EXPLORATORY BEHAVIOUR IN LABORATORY ZEBRAFISH: POTENTIAL BENEFITS OF EXPLORING THE UNKNOWN

by

Courtney Graham

B.Sc., The University of Victoria, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Applied Animal Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2017

Abstract

Zebrafish are one of the most used animals in scientific research. They are typically housed in barren conditions that greatly differ from their vegetated and fluctuating wild habitats. This disconnect has received increasing attention in recent years, particularly concerning the physical environment and the necessity of environmental enrichment. However, research investigating the psychological needs of zebrafish—a highly cognizant animal—is in its infancy. One method of addressing this gap is to investigate the use of cognitive enrichment—that is, providing a captive animal with appropriate cognitive challenge in effort to improve welfare. Therefore, the aim of this thesis was to assess the role of allowing laboratory zebrafish to engage in exploration on their behavioural responses. I provided zebrafish with an opportunity to explore novel tank space by offering them access to a previously inaccessible portion of their semi-natural home tanks, within which they had been housed for nine months. I examined their exploratory behaviour (latency and number of inspections of the novel space), anxiety behaviour (bottom-dwelling) and social behaviour (agonistic behaviour, cohesion and coordination) on the day before (baseline), the day of, the day after and two weeks after providing access to the novel area. Zebrafish were found to quickly move into the new space (on average within 9.7±7.6 seconds; mean±SD) and sustained their interest on each of the observation days ($P<0.003$). I found no evidence of bottom-dwelling ($P>0.73$), indicating that the exploration opportunity was likely not anxiety-provoking. Further, the opportunity to explore increased positive affiliative social behaviour: reducing agonistic behaviour ($P=0.02$), and increasing both cohesion ($P=0.04$) and coordination ($P=0.04$) relative to baseline. Considering their natural habitats would normally include such information-gain opportunities, I suggest the use of barren and stagnant laboratory conditions compromises the behavioural and psychological needs of zebrafish and reduces welfare. This thesis adds to the growing body of literature focusing on the role cognitive stimulation may play in welfare and indicates that zebrafish are good candidates for further cognitive enrichment research.

Preface

All research, husbandry and experimental procedures in this thesis were approved by the University of British Columbia's Animal Care Committee (protocol number: A14-0119).

C. Graham and Dr. B. Franks created the study and, with Dr. M.A.G. von Keyserlingk, designed the experimental procedure. C. Graham collected the data; undergraduate laboratory member C. Powell scored data for reliability. C. Graham and B. Franks conducted the statistical analyses. C. Graham prepared the manuscript. B. Franks and M.A.G. von Keyserlingk supervised and provided feedback on the manuscript.

A version of Chapter 1 has been submitted for publication: C. Graham, M.A.G. von Keyserlingk, B. Franks. 2017. Zebrafish welfare: natural history, social motivation and behaviour.

A version of Chapter 2 has been submitted for publication: C. Graham, M.A.G. von Keyserlingk, B. Franks. 2017. Free-choice exploration in laboratory zebrafish.

Table of Contents

List of Tables

List of Figures

Acknowledgements

This project would not have been possible without the ideas and expertise of Dr. Becca Franks, my mentor to which I owe more gratitude than I could ever express in words. She took a chance on me and allowed me to flourish under her wing, always providing inspiration, encouragement and endless support. Her passion for fish is intoxicating and her words of advice never failed (no matter how many questions I asked). I'm truly the lucky one to have been given the opportunity to learn from her.

I owe immeasurable thanks to my supervisor, Dr. Nina von Keyserlingk, who has provided unfailing guidance, support and freedom to conduct this project. She challenged me to grow and develop my skills as a graduate student and I'm so fortunate to have had the chance to learn under her renowned expertise.

I am also grateful to my esteemed supervisory committee members, Drs. David Fraser and Daniel Pauly, who provided thoughtful insight and knowledgeable support at various stages. I am thankful to the UBC Animal Welfare Program for allowing this type of work to be done and to its students, professors and staff who provide a support group unlike I've ever known. My experience as a graduate student would not have been the same without this stimulating group of people I'm honoured to call not only colleagues, but also friends.

I am also extremely grateful for the student and volunteer members of the zebrafish welfare laboratory; their commitment to the lab was inspirational. I also want to acknowledge the researchers and veterinarians of the UBC Aquatics Facility for their guidance and assistance with the lab set up and maintenance.

To my friends outside of school, I am so thankful to have them by my side (if not in body, always in spirit). When I needed a break, they were there to help me relax with a laugh or a beer.

And to my parents and family, I indisputably would not be standing where I am without them. They have been the upmost example of unconditional love and support and have instilled in me compassion and moral values for which I will be forever grateful. I love you from the bottom of my heart.

And, finally, to the zebrafish, who revealed to me both their subtlety and complexity. They have an unenviable place is science and truly deserve better.

Chapter 1: Introduction[1]

1.1 Zebrafish in science

In Canada, fish are one of the most used animals in scientific research and, of the fish species studied, zebrafish (*Danio rerio*) are one of the most popular (CCAC, 2015). They are used, for example, in the study of genetics, developmental biology, neurophysiology and biomedicine, and are common models for investigating complex brain diseases in humans, including autism and other social disorders (Gerlai, 2012; Spence et al., 2008). Zebrafish have a number of attributes that make them desirable for use in scientific research, including their robust and social nature, high year-round reproductivity, fast growth rates, transparent embryos and established genome (Lawrence, 2007; Spence et al., 2008). Also used in the study of animal pain (Correia et al., 2011), zebrafish have recently been proposed to replace rodents as the ideal model species to examine pain in vertebrates (Steenbergen and Bardine, 2014). Given the breadth to which they are used in science, these small freshwater fish are subjected to a wide range of laboratory conditions and experimental procedures.

There are millions of zebrafish in laboratories worldwide (Reed and Jennings, 2011), yet the question of their individual welfare is rarely, if ever, asked. Recently, better understanding of the link between animal welfare and scientific validity has highlighted concerns for the physical environment and ability to recognize abnormal behaviour in laboratory animals, particularly rodents (Bayne and Würbel, 2014; Garner, 2005; Sherwin, 2004). The knowledge of these concerns for zebrafish is diminutive in comparison. To improve the welfare of zebrafish used in science, we need to ask the difficult questions of how to infer and assess their species-specific welfare. Improving our understanding is an important first step towards enhancing the welfare of the countless individuals used in research and will also improve the validity of the results obtained from zebrafish studies and

---

[1] A version of Chapter 1 has been submitted for publication: C. Graham, M.A.G. von Keyserlingk, B. Franks. 2017. Zebrafish welfare: natural history, social motivation and behaviour.

allow for higher relevance for comparisons to other species, including humans (Miller and Gerlai, 2011a).

1.1.1 Current scientific standards

Zebrafish are relatively easy to maintain in simple conditions and can persist in a range of water quality parameters (Spence et al., 2008). However, the current standard for laboratory housing for zebrafish typically consists of small, barren and often overstocked tanks (Harper and Lawrence, 2011; Kistler et al., 2011; Matthews et al., 2002; Reed and Jennings, 2011), which is a drastic contrast from their vegetated and fluctuating natural habitats (Engeszer et al., 2007b; Spence et al., 2006). Tank wall colour is often transparent, providing no cover, or white, a colour known to elicit anxiety behaviour in zebrafish, such as freezing or bottom-dwelling (Blaser et al., 2010; Blaser and Rosemberg, 2012; Serra et al., 1999). These conditions are comparable to the 'shoebox cage' commonly used for housing laboratory rodents and appear to be a consequence of economic factors rather than biological, scientific or animal-based ones (Williams et al., 2009; Würbel, 2002).

The majority of zebrafish studies house or test fish within these 'standard' tanks; however, such inadequate conditions may provide an inaccurate representation of what constitutes 'normal' baseline behaviour—a discrepancy that reduces scientific validity and justification of the research (Balcombe, 2006; Garner, 2005; Sherwin, 2004; Würbel, 2002). The divergence in conditions has the potential to induce behavioural abnormalities and frustration from behavioural deprivation and, ultimately, results in reduced welfare (Dawkins, 1988; Mason and Burn, 2011; Olsson and Dahlborn, 2002).

1.2 Fish welfare

The term welfare is used in science to refer to the state of an animal—a characteristic *of* the animal, and not something that is given *to* it (Broom, 1996). Assessing animal welfare is generally considered through the asking of three questions: (1) is the animal healthy and functioning well?, (2) is the animal feeling well (e.g., is there freedom from negative emotions and the opportunity to experience positive emotions)? and, (3) is the animal able to live a reasonably natural life (e.g., able to perform natural behaviours that they are highly motivated to engage in)? (Fraser et al., 1997).

The study of animal emotions (also called affective states)—used to answer the question of how an animal is feeling—has received much attention in the last decade (e.g., Paul et al., 2005), and concern for animal health has long been of interest in the scientific community (Fraser et al., 1997). The study of natural behaviour, particularly for zebrafish, has received less attention arguably because it is the least clear academically. Given that little is known about zebrafish behaviour in the wild, more knowledge is needed to better identify behavioural discrepancies and to promote the display of natural behaviour in captivity.

The ability to live a reasonably natural life is key to sentient beings that can perceive and be affected by their surroundings, a group in which fish are arguably included (Broom, 2007). In general, moral consideration is rarely afforded to fish, as our assessment of welfare depends on our perception of an animals' intelligence or sentience (Kirkwood and Hubrecht, 2001). Although the debate on fish sentience persists (e.g., Rose et al., 2014), evidence in support of fish sentience, intelligence and the capacity to feel pain is growing (Braithwaite and Boulcott, 2007; Brown, 2015; Huntingford et al., 2006; Sneddon, 2015, 2006, 2003) and deserves attention.

1.2.1 Cognitive ability, sentience and capacity to feel pain

Sentience is described as having the capacity to feel and involves the awareness and cognitive ability to do so (Broom, 2014). Many studies have been conducted highlighting the capacity of zebrafish to learn and testing their cognitive abilities. For example, zebrafish are able to learn to associate different coloured arms of a T-maze with a food reward, learn to reverse their choice when the food reward is changed to the opposite colour, and remember the correct colour for at least 24 hours (Colwill et al., 2005). Zebrafish have been shown to learn to use a tunnel maze to access a group of conspecifics (Gómez-Laplaza and Gerlai, 2010) and learn, in as little as one week, the correct chamber in a food-rewarded colour chamber choice test (Mueller and Neuhauss, 2012). Zebrafish are also able to learn a spatial alteration task when a food reward is delivered on alternating sides of a test tank and recall the pattern after a period of 10 days (Williams et al., 2002). Further, they can use knowledge socially transmitted from conditioned shoalmates about the location of a food reward (Zala and Maattanen, 2013) and an escape route (Lindeyer and Reader, 2010). These examples highlight the adept cognitive abilities of zebrafish and thus also underline their eligibility for welfare consideration (Broom, 2014).

Additional evidence of consciousness that has been described for mammals, birds and reptiles is the ability to exhibit psychological stress-induced hyperthermia, or emotional fever, quantified by a temporary rise in body temperature in response to a psychological stressor (Cabanac et al., 2009). This phenomena is thought to result from psychological processing and assessment of a stimulus through some level of conscious thought and appraisal (Bouwknecht et al., 2007). The first evidence that zebrafish are also susceptible to emotional fever was in response to a psychological stressor where the body temperature of zebrafish increased 2 to 4°C following what was initially thought to be a biologically harmless confinement period (Rey et al., 2015). Interestingly, in this study the zebrafish took up to eight hours to return to normal thermal conditions, suggesting confinement may be a particularly intense stressor for this species.

In addition to cognitive abilities and consciousness, there is a growing body of evidence showing that fish have the capacity to feel pain. Despite some critics (e.g., Rose et al., 2014) arguing that the absence of a neocortex prevents fish from experiencing pain, it has been established that fish indeed can perceive pain (i.e., nociception) (reviewed by Sneddon, 2006). Fish have analogous brain areas that are activated in response to noxious stimuli and whose function is to learn from and control emotional reactions (reviewed by Broom, 2016); moreover, analgesics reduce the symptoms and pain-related behaviours (Sneddon, 2003). Available evidence indicates that fish will display behavioural and physiological responses to pain, such as abnormal behaviour and elevated ventilation or respiration, will show avoidance behaviour, and will seek relief of pain from analgesics (Sneddon, 2015). For example, zebrafish injected with acid in their tails as a noxious stimulus display abnormal tail wafting and avoidance behaviour (Maximino, 2011). Furthermore, when housed in an environment with the choice of a barren or enriched condition, zebrafish exposed to painful acid will override their preference for enrichment and self-administer analgesics offered only on the barren side to ease the painful experience (Sneddon, 2015). Detailed reviews describing pain in fish (e.g., Braithwaite, 2010; Chandroo et al., 2004; Sneddon, 2015, 2006, 2003) provide robust evidence that sensory pain is likely to occur in fish, that there is the possibility for psychological suffering, that behavioural responses are prolonged in relation to stimulus intensity, and that these responses are not automatic reflexes but are instead the result of conscious experience.

1.2.2 Natural living and welfare

The ability to live a reasonably natural life is a central component to the assessment of welfare (Fraser et al., 1997). In reference to farm animals, Špinka (2006) suggested that allowing animals to behave freely in an environment featuring important natural elements will aid in achieving several welfare goals. First, by allowing animals to behave naturally, they can achieve good biological functioning and in turn meet the goals of the farmer, such as high production rates—or in the case of laboratory zebrafish, meet the needs of the scientist by producing reliable and robust data. Second, the exhibition of natural behaviour patterns is often associated with positive affective states, namely the capacity to experience positive emotions, an aspect that may directly enhance the animals' quality of life. Finally, the ability to engage in natural behaviour arguably allows for a rich and complex life that could provide long-term benefits, including improved aptitude in coping with social and physical challenges. From this perspective, natural behaviour is possibly the most essential component necessary for good welfare to be achieved.

Many research animals are a consequence of generations of captive breeding; thus, the degree to which 'naturalness' is required in their environment is frequently questioned (Newberry, 1995). If the behavioural and physiological needs of domesticated animals are met in captivity, they can adapt better; but adaptation does not necessarily imply good welfare (Broom, 2014). Even highly domesticated animals display a preserved behavioural repertoire shaped by their wild ancestors and will exhibit these behaviours when given the chance (e.g., Stolba and Wood-Gush, 1989), and thus should be given precautionary consideration (Bekoff, 2007; Sneddon et al., 2014). For example, small groups of domestic pigs housed in diverse semi-natural habitats exhibited behaviours described as identical to their native ancestor, the wild boar (Stolba and Wood-Gush, 1989). Further, when released into the wild, laboratory rats bred for hundreds of generations in captivity immediately adapted to a natural lifestyle by seeking shelter, food and water, avoiding predators and establishing social groups (Berdoy, 2002). In addition, recent work has shown that even in captivity, laboratory rats will readily and frequently burrow in soil substrate when given the opportunity to do so (Makowska and Weary, 2016). These examples underline the need to fully understand an animal's evolutionary history when considering the design of laboratory housing, as this information will provide valuable insights regarding features and behaviours that may be critical for good welfare. Ideally, captive housing designs would allow for the display of normal behaviour (Mayer, 2004), but first, we must understand what that behaviour looks like under naturalistic conditions.

Natural behaviour is an important indicator for welfare because it can be used to assess health (e.g., disease, injury) and an animal's motivation (or preference) for certain resources or environments (Dawkins, 2004, 2003). It can be used to non-invasively investigate the extent of an animal's behavioural repertoire without husbandry or experimental interruptions and will aid in identifying which elements of the wild habitat might help fulfill an animal's behavioural needs (Stolba and Wood-Gush, 1989), thereby, highlighting the animal's behavioural integrity (Würbel, 2009).

To be useful as a baseline for assessing welfare, natural behaviour must be well defined (Bracke and Hopster, 2006). We must also have a good understanding of what may be required for the behaviour's performance (e.g., environmental or spatial needs), consequences of its prohibition (e.g., welfare or health concerns) and to what extent the environment meets the behavioural needs (Bracke and Hopster, 2006). Allowing or promoting animals to perform natural behaviour patterns that are associated with a positive emotional state undoubtedly improves their welfare and aids the development of skills needed to lead a satisfying life (Špinka, 2006). It also allows for better identification of discrepancies from normal (i.e., abnormal or stereotypic behaviour), and produces less variability and more robust and valid data (Garner, 2005; Pagnussat et al., 2013; Sherwin, 2004).

As the environments of wild zebrafish are often vegetated and fluctuate with the monsoon seasons (Engeszer et al., 2007b), like many animals (Berlyne, 1960), inhabitants can continually explore and search for information about their surroundings, for example, in efforts to find new sources of food or mates, conduct patrolling to maintain familiarity, or even to seek out novelty.

Knowledge of what exploration may look like in zebrafish, or of what stimuli they may prefer to explore, is essentially absent in the scientific literature, especially under natural or semi-natural conditions. Further, discovering which dimensions of natural living produce the greatest changes in baseline behaviour can help animal welfare scientists and managers determine which features are most likely to improve welfare in captive settings (Fraser, 2008; Stolba and Wood-Gush, 1989).

1.3 Zebrafish social behaviour

One characteristic central to zebrafish behaviour is their social conduct. Zebrafish are well known to aggregate in a process known as shoaling, demonstrated by loose aggregation of a group of fish, and are also known to school, when a shoal expresses coordinated movement in the form of synchronized ordered swimming, especially under threat of predation (Delcourt and Poncin, 2012; Pitcher, 1983).

1.3.1 In nature

In the wild, zebrafish are found in various riverine and stream environments on the Indian subcontinent across a wide range of temperatures, pH levels, turbidity, levels of water flow and vegetation cover, as well as in still pools and rice paddies (Engeszer et al., 2007b; Spence et al., 2006). A recent field study reported that wild zebrafish exhibit a range of natural group sizes with differing levels of water flow (Suriyampola et al., 2015). In still water, zebrafish were found in relatively small groups of up to 22 fish. In a slow-flowing river, they were found in even smaller groups (6 to 7 fish), whereas in a fast-flowing river, they were found in very large groups (up to 300 fish). Thus in the wild, zebrafish aggregate in groups and display a wide range of natural group sizes that may depend on various environmental parameters (Suriyampola et al., 2015). Other field studies have documented geographic range, biotic and abiotic habitat features, co-occurring fish species, diets and growth rates of wild zebrafish (Engeszer et al., 2007b; McClure et al., 2006; Spence et al., 2007); however, there is limited knowledge of their natural social behaviour. The sparse knowledge of zebrafish behaviour in the wild—further hindered by the challenges associated with monitoring behaviour in highly turbid water (Spence et al., 2008; Ward et al., 2007)—prevents our understanding of their natural behavioural repertoire thereby limiting our use of a valid control for laboratory investigation (Spence et al., 2008). This lack of knowledge impedes our understanding of the elements of their environment required for optimal welfare and our ability to recognize behavioural modification or deprivation in captivity. The limited information regarding the appropriate natural behavioural baseline is especially concerning given that we house laboratory zebrafish in environmental and social conditions that drastically contrast with their natural history.

1.3.2 In the laboratory

The social behaviour of captive zebrafish, particularly shoaling, has been investigated in many laboratory studies. Reflecting its instinctive nature, experimentally inducing shoaling in zebrafish is relatively simple, for example, by presenting them with conspecifics or even computer animations (Gerlai, 2014). The defining factor of shoal membership is often measured using the distance between individuals within a group (i.e., distance between 'nearest neighbours') (Clark and Evans, 1954; Miller and Gerlai, 2011b). In laboratories, zebrafish social behaviour has been found to range from a shoaling to schooling formation (Miller and Gerlai, 2012) and, interestingly, the group's cohesion (i.e., nearest-neighbour distance) seems to oscillate over short periods of time (Miller and Gerlai, 2008).

Zebrafish have been found to adjust the dimensions of cohesion and group diameter according to the relative amount of available space in their environment depending on density and group size (Shelton et al., 2015). For example, by varying the number of individuals in a group and the size of the testing arena, researchers were able to influence shoal dynamics. Groups of fish in larger tanks spread out more (had greater distances between nearest neighbours) and larger groups (8 fish versus 4) had greater shoal diameters (Shelton et al., 2015). These two measures of social behaviour did not respond in the same way to spatial manipulation tests, emphasizing the importance of addressing not only individual factors (e.g., nearest-neighbour distance) but also group factors (e.g., shoal diameter) when analyzing complex social dynamics (Shelton et al., 2015). Similarly, the closely related giant danios (*Devario aequipinnatus*), when tested against a current, have been found to adjust their group activity depending on water temperature. When tested at high temperatures (28°C), giant danios display less cohesion and higher activity compared to low temperatures (22°C) (Bartolini et al., 2014). This result echoes the finding in wild zebrafish that water flow affects group dynamics (Suriyampola et al., 2015). These examples illustrate the sensitivity of the social behaviour of zebrafish and how different social or physical environments can influence their behavioural conduct.

Behavioural preference can be assessed by offering an animal a choice of, for example, resources or environments and examining the amount of time spent near or within their choice (Fraser and Nicol, 2011). There have been several studies exploring the shoaling preferences of zebrafish for certain visual characteristics when fish are given a choice of options. Experimentally, zebrafish have shown preference, for example, for fish with similar pigment types or for pigmentation similar to those of the fish with which they were raised, such as striped wild-type versus genetically modified stripeless strains of either live or computer-animated shoals (Engeszer et al., 2007a, 2004; Rosenthal and Ryan, 2005). These findings make sense according to theoretical models of the value of shoals: researchers have argued that the reason that shoaling fish in the wild tend to adopt groups of similar visual characteristics is that groups of similar individuals appear more cohesive and can thus confuse potential predators (Landeau and Terborgh, 1986). Zebrafish have also shown preference for larger group sizes (Pritchard et al., 2001) and for well-fed groups (Krause et al., 1999). Further, males prefer female groups over male groups, but show no preference when given the choice of mixed sex groups; females show no preference for sex, but for the larger group of their experimental choices (Ruhl and McRobert, 2005).

Taken together, this evidence suggests that zebrafish can decipher and actively choose between subtle (e.g., nutritional state) or explicit (e.g., pigment pattern) differences in conspecifics and that we have some knowledge of group preferences shown by zebrafish in the laboratory. However, there is considerable diversity in their social preferences and induction of shoaling behaviour. Most concerning is that these studies were primarily conducted in standard barren laboratory tanks or tested fish in social isolation, with the exception of a few that used gravel substrate as an enrichment feature. Thus, while these studies provide evidence that zebrafish are very social and environmentally responsive animals, there is risk that the findings arising from experiments that use zebrafish housed in barren conditions are unreproducible and unreliable, due to, for example, animals demonstrating more variable behaviour or heightened anxiety (Kistler et al., 2011; Richter et al., 2009; Sherwin, 2004). These issues highlight the need for research under more naturalistic conditions that include features of the physically and cognitively stimulating environments of wild zebrafish.

1.4 Exploration

Though difficult to define precisely, in its broadest sense, exploratory behaviour refers to any activity that involves the gathering of information about the environment (Mills et al., 2010). A number of classic distinctions of types of exploratory behaviour were made popular in the mid-1900s by Berlyne (1960) and are still used today. Obtaining information about conventional resources, such as food or nesting sites, is characterized as extrinsic (external) exploration, while intrinsic (internal) exploration is directed at stimuli that may seemingly have no biological significance, such as investigating a novel object (Archer and Birke, 1983; Berlyne, 1960). There is also distinction between specific exploration, that is behaviour directed at an explicit stimulus object or event, and diversive exploration, when behaviour is directed at obtaining stimulus change and information from any environmental source (Berlyne, 1960; Hughes, 1997). The motivation for specific exploration has been linked to curiosity, whereas diversive exploration has been linked to boredom due to a general lack of stimulation (Mills et al., 2010).

Berlyne (1960) also distinguished between categories of behavioural response to exploration. These include orienting responses (in which the animal changes posture or other sense state; e.g., turning to gaze), locomotor exploration (in which the animal adjusts its body positioning), and investigatory responses (in which the animal manipulates or alters the stimulus). Stimulus intensity, novelty, surprise, complexity, incongruity and conflict all play a role in determining the response of the animal and, ultimately, the motivation for exploration (Berlyne, 1960). In addition to Berlyne's renowned work, there have been other influential reviews of exploratory behaviour (e.g., Archer and Birke, 1983; Fowler, 1965; Russell, 1983) as well as of the use of cognitive stimulation to activate the exploratory response of captive animals (e.g., Meehan and Mench, 2007). Exploration is a driving principle for many other behaviours necessary for survival, such as food acquisition and reproduction (Fowler, 1965), is a foundation of intelligent behaviour (Barnett and Cowan, 1976), and has been found, in and of itself, to be rewarding for animals (e.g, Franks et al., 2013; Wood-Gush and Vestergaard, 1989). Still, how exploration might influence welfare—for example, is the motivation to explore driven by curiosity or boredom?—is an important question to ask, especially for animals in captivity (Wood-Gush and Vestergaard, 1989).

1.4.1 Cognitive enrichment

Providing animals in captivity with opportunities for cognitive stimulation or engagement with the environment allows control over behavioural outcomes and promotes successful coping of environmental demands (Broom, 2014; Franks and Higgins, 2012; Meehan and Mench, 2007). In order to be an effective enriching experience for the animals, cognitive stimulation or challenge must match the skills of the animal in question (Franks and Higgins, 2012; Meehan and Mench, 2007)—too low challenge can lead to boredom or anhedonia (Fureix and Meagher, 2015; Meagher and Mason, 2012), while too high challenge can lead to frustration or even learned helplessness (Maier and Seligman, 1976). An early investigation into the use of cognitive stimulation as enrichment examined responses of rhesus macaques provided with a complex puzzle apparatus and found that they actively engaged with the puzzle (Harlow, 1950). However, over time the macaques became satiated to the challenge and their interest waned. Therefore, tasks that require ongoing learning and that vary in the degree of challenge are required to minimize habituation, maintain the animals' interest and achieve enduring welfare benefits; but implementation of these types of tasks may also pose difficulties for animal managers and caretakers (Manteuffel et al., 2009; Meehan and Mench, 2007). Other examples of successful cognitive enrichment include learning of auditory cues to complete feeding challenges (e.g., pigs: Puppe et al., 2007; Zebunke et al., 2013) and positive reinforcement training (e.g., marine mammals: Clark, 2013). Additionally, environmental variation early in life has been shown to promote greater cognitive ability later in life. For example, cichlid fish that were offered a varied food ration as juveniles outperformed those fish that were kept on a constant diet when tested in a learning task as adults, one year later (Kotrschal and Taborsky, 2010).

The use of cognitive enrichment has been shown to enhance the welfare of captive animals, including positive emotional states from the process of learning (i.e., pleasure) and greater cognitive abilities allowing easier or faster adaptability or superior coping skills (Boissy et al., 2007; Meehan and Mench, 2007). The opposite is also true: an environment that provides unsatisfactory stimulation has been shown to have a number of repercussions for welfare across species, such as behavioural pathologies, increased sensitivity to stress, impaired brain function, lethargy, restriction of normal behavioural repertoire, increased aggression and decreased growth rates or immunity (Dawkins, 1988; Hughes and Duncan, 1988; Kistler et al., 2011; Lawrence, 2007; Olsson et al., 2011; Reinhardt, 2004; Salvanes et al., 2007).

As the behaviour of zebrafish has been found to be influenced by their environment (Basquill and Grant, 1998; Spence et al., 2008) and because they have high cognitive function, zebrafish may be good candidates for research investigating the benefits of cognitive stimulation as a form of enrichment. Zebrafish have also been shown to have neophilic tendencies, i.e., love of novelty (Lucon-Xiccato and Dadda, 2014), and therefore may benefit from being offered something new to investigate. Having the opportunity to explore may have potential as a form of cognitive enrichment. Furthermore, this type of enrichment has been considered by researchers to be a fundamental motivation in healthy animals given that animals will work for the chance to explore (Franks et al., 2013; Franks and Higgins, 2012). Developing and improving our understanding of beneficial environmental conditions and psychological challenges for zebrafish will aid in discovering behaviours they may be motivated to perform and, ultimately, in achieving good welfare in captivity.

1.5 Divergence of environments

In effort to mitigate some of the negative effects of living in an under-stimulating environment, barren housing conditions can be enhanced by the introduction of structural modifications, often referred to as environmental enrichment (e.g., Williams et al., 2009). For zebrafish, common types of environmental features used as enrichment include substrate (e.g., gravel, sand), plants (e.g., artificial, live) or other features (e.g., pipes, caves) that can be used for hiding (Williams et al., 2009). Currently much of the enrichment work focuses on aspects that provide a more stimulating environment (Weed and Raber, 2005). For example, giving captive animals some control over their environment, such as by providing hiding places to avoid aggressive conflicts, is suggested to be beneficial to their welfare (Matthews et al., 2002; Näslund and Johnsson, 2014; Olsson and Dahlborn, 2002; Reed and Jennings, 2011). Though the term 'enrichment' is intended to mean an improvement, it should be used with care. In some cases, it may be synonymous with simply an increase in complexity rather than a biologically relevant improvement in the well-being of the animal (Newberry, 1995; Wilkes et al., 2012). Further, it has been argued by some that 'enrichment,' as the term is commonly used, is misleading and creates the impression that the environment already meets basic behavioural needs. To be considered beneficial as a means to improve welfare, enrichment features must be above and beyond meeting basic needs and should promote positive states (Duncan and Olsson, 2001; Leach et al., 2000). Understanding what aspects of the environment are important to the animal or what features would be found in their natural habitat is key to implementing suitable and beneficial improvements (Näslund and Johnsson, 2014). Several experiments have investigated zebrafish preference for certain environmental features and have reported a wide array of behavioural responses, including reductions in behaviours such as aggression, food monopolization and anxiety-like behaviours, i.e., darting or escape behaviour or reduced exploration (Basquill and Grant, 1998; Collymore et al., 2015; Hamilton and Dill, 2002; Keck et al., 2015), reduced stereotypic behaviour (Kistler et al., 2011), and increases in fecundity (Carfagnini et al., 2009) when zebrafish are housed within enriched compared to barren environments.

1.5.1 Zebrafish preference

When given the choice between barren or enriched conditions, zebrafish show a strong preference for (i.e., spend more time in) environments furnished with plants, clay pots and substrate, over the barren option (Kistler et al., 2011; Schroeder et al., 2014). Further, when given the choice of a range of enrichment features, groups of zebrafish consistently prefer enrichment over a barren choice. Groups of both females and males have been shown to prefer floating plants over barren conditions and females also show preference for submerged plants (Schroeder et al., 2014). When given the choice of substrate (sand or gravel) over barren conditions, groups of zebrafish repeatedly prefer the substrate, but also prefer gravel over sand.

When testing in pairs, however, social status has been reported to play a role in individual preference: dominant fish have been shown to patrol enrichment and restrict subordinates to the barren side of the test tank regardless of subordinate choice, a finding that highlights the importance of considering the effect of social situation (Schroeder et al., 2014). Interestingly, zebrafish prefer images of gravel placed underneath the transparent tank bottoms and occupy the image space almost as much as they do when actual substrate is present, a finding that arguably improves welfare within the captive housing environment (Schroeder et al., 2014).

It has also been suggested that tank wall colour affects behaviour. Zebrafish have been shown to prefer dark environments and, when confined to a white tank, display anxiety-like behaviours, such as freezing or bottom-dwelling (Blaser et al., 2010; Blaser and Rosemberg, 2012; Serra et al., 1999). Similarly, Coho salmon (*Oncorhynchus kisutch*), a popular aquaculture species, have been found to prefer and show markedly reduced aggression in darker tanks compared to lighter-coloured tanks, including the industry-standard light blue (Gaffney et al., 2016). Most experiments exploring zebrafish behaviour use white walls to enhance video tracking of the location and movement of individuals (e.g., Miller and Gerlai, 2012), but using environments that are aversive to zebrafish could have a confounding impact on scientific results.

Additional benefits of housing or rearing zebrafish in enriched environments include, for example, greater brain cell proliferation (von Krogh et al., 2010) and faster learning of a food location (Spence et al., 2011) than their barren-kept counterparts, suggesting environment plays an important role in cognitive and emotional processes—from development through adulthood—and potentially improves welfare. These benefits, together with zebrafish preference for enrichment and reduced agonistic behaviour within enriched environments, provide evidence to support the use of environmental enrichment in captivity. Unfortunately, despite a substantial body of research supporting this evidence, current laboratory zebrafish housing trends have failed to take this into consideration.

1.5.2 Impact on zebrafish welfare

A first step toward improving the welfare of zebrafish is gaining better knowledge of their behaviour in natural and semi-natural environments. Such information would help us develop a more accurate baseline for the types of behaviour that are characteristic of zebrafish, inform future investigations into how motivated zebrafish are to perform these behaviours, and inspire tests assessing the potential emotional and biological costs associated with housing this popular research animal in environments that restrict or alter their behavioural repertoire. Furthermore, understanding what proportion of a captive animal's behavioural repertoire should be considered normal versus abnormal is an important indicator for assessing welfare (Bracke and Hopster, 2006; Špinka, 2006). Finally, accurate knowledge of natural behaviour can help ensure that laboratory animals are generating valid and robust scientific data (Balcombe, 2006; Garner, 2005; Sherwin, 2004).

There are some established behavioural indicators that are used to assess fish welfare, including changes in foraging, aggressive or shoaling behaviour, or the presence of abnormal behaviour, such as waving (similar to pacing observed in some zoo animals) (Huntingford and Kadri, 2014; Martins et al., 2012). Spatial distribution is another measure that provides a valuable non-invasive method to reveal how animals respond to each other and their environment (Dawkins, 2004). Knowledge of an animal's natural behaviour allows for easier identification of reduced behavioural repertoire or behavioural deprivation (Dawkins, 1988).

In addition to the physical environment, psychological experiences can play an important role in welfare on both ends of the spectrum—by promoting positive emotional states with sufficient cognitive stimulation, or by inducing negative emotional states from a lack of stimulation. Appropriate levels of cognitive challenge promote a greater behavioural range and allow animals to arguably have more control over their environment, but it also requires knowledge of an animal's natural behaviour and cognitive skills (Meehan and Mench, 2007).

1.6 Thesis objectives

Knowledge of zebrafish behaviour within wild or naturalistic environments is limited. The overall aim of this thesis project was to describe the behaviour of small groups of zebrafish living in large semi-natural conditions, furnished with a sloped gravel substrate, rocks and artificial plants that provided hiding and open spaces to mimic their wild habitats.

Cognitive stimulation as a form of enrichment is suggested to promote a greater range of behaviours and, ultimately, enhance welfare (Meehan and Mench, 2007). Therefore, the specific aim of this thesis was to describe the relative changes in zebrafish behaviour following an opportunity to explore, by providing access to a novel space within their home tanks, as a form of cognitive enrichment. This thesis strives to add to the evidence in support of providing more naturalistic environmental conditions and psychological experiences to improve the welfare of laboratory zebrafish.

Chapter 2: Free-choice exploration in laboratory zebrafish[2]

2.1 Introduction

Cognitive stimulation has been shown to be rewarding (e.g., Wood-Gush and Vestergaard, 1989) and capable of eliciting positive emotions in several species (e.g., Ernst et al., 2005; Franks et al., 2013). All natural environments are subject to at least some degree of change over time; ongoing exploration of habitats provides animals benefits in terms of maintaining familiarity (Russell, 1983; Wood-Gush and Vestergaard, 1991) and provides information essential for survival (Berlyne, 1960; Russell, 1983). Also, an animal's tendency to explore and sustain curiosity about their environment is a driving principle for many other behaviours necessary for survival, such as food acquisition and reproduction (Fowler, 1965), and is a foundation of intelligent behaviour (Barnett and Cowan, 1976). Thus, how exploration opportunities (or the lack thereof) might influence an animal's quality of life is an important question to ask when assessing the welfare of animals in captivity (Wood-Gush and Vestergaard, 1989).

There is evidence that under-stimulating captive environments have the potential to induce boredom or anhedonia thereby compromising welfare (Fureix and Meagher, 2015; Meagher and Mason, 2012). In many captive habitats, for example intensive confinement systems for farm animals or barren laboratory environments for research animals, an animal is often denied cognitive stimulation which can increase aggression towards conspecifics resulting in damage or injury, or induce mental states that are indicative of frustration or aversion (Wood-Gush and Vestergaard, 1989). In these types of situations animal welfare is clearly compromised but little is known regarding the implications of depriving animals of the opportunity to explore.

Zebrafish (*Danio rerio*) are native to the Ganges and Brahmaputra river basins in habitats that fluctuate drastically with the seasons and provide many opportunities for exploration (Engeszer et al., 2007b; Spence et al., 2006). Zebrafish are now one of the most widely used animal research

---

[2] A version of Chapter 2 has been submitted for publication: C. Graham, M.A.G. von Keyserlingk, B. Franks. 2017. Free-choice exploration in laboratory zebrafish.

models worldwide (Spence et al., 2008), in part because of their high cognitive function (e.g., Williams et al., 2002) and sensitivity to environmental manipulations (e.g., Basquill and Grant, 1998). In stark contrast to their natural habitats, laboratory zebrafish are commonly housed in barren or stagnant conditions, with little change in their environment or opportunity to explore new stimuli (Kistler et al., 2011). While the effects of barren environments on animal welfare have previously been described for some domesticated terrestrial animals (e.g., Mellor, 2016; Olsson et al., 2003; Reinhardt, 2004; Würbel, 2001), little is known regarding the motivation of zebrafish to interact with, and respond to, cognitive stimulation opportunities as a form of enrichment and the potential effects on their welfare.

Neophilia, the love of novelty, has been suggested to provoke quick entry and repeated visits when an animal is given access to a new place or stimulus (Barnett and Cowan, 1976). Zebrafish have been found to have strong neophilic tendencies in novel situations (Lucon-Xiccato and Dadda, 2014) and, together with their high cognitive capacity, may be particularly susceptible to the negative effects of living in barren conditions. They therefore may benefit greatly from cognitive stimulation in their captive environments, such as having the opportunity to explore.

Responses to novel tank or open field tests are often used to assess neophilia in zebrafish. Both of these tests are forms of forced exploration in which the animal is involuntarily exposed to the novel stimuli. In contrast, free-choice exploration allows an animal to freely choose between exploring stimuli differing in degrees of temporal and/or spatial novelty and is often conducted in home, or at least familiar, environments. Free-choice tests are deemed superior to forced tests for assessing behavioural responses and are considered powerful methods to analyze exploratory behaviour (Barnett and Cowan, 1976; Hughes, 1997).

Our aim was to broaden our understanding of the potential role that cognitive enrichment may play in zebrafish welfare. To do so we used zebrafish housed in large semi-natural environments and monitored their behaviour after they were allowed to freely explore an additional novel space in their tanks.

We hypothesized that if the exploration opportunity provided a form of cognitive enrichment for the zebrafish, we would observe quick and sustained interest in the novel space, little or no signs of anxiety, and increased affiliative behaviour (i.e., low agonistic behaviour and high group cohesion and coordination). Alternatively, the exploration opportunity could have little apparent effect on the fish (e.g., low interest in the novel space and/or no change to social behaviour) or even a negative effect (e.g., avoidance of the novel space and increased anxiety behaviour), which would indicate that zebrafish are unlikely to benefit from being provided novel space as a form of cognitive enrichment.

2.2 Materials and methods

2.2.1 Animals and housing

Wild-type adult zebrafish were obtained from a local pet store (Aquariums West, Vancouver BC) in August 2014 and were estimated to be three to four months of age. Fish were housed for approximately nine months in groups of 10 within large glass tanks (110 L; 91 x 30.5 x 40.5 cm) (n=6) filled with conditioned water maintained at 26–28°C. Tanks were furnished with a sloped gravel substrate, rocks and five artificial plants placed to create a structurally enriched environment (Fig. 1). Slopes were offset across tanks to reduce any side bias. Opaque black plastic covered the back and side-walls of the tank to prevent visible interaction with other tanks and to minimize noticeable movements and activity within the laboratory. A black opaque divider was installed in the tanks during initial set up (before the fish were introduced), prohibiting entrance to 10 cm of tank space on the far side of the deep end of the tanks. The bottom half of the divider had a rectangular hole cut out (approximately 25 x 20 cm) that was covered until the time of removal for this experiment ('divider removal'). Security cameras (Swann NVR8-7200; resolution: 1000 TVL/1080p), fastened to the front of each tank located approximately 1 m from the glass, continuously recorded fish behaviour.

Room and tank lighting were set on a gradual 12L:12D schedule to simulate sunrise and sunset (i.e., room lights came on half an hour before tank lights and turned off half an hour after tank lights). Fish were fed a diet of flake food (Nutrafin Max Tropical Fish Flakes, Hagen, Canada) in the mornings and thawed frozen blood worms (Hikari Bio-Pure, USA) in the afternoons; both meals were delivered through a standard feeding ring in the shallow end of the tanks. Every week, tank water was changed 15–20% and water testing was done two days after water changes for pH, ammonia, nitrate, nitrite, carbonate hardness, general hardness and dissolved oxygen, to maintain water quality. All experimental and husbandry procedures were approved by the University of British Columbia's Animal Care Committee (protocol A14-0119).

2.2.2 Experimental procedure

On the morning of 1 June 2015, the piece covering the hole of the black opaque dividers was removed from each tank (see Section 2.2.1), providing access to an additional 10 cm of novel space located at one end of the tank (Fig. 1). All data collection took place from video footage from each tank. Video files were transferred to a hard drive and viewed using VLC media player (version 2.2.2 for Mac).

2.2.3 Data collection of behavioural measures

To assess free-choice exploratory behaviour we evaluated the latency to enter the novel space and the number of inspections into the novel space, as well as the number of fish engaged in bottom-dwelling (a proxy for anxiety) and social behaviours, including agonistic behaviour, group cohesion and coordination. High group cohesion and low agonistic behaviour can be indicative of affiliative or socio-positive behaviour and may play a major role in achieving positive emotional states (Boissy et al., 2007). However, directly measuring positive emotion also requires some insight of perceived threat by the animal—incorporating a measure of anxiety aids in the interpretation of the connection between social behaviour and positive emotional state (Boissy et al., 2007).

Four days of video were selected for behavioural analysis: the day before (baseline), the day of, the day after, and two weeks after the divider removal.

Exploratory behaviour was measured using latency to begin exploring and number of inspections of the novel area. Latency was measured as the time (seconds) it took the first fish to approach and inspect the novel area immediately after the removal of the divider. An inspection was counted when a fish swam into and/or faced or came into contact with the novel area and explicitly inspected or entered through the divider opening itself. A fish was required to leave and return to the divider entrance to count as a separate inspection. Sustained inspection (i.e., when a fish remained in the novel area for an extended period of time) counted as one inspection when the fish first entered the novel area and would only count as a second inspection if the fish left and returned. Given that the divider was still in place during baseline observations, an inspection was counted when a fish touched the divider with its head. Inspections were scored in 30-second bins for one hour from the time immediately after removal of the dividers.

Anxiety was measured using bottom-dwelling (i.e., the number of fish in the bottom portion of the tank) based on the spatial location of the fish. A grid system was used to split the tank into three vertical areas—surface, middle and gravel—and two horizontal areas—deep end and shallow end (Fig. 1) and fish were counted in each area. Spatial locations were recorded every two minutes for one hour from the time immediately after removal of the dividers.

To assess social behaviour, we measured agonistic behaviour, cohesion and coordination. Agonistic behaviour was scored as either 0 for no agonistic act observed or 1 for any agonistic act observed. An agonistic act consisted of a chase or charge by an aggressor and a retreat by an opponent, or a lateral display in which two zebrafish swim parallel to each other in opposite directions and circle with their dorsal fins raised and caudal fins extended. Cohesion and coordination were scored on Likert scales from 0 to 4 (see Table 1 for description of scores). Video footage was viewed from the exact time the dividers were removed until 18:00 h when the tank lights were turned off. Social behaviours were scored every two minutes for a total of three seconds: the video was paused at each of the three seconds and each behaviour was scored. The final score was an average of each of the three second scores and rounded to the nearest full number.

Table 1. Description of scores for the social behaviours of cohesion and coordination of zebrafish housed in groups of 10 in large (110 L) semi-natural tanks.

Behaviour	Score	Criteria	Description
Cohesion (i.e. the proximity of the individuals in the group)	0	no cohesion	fish are scattered throughout the tank for the entire coding period
	1	low cohesion	fish are scattered throughout the tank for most of the coding period
	2	moderate cohesion	fish are occasionally cohesive (within 4 BLs* of each other)
	3	high cohesion	majority of fish are cohesive often throughout the coding period (up to 2 BLs*)
	4	extreme cohesion	majority of fish are in a very tight group for most (>50%) of the coding period (less than 2 BLs*)
Coordination (i.e. the alignment of the individuals in the group on the same horizontal plane)	0	no coordination	fish are pointing in different directions for the entire coding period
	1	low coordination	fish are pointing in different directions for most of the coding period with some brief periods of coordination/synchrony
	2	moderate coordination	some fish are occasionally coordinated
	3	high coordination	majority of fish are very coordinated with each other for periods throughout the coding period
	4	extreme coordination	majority of fish are highly coordinated for long periods throughout the entire coding period

*BLs = body lengths (1 BL = approximately 3 cm)

2.2.4 Statistical analysis

The tank was defined as the observational unit in all analyses. To evaluate exploratory behaviour, latency to approach the novel space was measured in seconds. The numbers of inspections were aggregated and averaged to create one score (i.e., average number of inspections) for each day for each tank (24 data points total). Average inspections were analyzed using a linear mixed model with a random effect to control for the repeated measures of the tanks.

To analyze anxiety behaviour from spatial location data, horizontal areas (deep end and shallow end) were added together to create three variables of vertical space: bottom, middle and top. Counts were aggregated and averaged to create one score (i.e., average number of fish) in each area for each tank (24 data points total). We again used a linear mixed model with tank as a random effect to analyze average spatial location.

To analyze the social behaviours of agonistic behaviour, cohesion and coordination, the two-minute scan samples were aggregated into 10-minute bins, thus an aggregated data point consisted of the average of five scan samples. For example, data from 12:00 to 12:10 h:m had five original scores and, once aggregated, had one average score for the 10-minute period. We modeled each aggregated behaviour variable with a linear mixed model with tank and day as nested random effects and time of day and experimental day as fixed effects and used the Satterthwaite method to approximate the degrees of freedom.

Inter-observer reliability to confirm adequate behaviour scoring was conducted with an external observer (ICC=0.83 for agonistic behaviour and coordination, and 0.85 for cohesion). All statistical analyses were conducted in RStudio (Version 0.99.484 – © 2009-2015 RStudio, Inc.; R Core Team, R Foundation for Statistical Computing, Vienna, Austria). The following R packages were used: *lme4* (Bates et al., 2015), *lmerTest* (Kuznetsova et al., 2015), *ggplot2* (Wickham, 2009), *doBy* (Højsgaard and Halekoh, 2016), *gridExtra* (Auguie, 2015), and *tidyverse* (Wickham, 2017).

2.3 Results

Exploratory behaviour was measured using latency to start exploring and the number of inspections of the novel space in the hour after the removal of the divider. The latencies to start exploring the novel space ranged from zero to 19 seconds across tanks (9.7±7.6 seconds; mean±SD). Relative to baseline, the number of inspections of the novel space increased on the day of ($t(15)=3.47$; $p=0.003$) and day after ($t(15)=5.38$; $p<0.0001$) and continued to increase up to two weeks after the removal of dividers ($t(15)=7.62$; $p<0.0001$) (Fig. 2a).

Anxiety behaviour was measured based on the fishes' spatial locations using the number of fish in the bottom portion of the tank and was not different from baseline on any day examined (day of: $t(15)=0.35$; $p=0.73$; day after: $t(15)=0.08$; $p=0.94$; two weeks after: $t(15)=0.15$; $p=0.88$).

The opportunity to explore the novel space after the removal of dividers increased the affiliative behaviour of zebrafish. Agonistic behaviour was lower on the day of divider removal ($t(14.6)=2.58$; $p<0.02$; Fig. 2b) and the day after ($t(15.7)=2.49$; $p<0.02$), but returned to baseline levels by two weeks later ($t(14.7)=0.41$; $p>0.69$). Cohesion was higher on the day of the removal of dividers ($t(14.8)=2.29$; $p<0.04$; Fig. 2c) and returned to baseline by the day after ($t(15)=1.31$; $p>0.21$; two weeks later: $t(14.9)=0.70$; $p>0.49$). Coordination tended to be higher on the day of divider removal ($t(14.8)=2.05$; $p<0.06$; Fig. 2d), was higher on the day after ($t(15)=2.20$; $p<0.04$), but returned to baseline by two weeks later ($t(14.8)=1.45$; $p>0.17$).

2.4 Discussion

Advances in animal welfare science have shown that, in many species, the quality of life led by an animal is influenced by the level of cognitive stimulation in the environment (Meehan and Mench, 2007). Within captive enrichment programs, the provision of exploration opportunities has been deemed an important, and even necessary, feature to be beneficial for improving welfare

(Meehan and Mench, 2007). Exploration may promote positive emotions because (i) most animals are motivated to perform such behaviour (i.e., it fills a behavioural need) and, (ii) the gathering of information allows control over coping with challenges and, in and of itself, appears to be reinforcing (Barnett and Cowan, 1976; Boissy et al., 2007; Franks et al., 2013). One essential component for cognitive enrichment to be effective is that the problems presented to an animal do not exceed its skills and resources (Boissy et al., 2007; Meehan and Mench, 2007).

In the present study, zebrafish were quick to move into the new area after the removal of a divider and this interest was sustained, and actually increased, over the days observed. Moreover, the fish did not express any anxious behaviour but did engage in higher levels of affiliative behaviour: decreased agonistic behaviour and increased group cohesion and coordination. Collectively these findings provide evidence suggesting the opportunity to explore may have induced a positive emotional state in the fish (Boissy et al., 2007).

Zebrafish are known to exhibit anxiety- or fear-like behavioural responses after exposure to novelty, such as when placed in a novel tank (e.g., Blaser et al., 2010; Egan et al., 2009; Maximino et al., 2010). A common measure of anxiety is spatial location within the tank: swimming lower in the vertical water column (nearer to the bottom) is suggested to indicate a more anxious or stressed fish, whereas swimming higher in the water column (nearer to the surface) suggests a less anxious fish (Cachat et al., 2009). Our study is the first to examine zebrafish behaviour in their home tanks when provided access to a previously unexplored 10 cm of tank space. Given that we observed no change in bottom-dwelling behaviour combined with the short latencies to enter the novel area, our findings suggest the fish did not experience any anxiety in relation to the novel stimulus.

Zebrafish are also well known to be gregarious and spend much of their time in social groups called shoals, both in the wild and in captivity (Miller and Gerlai, 2012; Spence et al., 2008; Suriyampola et al., 2015). Shoaling behaviour is often quantified using group cohesion (distance between nearest neighbours) and swimming coordination (polarization) (Clark and Evans, 1954; Miller and Gerlai, 2012, 2011b). Zebrafish also exhibit agonistic behaviour, such as lateral displays, chasing and charging, especially around times of breeding (Schneider, 2011; Suriyampola et al., 2015; Way et al., 2015). Our finding that the provision of additional space increased group cohesion is interesting given it contradicts previous work. Researchers compared the nearest-neighbour distances of groups of zebrafish within larger and smaller tanks and found that groups of fish in the larger tanks spread out more, resulting in lower group cohesion, compared to fish in the smaller tanks (Shelton et al., 2015). The fact that cohesion *increased* (i.e., the fish were closer together) in our study after the provision of the additional space within the home tank therefore points toward a cognitive (information gain) rather than a physical (more space) mechanism.

Many researchers have investigated shoaling behaviour in zebrafish and, collectively, these studies suggest this behaviour is largely in response to threatening situations, such as when evading a predator (Pitcher, 1986; Pitcher and Parrish, 1993). Further, it has been suggested that increased levels of distress (e.g., from greater perceived threat) lead individuals to examine their relative position and orientation within a group at a greater rate and thus induce greater degrees of synchronization in the group's coordination (Bode et al., 2010). However, our finding of increased cohesion and coordination in the absence of perceived threat suggests that distress may not be the only driver of group dynamics. Considering the highly social nature of zebrafish—they live in groups (Engeszer et al., 2007b; Suriyampola et al., 2015), readily perform shoaling or schooling behaviour (Spence et al., 2008), transmit information socially (Lindeyer and Reader, 2010), and are influenced by social cues from conspecifics (Hall and Suboski, 1995; Speedie and Gerlai, 2008)—it could be argued that their social dynamics are sensitive to environmental context, whether negative (e.g., threat) or positive (e.g., foraging). As such, it is possible that zebrafish may also shoal or school when exploring, and there is reason to consider these behaviours under different, possibly positive, contexts, such as information gathering (Franks and Higgins, 2012).

It follows that situations where zebrafish are denied the opportunity to explore may negatively influence their social conduct. The typical laboratory housing for zebrafish consists of small and barren tanks that greatly contrast their wild habitats and likely impact the behaviours of the fish within them (Kistler et al., 2011). Without opportunities to explore, the behaviour exhibited by standard-housed laboratory zebrafish or those tested in barren experimental tanks may represent inaccurate baselines of 'normal' behaviour (Sherwin, 2004). Thus, we strongly suggest that the range of situations that influence social dynamics also be extended to include positively or ambiguously valenced situations, such as information gathering.

In the wild, many animals have numerous free-choice options to explore and employ agency over their environment. Depriving an animal of the opportunity to exhibit control ultimately denies them a source of naturally sustained positive experience, which can cause an underdeveloped ability to cope with environmental or social changes, and may result in reduced health (reviewed by Špinka and Wemelsfelder, 2011). If having the opportunity to explore is a behavioural need that may influence emotional state—that is, a behaviour an animal is highly motivated to perform—then denying animals this opportunity can result in reduced welfare (Bracke and Hopster, 2006; Špinka, 2006; Wood-Gush and Vestergaard, 1989).

The standard laboratory environment has been shown to be associated with behavioural abnormalities (e.g., Balcombe, 2010; Kistler et al., 2011; Olsson and Dahlborn, 2002) which have been shown to produce variable or unreliable scientific results (Balcombe, 2006; Garner, 2005; Sherwin, 2004). It has frequently been suggested that psychological, physiological and behavioural well-being develops from the ability to successfully cope with life's problems, not from the complete absence of challenge (Broom, 2014; Franks and Higgins, 2012; McMillan, 2004; Meehan and Mench, 2007). Natural behaviour can be promoted by a deeper understanding of the features of the natural environment that are likely to meet the animal's needs (Špinka, 2006; Würbel, 2002, 2001). Considering their wild habitats would normally include many such information-gain opportunities, our findings suggest that high agonistic behaviour and low group cohesion and coordination—behaviours typical within standard laboratory housing—may be signs of abnormal behaviour in captive zebrafish.

The relationship between environment, cognition and welfare is interconnected: the environment influences cognitive capacity which in turn affects the ability to effectively cope, and ultimately reflects emotional state, thus influencing their quality of life (Ebbesson and Braithwaite, 2012). Emotions can also influence cognition by affecting the value attached to a behavioural outcome by an animal; for example, frustration from behavioural deprivation may cause an animal to be less sensitive to small or subtle environmental changes (Braithwaite et al., 2011). Our findings suggest not only is a more natural physical environment important for animal welfare, but the provision of natural psychological experiences—such as the opportunity to explore—may also be required to achieve a reasonably good life for captive animals.

As a first step in addressing the role cognitive enrichment may play in laboratory zebrafish welfare, we examined the fishes' response to the opportunity to explore a novel space and found evidence that they responded to the cognitive stimulation and that they had a sustained interest in this exploration opportunity. The provision of environmental enrichment has previously been suggested to have numerous benefits in many species of fish (e.g., Kotrschal and Taborsky, 2010; Salvanes et al., 2013; Strand et al., 2010). Examining the motivation of zebrafish (i.e., how hard they would work) to freely explore within their environment would shed further light on the welfare implications of cognitive stimulation as a form of enrichment for laboratory zebrafish and provides a promising area of future research. For instance, the motivation of goldfish to access a tank space with real or artificial plants was recently tested using increasingly stronger water currents; goldfish indeed will work for this opportunity (Sullivan et al., 2016). This or similar types of experimental paradigms (e.g., training fish to swim through a ring: Hogan, 1967) could be applied in future studies to test the motivation of zebrafish to access novel space or other opportunities for exploration or information gain.

A limitation of the current study was our confined scoring period. Given the comprehensive scoring methods, we only looked at one hour of video for exploratory and anxiety behaviour on each of the observational days. Additional video observations would have also allowed us to determine any pattern to the fish using the novel space, for instance if they refrained from the novel space at certain times of the day. Another potential limitation was the challenges associated with video recording fish in water which may have resulted in missing of counts for spatial location due to, for example, hiding behind a plant during the scoring period. Further, our measure of exploration (i.e., the number of inspections) may have underestimated actual exploration, as we did not score for duration of the fishes' investigation of the novel space. Additionally, the greater number of inspections observed on the day examined two weeks later may not have actually been indicative of greater exploration, but simply a change in how the fish interacted with the space—for example, on the first day, fish may have entered and stayed in the novel area, spending more time exploring, whereas by two weeks later, fish may have been simply entering and leaving continually, hence the greater number of inspections scored. Future research could, for example, utilize sophisticated tracking software to ensure all fish within a tank are recorded, as well as consider additional measures of exploration, such as duration. Such data would provide better interpretation of the effects of exploration opportunities. The fact that fish were not individually identified can also be viewed as a limitation. Software that allows for the tracking of individuals could provide even further insight of the influence on complex social dynamics (currently available only for unobstructed video (e.g., Pérez-Escudero et al., 2014), that is, without plants or obstructed views).

Future research could also implement a standard control treatment, such as a tank of similar size (including the extra space) but furnished in the standard barren condition. Such a treatment would allow disentangling of the effect of having more space *per se* on the behaviour of the zebrafish from behaviours in direct response to being provided cognitive enrichment in the form of additional novel space. Nevertheless, monitoring behaviour in a semi-natural environment allows for observation of a behavioural richness that may not be possible with strict experimental manipulation (Martin and Bateson, 2007) and arguably facilitates external validity and relevance to the natural world (Altmann, 1974). We strongly encourage future research to expand on our findings to further elucidate the potential benefits of providing cognitive enrichment on the behaviour and welfare of laboratory zebrafish.

In summary, we found that zebrafish readily engage in free-choice exploration and that such opportunities do not induce anxiety behaviour in semi-natural environments. Moreover, these opportunities increased positive affiliative behaviour: reducing agonistic behaviour and increasing group cohesion and coordination. These findings suggest that having the opportunity to explore within semi-natural home environments promotes a positive emotional state and thereby improves welfare (Boissy et al., 2007). This study adds to the growing body of literature on the role cognitive stimulation plays in welfare and indicates that zebrafish are good candidates for further cognitive enrichment research.

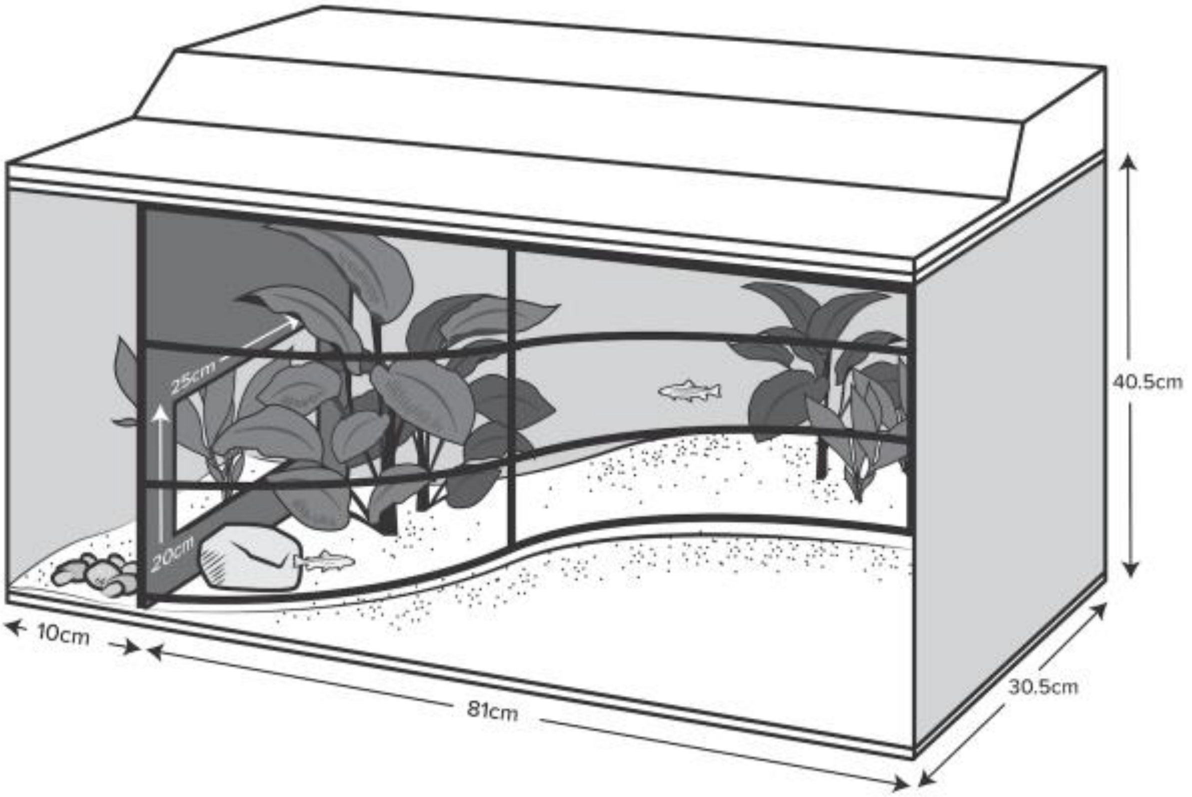

Figure 1. Layout of tank environment. A semi-natural habitat was created by including artificial plants, allowing hiding and open spaces, sloped gravel substrate and rocks. The cover over the divider opening, on the far side of the deep end (far left above), was removed to allow an opportunity to explore novel space. Lines are the grid system used for measuring anxiety, using three vertical areas—surface, middle and gravel—and two horizontal areas—deep end and shallow end—fish were counted in each area. The number of fish in the lower sections (gravel) was used to identify bottom-dwelling, a common measure used to assess anxiety.

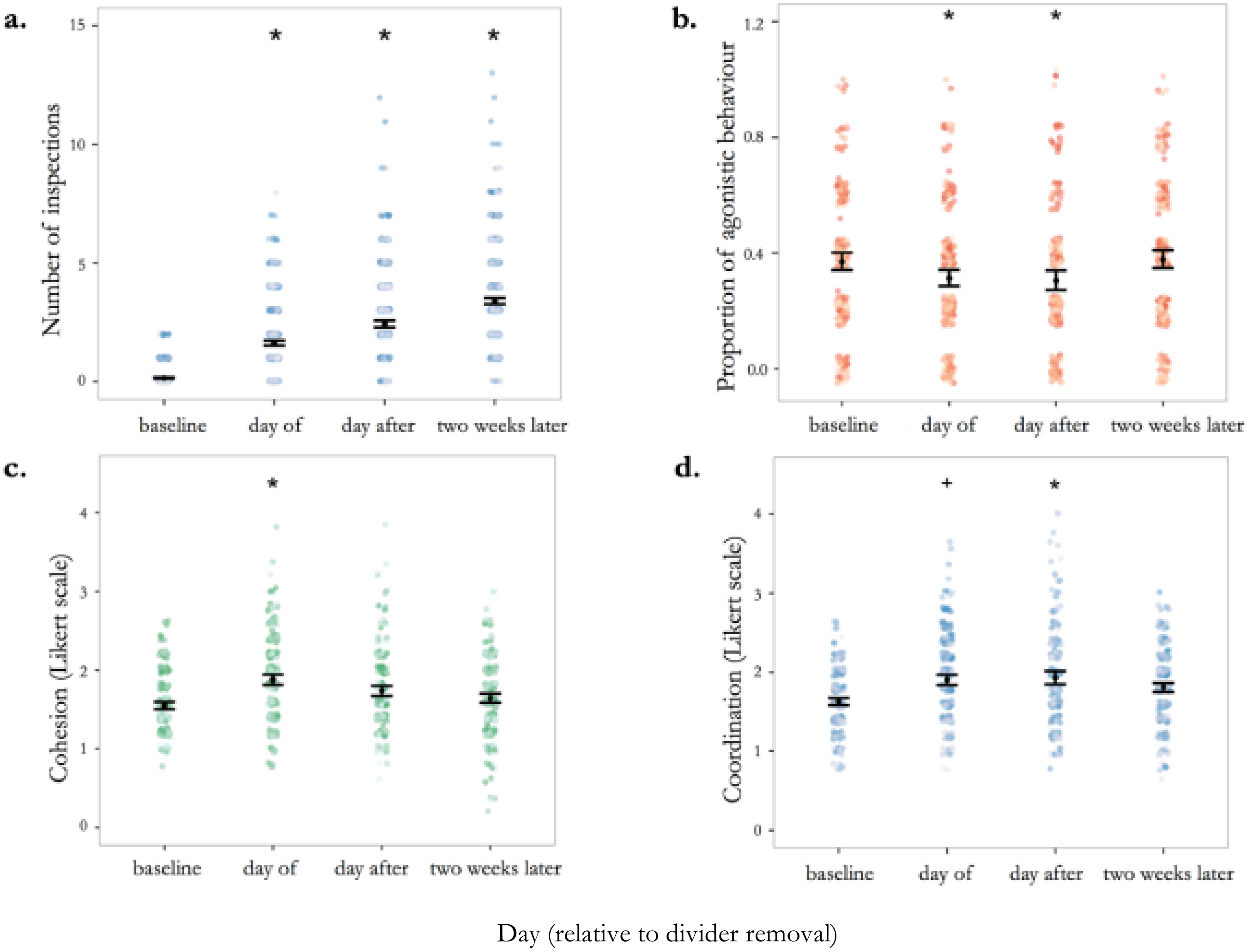

Figure 2. Exploration and social behaviours of zebrafish following the removal of a divider allowing the opportunity to explore additional novel space in their semi-natural home tank environments. Shade of data point colours represents the time of observation relative to the divider removal: darker means immediately after divider removal, lighter means later in the scoring period.

a. Exploration of the novel area measured using the number of inspections. Each day examined had a greater number of inspections (day of: $t(15)=3.47$; $p=0.003$; day after: $t(15)=5.38$; $p<0.0001$; day two weeks later: $t(15)=7.62$; $p<0.0001$) compared to baseline. Errors bars represent 95% confidence. (***** $p<0.05$; n=6)

b. Agonistic behaviour was scored by presence/no presence of an agonistic act (i.e., charge, chase, lateral display). Agonistic behaviour was reduced on the day of and day after the divider removal ($t(14.6)=2.58$; $p=0.02$ and $t(15.7)=2.49$; $p=0.02$, respectively), but returned to baseline by the day examined two weeks later ($t(14.7)=0.41$; $p=0.69$). Errors bars represent 95% confidence. (***** $p<0.05$; n=6)

c. Cohesion was scored on a Likert scale from 0 to 4 (0 meaning fish were spread throughout the tank, 4 meaning fish were very cohesive; see Table 1). Cohesion was greater on the day of divider removal ($t(14.8)=2.29$; $p=0.04$) but returned to baseline by the day after ($t(15)=1.31$; $p=0.21$; two weeks later: $t(14.9)=0.70$; $p=0.49$). Errors bars represent 95% confidence. (***** $p<0.05$; n=6)

d. Coordination was scored on a Likert scale from 0 to 4 (0 meaning no coordination and the fish were swimming in different directions, 4 meaning the fish were very coordinated, see Table 1). Coordination tended to be greater on the day of divider removal ($t(14.8)=2.05$; $p=0.06$) and was greater on the day after ($t(15)=2.20$; $p=0.04$), however returned to baseline by the day examined two weeks later ($t(14.8)=1.45$; $p=0.17$). Errors bars represent 95% confidence.
(**+** $0.05>p<0.01$; ***** $p<0.05$; n=6)

Chapter 3: Conclusion

3.1 Thesis findings

The specific aim of this thesis was to analyze the behavioural responses of zebrafish offered an opportunity to explore additional novel space within their semi-natural home tank environments as a form of cognitive enrichment. The results described in this thesis provide evidence that zebrafish are good candidates for cognitive enrichment research: as the fish readily explored the novel space, did not exhibit anxiety behaviour following the novelty, and their social dynamics were altered in what can be viewed in a positive way (i.e., reduced agonistic behaviour and increased cohesion and coordination).

Zebrafish in the current study were quick to move into the new area following the provision of the opportunity to explore. Most interesting was that this exploration was sustained on each of the observation days after the divider was removed. Moreover, the number of inspections was three times greater after two weeks than on the day the dividers were removed. This finding is in contrast to other research that has shown zebrafish quickly habituate to various novel stimuli (Wong et al., 2010). The higher group cohesion observed after the additional space was offered also contrasts the results of previous work that reported that groups of zebrafish in a larger arena were found to spread out more than groups in a smaller arena (Shelton et al., 2015; see also Introduction Section 1.3.2), suggesting a different mechanism was at play, such as a cognitive (information gain) rather than a physical (more space) mechanism.

The findings from this thesis suggest the social behaviour of zebrafish is affected by positively or ambiguously valenced situations, such as information-seeking, and not only negatively valenced threat responses, such as predator evasion, as commonly reported in the literature (Pitcher, 1986; Pitcher and Parrish, 1993). These results suggest that there is need to broaden our approach to studying the social dynamics of zebrafish, particularly for situations that may influence their welfare. High agonistic behaviour and low cohesion and coordination are common in laboratory zebrafish maintained in 'standard' housing, often due to overcrowding, but the results in this study suggest that they may actually be indicative of abnormal behaviour.

Free-choice exploration allows animals to employ agency over their environment (Špinka and Wemelsfelder, 2011) and is frequently available to many wild animals, including zebrafish. Having the freedom to choose between investigating the novel space, and staying in or returning to a familiar part of the tank, likely eased any potential anxiety responses that may have been present when the additional space was made available (Wood-Gush et al., 1990). Incorporating opportunities for free-choice exploration in laboratory zebrafish environments may induce a more naturally representative behavioural repertoire which may in turn impact the emotional state of the fish, and may ultimately enhance their ability to live a reasonably natural life in captivity (Boissy et al., 2007; Ebbesson and Braithwaite, 2012; Fraser et al., 1997).

In summary, the conclusions from this thesis indicate that opportunities to explore in semi-naturalistic housing may provide positive effects for zebrafish welfare by (1) reducing agonistic behaviour that can lead to physiological or health issues, (2) promoting positive emotional states with increased affiliative behaviour and, (3) allowing the performance of important natural behaviours.

3.2 Implications for fish welfare

Housing for laboratory zebrafish is typically designed to maintain hygiene and physical health and to standardize behaviour between experimental groups, resulting in small, barren and unchanging environmental conditions (Kistler et al., 2011; Olsson and Dahlborn, 2002; Reinhardt, 2004). In contrast, the natural habitats of zebrafish are vegetated and fluctuate with monsoon seasons, thus providing many opportunities to explore (Engeszer et al., 2007b; Spence et al., 2006). This thesis provides evidence that exploration may be particularly important for captive zebrafish and suggests, because the opportunity to explore is rarely, if ever, provided in laboratories, standard barren environments could be denying them an important behavioural need.

The three concepts of animal welfare—natural living, biological health and emotional state—are fundamentally interconnected (Fraser et al., 1997); an effect on one can have effects on the others. Having the opportunity to explore may bring a feeling of pleasure or satisfaction indicative of positive emotional state and improve biological functioning by reducing agonistic behaviour towards conspecifics (Boissy et al., 2007; Fraser et al., 1997). The inability to express species-specific natural behaviours may affect biological functioning, potentially causing increased agonistic behaviour or affect the emotional state, by causing frustration, boredom or anxiety (Dawkins, 1988). An animal's emotional state can influence behaviour, for example, by inducing abnormal stereotypies, and health by negatively affecting the immune system (Boissy et al., 2007). Further, poor health can elicit sickness behaviour, for example, by causing lethargy (Hart, 1988), and affects emotional state by eliciting fear, distress or impaired threat perception (Cockram and Hughes, 2011). Therefore, addressing these concepts in a unified approach is crucial for understanding an animal's welfare. As reviewed previously (see Section 1.2.2), gaining a better understanding of the natural behaviour of zebrafish will greatly aid in our ability to decipher behavioural divergences due to inadequate housing conditions or the inhibition of motivated behaviours.

As suggested from the work described in this thesis, the use of cognitive enrichment may aid in promoting good welfare for zebrafish by meeting psychological needs, allowing the performance of motivated and complex behaviours, and improving the ability to cope with environmental challenge (Meehan and Mench, 2007).

3.3 Implications for the quality of science

Studies using zebrafish housed in 'standard' conditions arguably may compromise the validity of scientific results, as has been shown in other species (rodents: Bayne and Würbel, 2014; Garner, 2005; Sherwin, 2004). It is assumed that animals housed in barren conditions are 'normal', such as having normal sensory development, physiology and behaviour, and that any responses dependent on these aspects are also normal (Sherwin, 2004). However, discrepancies from normal due to being housed in inadequate environments (e.g., frustration, heightened sensitivity, boredom, etc.) may instead lead animals to generate abnormal responses that are not experimentally reproducible (Sherwin, 2004). The barren housing used to standardize behaviour may in fact be leading to idiosyncrasies, both within and across laboratory facilities or even within a single study, and may produce more variable, less reliable results. A recent proof of principle based on data from a multi-laboratory study investigating differences across mouse strains suggests that standardization actually causes—rather than remedies—poor reproducibility (Richter et al., 2009). Consequently, reduced normality of the animals being studied also reduces the accuracy of translating animal results to human mechanisms for which many animal models, including zebrafish, are used to investigate (such as the processes for biogenetics or disease).

Environmental enrichment is suggested to improve scientific results by reducing the number of abnormal behaviours expressed by animals and responses affecting experimental outcomes (Garner, 2005). The effects of cognitive enrichment are less understood, especially in zebrafish, but it is suggested that introducing appropriate cognitive challenge may prove to be an effective tool in managing some stereotypies by improving the cognitive involvement of the environment and preventing a shift toward processes that promote abnormal behaviour (Meehan and Mench, 2007). The behaviour observed in this study may be more representative of the natural behavioural repertoire of zebrafish (compared to behaviour observed from barren-housed fish) and these findings improve our understanding of what zebrafish behaviour may look like in more naturalistic environments. In and of itself, this provides invaluable benefits to science by providing a more accurate behavioural baseline and allowing for clearer identification of behavioural discrepancies from unsatisfactory laboratory environments.

3.4 Limitations

A limitation of this thesis study was the confined period of behavioural scoring. Given the comprehensive scoring methods, only one hour of video was viewed for exploratory and anxiety behaviour on each of the observational days. Additional video observations may have indicated any patterns to the fish using the novel space, for instance if they refrained from the novel space at certain times of the day. Another limitation was the challenge associated with video recording fish in water. Visibility was limited which may have resulted in missing of counts for fishes' spatial location due to, for example, hiding behind a plant during the scoring period. Utilizing sophisticated tracking software, ideally that can identify individuals, could reveal deeper insight into effects of exploration on complex social behaviour. Further, the measure of exploration used (i.e., the number of inspections) may have underestimated actual exploration, as I did not measure duration of investigation of the novel space. However, even with the limited measure of number of inspections, I found clear evidence that zebrafish were interested and engaged when provided with a novel space. Measuring the duration of their exploration could provide stronger evidence of their motivation to explore.

The measure of bottom-dwelling used for anxiety is well established for zebrafish in a novel tank test (Champagne et al., 2010; Egan et al., 2009). However, I did not remove fish from their home tank or place them into a novel arena, thus the extra space provided may have been insufficient to induce novelty that elicits an anxiety response. Another measure used to quantify anxiety in zebrafish (as well as rodents) is thigmotaxis, when the animal remains along the edges and avoids the middle of its environment (Champagne et al., 2010). Other measures of anxiety include freezing, jumping and erratic movement, though their efficacy is not as well established as bottom-dwelling and thigmotaxis (Egan et al., 2009). Potentially even more problematic is that the display of these behaviours could also represent something other than anxiety, for example anticipation before feeding. The use of a single measure of anxiety may be viewed as a limiting factor and thus I strongly encourage future studies to include additional measures of anxiety-related behaviours.

In contrast, benefits of this work include that it was conducted in home tank environments and that it offered a free-choice opportunity to explore (versus forced exploration). These aspects likely reduced many potential confounds associated with, for example, stress from handling and transport and anxiety responses from being placed in a new environment or being forced to explore.

3.5 Future research

The work described in this thesis provides a starting point for investigating the effects of cognitive enrichment and the implications for welfare of zebrafish living in semi-natural laboratory environments. Because field studies of zebrafish are difficult, semi-naturalistic conditions may offer the next best thing for quantitative (and qualitative) research of the natural behaviour of zebrafish.

Most interesting to me from this project was the clear exploratory response of the zebrafish and their sustained interest on all of the testing days investigated after the fish were provided access to the novel space. To further quantify the importance of providing access to a novel space as a proxy for cognitive stimulation opportunities for laboratory zebrafish, future studies could apply measures of motivation, that is making the fish work for access to such an opportunity (Fraser and Nicol, 2011; Jensen and Pedersen, 2008). There has been some research into measuring motivation in fish and the techniques used may or may not be suitable for zebrafish.

The motivation of Mozambique tilapia (*Oreochromis mossambicus*) to access food, a social partner and additional space has been tested using a push-door mechanism. Tilapia were found to be motivated at increasing costs (i.e., more weight on the push-door) to access both food and a social partner (they were less inclined to work for additional space), thus the use of the push-door was successful at measuring motivation in this species (Galhardo et al., 2011). At nearly 10 times the size of zebrafish, tilapia have strong snouts that enable them to push a door; however, this mechanism is likely unfit for the much smaller zebrafish. More recently, the motivation of goldfish (*Carassius auratus*) to access real or artificial plants was tested using increasingly strong water currents. Goldfish showed robust motivation to access vegetation with increasing currents and did not discriminate between real or artificial plants (Sullivan et al., 2016). It is not presently known if zebrafish would respond to water currents in the same way; however, in the wild they have been shown to occupy regions of both still water and differing water flows (Engeszer et al., 2007b; Suriyampola et al., 2015), thus it is possible this mechanism may be useful for experimentally measuring the motivation of zebrafish to access opportunities to explore.

As alluded to above, a limitation of this work is that I only measured one type of exploration opportunity (novel space). Future studies could implement different types of cognitive stimulation, for example, dynamic novel objects, or offer multiple choices of exploration opportunities to gain insight into the types of features zebrafish prefer to explore. Also, having the ability to manipulate or induce an exploration opportunity (i.e., diversive exploration) may produce different results than having the chance to explore a particular stimulus (i.e., specific exploration, such as that within this thesis) (Hughes, 1997). Having been linked to boredom (Mills et al., 2010), the novelty of diversive exploration is less rewarding when an animal is previously deprived (Berlyne et al., 1966). However, having the chance to manipulate a feature within their environment (or self-induce a learning experience; similar to a rodent learning to press a bar for rewards, e.g., Berlyne et al., 1966) may be something zebrafish are motivated to engage in. This could be especially likely for zebrafish kept in barren conditions where stimulation is generally low, but stimuli may need to be introduced subtly so as to not overwhelm the fish or cause overstimulation to the point of avoidance (Hughes, 1997).

To further examine the theory that exploration leads to positive emotional state, judgement bias tests may be of use (i.e., judgements about the reward value of ambiguous cues) (Harding et al., 2004; Paul et al., 2005). To my knowledge no judgement bias test has been conducted in fish (Bethell, 2015). Such a test would require training fish to respond to both a positive and a negative stimulus, and then assessing their response to an ambiguous stimulus. Latency or changes in response times to cues could act as measures for assessing emotional state; however, cues (e.g., visual, auditory) as well as behavioural responses (e.g., aggression, redirected activities, stereotypies) of zebrafish would require validation for their appropriate use as a test of judgement bias (Bethell, 2015).

The issue of practical implementation of appropriate challenge or exploration opportunities into laboratories, especially those favouring 'standard' conditions, also requires future investigation. One suggestion is incorporating challenge into the feeding regime (e.g., alternating food locations, a task zebrafish have been shown to successfully learn; Williams et al., 2002). Another suggestion is manipulating or changing the environment following water changes or tank cleaning as part of regular laboratory husbandry. Again, it is important that the challenge does not exceed the abilities of the fish (Meehan and Mench, 2007); nevertheless, simple alterations may provide sufficient challenge or variation to engage the fishes' interest and improve their welfare in captivity.

The moral consideration for the welfare of zebrafish is far behind that for other laboratory species (e.g., rodents). A driving factor for welfare consideration is our perception of the cognitive abilities and intelligence or sentience of an animal (Broom, 2007; Brown, 2015). Better understanding of the behavioural and cognitive extent of zebrafish and their response to cognitive stimulation would promote greater consideration for their welfare. Future studies that broaden the exposure of the cognitive abilities of zebrafish may help diminish these perceptions and promote increased consideration for their welfare in laboratories.

3.6 Final conclusions

Based on this project and the literature reviewed in this thesis, I encourage laboratories researching zebrafish to consider the psychological, behavioural and emotional needs of the fish. A shift in the way we think about the animals we use can profoundly affect their welfare, the quality of the research and, ultimately, the advancement of science. Observing zebrafish display their natural behavioural repertoire could in turn influence the researchers' perception of the intelligence and complexity of a zebrafish—that they deserve individual consideration. More accurate representation of natural behaviour will also improve scientific quality by producing more reliable data, a crucial aspect to appropriate translation to humans and the advancement of research using zebrafish as models.

Overall, this thesis provides evidence that zebrafish are good candidates for future research on the role cognitive enrichment may play in welfare, and adds to the growing body of literature focusing on the improvement of the welfare of laboratory zebrafish.

References

Altmann, J., 1974. Observational study of behavior: sampling methods. Behaviour 49, 227–267. doi:10.1080/14794802.2011.585831

Archer, J., Birke, L., 1983. Some issues and problems in the study of animal exploration, in: Archer, J., Birke, L. (Eds.), Exploration in Animals and Humans. Van Nostrand Reinhold Co. Ltd., Wokingham, UK, pp. 1–21.

Auguie, B., 2015. gridExtra: miscellaneous functions for "grid" graphics.

Balcombe, J., 2010. Laboratory rodent welfare: thinking outside the cage. J. Appl. Anim. Welf. Sci. 13, 77–88. doi:10.1080/10888700903372168

Balcombe, J.P., 2006. Laboratory environments and rodents' behavioural needs: a review. Lab. Anim. 40, 217–235. doi:10.1258/002367706777611488

Barnett, S.A., Cowan, P.E., 1976. Activity, exploration, curiosity and fear: an ethological study. Interdiscip. Sci. Rev. 1, 43–62. doi:10.1179/030801876789768534

Bartolini, T., Butail, S., Porfiri, M., 2014. Temperature influences sociality and activity of freshwater fish. Environ. Biol. Fishes 98, 825–832. doi:10.1007/s10641-014-0318-8

Basquill, S.P., Grant, J.W.A., 1998. An increase in habitat complexity reduces aggression and monopolization of food by zebra fish (Danio rerio). Can. J. Zool. 76, 770–772. doi:10.1139/cjz-76-4-770

Bates, B., Maechler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw. 67, 1–48. doi:doi:10.18637/jss.v067.i01

Bayne, K., Würbel, H., 2014. The impact of environmental enrichment on the outcome variability and scientific validity of laboratory animal studies. Rev. Sci. Tech. 33, 273–80.

Bekoff, M., 2007. Aquatic animals, cognitive ethology, and ethics: questions about sentience and other troubling issues that lurk in turbid water. Dis. Aquat. Organ. 75, 87–98. doi:10.3354/dao075087

Berdoy, M., 2002. The laboratory rat: a natural history. Oxford University, UK.

Berlyne, D.E., 1960. Conflict, arousal, and curiosity. McGraw-Hill Book Company, Inc., New York, USA.

Berlyne, D.E., Koenig, I.D., Hirota, T., 1966. Novelty, arousal, and the reinforcement of diversive exploration in the rat. J. Comp. Physiol. Psychol. 62, 222–226. doi:10.1037/h0023681

Bethell, E.J., 2015. A "how-to" guide for designing judgment bias studies to assess captive animal welfare. J. Appl. Anim. Welf. Sci. 18, S18–S42. doi:10.1080/10888705.2015.1075833

Blaser, R.E., Chadwick, L., McGinnis, G.C., 2010. Behavioral measures of anxiety in zebrafish (Danio rerio). Behav. Brain Res. 208, 56–62. doi:10.1016/j.bbr.2009.11.009

Blaser, R.E., Rosemberg, D.B., 2012. Measures of anxiety in zebrafish (Danio rerio): dissociation of black/white preference and novel tank test. PLoS One 7, 1–9. doi:10.1371/journal.pone.0036931

Bode, N.W.F., Faria, J.J., Franks, D.W., Krause, J., Wood, A.J., 2010. How perceived threat increases synchronization in collectively moving animal groups. Proc. R. Soc. B 277, 3065–3070. doi:10.1098/rspb.2010.0855

Boissy, A., Manteuffel, G., Jensen, M.B., Moe, R.O., Spruijt, B., Keeling, L.J., Winckler, C., Forkman, B., Dimitrov, I., Langbein, J., Bakken, M., Veissier, I., Aubert, A., 2007. Assessment of positive emotions in animals to improve their welfare. Physiol. Behav. 92, 375–97. doi:10.1016/j.physbeh.2007.02.003

Bouwknecht, J.A., Olivier, B., Paylor, R.E., 2007. The stress-induced hyperthermia paradigm as a physiological animal model for anxiety: a review of pharmacological and genetic studies in the mouse. Neurosci. Biobehav. Rev. 31, 41–59. doi:10.1016/j.neubiorev.2006.02.002

Bracke, M.B.M., Hopster, H., 2006. Assessing the importance of natural behavior for animal welfare. J. Agric. Environ. Ethics 19, 77–89. doi:10.1007/s10806-005-4493-7

Braithwaite, V.A., 2010. Do fish feel pain? Oxford University Press, Oxford, UK.

Braithwaite, V.A., Boulcott, P., 2007. Pain perception, aversion and fear in fish. Dis. Aquat. Org. 75, 131–138.

Braithwaite, V.A., Huntingford, F.A., van den Bos, R., 2011. Variation in emotion and cognition among fishes. J. Agric. Environ. Ethics 26. doi:DOI 10.1007/s10806-011-9355-x

Broom, D.M., 2016. Fish brains and behaviour indicate capacity for feeling pain. Anim. Sentience 1–6.

Broom, D.M., 2014. Sentience and Animal Welfare. CABI, Oxfordshire, UK.

Broom, D.M., 2007. Cognitive ability and sentience: which aquatic animals should be protected? Dis. Aquat. Organ. 75, 99–108. doi:10.3354/dao075099

Broom, D.M., 1996. Animal welfare defined in terms of attempts to cope with the environment. Acta Agric. Scand. Sect. A—Animal Sci. Suppl. 27, 22–28.

Brown, C., 2015. Fish intelligence, sentience and ethics. Anim. Cogn. doi:10.1007/s10071-014-0761-0

Cabanac, M., Cabanac, A.J., Parent, A., 2009. The emergence of consciousness in phylogeny. Behav. Brain Res. 198, 267–272. doi:10.1016/j.bbr.2008.11.028

Cachat, J.M., Canavello, P.R., Elegante, M.F., Bartels, B.K., Elkhayat, S.I., Hart, P.C., Tien, A.K., Tien, D.H., Beeson, E., Mohnot, S., Laffoon, A.L., Stewart, A.M., Gaikwad, S., Wong, K., Haymore, W., Kalueff, A. V., 2009. Modeling stress and anxiety in zebrafish, in: Kalueff, A. V., Cachat, J. (Eds.), Zebrafish Models in Neurobehavioral Research. Humana Press, New York, USA, pp. 73–88. doi:10.1007/978-1-60761-922-2_3

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

CCAC, 2015. Canadian Council on Animal Care Animal Data.

Champagne, D.L., Hoefnagels, C.C.M., de Kloet, R.E., Richardson, M.K., 2010. Translating rodent behavioral repertoire to zebrafish (Danio rerio): relevance for stress research. Behav. Brain Res. 214, 332–342. doi:10.1016/j.bbr.2010.06.001

Chandroo, K.., Duncan, I.J.., Moccia, R.., 2004. Can fish suffer?: perspectives on sentience, pain, fear and stress. Appl. Anim. Behav. Sci. 86, 225–250. doi:10.1016/j.applanim.2004.02.004

Clark, F.E., 2013. Marine mammal cognition and captive care: a proposal for cognitive enrichment in zoos and aquariums. J. Zoo Aquarium Res. 1, 1–6.

Clark, P.J., Evans, F.C., 1954. Distance to nearest neighbor as a measure of spatial relationships in populations. Ecology 35, 445–453.

Cockram, M.S., Hughes, B.O., 2011. Health and Disease, in: Appleby, M., Mench, J., Olsson, I.A.S., Hughes, B.O. (Eds.), Animal Welfare. CABI, Oxfordshire, UK, pp. 120–137.

Collymore, C., Tolwani, R.J., Rasmussen, S., 2015. The behavioral effects of single housing and environmental enrichment on adult zebrafish (Danio rerio). J. Am. Assoc. Lab. Anim. Sci. 54, 280–5.

Colwill, R.M., Raymond, M.P., Ferreira, L., Escudero, H., 2005. Visual discrimination learning in zebrafish (Danio rerio). Behav. Processes 70, 19–31. doi:10.1016/j.beproc.2005.03.001

Correia, A.D., Cunha, S.R., Scholze, M., Stevens, E.D., 2011. A novel behavioral fish model of nociception for testing analgesics. Pharmaceuticals 4, 665–680. doi:10.3390/ph4040665

Dawkins, M.S., 2004. Using behaviour to assess animal welfare. Anim. Welf. 13, 3–7. doi:10.1177/1091581812471490

Dawkins, M.S., 2003. Behaviour as a tool in the assessment of animal welfare. Zoology 106, 383–387. doi:10.1078/0944-2006-00122

Dawkins, M.S., 1988. Behavioural deprivation: a central problem in animal welfare. Appl. Anim. Behav. Sci. 20, 209–225. doi:10.1016/0168-1591(88)90047-0

Delcourt, J., Poncin, P., 2012. Shoals and schools: back to the heuristic definitions and quantitative references. Rev. Fish Biol. Fish. 22, 595–619. doi:10.1007/s11160-012-9260-z

Duncan, I.J.H., Olsson, I.A.S., 2001. Environmental enrichment: from flawed concept to pseudo-science, in: Proceedings of the 35th Congress of the International Society of Applied Ethology. Davis, CA, USA.

Ebbesson, L.O.E., Braithwaite, V. a, 2012. Environmental effects on fish neural plasticity and cognition. J. Fish Biol. 81, 2151–2174. doi:10.1111/j.1095-8649.2012.03486.x

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

Engeszer, R.E., Da Barbiano, L.A., Ryan, M.J., Parichy, D.M., 2007a. Timing and plasticity of shoaling behaviour in the zebrafish, Danio rerio. Anim. Behav. 74, 1269–1275. doi:10.1016/j.anbehav.2007.01.032

Engeszer, R.E., Patterson, L.B., Rao, A.A., Parichy, D.M., 2007b. Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish 4, 21–40. doi:10.1089/zeb.2006.9997

Engeszer, R.E., Ryan, M.J., Parichy, D.M., 2004. Learned social preference in zebrafish. Curr. Biol. 14, 881–884. doi:10.1016/j

Ernst, K., Puppe, B., Schön, P.C., Manteuffel, G., 2005. A complex automatic feeding system for pigs aimed to induce successful behavioural coping by cognitive adaptation. Appl. Anim. Behav. Sci. 91, 205–218. doi:10.1016/j.applanim.2004.10.010

Fowler, H., 1965. Curiosity and exploratory behavior. The MacMillan Company, New York, USA.

Franks, B., Champagne, F.A., Higgins, E.T., 2013. How enrichment affects exploration trade-offs in rats: implications for welfare and well-being. PLoS One 8, 8–12. doi:10.1371/journal.pone.0083578

Franks, B., Higgins, E.T., 2012. Effectiveness in humans and other animals: a common basis for well-being and welfare, in: Olson, J.M., Zanna, M.P. (Eds.), Advances in Experimental Social Psychology. Elsevier Inc. Academic Press, New York, pp. 285–346. doi:10.1016/B978-0-12-394281-4.00006-4

Fraser, D., 2008. Understanding Animal Welfare: The Science in its Cultural Context. Wiley-Blackwell, Oxford, UK.

Fraser, D., Nicol, C., 2011. Preference and Motivation Research, in: Appleby, M.C., Mench, J.A., Olsson, I.A.S., Hughes, B.O. (Eds.), Animal Welfare. CABI, Oxfordshire, UK, p. 344.

Fraser, D., Weary, D.M., Pajor, E.A., Milligan, B.N., 1997. A scientific conception of animal welfare that reflects ethical concerns. Anim. Welf. 6, 187–205.

Fureix, C., Meagher, R.K., 2015. What can inactivity (in its various forms) reveal about affective states in non-human animals? A review. Appl. Anim. Behav. Sci. 171, 8–24. doi:10.1016/j.applanim.2015.08.036

Gaffney, L.P., Franks, B., Weary, D.M., Von Keyserlingk, M.A.G., 2016. Coho salmon

(Oncorhynchus kisutch) prefer and are less aggressive in darker environments. PLoS One 11, 1–12. doi:10.1371/journal.pone.0151325

Galhardo, L., Almeida, O., Oliveira, R.F., 2011. Measuring motivation in a cichlid fish: an adaptation of the push-door paradigm. Appl. Anim. Behav. Sci. 130, 60–70. doi:10.1016/j.applanim.2010.12.008

Garner, J.P., 2005. Stereotypies and other abnormal repetitive behaviors: potential impact on validity, reliability, and replicability of scientific outcomes. ILAR J. 46, 106–117. doi:10.1093/ilar.46.2.106

Gerlai, R., 2014. Social behavior of zebrafish: from synthetic images to biological mechanisms of shoaling. J. Neurosci. Methods 234, 59–65. doi:10.1016/j.jneumeth.2014.04.028

Gerlai, R., 2012. Using zebrafish to unravel the genetics of complex brain disorders. Curr. Top. Behav. Neurosci. 3–24. doi:10.1007/7854

Gómez-Laplaza, L., Gerlai, R., 2010. Latent learning in zebrafish (Danio rerio). Behav. Brain Res. 208, 509–515. doi:10.1016/j.bbr.2009.12.031

Hall, D., Suboski, M.D., 1995. Visual and olfactory stimuli in learned release of alarm reactions by zebra danio fish (Brachydanio rerio). Neurobiol. Learn. Mem. doi:10.1037/0735-7036.109.1.76

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

Harding, E.J., Paul, E.S., Mendl, M., 2004. Cognitive bias and affective state. Nature 427, 312. doi:10.1038/427312a

Harlow, H.F., 1950. Learning and satiation of response in intrinsically motivated complex puzzle performance by monkeys. J. Comp. Physiol. Psychol. 43, 289–294. doi:10.1037/h0058114

Harper, C., Lawrence, C., 2011. The Laboratory Zebrafish. CRC Press, Boca Raton, FL, USA.

Hart, B.L., 1988. Biological basis of the behavior of sick animals. Neurosci. Biobehav. Rev. 12, 123–137. doi:10.1016/S0149-7634(88)80004-6

Hogan, J.A., 1967. Fighting and reinforcement in the Siamese fighting fish (Betta splendens). J. Comp. Physiol. Psychol. 64, 356–359. doi:10.1037/h0088042

Højsgaard, S., Halekoh, U., 2016. doBy: groupwise statistics, lsmeans, linear contrasts, utilities.

Hughes, B.O., Duncan, I.J.H., 1988. The notion of ethological "need", models of motivation and animal welfare. Anim. Behav. 36, 1696–1707. doi:10.1016/S0003-3472(88)80110-6

Hughes, R.N., 1997. Intrinsic exploration in animals: motives and measurement. Behav. Processes 41, 213–226. doi:10.1016/S0376-6357(97)00055-7

Huntingford, F.A., Adams, C., Braithwaite, V.A., Kadri, S., Pottinger, T.G., Sandøe, P., Turnbill, J.F., 2006. Current issues in fish welfare. J. Fish Biol. 68, 332–372. doi:10.1111/j.1095-8649.2005.01046.x

Huntingford, F.A., Kadri, S., 2014. Defining, assessing and promoting the welfare of farmed fish. Rev. Sci. Tech. Off. Int. Epiz 33, 233–244.

Jensen, M.B., Pedersen, L.J., 2008. Using motivation tests to assess ethological needs and preferences. Appl. Anim. Behav. Sci. 113, 340–356. doi:10.1016/j.applanim.2008.02.001

Keck, V.A., Edgerton, D.S., Hajizadeh, S., Swift, L.L., Dupont, W.D., Lawrence, C., Boyd, K.L., 2015. Effects of habitat complexity on pair-housed zebrafish. J. Am. Assoc. Lab. Anim. Sci. 54, 387–383.

Kirkwood, J.K., Hubrecht, R., 2001. Animal consciousness, cognition and welfare. Anim. Welf. 10, S5–S17. doi:10.1016/j.tins.2009.05.008

Kistler, C., Hegglin, D., Wurbel, H., Konig, B., 2011. Preference for structured environment in zebrafish (Danio rerio) and checker barbs (Puntius oligolepis). Appl. Anim. Behav. Sci. 135, 318–327. doi:10.1016/j.applanim.2011.10.014

Kotrschal, A., Taborsky, B., 2010. Environmental change enhances cognitive abilities in fish. PLoS Biol. 8, e1000351. doi:10.1371/journal.pbio.1000351

Krause, J., Hartmann, N., Pritchard, V.L., 1999. The influence of nutritional state on shoal choice in zebrafish, Danio rerio. Anim. Behav. 57, 771–775. doi:10.1006/anbe.1998.1010

Kuznetsova, A., Brockhoff, P.B., Bojesen, R.H.C., 2015. lmerTest: tests in linear mixed effects models.

Landeau, L., Terborgh, J., 1986. Oddity and the "confusion effect" in predation. Anim. Behav. 1372–1380. doi:10.1016/S0003-3472(86)80208-1

Lawrence, C., 2007. The husbandry of zebrafish (Danio rerio): a review. Aquaculture 269, 1–20. doi:10.1016/j.aquaculture.2007.04.077

Leach, M., Ambrose, N., Bowell, V., Morton, D., 2000. The development of a novel form of mouse cage enrichment. J. Appl. Anim. Welf. Sci. 3, 117–125. doi:10.1207/S15327604JAWS0302

Lindeyer, C.M., Reader, S.M., 2010. Social learning of escape routes in zebrafish and the stability of behavioural traditions. Anim. Behav. 79, 827–834. doi:10.1016/j.anbehav.2009.12.024

Lucon-Xiccato, T., Dadda, M., 2014. Assessing memory in zebrafish using the one-trial test. Behav. Processes 106, 1–4. doi:10.1016/j.beproc.2014.03.010

Maier, S.F., Seligman, M.E., 1976. Learned helplessness: theory and evidence. J. Exp. Psychol. Gen. 105, 3–46. doi:10.1037/0096-3445.105.1.3

Makowska, I.J., Weary, D.M., 2016. The importance of burrowing, climbing and standing upright for laboratory rats. R. Soc. Open Sci. 3, 160136. doi:10.1098/rsos.160136

Manteuffel, G., Langbein, J., Puppe, B., 2009. From operant learning to cognitive enrichment in farm animal housing: bases and applicability. Anim. Welf. 18, 87–95.

Martin, P., Bateson, P., 2007. Measuring Behaviour: An Introductory Guide, 3rd ed. Cambridge University Press, Cambridge, UK.

Martins, C.I.M., Galhardo, L., Noble, C., Damsgård, B., Spedicato, M.T., Zupa, W., Beauchaud, M., Kulczykowska, E., Massabuau, J.C., Carter, T., Planellas, S.R., Kristiansen, T., 2012. Behavioural indicators of welfare in farmed fish. Fish Physiol. Biochem. 38, 17–41. doi:10.1007/s10695-011-9518-8

Mason, G.J., Burn, C.C., 2011. Behavioural Restriction, in: Appleby, M.C., Mench, J.A., Olsson, I.A.S., Hughes, B.O. (Eds.), Animal Welfare. CABI, Oxfordshire, UK, pp. 98–119.

Matthews, M., Trevarrow, B., Matthews, J., 2002. A virtual tour of the guide for zebrafish users. Lab Anim. 31, 34–40.

Maximino, C., 2011. Modulation of nociceptive-like behavior in zebrafish (Danio rerio) by environmental stressors. Psychol. Neurosci. 4, 149–155. doi:10.3922/j.psns.2011.1.017

Maximino, C., Marques, T., Brito, D., Waneza, A., Manoel, A., Morato, S., Gouveia, A., 2010. Measuring anxiety in zebrafish: a critical review. Behav. Brain Res. 214, 157–171. doi:10.1016/j.bbr.2010.05.031

Mayer, J., 2004. The importance of understanding the natural history of novel species used in research. Lab Anim. 33, 32–33.

McClure, M.M., McIntyre, P.B., McCune, A.R., 2006. Notes on the natural diet and habitat of eight danionin fishes, including the zebrafish Danio rerio. J. Fish Biol. 69, 553–570. doi:10.1111/j.1095-8649.2006.01125.x

McMillan, F., 2004. Psychological well-being, in: Bekoff, M. (Ed.), Encyclopedia of Animal Behaviour. Greenwood Press, Westport, CT, pp. 1133–1144.

Meagher, R.K., Mason, G.J., 2012. Environmental enrichment reduces signs of boredom in caged mink. PLoS One 7, e49180. doi:10.1371/journal.pone.0049180

Meehan, C.L., Mench, J.A., 2007. The challenge of challenge: can problem solving opportunities enhance animal welfare? Appl. Anim. Behav. Sci. 102, 246–261. doi:10.1016/j.applanim.2006.05.031

Mellor, D., 2015. Positive animal welfare states and encouraging environment-focused and animal-to-animal interactive behaviours. N. Z. Vet. J. 63, 9–16. doi:10.1080/00480169.2014.926800

Mellor, D.J., 2016. Updating animal welfare thinking: moving beyond the "five freedoms" towards "a life worth living." Animals 6, 1–20. doi:10.3390/ani6030021

Miller, N., Gerlai, R., 2012. From schooling to shoaling: patterns of collective motion in zebrafish (Danio rerio). PLoS One 7. doi:10.1371/journal.pone.0048865

Miller, N., Gerlai, R., 2011a. Shoaling in zebrafish: what we don't know. Rev. Neurosci. 22, 17–25. doi:10.1515/RNS.2011.004

Miller, N., Gerlai, R., 2011b. Redefining membership in animal groups. Behav. Res. Methods 43, 964–970. doi:10.3758/s13428-011-0090-z

Miller, N., Gerlai, R., 2008. Oscillations in shoal cohesion in zebrafish (Danio rerio). Behav. Brain Res. 193, 148–151. doi:10.1016/j.bbr.2008.05.004

Mills, D., Marchant-Forde, J., McGreevy, P., Morton, D., Nicol, C., Phillips, C., Sandøe, P., Swaisgood, R. (Eds.), 2010. The Encyclopedia of Applied Animal Behaviour and Welfare. CABI, Wallingford, U.K.

Mueller, K.P., Neuhauss, S.C.F., 2012. Automated visual choice discrimination learning in zebrafish (Danio rerio). J. Integr. Neurosci. 11, 73–85. doi:10.1142/S0219635212500057

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

Newberry, R.C., 1995. Environmental enrichment: increasing the biological relevance of captive environments. Appl. Anim. Behav. Sci. 44, 229–243. doi:10.1016/0168-1591(95)00616-Z

Olsson, I.A.S., Dahlborn, K., 2002. Improving housing conditions for laboratory mice: a review of "environmental enrichment." Lab. Anim. 36, 243–270. doi:10.1258/002367702320162379

Olsson, I.A.S., Nevison, C.M., Patterson-Kane, E.G., Sherwin, C.M., Van De Weerd, H.A., Wurbel, H., 2003. Understanding behaviour: the relevance of ethological approaches in laboratory animal science. Appl. Anim. Behav. Sci. 81, 245–264. doi:10.1016/S0168-1591(02)00285-X

Olsson, I.A.S., Wurbel, H., Mench, J.A., 2011. Behaviour, in: Appleby, M.C., Mench, J.A., Olsson, I.A.S., Hughes, B.O. (Eds.), Animal Welfare. CABI, pp. 138–154.

Pagnussat, N., Piato, A.L., Schaefer, I.C., Blank, M., Tamborski, A.R., Guerim, L.D., Bonan, C.D., Vianna, M.R.M., Lara, D.R., 2013. One for all and all for one: the importance of shoaling on behavioral and stress responses in zebrafish. Zebrafish 10, 338–342. doi:10.1089/zeb.2013.0867

Paul, E.S., Harding, E.J., Mendl, M., 2005. Measuring emotional processes in animals: the utility of a cognitive approach. Neurosci. Biobehav. Rev. 29, 469–91. doi:10.1016/j.neubiorev.2005.01.002

Pérez-Escudero, A., Vicente-Page, J., Hinz, R.C., Arganda, S., de Polavieja, G.G., 2014. idTracker: tracking individuals in a group by automatic identification of unmarked animals. Nat. Methods 11, 743–8. doi:10.1038/nmeth.2994

Pitcher, T.J., 1986. Functions of shoaling behaviour in teleosts, in: The Behaviour of Teleost Fish. Springer US, London, UK, pp. 294–337.

Pitcher, T.J., 1983. Heuristic definitions of fish shoaling behaviour. Anim. Behav. 31, 611–613. doi:10.1016/S0003-3472(83)80087-6

Pitcher, T.J., Parrish, J.K., 1993. Functions of shoaling behaviour in teleosts, in: Pitcher, T.J. (Ed.), Behaviour of Teleost Fishes. Chapman & Hall, London, UK, pp. 363–439.

Pritchard, V., Lawrence, J., Butlin, R.K., Krause, J., 2001. Shoal choice in zebrafish, Danio rerio: the influence of shoal size and activity. Anim. Behav. 62, 1085–1088. doi:10.1006/anbe.2001.1858

Puppe, B., Ernst, K., Schon, P.C., Manteuffel, G., 2007. Cognitive enrichment affects behavioral reactivity in domestic pigs. Appl. Anim. Behav. Sci. 105, 75–86. doi:10.1016/j.applanim.2006.05.016

Reed, B., Jennings, M., 2011. Guidance on the housing and care of zebrafish Danio rerio. West Sussex, UK.

Reinhardt, V., 2004. Common husbandry-related variables in biomedical research with animals. Lab. Anim. 38, 213–235. doi:10.1258/002367704323133600

Rey, S., Huntingford, F.A., Boltana, S., Vargas, R., Knowles, T.G., Mackenzie, S., 2015. Fish can show emotional fever: stress-induced hyperthermia in zebrafish. Proc. R. Soc. B 282, 1–7. doi:10.1098/rspb.2015.2266

Richter, S.H., Garner, J.P., Würbel, H., 2009. Environmental standardization: cure or cause of poor reproducibility in animal experiments? Nat. Methods 6, 257–261. doi:10.1038/NMETH.1312

Rose, J.D., Arlinghaus, R., Cooke, S.J., Diggles, B.K., Sawynok, W., Stevens, E.D., Wynne, C.D.L., 2014. Can fish really feel pain? Fish Fish. 15, 97–133. doi:10.1111/faf.12010

Rosenthal, G.G., Ryan, M.J., 2005. Assortative preferences for stripes in danios. Anim. Behav. 70, 1063–1066. doi:10.1016/j.anbehav.2005.02.005

Ruhl, N., McRobert, S.P., 2005. The effect of sex and shoal size on shoaling behaviour in Danio rerio. J. Fish Biol. 67, 1318–1326. doi:10.1111/j.0022-1112.2005.00826.x

Russell, P.A., 1983. Psychological studies of exploration in animals: a reappraisal, in: Archer, J., Birke, L. (Eds.), Exploration in Animals and Humans. Van Nostrand Reinhold Co. Ltd., Wokingham, UK, pp. 22–54.

Salvanes, A.G. V., Moberg, O., Ebbesson, L.O.E., Nilsen, T.O., Nilsen, O., Jensen, K.H., Braithwaite, V.A., 2013. Environmental enrichment promotes neural plasticity and cognitive ability in fish. Proc. R. Soc. B 280, 20131331.

Salvanes, A.G. V, Moberg, O., Braithwaite, V.A., 2007. Effects of early experience on group behaviour in fish. Anim. Behav. 74, 805–811. doi:10.1016/j.anbehav.2007.02.007

Schneider, H., 2011. Measuring agonistic behavior in zebrafish, in: Kalueff, A. V., Cachat, J.M. (Eds.), Zebrafish Neurobehavioral Protocols, Neuromethods. Springer Science+Business Media, LLC, pp. 125–134. doi:10.1007/978-1-60761-953-6

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

Serra, E.L., Medalha, C.C., Mattioli, R., 1999. Natural preference of zebrafish (Danio rerio) for a dark environment. Brazilian J. Med. Biol. Res. 32, 1551–1553. doi:10.1590/S0100-879X1999001200016

Shelton, D.S., Price, B.C., Ocasio, K.M., Martins, E.P., Shelton, D.S., Price, B.C., Ocasio, K.M., Martins, E.P., 2015. Density and group size influence shoal cohesion, but not coordination in zebrafish (Danio rerio). J. Comp. Psychol. 129, 72–77. doi:10.5061/dryad.90n5f

Sherwin, C.M., 2004. The influences of standard laboratory cages on rodents and the validity of research data. Anim. Welf. 13, 9–15.

Sneddon, L.U., 2015. Pain in aquatic animals. J. Exp. Biol. 218, 967–976. doi:10.1242/jeb.088823

Sneddon, L.U., 2006. Ethics and welfare: pain perception in fish. Bull. Eur. Assoc. Fish Pathol. 26, 6–10.

Sneddon, L.U., 2003. The evidence for pain in fish: the use of morphine as an analgesic. Appl. Anim. Behav. Sci. 83, 153–162. doi:10.1016/S0168-1591(03)00113-8

Sneddon, L.U., Elwood, R.W., Adamo, S.A., Leach, M.C., 2014. Defining and assessing animal pain. Anim. Behav. 97, 201–212. doi:10.1016/j.anbehav.2014.09.007

Speedie, N., Gerlai, R., 2008. Alarm substance induced behavioral responses in zebrafish (Danio rerio). Behav. Brain Res. 188, 168–177. doi:10.1016/j.bbr.2007.10.031

Spence, R., Fatema, M.K., Ellis, S., Ahmed, Z.F., Smith, C., 2007. Diet, growth and recruitment of wild zebrafish in Bangladesh. J. Fish Biol. 71, 304–309. doi:10.1111/j.1095-8649.2007.01492.x

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

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biol. Rev. Camb. Philos. Soc. 83, 13–34. doi:10.1111/j.1469-185X.2007.00030.x

Spence, R., Magurran, A.E., Smith, C., 2011. Spatial cognition in zebrafish: the role of strain and rearing environment. Anim. Cogn. 14, 607–612. doi:10.1007/s10071-011-0391-8

Špinka, M., 2006. How important is natural behaviour in animal farming systems? Appl. Anim. Behav. Sci. 100, 117–128. doi:10.1016/j.applanim.2006.04.006

Špinka, M., Wemelsfelder, F., 2011. Enrivonmental Challenge and Animal Agency, in: Appleby, M., Mench, J., Olsson, I.A.S., Hughes, B.O. (Eds.), Animal Welfare. CABI, Oxfordshire, UK, pp. 27–43.

Steenbergen, P.J., Bardine, N., 2014. Antinociceptive effects of buprenorphine in zebrafish larvae: An alternative for rodent models to study pain and nociception? Appl. Anim. Behav. Sci. 152, 92–99. doi:10.1016/j.applanim.2013.12.001

Stolba, A., Wood-Gush, D.G., 1989. Behaviour of pigs in a semi-natural environment. Anim. Prod. 48, 419–425.

Strand, D., Utne-Palm, A., Jakobsen, P., Braithwaite, V., Jensen, K., Salvanes, A., 2010. Enrichment promotes learning in fish. Mar. Ecol. Prog. Ser. 412, 273–282. doi:10.3354/meps08682

Sullivan, M., Lawrence, C., Blache, D., 2016. Why did the fish cross the tank? Objectively measuring the value of enrichment for captive fish. Appl. Anim. Behav. Sci. 174, 181–188.

Suriyampola, P.S., Shelton, D.S., Shukla, R., Roy, T., Bhat, A., Martins, E.P., 2015. Zebrafish social behavior in the wild. Zebrafish 13, 1–8. doi:10.1089/zeb.2015.1159

von Krogh, K., Sørensen, C., Nilsson, G.E., Øverli, Ø., 2010. Forebrain cell proliferation, behavior, and physiology of zebrafish, Danio rerio, kept in enriched or barren environments. Physiol. Behav. 101, 32–9. doi:10.1016/j.physbeh.2010.04.003

Ward, A.J.W., Webster, M.M., Hart, P.J.B., 2007. Social recognition in wild fish populations. Proc. R. Soc. B 274, 1071–1077. doi:10.1098/rspb.2006.0231

Way, G.P., Ruhl, N., Snekser, J.L., Kiesel, A.L., McRobert, S.P., 2015. A comparison of methodologies to test aggression in zebrafish. Zebrafish 12, 144–151.

Weed, J.L., Raber, J.M., 2005. Balancing animal research with animal well-being: establishment of goals and harmonization of approaches. ILAR J. 46, 118–28. doi:10.1093/ilar.46.2.118

Wickham, H., 2017. tidyverse: easily install and load "tidyverse" packages.

Wickham, H., 2009. ggplot2: elegant graphics for data analysis. Springer New York.

Wilkes, L., Owen, S.F., Readman, G.D., Sloman, K.A., Wilson, R.W., 2012. Does structural enrichment for toxicology studies improve zebrafish welfare? Appl. Anim. Behav. Sci. 139, 143–150. doi:10.1016/j.applanim.2012.03.011

Williams, F.E., White, D., Messer, W.S., 2002. A simple spatial alternation task for assessing memory function in zebrafish. Behav. Processes 58, 125–132.

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

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K.M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N., Cachat, J., Kalueff, A. V., 2010. Analyzing habituation responses to novelty in zebrafish (Danio rerio). Behav. Brain Res. 208, 450–457. doi:10.1016/j.bbr.2009.12.023

Wood-Gush, D.G., Vestergaard, K., 1989. Exploratory behavior and the welfare of intensively kept animals. J. Agric. Ethics 2, 161–169.

Wood-Gush, D.G.M., Vestergaard, K., 1991. The seeking of novelty and its relation to play. Anim. Behav. 42, 599–606. doi:10.1016/S0003-3472(05)80243-X

Wood-Gush, D.G.M., Vestergaard, K., Petersen, H. V., 1990. The significance of motivation and environment in the development of exploration in pigs. Biol. Behav. 15, 39–52.

Würbel, H., 2009. Ethology applied to animal ethics. Appl. Anim. Behav. Sci. 118, 118–127. doi:10.1016/j.applanim.2009.02.019

Würbel, H., 2002. Behavioral phenotyping enhanced - beyond (environmental) standardization. Genes, Brain Behav. 1, 3–8. doi:10.1046/j.1601-1848.2001.00006.x

Würbel, H., 2001. Ideal homes? Housing effects on rodent brain and behaviour. Trends Neurosci. 24, 207–211. doi:10.1016/S0166-2236(00)01718-5

Zala, S.M., Maattanen, I., 2013. Social learning of an associative foraging task in zebrafish. Naturwissenschaften 100, 469–472. doi:10.1007/s00114-013-1017-6

Zebunke, M., Puppe, B., Langbein, J., 2013. Effects of cognitive enrichment on behavioural and physiological reactions of pigs. Physiol. Behav. 118, 70–79. doi:10.1016/j.physbeh.2013.05.005