition in vertebrates (Gerlach & Wullimann, 2021). There is strong evidence that zebrafish prefer to associate with kin, and do so by using olfactory and visual cues that develop by imprinting at 6 dpf (Hinze *et al.*, 2013). As fish mature, their preference for kin changes: adult females prefer unrelated males while adult males show no preference for related or unrelated females (Gerlach & Wullimann, 2021).

Larvae reared in kin groups have been shown to grow significantly faster than larvae reared with non-kin (Gerlach *et al.*, 2007). In that study, similar shoaling behaviour and rates of aggression were observed in both groups, and the authors suggested that the difference in weight gain was due to increased stress levels in the larvae housed with non-kin (Gerlach *et al.*, 2007). Given that young zebrafish prefer to associate with kin (Gerlach & Lysiak, 2006) and have reduced growth (a proven indicator of stress in fish) when housed with non-kin (Gerlach *et al.*, 2007), the welfare of juvenile zebrafish might be improved by housing them in kin groups until sexual maturity, which occurs from 2 to 4 months depending on husbandry conditions.

(4) Stocking density

Zebrafish stocking density should be considered when interpreting study results. For example, intermittent crowding of adult zebrafish has been shown to increase cortisol secretion and reduce their capacity to renew heart tissue after injury (Sallin & Jaźwińska, 2016), with ramifications for studies of stress responses and tissue regeneration. Temporary crowding has been reported to improve zebrafish working memory (Fontana *et al.*, 2021) with

implications for neurobiology, spatial learning and memory research. Further, crowding embryos may lead to hypoxia, which has been shown to result in a higher percentage of malformations and a male-biased F1 generation (Wu, 2009).

Recommended stocking densities from research studies range from 4 to 10 fish per litre (Lawrence & Mason, 2012; Alestrom *et al.*, 2019; Cockington, 2020) while manufacturers of zebrafish housing systems recommend 6–15 fish per litre (Castranova *et al.*, 2011). Respondents to an international survey of husbandry practices in research laboratories revealed that stocking densities in their establishments range from <1 to >5 fish per litre (Lidster *et al.*, 2017). Density has been found to affect various aspects of physiology and behaviour in laboratory zebrafish and low as well as high stocking density can have a negative effect on fish welfare (Turnbull *et al.*, 2005). Increased density has been shown to reduce survival and growth (Ribas *et al.*, 2017) and egg batch size (Spence & Smith, 2005) but not in all cases (Castranova *et al.*, 2011). Stress levels have been shown to be elevated by both high and low stocking densities (Spagnoli, Lawrence & Kent, 2016). Furthermore, high stocking density causes invasion of individuals' space and a reduction in the duration of sleep, with negative implications for welfare (Ali & Nicholson, 2018). Changes in aggression, breeding behaviour and foraging behaviour have been linked with stocking density. Aggression increases when zebrafish are held in small groups of six or less (Paull *et al.*, 2010). Density also interacts with tank size and availability of spawning sites to affect courtship and breeding behaviour (Spence & Smith, 2005; Hutter *et al.*, 2010). Density too affects foraging behaviour by reducing the effectiveness of aggression in excluding competitors from food patches (Gillis & Kramer, 1987). Increased density furthermore creates practical welfare issues. At high densities, it becomes increasingly difficult to observe and manage the health of fish or to remove sick fish. Further investigations into optimum stocking densities are needed to define the components of stocking density and social hierarchy that affect welfare, provide insight for researchers, and guide improvements to welfare.

V FUTURE DIRECTIONS

Past and present drivers for housing laboratory fish are maximising growth and reproduction in a small space. Except for water quality and diet, other factors that may affect welfare have been more of an afterthought rather than a major consideration in tank and facility design. As we learn more about husbandry-related effects on science, welfare is set to become more integral to research needs. We have optimised the production of zebrafish from eggs to breeding adults in the least amount of time and managed our fish to produce as many eggs as possible, but we have not engaged with aspects of welfare to help inform, for example, tank and facility design to best fit scientific needs.

The use of zebrafish as a research model to study human disease, develop new therapies, understand environmental

health and assess the safety of chemicals continues to grow (<https://apps.webofknowledge.com>) with additional fields of research, such as neuroscience, behavioural science, and epigenetics, also now widely employing embryonic, larval and adult zebrafish as study models. Research questions using zebrafish include those requiring accurate measurements of subtle and sensitive endpoints which are likely to be influenced by the housing and social environments in which experimental zebrafish are produced and maintained. Challenges for future research to help ensure consistent, high-quality experimental research with zebrafish requires that they are maintained under the best possible practicable conditions for their welfare and this in turn will help reduce the numbers of animals required for any given purpose as experimental repeatability will inevitably be improved.

VI CONCLUSIONS

- (1) Good animal welfare is widely accepted as being important for high-quality science, but understanding and evaluating the welfare of zebrafish is challenging. Legislation and guidelines for their care differ from country to country, and there is no single litmus-test measure of fish welfare. Indeed, in some cases it is unclear whether a particular measure is reliable.
- (2) To promote welfare for zebrafish, it is essential that housing and social conditions are tailored to the needs of the species and that features of its life history and ecology are considered when designing housing systems and interpreting study results.
- (3) This review identifies, in particular, the need for more fundamental knowledge of how zebrafish interact with the biotic and abiotic features of their natural environment and which of these features are most important for their welfare.
- (4) More focus is needed on what captive zebrafish need by way of housing and social interaction to ensure they are physiologically and behaviourally ‘best suited’ for the research they are purposed for. Improving zebrafish welfare is not a passive process. It needs to be continuously assessed, revised and implemented, while taking into account the competing priorities of researchers and animal care staff.
- (5) As a research community using zebrafish as an experimental model we also have a duty to increase our knowledge and understanding of welfare to enable evidence-based decisions on how best to improve the quality of life for laboratory zebrafish.

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