Potential environmental enrichment for zebrafish used in regulatory toxicology

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

.....(Luanne Wilkes)

ABSTRACT

The aim of environmental enrichment is to alter the environment of a captive animal in a way that results in improved mental and physical welfare. The technique has been utilised effectively for many years for captive mammals in a variety of settings. However, until now it has never been considered as a way of improving the welfare of aquatic animals such as fish.

Fish that are used in regulatory toxicology studies are at present maintained solely in barren tank environments. Little is known about how these types of environments affect the well-being of the animals residing there and whether they impact either physiological heath or behavioural repertoire. This thesis aims to address this gap in the knowledge regarding the potential for environmental enrichment to improve the welfare of fish used in regulatory toxicology. More specifically it looks at two types of enrichment and the effects of these on the commonly used model species, the zebrafish (*Danio rerio*).

The first type of enrichment studied was glass rod structures of varying heights provided to increase tank complexity and provide refuge. The glass structures did not produce any quantifiable benefits in unstressed fish and appeared to delay the formation of stable social hierarchies. When fish were stressed by a period of chasing, the presence of the glass rods appeared to reduce the magnitude of the cortisol response. Whilst this could be viewed as a potential benefit, it was felt that it would not outweigh the costs of this type of enrichment.

The second type of enrichment studied was provision of airstones. Again, no clear evidence was found that fish in tanks with airstones experienced an improvement in welfare. The main observation was the vast increase in mortality in tanks containing these airstones, in particular, those of a smaller size. Regardless of the physiological cause underlying this result, this can only be viewed as a negative consequence and one that appears to rule out airstones as an effective form of enrichment for this species and strain of fish.

It was also observed that both stress and the presence of enrichment influenced the absolute deviation from the mean in several endpoints. Since changes in endpoint variation will have effects both on the number of animals required to statistically measure environmentally relevant effects this is a factor that should be considered when researching methods of environmental enrichment.

Finally, results from these studies suggest the possibility that laboratory zebrafish do not require the addition of environmental enrichment to tanks in order to promote maximum welfare. Furthermore, as considerable costs would be involved in implementing many types of enrichment (relating to manufacture, cleaning, incompatibility of results with previous studies etc.) it is likely that observed benefits would have to be both substantial and well established in order for changes in regulatory guidelines to take place. For a species such as zebrafish that are extremely easy to breed and maintain in the laboratory with minimal amounts of disease, social problems or mortalities, it may be that current conditions are satisfactory.

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Table 5.1 Mean absolute deviation observed in all endpoints.

LIST OF GENERAL ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ASV	Air saturation value
BCE	Before the Common Era
cDNA	Complimentary deoxyribonucleic acid
cm	Centimetre
CO_2	Carbon dioxide
CRF	Corticotropin releasing factor
DA	Dopamine
dB	Decibels
DDT	DichloroDiphenylTrichloroethane
DEFRA	Deparment for Environment, Food and Rural Affairs
dpf	Days post fertilisation
ELISA	Enzyme linked immunosorbent assay
FAWC	Farm Animal Welfare Council
FSBI	Fisheries Society of the British Isles
g	Gravity
g	Grams
G6Pase	Glucose-6-phosphase
GR	Glucocorticoid receptor
h	Hours
HPA	Hypothalamic pituitary adrenal
HPI	Hypothalamic pituitary interenal
HSP	Heat Shock Protein
Hz	Hertz
ISAE	International Society for Applied Ethography
l	Litres
L:D	Light:Dark
LD_{50}	Lethal dose, 50% (median lethal dose)
MAD	Mean absolute deviation
mg	Milligrams
min	Minutes
ml	Millilitres
mm	Millimetres
mM	Millimolar
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NE	Norepinephrine
ng	Nanograms
O_2	Oxygen
OECD	Organisation for Economic Co-operation and Development
Oligo-dT	Oligodeoxythymidylic acid
PBS	Phosphate buffered saline
P/C	Predictability/Control ratio
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyrubate carboxykinase
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RSPCA	Royal Society for the Prevention of Cruelty to Animals

s	Seconds
UK	United Kingdom
WIK	Wild Indian Karyotype
5-HIAA	5-hydroxyindoleacetic acid (serotonin metabolite)
5-HT	5-hydroxytryptamine (serotonin)
%	Percent
°	Degrees
°C	Degrees centigrade
µl	Microlitres
µM	Micromolar

LIST OF SPECIES NAMES

African catfish (*Clarias gariepinus*)
American mink (*Mustelidae vison*)
Arctic charr (*Salvelinus alpinus*)
Atlantic cod (*Gadus morhua*)
Atlantic salmon (*Salmo salar*)
Australian crimson-spotted rainbow fish (*Melanotaenia fluciatilis*)
Brown trout (*Salmo trutta*)
Butterfly splitfins (*Ameca splendens*)
Carp (*Cyprinus carpio*)
Coho salmon (*Oncorhynchus kisutch*)
Crucian carp (*Carassius carassius*)
Eurasian perch (*Perca fluviatilis*)
Fighting fish (*Betta splendens*)
Great sturgeon (*Huso huso*)
Greenback flounder (*Rhombosolea tapirina*)
Nile tilapia (*Oreochromis niloticus*)
Paddlefish (*Polyodon spathula*)
Pearl cichlids (*Geophagus brasiliensis*)
Pig (*Sus scrofa*)
Rainbow trout (*Oncorhynchus mykiss*)
Red porgy (*Pagrus pagrus*)
Sea bass (*Decentrarchus labrax*)
Zebrafish (*Danio rerio*)

CHAPTER 1

GENERAL INTRODUCTION

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1.1 Animal welfare

"The greatness of a nation and its moral progress can be judged by the way its animals are treated" Mahatma Gandhi

This introduction aims to give a brief synopsis of animal welfare prior to elaborating further on the concept of environmental enrichment. Furthermore, I will provide some information on both the study species and regulatory toxicology and give some background regarding the aims of this PhD. For clarification, section 1.1 deals with the overall topic of animal welfare and mostly focuses on how this has been applied to mammalian subjects. This is for the simple reason that most efforts to improve welfare, and therefore the associated research and legislations, are at present involved with these types of animals. Sections 1.2 and 1.3 will focus entirely on issues associated with fish welfare.

I would also briefly like to add a note regarding the terminology used within this introduction. Whilst I have attempted, wherever possible, to refrain from using anthropomorphic terms, particularly regarding emotions and feelings of non-human animals, in some cases I feel they are appropriate, or are the most understandable term to use. This does not mean, however, that I consider the word to mean the same thing in terms of animal and human experience. The "feelings" and subjective experiences of non-human animals, particularly fish, is

a subject that arouses much debate in the current scientific community. Although issues of sentience and consciousness are, of course, very pertinent to the research conducted herein, I feel that a detailed philosophical discussion upon this topic does not fall within the remit of this PhD.

I would also like to make a short comment regarding the use of inverted commas within this introduction. The purposes of these have been to denote words or phrases that may be potentially controversial. However, in the circumstances in which they have been used I feel that they are the most suitable or logical words to use.

1.1.1 Defining welfare

Animal welfare is concerned with the way that individuals cope within different environments (Broom and Johnson, 1993). This being true, then good welfare is only achieved when the state of mental and physical health of the individual indicate that it is living in harmony with its environment (Wiepkema and Koolhaas, 1992). However, many people feel that the issues of animal welfare run outside those of mainstream biology and question the possibility of welfare studies obtaining anything more than an unscientific collection of assumptions that are impossible to test, about what animals might be feeling (Dawkins, 1998). In some cases, animal welfare is equated with biological fitness implying that, for welfare to be good, the survival and subsequent reproductive potential of the individual must be optimised. Most often, however, it is the physical and mental health of the animal that is of concern. If welfare is poor these factors

may all be accompanied by similar symptoms, such as poor physical health, behavioural abnormalities and the activity of the pituitary-adrenocortical system, resulting in the production of 'stress' hormones (Mason and Mendl, 1993).

In humans, there are several theories relating to what constitutes good welfare. The 'mental-state theory' suggests that for welfare to be good a positive mental state, resulting from pleasurable experiences, must be created. The 'desire' or 'preference' theory similarly suggests that good welfare demands that the intrinsic desires of the individual are met. Finally, the 'objective-list theory' is slightly different in that it assumes that there are certain things required by the individual for good well-being, whether or not they are desired or pleasurable (Appleby and Sandoe, 2002). These theories have been compared to those applied to animal welfare by Fraser (1997), who states that the three main approaches to animal welfare can be described as feelings, functioning and natural living. The term feeling suggests that animals should 'feel well' as a result of being free from pain, distress and other negative mental states. Functioning, conversely, refers to the physical and behavioural needs of the animal being met and is a theory that is widely attributed to Broom (1991). The final concept stresses the importance of living in "natural environments" which provide the animal with the opportunity to fulfill their range of natural behaviours and functions. The ideal approach to animal welfare would therefore seem to be one that encompasses all three of the concepts mentioned. Provided that the natural behaviour and subjective feelings of the individual promote positive biological functioning, then it is likely that all three approaches will lead to

similar conclusions regarding welfare of that individual (Duncan and Fraser, 2003).

It would seem apparent that a brief definition of welfare is simply not possible and efforts to define it depend largely on the context in which it is being used (Appleby and Sandoe, 2002). Subjective terms such as ‘well-being’ and ‘quality of life’ are often used synonymously with ‘welfare’. For the purposes of this study, we will consider welfare to involve both physical and mental well-being. This means that, in addition to being free from injury, disease and deformity, animals experiencing good welfare must also possess a positive mental state. This includes not being fearful, not attempting to get away from situations that they find aversive and not frustrated as a result of deprivation (Dawkins, 2004).

1.1.2 Philosophy and ethics of animal welfare

For the study of animal welfare to be deemed relevant, or required, then we must accept that animals have the mental capacity for experiencing pain, hunger, thirst, boredom and other negative conscious states (Duncan, 1993). If we accept this, then the issues concerned become ones related to moral behaviour and can be studied within the field of ethics (Broom, 2003). As such, philosophy has contributed considerably to the discussion of animal welfare, and philosophers have done much to raise awareness and the public profile of issues concerned (Appleby and Sandoe, 2002). As scientific knowledge of animal physiology and behaviour has increased over recent years, the unique and sophisticated abilities of different animals have begun to be appreciated,

both within and outside of the scientific community. Increasingly people are beginning to include non-humans within the category of “sentient beings who share characteristics with me”, and are subsequently increasingly likely to direct moral actions towards them (Broom, 2006). Incidentally, attitudes of human societies towards the animals with which they are in contact appear to be correlated with the education level of the people, rather than their affluence. The majority, though not all, of educated people believe that if we use an animal in a way that benefits us, then we have an obligation to that animal. This obligation is most commonly represented in avoidance of poor welfare (Broom, 2006).

Sandoe *et al.* (2003) suggests that attitudes towards animal rights can be separated into four main groups. The first, ‘utilitarianism’ takes the point of view that each individual, whether human or non-human, is equally important and so deserves equal consideration regarding welfare. The second, referred to as the ‘animal rights view’ goes further to say that it is never justified to sacrifice one individual to benefit another. The ‘species-integrity’ view maintains that humans have a responsibility both to the individual animal and to the species as a whole. Finally, the ‘agent-centred view’ takes a much different approach in suggesting that what we do to animals is important purely because of how it affects us as moral agents. For example, if we inflict harm on animals, then it exposes a flaw of character within ourselves. These four approaches are by no means independent from each other, and belief in any of them may lead to the same conclusions regarding specific welfare issues. Reason would suggest that a hybrid of all four views would be the optimum approach (Sandoe *et al.*, 2003).

This thesis is not directly concerned with the philosophy behind animal welfare and rights, although a short background has been provided here. For the purposes of the work, it will be assumed that good animal welfare is both desirable and necessary for all animals that are used by humans for our own benefit. Justification for this assumption will be addressed in the following sections.

1.1.3 Why are welfare studies needed?

Organisms have evolved and adapted over millions of years to survive the fluctuation of conditions within their natural environments. As such they are perfectly able to survive in life conditions that are not entirely stable or ideal (Wiepkema and Koolhaas, 1993). Every animal has a suite of behavioural and physiological mechanisms, which enable them to deal with such changing conditions within their surroundings, The effectiveness of these mechanisms is aided considerably by the reliability of knowledge possessed by the animal regarding existing causal and spatial relationships. However, within captivity, this knowledge is either reduced or absent, and adaptive mechanisms may no longer be suitable (Wiepkema and Koolhaas, 1993).

In 1987, Wiepkema devised a conceptual framework for the scientific assessment of animal welfare. In this model he labelled “Istwerte” the actual values of the external world as perceived by the animals. The conditions that are actually wanted or preferred by the animal were designated as “Sollwerte”. When the animal perceives a mismatch between Istwerte and Sollwerte,

behavioural and physiological mechanisms are activated to resolve the mismatch. Responses of small magnitude result in rapid restoration of homeostasis. However, mismatches of greater magnitude, requiring large behavioural or physiological responses result in stress and possible pathological problems. As Sollwerte are the product of the organism's life history and evolutionary history, a relatively sudden change to conditions within captivity means that these 'preferred' values are no longer applicable to their current situation (Bracke and Hopster, 2006). As such, it is highly likely that an action of physiological or behavioural response that is effective in the wild may become pathological and counterproductive within the confines of a captive environment (Dawkins, 1998).

Wiepkema and Koolhaas (1993) suggest that the predictability and control of positive and negative reinforcers within a particular environment are important for the welfare of the individuals living within it. In other words, animals appear to be under least stress when they can foresee or control negative or positive events. Within captivity, where conditions may be very different from those in which they evolved, it may be that the ratio between predictability and control (P/C) is no longer suitable. Studies performed by Wiepkema and Koolhaas seem to suggest that a moderate P/C ratio should be optimum, as mild and temporary stress may be beneficial in optimising vigilance of the animals. This would suggest that stress is not inherently detrimental, as animals have evolved behavioural and physiological mechanisms to deal with it. Only when the stress is prolonged and cannot easily be predicted and controlled, such as in unsuitable captive environments, does it become a problem (Moberg, 2000).

1.1.4 History of animal welfare

Since the dawn of civilisation, people's lives have been inextricably linked with those of non-human animals, as food, working machines and companions. Many religions have sustained beliefs which pertain to animals as being sacred. As early as 3300 BCE in the Indus Valley Civilisation, it was believed that after death, humans returned as animals, a belief that still survives in some modern Indian religions. As people have become more educated and scientific knowledge has progressed, however, it has become apparent how sophisticated and advanced many animals are. Perhaps because of this, many more people are now concerned with animal welfare and well-being.

Until the 19th century there was no legislation in the U.K. concerning the treatment of animals, and people were free to do as they pleased with animals that they owned. However, in 1822, the MP Richard Martin put a bill through parliament that gave protection from cruelty to cattle, horses and sheep. Two years later, in 1824, he co-founded the Society for the Prevention of Cruelty to Animals (SPCA), which later became the RSPCA when it was given royal support from Queen Victoria. Many years later, in 1964, Ruth Harrison wrote her groundbreaking and startling book entitled Animal Machines, which was an indignant assault on the excruciatingly intensive housing of veal calves, chickens and pigs. In response to this, the government set up the Farm Animal Welfare Advisory Committee in 1967, which later became the Farm Animal Welfare Council (FAWC) in 1979.

The FAWC considers that an animal's welfare should be considered in terms of 'five freedoms', which define ideal states, rather than standards for acceptable welfare (see www.fawc.org.uk). These are:

1. Freedom from hunger and thirst
2. Freedom from discomfort
3. Freedom from pain, injury or disease
4. Freedom from fear and distress
5. Freedom to express normal behaviour

These five freedoms are closely related to the five domains in which welfare can be compromised as stated by Mellor and Staffords (2001) and reviewed in Huntingford *et al*. (2006). Now there are both governmental and non-governmental bodies whose sole responsibility is to monitor and regulate the use of animals by humans. The abuse of animals in the U.K. is a punishable offence and is strictly controlled (www.legislation.gov.uk).

1.1.5 Which animals should be protected?

Due to the variety and complexity of responses and behaviour seen in animals, humans generally consider ourselves to have more obligations to an animal than to a micro-organism or a plant. This is especially true with the more complex animals (Broom, 2007). However, it is still the case that we feel more of a kinship with some animals (such as companion species or primates most similar to ourselves) than we would for a fish or reptile for example. For this reason, there needs to be a scientific basis to determine which animals require protection (Feinberg, 2009).

Broom and Littin (Unpublished, see Broom, 2007) have developed a list of criteria by which we should decide on the animals that should be protected. This includes: complexity of life and behaviour, learning ability, functioning of the brain and nervous system, indications of pain or distress, studies illustrating the biological basis of suffering and other feelings such as fear and anxiety and finally, indications of awareness based on observations and experimental work. Increased scientific knowledge has tended to show that the abilities and functioning of non-human animals are more complex than have previously been assumed, and many animals respond to distress and painful experiences in ways that are comparable to those of humans. For this reason the issue of animal welfare has become one of huge public concern.

In determining which animals should be the focus of welfare efforts, many references are made to the quality of “sentience”. Broom (2007) describes a sentient being as one that has some ability to evaluate the actions of others in relation to itself and third parties, to remember some of its own actions and their consequences, to assess risk, to have some feelings, and to have some degree of awareness. In particular, the ability to feel pain as an aversive sensation and feeling associated with actual and potential tissue damage should be of huge concern. The ability to feel pain is generally included amongst the capabilities of sentient animals, and pain is an important cause of poor welfare (Broom, 2001). As stated in the famous quote by Jeremy Bentham “The question is not can they reason? nor can they talk? but can they suffer?” (Bentham, 1789).

1.1.6 How should welfare be measured?

The scientific framework for welfare assessment regards animals as having cognitive-emotional control systems that have evolved in the course of evolution to deal with a variable environment (Bracke and Hopster, 2006). Where poor welfare conditions are present, the animals are provided with an environmental challenge, which results in both physiological and behavioural responses that are often highly integrated (Wiepkema and Koolhaas, 1993).

Studying the behavioural responses to poor welfare is often seen as beneficial due to the non-invasive and non-intrusive nature of the methods required (Dawkins, 2004). In addition to this, they are often quicker and easier to obtain than physiological measures and are considered to more directly reflect the animals' subjective interpretation of their environment (Rushen, 2000). In humans we would use the term “feelings” or “emotions”. Even as early as 1872, Darwin described behaviour as the “expression of the emotions”.

Since 1969, when Thorpe stated that the ability to perform natural patterns of behaviour was an important criterion of welfare, the concept of “natural behaviour” has become a widely used template for determination of what constitutes good versus poor welfare conditions. Bracke and Hopster (2006) describe natural behaviour as “behaviour that animals tend to perform under natural conditions, because it is pleasurable and promotes biological functioning”. This is incorporated into the fifth of the five freedoms, which states that animals should be free to express natural behaviour (FAWC, 1992). For

these purposes we interpret the term natural to define behaviour shown in natural surroundings as opposed to that shown in artificial or high-tech environments. Some types of natural behaviour are intrinsically motivated, and also include behavioural needs which must be satisfied for the animal to avoid frustration. This means that these needs are controlled and satisfied by the performance of the behaviour, rather than by the functional or physiological consequences resulting from this. As a result, foraging and the consumption of food could, in some cases, be classified as two separate and distinct needs, both of which must be satisfied for good welfare of the animal (Bracke and Hopster, 2006).

This, however, leads us to the question of what constitutes unnatural behaviour. Often quoted are those behaviours such as stereotypies, displacement, vacuum and substitute activities, all of which are seen in various animals within captive environments, but rarely observed in the wild (Dawkins, 2004). Due to this fact, they are generally viewed as signs of poor welfare conditions. Stereotypies in particular, which involve the animal continuously repeating a seemingly functionless behavioural pattern for extensive periods of time, are usually seen as unnatural and problematic. It has been speculated that they stem from previous attempted escape behaviour and represent a ritualised form of this behaviour. They may also reflect the absence of exploratory behaviour and or normal social contacts (Wiepkema and Koolhaas, 1993). However, Dawkins (2004) argues that it is not the naturalness of the behaviour that is important factor, but the effect it has on the animal's physical health. For example, stereotypies may indicate that welfare has been poor in the past, but conditions

may be optimal in the present. If the behaviour has no negative consequences for the animal's health then it is debatable whether this should be seen as a problem.

Aside from studies of whether behaviour should be classed as 'natural', behavioural measures have a variety of other applications. Preference tests in particular can be extremely useful, as knowledge of situations that the animal prefers or wishes to get away from have an important role in welfare. It may also be the case that animals find certain circumstances negatively reinforcing, even when their health or fitness is not actually threatened, and so such tests can give us insight into welfare "as perceived by the animal" not just as observed by ourselves (Dawkins, 1998). Similarly, changes in other behavioural indices such as activity levels, and aggression (Dawkins, 1998), together with observed increases in self-harming and over-grooming, poor maternal care and infanticide and apathy may be extremely useful to assess for some species, especially when compared to those observed in the wild (www.defra.org.uk).

Increasingly so, behavioural measures are being acknowledged as important tools in the study of animal welfare (Gonyou, 1994). The International Society for Applied Ethology (ISAE), which was created in 1966 as the Society for Veterinary Ethology, is devoted to the study of ethology and other behavioural sciences relevant to human-animal interactions such as farming, wildlife management, the keeping of companion and laboratory animals and the control of pests (www.applied-ethology.org). Similarly, the FAWC now recommends to

the British Government that ethological studies be an integral part of animal welfare decisions (Gonyou, 1994).

Physiological measures used in the study of animal welfare most notably include the measurement of plasma levels of catecholamines or corticosteroids, often labelled “stress hormones”. These are used as indicators of stress and consequently of a lack of well-being (Wiepkema and Koolhaas, 1993). However, the problem with such physiological measures is that they may merely reflect the normal activities of the physiological mechanisms of an organism when adapting to existing conditions. Equally problematic is the generality of the stress response in situations regarded either as pleasant or unpleasant (Dawkins, 1998). For example, high levels of the hormone cortisol may be present both when an animal is experiencing fear and pain or excitement and pleasure.

Other physiological indicators of poor welfare include monitoring of the heart rate which can be measured using radio telemetry. Production of prolactin has also been found to vary in response to stress, and the ratio of neutrophils to lymphocytes can give an index of adrenal cortex activity. Similarly, measurement of reproductive hormones such as testosterone, oestrogen and progesterone can give information on the reproductive state and health of the individual. Non-invasive physiological indicators include body temperature, which often rises in response to stress due to the Sympathetic-Adrenal-Medulla axis. Changes in body mass and condition can also indicate stress or disease.

Any simple conclusion based on just one physiological stress variable is bound to be suspect, and would ideally be supplemented with behavioural measures of aversion (Dawkins, 1998). As previously mentioned, an environmental challenge results in an integrated physiological and behavioural response, and so it would seem sensible to include measures of both when studying welfare conditions (Wiepkema and Koolhaas, 1993). Even with a variety of tools to consider, however, it is often the case that results do not co-vary and the significance of different measures is often hard to interpret (Mason and Mendl, 1993). In addition to the difficulties posed by individual variation, species differences mean that cross-species generalisations are often not valid.

Dawkins (2004) maintains that there are only two questions that are of any importance in the study of animal welfare: “Are the animal’s healthy and do they have what they want?”. Similarly, Appleby and Hughes (2003) state that animal health is the foundation of all good welfare. As with humans, however, what an animal wants will not always agree with what is good for their health. It is also very difficult to establish the exact nature of these “wants” for many animal species. Furthermore, although different measures of animal welfare are important, it is always difficult to relate them precisely to what the animal is actually feeling. For these reason, the study of animal welfare is an inherently tricky area of study. Through scientific investigation, however, we can only collect evidence from which inferences must then be made (Mason and Mendl, 1993).

1.2 Fish welfare

1.2.1 Scientific basis for its necessity

The traditional *Scala naturae* regarding the evolution of intelligence has always regarded fishes as 'primitive' and 'less evolved' than the other higher organisms. This point of view considers that the telencephalon of fishes consists of a subpallium ('paleostratum') and a primitive pallium ('paleocortex') made up of simple neural circuits. This gives rise to what has generally been assumed to be essentially reflex or instinctive behaviour (Salas *et al.*, 2006). The reasons for regarding fish as more 'primitive' therefore stem largely from observations of a less complex physiology and simple behavioural responses. Techniques for studying the neuropsychology of learning and memory in fish have, however, in recent years become increasingly sophisticated (*e.g*. Portavella *et al.* 2004; Salas *et al.*, 2006). It is now known that the forebrain of teleost fish is far from the 'primitive' organ as previously thought, and is involved in a variety of emotional, social and reproductive behaviours, including aspects of learning and memory (Salas *et al.*, 2006).

In fact, it seems likely that an area of the teleost forebrain, known as the lateral pallium, is analogous to the avian and mammalian hippocampus (Broglio *et al.*, 2003). The hippocampus is the brain area associated with long-term memory and spatial relationships, and indeed, many fish species have been shown to generate internal map-like representations and display topographical learning (Rodríguez *et al.*, 1994). It would seem that all extant fish and tetrapods may

have evolved from an ancestral fish group 400 million years ago, and inherited behavioural and cognitive traits that have been retained during phylogenesis (Salas *et al.*, 2006).

This knowledge concerning complexity of the fish brain and cognitive capabilities has led to theories suggesting sentience in fish species and one of the key issues in determining the need for welfare criteria for fish, or indeed any group of animals, is that of sentience. Most people would agree that for welfare to be of concern, the animal of concern must have at least a certain degree of sentience (Broom, 2007).

1.2.2 Sentience in fish

A sentient being is described as one that has some ability to evaluate the actions of others in relation to itself and third parties, to remember some of its own actions and their consequences, to assess risk, to have some feelings and to have some degree of awareness (Broom, 2007). It would therefore seem obvious that sentience is an appropriate prerequisite to having a welfare status (Wood-Gush *et al.*, 1981).

Some scientists are resolutely critical regarding the possibility of sentience in fish. Rose (2002) states that it is impossible for fish to experience pain and suffering, because they do not possess the brain structures required for

conscious pain perception. He suggests that for pain to be experienced as a psychologically unpleasant stimulus, a highly developed neocortex, such as that found in humans, would be required. He further states that observed fish responses to noxious stimuli are purely non-conscious reactions and that the capacity for learning demonstrated by many species does not require or imply the presence of a conscious awareness. In part, he makes a compelling argument in that pain is indeed a sensory and emotional experience. Unless the neural activity resulting from nociception is consciously perceived, then it cannot result in 'pain' as we understand it ourselves.

However, increasing numbers of scientists are willing to disagree with Rose's point of view. Braithwaite and Huntingford (2004) suggest that perception and processing of the same kind of information can occur via different pathways in different taxonomic groups. In particular, the fact that many fish species can learn from observation and retain memories as declarative representations (*i.e.* the conscious recollection of events, locations etc.) provides strong evidence of conscious cognition within these species (Chandroo *et al.*, 2004).

In 1999, Gregory stated that "the appropriate question appears not to be do fish feel pain?, but rather, what types of pain do fish experience?". Anatomical and physiological studies have confirmed the existence of both A-delta and C-nociceptor fibres in several species of fish, including rainbow trout (*Oncorhynchus mykiss*). These receptors are, as in humans, polymodal, and are activated by a range of mechanical, thermal and chemical stimuli (Broom, 2007). What is more relevant, however, is that fish have been observed to avoid

specific situations or locations where they have previously experienced noxious stimuli that, in human terms, we would describe as painful. Incidentally, mechanical thresholds of nociceptors within fish have been found to be lower than those found in humans, possibly because of the more easily damaged nature of fish skin (Sneddon *et al.*, 2003).

Physiological and behavioural features of fish also show evidence that they possess functional limbic and dopaminergic structures homologous to those found in tetrapods, and which were probably inherited from a common ancestor (Chandroo *et al.*, 2004). Such limbic and dopaminergic systems are used to motivate specific behaviours and allow for a flexible or learned response in situations where a rigid or reflexive one may be less appropriate (Fraser and Duncan, 1998). As summarised in the intensive review of fish anatomy, physiology and behaviour by Chandroo *et al.* (2004) it is suggested that fish are more likely to be sentient than not. However, this hypothesis of fish as sentient beings serves to raise some further issues involving perception of stress and fear, together with their role in fish welfare.

Often associated with the concept of pain, is that of fear. Fear itself is a learned response which acts as a motivator to evade perceived threats (Jones, 1997). Within fish, this experience of 'fear' appears to be mediated by the limbic neural system, and several studies have suggested that fish can experience fear in similar ways to other vertebrate animals (Portavella *et al*., 2004; Yue *et al.,* 2004).

Much attention has been given to the stress response in fish (*e.g.*Wendelaar-Bonga, 1997) particularly in relation to conditions within aquaculture facilities, such as the effects of stocking densities and crowding on fish physiology (*e.g*. Ruane and Komen, 2003). The primary stress response involves the activation of the hypothalamic-pituitary-interenal (HPI) axis which stimulates the release of glucocorticoid hormones such as cortisol. Substantial and sustained increases in such hormones have been linked to reductions in growth, reproduction and the immune response (Ramsay *et al.*, 2006). As with most organisms, there is considerable evidence that fish do not respond well to prolonged periods of stress (Braithwaite and Huntingford, 2004).

To summarise, most of the evidence points to the fact that many species of fish show substantial perceptual ability, the presence of pain and adrenal systems, together with emotional responses, long- and short-term memory, complex cognition and social learning (Broom, 2007). Although it is true that fish are a highly divergent group and so would also be expected to show a large variability in brain structure and function, numerous species studied have fulfilled many, if not all of the requirements of sentience. It would seem apparent, therefore, that the case for protecting these animals and considering their welfare is substantial (Chandroo *et al.*, 2004).

1.2.3 How can fish welfare be compromised?

Fish are utilised by humans in many different ways. These include fishing (both for food and sport), intensive production for food, keeping fish in aquaria or as pets and through scientific research (FSBI, 2002). All of these uses may potentially have negative impacts upon fish welfare. Even those fish that are not directly used by humans may be affected by our actions through environmental degradation and development and pollution of the aquatic environment (FSBI, 2002). In particular, we will focus here on welfare issues within research, as these are most relevant to the thesis.

All animals have physical and chemical requirements which must be met in order for basic welfare conditions to be satisfied. For fish species these include a nutritionally complete diet, appropriate physico-chemical parameters of the water (*e.g*. temperature, oxygen, salinity, pH, dissolve nutrients and waste products etc.), suitable levels and duration of light intensity and sufficient space to provide freedom of movement (FSBI, 2002). If any or several of these factors are less than adequate, then the implications for welfare may be severe. Although fisheries biologists have constructed large databases covering the biological needs of commonly used fish, these often do not cover species that are used in laboratory research (Conte, 2004).

The rearing and living environment is obviously a critical component in animal welfare. Despite this, however, little is known about the optimal conditions required by many fish species used within laboratory procedures (Ramsay *et*

al., 2006). Sub-optimal conditions can lead to stress, and the management of stress is a key principle in ensuring animal welfare since, as previously mentioned, stressed fish have a much reduced ability to resist attack from disease and parasites and show reductions in other biological parameters such as growth and reproduction (Conte, 2004).

One of the most studied ways that fish welfare can be compromised is through inappropriate stocking densities. Density incorporates the number (or mass) of fish per unit of static water volume, and/or the fish biomass per volume of flowing water per unit of time (Conte, 2004). These factors influence water quality and fish-to-fish interaction, and subsequently affect the animal's welfare (Conte, 2004).

The concept of defining a minimum space for a fish is more complex than for terrestrial species because of their utilisation of 3-dimensional space (Ashley, 2007). Keeping fish at densities which are too high can cause problems with water quality and increase negative social interactions. Reduction in water quality, however, is a much more common problem in intensive husbandry conditions rather than within laboratory settings (Conte, 2004). In some cases it may be just as stressful for fish to keep them at unsuitably low densities, especially for social species that require interaction with conspecifics (FSBI, 2002). Stocking densities, therefore, are very species-specific. Whilst many fish appear to experience stress when densities are high, some such as the Arctic charr (*Salvelinus alpinus*), exhibit most dramatic stress-related behavioural responses at low densities (Conte, 2004). With some species it may be that

intermediate densities are most problematic. In rainbow trout, the highest plasma cortisol levels are observed when fish are stocked in pairs, rather than in isolation or larger groups. This is apparently due to the intense social interactions that occur under these dyadic conditions (Pottinger and Pickering, 1992).

As well as species-specific differences in response to tank conditions, there may also be differences related to life-stage and ontogeny (Huntingford *et al.*, 2006). Ramsay *et al.* (2006) observed that the stress response increased in relation to development in the zebrafish (*Danio rerio*). Incidentally, this particular study also reached another important conclusion, namely that cortisol, albeit at low non-stress levels, is needed for normal development and growth. Further studies in which cortisol levels are correlated with fitness parameters would help to establish baseline cortisol levels for optimal health and welfare.

Fish may also show a preference for other environmental factors, including lighting, tank colour and methods of food presentation. For example, Papoutsoglou *et al*. (2000) showed clearly that fish can be stressed by the background colour of tanks and that they show a marked preference, accompanied by an increase in growth rate, for specific tank colours. Fish within aquaculture facilities and laboratory settings may also be exposed to acoustic stress. Despite the fact that background noise can contribute to physiological and behavioural stress responses in fishes similar to those found in mammals, little or no concern has been directed to determining the appropriate acoustic environment for optimal growth and development.

It is important to remember that animals have not only physiological needs, but also behavioural ones, both of which need to be satisfied for good welfare (Bracke and Hopster, 2006). Particularly for satisfaction of behavioural requirements, a good knowledge of species-specific behaviour is essential. Stress can initiate behavioural changes and forced behavioural changes can cause stress, both of which are important welfare concerns (Iwama *et al.*, 1995). For most species, beyond the basic physiological parameters required to keep them alive, little is known about their specific environmental preferences (Huntingford *et al.*, 2006).

1.2.4 Current standards in fish welfare

The guidelines and legislations for fish used for research, teaching and testing are generally much more liberal than those in place for mammals. This can be demonstrated in particular by the lack of focus on humane endpoints in fish models. For example, the LD_{50} test is still allowed in fish, but banned in mammals. In fact, in many countries fish are not included in national laws, and reporting of the health and welfare of fish used in research is often sparse (Johansen *et al.*, 2006).

However, several countries have published guidelines on the care and use of fish in research. The Canadian Council on Animal Care, for example, provides recommendations for facilities, management and husbandry, although they are general for all fish species in all types of research (www.ccac.ca). Similarly, the

Norwegian School of Veterinary Science provides extensive resources covering the care and use of fish in research (oslovet.veths.no/fish). Within Britain, fish species used in research were, until recently, subject to legal protection under the Animals (Scientific Procedures) Act, 1986, enforced by the Home Office Inspectorate (UK Animals Act, 1986). This has recently been replaced by European Directive 2010/63/EU which came into force across the EU in November 2010. This revised directive is intended to strengthen the protection of animals in line with the EC Treaty of Rome Protocol on Animal Welfare, along with reducing disparities between the welfare standards employed by different member states. All members have been given two years in which to implement the required changes (www.understandinganimalresearch.org.uk).

It is only relatively recently that authorities have come to accept the fact that fish are different from other vertebrates in ways that have important implications for their welfare. In an attempt to address this lack of information, Huntingford *et al*. (2006) considered the framework for animal welfare based on the five freedoms (used by the UK Farm Animal Welfare Council, 2005) or five domains in which welfare may be compromised (Mellor and Staffords, 2001) and discussed how it might be applied to fish.

Domain 1 refers to the provision of water and food. Although seemingly obvious, the exact nutritional requirements and preference for feeding method is not well known for many fish species. Domain 2 covers environmental challenge, which states that a living area should comprise of a suitable space with a resting facility if required. Because fish are in complete contact with the

water they reside in, the problem of contaminants and quality of this water are crucial for welfare. This category also covers flow rates and the possibilities of enrichment. Domain 3 involves issues relating to disease and injury, and is mainly concerned with effective prevention and diagnosis. The fourth domain refers to behavioural and interactive restriction. In fish this mostly involves keeping species at a density that is appropriate for them to perform natural social behaviours such as shoaling. The final and fifth domain covers the rather more difficult area of mental and physical suffering and relates unsuitable conditions to emotional response. This is undeniably more difficult to measure in fish in comparison with other mammals and remains a critical area for further study.

To conclude, Casebolt *et al*. (1998) in their review of the care of laboratory fish state that, whilst water quality parameters such as temperature, O_2 saturation, nitrogen compounds, CO_2, pH and salinity are the most important elements for maintaining healthy animals and ensuring valid experimental results, other factors such as light levels, noise levels, stocking density, water flow and feeding regime are also of importance. In fact, many hatchery operators have reported the linkage between accommodating for both fish physiology and behaviour and improvement of culture performance (Conte, 2004).

1.3 Indicators of welfare in fish

Welfare assessment of any species should be based upon a thorough understanding of the biology of the species and their related requirements. Ideally, the assessment process should include measures that are

physiological, behavioural and pathological. A combination of such measures will usually provide a more accurate assessment of welfare because of the range of coping mechanisms used by animals, and the various effects of the environment on individual species.

1.3.1 Physiological indicators

The response to stress in fish is often grouped into primary, secondary and tertiary functions. Primary responses consist of the initial neuroendocrine response including the release of catecholamines and activation of the hypothalamic-pituitary-interrenal (HPI) axis, which stimulates the synthesis and mobilisation of glucocorticoid hormones such as cortisol. These initiate the secondary responses which include changes in plasma and tissue ion metabolite levels, hematological features and heat shock or stress proteins (Barton, 2002). This also includes increases in blood glucose levels which all prepare the individual for a flight or fight response in an emergency situation (Martínez-Porchas *et al.*, 2009). Tertiary responses relate to changes in whole-animal functions such as metabolic scope for growth, immunity, reproduction and behaviour (Wendelaar-Bonga, 1997).

Due to reliable patterns of increased plasma cortisol in response to stress, changes in this variable are often used as an indicator of welfare in fish. Cortisol can be measured from blood samples (Pottinger and Moran, 1993), whole-body homogenates (Ramsay *et al.*, 2006) or water samples (Ellis *et al.*, 2004) of fish using readily available commercial enzyme linked immunoassay kits, or by

radioimmunoassay. It is important to remember, however, that stress responses are adaptive, and in the short term they play a naturally important role in preservation of the individual. However, under conditions of prolonged, repeated or unavoidable stress which lead to persistent activation of primary responses, the resulting tertiary effects can be greatly maladaptive, including reductions in growth, suppressed reproductive function, diminished immune function and disease resistance (Ashley, 2007). As a result, a measurable increase in plasma cortisol will often provide an indication that welfare is less than optimal (Broom, 1988; see Ashley, 2007).

As a consequence of the release of the aforementioned “stress hormones”, a number of secondary stress responses can also be measured, such as an increase in blood glucose and associated decrease in hepatic glycogen. As hepatic glycogen is rapidly converted to glucose, both of these factors prepare the individual for a flight or fight response in an emergency situation (Martínez-Porchas *et al.*, 2009). Plasma glucose and liver glycogen can be determined spectrophotometrically.

Other physiological methods which play a role in welfare assessment are measurement of brain monoamine neurotransmitters such as serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA) and norepinephrine (NE) (DiBattista *et al*. 2005; Øverli *et al*. 2001). Brain monoamines play a vital role in the control of behavioural and physiological response to stress (Ashley, 2007) in that the modulatory action of cortisol may be mediated through monoaminergic systems (DiBattista *et al.*, 2005). Several lines of evidence also suggest that

hypothalamic 5-HT may be involved in the regulation of the HPI axis (Lepage *et al.*, 2002). Dibattista *et al*. (2005) found that fish under greater stress, and subsequently exhibiting higher cortisol levels, showed increases of brain 5-HT and other brain monoamine activity, which seemed to be responsible for behavioural inhibition of rainbow trout. Similarly, Øverli *et al*. (2001) observed that stress increased the concentrations of serotonin and dopamine in the brain stem, and norepinephrine in the optic tectum and telencephalon of rainbow trout. Incidentally, they also measured an increased concentration of monoamine metabolites, suggesting that under stressful conditions, both synthesis and metabolism of the monoamines increases.

Other possibilities include observation of heart rate and respiration through operculum ventilation or smart-tags and observation of skin condition (as stress can cause skin ulceration without the occurrence of physical trauma or pathogens; Johansen *et al.* 2006). Molecular biological methods may also be a possibility, such as to assess the production of stress proteins in fish. For example, a number of heat shock proteins (HSP's) in fish have been shown to exhibit a significant change in expression in response to a range of abiotic and biological stressors (Iwama *et al.*, 1999).

1.3.2 Behavioural indicators

Behavioural studies are important within welfare research for a number of reasons, particularly as changes in behaviour can reflect how a fish is sensing and responding to its environment (Conte, 2004). In addition to this, altered

behaviour is often an early response to adverse conditions that can be used as an indicator of impaired welfare (Huntingford *et al.*, 2006). Knowledge of behaviour that is relevant to individual species can also benefit welfare studies, in that natural activity patterns of fish can be compared to those observed in captivity (Ashley, 2007). With the additional benefit that behavioural tools are generally non-invasive and non-intrusive, they may even provide an 'early warning' system of poor conditions that haven't yet initiated a measurable physiological response (Dawkins, 2004). One of the only minor drawbacks is that the nature of recording behavioural observations can be subjective if not properly controlled, and its interpretation is often problematic (Dawkins, 2004).

As it has been previously stated, there is a widespread belief that animals may suffer if they are prevented from performing their full behavioural repertoire. This has led to the theory that behavioural deficits can be used to identify conditions that detract from welfare (Mench and Mason, 1997). Dawkins (1998), however, warns that there is not necessarily a connection between the naturalness of behaviour and good welfare. What is important is whether it is the behaviour itself which is the reinforcing stimuli, or if it is purely the endpoint that matters. In cases where behaviour is performed purely as a means to an end, then deprivation of this behaviour may not necessary affect welfare.

The study of 'abnormal behaviours' has had a huge part in the assessment of bird and mammal welfare, and indeed, changes in behaviour can be useful welfare measures (Ashley, 2007). Expression of abnormal behaviours, such as stereotypes, has been documented for fish kept in high-density aquaculture

facilities. For example, the circular shoaling behaviour performed by Atlantic salmon (*Salmo salar*) within sea cages has been compared to the pacing observed in various terrestrial zoo animals (Oppedal *et al.*, 2001). In such cases, however, if the behaviour appears to have no negative consequences on the animal's health, it may not necessarily be a concern. At the very least, however, the observation of abnormal behaviours may just alert us to the fact that additional welfare assessment is required (Ashley, 2007).

Changes in social behaviour, such as as shoaling, aggression and dominance hierarchy structure can also be used as assessment of welfare status as all can be affected by stress. Sloman *et al.* (2001) studied the aggression and dominance of brown trout in relation to environmental perturbation. Principal Component Analysis (PCA) was used to form a total dominance score from several behavioural measures, and from this create a dominance hierarchy model. They found that the environmental stress, in this case simulated drought conditions, reduced the amount of aggressive interactions and changed both the social behaviour and the hierarchy structure of the group.

One of the most important ways in which behaviour can be used to study welfare, however, is through choice or preference tests. By allowing animals to express their natural preferences, we can potentially identify things that may promote or detract from their welfare (FSBI, 2002). In fact, Dawkins (2004) emphasises that, far from being just another measure of welfare, choice tests should be a necessary piece of evidence that give clarity and meaning to the more traditional physiological measures. An example of how preference tests

have been used with fish is shown by Serra *et al*. (1999). They studied the natural preference of the zebrafish for a dark environment using a two chambered aquarium consisting of one black and one white compartment. An animal was placed into the tank and then its position observed for ten minutes. The study was combined with an exit latency test where the time taken to leave its initial compartment and to enter the other was recorded. More time was spent by fish in the black environment and fish took more time to go from the black to the white compartment, indicating an overall preference for a dark environment.

Such preference tests rely on the reinforcement mechanisms that have developed in the species throughout its evolution. We can directly assess the strength of a reinforcer by seeing how much effort an individual will put into either obtaining or getting away from it. However, these mechanisms may not be appropriate in unnatural conditions. For example, animals may find some circumstances very positively or negatively reinforcing, despite the fact that they pose no direct benefit or threat to their health or fitness within captivity. Similarly, the underlying assumption that animals choose what is good for them is not always valid (Huntingford *et al.*, 2006).

1.4 Environmental enrichment

1.4.1 Introduction to environmental enrichment

The study of environmental enrichment was initially introduced as a research tool for understanding the effects of experience upon the brain (Benefiel *et al.*,

2005). It was first considered in 1949 by the Canadian psychologist Donald Hebb, who brought laboratory rats into his own home, where they were treated as pets. He then proceeded to study how environmental complexity affected their subsequent behaviour in learning tasks. It was not until 1962, however, that Krech, Rosenzweig and Bennet used the term 'environmental enrichment' whilst studying the biochemical changes in the brains of rats reared in complex housing environments and supplemented with daily exposure to novel items.

It is now commonly accepted that many forms of environmental enrichment have beneficial cognitive effects in both healthy and diseased animals, often corresponding to higher brain mass and cortical thickness. There is also a wide range of data showing effects of enrichment on behavioural tasks involving spatial memory, anxiety, exploratory activity and aggressiveness (Amaral *et al.*, 2008). However, the use of enrichment as a means of improving husbandry and well-being for animals aside from non-human primates, has only been studied extensively since the mid- to late 1990's (Benefiel *et al.*, 2005).

Similarly to other concepts such as 'animal welfare' and 'stress', the term 'environmental enrichment' is one that is difficult to define, and which has been used to mean several different things within the literature. Often, environmental enrichment is used merely to describe an increase in complexity of an animal's habitat (Newberry, 1995). However, when used in the study of animal welfare, the term is only appropriate if a quantifiable improvement in the well-being and biological functioning of the animal is observed which has occurred as a result of modifications to the environment (Olsson and Dahlborn, 2002). A common

misconception when considering environmental enrichment as a means towards improving welfare is that enrichment always implies good welfare, whereas the relationship between the two should be considered to be a testable hypothesis rather than an implicit thing (Benefiel *et al.*, 2005). Within enrichment studies, however, 'control' environments are rarely standardised, and there are few published methods or criteria for assessing whether enrichment has been achieved (Newberry, 1995).

The aims of enrichment may be varied. Some authors, particularly those concerned with primate welfare, advocate its use in order to provide an environment which promotes natural behaviour (Gilloux *et al.*, 1992). For example, Mason (2001) found that fur-farmed mink were still strongly motivated to perform their natural swimming behaviour in pools, despite being bred in captivity for over seventy generations. Furthermore, they experienced high levels of stress from being denied access to such a resource. With the use of weighted entrance doors, Mason discovered that the effort they would voluntarily invest in accessing a swimming pool was considerable. In this case, it appears that it is important for the mink to perform this natural behaviour.

However, in many cases it is difficult to decide what constitutes natural behaviour. Additional issues arise where animals are being kept indefinitely within captivity. For animals that are due to be released back into their natural, wild environments, it makes sense for their captive environment to be suitably equipped for them to display their full range of natural behaviours. However, for animals which are to remain in captivity for the rest of their lives, their well-being

depends on their ability to adapt to the captive condition (Newberry, 1995). In other cases, most notably in zoos or in farming production, enrichment studies have been used to improve the public image or 'holding power' of exhibits (Shepherdson *et al.*, 1993). However, most commonly the purpose of environmental enrichment is to improve the well-being of animals by promoting good emotional and physical health (Newberry, 1995).

Within farming, there have been several cases in which environmental enrichment appears to have had beneficial effects on animal welfare. In pig pens, for example, increasing both the size and complexity of pens appears to reduce the number of harmful social behaviours and subsequently the levels of aggression displayed by the residents (Beattie *et al.*, 1996). This is thought to be because the additional complexity provides alternative objects towards which such behaviours can be directed towards. A similar situation has been observed with chickens. When provided with pecking stimuli, such as hanging pieces of knotted twine, the chickens show a marked decrease in fearfulness, depression and feather pecking (Jones *et al.*, 2000).

In the case of zoo animals, much work has been done to 'enrich' the enclosures of residents, often with the use of feeding enrichment. For apes, the provision of food in puzzle feeders, or feeding in unpredictable places and times, have been used as a way of minimising stereotypical or unnatural behaviours and encouraging the animals to utilise their natural inquisitiveness (Gilloux *et al.*, 1992). Similar techniques have been used with other large mammals, such as brown bears, where providing honey-filled logs has been shown to reduce the

daily duration of stereotypical behaviours, replacing them with more 'natural' investigatory behaviour (Carlstead *et al.*, 1991).

1.4.2 Enrichment in the laboratory

There are opposing arguments concerning the use of environmental enrichment techniques within the research laboratory, and a wide range of people including scientists, animal review committees, care staff, veterinarians and financial administrators all have a vested interest in decisions concerning animal care (Wolfle, 2005). Reinhardt (2004) suggests that isolated animals, with no forms of habitat enrichment, may suffer from boredom, depression, distress or a lack of opportunity to express essential species-appropriate behaviours. Such a lack of stimulation may lead to increased variability in the animals, which is detrimental to scientific studies. Garner (2005) supports this argument by stating that if environmentally enriched enclosures help to avoid the formation or expression of stereotypical or repetitive behaviours, then this should lead to increased scientific validity, reliability and repeatability. For the many scientists who support these views, "good welfare is good science".

Others disagree. Mering *et al*. (2001) suggest that increasing stimulation by altering standard 'barren' housing may introduce unpredictable variation in laboratory studies. Similarly, Wolfle (2005) suggests that changing an animal's environment can lead to important behavioural and biological changes. As change is often a precursor to variability in results, increased numbers of animals will be required within research studies to reduce this effect of

individual variability. Similarly, the change in study conditions may make new research incomparable with research conducted under previously standardised conditions. Unfortunately, this is at odds with the common goal of reducing the numbers of animals in research to a minimum (Wolfle, 2005).

Nevertheless, the use of environmental enrichment to improve the welfare of animals used in laboratory studies has remained an important issue, and one that has recently been pursued by various national legislative organisations, including the UK Home Office. Several studies have looked at the effect of environmental enrichment on brain anatomy, hormonal response and environmental adaptation in rats, with variable and often surprising results. Moncek *et al.* (2004) found that environmental enrichment was associated with increased basal secretions of corticosterone and increased adrenocortical functions, which are usually considered to be indications of chronic stress. However, it was also observed that the rats did not develop the negative health consequences that generally accompany chronic stress, and it is thought that the observed changes may have been induced by an increase in physical activity. Importantly, it was also found that rats that were provided with environmental enrichment showed a much smaller stress response when handled, suggesting that it may improve the animal's ability to cope with environmental change and stress. Several studies, however, have documented no significant differences in corticosteroid production in relation to enrichment of their cages (Pham *et al*. 1999; Larsson *et al*. 2002; Schrijver *et al*. 2002). Interestingly, all studies showed that rats from enriched environments

demonstrated faster emotional adaptation in novel situations and better performance in spatial learning tasks.

With mice, results from enrichment studies have also proven to be conflicting. Haemisch *et al.* (1994) found that mice from enriched cages showed significantly more aggressive behaviour, and that social organisation was less stable. Similarly, Marashi *et al.* (2003) found that mice from enriched environments showed increased levels of both play and aggression which was associated with a rise in plasma cortisol. Once again, however, it was unclear if this was simply a result of the increased physical activity of the animals. Van de Weerd *et al.* (1994) reported that mice from enriched cages were more dynamic in their reactions to novel situations and were more alert in general. However, it was noted that behavioural effects differed greatly between strains. A later study by Van de Weerd *et al.* (1997) found that mice from cages enriched with nesting material weighed more than mice from barren cages, despite eating less food, which suggests that the enriched conditions allowed them to invest more energy in growth by regulating their body temperatures behaviourally. They concluded that there were no significant differences in behavioural and physiological parameters in mice from enriched or barren cages, although subjects appeared to show a marked preference for cages with bedding. This study also stressed the importance of assessing whether the animals benefit from the enrichment over a long- or short-term period.

Concerns about negative consequences of environmental enrichment are often overlooked or unconsidered. These may range from inadvertent physical harm

to the individual, physiological changes that may impact study results and also changes in variability of both physiological and behavioural results. In terms of animal welfare, of most concern would be the potential for new forms of enrichment to provide a situation capable of incurring physical harm on the animal for which it is provided. However, it is all too common that incidents of harm resulting from the provision of enrichment are not published, a situation which allows the perpetuation of similar mistakes. For this reason, all consequences of enrichment, whether positive or harmful should ideally be reported within the scientific literature.

In some cases it has been found that alteration of the social environment, in an attempt to improve the welfare of captive primates, has resulted in increases in both aggressive incidences and cases of self-injurious harm. Line *et al.* (1990), for example, reported numerous injuries and one mortality in a newly formed group of 13 Rhesus monkeys. Although the formation was an attempt to enhance the social lives of the animals, the results of the “enrichment” were far from ideal. In other cases, the provision of physical enrichment items has proven to be problematic. Hahn *et al.* (2000) reported a case of intestinal linear foreign body in a cynomolgus monkey as a result of ingesting rope that was suspended on the outside of its cage for enrichment purposes. Shomer *et al*. (2001) describe a case involving a New Zealand white rabbit provided with a whiffle ball (a perforated hollow ball made of hard plastic) for entertainment and enrichment. In this case the ball became trapped in the incisors of the animal, resulting in the animal not being able to eat or drink for 12 hours. There was also trauma to the gums.

As environmental enrichment involves an alteration to an animal's environment it is likely that it will produce changes in the animal the same way that a change in any environmental component would. For example, significant increases in both growth and food consumption have been observed in mice that are reared with enrichment (*e.g*. addition of nest bows, nesting material, climbing bars; Bayne, 2005) in comparison to those in barren cages. Bonnet *et al.* (2004) have also documented significant differences in adrenal and thymus weights of mice housed with physical enrichment (hemp fibre mat) or social enrichment respectively. This highlights an important issue. As enrichment is a method of improving welfare, often the only endpoints considered when studying the effects of such enrichment are those relating to physiological and "mental" health. It is highly likely, however, that such changes in the animal's environment will result in alteration of other physiological variables which we may not feel contributes to their overall welfare picture, but which may impinge on study results.

A final concern is the impact that enrichment may have on the variability of research results. Even as early as the 1950's it was recognised that the magnitude of the variance in results is related to the nature of the subject's conditions, whether that be housing, treatment or social situation (Chance, 1956). One of the desirable consequences of enrichment provision is that animals perform a more diverse range of behaviours. These animals may therefore show more variability in their responses to experimental procedures (Van de Weerd *et al.*, 2002). However, an alternative argument is that, as

enriched animals can perform more of their “natural” repertoire of specific-specific behaviours, they may be able to cope more effectively with novel and unexpected changes, therefore showing a more uniform response within studies. Furthermore, enriched animals may have a less sensitive and reactive nervous system, as they have not been subjected to restricted sensory input in the way that is common with animals housed in barren accommodation. Therefore, such enriched animals may exhibit improved physiological and psychological stability, providing us with more refined models and subsequently a better quality of experimental results. Some scientists have reported a reduction in variability of research results in response to enrichment. Others have demonstrated enhanced variation. What seems apparent is that the effects of enrichment may differ depending on both the enrichment type and the variable studied. A study by Eskola *et al*. (1999), for example, reported increased, decreased or unchanged variation in a large range of physiological parameters in a study with Wistar rats.

The main consequence of alteration of endpoint variability is that it will affect the number of subjects required within a study to maintain adequate statistical power. Of primary concern for all researchers should be the reduction, refinement and replacement of live subjects used within the laboratory. Whilst provision of environmental enrichment goes some way to achieving the “refinement” part of this goal, the results of such efforts may not satisfy the “reduction” requirement if resulting increases in the variation of experimental variables mean that more subjects have to be utilised or studies duplicated. For this reason, researchers need to balance the issues of enhanced animal welfare

with the potential for reduced animal numbers. In addition to this, more detailed disclosure within peer reviewed journals regarding the living environment of animals within experimental studies will assist with standardisation and comparison of results.

1.5 Regulatory toxicology

Regulatory toxicology is the study of the adverse effects of chemicals on organisms and the environment. Laboratory animal tests are used to identify the toxicological properties of chemicals to which humans or wildlife are exposed when the chemicals are used in a specific product or for a specific purpose. Such chemicals include industrial products and wastes, pesticides and cosmetics, drugs and medical devices, hormones, vaccines and other immunological products. Within the UK, development and testing of such chemicals is monitored by the Organisation for Economic Co-operation and Development (OECD). OECD Test Guidelines cover four main sections: 1, physicochemical properties, 2, effects on biotic systems, 3, degradation and accumulation and 4, health effects. These guidelines comprise over 100 internationally agreed testing methods used by government, industry and independent laboratories to identify and characterise potential hazards of new and existing chemical substances, chemical preparations and chemical mixtures. They are a set of tools for professionals, used primarily in regulatory safety testing and subsequent chemical and chemical product notification and chemical registration. They can also be used for the selection and ranking of candidate chemicals during the development of new chemicals and products and in toxicology research. Sections 2 covers effects on biotic systems and

involves a number of tests on fish, including reproduction and growth tests as well as acute, prolonged and early-life stage toxicity tests.

1.5.1 Enrichment for fish used in regulatory toxicology

At present the conditions required for fish used in scientific studies is covered under the European Directive 2010/63/EU which came into force across the EU in November 2010. Further regulations are given in Appendix A of the European Convention for the protection of vertebrate animals used for experimental and scientific purposes. However, advice concerning enrichment for fish merely states that a form of enrichment may be required for some species to take account of their specific behavioural traits. Little other information exists concerning the application of environmental enrichment procedures to aquatic organisms such as fish (Williams *et al.*, 2009).

The issue is further complicated by the limitations posed by the need for studies to meet regulatory compliance with test guidelines. This means that many procedures which could potentially be beneficial for fish welfare cannot be implemented (Handley, 2001). Enrichment techniques must be successfully transferable from husbandry to regulatory study laboratories without compromising study validity or robustness. For regulatory based toxicological testing using fish, the container vessel must be inert, efforts must be made to avoid excessive microbial growth, and behavioural observations must be conducted (for comparison with controls). These requirements are generally not compatible with the addition of objects to the tank, as increased cover (*e.g.*

plants) can make observation difficult, the required regular cleaning ineffective, in addition to leaching of confounding chemicals into the test environment. Increased surface area can lead to absorption of the test chemical, and increased microbial load can potentially lead to chemical degradation.

Furthermore, any manipulation of the environment that results in alteration of the behaviour and/or physiology of test fish, while being beneficial in terms of welfare, may serve to increase innate variability within the study. Several studies, for example, have shown that fish exhibiting different behavioural phenotypes can show dramatically different chemical uptake profiles (*e.g.* 20-fold differences in copper uptake between dominant and subordinate fish; Sloman and Armstrong, 2002). This variability could make it necessary to utilise more animals to attain comparable statistical power and would not be in accordance with current attempts to replace, refine and reduce the use of animals in research (www.nc3rs.org.uk). Of course, a counter argument is that studies with a degree of behavioural/physiological variability in the fish may be more comparable to the situation in the natural environment and so may increase the relevance of such studies.

There are some exceptions where it is necessary for materials to be introduced within the tank, such as spawning materials (OECD 203 annex 4A, 2009). However, in such cases, leachates from these materials such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls from plastics must be measured and maintained below a threshold concentration. All of these factors represent the perceived costs of environmental enrichment for laboratory fish.

Some, such as the change in chemical nature of the water and potential interaction with the test chemical, are likely to challenge the validity of the overall test and so this must be balanced against the benefits in terms of improved welfare for the test fish. Unfortunately, little information is available to make a balanced scientific assessment of the value of the improved welfare of including an item in the test tank to improve welfare.

Few experimental studies under laboratory conditions have been conducted to research the preferences of fish species for particular environments. Furthermore, it is apparent that the huge diversity of species and habitats utililised by different fish types means that studies will rarely be applicable to species others than those targeted. For example, Strand (2007) studied the effect of tank colour and light intensity on the feeding success, behaviour and activity levels of the Eurasian perch (*Perca fluviatilis* L.). It was found that food intake and growth was higher in individuals kept in white tanks compared to those in grey or black, and differences in growth were most pronounced in dark tanks compared to light. Conversely, Appelbaum and Kamler (2000) found that the African catfish (*Clarias gariepinus*) showed decreased locomotion and increased growth rates within dark conditions compared to light. It should be mentioned, however, whilst this provides useful information for the productivity of fish-farming, it does not necessarily indicate good welfare.

1.6 The model species - Zebrafish (*Danio rerio*)

Figure 1.1 The zebrafish (*Danio rerio*)

1.6.1 Background and life history

Zebrafish are indigenous to South Asia and can be found across parts of India, Bangladesh, Nepal, Myanmar and Pakistan (Lawrence, 2007). Within these regions they occur in a wide variety of habitat types including irrigation ditches and rice fields, man-made fish ponds, upper reaches of rivers and fast flowing hill streams (Spence, 2006). In general, zebrafish have been found to prefer still or slow moving, slightly alkaline (pH ~ 8.0) water of relatively high clarity (~ > 35 cm) (McClure, 2006; Spence *et al*. 2007). Both in field and laboratory based behavioural studies, wild and domestic zebrafish have shown a preference for spawning in sites associated with aquatic vegetation (Spence *et al.*, 2007b). They are generalist feeders, consuming a variety of benthic and planktonic crustaceans, worms and insect larvae.

Zebrafish appear to be primarily an annually spawning species, with the main period of recruitment being from April to August (Spence *et al.*, 2007a). Growth and reproduction are most likely controlled by the season, with the main period of rapid growth occurring during the monsoon season. The commencement of spawning is most often just before the onset of the monsoon due to increases in food availability at this time. They are asynchronous batch spawners that breed in small groups and most commonly in small pools adjacent to streams. Here, eggs are scattered over the substratum by females and the species shows no evidence of providing parental care. Male zebrafish demonstrate territoriality around spawning sites and have also been observed chasing females (Spence and Smith, 2006). Larvae and juveniles remain in seasonal waters as they develop then move back into streams as these seasonal waters recede (Engeszer *et al.*, 2007).

1.6.2 Use of zebrafish in the laboratory

The zebrafish was introduced as an animal model by George Streisinger in the 1970s when he sought an alternative model for the study of genetics and development in vertebrates. This decision was motivated, in part, based on the relative ease with which the animal could be maintained and bred. In fact, many features of this species make it suitable for widespread use in the laboratory. These include high fecundity, transparent embryos and the fact that embryos develop quickly outside of the mother (unlike mammals) where they can be easily observed and manipulated.

The zebrafish is a much favoured model species for embryologists and the analysis of mutations. There is also a large base of established knowledge on the developmental biology and genetics of the zebrafish and mutations are studied to identify genes required for a wide variety of biological processes. The zebrafish genome project has also facilitated this progress. Similarly there is now a large amount of work being conducted on transgenic zebrafish and the study of gene regulation and function.

1.7 Aims of the study

Fish are commonly used in the laboratory, and numbers of fish used in regulatory toxicology are increasing yearly. The maintenance of good welfare for these fish is not only a moral and ethical concern, but should also ensure that results from studies are realistic and robust. The use of environmental enrichment to improve welfare has been well documented for many species. However, there is currently little information available concerning the use of such techniques for fish, and in particular how these may or may not be compatible with the strict conditions required for ecotoxicological research.

The aim of this PhD was therefore to study the effects of environmental enrichment on the welfare of a commonly used species of fish, the zebrafish (*Danio rerio*). The limitation imposed on this research is that enrichment must be compatible with regulatory toxicology guidelines as enforced in the UK. In order to fulfil these aims I looked at two types of physical enrichment. The first consisted of groups of vertical black glass rods of varying heights designed to

increase environmental complexity and provide an element of refuge. The second consisted of airstones of two different sizes. The first enrichment type was used in two main studies. The first looked at the effects of providing these structures on a range of behavioural endpoints alongside whole-body cortisol concentrations. This was observed in both juvenile and adult fish. The second study utilising the rod structures looked at the effects of these on both chronic and acute stress responses of adult zebrafish. The third and final study looked at the effects of providing airstones of different sizes on behavioural and physiological endpoints relating to stress and anxiety.

CHAPTER 2

GENERAL METHODS

CHAPTER 2 – GENERAL MATERIALS AND METHODS

2.1 Source and maintenance of zebrafish

Zebrafish (Wild Indian Karyotype - WIK) were bred at the AstraZeneca, Brixham Environmental Laboratories and kept under conditions compatible with OECD guidelines throughout. (fFurther discussion of the strain of fish used is included in Chapter 6). Dissolved oxygen was maintained at ≥ 80 % of air saturation Prior to all experiments, fish were kept in flow-through fresh water at 28 °C and under a photoperiod of 14L:10D (light:dark) with a 20 minute phased sunrise/sunset. From 4 days post-fertilisation (dpf) fish were fed daily to excess with ZM000 infusoria grade food (Special Diet Services, Essex UK), and live rotifers. From 10 dpf, in addition to the rotifers and dry food, fish were fed live *Artemia* 24 h nauplii. At the beginning of experiments juvenile fish were 35 dpf and adult fish were approximately one year old. From 4 dpf and at all points prior to transfer to experimental tanks, fish were maintained in polycarbonate tanks on a zebrafish culture system (Techniplast, IWT, Italy).

2.2 Experimental exposures of zebrafish

2.2.1 Responses of zebrafish to a structured environment

Prior to the first study, a number of types of enrichment were considered. These included the use of opaque barriers in tanks to allow visual isolation of individuals, as well as arc-shaped hides situated on the bottom of tanks. The glass rod structures (described in more detail in the following section) were

chosen due to the desire for structures that would both mimic aquatic vegetation and also allow fish to be observed from in front of and above tanks.

2.2.1.1 Behavioural and physiological responses of juvenile zebrafish to a structured environment

On day 1 of the study, 120 juvenile zebrafish (wet mass = 0.18 ± 0.07 g) were transferred to 20 experimental glass tanks (*i.e.* 6 fish per tank) measuring 30 x 20 x 20 cm with a capacity of 12 l. The stocking density of test tanks was lower than that required by OECD guidelines (0.2 – 1.0 g/l or 5 – 10 fish/litre), and also lower than that used in husbandry, as the suggested densities would make behavioural analysis impossible. During the experimental period, the photoperiod was maintained at 14L:10D and the temperature 28 ± 1 °C. Dissolved oxygen was maintained at ≥ 80 % of air saturation and pH was 7 throughout the study. Water flow was 50 ± 1 ml/min/tank throughout the experiments (six volume changes per day). Fish were fed twice daily with live *Artemia* 24 h nauplii in the morning and SDS 300 dry food in the afternoon, following filming. Uneaten food and faecal material were manually removed from the bottom of test tanks each day by siphon.

Ten of these tanks were fitted with three glass base plates each measuring 70 x 85 mm. To approximate vertical stems of aquatic plants, twelve black opaque glass rods were attached to each base plate using aquarium silicone sealant (Dow Corning) in a 3 x 4 grid. Each of the three structures within a tank had glass tubes of different heights measuring 180 mm, 100 mm and 50 mm.

Structures were positioned as shown in Figures 2.1 and 2.2. Placement of control and structured tanks within the laboratory was determined randomly and ends of the tanks masked with black paper to ensure that fish in adjacent tanks had no visual contact with each other.

To facilitate behavioural analysis, a 3 x 3 grid was drawn on the front and lid of each tank, resulting in 27 three-dimensional cells that could be occupied by fish. For information regarding behavioural endpoints studied see sections 2.3 and 3.2. The experiment was repeated four times, lasting for either 1, 2, 4 or 7 days (with behavioural recording taking place between 12:00 and 16:00 h on alternate days throughout the 7 day study). The length of the study was determined primarily due to time constraints and availability of aquaria space. However, one week was deemed to be a ~~not vastly dissimilar~~representative length in comparison to regulatory studies which typically last for 28 days or less. At the end of each experiment, fish were rapidly removed from tanks and terminated, and water allowed to flow through tanks for a minimum of a day (six water changes) before the next batch of fish was added. All fish were killed humanely by an overdose of anaesthetic (100 mg/l of MS-222 buffered to pH 7 with $NaHCO_3$ and aerated for 30 minutes), weighed to the nearest 0.0001 mg (Mettler AE 163 balance), and then snap frozen in liquid nitrogen. Whole-fish samples were stored at -80 °C until analysis.

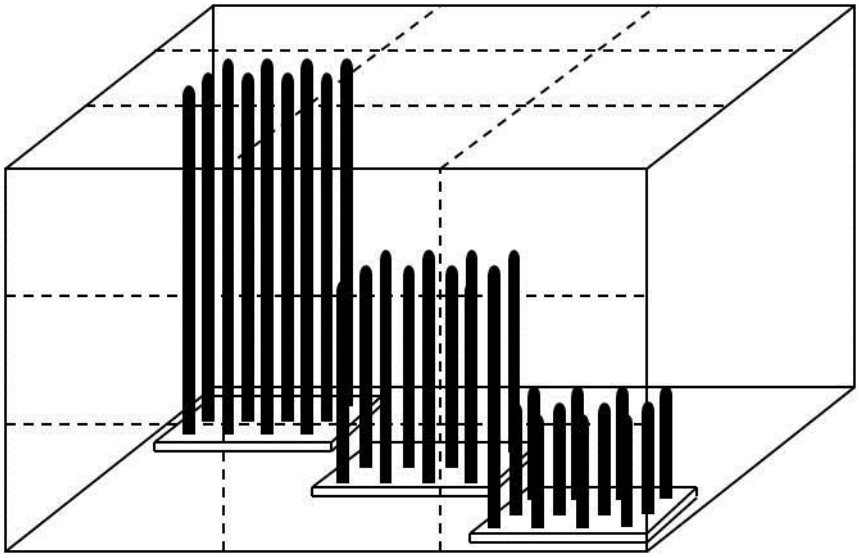


Figure 2.1. Structured tanks used in studies one and two containing three clusters of glass rod structures. Dotted lines indicated lines drawn on tank surfaces to allow behavioural analysis.

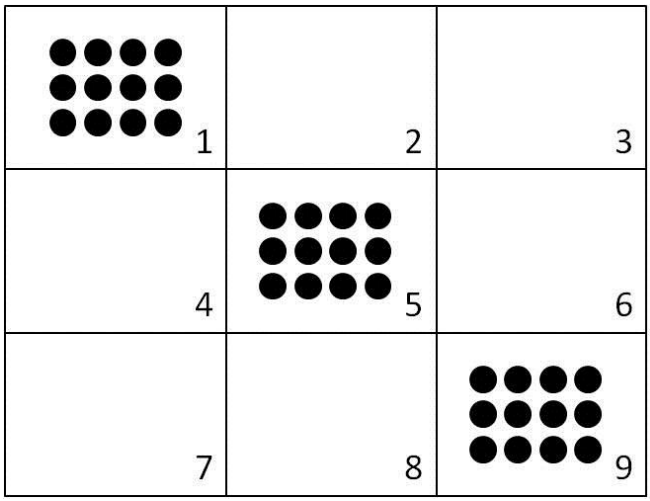


Figure 2.2 Plan view of structured tanks used in studies one and two. Numbers denote labelling of grids used for behavioural analysis. Long, medium and short rods are located in grids 1, 5 and 9 respectively.

2.2.1.2 Behavioural responses of adult zebrafish to a structured environment

A second study was conducted using the same enrichment rods as described in section 2.2.1.1., but using adult zebrafish. These zebrafish were from the same stock and were one generation removed from the fish used in the juvenile study (*i.e.* that described in 2.2.1.1). All conditions were maintained the same as the juvenile study, but the experiment was conducted a single time for a duration of seven days. At the beginning of the study fish were approximately one year old and therefore fully mature. Each tank was stocked with an equal number of male and female fish (*i.e.* 3 females and 3 males per tank). For more information on behavioural observations see section 2.3 and 3.2.

2.2.2 Effects of tank structure on acute and chronic stress responses of zebrafish

Design of experimental tanks and enrichment was identical to that described for the previous study (see 2.2.1). During the experimental period, the photoperiod was maintained at 14L:10D and the temperature 28 ± 1 °C. Dissolved oxygen was kept ≥ 80 % of air saturation and pH was 7 throughout the study. Water flow was 50 ± 1 ml/min/tank throughout the experiments (six volume changes per day). Fish were fed twice daily, with live *Artemia* in the morning and SDS 300 dry food in the afternoon, following filming. Uneaten food and fecal material were removed from test vessels each day by carefully cleaning the bottom of each tank using a siphon.

On day 1 of the study 120 adult zebrafish (wet mass 0.71 ± 0.02 g) were transferred to 20 tanks, 10 of which were barren as a control, and 10 of which contained glass rod structures. Control and structured groups were split into two further treatments, the first of which were unstressed and the second, stressed. The experiment ran for two weeks. In the first week, all tanks were unstressed. In the second week, tanks in both “barren-stressed” and “structured-stressed” treatments were subjected to a daily physical disturbance, which consisted of chasing fish with a net for 30 seconds. Behavioural observations took place on alternate days throughout the study and took place between 14:00 and 17:00 h. On days in which behavioural observations were recorded, fish were stressed directly prior to filming. As in the previous study, a 3 x 3 grid was drawn on the front and lid of each tank to facilitate behavioural analysis. For more information regarding behavioural analysis, see sections 2.3 and 4.2.

At the end of the study fish were rapidly removed from tanks and terminated. In all tanks in the stressed treatments three fish were sampled immediately. The remaining three fish were then subjected to the chasing stress 10 minutes prior to termination. Treatments were labelled “Unstressed” for fish unstressed throughout the whole study. “Chronic” were fish stressed throughout the second week of the study. “Chronic+Acute” were fish receiving both the daily stress and the additional stress immediately prior to sampling.

All fish were killed humanely by an overdose of anaesthetic (100 mg/l of MS-222 buffered to pH 7 with $NaHCO_3$ and aerated for 30 minutes), weighed to the nearest 0.0001 mg (Mettler AE 163 balance). Brains and livers were collected

then all tissues and remaining fish samples snap frozen in liquid nitrogen before being stored at -80 ºC until required for analysis.

2.2.3 Behavioural and physiological responses of zebrafish to airstones

On day 1 of the study, 121 adult fish (wet mass 0.65 ± 0.01 g) were transferred to 21 experimental glass tanks (*i.e.* 6 fish per tank) measuring 30 x 20 x 20 cm with a capacity of 12 l. During the experimental period, the photoperiod was maintained at 14L:10D and the temperature 28 ± 1 °C. Dissolved oxygen was kept at ≥80 % of air saturation and pH was 7 throughout the study. Water flow was 50 ± 1 ml/min/tank throughout the experiments (six volume changes per day). Fish were fed twice daily, with live *Artemia* in the morning and SDS 300 dry food in the afternoon, following filming. Uneaten food and fecal material were removed from test vessels each day by carefully cleaning the bottom of each tank using a siphon.

The study lasted for 18 days. During the first nine days, all tanks were barren. On day 10, seven tanks were left barren, seven tanks were provided with a small airstone, (low airflow treatment; 14 x 25 mm blue cyclinder airstone, Elite) and seven with a large airstone (high airflow treatment; 100 x 15 mm blue longstone airstone, Elite). All airstones were placed in the rear left-hand corners or tanks (see Figure 3.3) and arrangement of tanks within the laboratory was random. Ends of the tanks were masked with black paper to ensure that fish in adjacent tanks had no visual contact with each other. Behavioural recordings

took place on alternate days throughout the study between 12:00 and 16:00 h. For more information regarding behavioural analysis see sections 2.3 and 5.2.

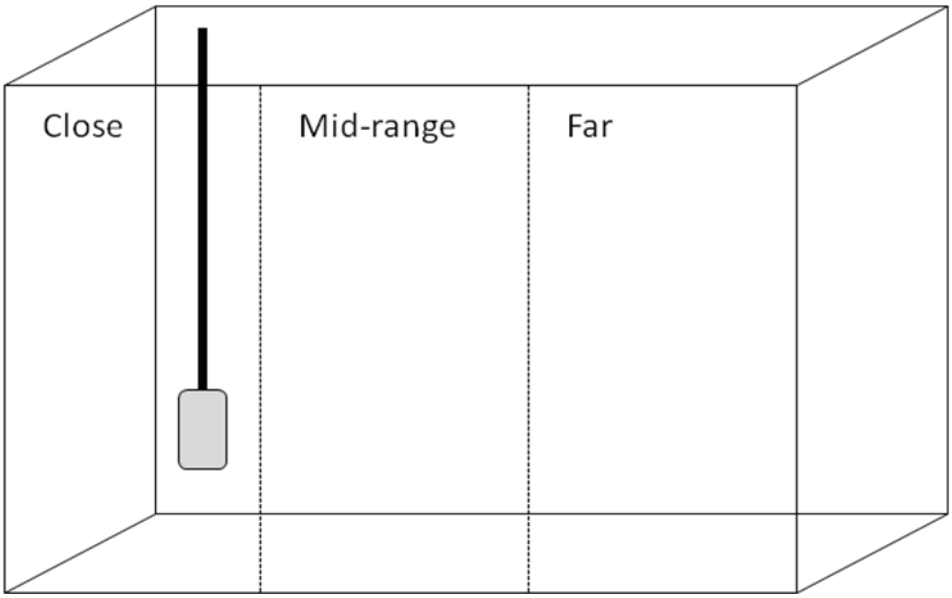


Figure 2.3 Location of airstone in tanks used in third study. (Small airstone shown in diagram). Labels denote names of tank compartments as used for behavioural analysis.

2.3 Behavioural measurements

In the first two studies, a 3 x 3 grid was drawn on the front and lid of each tank, resulting in 27 three-dimensional cells that could be occupied by fish. In the final study, moveable lines were attached to the computer screen during analysis.

In study one, examining potential structural enrichment for juvenile zebrafish, behavioural analysis was conducted beginning on day 1 (when fish were introduced to experimental tanks), and on alternate days until termination (*e.g.* days 3, 5 and 7), with 10 minutes of digital video footage being recorded for each tank (Samsung VP-MX10). During recording the camera operator was

concealed from view behind a screen. All recording took place between 12:00 and 16:00 h (4 and 8 hours following simulated sunrise). To provide a 3-dimensional view of fish movements using a single camera, a mirror was secured above the tank at an angle of 45°. Videos were analysed between minutes 6 and 10 of each recording, and the first 5 minutes discounted, to minimise effect of disturbance by the camera operator. This 5 minute period was determined to be suitable in a pilot study which looked at behavioural variables during a 20 minute period following typical disturbance from a camera operator. Videos were analysed for activity level, shoaling density, aggression, time spent in the bottom third of the tank and use of areas containing physical structures.

In study two, examining potential structural enrichment for adult zebrafish, behavioural analysis was conducted on days 1, 3, 5, 7, 8, 10, 12, and 14, with 10 minutes of digital video footage being recorded for each tank on each observation day (Samsung VP-MX10). As in the previous study, the camera operator was concealed from view behind a screen and a mirror was secured above the tank to provide a 3-dimensional view. All recording took place between 14:00 and 17:00 h (6 and 9 hours following simulated sunrise). On days and in tanks where fish were not subjected to the stressor, videos were analysed between minutes 6 and 10 of each recording, and the first 5 minutes discounted, as it was assumed that fish would still be disturbed from the movement of the camera operator. On observation days where tanks were subject to the chasing stress, recording and analysis was conducted for 5 minutes directly following the stress. As in the previous study, videos were

analysed for activity level, shoaling density, aggression, time spent in the bottom third of the tank and use of areas containing physical structures.

In the third study, examining the effects of tank aeration on adult zebrafish, behavioural analysis was conducted on day 1 (when fish were transferred to experimental tanks) and on alternate days until termination. Camera equipment was identical to that previously stated and recording took place between 12:00 and 16:00 (4 and 8 hours following simulated sunrise). Behaviours quantified were activity level, aggression and use of tank space (*i.e.* proximity to airstone).

2.3.1 Activity level

Activity level was recorded as the total number of horizontal and vertical lines, viewed from the front of the tank, crossed within a minute. This was averaged for all fish within the tank and counts for all fish took place between minutes 6 and 7. In the second study (see section 2.2.2), for tanks receiving the handling stress, activity was quantified between minutes 1 and 2 (*i.e.* directly following the period of stress).

2.3.2 Shoaling density

Shoaling density was calculated using the 27 three-dimensional cells of water created by the 3 x 3 grids on the front and lid of the tanks. The number of fish located in the cell containing the most individuals was recorded every 30

seconds over the 5 minute period, and these values averaged to give a mean value for shoaling density within the tank.

2.3.3 Aggression

Aggression was measured as the total number of aggressive advances made by all individuals in the tank over one minute (observed between minutes 6 and 7, or 1 and 2 following the chasing stress in study 2 (see section 2.2.2). An aggressive advance is defined as either a chase or a bite, with chases being one animal pursuing another over a distance of greater than two body lengths. If a chase continued after the retreating animal changed direction this was counted as an additional aggressive interaction.

In study 3 (the effect of aeration), this is expressed as number of aggressive advances per fish. This is for standardisation due to fluctuating numbers of animals in tanks as a result of some mortalities throughout the study. (Total mortality at the end of the study was 0, 21 and 10 % in the control, low airflow and high airflow treatments respectively).

2.3.4 Percent time in bottom third of tank

Percentage time that each zebrafish spent in the lower third of the tank was determined over a five minute period, and the mean value for the six fish calculated.

2.3.5 Proximity to tank structures/airstones

To quantify use of space within the tanks in studies one and two, the nine two-dimensional areas created by a 3 x 3 grid drawn on the tank lid (viewed from the top of the tank) were numbered 1 to 9. In tanks containing glass structures, long, medium and short rods were located in grids 1, 5 and 9 respectively (see figure 2.2). The position of each fish was recorded every 30 seconds for five minutes and a total for the whole observation period calculated for each grid.

In study three, proximity to the airstone was recorded. The tank was divided into 3 sections (“close”, “mid-range”, and “far”, being left, central and right as viewed from the front of the tank – see figure 2.3). This was done on the computer screen during behavioural analysis following the study so lines did not have to be drawn on tanks. Airstones were located in the left hand of the three compartments, (labelled “close”). Every 30 seconds over a 5 minute observation period, the number of fish in each section was recorded and the average calculated for the entire observation period. This was then expressed relative to the number of fish in the tank for standardisation due to some mortalities throughout the study.

2.4 Physiological measurements

2.4.1 Whole-body cortisol

The cortisol extraction procedure was modified from Ramsay *et al*. (2006). Whole zebrafish were thawed on ice, weighed and placed in individual 15 ml microcentrifuge tubes (Falcon). Each fish was homogenised in 0.5 ml of phosphate buffered saline (PBS) for one minute using a bench-top homogeniser (Ultra-Turrax T25) and the probe rinsed into the sample tube with a further 0.5 ml of PBS. Homogenised samples were vortexed briefly and placed on ice. Three ml of diethyl ether solvent was added to each sample, briefly vortexed then centrifuged at 500 x *g* (Sanyo Mistral 3000i) for 2 minutes to separate aqueous and diethyl ether layers. Samples were stored at -80 °C for 15 minutes and the unfrozen diethyl ether layer poured into a clean test tube. This process was repeated with a further 3 ml diethyl ether, and then combined diethyl ether portions were dried under a gentle stream of nitrogen gas for 2 hours. These were stored at -20 °C for a maximum of 48 hours. After thawing on ice, samples were reconstituted in 250 µl assay buffer (Tris buffered saline containing proteins and sodium azide as a preservative; Assay designs, U.K. 80-0010) and vortexed thoroughly before use. Cortisol concentrations were measured using a commercial cortisol enzyme linked immunoassay (ELISA) kit (Assay Designs, U.K. 900-071). Cortisol levels were normalised based on the mass of the whole-body sample and reported as ng/g.

Prior to use the kit was validated for use with zebrafish whole-body samples, with average linearity and recovery of spiked samples of 96 and 81% respectively.

2.4.2 Quantification of gene expression in zebrafish brain and liver relating to stress.

2.4.2.1 RNA extraction

Total RNA was extracted from liver and brain tissue, using 1 ml TRIzol RNA isolation reagent (Invitrogen). TRIzol was combined with the sample and homogenized using a pellet pestle. Samples were then incubated with 0.2 ml chloroform for 15 minutes and centrifuged at 12,000 x *g* for 15 minutes at 4 °C for phase separation. The uppermost aqueous layer was transferred to a fresh microcentrifuge tube and the RNA precipitated with 0.5 ml isopropanol and centrifugation at 12,000 x *g* for 10 minutes at 4 °C. Following washing with 1 ml 75 % ethanol and further centrifugation at 7,500 x *g* for 5 minutes at 4 °C, the pellet was re-suspended in 100 µl RNAse-free water. Sample quantity and quality was checked using a NanoDrop ND-1000 spectrophotometer. A minimum 260/280 ratio of 1.8 was accepted to ensure that there was no DNA contamination within the RNA samples..

2.4.2.2 cDNA synthesis

cDNA was synthesised using 1 µl total RNA, 2 µl 10x buffer, 2 µl dNTP mix (5 mM each dNTP), 2 µl Oligo-dT primer (10 µM), 1 µl RNase inhibitor, and 1 µl Omniscript Reverse Transcriptase (Qiagen) in a final volume of 20 µl. The mixture was incubated at 36 °C for 30 minutes.

2.4.2.3 qPCR

Primers specific to zebrafish Glucocorticoid Receptor (GR) and Phosphoenolpyruvate Carboxykinase (PEPCK) were designed with Primer3 v.0.4.0 software (http://frodo.wi.mit.edu/primer3) and purchased from Invitrogen. Primers are shown in Table 2.1 and were used to amplify a ~100 bp product. Primer pair annealing temperatures were optimised for real-time PCR on a temperature-gradient program. Primer specificity was confirmed by melt curve analysis. To determine the detection range, linearity and real-time PCR amplification efficiency ($E = 10^{[-1/slope]}$) of each primer pair, real time PCR amplifications were run in triplicate on a 10-fold serial dilution series of zebrafish cDNA and standard curves were created referring to the threshold cycle (Ct: the PCR cycle at which fluorescence increased above background levels) to the logarithm of the cDNA dilution.

Real time PCR was performed with the i-Cycler iQ Real-Time Detection System. Each sample was amplified in triplicate using 96-well optical plates (ABgene, Epsom, U.K.) in a 20µl reaction volume using 0.25 µl specific primers (see Table 2.1), 1 µl cDNA, and 10 µl 2 x Absolute QPCR SYBR Green Fluorescein mix (ABgene). PCR conditions were as follows: Fifteen minute

activation step at a temperature of 95 °C, followed by 40 cycles of the following: 10 seconds denaturation at 95 °C and 60 seconds annealing and extension at 55 °C. Template-minus and reverse transcriptase-minus negative controls were run for each plate and each sample respectively. Aliquots of zebrafish liver cDNA were repeatedly quantified on each plate to assess intra- and interassay variability.

To ~~confirm the integrity of cDNA and to quantify~~normalise for differences in RNA load between samples, GR and PEPCK expression values were normalised to a housekeeping gene. In a preliminary study, the levels of two potential housekeeping genes (β-actin and elongation factor 1α) were measured in each sample (primer sequences are shown in Table 2.1). β-actin exhibited the least variation between control/experimental fish and were, therefore, used for these normalisations. Fold changes in gene expression were determined using a development of the arithmetic comparative method ($2^{-\Delta\Delta Ct}$) which includes a correction for differences in efficiency (E) between the target and housekeeping gene. The formula was as follows:

$$\text{Fold change} = \frac{(E_{target})^{\Delta Ct \text{ target (control-treated)}}}{(E_{reference})^{\Delta Ct \text{ reference (control-treated)}}}$$

Target gene	Genbank Acc. No.	Product length (bp)	Annealing temp	Efficiency	Primers
β-actin	AF025305	121	55 °C	2.08	Forward: 5'-GTA AGG ACC TGT ATG CCA AC-3' Reverse: 5'-ATG TGA TCT CCT TCT GCA TC-3'
GR	BC164545	116	55 °C	1.98	Forward: 5'-AGA CCT TAA CAA CCC CTC TC-3' Reverse: 5'-GGG AGA AAA GTC CTC TGT TT-3'
PEPCK	BC053122	115	55 °C	2.11	Forward: 5'-TCA TCA TCA TCA CCA CAG AC-3' Reverse: 5'-CTG ACC GAG AGA GAG AGA GA-3'

Table 2.1 Primers used for qPCR

2.5 Statistical analysis

All statistical analyses were performed using Minitab v. 15. Specific details regarding transformation of data are included within relevant chapters. In all studies, repeated measures analysis of variance (ANOVA) were used to detect any differences in activity level, shoaling density, time spent in the bottom third of the tank and total aggressive interactions between different treatments. A Tukey post hoc test was used to determine where significant differences lay. One-way ANOVA was used to compare time spent in different areas of tank between control and enriched treatments. For analysis of whole-body cortisol values ANOVA was used to detect any differences in cortisol between fish from

structured and control tanks. In all cases, statistical significance was accepted at $p < 0.05$.

In the second study (see section 2.2.2 and chapter 4) non-paired t-tests were used to compare levels of GR expression between different treatments. In the third study (see section 2.2.3 and chapter 5) levels of GR and PEPCK expression were compared using one way ANOVA.

Mean absolute deviation (MAD) was calculated for all behavioural and physiological endpoints and comparisons made between treatments using one-way ANOVA. Further information regarding these specific comparisons are given in sections 3.2, 4.2 and 5.2.

CHAPTER 3

BEHAVIOURAL AND PHYSIOLOGICAL RESPONSES OF ZEBRAFISH TO A STRUCTURED ENVIRONMENT

CHAPTER 3 – BEHAVIOURAL AND PHYSIOLOGICAL RESPONSES OF ZEBRAFISH TO A STRUCTURED ENVIRONMENT

3.1 Introduction

Fish are increasingly used in research relating to their fundamental physiology and behaviour, but also biomedical and regulatory toxicology studies. Commitment to the principles of the 3Rs (Reduction, Replacement, Refinement) are continually being made to reduce the number of mammalian vertebrates used within research laboratories, and it is likely that one consequence of this will be that the numbers of fish being used will continue to rise in the coming years. In the United Kingdom (UK), the total number of vertebrates used in animal experiments is recorded in such a way that the numbers and class of vertebrates and the purpose for which the study was conducted can be obtained from the Home Office (http://www.homeoffice.gov.uk/). In 2010 more than twice as many fish were used than rats, and fish represented 13 % of all vertebrate studies. These numbers represent a 23% increase on the previous year which has increased year on year over the last decade. It is axiomatic that the welfare of fish is of importance, and this has led to specific legislation in the European Union (EU). In part, this is due to several studies detailing the potential for sentience, nociception and the perception of fear in fish (Braithwaite and Boulcott, 2007; Chandroo et al., 2004; Sneddon et al., 2003) and it is now thought that fish possess forebrains that are considerably more developed than previously assumed (Salas et al., 2006). However, reasons for concern relate to the potential effects of poor welfare on scientific validity and repeatability in experimental work (Williams et al., 2009). At present, fish used in the laboratory are commonly housed in barren tanks with only the addition of

spawning substrate when required. Often the simple reason for this is to ensure that test conditions between research locations are standardised (Olssen and Dahlborn, 2002). However, there are now widespread concerns that barren conditions may compromise the health of animals generally (Dawkins, 1998). For this reason, there is much interest in how environmental enrichment may be used to improve the welfare of fish used in the laboratory, and indeed some welfare organisations recommend enrichment for fish despite the lack of positive scientific evidence of benefits for the fish (Reed and Jennings, 2010).

Environmental enrichment is the alteration of a captive environment in a way that promotes a positive change in welfare of the animals within it (Olssen and Dahlborn, 2002). This can be through manipulation of the social, physical or chemical environment, and may encompass types and methods of food provision. Such enrichment can have beneficial cognitive effects in both healthy and diseased animals, and has been found to correspond with higher brain mass and cortical thickness relative to body size in studies with rats (Bennett et al., 1964; Diamond, 2001). Significantly higher relative brain mass has also been observed in wild salmon and rainbow trout, in comparison to their hatchery reared counterparts (Kihslinger and Nevitt, 2006; Marchetti and Nevitt, 2003, respectively) and this effect has also been seen in fish that are reared in enriched tanks in comparison to those reared in barren tanks (Kihslinger and Nevitt, 2006). There is also a wide range of data showing effects of enrichment on behavioural tasks involving spatial memory, anxiety, exploratory activity and aggressiveness, particularly in laboratory rodents (Amaral et al., 2008). As

such, environmental enrichment is widely used to promote good welfare in farms, zoos and mammalian laboratory settings.

Very few studies have conducted research into the environmental preferences of fish. Types of enrichment techniques previously studied have included tank colour (Appelbaum and Kamler, 2000; Serra et al., 1999; Strand et al., 2007), addition of artificial vegetation (Basquill and Grant, 1998; Hamilton and Dill, 2002) and even music (Papoutsoglou et al., 2007). However, the huge diversity of fish species (more than 25,000 discovered so far; Nelson, 2006), their varied life histories and habitat preferences, means that results from such studies are often not applicable to species others than those directly investigated. Furthermore, the purpose of the research and interests of the various stakeholders will have a major effect on the type of enrichment that is appropriate. Within aquaculture, for example, the primary target will be to improve growth and overall condition of fish, while in public aquaria, visitor perception of the fish and its environment, along with reduction of negative behaviours such as aggression is often the main concern. For fish used in regulatory studies, for example to establish safe levels of contaminant chemicals in the environment, there is currently no information available regarding the application of specific enrichment techniques . However, Williams et al., (2009) have advocated appropriate species and life stage specific environmental enrichment including the physical parameters (temperature and light), relevant feeding techniques including live foods, water movement, con-specifics and co-culture as potential methods to increase welfare and enrichment of laboratory fish.

Within regulatory toxicology, the potential for tank enrichment is complicated by the prescriptive nature of regulatory guidelines and their strict criteria to ensure validity and international compatibility (http://puck.sourceoecd.org). In Europe, laboratory experiments with vertebrates are regulated within the European Convention for the Protection of Vertebrate Animals used for experimental and other purposes (1986, ETS 123) which was updated via the new Directive 2010/63/EU (http://eur-lex.europa.eu). Within the UK, Home Office Inspectorate Guidance (ASPA, 1986) (and therefore licence to conduct scientific investigations with vertebrates) require that the fish are inspected regularly and must be clearly visible within the tank. This may not be appropriate for all species, those using cryptic camouflage may well be 'stressed' in an arena in which they can be clearly seen. For many types of regulatory-based toxicological testing using fish, the container vessel must be inert, efforts must be made to avoid excessive microbial growth, and behavioural observations must be conducted (for comparison with controls). These requirements are generally not compatible with the addition of objects to the tank, as increased cover (e.g. plants) can make observation difficult, increased surface area can lead to absorption of the test chemical and increased microbial load (potentially leading to chemical degradation) could make required regular cleaning ineffective, in addition to leaching of confounding chemicals into the test environment. Furthermore, any manipulation of the environment that results in alteration of the behaviour and/or physiology of test fish, while being beneficial in terms of welfare, may serve to change innate variability and potentially prevent useful comparison with previously published data. Several studies, for example, have shown that fish exhibiting different behavioural phenotypes can

show dramatically different chemical uptake profiles (e.g. 20-fold differences in copper uptake between dominant and subordinate fish; Sloman et al., 2002). This variability could make it necessary to utilise more animals to attain comparable statistical power and would not be in accordance with current attempts to replace, refine and reduce the use of animals in research (www.nc3rs.org.uk). Of course, a counter argument is that studies with a degree of behavioural/physiological variability in the fish may be more comparable to situations in the natural environment and so may increase the relevance of such studies. Several factors, therefore, present a perceived cost of environmental enrichment for laboratory fish. Some, such as the change in chemical nature of the water and potential interaction with the test chemical, are likely to challenge the validity of the overall test and so this must be balanced against the benefits in terms of improved welfare for the test fish. Unfortunately, little information is available to make a balanced scientific assessment of the value of the improved welfare of including an item in the test tank to improve welfare. There is an additional perceived constraint for regulatory testing in that the conditions in the husbandry and rearing of the fish should be the same as in the tests themselves. This is true for the quality of the water, and best practice applies this to the housing in most establishments.

There continues to be a drive from regulatory sources (e.g. EU 2010 and UK HO inspectorate and welfare organisations such as the RSPCA) that enrichment for fish is the same as for mammals. This is widely interpreted as objects being placed in the tank as enrichment for the fish as toys or “chews” are used with mammals. We are unable to identify any evidence from sound

scientific study in the literature that would agree with this concept for fish. Although a precautionary principle should be applied, it should also be considered that some forms of enrichment could provide defendable territory and potentially harm the welfare rather than improve it.

The aim of the present study was to address this lack of knowledge concerning the potential welfare benefits of hypothesised environmental enrichments for zebrafish (*Danio rerio*) used in regulatory studies such as those described by the OECD (http://www.oecd.org). We therefore designed a simple form of physical enrichment consisting of vertical glass rods of varying heights within fish tanks to provide a degree of three-dimensional complexity and potential refuge. As zebrafish are generally found in areas of vegetation which provide cover from predators and microhabitats for spawning and foraging (Engeszer et al., 2007). Our hypothesis was that this type of artificial enrichment might approximate such vegetative cover, but in a way that would be most compatible with regulatory toxicology studies. The glass rods meet the inert requirement and appear to increase environmental complexity, but do have the drawback of increasing surface area and are difficult to clean *in situ*, but could be easily removed and replaced for daily cleaning. Behavioural parameters were examined in juvenile zebrafish during a 7 day exposure to either barren tanks or enriched tanks. Activity level, shoaling density, aggression and time spent in the bottom third of the tank were measured as these key behaviours have all been associated with stress and anxiety in zebrafish (Egan et al., 2009; Rehnberg and Smith, 1988). In addition to this we measured whole-body cortisol concentration. Cortisol is a hormone which has been well documented in many

animals, primarily due to its increase in response to physical and environmental stress (for review: Wendelaar Bonga 1997; Mommsen et al., 1999).

3.2 Materials and methods

Juvenile zebrafish were obtained from AstraZeneca, Brixham Environmental Laboratories and kept under conditions compatible with OECD guidelines throughout. Prior to the experiment, fish were kept in flow-through fresh water at 28 °C and under a photoperiod of 10L:14D (light:dark) with a 20 minute phased sunrise/sunset. From 4 days post-fertilisation (dpf) fish were fed daily to excess with ZM000 infusoria grade food (Special Diet Services, Essex UK), and live rotifers, with the addition of live *Artemia* 24 h nauplii at 10 dpf. At the beginning of experiments fish were 35 dpf. Adult zebrafish were similarly obtained from Brixham Laboratories. At commencement of the study, adult fish were approximately one year old. Prior to the study they were maintained under identical conditions as previously stated for juvenile fish, and fed a diet of *Artemia* 24 h nauplii and SDS 300 dry food.

For the juvenile study, groups of six fish were provided with either a control (barren) or a structured environment for a duration of either 1, 2, 4 or 7 days. Fish were terminated by over-anaesthesia in accordance with UK home office guidelines at the end of each of these periods. Behavioural endpoints consisting of: activity level, shoaling density, aggression, time spent in the bottom third of the tank and use of areas containing physical structures were analysed on alternate days during the 7-day exposure. The same protocol was conducted for adult fish. It was not possible to determine sex differences in juveniles and so

fish were allocated to tanks randomly. However, in the adult study, all experimental tanks contained three male and three female fish. Whole-body cortisol concentrations were measured from individual fish following termination at the end of the juvenile study only. This was accomplished using a commercial Enzyme Linked ImmunoAssay kit. (Cortisol analysis was not conducted on adult fish due to the lack of significant differences observed in juveniles, combined with financial constraints).

For further information on the experimental design looking at the effects of a structured environment on juvenile and adult zebrafish see sections 2.2.1.1 and 2.2.1.2 respectively, and for details on the behavioural and physiological endpoints used see sections 2.3 and 2.4.

All statistical analyses were performed using Minitab v. 15. Activity and shoaling density data were log transformed and data regarding time spent in areas containing structures was square root transformed to meet the assumptions of normality. Repeated measures analysis of variance (ANOVA) was used to detect any differences over the four observation days in activity level, shoaling density, time spent in the bottom third of the tank and total aggressive interactions between control and structured tanks. A Tukey post hoc test was used to determine where significant differences lay. One-way ANOVA was used to compare time spent in areas containing structures (or corresponding areas in control tanks). For analysis of whole-body cortisol values, ANOVA was used to detect any differences in cortisol between fish from structured and control tanks. Mean absolute deviation was calculated for all measured parameters to determine the variation of individual endpoints and the effects of enrichment

upon this measure. In all cases, MAD was calculated for both barren (control) and structured treatments, combining values from all observation days. Statistical significance was accepted at $p < 0.05$.

3.3 Results

3.3.1 Behavioural responses to tank structures and observation day

3.3.1.1 Juveniles

In both treatments mean activity values varied between approximately 15 and 22 lines crossed per minute, and there was no effect of treatment ($F_{1,68} = 0.24$, $p = 0.63$) or observation day ($F_{3,68} = 0.98$, $p = 0.41$), but there was a significant interaction effect ($F_{3,68} = 3.13$, $p < 0.05$) (Figure 3.1). This means that the effect of the observation day on activity level was different for control tanks than it was for structured tanks. There was also no effect of either environment ($F_{1,68} = 0.44$, $p = 0.51$) or observation day ($F_{3,68} = 1.54$, $p = 0.21$) on the shoaling density. Typical shoaling size throughout the study was a maximum of 2 fish per cell (*i.e.* one of the 27, three-dimensional cells would typically contain two or fewer fish – see Figure 3.2). There was a significant interaction effect of time and treatment on the level of aggression observed within tanks ($F_{3,72} = 2.37$, $p < 0.05$) indicating that observation day had a different effect on aggression in control and structured tanks. Aggression in control tanks was sustained at 5-7 acts per minute on days 1 and 3 but then dropped significantly to less than 3 acts per minute on days 5 and 7. Aggression in structured tanks was similarly high at the start compared to controls (6-7 acts per minute) and remained at this high level on days 3 and 5 only dropping to a significantly lower level (< 2 per

minutes) on day 7 (Figure 3.3). The observation day had a strong effect on the time spent by fish in the bottom of the tank with fish from both treatments spending significantly less time in this lower zone on day 1 than on any of the following days ($F_{3,72}$ = 45.71, p < 0.001). On day 1, fish in structured tanks spent significantly more time (three times longer) in the bottom third than fish from control tanks that spent less than 8 % of their time there ($F_{1,18}$ = 28.03, p < 0.05). In contrast, after day 1, fish in both treatments spent the majority (50-60 %) of their time in this lower third of the tank (Figure 3.4). Neither tank environment ($F_{1,68}$ = 0.07, p = 0.80) nor observation day ($F_{3,68}$ = 1.22, p = 0.31) had a significant effect on the amount of time spent in areas containing enrichment (as compared with the corresponding areas within control tanks) (Figure 3.5 A-C).

Mean absolute deviation (MAD) of activity levels of control and structured tanks were 2.6 and 3.0 lines crossed respectively. These values were not significantly different ($F_{1,35}$ = 0.37, p = 0.55). Mean absolute deviation of shoaling density of control and structured tanks were 0.35 and 0.32 fish, also not significantly different ($F_{1,35}$ = 0.10, p = 0.76). Mean absolute deviation of percentage time spent in the bottom third of tanks was 33.73 % in control tanks and 22.83 % in structured. The difference was again not significant ($F_{1,36}$ = 3.59, p = 0.07). Finally, mean absolute deviation of aggression was 0.35 acts per minute in control tanks and 0.32 acts per minute in structured tanks, values that showed no statistical difference ($F_{1,34}$ = 0.14, p = 0.29). (All MAD results are summarized in Table 3.1).

3.3.1.2 Adults

Activity of adult fish was extremely stable over time ($F_{3,32}$ = 0.98, p = 0.98) and there was no effect of treatment on this behavioural variable ($F_{1,32}$ = 0.09, p = 0.76). Typical shoaling density throughout the study ranged from 2 to 3 fish per cell, and there was no effect of observation day ($F_{3,32}$ = 1.1, p = 0.36). However, the presence of enrichment had a strong affect on this measure ($F_{1,32}$ = 8.7, p < 0.01) with fish from structured tanks exhibiting significantly lower shoaling density on day 7 than those from control tanks which exceeded 3 fish per cell on day 7. There was a significant effect of observation day on total aggression in both control ($F_{3,16}$ = 7.97, p < 0.01) and structured ($F_{3,16}$ = 4.03, p < 0.05) treatments. Aggression in control tanks dropped steadily from about 25 attacks per minute on day 1 to less than 5 attacks per minute on day 7. Aggression levels in structured tanks similarly started at about 28 attacks per minute on day 1, and dropped to a value of about 13-14 on day 5 but then remained at this level for the remainder of the study. There was a significant effect of both treatment ($F_{3,32}$ = 3.1, p < 0.05) and observation day ($F_{1,32}$ = 36.6, p < 0.01) on the time spent by fish in the bottom third of the tank. On days 3, 5 and 7 fish in control tanks spent significantly more time here compared to both control fish on day 1, and compared to fish from structured tanks on the same days.

On all days, except for day 1, fish from structured tanks spent a significantly greater proportion of time in areas of tanks containing long rods than fish from control tanks did in corresponding areas ($F_{1,32}$ = 11.7, p < 0.01). In areas of tanks containing short rods, the opposite trend was observed. Except for day 1, fish from structured tanks spent a significantly lower proportion of time in areas

of tanks containing short rods than fish from control tanks did in corresponding areas ($F_{1,32}$ = 0.52, p < 0.01). There was no significant difference in the proportion of time that fish spent in the centre portion of tanks (containing medium rods in structured treatments) between control or structured tanks ($F_{1,32}$ = 1.8, p = 0.19).

Mean absolute deviation of activity levels was 12.05 lines crossed in control tanks and 8.53 lines crossed in structured, not significantly different ($F_{1,38}$ = 2.72, p = 0.11). Mean absolute deviation of shoaling densities were 0.44 and 0.32 fish in control and structured tanks respectively. Again, these values were not statistically different ($F_{1,38}$ = 1.68, p = 0.20). Mean absolute deviation of aggression was 9 acts in control tanks and 7 acts in structured tanks, again not significantly different ($F_{1,38}$ = 0.73, p = 0.40). Deviation in time spent in the bottom third of tanks was 9 % and 7 % in control and structured tanks respectively. This was also not a significant difference ($F_{1,38}$ = 0.73, p = 0.40).

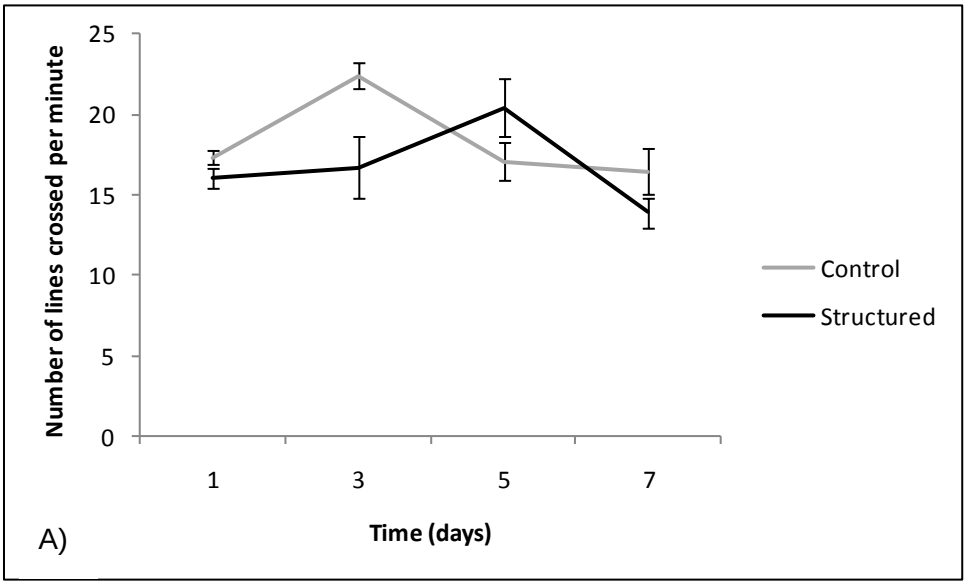
3.3.1.3 Comparison of adult and juvenile behavioural results

Activity level of adults was significantly greater than juveniles when measured as number of tank lines crossed per minute (see methods) ($F_{1,76}$ = 52.8, p < 0.01), averaging about double the activity of juvenile fish. On all days adult fish showed a significantly greater shoaling density than juvenile fish ($F_{1,76}$ = 21.2, p < 0.01), averaging about 50 % higher than juveniles. Adult fish also showed a significantly greater number of aggression interactions per minute (approximately three times as many) in comparison to juveniles ($F_{1,76}$ = 35.0, p < 0.01). Adults spent a greater percentage of time in the bottom of the tank in comparison to adult fish ($F_{1,76}$ = 44.0, p < 0.01), with adults spending up to 90 %

of their time in this zone compared to less than 60 % of their time in juveniles. In structured tanks, there were no differences between the time spent by adults and juveniles in areas containing short ($F_{1,38}$ = 2.01, p = 0.17), medium ($F_{1,38}$ = 0.03, p = 0.87) or long ($F_{1,38}$ = 2.54, p = 0.12) enrichment rods.

3.3.2 Whole-body cortisol content

Whole body cortisol concentrations (juvenile fish only) were significantly higher on day one than any of the following sampling days ($F_{3, 68}$ = 6.12, p < 0.01). However, there was no effect of treatment at any time point ($F_{1, 68}$ = 2.28, p = 0.14) (fig 3.6). Mean absolute deviation was not significantly different between treatments on any of the days tested ($F_{1,72}$ = 0.6, p = 0.45) although there was a significant effect of observation day, with variation being significantly greater on day one than any other sampling days ($F_{3,72}$ = 15.5, p < 0.01).



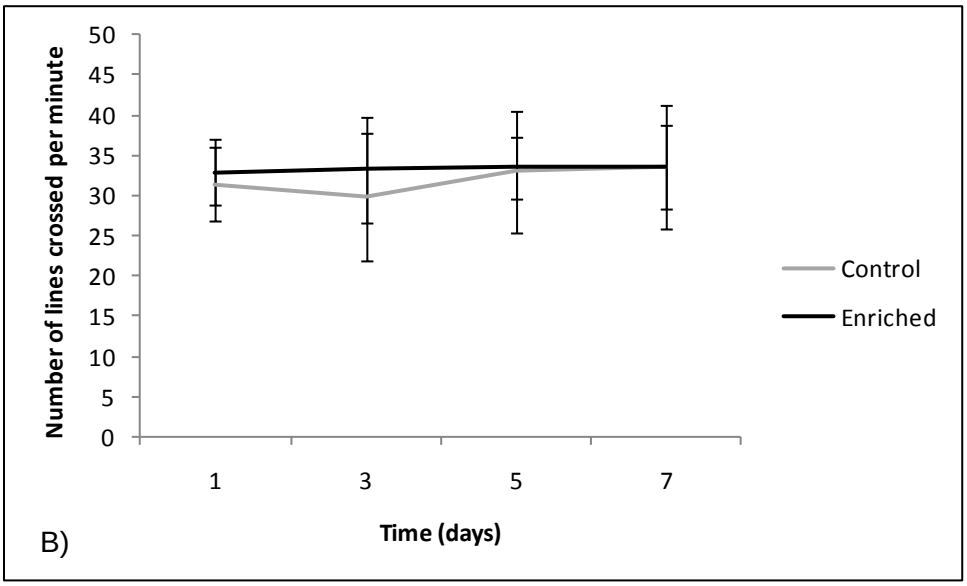


Figure 3.1 Mean activity level of juvenile (A) and adult (B) fish in control and structured tanks. (Mean ± SE, n = 10).

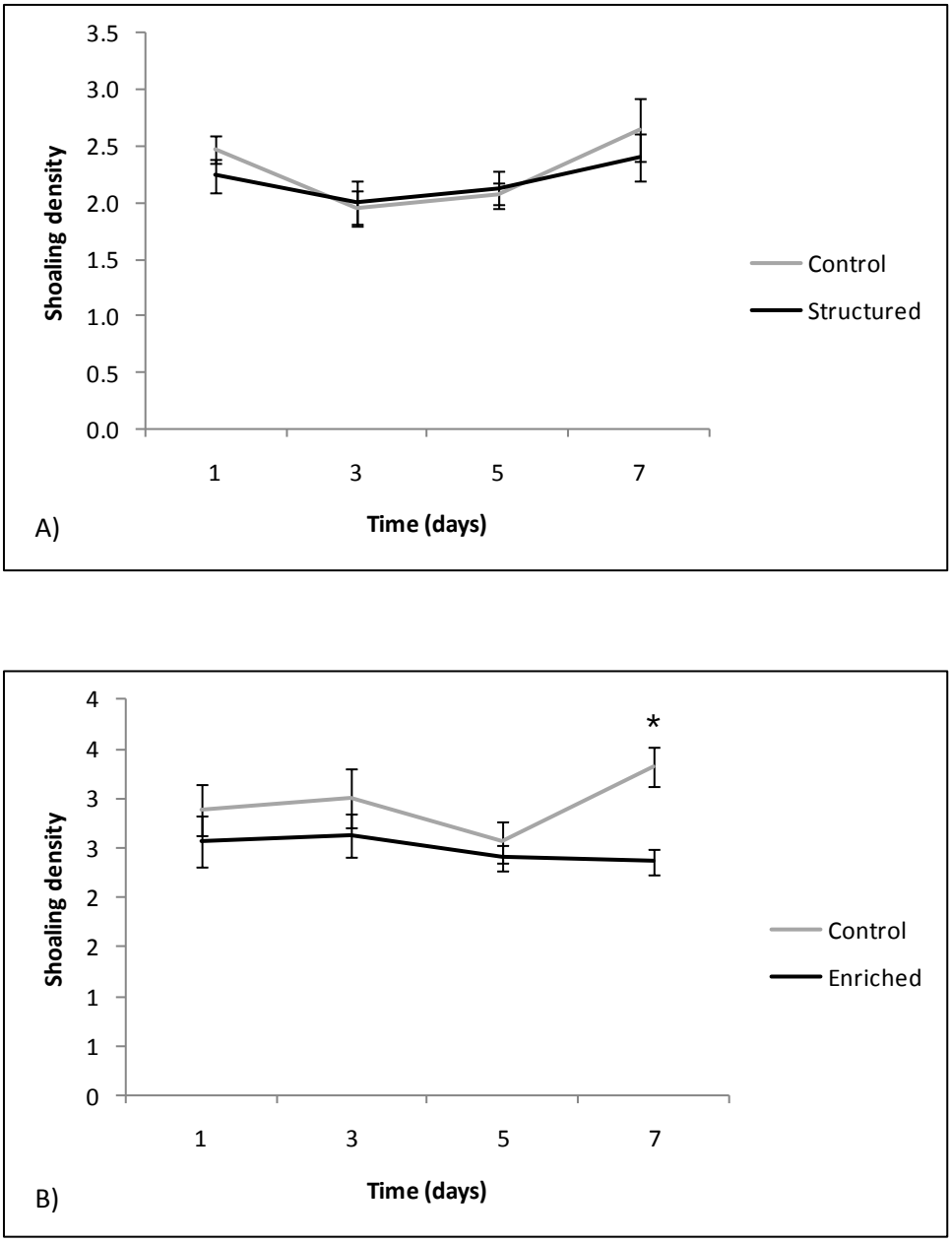
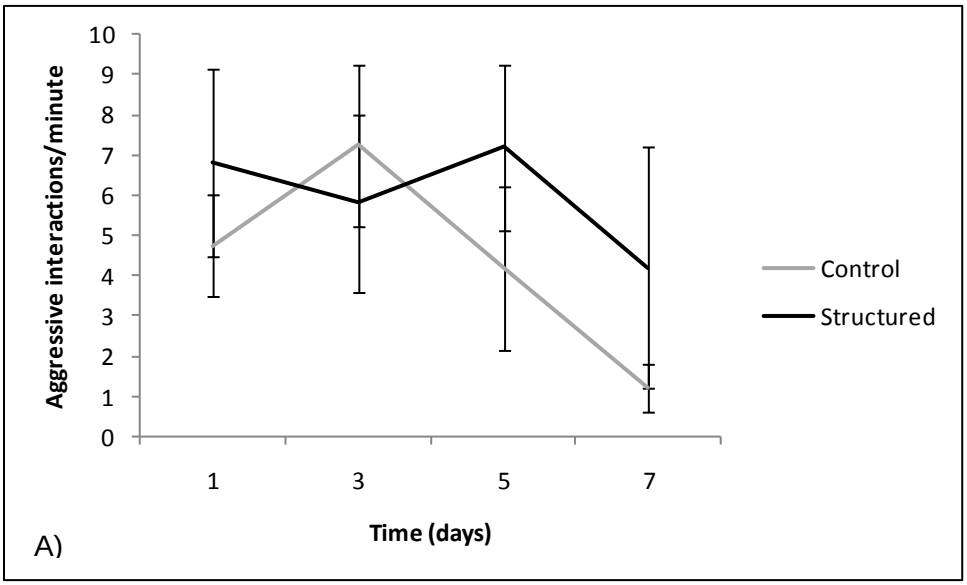


Figure 3.2 Mean shoaling density of juvenile (A) and adult (B) fish in control and structured tanks. (Mean ± SE, n = 10). * denote a significant difference between treatments. Statistical significance accepted at $p < 0.05$.



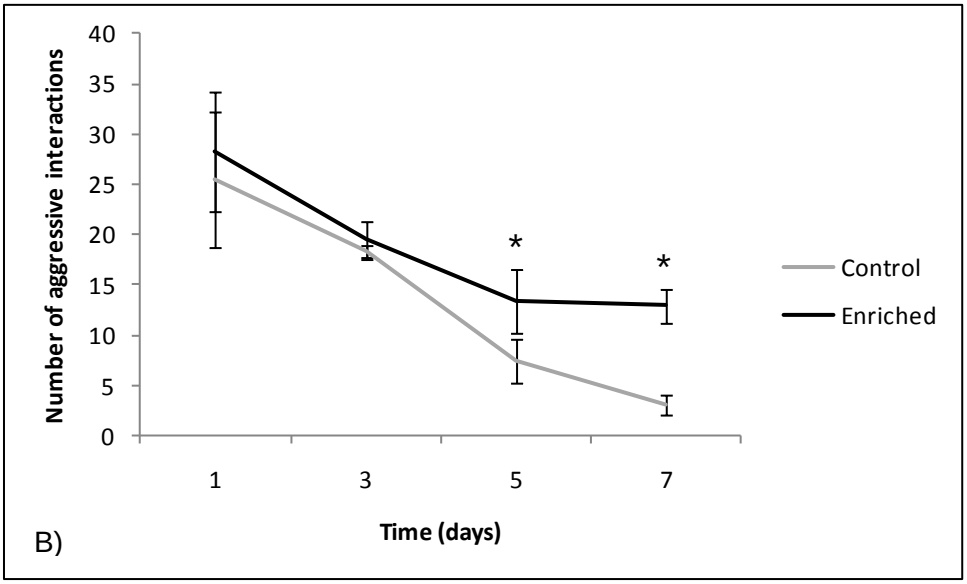
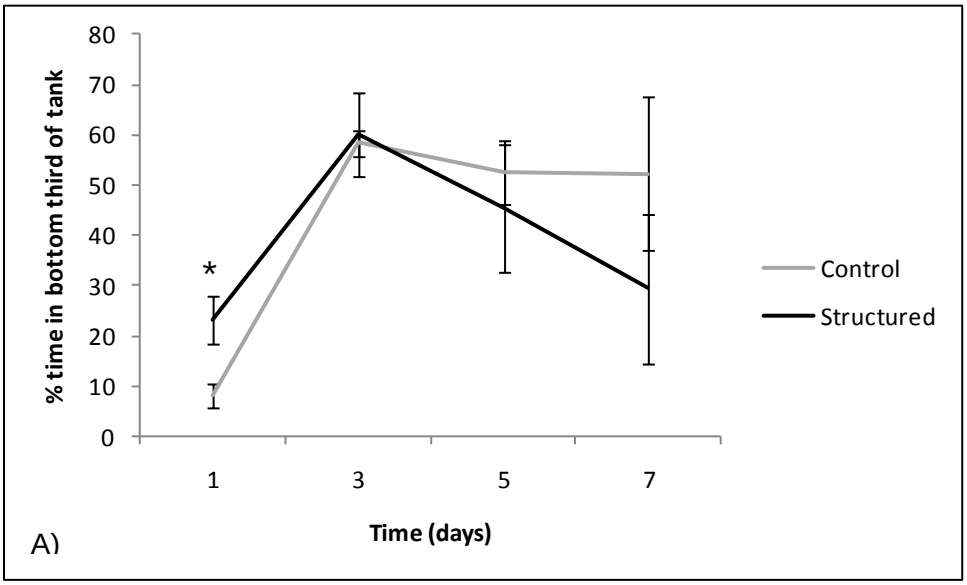


Figure 3.3 Mean whole-tank aggression levels in juvenile (A) and adult (B) fish in control and structured tanks. (Mean ± SE, n = 10). * denote a significant difference between treatments. Statistical significance accepted at $p < 0.05$.



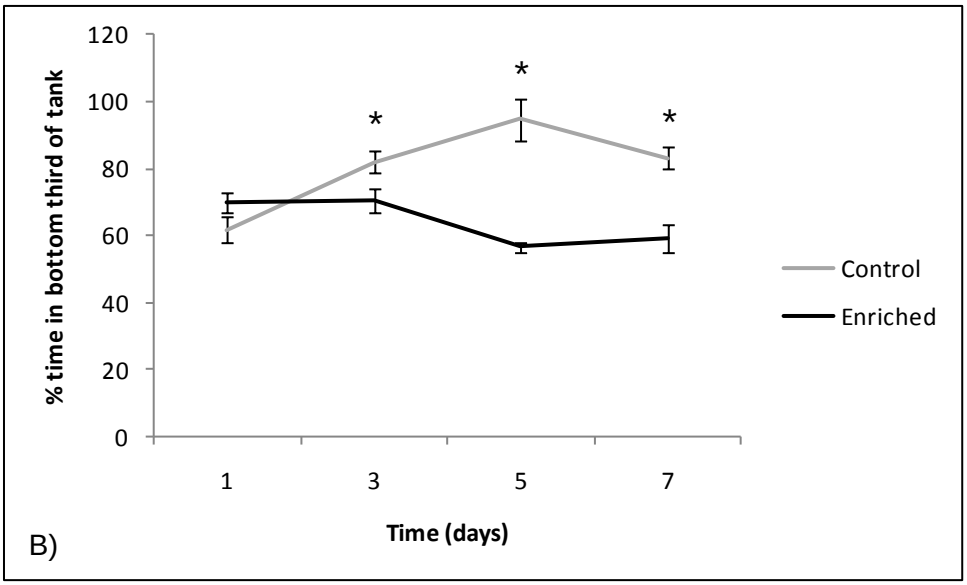


Figure 3.4 Mean percentage time spent by juvenile (A) and adult (B) fish in the bottom third of the tank in control and structured tanks. (Mean ± SE, n = 10). * denote a significant difference between treatments. Statistical significance accepted at $p < 0.05$.

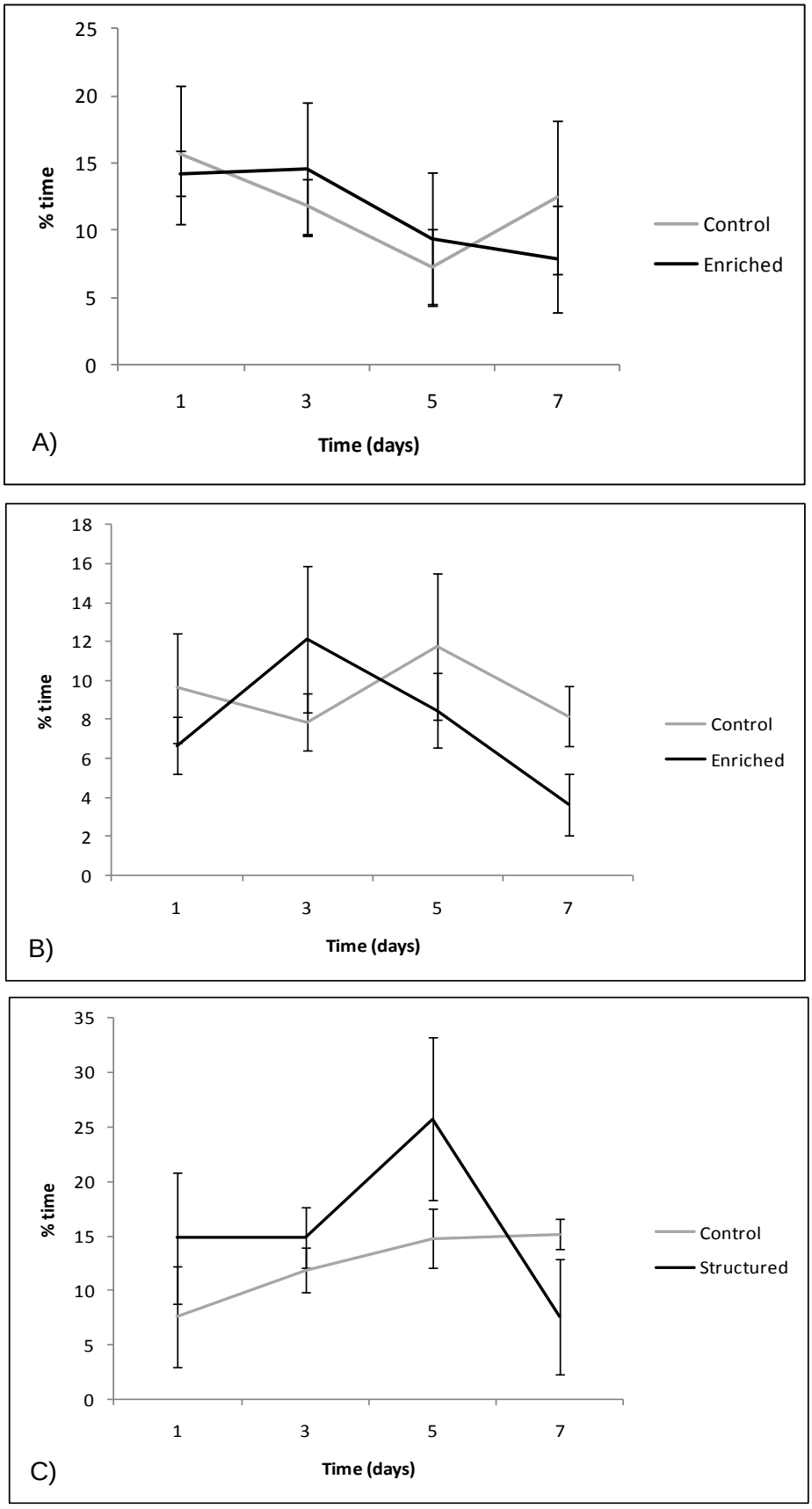


Figure 3.5 Mean percentage time spent by juvenile fish in areas of tank containing short (A), medium (B) and long (C) rods. (Mean ± SE, n = 10).

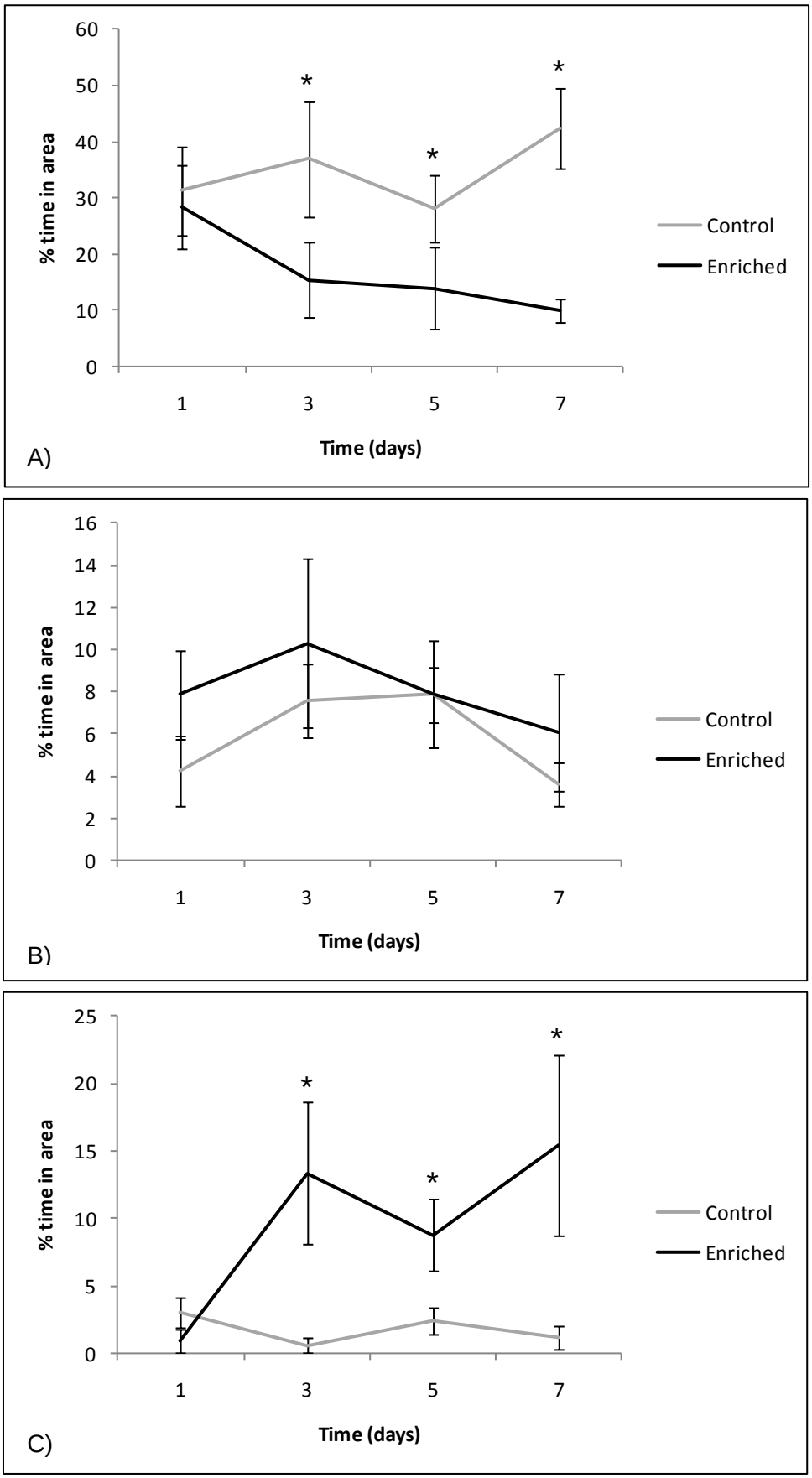


Figure 3.6 Mean percentage time spent by adult fish in areas of tank containing short (A), medium (B) and long (C) rods. (Mean ± SE, n = 10). * denote a significant difference between treatments. Statistical significance accepted at $p < 0.05$.

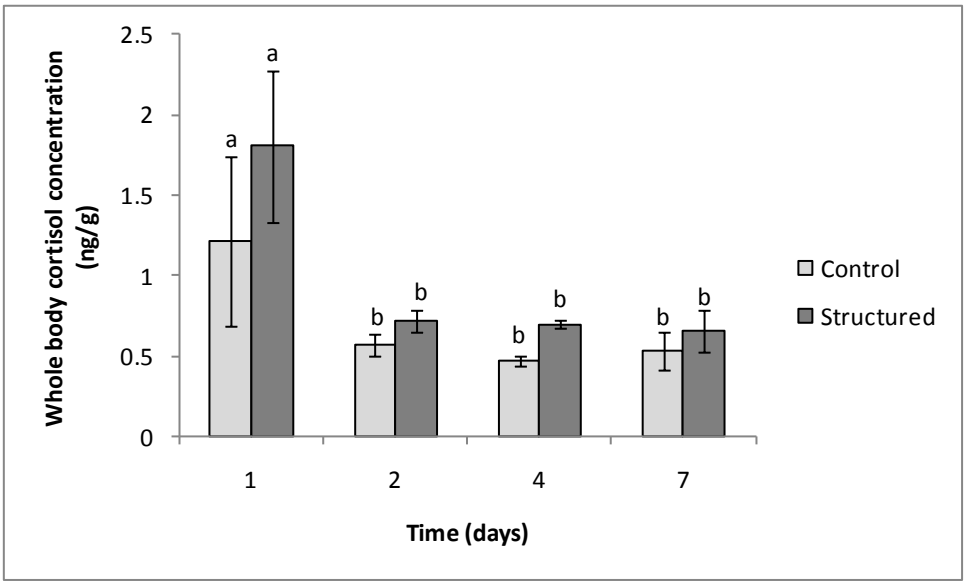


Figure 3.7 Mean cortisol values of juvenile fish from control and structured tanks at four time periods. (Mean ± SE, n = 10). Different letters denote a statistical difference. (Significance accepted at $p < 0.05$).

Table 3.1 Mean absolute deviation (MAD) observed in all behavioural endpoints and whole body cortisol from juvenile and adult fish maintained in either control or structured tanks.

	Juvenile		Adult	
	Control	**Structured**	**Control**	**Structured**
Activity level	2.6	3	12.05	8.53
Shoaling density	0.35	0.32	0.44	0.32
Aggression	0.53	0.61	8.84	6.94
% time in tank bottom	33.73	22.83	30.86	23.17
Whole-body cortisol	0.36	0.45	-	-

3.4 Discussion

Within both juvenile and adult studies, there were no observed differences in locomotory activity between treatments or on different observation days. Increased activity level or locomotion is a behaviour that is associated with an anxiety response in fish (Gerlai *et al.*, 2000). This has been observed in fish exposed to drugs noted for their anxiogenic or anxiolytic properties, and indeed is a key behavioural endpoint in several OECD type regulatory studies, as well as in wider research. For example, zebrafish subjected to cocaine withdrawal, a documented source of anxiety both in fish as well as humans, show increased locomotion (López-Patiño *et al.*, 2008). Conversely, fish exposed to nicotine, which is commonly observed to attenuate anxiety in a variety of subjects, show reduced levels of activity (Levin *et al.*, 2007). Locomotory activity has also been shown to change in response to other stressors. Blaser and Gerlai (2006) found that fish introduced to a novel tank showed increased activity in comparison to latent levels, and the same response was also seen for fish that were monitored for aggression towards a mirror image. Both of these situations were expected to cause the fish a degree of stress. Similarly, McFarlane *et al.* (2004) showed increased locomotion in rainbow trout that were subjected to a crowding stress, suggesting that this is a more general fish response. (However, see below for the opposite response to stress in some fish).

There are several scenarios that might explain why there were no observed differences in activity level. Firstly, it is possible that the physical structures simply had no effect on this behaviour. The primary intention of this treatment design was to provide a refuge and therefore a less “stressful” or “risky” environment than the control (barren) tanks. However, present data imply that

control tanks were not necessarily stressful or anxiogenic, and definitely not to the same extent as the toxicant exposure or crowding reported by others. The addition of a proposed enrichment, therefore, may not have changed the anxiety levels of fish at all, or at least not sufficiently that it was reflected in their locomotion rate. Another option to be considered relates to the way the data was analysed. To avoid pseudoreplication, activity levels of all fish within one tank were averaged. Several studies have observed that both innate activity levels and anxiety-related responses of zebrafish vary greatly between individuals, with some showing “freezing” and others more erratic movements (López-Patiño *et al.*, 2008). Similarly, natural variations in behaviours relating to dominance and subordinance are common (Larson *et al.*, 2006). An average of all activity levels within one tank would, therefore, explicitly lose this level of information and allow us to test the effect of the treatment. However, there were no significant differences in either standard deviation or maximum range of individual activity levels between tanks.

In comparison with juveniles, adults showed a significantly higher amount of activity level. The method of quantifying this behaviour counted the number of times fish moved between different areas of the tank. This is effective as a crude, absolute measure of activity for comparison of treatments and over time. However, as a relative measure of swimming speed, this measure is less worthwhile as it does not take into account 3-dimensional movement or body size of the fish. In this instance it is likely that the higher activity levels recorded in adult fish are a reflection of their larger body size rather than a relevant response to the experimental conditions. In support of this, a rough estimation of swimming speed calculated from the distance between tank lines suggested

that juvenile and adult fish were swimming at approximately 57 and 60 body lengths per minute respectively.

In the juvenile study, shoaling cohesion did not vary between treatments or with time. In the adult study, however, on day 7, the shoaling density within structured tanks was significantly lower than that of control tanks. Other laboratory studies have observed that "tightness" of a shoal varies in response to perceived risk and anxiety (Rehnberg and Smith, 1988; Egan *et al.*, 2009). This is an adaptive response which, in the wild, would confer increased protection from predators (Speedie and Gerlai, 2008). When transferred to a new environment, we would expect fish to swim closer together and that this increase in cohesion would diminish over time (Miller and Gerlai, 2007). The results from the adult study may at first appear to support this hypothesis, due to the difference observed on day 7. However, on closer inspection of this data, this difference appears to be due to an increase in shoaling density in the control treatment rather than any significant change in the structured tanks. Incidentally, it is interesting to note that shoaling density of adults in control tanks was greater than adults in structured tanks on all observation days, although not significantly so. The study, therefore, did not confirm the hypothesis that transfer to a novel environment causes increased cohesion which subsequently decreases. Indeed shoal density did not change over the seven day period apart from in the adult study where shoal cohesion in control tanks increased on the final day of observation. However, it may be that the initial tighter cohesion could be present over very short time periods (*i.e.* less than 24 hours) and so would not have been detected by this design. A further drawback of the design relates to evidence suggesting that shoal cohesion in

this species oscillates with a period of 5 to 15 seconds. This presumably gives fish a balance between protection from predation and foraging by compromising on a particular density of shoaling (Miller and Gerlai, 2008). As measures of shoaling were taken every 30 seconds throughout the observation period, it may be that these natural fluctuations may have affected the results.

A second hypothesis was that if the presence of rod structures did provide a refuge, this could decrease the amount of time taken for shoaling density to return to former levels. Similar to the results reported here, Miller and Gerlai (2007) found that shoal cohesion in zebrafish was constant, both upon introduction to a novel environment and following a period of habituation. They suggested the possibility that shoaling density is insensitive to novelty (as opposed to more significant and overtly life-threatening stressors such as predation), or that longer periods of exposure to a novel environment would be required to see an effect. In that study the observation tank they used, being white and devoid of hiding places, probably remained aversive to the fish throughout the experiment. Within this study it is possible that the size of the tanks did not allow us to observe patterns of shoaling as effectively as we would have been able with bigger tanks, however these tanks were four times larger, and a stock density 12 times lower than the minimum required for a valid OECD study.

It was also found that adult fish appeared to shoal more densely than juvenile fish. (approximately three fish and two fish per cell respectively). Due to the size differences of the fish, this result is counterintuitive, as we would expect a larger number of smaller fish within a given volume of water for relative shoaling

cohesion. Zebrafish shoal from the last (postflexionic) larval stage prior to becoming juveniles and it has been found that juveniles and adult spend a similar amount of time shoaling (Engeszer *et al.*, 2007). However, there is little information available in published literature regarding the density of typical shoals and in particular how this changes with life stage. The higher cohesion observed in adult fish in comparison to juveniles may have been due to behaviours specific to adult fish, such as those involved in reproduction and courtship, which most certainly require a degree of interaction. However, observation of reproductive behaviours was not deemed to be relevant to the present study and so this suggestion can only be speculative.

Despite being regarded as a shoaling fish, zebrafish also show high levels of aggression, both in males and females (Moretz *et al.*, 2007). Upon initial introduction a new group of fish will typically show high levels of aggression which gradually decline over several days as stable dominant/subordinate relationships are formed (Larson *et al.*, 2006). This pattern was supported by the results of both the juvenile and adult studies, but importantly, it appeared that the presence of the physical structures used here increased the amount of time taken for aggression to decline.

Environmental enrichment has been found to alter aggression in many species of captive animals. Haemisch and Gärtner (1997) found that male laboratory mice reared in enriched cages exhibited increased territorial aggression in comparison to than those reared in conventional cages. Van Loo *et al*. (2002) however found that, whilst provision of a shelter within mice cages increased aggression, addition of nesting material had the opposite effect. It was

hypothesised that this was due to the moveable nature of nesting substrates which allow an element of environmental control for the animals. As control and predictability are important features contributing to welfare, allowing mice to actively structure their environment therefore has a beneficial effect and is accompanied by a decrease in intraspecific aggression. In a study looking at the design of enclosures for growing pigs, Beattie *et al*. (1996) found that pigs raised in enriched enclosures showed less frequent harmful social behaviours such as nosing and tail biting. Similarly, provision of coloured rings as enrichment for caged laying hens significantly reduced aggressive head-pecking behaviours alongside overall mortality rates. Honess and Marin (2006) in their review of enrichment techniques for primates state that environments without enrichment have serious consequences, including increased likelihood of becoming subordinate, together with increased frequencies of aggression and abnormal behaviours.

There have been conflicting results found regarding the effects of environmental enrichment on aggression in fish. In a study by Kelley *et al*. (2006) butterfly splitfins (*Ameca splendens*) demonstrated higher levels of aggression in structured tanks, where dominant fish used enrichment to establish territorial boundaries. A similar finding was reported by Mikheev *et al*. (2005) in a study providing sections of pipe to juvenile perch (*Perca fluviatilis*). Conversely, Basquill and Grant (1998) found that aggression was reduced in a complex habitat containing simulated vegetation compared to a simple one. This was thought to be related to the difficulty of defending such a habitat, and the affect of the vegetation on visibility, a hypothesis supported by both Höjesjö and Johnsson (2004) in a study looking at the growth of dominant zebrafish in

complex and simple environments and by Kadry and Barreto (2010) in a study with pearl cichlids (*Geophagus brasiliensis*) looking at the defensive value of enriched versus barren territories. Such differences between studies may be due to the design and placement of proposed physical enrichments. The simulated vegetation used by Basquill and Grant (1998) allowed visual isolation of individuals. Provision of woody debris on the bottom of tanks holding brown trout (*Salmo trutta*) has been found to reduce both swimming activity and aggression (Sundbaum and Näslund, 1998). This is thought to be a result of reduced visual isolation. The design and location of enrichment used by Kelley *et al*. (2006) and Mikheev *et al*. (2005), however, allowed no such visual isolation but instead permitted monopolisation by dominants. This highlights an important consideration concerning the design of enrichment, and shows that different types of tank structure can have vastly different effects on the behaviours of different species.

A further explanation for the effect of enrichment and tank structures on aggression is that it increases the amount of resources that a territorial animal must defend, and thereby affects its aggressive dynamics (Barreto *et al.*, 2011). Game theory predictions concerning resource value suggest that as an individual perceives the value of its environment to increase, it will be more likely to behave aggressively in order to defend its territory (Enquist and Leimar, 1987). If this is the case within captive environments, where aggressive behaviour serves less of a crucial role for fitness, then enrichment of the environment is not likely to serve welfare purposes.

Within studies looking at the effects of standard types of hatchery tank compared to more naturally designed or enriched hatchery tanks (most usually for fish to be released as part of restocking practices) it appears that fry reared in enriched tanks are generally more aggressive (Berejikian *et al.*, 1996), possess greater competitive ability and usually obtain social dominance over individuals raised in standard laboratory tanks (Berejikian *et al.*, 2001). Other studies, however, have found little difference in aggression, foraging and territoriality between fry raised in natural, enriched or conventional tanks (Riley *et al.*, 2008).

The glass rods were intended to provide a refuge for the shoal. However, they may have allowed subordinate fish to escape from dominant individuals more easily and it is possible that this increased the amount of aggressive interactions required for dominant/subordinate relationships to be established. It is also possible that dominant fish did use enrichment as a way of establishing territories. However, these explanations can only be speculative, as analysis of the social hierarchy and relationship between specific individuals was not covered by the present study.

It was also observed that adult fish exhibited a much greater number of aggressive interactions in comparison to juvenile fish. It is interesting to note the correlation in increased aggression and increased shoaling density in adults compared to juveniles. This has been observed by others (*e.g.* Gerlach *et al.,* 2007) which they suggest show that the increased shoaling cohesion is not indicative of amicable behaviour. Instead it may represent increased competition for something, possibly territory or reproductive partners.

Within the juvenile study, the artificial structures had an effect on the time spent in the bottom of the tank on day one only, where fish from structured tanks spent more time in the bottom than those in barren tanks. However, in all tanks fish spent a much lower proportion of time in the bottom third on the first observation day in comparison to all other days. In the adult study, on days 1-5 fish in control tanks spent a significantly greater proportion of time in the bottom of the tank than those in structured tanks. The amount of time away from the surface of the tank (or nearer to the bottom) is another frequently cited measure of fear and anxiety in zebrafish (Gerlai *et al.*, 2000; Blaser and Gerlai, 2006; Egan *et al.*, 2009) and is reduced significantly by exposure to alcohol and nicotine, both of which have anxiety-reducing effects in many species, including zebrafish (Gerlai *et al.*, 2000 and Levin *et al.*, 2007 respectively). Similarly, Lopez-Patino *et al.* (2008) found that fish showing stereotypies also move closer to the bottom of the tank, similar to the manner reported as anxiety-like behaviour in zebrafish. Blaser and Gerlai (2006) found that zebrafish introduced to a novel tank initially spent a large proportion of time in the bottom third of the water column and over time, use of the upper water increased. If the physical structures used in the present study were providing a refuge, and thus reduced the perceived risk of the novel environment, we would have predicted fish to utilise the upper water column at an earlier point than fish in barren tanks. Only in the adult structured tanks did we see the vaguest indication of this trend. The juvenile results do not support this hypothesis and in fact show the reverse of that reported by other studies, *i.e.* they spent more time in the bottom third as time progressed, and more time on the bottom on the first day in the structured compared to barren tanks.

At this point it is not possible to say why this result is different from that observed in other studies, particularly with reference to the juvenile study. A possible explanation is that these fish were initially displaying exploratory behaviour of the novel environment and this behaviour then decreased in the following days. Another hypothesis might be that fish were responding to their own reflection, which was clearly visible to the on the bottom of the tank. Prior to the exposure, fish were maintained in polycarbonate tanks on a zebrafish culture system (Techniplast, IWT, Italy) in which reflections of the fish might not be seen in the same way as observed in experimental tanks. Interestingly, it was observed that fish in husbandry tanks do not show the same 'nose-down' swimming pattern near to the bottom of the tanks as shown by fish in the glass experimental tanks of the present study and this difference in environmental history may have contributed to the results that observed. In other words, the reflective bottom of tanks was perceived as more of a novelty or danger than the surface. As fish reflections were not visible in areas with rods, this might explain why juvenile fish in structured tanks containing rods spent significantly more time in the bottom third compared to control tanks without rods ($F_{(1,36)} = 5.12$, $p < 0.05$) *i.e*. there was less floor area with reflections to be perceived as novel or aversive. Similarly in the adult study, greater use of the upper water column in structured tanks in comparison to control tanks may indicate that the enrichment was effective in reducing the perceived risk of the environment.

Time spent by juvenile fish in the areas of tank containing glass rods, and the corresponding areas within control tanks did not differ significantly between treatments or observation days. However, adult fish spent more time in areas of

tanks with long rods and less time in areas containing short rods than fish spent in corresponding areas in control tanks. In a study by Delaney *et al*. (2002), zebrafish spent 99 % of time in areas containing plastic vegetation which, in anthropocentric terms, more closely resembled natural plant matter than did our glass rods. In the Delaney study the aquarium was an attempt to provide a mesocosm-type environment to closely match wild habitat. The intention in this study was to increase the environmental complexity rather than mimic plants, hence the design and colour choice (black), with the overall aim to address the hypothesis that increased environmental complexity reduces stress. There are several possible explanations why juveniles showed no clear increase in time spent in areas containing enrichment, the first being simply that the fish had no preference for these structured areas. It is also possible that the size of the tank meant that fish were always relatively close to enrichment, regardless of their position in the tank. Standard lengths of juvenile fish were 24 ± 4 mm, and so all individuals were within a maximum of 2 body lengths of the nearest rod structure at all times.

Although the data could initially appear to suggest that adult fish showed a preference for the long rods alongside an aversion to the short rods, this difference is in fact more due to an apparent aversion and preference of fish in control tanks for the corresponding areas. *i.e.* control fish spent much more time than expected (34 % in comparison to the expected 11 % if we assume equal use of all tank spaces) in areas corresponding to short rod sections and less time than expected (only 1.8 %) in areas corresponding to those with long rods. Fish in structured tanks, however, spent a relatively similar amount of time in tanks with long and short rods than we would expect (9.6 and 17.0 %

respectively). Whilst it is difficult to speculate why fish in control tanks showed these apparent preferences/aversions, it is still evident that the presence of the enrichment rods alters this pattern of tank space use. However, the main point of this study was to use enrichment rods to make positive welfare changes for this species, and these results, whilst interesting to ponder, do not clearly indicate any change in welfare status.

The concentration of cortisol measured in fish within the present study is within the same range as recorded in other studies with zebrafish (Pottinger and Calder, 1995; Egan *et al.*, 2009) although it is lower than that cited in some studies (Ramsay *et al.*, 2006; Barcellos *et al.*, 2007). Cortisol was significantly higher on day one than on any of the subsequent days. This was most likely because fish were still stressed from the transfer to experimental tanks, which has been documented as a significant source of stress affecting laboratory fish (Pottinger and Calder, 1995). On days 2, 4 and 7, sufficient time had passed for the cortisol concentration to return to a lower level. Importantly there was no difference in whole-body cortisol concentration between fish in control and structured tanks at any of the time points measured, indicating that the presence of the glass rods did not appear to either reduce or increase the stress levels of the fish via this simplistic measurement.

There were no differences in mean absolute deviation of any of the behavioural parameters measured in either juvenile or adult studies. Similarly there was no difference in mean absolute deviation of measured whole-body cortisol concentrations. Mean absolute deviation shows the average deviation of individual sample values in comparison to the sample population mean. High

mean absolute deviation values therefore indicate a great amount of variation around the mean. The amount of variation measured in any biological parameter is partly determined by internal factors such as genotype and health status (Mering *et al.*, 2001). Within a scientific study, however, the magnitude of variation in results is further altered by the nature of the subject's conditions. This includes differences in housing, treatment and social situation (Chance, 1956). Changes in variation of biological parameters, due to the addition of environmental enrichment have been reported in several studies. For example, Van de Weerd *et al*. (2002) report inconsistent changes in variation of several parameters in response to an enriched environment. For example, variation in blood corticosterone was lower in mice housed in enriched enclosures in comparison to mice in standard enclosures. Conversely, variation in freezing behaviour (*i.e*. zero activity) was higher in enriched housed mice. In other parameters (*e.g.* body weight and open-field behaviours) variability did not differ. These results have been mirrored by those of other researchers in studies with rats (*e.g.* Eskola *et al.,* 1999) and mice (*e.g.* Tsai *et al.* 2002). It has also been suggested that effects of enrichment on variation differ between strain and test (Tsai *et al.,* 2002).

The implications of the potential impact of enrichment on the variability of endpoints is, firstly that results may not be comparable to other studies in which the environmental conditions are not identical. This is particularly problematic as definition of what constitutes enrichment differs greatly between laboratories and testing facilities. With the current knowledge that environmental design can have a huge impact, not only on individual biological responses, but also on the amount of variation observed within them, should indicate to researchers the

importance of standardising testing environments. Furthermore, studies that measure parameters with large amounts of variation would be required to use greater number of individuals in order to obtain suitable statistical significance (Fitzmaurice, 2002), and this in itself would not be in accordance with the current attempts to reduce the numbers of live animals used in research. Within this study it appears that this simple form of potential enrichment has no measurable effect on the variation of all of the endpoints measured.

3.5 Summary

Although it is widely accepted that increased environmental complexity can improve the welfare of captive animals, I have not found this to be reflected in either the behaviours of juvenile and adult zebrafish or in the whole-body cortisol levels of juvenile fish provided with simple physical structures. The presence of the vertical glass rods had no effect on activity level or shoaling density and fish did not spend a greater amount of time in areas of tanks containing these physical features. It did, however, increase the amount of time taken for dominant/subordinate relationships to be established and for levels of aggression to decrease. This in itself may be viewed as a negative consequence. It is generally deemed undesirable to have a situation where subordinate individuals have limited means of escape, and hence one of the main drivers for enrichment is the anthropocentric view that we should provide a place of refuge. However, provision of these structures (as refuge) resulted in prolonged aggression, which was the opposite of the intended effect. Enrichment also had no effect on whole-body cortisol levels of juvenile fish, aside from day one when levels were increased in fish from both barren and

structured treatments following transfer to experimental tanks, and remained low throughout all subsequent days and treatments. Therefore there is a little tension between the simple physiological measure of stress (cortisol) and the behavioural measures. In this case it appears that behaviour is the more sensitive endpoint.

Provision of enrichment for adult zebrafish also had no effect on most of the behavioural endpoints, and few that would clearly indicate an improvement in welfare. Similarly to the juvenile study, aggression remained high for a longer period of time in tanks containing enrichment in comparison to control tanks which, as previously stated, I consider to be a negative result. Particularly as adult levels of aggression were observed to be significantly higher than those measured in the groups of juveniles, this could be a more serious problem for fish of a later life stage.

A limitation of the conclusions from our present study is that all behavioural measurements and terminal sampling occurred at times when disturbance of the fish was kept to a minimum. As the nature of the hypothesised enrichment was to provide fish with a form of refuge, it is possible that potential benefits may only be apparent when fish are stimulated or feel threatened. A logical progression of this work would therefore examine the effect of similar enrichment on the response of fish to typical laboratory stressors, and to look at a wider variety of enrichment types. Both of these areas are dealt with in Chapters 4 and 5 using the same model species, zebrafish.

CHAPTER 4

EFFECTS OF TANK STRUCTURES ON ACUTE AND CHRONIC STRESS RESPONSES OF ZEBRAFISH

CHAPTER 4 – EFFECTS OF TANK STRUCTURES ON ACUTE AND CHRONIC STRESS RESPONSES OF ZEBRAFISH

4.1 Introduction

The response to stress in fish, as with any animals, is an adaptive mechanism which allows individuals to deal with real or perceived stressors and so maintain normal functioning at such times (Barton, 2002). The physiological stress response in fish is often grouped into primary, secondary and tertiary functions. Primary responses consist of the initial neuroendocrine response including the release of catecholamines and activation of the hypothalamic-pituitary-interrenal (HPI) axis, which stimulates the synthesis and mobilisation of glucocorticoid hormones such as cortisol. A second neuroendocrine axis involved in the primary stress response is the hypothalamus-sympathetic-chromaffin cell axis which produces catecholamines (adrenalin and noradrenaline) from the chromaffin cells. Activation of both of these axes initiate secondary responses which include changes in plasma and tissue levels of ion and metabolites, haematological features and heat shock or stress proteins (Barton, 2002). The subsequent tertiary responses relate to changes in whole-animal functions such as metabolic scope for growth, immunity, reproduction and behaviour (Wendelaar-Bonga, 1997) and therefore also involve consequences at the larger population level.

The behavioural response to stress begins with an initial alarm response which narrows the attention and promotes a state that favours both the retrieval of acquired memories and the acquisition of new ones (Galhardo and Oliveira, 2009). In fish, the most common response of this type is “freezing” behaviour, during which the fish remains motionless. However, also common is the fight-or-

flight defensive mechanism by which activity level, most often near the bottom of the water column, is increased. The purpose of this is to remove the threat of the stressor by moving away from it or making it more difficult for a potential predator to direct its attention to an individual fish (Schreck *et al.*, 1997). Either or both of these behaviours may be exhibited by fish depending on the type, duration and context of the stress.

Persistent stressors can have huge impacts on disease levels and subsequent mortality in fish. Even single or rare episodes of stress can have negative effects on their physiology and behaviour (Pottinger and Calder, 1995). Coping effectively with stress requires that an animal appropriately adapt its response by altering its behaviour or learning about its situation (Levine, 1985). However, because of the large variety of factors that can induce the stress response in fish and the speed with which the aquatic environment can alter (*e.g.* through hypoxia or point source pollution events), it may be that exposure of laboratory fish to stress is more important than common mammalian laboratory species (Casebolt *et al.*, 1998).

Many of the existing guidelines relating to fish husbandry are extrapolated from the literature on commercial aquaculture production, so there is limited information available regarding environmental preferences of many species used in the laboratory. Husbandry techniques are often aimed at increasing biomass and production rather than minimising stress and improving welfare. Within the laboratory, capture, handling, crowding, confinement, transport and anaesthesia can all provide a source of stress for fish, and it is generally agreed that husbandry practices should be managed to minimise this (Barnett and

Pankhurst, 1998). In addition, environmental changes such as temperature fluctuation, water quality alterations, food availability, environmental noise, human activity and social hierarchy pressures can provide additional issues (Casebolt *et al.*, 1998).

A further concern is that fish in the laboratory are deliberately exposed to a number of adverse physiological and behavioural states, in the interests of research (Huntingford *et al.*, 2006). Within aquatic toxicology in particular, where fish are commonly exposed to a variety of conditions and chemicals, protocols are inherently stressful for the fish involved (Pottinger and Calder, 1995). There is now increasing concern that response to these types of stresses, that are inherent in the testing procedure, may impinge significantly on the results of such studies and may result in the collection of atypical data (Vogel, 1993). This has resulted in the need for improved and tested methods of minimising the effects of such procedures.

There is an increasing body of evidence suggesting that enrichment may temper animals' emotional reactivity to stressors, particularly in strains which are more reactive to stress (Fox *et al.*, 2006). In addition to this, enrichment has been shown to decrease the time taken to recover from exposure to a stressor (Braithwaite and Salvanes, 2005) and to prevent the impairment of spatial learning and memory in animals which have been chronically stressed (Wright and Conrad, 2008). Since the aversive effect of many stressors may be relieved by the opportunity to huddle, move away, flee or hide (Moberg and Mench, 2000), it is reasonable to assume that enrichment of the environment may be critical in allowing such behaviours to occur.

In studies with laboratory rats, it has been found that environmental enrichment reduces the adrenocorticotropic hormone (ACTH), corticosterone and adrenaline responses to acute handling suggesting that enriched individuals show increased habituation to the stressor (Moncek *et al.*, 2004). Similarly in a study by Belz *et al.* (2003), provision of toys and nesting material appeared to provide rats with a diversion from monotonous cage life, thereby resulting in lower hypothalamic pituitary adrenal (HPA) axis activity both before and after a mild stress. In studies with laboratory mice, similar results have been found, with individuals housed in enriched enclosures exhibiting decreased anxiety-like behaviours (as indicated by reduced time spent in a "frozen" state and increased exploration) alongside minimal corticosterone reactivity in response to a novel environment (Benaroya-Milshtein *et al.*, 2004).

In a study with growing pigs (*Sus scrofa*), however, Beattie *et al.* (2000), found that plasma cortisol responses to both a novel pen test and at slaughter were higher in individuals from enriched environments. Instead of this being viewed as a negative consequence of the enrichment, however, it was suggested that chronic activation of the HPA axis in barren environments led to the subsequent suppression of cortisol response to acute stress. This study highlights an important consideration, in that we should be wary of always viewing increases in cortisol to be evidence of reduced welfare. (Particularly as the ultimate effect of any hormone is dependent on both the local concentration of the hormone itself combined with the density and activity of the receptors in the target tissue).

The activation of the HPA system (or the equivalent HPI axis in fish) is an appropriate response to many types of stress (such as that which would likely be provided by the prospect of slaughter in the aforementioned study) and the absence of this physiological change, at times when it would be appropriate, could therefore equally be viewed as a negative trait. It is important that we remember that stress is a vital part of being alive. As Hans Selye (1956) famously stated: “The absence of stress is death”. Our aim, therefore, should not be to eliminate all stress, but to provide animals with a suitable opportunity to deal with appropriate levels and types of stress.

Animals from enriched environments have also been shown to exhibit increased glucocorticoid receptor (GR) expression (Olsson *et al.* 2002). The glucocorticoid receptor, along with the mineralocorticoid receptor (MR) is a receptor to which cortisol binds, and which regulates genes controlling metabolism, development and the immune response. In mammals, the MR is sensitive to much lower concentrations of cortisol than the GR. This means that, while the MR is responsible for osmotic balance and negative feedback of cortisol throughout the diurnal cycle, the GR is more involved with the stress response and following recovery from stressful events (Galhardo and Oliveira, 2009). In fish, both receptors appear to have a similar affinity for cortisol, and the role of the MR ligand is still not well defined (Stolte *et al.*, 2008).

It has been suggested that differences in expression of the GR may explain some of the effect of enrichment on stress-affected behaviours. Increased GR expression in enriched animals provides more effective negative feedback on the paraventricular nucleus in the hypothalamus, thereby inhibiting further

secretion of corticotrophin-releasing factor (CRF) (de Kloet *et al.*, 1998). CRF has an effect on animal behaviour, in that reduced secretion of generally has an anxiolytic effect such as increased exploration. Therefore, variations in GR expression may explain some of the behavioural responses observed in animals maintained in different housing conditions (Larsson *et al.*, 2002).

There are few published cases looking at the effects of environment on the stress response of captive fish. In a study with red porgy (*Pagrus pagrus*) it has been observed that alteration of tank background colour can speed up the recovery of homeostasis in cortisol levels following a stressful event (Rotllant *et al.*, 2003). Furthermore, in a study by Höglund *et al*. (2005), crucian carp (*Carassius carassius*) maintained in aquaria without hiding material showed a greater physiological response to stress when compared with fish with available hiding material. This is thought to be because fish were allowed to perform the type of avoidance behaviour natural to this species when perceiving a stressor. As we can see, whilst the effect of enrichment in improving the welfare of captive animals in general is fairly well documented, particularly for laboratory rodents and farmed species, there is relatively little information available for fish species used in the laboratory. Importantly, there has been little research conducted to study the possibility of using enrichment to alleviate the negative effects of typical laboratory stressors.

Use of enrichment within regulatory toxicological studies may be complicated by the prescriptive and strict nature of the test guidelines. Within studies that comply with Organisation for Economic Co-operation and Development (OECD) testing standards, the container vessel must be inert and microbial growth kept

to a minimum and visual observations of fish must be possible at all times. Furthermore, items added to tanks must not interfere with either the test chemical or the behaviour and physiology of study fish. Due to these restrictions, the addition of environmental enrichment in many types of studies may be problematic. It is important, therefore, that a balanced assessment be made that takes into account both the benefits and costs of potential forms of enrichment. Costs would obviously include production of such enrichment materials, but would also incorporate labour costs relating to cleaning and maintenance. Other, possibly more serious, costs would include incompatibility of results with those from previous studies and unmeasured physiological or behavioural responses of fish affecting the uptake and assimilation of test chemicals.

The aim of the current study was to assess the effects of a hypothesised form of enrichment, consisting of glass rod structures of varying heights that added structural complexity and provided potential refuge areas, on the acute and chronic responses of adult zebrafish to a repeated chasing stressor. The behavioural parameters measured were activity level, shoaling density, aggression and time spent in the bottom third of the tank, as these behaviours have been associated with stress and anxiety in zebrafish (Rehnberg and Smith, 1988; Egan *et al.*, 2009). The amount of time fish spent in areas of tanks containing enrichment in comparison to equivalent areas in control tanks was also quantified. In addition to this whole-body cortisol concentration and expression of the glucocorticoid receptor (GR) in brain and liver tissue was measured. As previously mentioned, cortisol is a hormone which has been well documented in many animals, primarily due to its short-term increase in

response to physical and environmental stress (see Mommsen *et al.* (1999) for a review). As the associated cortisol receptor, the expression of the GR has been found to be reliably correlated with long-term patterns in cortisol production (Terova *et al.*, 2005) and can therefore provide more information about this particular physiological response to stress.

4.2 Materials and Methods

Adult zebrafish were obtained from AstraZeneca, Brixham Environmental Laboratories and kept under conditions compatible with OECD guidelines throughout. Prior to the experiment, fish were kept in flow-through fresh water at 28 ºC and under a photoperiod of 14L:10D (light:dark) with a 20 minute phased sunrise/sunset. Fish were fed SDS dry food in the morning live *Artemia* 24 h nauplii in the afternoon. At the beginning of experiments fish were approximately one year old.

Groups of six fish were provided with either a control (barren) or a structured environment for a duration of 14 days. These groups were further divided into unstressed and stressed treatments. Tanks in the stressed treatment received a chasing stressor on days 8 to 14 inclusive, which consisted of chasing with a net for 30 seconds. Fish were terminated on day 15 by over-anaesthesia in accordance with UK home office guidelines. Half of the individuals from each tank (*i.e*. 3 fish) in the stressed treatments received a 30-second chasing stressor immediately prior to sampling.

Behavioural endpoints consisting of: activity level, shoaling density, aggression, time spent in the bottom third of the tank and use of areas containing physical

structures were analysed on alternate days during the study. Whole-body cortisol concentrations were measured from individual fish following termination at the end of the study using a commercial Enzyme Linked ImmunoAssay kit. Expression of the Glucocorticoid Receptor was quantified in brains and livers of fish using quantitative PCR. GR expression was not quantified from chronic + acute stressed fish as it was assumed this measure would not be affected by such a short-term stress.

For further information on the experimental design looking at the effects of a structured environment on the chronic and acute stress responses of adult zebrafish see section 2.2.2, and for details on the behavioural and physiological endpoints used see sections 2.3 and 2.4.

Statistical analyses used are discussed in section 2.5. All behavioural data satisfied the criteria for parametric tests and so was analysed in its original form. Cortisol data, however, was log transformed to meet the assumptions of normality. Within this study, mean absolute deviation (MAD) of all endpoints were analysed as follows. Results from week one and week two were analysed separately so that we could observe the effects of the daily stressor (which occurred only in week two). For week one data (*i.e*. days 1-7 inclusive), observations from all control tanks (*i.e*. control unstressed and control stressed treatments) were combined as were observations from all enriched tanks. As no tanks were stressed during this time we therefore treated the data as though there were two treatments rather than four. For week two data, (days 8-14 inclusive), observations were combined and compared for each of the four treatments.

4.3 Results

4.3.1 Behavioural responses to tank structures and chasing stress

On day one tanks containing structures showed a higher level of activity than control tanks ($F_{(1,18)} = 17.32$, $p < 0.01$). On days 8, 10, 12 and 14 fish in the stressed treatments showed an increase in activity level in comparison to those from unstressed treatments. However, only on day 8 was this difference significant ($F_{(3,16)} = 6.50$, $p < 0.01$) and the difference between stressed and unstressed treatments, in general, became less with each observation day (Figure 4.1). There were no significant differences in shoaling density between treatments or observation days ($F_{31,127} = 1.08$, $p = 0.27$) (Figure 4.2). In both unstressed treatments, there were no significant differences in aggression throughout the 14-day study period. However, in both stressed groups, aggression was significantly lower on days in which fish received the handling stress (*i.e.* days 8, 10, 12 and 14) than on previous observation days (Control: $F_{7,31} = 6.65$ $p < 0.01$; Structured: $F_{7,32} = 5.54$, $p < 0.01$) (Figure 4.3). There were no significant differences in time spent in the bottom of the tank between treatments or observation days ($F_{27,111} = 1.08$, $p = 0.38$) (Figure 4.4).

When comparing the areas of tanks containing short rods and the corresponding areas in control tanks, there was a significant effect of the treatment ($F_{3,128} = 8.0$, $p < 0.01$) on the time spent by fish in these regions, but no effect of observation day ($F_{7,128} = 1.8$, $p = 0.09$). There was also a significant interaction effect ($F_{21,128} = 2.0$, $p < 0.05$) suggesting that the observation day affected the treatments in different ways. Further analysis showed that significant differences were observed on days one, three and fourteen, but there

were no consistent patterns in the use of this area of the tank (Figure 4.5 A). In the areas of tanks containing medium sized rods, there was a significant effect of treatment ($F_{3,128} = 3.5$, $p < 0.05$) but not observation day ($F_{7,128} = 1.7$, $p = 0.11$). Differences between treatments were only observed on day one, in which time spent in this area was significantly lower in the control stressed treatment than all other treatments (figure 4.5 B). In areas of tanks that contained long rods, again there was no effect of day upon the amount of time spent by fish there ($F_{7,127} = 1.4$, $p = 0.22$) but there was a significant effect of treatment ($F_{3,128} = 13.3$, $p < 0.01$). When examined in more detail, differences were observed between treatments on days one, two and fourteen. On day one, fish in the control unstressed group spent more time in this area of the tank than any of the other treatments. On day two, a greater percentage of time was spent in this area by fish in the enriched stressed group than any other treatment. Finally, on day fourteen a greater percentage of time was spent in this area by fish in the enriched unstressed group than any other treatment (Figure 4.5 C).

In the first week of the study, when all tanks were unstressed, mean absolute deviation (MAD) of control and enriched tanks was not significantly different ($F_{1,58} = 0.03$, $p = 0.87$). MAD was approximately 9 lines crossed per minute. In week 2, fish in tanks receiving the daily stressor (*i.e*. tanks in the 'control stressed' and 'enriched stressed' treatments) showed a significantly reduced amount of variation in activity ($F_{3,76} = 3.80$, $p = 0.01$). Variation was roughly halved from 10-11 lines crossed per minute in unstressed tanks, to 5-6 lines crossed per minute in stressed. There were no differences in MAD in shoaling between control and enriched tanks during the first week of the study ($F_{1,58} = 0.192$, $p = 0.66$). Deviation from the mean was approximately 0.4 fish. In week

two, there was significantly less variation in shoaling density in stressed tanks in both control and enriched treatments ($F_{3,76} = 5.63$, $p < 0.01$). In week one, there was no significant difference in variation of aggression between control and enriched tanks ($F_{1,58} = 0.68$, $p = 0.41$). In the second week of the study, however, we observed significantly lower variation in aggression in stressed tanks in comparison to unstressed, as well as lower variation in control tanks in comparison to enriched ($F_{3,76} = 8.00$, $p < 0.01$). Neither presence of enrichment nor daily stressing had any effect on the variation observed in percentage time spent by fish in the bottom of the tank in either week one or week two of the study ($F_{1,58} = 1.05$, $p = 0.31$ and $F_{3,76} = 1.35$, $p = 0.26$ respectively).

There was no significant difference in the variation of time spent in areas of tanks containing short rods (or equivalent areas in control tanks) between any of the treatments in either week one ($F_{1,58} = 1.66$, $p = 0.20$) or week two ($F_{3,76} = 1.03$, $p = 0.39$). Average deviation from the mean was between 5 and 8 % throughout the study. When we looked at the amount of time spent in areas containing medium sized rods, variation was approximately two times greater in enriched tanks compared to control tanks in the first week of the study ($F_{1,58} = 17.80$, $p < 0.01$). In the second week, however, this difference was no longer apparent ($F_{3,76} = 1.83$, $p = 0.15$). Similarly, variation in time spent in areas containing long rods was approximately four times greater in enriched tanks compared to control tanks during week one ($F_{1,58} = 24.64$, $p < 0.01$). In the second week of the study, variation remained high in the enriched unstressed tanks. However, it was significantly reduced in enriched tanks that received the daily stress to approximately the same level as observed in both control treatments ($F_{3,76} = 24.98$, $p < 0.01$).

All results from calculations of the mean absolute deviation (including those for cortisol analysis and qPCR results) are summarised in Table 4.1.

4.3.2 Whole body cortisol content

There was no correlation between whole-body cortisol concentration and body mass (R^2 = 0.05) or standard length (R^2 = 0.02). There was also no significant difference in cortisol concentration between males and females ($F_{(1,107)}$ = 0.52, p = 0.47). In control tanks, fish from the chronic+acute stress group, showed significantly higher cortisol levels than those from the unstressed treatment ($F_{(2,53)}$ = 4.67, p = 0.01). However, in structured tanks, there were no significant differences between any of the treatments ($F_{(2,51)}$ = 0.04, p = 0.96) (Figure 4.6).

The acute stress immediately prior to termination produced a significant increase in variability of the cortisol response in fish from control tanks ($F_{2,53}$ = 6.27, p < 0.01). However, this increase in variation was not seen in the enriched treatments where MAD was comparable between unstressed, chronic stress and chronic + acute stress groups ($F_{2,51}$ = 1.65, p = 0.20).

4.3.3 Glucocorticoid receptor expression

In both brain and liver samples, expression of the glucocorticoid receptor was approximately twofold lower in the control stressed treatment in comparison with all other treatments. This difference was significant in comparison to both the structured unstressed and structured stressed groups (brain: t(8), p = 0.04 and liver: t(8), p = 0.01), although not to the control unstressed group. (brain: t(7) = 2.23, and liver: p = 0.06) (Figures 4.7 A and B).

There was no significant difference in mean absolute deviation of GR expression in liver samples between treatments ($F_{3,13} = 0.80$, $p = 0.51$). Similarly there was no difference in variation in GR expression observed in brain samples ($F_{3,15} = 2.19$, $p = 0.13$).

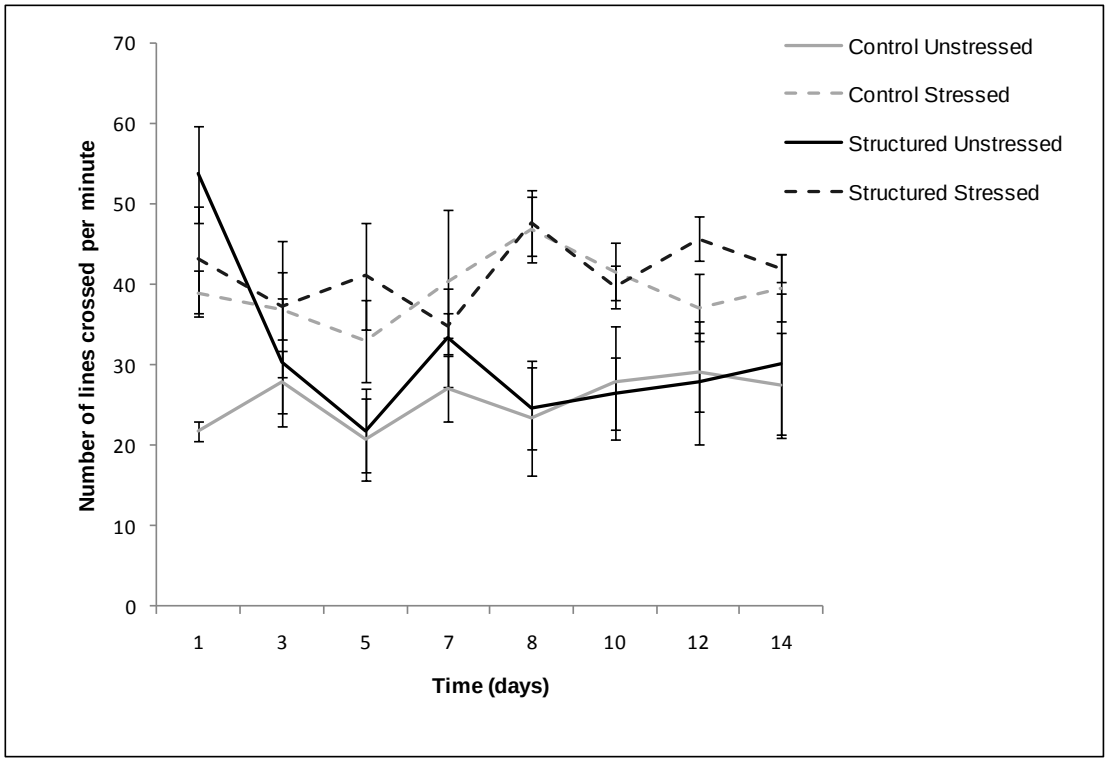


Figure 4.1 Mean activity level of fish in control and structured tanks that were unstressed or stressed immediately prior to observation. (Mean ± SE, n = 5).

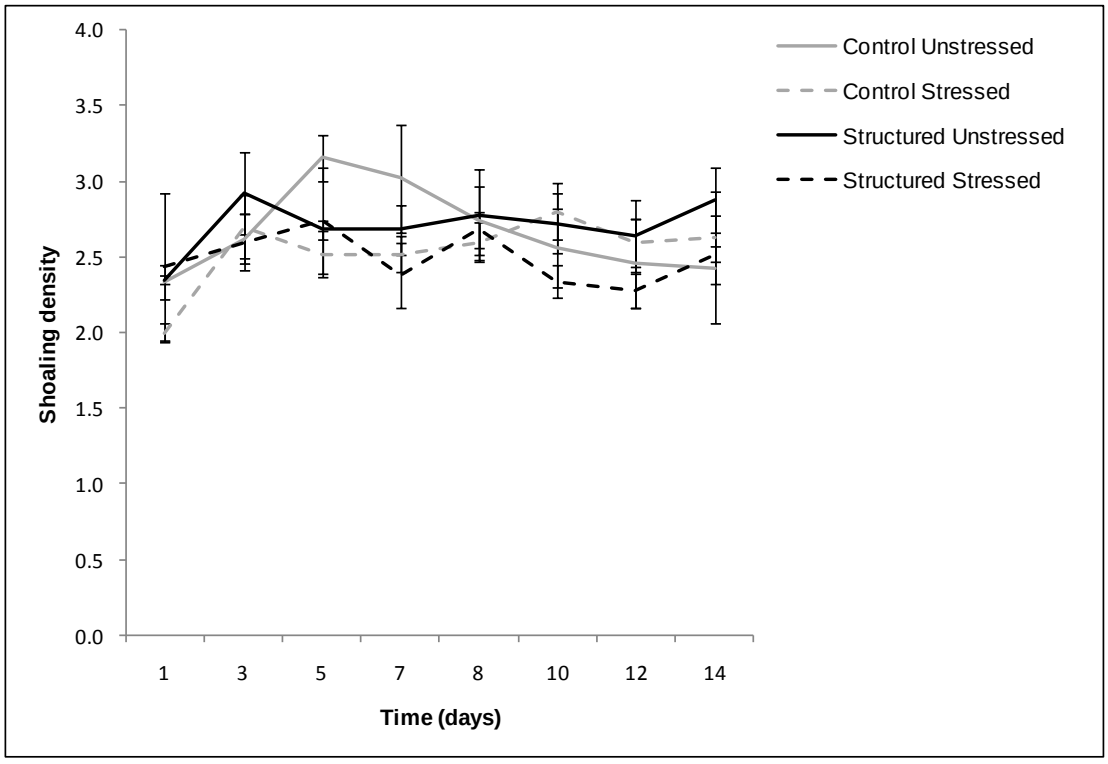


Figure 4.2 Mean shoaling density of fish in control and structured tanks that were unstressed or stressed immediately prior to observation. (Mean ± SE, n = 5).

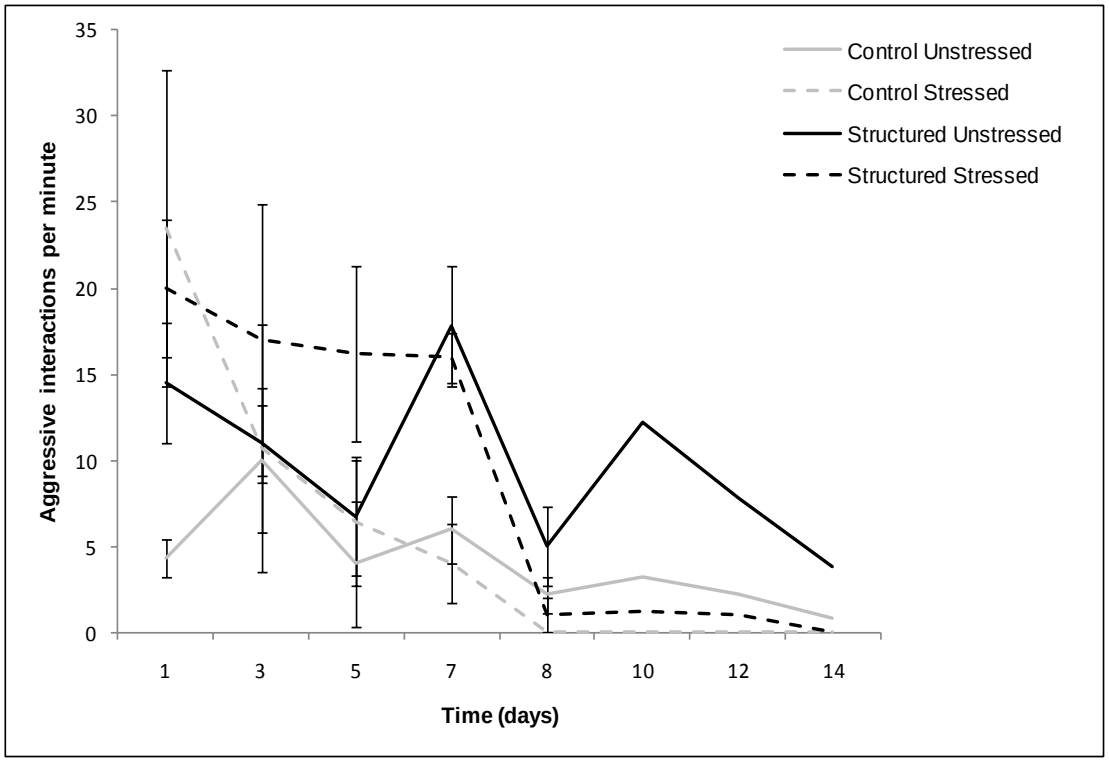


Figure 4.3 Mean whole-tank aggression levels of fish in control and structured tanks that were unstressed or stressed immediately prior to observation. (Mean ± SE, n = 5).

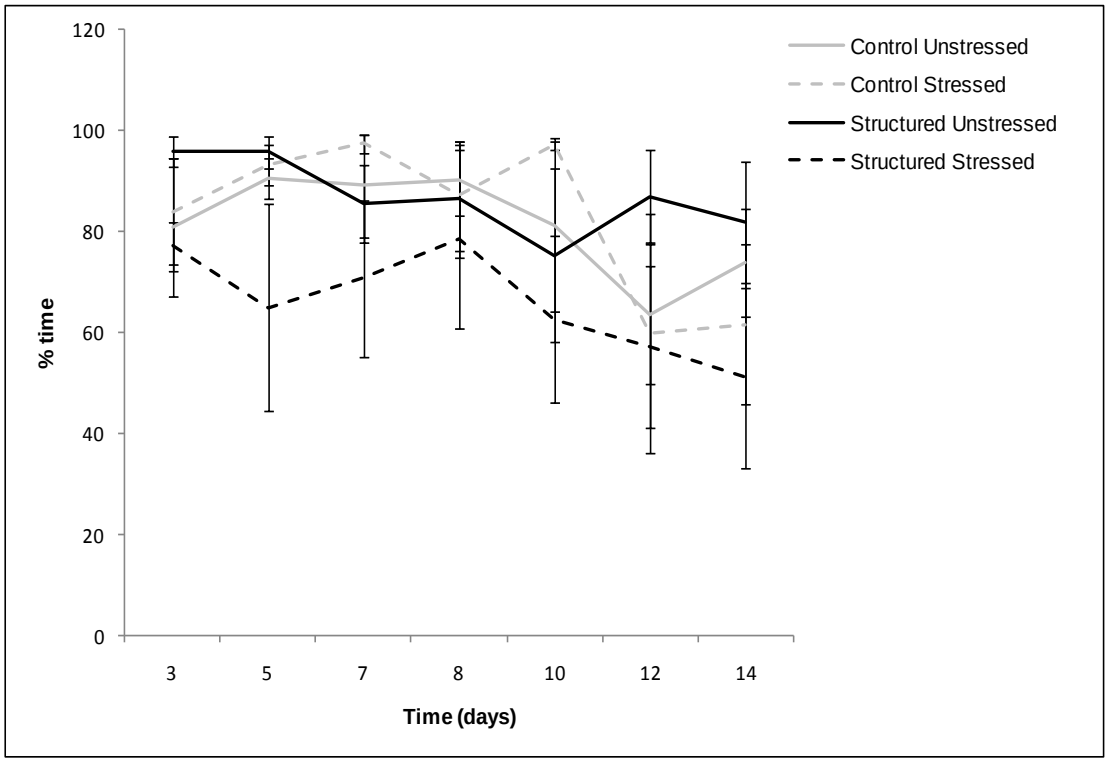


Figure 4.4 Mean percentage time spent by fish in control and structured tanks that were unstressed or stressed immediately prior to observation. (Mean ± SE, n = 5).

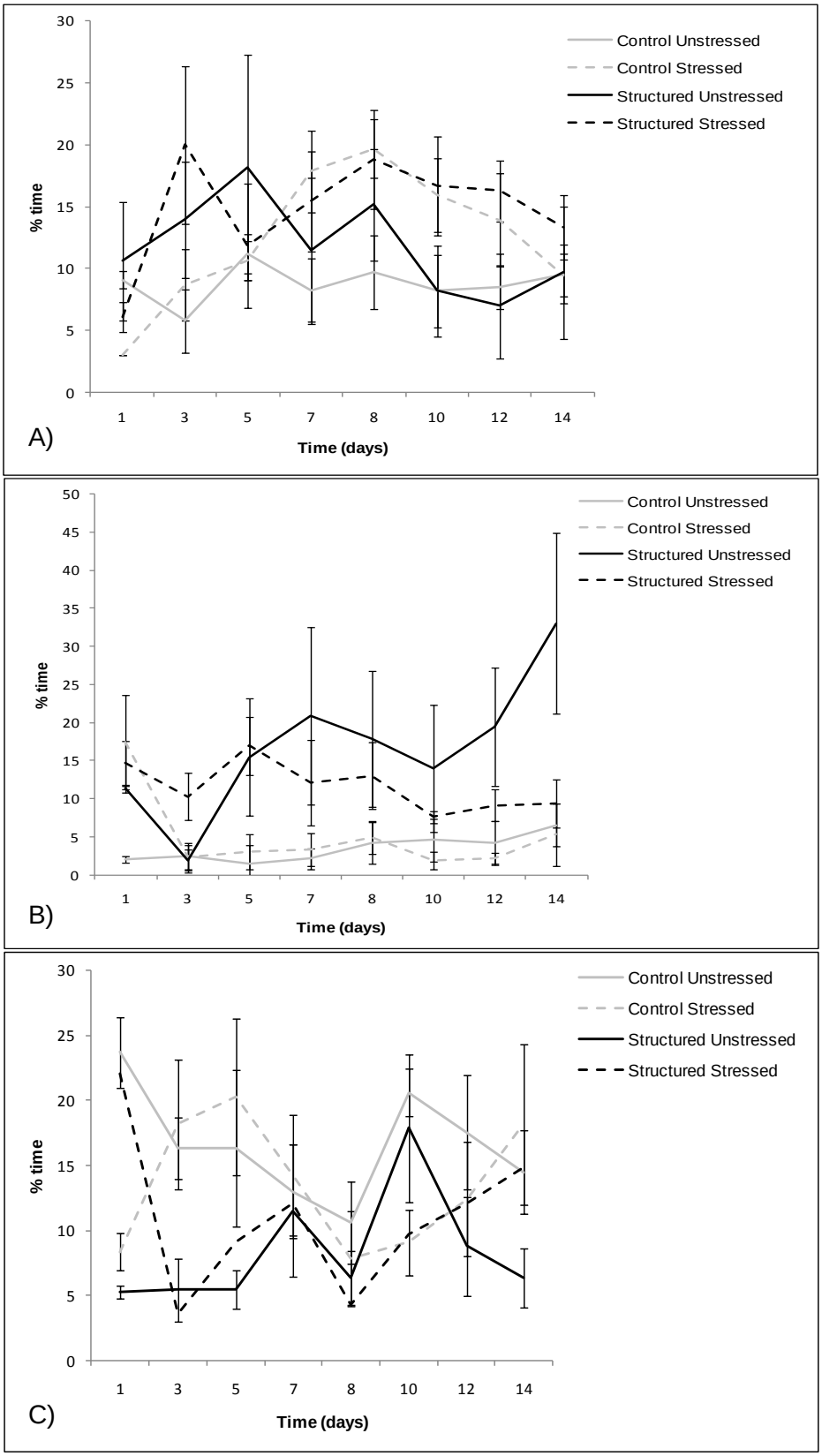


Figure 4.5 Mean percentage time spent by fish in areas of tank containing short (A), medium (B) and long (C) rods in control and structured tanks that were unstressed or stressed immediately prior to observation.

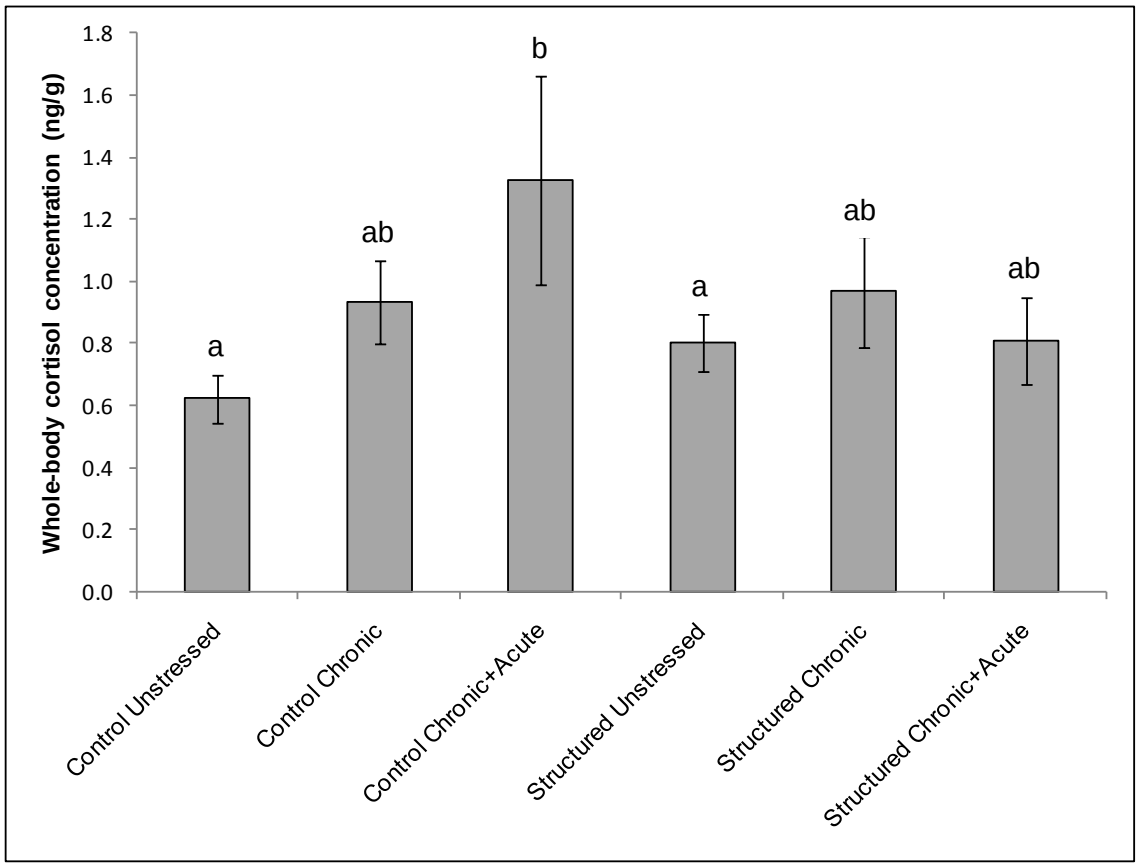


Figure 4.6 Mean cortisol values of fish from control and structured tanks subjected to either no stress (Unstressed) daily stress for 9 days prior to sampling (Chronic) or daily stress plus a 30 s stress immediately prior to sampling (Chronic+Acute). (Mean ± SE, n = 30). Different letters indicate a significant difference. Statistical significance accepted at $p < 0.05$.

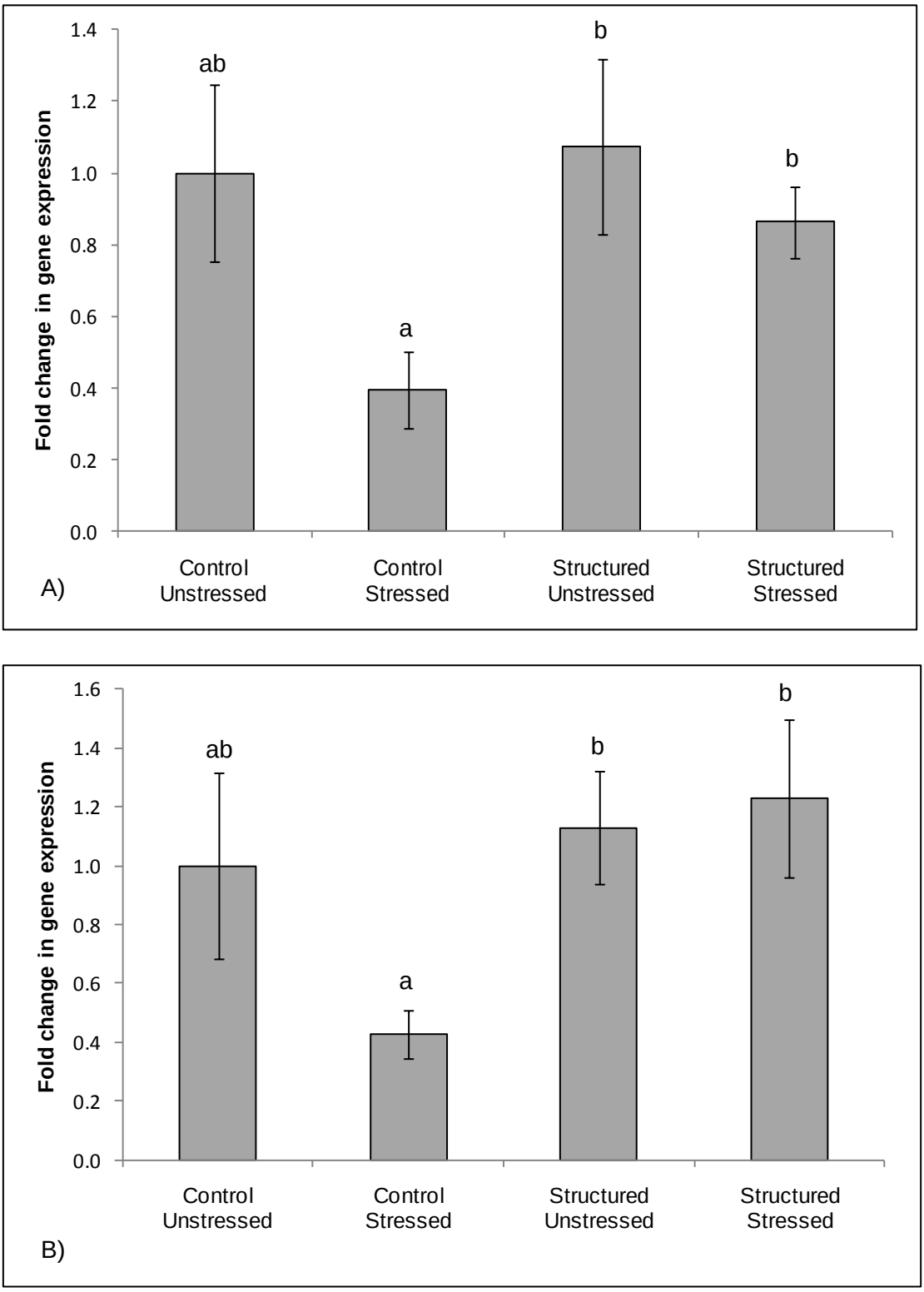


Figure 4.7 Expression of GR in brain (A) and liver (B) tissue from control and structured tanks subjected to either no stress (unstressed) or daily stress for 9 days prior to sampling (stressed) – fold change from control unstressed group. (Mean ± SE, n = 5). Different letters denote significant differences. Statistical significance accepted at $p < 0.05$.

Table 4.1 Mean absolute deviation of all endpoints

	Week 1		Week 2			
	Control	Structured	Control Unstressed	Control Stressed	Structured Unstressed	Structured Stressed
Activity level	9.3	9.6	11.4	6.9	10.5	5.5
Shoaling density	0.4	0.4	0.5	0.3	0.4	0.2
Aggression	5.7	7.0	2.4	0.0	5.0	1.1
% time in tank bottom	13.5	17.2	23.7	27.9	20.6	30.8
Use of small rods (%)	7.0	5.3	6.8	8.1	7.4	5.6
Use of medium rods (%)	5.5	10.9	4.6	5.7	7.6	5.2
Use of long rods (%)	3.0	11.3	4.5	3.5	17.6	4.7

	Control Unstressed	Control Chronic	Control Chronic+Acute	Structured Unstressed	Structured Chronic	Structured Chronic+Acute
Whole-body cortisol	0.30	0.41	0.87	0.37	0.55	0.39
GR expression - Brains	0.51	0.19	-	0.42	0.19	-
GR expression - Livers	0.52	0.10	-	0.35	0.43	-

4.4 Discussion

In zebrafish, cortisol is biosynthesised by various microsomal enzymes located in the interrenal cells of the head kidney tissue, and their production is under the strict control of the pituitary gland (Donaldson, 1981). Corticosteroid-mediated gene induction can influence a variety of physiological functions related to metabolism, immunity, behaviour, osmoregulation and cardiovascular transport (Mommsen *et al.*, 1999). Consistent elevation of this hormone though environmental perturbations, is commonly viewed as a negative response.

Within the current study, cortisol concentrations measured in fish from control tanks were significantly higher in fish that were stressed immediately prior to sampling (chronic+acute stress group) than those from the unstressed or chronic stress groups. In structured tanks, there was no significant difference in cortisol concentration between any of the treatments. King and Berlinsky (2006) found that both whole-body immunoreactive cortisol and plasma cortisol in Atlantic cod (*Gadus morhua*) increased significantly in response to a 30 second net stressor. Barton and Rahn (1998) also reported a significant rise in plasma cortisol in juvenile Paddlefish (*Polyodon spathula*) in response to a 30 second aerial stressor. Cortisol was also increased by 1 hour of chasing within the home tank. Similarly Barnett and Pankhurst (1998) found that crowding and chasing of Greenback flounder (*Rhombosolea tapirina*) resulted in elevated cortisol levels for up to 48 hours. Pottinger and Moran (1993) showed an increase in both cortisol and cortisone in rainbow trout in response to confinement stress and in a study by Falahatkar *et al.,* (2009), with juvenile Great sturgeon (*Huso huso*) it was found that a 60 second aerial stressor

increased plasma cortisol in fish previously maintained at high densities, but not in fish kept at lower densities.

It is therefore apparent that standard husbandry and laboratory procedures can have a measurable and biologically relevant effect on the cortisol response in a variety of species. However, there are very few studies for which comparison can be made regarding the efficacy of enrichment or tank environment in alleviating the effects of such stressors. In one such experiment, Rotllant *et al*. (2003), observed that tank background colour played a role in altering the recovery of baseline cortisol levels following stress in red porgy (*Pagrus pagrus*). Fish that were subjected to a crowding stressor all showed an elevated cortisol response as expected. Those kept in tanks with a black background, however, showed a quicker return to pre-stress cortisol levels than did those kept in tanks with a white background. It has also been found that social environment can influence the stress response. In a study by Barcellos *et al*. (1999), Nile tilapia (*Oreochromis niloticus*) that were maintained in groups of 10 fish, showed a more intense cortisol response than those kept in groups of smaller sizes. The results reported here appear to suggest that the presence of glass rod structures reduced the increase in cortisol produced in response to a brief chasing stress.

A possible explanation for this could be that the rods were providing fish with a way of avoiding the net in the tank and allowed them to rest in an area of relative safety. Fish in control tanks did not have this refuge option and so perhaps showed a more severe physiological reaction to the chasing. An alternative explanation, however, could be that fish in enriched tanks actually

demonstrated an impaired response to the chasing stress. As previously mentioned, a significant and temporary rise in cortisol in response to stress is an adaptive response and one that allows and stimulates the required physiological and behavioural changes within the individual. This temporary rise in cortisol is considered “natural” and “healthy” provided the stressor is not too large or sustained. The absence of this response could, therefore, equally be viewed as a negative observation. It is possible that previous chronic activation of the cortisol response (*i.e.* in the week preceding termination) led to the suppression of the cortisol response to acute stress (Beattie *et al.*, 2000).

Similar to the previous study, however, (see chapter 3) there were no observed differences in baseline levels of cortisol. By this I mean that fish that were maintained in either control or enriched tanks but not provided with any stress, did not show any differences in this parameter. Similarly, fish that were provided with a chasing stress daily for a week prior to termination, but were not stressed directly before sampling, also showed no significant increase in cortisol levels at the time of sampling. This illustrates an important point. Many studies and published articles cite cortisol, measured either from blood plasma, whole-body extractions or tank water as a measure of stress and therefore welfare (*e.g.* Ramsay *et al.,* 2006, Barcellos *et al.*, 2007 and Ellis *et al.*, 2007 respectively). However, it is fairly well understood that this response is relatively short-term. Within this species (*Danio rerio*) cortisol values have been found to increase within 3 minutes of an acute stress such as netting and air exposure. Following a peak at approximately 6 minutes, a return to pre-stress levels has been observed at 1 hour post stress (Ramsay *et al.*, 2006). For this reason, this parameter is perhaps not ideal as a measure of longer-term stress.

As previously stated, glucocorticosteroids play a key role in stress responses, growth and general metabolism, reproduction and immunity (Mommsen *et al.*, 1999). The physiological effects of corticosteroids are regulated by the cellular glucocorticoid receptor (GR); a ligand inducible transcription factor that can activate or repress target genes (Adcock, 2000). Many studies have shown GR abundance to be transient and closely following cortisol profiles (Maule and Schreck, 1991; Mommsen *et al.*, 1999; Terova *et al.*, 2005; Alsop and Vijayan, 2008; Stolte *et al.*, 2008).

The present study demonstrated that fish kept in control tanks and exposed to a daily stressor showed an approximately two-fold lower GR expression than all other treatment groups. This result was consistent in both liver and brain samples. Terova *et al*. (2005), reported a down-regulation of glucocorticoid receptor mRNA levels in the liver of sea bass (*Decentrarchus labrax)* reared at increased population density. This abundance was inversely correlated with blood cortisol levels which were seen to rise under these conditions. Stolte *et al*. (2008) subjected carp (*Cyprinus carpio*) to restraint and repeated temperature drops. Whilst long-term restraint resulted in increased plasma cortisol but no change in glucocorticoid receptor expression, repeated temperature reductions resulted in increased cortisol, accompanied by down-regulation of glucocorticoid receptors in the brain. This suggests that, in some cases, prolonged or severe stressors are required for glucocorticoid receptor expression to be altered. Mild or short term stressors, whilst producing a measurable cortisol response, appear to have little effect on gene expression. Maule and Shreck (1991) found that chronic stress in coho salmon (*Oncorhynchus kisutch*) resulted in increased

numbers of glucocorticoid receptor in whole leucocytes but decreased numbers in gills. This suggests that more than one mechanism is responsible for the changes in glucocorticoid receptor in response to stress within different tissues.

A physiological explanation for the down-regulation of GR due to cortisol is that repeated and consistent production of the hormone will result in negative feedback mechanisms which reduce the numbers of receptors available in tissues (Sapolsky *et al.*, 1985; Oakley and Cidlowski, 1993). This down-regulation provides an effective way of countering the effect of repeated exposure to high concentrations of glucocorticoids (Lee *et al.*, 1992). The implications of this within the present study, are that fish from control tanks that were exposed to a daily stressor may have been producing higher levels of cortisol in the long-term than any of the other treatment groups. This is particularly interesting when we compare this group to the stressed fish from enriched tanks, which do not show the same down-regulation of the glucocorticoid receptor. In accordance with the cortisol results this would also seem to suggest that presence of the tank structures appear to have alleviated the affects of this type of stress for adult zebrafish as indicated indirectly by changes in GR expression that suggest reduced cortisol production.

In contrast to the results stated above, however, several other studies have reported up-regulation of GR in response to increased cortisol. Sathiyaa and Vijayan (2003), in a study with rainbow trout observed significantly elevated GR mRNA content over a 24-hour period following glucocorticoid stimulation. A following study by Vijayan *et al.* (2003) also noted significantly higher hepatic GR mRNA abundance as a result of long term elevated cortisol (achieved via

implants). A similar result was also found in another study with rainbow trout by Wiseman *et al.* (2007). This is not comparable to the results observed within the current study and previously mentioned works. Incidentally, it is well documented that in mammals GR expression is down-regulated by an increase in glucocorticoids (Rosewicz *et al.* 1988; Yudt and Cidlowski, 2002). Such differences in GR regulation (*i.e.* between both mammals and various fish species) may be at the level of the transcriptional control mechanisms and are therefore possibly species specific (Vijayan *et al.*, 2003). Alternatively, they may be dependent on the type, intensity and duration of the stressor (Wiseman *et al.*, 2007). As comparable GR expression was measured between both unstressed groups and enriched stressed groups in this study, it would be logical to assume that the down-regulation observed in the control stressed group was due to increased cortisol in this group throughout the second week of the study. This would, in turn, lead us to suggest that zebrafish are comparable to carp and sea bass, in that production of cortisol down-regulates liver GR mRNA in this species. Further studies looking at effects of different types of stress on different species and the associated chances in GR expression would be recommended to further elucidate this issue.

Production of corticosteroids during stress serve to maintain energy balance by stimulating some processes, such as gluconeogenesis, and inhibiting others, such as digestion and the immune response (Schjolden *et al.*, 2009). However, they also play a large role in the regulation of behaviours through both genomic and non-genomic mechanisms in the central nervous system (Haller *et al.*, 1998). In mammals it is fairly well documented that acute increases in corticosteroids lead to the stimulation of particular behaviours, such as

escalated aggression (Kruk *et al.*, 2004) whilst continued elevation lead to inhibition of many behaviours (Gregory and Wood, 1999). The same pattern in behavioural responses has also been observed in fish, in that both locomotion and aggression have been found to be inhibited by long-term cortisol treatment, but not by short-term (Øverli *et al.*, 2002).

In the present study an increase in activity levels was observed in tanks and on days that fish received the chasing stress. However, only on the first day that fish received the stress was this significantly different to pre-stress levels. Increased activity level or locomotion is a behaviour that is associated with an anxiety response in fish (Gerlai *et al.*, 2000). As discussed in more detail in the previous study (see chapter 3), a increase or decrease in this parameter has been observed in fish treated with various drugs known for their anxiogenic or anxiolytic properties respectively (*e.g.* Levin *et al.*, 2007; López-Patiño *et al.*, 2008). In a study looking at the effects of fasting and crowding stress on the activity of rainbow trout, McFarlane *et al*. (2004) found that stress induced obvious changes in swimming behaviour including increases in activity level. Cooke *et al*. (2000) in a study also looking at rainbow trout similarly found that stress resulting from increased stocking density raised activity levels.

This type of behavioural response involving an increase in overall activity is often referred to as the fight-or-flight defensive mechanism. This increase in movement, most often near the bottom of the water column, has the purpose of removing the threat of the stressor by moving away from it or, in more natural environments, making it more difficult for potential predators to direct its attention to an individual fish (Schreck *et al.*, 1997). By providing fish with a

refuge, we might have expected that, instead of utilising the “fight or flight” response to a stressor, they may have instead used the rods as a place to “freeze” and effectively hide from the net. In this case we would have expected the fish to have exhibited a much lower activity level directly following the stress. However, as this was not observed, it appears that either the rods were not viewed by fish as a suitable place to hide or that increasing activity was viewed as the most appropriate response.

The impact of the stress, however, appeared to have the opposite effect on observed levels of aggression. In both stressed treatments, aggression was significantly lower on days where fish received the handling stress (*i.e.* days 8, 10, 12 and 14) than on previous observation days. Comparable to the results reported here, in a study with rainbow trout, (Schjolden *et al.*, 2009) found that fish treated with cortisol did not become more aggressive than control fish and in fact became slower in initiating social confrontation. Similarly Øverli *et al*. (2002) found that rainbow trout acutely treated with cortisol, were not significantly more aggressive towards an intruder than control fish. In times of stress it is likely that it is more instinctive to devote energy to hiding or escaping from the stress, rather than initiating aggressive interactions with conspecifics. For this reason it was not an unexpected observation that aggression decreased directly following the daily stress.

The physiological mechanism behind this response may involve the serotonergic system. Within mammals, both stress and food deprivation (Fuenmayor and Garcia, 1984) are known to activate the serotonergic system as indicated by increased serotonin metabolite (5-hydroxyindoleacetic acid or 5-

HIAA) levels. In their review of monoamine neurotransmitters and behaviour in fish, Winberg and Nilsson (1993), report that stress similarly increases brain serotonergic activity in fish (increases measured in both 5-HIAA and in the ratio of the metabolite to serotonin (*i.e.* 5-HIAA/5-HT). Höglund *et al*. (2001) found that the stress of social subordination resulted in behavioural inhibition through elevation of brain serotonergic activity. A study by Clotfelter *et al.* (2007) also found that acute treatment with 5-HT decreased aggression in the fighting fish, *Betta splendens* whilst a study by Winberg *et al.* (2001) found that aggression was suppressed in rainbow trout by provision of the serotonin precursor, L-tryptophan, in the diet.

Although the links between the serotonergic system, changes in glucocorticoids and behaviour have been briefly mentioned, these links are extremely complex and often it can be difficult to determine exact causes and effects. However, the present study is primarily concerned with the effects of potential enrichment on the physiological and behavioural responses to stress. Therefore, regardless of the physiological aetiology of the behavioural changes we observed, it appears that the presence of the glass rods had no effect on these responses, as they were comparable between fish from both control and enriched tanks.

Within the present study, there were no differences observed in shoaling density either between treatments or on different observation days. Shoaling within a group of fish is a behaviour which, in the wild, results in protection from predation, and increased chances of sexual encounters (Pitcher and Parrish, 1993). Despite its diminished function within captive environments, due to the absence of predation threat, we would expect this behaviour to remain. In fact,

several laboratory studies have observed that “tightness” of a shoal varies in response to perceived risk and anxiety (Rehnberg and Smith, 1988; Egan *et al.*, 2009). This led me to the hypothesis that following a stressor, shoaling density of fish would be significantly greater. However, no evidence was found to support this hypothesis and instead it was found that shoaling density remained relatively consistent throughout the study and between treatment groups. The fish utilised within this study were Wild Indian Karyotype (WIK) strain, which have been domesticated for many hundreds of generations and have long since been maintained in the absence of any predators. It is therefore possible that, within this particular strain, manipulation of shoaling density as a response to an external threat is no longer apparent.

No differences in the percentage of time spent in the bottom third of the tank were found either between treatments or observation days. As stated in chapter 3, the amount of time away from the surface of the tank (and so nearer to the bottom) is a frequently cited measure of fear and anxiety in zebrafish (Gerlai *et al.*, 2000; Blaser and Gerlai, 2006; Egan *et al.*, 2009). As I did not see any effect of the enrichment structures on this measure in the previous study (see chapter 3) it was not surprising that there were also no significant differences observed in this endpoint during the first week of the current study, when all tanks were unstressed. However, I hypothesised that during the second week, tanks in the stressed treatments would show an increase in amount of time spent in the bottom third of the tank, particularly as behavioural observations occurred directly following the chasing stress. (One of the concerns mentioned in the previous study was that the time between the most recent “stressful” event, *i.e.* the transfer to experimental tanks, and the recording for behavioural analysis

was greater than the time taken for fish to begin utilising upper parts of the water column). Furthermore, I hypothesised that, if the enrichment was providing fish with an element of refuge, then the amount of time spent in the bottom section of the tank would be less in enriched tanks in comparison to control tanks. However, based on the results of this study, it was not possibble to accept either of these hypotheses. Whilst we can be assured that the chasing stress did indeed pose a degree of "stress" on the fish due to the change observed in other behavioural parameters, we cannot state whether the type or extent of the stress was enough to illicit a response in vertical tank usage. And as this response could not be induced, then I was unfortunately unable to discern any affect of the enrichment in alleviating it.

The observations regarding the use of various tank spaces and the comparison of this parameter between control and enriched treatments appeared to be fairly random and it was not possible to discern any consistent patterns or obvious preferences in the data gathered. I ideally wished to test two hypotheses. The first of these would be that fish from enriched tanks would spend a greater amount of time in areas of tanks containing enrichment than fish from control tanks would spend in equivalent areas. Second was the hypothesis that, if fish were utilising the glass rod structures as a form of refuge during the period that they were stressed, then the amount of time spent in these "enriched" areas directly following the stress (and therefore during the period of behavioural observation) would be greater in enriched tanks in comparison to control tanks. We would also expect this time to be greater than the time spent there in the first week of the study when all tanks were unstressed. Based on observations it was not possible to accept any of these hypotheses. Fish in enriched tanks did

not show any clear preferences for spending time in areas containing glass rods, nor did fish in stressed treatments obviously find refuge in these regions. Incidentally, whilst chasing fish in enriched tanks, I did not observe fish “hiding” within the glass rod structures but rather fish seemed more inclined to swim around the tank at an increased rate in attempts to avoid the net. However, this observation was not quantifiable.

Presence of the enrichment and provision of the daily stress had different effects on the variability of results depending on the endpoint tested. In both activity and shoaling density data, the daily stress reduced the variability seen. However, neither was affected by enrichment. Variation in percentage time spent in the bottom of the tank, however, remained relatively constant throughout the study and between all treatments. When we considered the aggression response, stress appeared to reduce the variation exhibited, whilst presence of the enrichment had the opposite effect. The variation in time spent by fish in areas of tanks containing enrichment rods was also affected by the presence of the enrichment. In structured tanks, there was much more variation in time spent in areas containing medium and long rods than in the time spent by fish in corresponding areas of control tanks. This pattern was not seen in the areas containing short rods. Variation in the cortisol response was increased by acute stress (immediately prior to sampling) in control tanks but not in enriched. However, variation in glucocorticoid receptor expression was not affected by stress or tank environment.

These results illustrate an important point in that experimental manipulations and test environment can have a significant effect, not only on absolute

endpoint measurements, but also on the variability seen in each response. Furthermore, the direction and magnitude of the effect it has on this variability may vary between endpoints studied and also with the type of manipulation or environmental alteration. I would also suggest that such measures could differ between test species or strain.

There are various studies, mainly with strains of laboratory rats and mice, illustrating the different effects that enrichment can have on the variability of physiological and behavioural responses. In a study by Augustsson *et al*. (2003), three different statistical methods (mean absolute deviation, coefficient of variation and power analysis) were employed to study possible changes in within-group variability in a 5 minute light/dark test, relating to housing condition (*i.e.* comparison between standard, enriched and super-enriched enclosures). None of the methods showed any significant differences between standard and enriched conditions in any of the parameters measured. Similarly, Wolfer *et al.* (2004) found that, of all parameters measured in O-maze, novel object and open-field tests with female mice, within-group variability was unaffected by environmental enrichment of prior housing. Furthermore, the presence of enrichment did not affect the reproducibility of such studies even between different testing facilities. Van de Weerd *et al.* (2002) in a study involving two routine laboratory testing procedures with mice found that, although mean values of most endpoints were affected by housing condition, enrichment did not alter the variability of any parameters measured.

Further analysis by Van de Weerd and colleagues of data from previous studies (Van de Weerd *et al.*, 1994; 1997), however, found that enrichment had

different effects on variability depending on the parameter measured. Whilst variation in cage emergence test behaviours and blood corticosterone values decreased with environmental enrichment, the opposite effect was seen in freezing behaviours. Other parameters measured, such as body mass and adrenal gland mass, however, showed no differences in within-group variation due to housing condition. In a study utilising three difference strains of mice, Tsai *et al.* (2002) found that, although almost all the test variables were not affected by environmental enrichment in their mean values, the enriched group showed higher coefficient of variation in many variables. Additionally, strain differences of housing conditions were not found to be consistent. Another study by Mering *et al.* (2001) with Wistar rats used SOLO power analysis to study the minimum number of animals required to detect an arbitrarily chosen effect size (20%) when significance was set at $p = 0.05$. For the quantification of some parameters, such as adrenal gland, interscapular brown adipose tissue and epididymal adipose tissue, this number varied greatly in response to enrichment of the cage environment. For other variables such as final body mass and overall growth, variation, and therefore the minimum number of animals required, was much less. It was concluded that variation of different parameters will vary between both experiments and environments meaning that quantification of animal requirements is thereby complicated.

Similarly to several of the studies mentioned here, I found variable evidence of enrichment affecting the variation observed in results. Stress appeared to reduce the variation seen in several of the behavioural endpoints whilst in the aggression and whole-body cortisol responses we observed an increase in variation associated with enrichment. In reference to the cortisol data this

increase was observed only in fish that were stressed immediately prior to sampling.

There have been two hypotheses suggested to explain effects of enrichment on parameter variability. The first suggests that enrichment will make individuals less reactive to stressful situation and will therefore produce a more homogeneous response, leading to decreased variability (Van de Weerd *et al.*, 2002). The second suggests that enrichment allows a higher diversity of behaviour thereby increasing the variation observed (Augustsson *et al.*, 2003). Results from the present study combined with those from other published experiments, however, would suggest that in reality the situation is not so simple. It would appear that several factors, including the endpoint measured, type of environmental enrichment and species/strain, will influence the variability of result to a greater or lesser extent.

This is a problem that appears to be often overlooked. Although attempts to improve the welfare of laboratory subjects through enrichment is an important step towards refining studies, potential benefits may be reduced or negated somewhat if more individuals have to be utilised as a result of the enrichment increasing the variation in results. This is obviously at odds with current attempts to fulfil the reduction criteria included in the 3R's (reduction, refinement and replacement).

4.5 Summary

Behavioural results from this study showed a significant response to the daily stress as we would expect. However, presence of the enrichment did not

appear to have any measurable effect on any of the behaviours measured. Whilst fish from control tanks showed a much higher rise in cortisol levels as a results of the acute stress prior to sampling, there are conflicting views as to whether this should be viewed as a positive or negative outcome of the enrichment. Initially we might suggest that the presence of the enrichment is obviously beneficial as it appeared to reduce the rise in cortisol in response to a mild stress. However, it may be that previous chronic activation of the HPI axis in fish in enriched tanks led to the suppression of the cortisol response when it would have been appropriate.

The observation of higher expression of the GR in chronically stressed fish from enriched tanks (in comparison to chronically stressed fish from control tanks) suggests that the enrichment reduced the effect of the daily stress on the cortisol response. I do not know the reason for this but hypothesise that the rod structures may provide fish with a refuge from the net and so allow a behavioural way of avoiding the stress. In some species of fish (*e.g.* rainbow trout) it has been reliably shown that increased cortisol leads to up-regulation of the GR. However, in other species (*e.g.* sea bass and carp) the opposite trend has been observed. The results we report here suggest that zebrafish respond in a similar way to sea bass and carp, in that increased cortisol results in down-regulation of the liver GR. However, I recommend that further research on this is required.

The variation seen in both behavioural and physiological results was affected by both the daily stress and presence of enrichment. However, the magnitude and direction of the change in variation (if any) varied depending on the endpoint

measured. I feel that this is an important issue that is often overlooked when considering changing the environment of animals used in research. Whilst effects of enclosure alterations may not be apparent when looking at the mean values of endpoints, the variation may be affected considerably. Of particular concern are changes which increase variation as these will have an impact on the statistical power of study results, as well as the number of animals required within such studies.

CHAPTER 5

BEHAVIOURAL AND PHYSIOLOGICAL RESPONSES OF ZEBRAFISH TO AIRSTONES

CHAPTER 5 – BEHAVIOURAL AND PHYSIOLOGICAL RESPONSES OF ZEBRAFISH TO AIRSTONES

5.1 Introduction

In aquaculture facilities the use of artificial forms of aeration are considered to be crucial. Within such facilities, levels of dissolved oxygen are affected by many variables. Macro/microorganisms in the water and sediment, consumption/production by vegetation, consumption by the fish themselves and natural diffusion caused by wind action will all have roles to play in the moderation of oxygen concentration found within the water column. This, combined with the desire to maximise the amount of fish maintained within a set volume of water (and thereby maximise production), means that artificial ways of maintaining optimum oxygen levels, such as the use of airstones or other forms of aeration, are required.

The poikilothermic nature of most teleost fish means that metabolism and oxygen demand are higher during the warmer, daylight periods within aquaculture facilities. Within the laboratory, however, constant temperatures are usually maintained throughout experimental periods, and so there are fewer problems with diurnal fluctuations in dissolved oxygen or metabolic demand as would be the case in outdoor aquaculture facilities. Furthermore, in the laboratory and particularly in regulatory studies, most of the factors that serve to significantly deplete dissolved oxygen are eliminated. Added to this that maximisation of biomass within tanks is not a requirement within regulatory studies, maintenance of suitable levels of oxygen can effectively be achieved by

using appropriate stocking densities. For this reason OECD guidelines state that dissolved oxygen concentrations of at least 80 % air saturation value (ASV) should be maintained without the use of aeration, *i.e*. through the provision of a sufficient inflow of fully aerated water.

Within ornamental or hobbyist fish-keeping literature, it is generally recommended that fish tanks are provided with one or more airstones. Obvious reasons for this include a direct aeration function, but many biofiltration systems also use a flow of air bubbles as a “gas-lift” system to maintain a circulation of water through the porous sediment at the bottom of the tank. Less obvious reasons include suggestions that they also serve to circulate aquarium water and in doing so may provide fish with a form of environmental enrichment. The idea that airstones might provide more than just essential gases to the water appears to be one that, to our knowledge, has not been tested scientifically.

Although the main purpose of airstones is, purportedly, to aerate the water, their design and function means that the tank environment can be altered in other ways. The stream of bubbles emitted by airstones can create a water flow within the tank that would not exist in their absence. Furthermore, the bubbles created can form a barrier which could be utilised to gain visual isolation from other individuals. It is also possible that the bubbles could prove to be aversive for fish. There are numerous noted cases of “bubble curtains” used to deter fish from unwanted areas such as power plant inlets, and evidence that many species will avoid an air bubble barrier (Patrick *et al.*, 1985). It has been suggested that the deterrent properties of such barriers may be due more to the noise produced by the bubbles, rather than their physical presence (Hocutt,

1980). This, therefore, suggests the possibility that the sounds produced by aeration within either laboratory or aquaculture tanks may also have a negative effect on the fish living there.

There is much data to suggest that noise can have a significant effect on growth, metabolism and survival in various aquatic species. Banner and Hyatt (1973), for example, analysed the effects of noise on two estuarine fishes. They showed that a 20 dB increase in sounds in the 40 to 1000 Hz frequency range was significantly lethal during embryonic development and slowed larval growth. A later study by Lagardère (1982) found that, for the brown shrimp (*Crangon crangon*) “noisy” tanks (similar to those experienced by shrimp cultured in a standard thermoregulated aquarium) resulted in decreased food consumption, a decrease in growth at sexual maturity and during reproduction together with lesser resistance to pathogenic microorganisms. A study by Craven *et al.* (2009) has recently looked at the impact of water borne sound(s) as a potential source of stress for fish in aquaculture facilities. It was found that airstones are one of the dominant acoustic sources in tanks, particularly within higher frequency ranges (> 2500 Hz). Furthermore, a variation in sound pressure level was noted between airstone types. This was suggested to be a function of the relationship between the particle size of the airstones and bubble size. Further study is now required to evaluate potential impacts of the soundscape environment on the welfare of fish. Although the aforementioned studies have been concerned with looking at the consequences of noise for aquaculture species, we suggest that similar impacts could be seen for fish used in the laboratory. However, this is an area of study that has not been explored at present.

It is therefore apparent that we know little about the positive or negative impacts of airstones on the welfare of captive fish. Particularly in laboratory tanks, where aeration to maintain oxygen levels may not be critical, the presence of airstones as a form of enrichment is a possibility that we feel needs exploring further. We are also aware of the possibility that the alteration of the overall tank soundscape caused by the addition of airstones may prove to have negative consequences. Therefore, this study focussed on establishing any clear positive or negative effects of providing groups of adult zebrafish with aeration via different sizes of airstones. To do this we observed two behaviours, activity level and aggression, that have been shown to vary in response to stress and anxiety within this species. Then we looked at whether fish preferentially spent time in the vicinity of the airstone, or preferred to be farther away from it. Furthermore, we looked at mRNA expression of the glucocorticoid receptor (GR), and phosphoenolpyruvate carboxykinase (PEPCK).

In the previous studies reported in this thesis (see chapters 3 and 4) whole-body cortisol was measured as an indicator of stress. However, it was apparent, both from the results obtained here and from knowledge of the action of cortisol within this species, that it is only a useful measure in the case of short-term, or acute, stress. Within zebrafish, cortisol values have been found to increase within 3 minutes of an acute stress such as netting and air exposure. Following a peak at approximately 6 minutes, a return to pre-stress levels has been observed at 1 hour post stress (Ramsay *et al.*, 2009). More severe stressors can have longer lasting effects, however, the response is still a relatively short term one. Because of this rapid return to pre-stress levels it was felt that any

potential effects that would be caused by the presence of the airstones would not be reflected in the whole-body cortisol concentrations of fish 9 days after the airstones were added. For this reason, I looked at expression of the glucocorticoid receptor (GR). This particular receptor is the main corticosteroid receptor in this species, and its expression has been found to be reliably correlated with longer-term patterns in cortisol production (Terova *et al.*, 2005). I also looked at one of the genes that code for proteins involved in glucose synthesis which is required to fulfil the energy demand at times of stress, phosphoenolpyruvate carboxykinase (PEPCK).

The hormonal stress response in fish is accompanied by various biochemical responses which allow for metabolic adjustments required to meet the associated energy demand. A large part of this involves an increase in plasma glucose concentration. Glucose is a fuel that is oxidised to meet increased energy requirements (Aluru and Vijayan, 2007), and the increase in glucose is mediated by both catecholamines and glucocorticoids which involves enhancement of the liver metabolic capacity. This incorporates increases in glycogenolysis (conversion of glycogen to glucose), glycolysis (conversion of glucose to pyruvate) and gluconeogenesis (synthesis of glucose from substrates such as lactate and pyruvate) (Wiseman *et al.*, 2007). The glycogenolytic pathway, which is thought to be stimulated by adrenergic signalling, allows for the rapid output of glucose from the liver. The subsequent depletion of liver glycogen stores is then replenished and plasma glucose levels maintained by up-regulation of the gluconeogenic pathway in the liver largely using lactate recycled from muscle via the Cori cycle. In combination, therefore, longer-term plasma glucose maintenance is coupled with glycogen repletion,

both of which are important during times of stress recovery (Wiseman *et al.*, 2007).

Within teleosts there are several genes encoding proteins that are known to be involved in glycolysis and gluconeogenesis. However, the key regulatory enzymes involved in gluconeogenesis are 1) phosphoenolpyruvate carboxykinase (PEPCK) which promotes the decarboxylation of oxaloacetate to phosphoenolpyruvate and CO_2, and 2) glucose-6-phosphatase (G6Pase) which hydrolyses glucose-6-phosphate into free glucose and inorganic phosphate (Aluru and Vijayan, 2007). Expression of PEPCK has been found to be reliably increased with both stress and cortisol treatment in fish (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003; Aluru and Vijayan, 2007; Wiseman et al., 2007) confirming the gluconeogenic role of cortisol in fish.

5.2 Materials and methods

Adult zebrafish were obtained from AstraZeneca, Brixham Environmental Laboratories and kept under conditions compatible with OECD guidelines throughout. Prior to the experiment, fish were kept in flow-through fresh water at 28 °C and under a photoperiod of 14L:10D (light:dark) with a 20 minute phased sunrise/sunset. Fish were fed SDS dry food in the morning and live *Artemia* 24 h nauplii in the afternoon. At the beginning of experiments fish were approximately one year old.

Twenty-one groups of six fish were provided with a barren environment for a duration of 10 days. On day 11, seven of the tanks were provided with a small airstone (low airflow treatment; 14 x 25 mm blue cyclinder airstone, Elite) and

another seven with a large airstone (high airflow treatment; 100 x 15 mm blue longstone airstone, Elite). The remaining seven were left without airstones. Fish were terminated on day 19 by over-anaesthesia in accordance with UK home office guidelines. Behavioural endpoints consisting of: activity level, whole tank aggression and use of tank spaces were analysed on alternate days throughout the study. Expression of Glucocorticoid Receptor (GR) and Phosphoenolpyruvate carboxykinase (PEPCK) were quantified from livers of fish using quantitative PCR.

For further information on of the experimental design looking at the behavioural and physiological responses of zebrafish to airstones see section 2.2.3 and for details on the behavioural and physiological endpoints used see sections 2.3 and 2.4.

5.3 Results

5.3.1 Behavioural responses to airstones

Activity level in control tanks was fairly consistent. However, levels were higher on day 17 than on days one and three ($F_{8,52} = 4.00$, $p < 0.01$). In low airflow tanks, activity levels rose significantly on day 11, the day of airstone addition, in comparison to previous days ($F_{8,52} = 2.52$, $p = 0.02$). Following this, levels remained elevated until day 17, although not significantly so. In high airflow tanks, activity levels also rose somewhat following the addition of airstones, however this was only significant on day fifteen ($F_{8,52} = 6.66$, $p < 0.01$) (Figure 5.1). Aggression levels were consistent throughout the study in control ($F_{8,52} = 0.65$, $p = 0.73$), low airflow ($F_{8.52} = 1.21$, $p = 0.31$) and high airflow ($F_{8,52} =$

1.00, p = 0.44) treatments. Only on day 13 did we observe any difference in aggression between treatments. On this day aggression in low airflow tanks was significantly higher than that observed in high airflow tanks ($F_{2,18}$ 4.67, p = 0.02) (Figure 5.2)

In control tanks, fish spent approximately equal times in all three sections of the tank. However, on days 15 and 17, fish spent more time in the “far” area than in the other two areas ($F_{2, 162}$ = 16.6, p < 0.01). In the low airflow treatment, on observation days 9, 13, 15, and 17, fish spent a significantly greater amount of time in the “close” area in comparison to either the “mid-range” or “far” areas ($F_{2, 162}$ = 5.4, p < 0.01). In the high airflow treatment, only on day 13 did the time spent in each of the three areas differ significantly. On this day, fish spent more time in the “close” area than in the other areas ($F_{2, 162}$ = 4.9, p < 0.01).

The mean absolute deviation (MAD) in activity prior to addition of airstones was similar, with an average of 4 lines per minute ($F_{2,102}$ = 1.10, p = 0.34). However, following the addition of airstones, the MAD in activity level in both the low and high airflow treatments rose threefold to approximately 12 lines per minute. This was significantly higher than the MAD measured in control tanks ($F_{2,81}$ = 3.28, p = 0.04). Mean absolute deviation in relative aggression prior to addition of airstones was significantly greater in the low airflow treatment than in the control tanks ($F_{2,102}$ = 4.88, p < 0.01), despite the fact that all tank environments were identical at this point. Following the addition of airstones on day 11, there was no differences in MAD in aggression between any of the treatments ($F_{2,81}$ = 1.09, p = 0.34). It is worth mentioning, however, that whilst the MAD observed in control and high airflow treatments remained the same following the addition

of airstones as that previously, the MAD in aggression measured in low airflow tanks was reduced by half following airstone addition.

5.3.2 Glucocorticoid Receptor and PEPCK expression

There were no significant differences in the expression of either the GR or PEPCK genes between any of the treatment groups ($F_{2,18} = 0.25$, $p = 0.79$ and $F_{2,18} = 0.82$, $p = 0.46$ respectively) (Figures 5.4 and 5.5). Mean absolute deviation of GR and PEPCK expression were comparable between all treatments ($F_{2,18} = 0.18$, $p = 0.84$ and $F_{2,18} = 3.32$, $p = 0.06$ respectively).

5.3.3 Mortality

In all treatments, survival from days 1-10 was 100%. However, from day 11, survival declined considerably in the low airflow treatment and continued to decline until the end of the study. In the high airflow treatment we also observed a decrease in survival between days 13 and 19. At the end of the study overall survival in the control, low airflow and high airflow treatments was 100 %, 79 % and 90 % respectively (Figure 5.6). In the low airflow treatment, mortalities occurred in four tanks out of seven. In the high airflow treatment mortalities were found in three tanks out of seven. Fish that died throughout the study showed no obvious external signs of trauma that could be attributed to injury resulting from aggression. Furthermore, dissection of individuals did not provide us with an obvious cause of death.

5.3.4 Dissolved oxygen content

Oxygen saturation on day 2 was 96 % (7.5 mg/L) in all tanks. On day 12, following the addition of airstones on the previous day, oxygen saturation had

decreased to an average of 91 ± 0.93 % (7.2 ± 0.07 mg/L) in control tanks and increased to an average of 101 ± 0.71 % (8.0 ± 0.06 mg/L) and 102 ± 0.80 % (8.1 ± 0.06 mg/L) in low air flow and high air flow treatments respectively.

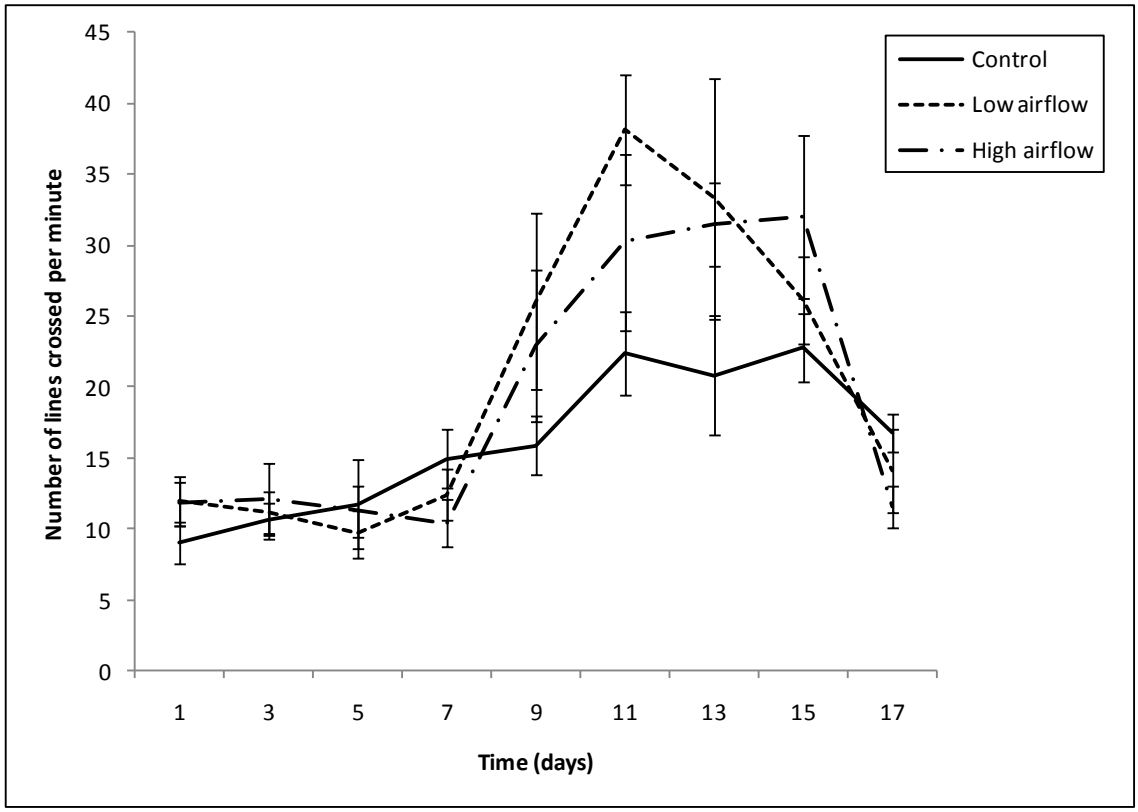


Figure 5.1 Activity level of fish (number of lines crossed per minute) in control, low airflow and high airflow treatments. (Mean ± SE, n = 7).

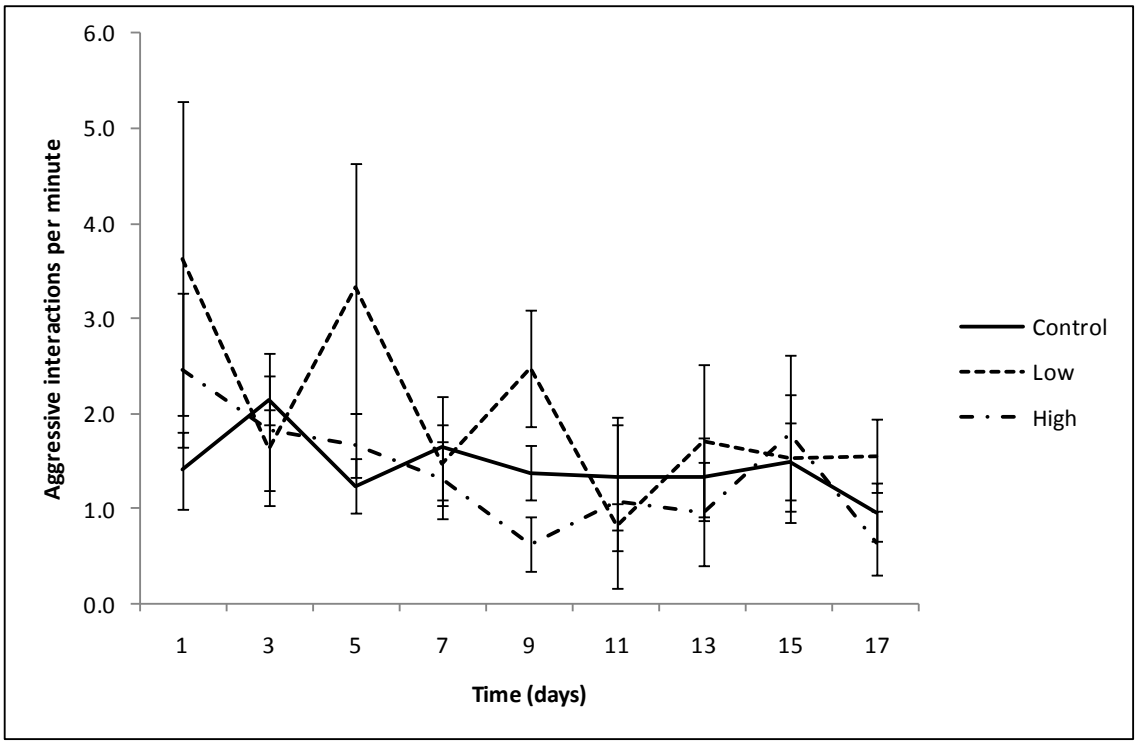


Figure 5.2 Aggressiveinteractions per minute between fish in control, low airflow and high airflow treatments. (Mean ± SE, n = 7).

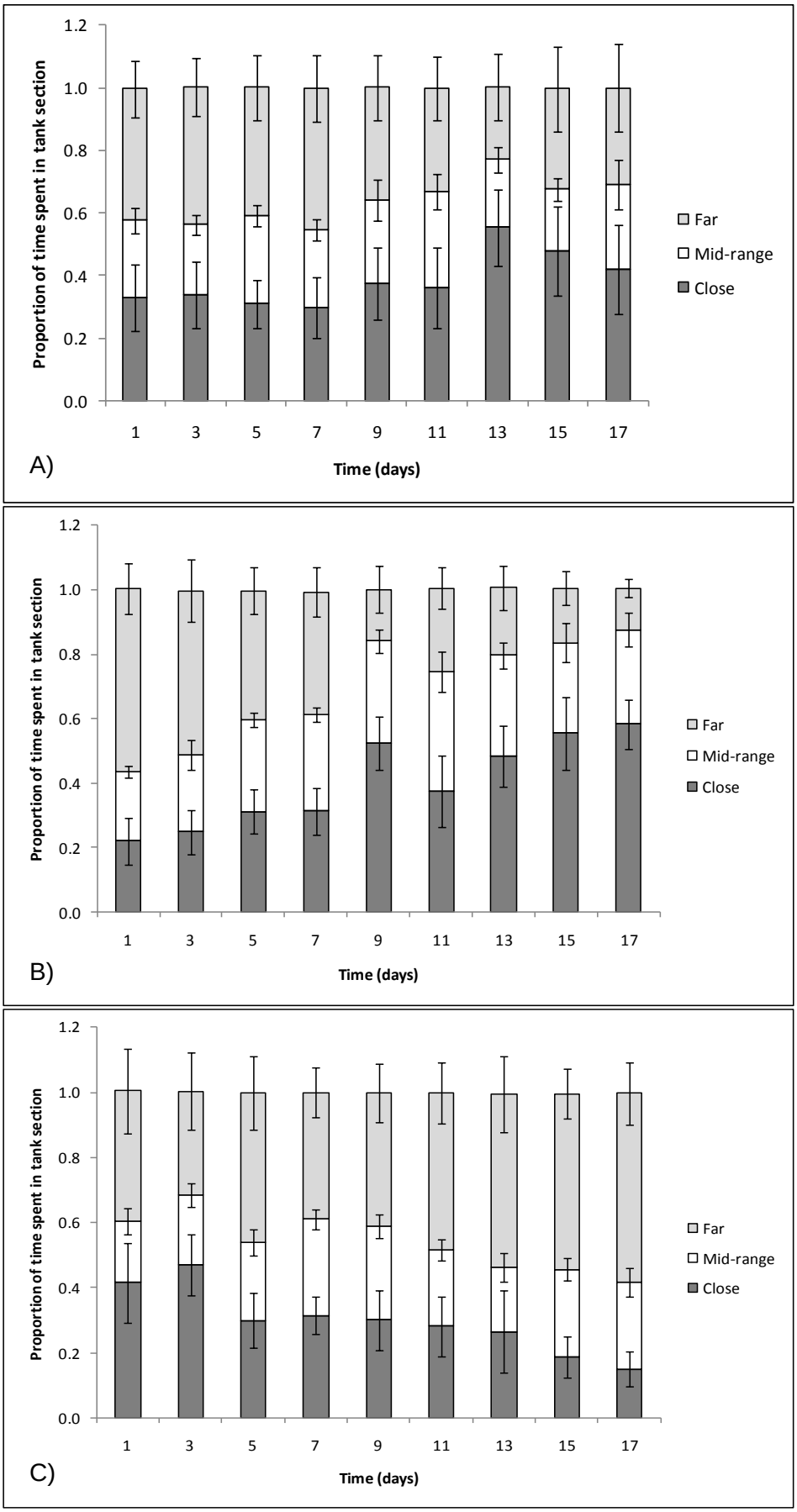


Figure 5.3 Proportion of time spent in three tank areas by fish in control (A), low (B) and high (C) airflow treatments. (Mean ± SE, n = 7).

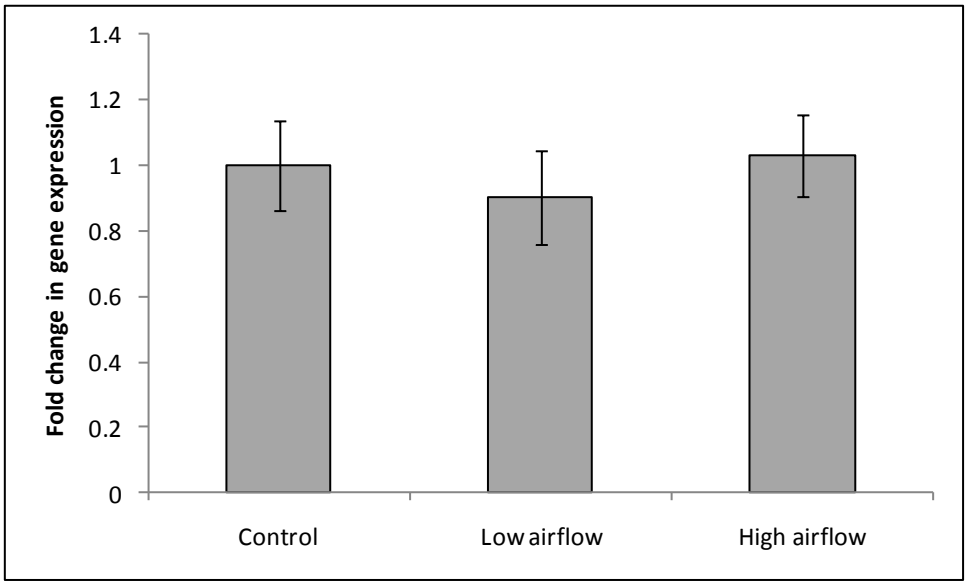


Figure 5.4 Expression of the glucocorticoid receptor in liver tissue of fish from control, low and high airflow tanks– fold change from control group. (Mean ± SE, n = 7).

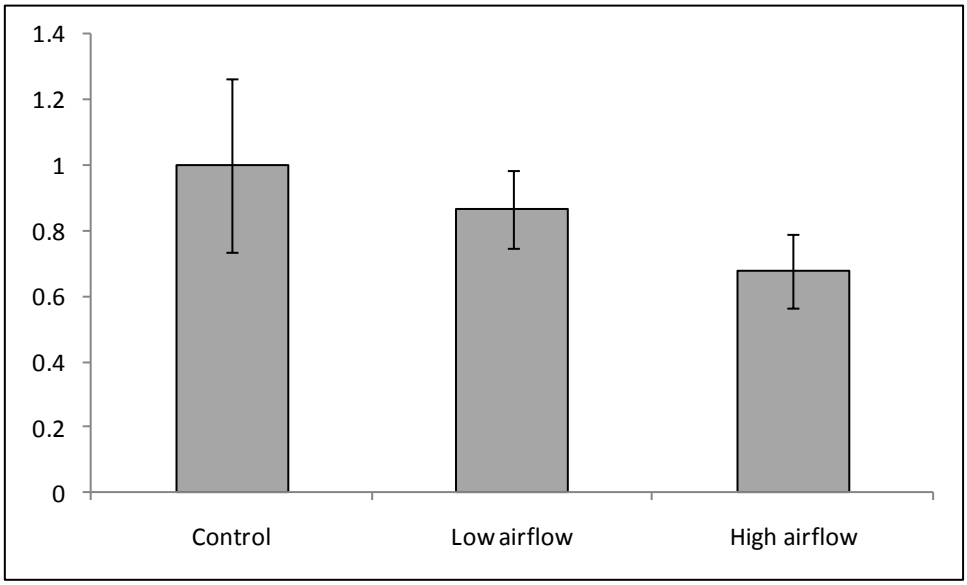


Figure 5.5 Expression of PEPCK in liver tissue of fish from control, low and high airflow tanks– fold change from control group. (Mean ± SE, n = 7).

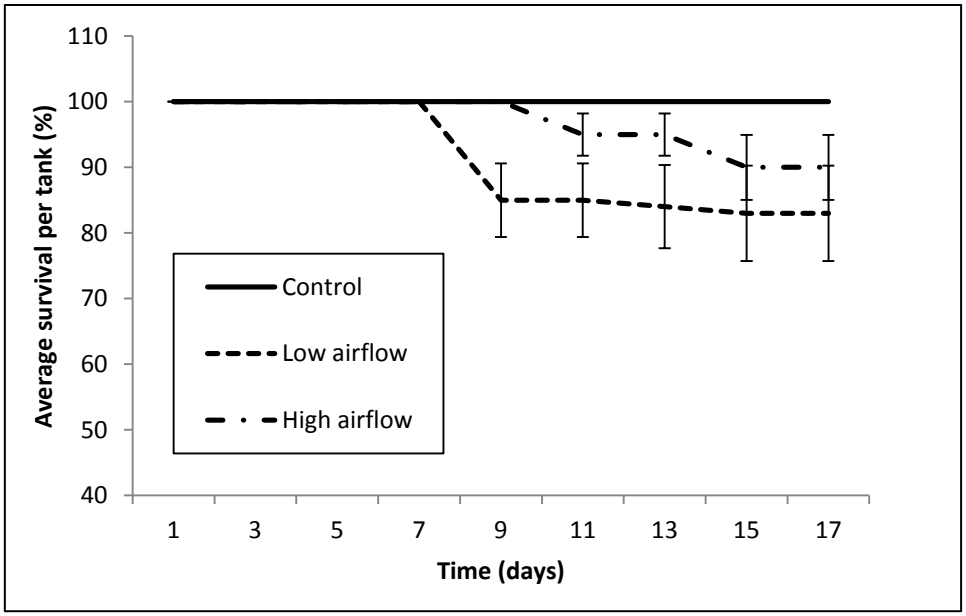


Figure 5.6 Average survival per tank (%) throughout the study in control, low airflow and high airflow treatments. (Mean ± SE).

Table 5.1 Mean absolute deviation of all endpoints

	Before addition of airstones			After addition of airstones		
	Control	Low airflow	High airflow	Control	Low airflow	High airflow
Activity	4.8	4	3.7	7.5	12	12.2
Aggression	0.8	1.8	1.2	0.8	1	1.2
Proportion of time in "dose" area	0.20	0.17	0.20	0.17	0.19	0.30
Proportion of time in "mid-range" area	0.07	0.08	0.08	0.09	0.10	0.11
Proportion of time in "far" area	0.23	0.19	0.20	0.20	0.12	0.28

	Control	Low airflow	High airflow
GR expression	0.26	0.30	0.24
PEPCK expression	0.54	0.22	0.23

5.4 Discussion

In the low air flow treatment, activity level was higher on the days following the addition of the airstone. A similar trend was observed in the high air flow treatment, although to a lesser extent. Significant increases in locomotion rate are often evidence of stress in zebrafish. Exposure to anxiogenic chemicals, such as alcohol, stimulation of aggression towards a mirror image and crowding stress have all been observed to induce an increase in activity (Gerlai *et al.*, 2000; Blaser and Gerlai, 2006 and McFarlane *et al*., 2004 respectively). The link between stress and activity level is discussed further in section 3.4.1. The increase in activity level observed in the current study may have been due to the specifically stressful nature of the air flow created by airstones. However, it may equally have been due to a non-specific change in the tank environment that the fish were not accustomed to. It is worth noting that these fish came from a husbandry facility that did not use airstones in the stock tanks.

An increase in activity level, however, will cause a subsequent increase in heart rate and breathing rate and therefore an increase in overall respiration (Stevens and Randall, 1967). Within fish the gills are one of the main routes of uptake for many chemicals (Murphy and Murphy, 1971). It is reasonable to assume, therefore, that an increase in activity and respiration will have a significant effect on chemical uptake. Several studies have looked at this in various species of fish. In 1971, Murphy and Murphy observed increased uptake of DichloroDiphenylTrichloroethane (DDT) when respiration was increased in the mosquito fish (*Gambusia affinis*). Barry *et al.* (1995), in a study with Australian crimson-spotted rainbow fish (*Melanotaenia fluciatilis*) discovered that the general use pesticide, Esfenvelerate, was more toxic to younger fish who have

a higher mass-specific respiration rate, than it was to older fish. Yang *et al.* (2000) exposed Coho salmon (*Oncorhynchus kisutch* and *Oncorhynchus mykiss*) to three separate organic compounds and found a significant correlation between toxicant uptake rate and fish oxygen consumption, regardless of fish size or species.

This takes on an increased relevance when we are considering fish to be used for regulatory toxicology. Any factors which affect the uptake of chemicals can be problematic, particularly if these factors are not consistent between different studies or testing facilities. As fish were only provided with air flow via airstones for a period of 8 days, it is not possible to say how long the effect on the fish would persist, or whether the increase in activity level observed was an initial response to the change in environment or a permanent one. However, as many regulatory tests last for only a short duration it could be argued that any change in behaviours, despite their apparent brevity, that could affect chemical uptake could impinge on experimental results.

Within the study there was observed no significant differences in aggression that could be attributed to the presence or size of air flow or the airstones that generated them. I initially hypothesised that fish may regard airstones as a defendable resource, being the only item present in the tank. In this case we would have expected higher levels of aggression in tanks containing airstones. Alternatively, if the airstones were providing fish with a degree of stress (as we suggested due to the rise in activity level in tanks with airstones) we might have expected to see the number of aggressive interactions decrease. In the previous study it was observed that fish subjected to a stressor showed

decreased levels of aggression directly following a stressor (see chapter 3). However, this was an acute stress and most likely more severe than a change to the tank environment.

In the low airflow treatment fish appeared to show a preference for the third of the tank containing the airstone (*i.e*. the "close" section). This was not observed in the high air flow treatment except on day 15. The intention of looking at this measure was to assess potential "preferences" of the fish for proximity to the airstone and associated bubble stream. Unfortunately there are no previous studies looking at the proximity of fish to airstones in either laboratory or ornamental fish. I could also find no studies that looked at tank space preferences of any fish and therefore could not make a comparison between our results and others.

It was initially assumed that if fish showed a "preference" for the area of tank closest to the airstone then this would indicate that that location was preferable to other, more distant, locations. However, it is also a possibility that the currents created by the airstone affected the position of individual fish. The upward motion of the bubbles may have created a current that dragged fish towards the left hand side of the tank where most fish were observed. This affect of the water current may also be helpful in explaining the high numbers of mortalities observed within the low air flow tanks in particular.

The number of mortalities in both the low and high air flow treatments was a surprising result within this study and was only observed following the addition of air flow via airstones. It was not possible to find any evidence in peer

reviewed literature of mortalities directly related to airstone use or oxygenation of the water within this species. However, as survival rates in the low and high airflow treatments were 79 and 90 % respectively at the end of the study (in comparison to 100 % survival in control tanks), this is obviously a large effect that cannot be explained as random deaths. Furthermore, in neither of the two previous studies (see chapters 3 and 4) did I observe any mortalities.

In searching for possible explanations for this mortality it was initially thought that this could be due to aggression. As fish in the low and high air flow treatments appeared to preferentially use the area of the tank containing the airstone it is possible that they were utilising it as a defendable resource. If this was the case then the most dominant fish would, most likely, have been located near the airstone and then instigating aggressive interactions with any less dominant fish that threatened his/her position. However, behavioural results show that there was not a significantly greater amount of aggression in tanks containing airstones than in control tanks. Furthermore, there was observed no significant increase in aggression following the introduction of either small or large airstones. In addition to this, the deceased fish showed no obvious external or internal signs of trauma which we would expect if injuries from aggressive interactions were severe enough to result in death.

Throughout the study dissolved oxygen (DO) concentration was measured on the day after addition of fish to tanks and a second time the day following addition of airstones (days 2 and 12 respectively). It was noted, not unexpectedly, that DO increased to more or less 100 % saturation in both low and high air flow treatments whilst DO in the control tanks decreased to

approximately 91 %. OECD guidelines state that oxygen concentration should be maintained at a minimum of 80 % air saturation value throughout studies. Whilst the dissolved oxygen concentrations required by zebrafish do not appear to have been determined, in general, small-bodied, tropical fish such as zebrafish have relatively high metabolic rates and therefore require more oxygen than larger, temperate species (Helfman *et al.*, 1997). It has therefore been recommended that dissolved oxygen levels for this species be maintained at or just below 100 % saturation to maintain optimal health of individuals (Lawrence, 2007). For this reason, we must assume that the mortalities we observed were not due to problems with oxygen levels (either too high or too low) within tanks.

Another option considered was that the water flow caused by the airstones was too great for the fish. It is possible that, when a strong water current is present in tanks, then fish will have to expend a lot of energy to maintain their position. If this energy cannot be replaced in a sufficient quantity or speed, then the fish may die of exhaustion. This hypothesis would also explain why there were no obvious injuries or cause of death. Although I found no evidence in the scientific literature of water flow from airstones causing problems for fish, there are numerous cases discussed in hobbyist fish keeping literature (*e.g.* internet sites such as www.myfishtank.net) that discuss problems relating to this. Suggestions that air bubbles disrupt both normal fish behaviour and feeding appear to be common. Overall there seems to be disagreement over whether airstones are provided for the benefit of fish (*i.e*. for provision of oxygen to water and reduction of tank thermoclines) or simply to improve the aesthetics of the tank environment. A consistent message, however, seems to be that certain

species find high water flow along with bubbles and turbulent water surfaces to be particularly aversive.

From these results it is difficult to determine the exact cause of the relatively high number of mortalities observed in tanks containing airstones. As zebrafish in general, and in particular the WIK strain used here, are so amenable to maintenance in both the laboratory and captive environment, it is surprising that so many were lost throughout the study. This is not an outcome that has been observed before, either in the previous studies presented here or in the work of colleagues. Furthermore, as mortalities were observed only in tanks containing airstones, the possibility that the airstones had some role in the low survival observed must be seriously considered. The most likely hypothesis to explain this is that the water flow created by the airstones was aversive and too strong for these relatively small fish. Subsequently they may have expended too much energy maintaining their position within the tank which possibly disrupted their feeding. Body mass of fish at the end of the study, however, were comparable between all treatments and also between tanks subject to mortalities and those without within each airflow treatment.

Other possibilities considered were either waterborne or airborne contamination. However, there were no other unexplained mortalities in the laboratory (*i.e.* other fish with the same water/air source) during the time of this study, and so we find this possibility unlikely. With the information available, unfortunately, it is not possible to prove or disprove any of these hypotheses.

As the conditions within both airstone treatments were such that survival was significantly reduced, it would be reasonable to assume that these conditions might have provided a substantial degree of stress on the fish housed there. For this reason, it was hypothesised that a difference in expression of the glucocorticoid receptor gene would be observed between control fish and those provided with airstones. In the previous study a significant decrease in GR expression was observed in fish that were subjected to a daily chasing stress and many other studies have reported changes in GR expression relating to stress or cortisol production. A decrease in GR expression was observed by Terova *et al*. (2005) in sea bass and by Stolte *et al.* (2008) in carp as a result of crowding and reduced temperature respectively. Conversely, Sathiyaa and Vijayan (2003), Vijayan *et al.* (2003) and Wiseman *et al.* (2007) all observed increased GR expression in rainbow trout in response to glucocorticoid stimulation.

Vijayan *et al*., (2003) also measured higher expression of PEPCK in cortisol implanted fish alongside increased expression of GR, indicating activation of the GR signalling pathway. The gene for cytosolic PEPCK is expressed primarily in the liver and kidneys and the level of PEPCK in a tissue in which the gene is expressed is controlled by a wide variety of physiological stimuli including dietary carbohydrate, hormones and cellular intermediates (Hanson and Reshef, 1997). In particular, glucagon, glucocorticoids and thyroid hormones all increase PEPCK, whilst insulin decreases its synthesis (Rajas *et al.*, 2000). When fish are under stress, glucose concentrations increase with increased plasma cortisol (Barton and Iwama, 1991) and PEPCK is responsible for catalysing the conversion of oxaloacetate to phosphoenolpyruvate, which is one

of the rate-limiting steps in gluconeogenesis (Rajas *et al.*, 2000). As such, the increased expression of PEPCK in fish that are exhibiting increased cortisol (*i.e.* as a result of genuine stress or achieved via implants) makes this a model stress responsive gene (Bears *et al.*, 2006).

With this knowledge in mind, it was hypothesised that fish in both airstone treatments, that were presumed to be under a greater degree of stress based on the number of mortalities observed, would show altered expression of the GR gene alongside increased expression of the PEPCK gene. However, results did not support this hypothesis, and expression of both of these genes was very similar between all treatment groups. Incidentally, an additional concern was that the water movement caused by airstones (which was very turbulent in comparison to the conditions previously experienced by these fish) would make feeding more difficult for the zebrafish. It was thought that lack of food as a result of this could also have contributed to the high levels of mortality observed. However, expression of PEPCK is also reliably induced by low carbohydrate and starvation diets so, again, there was no evidence in the qPCR results that would support this concern. It is particularly confusing that we observed no difference in the expression of such typically stress responsive genes when conditions were such that survival was significantly decreased in both airstone treatments.

The mean absolute deviation of activity level increased significantly in tanks equipped with airstones. The relevance of increasing deviation from the mean in any variable is discussed in greater detail in section 3.4. Whilst activity level is not generally an endpoint that is used in regulatory studies, the fact that the

tank environment has changed the variation in behaviour of fish should still be cause for concern. As the behaviour and physiology of fish (as with all species) are inextricably linked, we may therefore expect there to be additional effects on the variation in other responses not measured here. As explained previously, this would then affect the number of fish required in studies and the potential strength and reliability of any statistical analyses performed. MAD of aggression appeared to be affected by the airstones in the low air flow treatment only. In this case, variation was decreased following airstone addition. Although it is difficult to find an acceptable biological explanation for this result it nevertheless enforces the theory that, in cases where environmental changes have little or no effect on the mean value of a response, the variation observed may be altered significantly. Mean absolute deviation of GR and PEPCK expression, however, were comparable between treatments.

5.5 Summary

To summarize briefly these results, there was no evidence found to support the use of airstones as a form of environmental enrichment for adult zebrafish. Behavioural results suggested that fish found tanks with airstones more stressful than control tanks as activity levels increased significantly following airstone addition. Furthermore, although no evidence of stress was found to be reflected in the expression of GR and PEPCK in the zebrafish liver, the number of mortalities within both low and high air flow treatments was extensive. Regardless of the physiological cause behind these mortalities I feel that this is a definitive indicator that airstones of this type should not be provided for zebrafish in this size of tank, particularly as they are not required for aeration.

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6 – GENERAL DISCUSSION

Environmental enrichment is currently employed in a wide range of animal holding facilities including those which are publicly accessible, such as zoos and aquaria, as well as in research laboratories. The benefits of providing enrichment have been studied in depth, and although the results of many studies have proven to be either inconclusive or inconsistent, the general consensus appears to be that certain types of enrichment promote good welfare.

Most notably, particularly within farmed animals, enrichment has been found to reduce the occurrence and frequency of harmful behaviours such as social aggression, self-harming and stereotypies. However, it has also been found that animals from enriched environments show improvements in learning and memory based tasks alongside increased rates of neurogenesis and higher cortical thickness. Furthermore, enriched animals have been found to be less reactive to stressful events.

Within research laboratories the benefits of enrichment could be designated into two categories. Firstly, they assist with the moral and ethical obligation to provide a good standard of care for animals that are utilised for research. This is required of all research establishments and is enforced in the U.K., as in many countries, by prescriptive laws. Secondly, by maximising the welfare of research subjects, the standard of the research is also improved. As poor welfare can

have huge impacts on both the physiology and behaviour of individuals, this is likely to have effects on research results. Furthermore, improvement of welfare overall will ensure standardisation between studies and testing facilities.

Whilst enrichment has been employed within the laboratory for mammalian subjects for many years, there are currently no well established examples of its use for aquatic species. Furthermore, there is little available information as to the types of enrichment that could be beneficial and how these may differ between different test species. More so than mammals, the vast differences in life history traits and conditions required by different species of fish means that their enrichment requirements may show a similar level of variability.

The U.K. Home office, in recent and laudable attempts to improve the welfare of fish species used within the laboratory, has suggested the use of environmental enrichment to pursue this end. This PhD was therefore instigated to look at the potential benefits of a range of enrichment strategies for the zebrafish, an important model species that is frequently used in regulatory studies. In this chapter, a synopsis of the findings of this work will be provided, placing them in the context of current advances in this area.

6.1 Overview of findings

The first study (Chapter 3) examined the effects of tank structures (*i.e.* as physical enrichment) on various endpoints in juvenile and adult zebrafish. It was found that presence of enrichment had no effect on activity level or shoaling

density of either juvenile or adult fish. However, aggression levels were found to remain higher for a longer period in tanks containing structures than in groups of fish from barren tanks. This trend was observed both in the juvenile and adult studies. It was assumed that this result was due to the structures altering social interactions sufficiently that the process of establishing a hierarchy, as is usual when a group of fish are introduced to a tank environment, was subsequently extended. Fish from structured tanks did not spend less time in the bottom third of the tank suggesting that the presence of the rods did not make the novel environment less aversive or stressful. Importantly, the variation observed in all behavioural endpoints and for both age classes of fish were comparable between treatments. This is relevant as changes in endpoint variability can have important implications for the numbers of animals required for effective statistical analysis following regulatory testing.

A comparison of juvenile and adult behavioural results revealed that adults demonstrated significantly higher activity levels, shoaling densities and aggression levels than juvenile fish. Whilst this had no obvious impact on the results with respect to the relative effects of enrichment, it is still an important issue to be considered as differences in innate behaviours relating to age or size class of study individuals may alter their welfare and enrichment requirements.

Whole-body cortisol concentrations were measured in juvenile fish only. At no point throughout the study (measurements were taken on days 1, 2, 4 and 7) did the cortisol concentrations differ significantly between fish from barren and

structured tanks. On day one (approximately 24 hours following addition to study tanks) fish showed a significantly higher cortisol levels than at any other time during the study. This was presumably due to the stress of being moved to a novel environment only one day previously.

The second study (chapter 4) looked at the effects of the same design of tank structures on the acute and chronic stress responses of juvenile zebrafish. In general, the chasing stress applied to fish had the effect of increasing activity level, both in comparison to previous days and to unstressed, control fish. Aggression, shoaling density and amount of time spent in the bottom third of the tank were not affected by either the chasing stress or presence of enrichment.

In control tanks, fish from the chronic and acute stress group, showed significantly higher cortisol levels than those from the unstressed treatment. However, in structured tanks, there were no significant differences between any of the treatments. The reason for these results were not clear and could have been due to the rods providing fish with a refuge, thereby reducing their response to this stress. A further possibility is that fish from structured tanks were actually demonstrating an impaired response to the chasing stress. The acute stress immediately prior to termination produced a significant increase in variability of the cortisol response in fish from barren tanks. However, this pattern was not observed in fish from structured tanks that were similarly stressed before sampling.

In both brain and liver samples, expression of the glucocorticoid receptor was approximately twofold lower in the barren stressed treatment in comparison with all other treatments. It was concluded that this was due to the repeated and consistent production of cortisol in the previous week resulting in negative feedback mechanisms which reduced the numbers of receptors available in tissues. This result supports the previous theory that the enrichment rods alleviated the effects of the chasing stress on the cortisol response. At present, I do not know whether this was due to the rods providing fish with a refuge from the net (a possibility which was not supported by the behavioural results) or whether this was due to the observed reduction in whole-tank aggression levels.

6.2 Shortfalls and limitations

As with all research, there were various shortfall and limitations associated with this PhD. Here I will attempt to address these as fully as possible. The first of these relate to the species of fish used. The zebrafish was chosen due to its frequency of use within regulatory toxicology as well as its availability and ease of maintenance. Throughout this PhD I used only one strain of this species, the WIK (Wild Indian Karyotype) strain which is one of the most frequently used for this type of work. However, various strains do exist and are utilised in different facilities. Furthermore, these strains all have very different behavioural and physiological characteristics and, importantly, can vary in the magnitude of their response to stress. As a consequence of this, the results observed here may have been very different had the strain of fish used been altered. As a result, I

would hesitate in applying the advice given for WIK strain to any other strain of zebrafish without prior investigation.

In addition to this, it also should be taken into account that the zebrafish used in the vast majority of laboratories are highly domesticated and, in most cases, many generations removed from their most recent wild ancestors. Aside from the problem this causes in attempting to improve the environment and welfare of fish that have been selectively bred for generations for success in the laboratory environment, we also realise that some of the cited benefits of environmental enrichment may be inappropriate for such individuals. As discussed in chapter one, promotion of “natural behaviours” is seen to be one of the highly desirable outcomes of providing enrichment. For these fish, however, who are not only destined to spend their whole life within the captive environment, but also selected over many generations to be successful within this environment, promoting so called “natural behaviours” may not be either desirable or indeed possible.

Studying the behaviour of groups of zebrafish was also slightly problematic, in part due to the small size of individuals. Furthermore, the stocking densities recommended by the OECD for use in regulatory studies proved to be much greater than those enabling successful behavioural analysis (guidelines recommend stocking densities 10-20 times greater than those used in the current PhD). In an ideal study I would have preferred to maintain all standards in accordance with OECD guidelines, for obvious comparison reasons. However, I found this not to be possible. Furthermore, despite this lower

stocking density used within the studies reported here, I still found it difficult to determine shoaling density in a completely acceptable way, as the small tank size meant that fish were relatively close together regardless of their position.

The greatest limitation to the behavioural analysis, however, involved the time constraints involved in observation and analysis. Ideally I would have liked to employ video-tracking software which could have analysed increased durations and volumes of video footage in a much shorter space of time. Unfortunately, extensive research into commercially available software proved to be unsuccessful, as the presence of glass rods and air stones in tanks, alongside the depth of water in experimental tanks (many software programmes require fish to be in a shallow depth of water) proved to be problematic. Further knowledge and investigation into this area would be of great assistance to this kind of work but was considered to be outside the remit of the current PhD. For this reason, manual behavioural scoring from watching video footage was deemed to be the best method for my purposes. Any problems associated with subjectivity were hopefully negated by the fact that all observations were conducted by a single researcher.

I also had a number of reservations regarding the use of cortisol as an indicator of stress or welfare. Commonly referred to as the “stress hormone”, cortisol is often viewed as an easily measured and reportable indicator regarding the welfare status of any individual. I have attempted to stress throughout this PhD, however, the importance of the cortisol response in any healthy animal and how the stimulation of this hormone is a suitable and normal response to a wide

range of stressors. Only when cortisol production becomes consistently elevated over prolonged periods does the response become maladaptive and the individual experience the negative consequences of this. This being said, I also feel that, in combination with other endpoints both physiological and behavioural, a measure of cortisol within an individual can provide us with useful information regarding its physiological status alongside knowledge of how it is responding to its environment.

A final limitation of this PhD is the number of enrichment types studied. Obviously, it would have been beneficial to test a maximum number of enrichment designs to determine their individual benefits and limitations. However, both time constraints and restrictions posed by regulatory guidelines meant that this was not possible. As I have mentioned throughout this thesis, my intentions were to look at types of enrichment that would be able to be used within regulatory toxicology studies. Therefore, I was very limited as to the types of physical additions we could place in experimental tanks. Furthermore, other types of enrichment, such as those pertaining to the social environment and feeding types/methods were not considered, again due to the limitations imposed by testing regulations.

6.3 Key issues and recommendations for future work

Research into the use of environmental enrichment as a way of improving welfare of captive fish is still in its infancy. Whilst there are various examples of studies looking at this subject (see section 1.5.1 for more details), few are

directed at fish that are used in the laboratory. The study conducted by Brydges and Braithwaite (2010) concerning the effects of enrichment on the behaviour of laboratory fish, and that by von Krogh *et al.* (2010) looking at the effects of enrichment on cell proliferation, behaviour and physiology in zebrafish appear to be the only papers currently addressing this topic. The review by Williams *et al*. (2010) is the only literature that focuses entirely on fish used in regulatory toxicology. This gap in the knowledge is understandable, as fish used in regulatory studies comprise only a small proportion of the overall numbers of fish used in research. However, this proportion is continually increasing and I believe that the data reported within this PhD will go some way towards filling this knowledge deficit. In this section I will attempt to summarise key recommendations based on this research.

Throughout this PhD I considered two types of physical enrichment. The first being glass rod structures of varying heights and the second being air stones of two different sizes providing different rates of air flow and water currents within the tanks. The glass structures, designed to provide spatial complexity and an element of refuge, did not produce any quantifiable benefits in unstressed fish and appeared to delay the formation of stable social hierarchies. When fish were stressed by a period of chasing, the presence of the glass rods appeared to reduce the magnitude of the cortisol response. Whilst this could be viewed as a potential benefit, we feel that it would not outweigh the costs of this type of enrichment.

The most obvious costs would be those relating to manufacture and maintenance (for example, structures would have to be cleaned regularly to prevent microbial build-up). Other substantial costs include the fact that results of regulatory studies utilising these enrichment structures may not be directly comparable to previous studies. This may be due to either subsequent alterations in fish physiology or through interference with the test chemical as a result of the greatly increased surface area within the tank. The combination of these factors lead me to suggest that the regulatory ecotoxicology community as a whole, both within and outside of the U.K., may not be amenable to such changes required in providing this type of enrichment. In the absence of strong and irrefutable evidence supporting benefits to the target species as a result of enrichment. it may be difficult to change either opinions or guidelines.

It is also worth mentioning that, although it is a common inclination to view a decreased stress response as a positive result (as I have done in this thesis to a certain extent), the purpose of regulatory studies are to assess the impact of chemicals on animals in a way that can be extrapolated to the natural environment. Within the natural environment fish would be subjected to stressors much more severe and frequent than those in the laboratory, and so an argument could be made that minimising stress within regulatory studies only serves to make these studies less environmentally relevant.

The second type of enrichment we looked at was the provision of airstones. Again, I found no clear evidence that fish in tanks with airstones experienced an improvement in their welfare. The main observation from this study, in fact, was

the vast increase in mortality in tanks containing these air stones, in particular, stones of a smaller size. I feel that, regardless of the physiological cause underlying this result, this can only be viewed as a negative consequence of the enrichment and one that appears to rule out airstones as an effective form of enrichment for this species and strain of fish. Particularly as these fish, maintained in conditions typical to the laboratory, do not require additional or artificial aeration to maintain adequate dissolved oxygen concentration.

This PhD looked at only a couple of forms of enrichment type. I would recommend that future work be aimed at testing a wider range of designs alongside a wider range of zebrafish strains. As mentioned previously, different strains show vastly different behavioural and physiological profiles and, therefore, their enrichment requirements may be similarly varied. It would also be of benefit to investigate further the use of video-tracking software to improve the speed and efficiency of behavioural analysis, as well as removing all problems associated with subjectivity.

Although this PhD has looked at only one species, the intention was that findings might be applied to other warm water species that are commonly used in the laboratory. Whilst we have stressed the problems of differing life history traits and behavioural/physiological characteristics of different species in developing a universally effective form of enrichment, it remains that the findings we have reported here may be useful for species other than the zebrafish. Other fish species, such as medaka, are of a similar size, form hierarchies and exhibit the same types of social behaviours as the zebrafish. It

would therefore be reasonable to assume that enrichment criteria might be similar for both of these species.

A second PhD that ran concurrently to this one was conducted by a fellow University of Exeter student, Jenny Landin, and was designed to similarly assess potential environmental enrichment strategies for rainbow trout. Studies looked at the effects of hides, opaque barriers and airstones on the welfare of juvenile trout and it was found that different enrichment designs had vastly different effects on welfare, and in particular, aggression. Whilst hides appeared to increase aggression and the subsequent stress levels of subordinate fish (due to dominant fish utilising these structures as a defendable resource), opaque barriers allowed subordinate fish a certain amount of refuge from dominant individuals and so appeared to reduce their stress levels and the amount of overall aggression observed. These results further support our theory that the particular behavioural and social features of different fish species will have a large influence on the types of enrichment that could be beneficial or detrimental.

Finally, I would suggest that researchers consider the possibility that laboratory zebrafish do not require the addition of environmental enrichment to tanks in order to promote maximum welfare. It remains a fact that these fish survive well in the laboratory and do not show a significant amount of anti-social or self-harmful behaviours. Similarly, mortality rates tend to be minimal and their overall health good. They are extremely easy to breed and maintain, this being one of the reasons for their popularity as a model species. It may be the case

that this species, and in particular the WIK strain, has been selectively and successfully bred for many generations for suitability to the laboratory environment and additions or alterations to this are not required.

CHAPTER 7

REFERENCES

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CHAPTER 8

APPENDIX

Environmental complexity as potential enrichment does not reduce stress in zebrafish but slows the establishment of social hierarchies

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Abstract

Enrichment of the living environment for captive animals is aimed at producing beneficial effects on the behaviour and physiology of relevant species, and is commonly used to reduce harmful social behaviours and stereotypies. However, little work has been undertaken to develop enrichment strategies for fish, in particular those used in regulatory toxicology where strict criteria regarding holding conditions and experimental design can make implementing such techniques problematic. Here, we studied the effect of vertical rod structures, designed to increase environmental complexity and provide refuge, on several commonly cited anxiety-related behaviours and whole-body levels of the stress hormone cortisol in juvenile zebrafish measured over a 1 week period. Activity levels and shoaling density showed no response to tank structures and fish did not spend a significantly greater or lesser amount of time in areas of tanks containing glass rods. Aggression remained high during days 1-5 in tanks containing glass structures before falling to a lower level by day 7. In control tanks, this lower level was reached by day 5 ($F_{3,72} = 2.37$, $p = 0.048$), suggesting that the glass structures may have affected the rate of establishment of dominant/subordinate relationships. Laboratory fish reared in a 'barren' environment may find that transfer to a complex environment makes social structure more difficult to enforce, and so leads to an extended period of increased aggression. Overall, whole-body cortisol levels of fish were comparable to those reported in unstressed zebrafish in other studies. Levels were significantly higher in both treatments after 24 hours than on subsequent days ($F_{3,68} = 6.12$, $p = 0.001$), most likely due to the handling stress of the initial

transfer to experimental tanks. However, cortisol levels did not vary significantly between control and structured tanks at any point during the study ($F_{1,68} = 2.28$, $p = 0.136$). These results indicate that the addition of glass rod structures as hypothesized enrichment, via added refuge and increased environmental complexity, did not result in a measurable improvement in welfare.

Keywords

Anxiety; Behaviour; Cortisol; Toxicology; Welfare

1.0 Introduction

Fish are increasingly used in research relating to their fundamental physiology and behaviour, but also biomedical and regulatory toxicology studies. Commitment to the principles of the 3Rs (Reduction, Replacement, Refinement) are continually being made to reduce the number of mammalian vertebrates used within research laboratories, and it is likely that one consequence of this will be that the numbers of fish being used will continue to rise in the coming years. In the United Kingdom (UK), the total number of vertebrates used in animal experiments is recorded in such a way that the numbers and class of vertebrates and the purpose for which the study was conducted can be obtained from the Home Office (http://www.homeoffice.gov.uk/). In 2010 more than twice as many fish were used than rats, and fish represented 13 % of all vertebrate studies. These numbers represent a 23% increase on the previous year which has increased year on year over the last decade. It is axiomatic that the welfare of fish is of importance, and this has led to specific legislation in the European Union (EU). In part, this is due to several studies detailing the potential for sentience, nociception and the perception of fear in fish (Braithwaite and Boulcott, 2007; Chandroo et al., 2004; Sneddon et al., 2003) and it is now thought that fish possess forebrains that are considerably more developed than previously assumed (Salas et al., 2006). However, reasons for concern relate to the potential effects of poor welfare on scientific validity and repeatability in experimental work (Williams et al., 2009). At present, fish used in the laboratory are commonly housed in barren tanks with only the addition of spawning substrate when required. Often the simple reason for this is to ensure that test

conditions between research locations are standardised (Olssen and Dahlborn, 2002). However, there are now widespread concerns that barren conditions may compromise the health of animals generally (Dawkins, 1998). For this reason, there is much interest in how environmental enrichment may be used to improve the welfare of fish used in the laboratory, and indeed some welfare organisations recommend enrichment for fish despite the lack of positive scientific evidence of benefits for the fish (Reed and Jennings, 2010).

Environmental enrichment is the alteration of a captive environment in a way that promotes a positive change in welfare of the animals within it (Olssen and Dahlborn, 2002). This can be through manipulation of the social, physical or chemical environment, and may encompass types and methods of food provision. Such enrichment can have beneficial cognitive effects in both healthy and diseased animals, and has been found to correspond with higher brain mass and cortical thickness relative to body size in studies with rats (Bennett et al., 1964; Diamond, 2001). Significantly higher relative brain mass has also been observed in wild salmon and rainbow trout, in comparison to their hatchery reared counterparts (Kihslinger and Nevitt, 2006; Marchetti and Nevitt, 2003, respectively) and this effect has also been seen in fish that are reared in enriched tanks in comparison to those reared in barren tanks (Kihslinger and Nevitt, 2006). There is also a wide range of data showing effects of enrichment on behavioural tasks involving spatial memory, anxiety, exploratory activity and aggressiveness, particularly in laboratory rodents (Amaral et al., 2008). As such, environmental enrichment is widely used to promote good welfare in farms, zoos and mammalian laboratory settings.

Very few studies have conducted research into the environmental preferences of fish. Types of enrichment techniques previously studied have included tank colour (Appelbaum and Kamler, 2000; Serra et al., 1999; Strand et al., 2007), addition of artificial vegetation (Basquill and Grant, 1998; Hamilton and Dill, 2002) and even music (Papoutsoglou et al., 2007). However, the huge diversity of fish species (more than 25,000 discovered so far; Nelson, 2006), their varied life histories and habitat preferences, means that results from such studies are often not applicable to species others than those directly investigated. Furthermore, the purpose of the research and interests of the various stakeholders will have a major effect on the type of enrichment that is appropriate. Within aquaculture, for example, the primary target will be to improve growth and overall condition of fish, while in public aquaria, visitor perception of the fish and its environment, along with reduction of negative behaviours such as aggression is often the main concern. For fish used in regulatory studies, for example to establish safe levels of contaminant chemicals in the environment, there is currently no information available regarding the application of specific enrichment techniques . However, Williams et al., (2009) have advocated appropriate species and life stage specific environmental enrichment including the physical parameters (temperature and light), relevant feeding techniques including live foods, water movement, con-specifics and co-culture as potential methods to increase welfare and enrichment of laboratory fish.

Within regulatory toxicology, the potential for tank enrichment is complicated by the prescriptive nature of regulatory guidelines and their strict criteria to ensure validity and international compatibility (http://puck.sourceoecd.org). In Europe, laboratory experiments with

vertebrates are regulated within the European Convention for the Protection of Vertebrate Animals used for experimental and other purposes (1986, ETS 123) which was updated via the new Directive 2010/63/EU (http://eur-lex.europa.eu). Within the UK, Home Office Inspectorate Guidance (ASPA, 1986) (and therefore licence to conduct scientific investigations with vertebrates) require that the fish are inspected regularly and must be clearly visible within the tank. This may not be appropriate for all species, those using cryptic camouflage may well be 'stressed' in an arena in which they can be clearly seen. For many types of regulatory-based toxicological testing using fish, the container vessel must be inert, efforts must be made to avoid excessive microbial growth, and behavioural observations must be conducted (for comparison with controls). These requirements are generally not compatible with the addition of objects to the tank, as increased cover (e.g. plants) can make observation difficult, increased surface area can lead to absorption of the test chemical and increased microbial load (potentially leading to chemical degradation) could make required regular cleaning ineffective, in addition to leaching of confounding chemicals into the test environment. Furthermore, any manipulation of the environment that results in alteration of the behaviour and/or physiology of test fish, while being beneficial in terms of welfare, may serve to change innate variability and potentially prevent useful comparison with previously published data. Several studies, for example, have shown that fish exhibiting different behavioural phenotypes can show dramatically different chemical uptake profiles (e.g. 20-fold differences in copper uptake between dominant and subordinate fish; Sloman et al., 2002). This variability could make it necessary to utilise more animals to attain comparable statistical power and would not be in accordance with current attempts to replace, refine and

reduce the use of animals in research (www.nc3rs.org.uk). Of course, a counter argument is that studies with a degree of behavioural/physiological variability in the fish may be more comparable to situations in the natural environment and so may increase the relevance of such studies. Several factors, therefore, present a perceived cost of environmental enrichment for laboratory fish. Some, such as the change in chemical nature of the water and potential interaction with the test chemical, are likely to challenge the validity of the overall test and so this must be balanced against the benefits in terms of improved welfare for the test fish. Unfortunately, little information is available to make a balanced scientific assessment of the value of the improved welfare of including an item in the test tank to improve welfare. There is an additional perceived constraint for regulatory testing in that the conditions in the husbandry and rearing of the fish should be the same as in the tests themselves. This is true for the quality of the water, and best practice applies this to the housing in most establishments.

There continues to be a drive from regulatory sources (e.g. EU 2010 and UK HO inspectorate and welfare organisations such as the RSPCA) that enrichment for fish is the same as for mammals. This is widely interpreted as objects being placed in the tank as enrichment for the fish as toys or “chews” are used with mammals. We are unable to identify any evidence from sound scientific study in the literature that would agree with this concept for fish. Although a precautionary principle should be applied, it should also be considered that some forms of enrichment could provide defendable territory and potentially harm the welfare rather than improve it.

The aim of the present study was to address this lack of knowledge concerning the potential welfare benefits of hypothesised environmental enrichments for zebrafish (*Danio rerio*) used in regulatory studies such as those described by the OECD (http://www.oecd.org). We therefore designed a simple form of physical enrichment consisting of vertical glass rods of varying heights within fish tanks to provide a degree of three-dimensional complexity and potential refuge. As zebrafish are generally found in areas of vegetation which provide cover from predators and microhabitats for spawning and foraging (Engeszer et al., 2007). Our hypothesis was that this type of artificial enrichment might approximate such vegetative cover, but in a way that would be most compatible with regulatory toxicology studies. The glass rods meet the inert requirement and appear to increase environmental complexity, but do have the drawback of increasing surface area and are difficult to clean *in situ*, but could be easily removed and replaced for daily cleaning. Behavioural parameters were examined in juvenile zebrafish during a 7 day exposure to either barren tanks or enriched tanks. Activity level, shoaling density, aggression and time spent in the bottom third of the tank were measured as these key behaviours have all been associated with stress and anxiety in zebrafish (Egan et al., 2009; Rehnberg and Smith, 1988). In addition to this we measured whole-body cortisol concentration. Cortisol is a hormone which has been well documented in many animals, primarily due to its increase in response to physical and environmental stress (for review: Wendelaar Bonga 1997; Mommsen et al., 1999).

2.0 Materials and methods

2.1 Subjects and housing

Zebrafish (Wild Indian Karyotype (WIK) strain) were bred at the AstraZeneca, Brixham Environmental Laboratories (Devon, U.K.) and kept under conditions compatible with OECD guidelines throughout (food, water composition, temperature, light etc.). Prior to and during the experiments, fish were kept in flow-through fresh water at 28 °C and under a photoperiod of 14L:10D (light:dark) with a 20 minute phased sunrise/sunset. From 4 days post-fertilisation (dpf) fish were fed daily to excess with ZM000 infusoria grade food (Special Diet Services, Essex UK), and live rotifers (2.5 ml daily at a concentration of 5000/ml) with the addition of live *Artemia* 24h nauplii at 10 dpf. From 21 dpf fish were led live *Artemia* and SDS 300 to excess daily. At the beginning of experiments fish were 35 dpf.

On day 1 of the study, 120 fish (wet body mass 0.18 ± 0.07 g) were transferred to 20 experimental glass tanks (i.e. 6 fish per tank) measuring 30 x 20 x 20 cm with a working capacity of 12 l. To ten of these tanks three structures were added that were designed to approximate the vertical stems of aquatic plants. Each of the three structures consisted of a glass base plate measuring 70 x 85 mm with twelve black opaque glass rods attached using aquarium silicone sealant (Dow Corning) in a 3 x 4 grid. Each structure within a tank had glass rods of different heights measuring 180 mm, 100 mm and 50 mm. Structures were positioned as shown in Figure 1. Placement of control and

structured tanks within the laboratory was determined randomly and adjacent sides of tanks were masked with black paper to ensure that fish in neighbouring tanks had no visual contact. To facilitate behavioural analysis, a 3 x 3 grid was drawn on the front and lid of each tank, resulting in 27 different 3-dimensional cells that could be occupied by fish (Fig 1). Water flow was 50 ± 1 ml/min/tank throughout the experiments providing six volume changes per day. Fish were fed twice daily, with live *Artemia* in the morning and SDS 300 dry food in the afternoon, following filming.

2.2 Experimental protocol

The experiment was repeated four times, lasting for either 1, 2, 4 or 7 days. At the end of each experiment fish were rapidly removed from tanks and terminated, and tanks allowed to flow through for a minimum of a day (six water changes) before the next batch of fish was added. All fish were killed humanely by an overdose of anaesthetic (100 mg/l of MS-222 buffered to pH 7 with $NaHCO_3$ and aerated for 30 minutes), and then snap frozen in liquid nitrogen. Whole-fish samples were stored at -80 °C until analysis.

2.2.1 Behavioural assays

Behavioural analysis was conducted for the 7 day study only, beginning on day 1 (when fish were introduced to experimental tanks), and on alternate

days until termination (*i.e.*, days 3, 5 and 7), with 10 minutes of digital video footage (Samsung VP-MX10) being recorded for each tank. During recording the camera operator was concealed from view behind a screen. All recording took place between 12:00 and 16:00 h (*i.e.*, 4 and 8 hours following simulated sunrise, respectively). To provide a 3-dimensional view of fish movements using a single camera, a mirror was secured above the tank at an angle of 45°. Videos were analysed between minutes 6 and 10 of each recording, and the first 5 minutes discounted to minimise effect of disturbance by the camera operator. From these videos, activity level, shoaling density, aggression, time spent in the bottom third of the tank and use of areas containing physical structures were quantified (playback of digital recording was performed using VLC media player 0.9.8a). Activity level was recorded as the total number of horizontal and vertical lines, viewed from the front of the tank, crossed within a minute. This was averaged for all fish within the tank and counts for all fish took place between minutes 6 and 7. Shoaling density was calculated using the 27 discrete 3-dimensional cells of water artificially defined by the grids on the front and lid of the tanks (Figure 1). The number of fish located in the cell containing the most individuals was recorded every 30 seconds over the 5 minute period, and these values averaged to give a mean value for the tank. This value is referred to as a shoaling index. Percentage time that each zebrafish spent in the lower third of the tank was also determined over this five minute period, and the mean value for the six fish calculated. Aggression was measured as the total number of aggressive advances made by all individuals in the tank over one minute (observed between minutes 6 and 7). An aggressive advance is defined as either a chase or a bite, with chases being one animal pursuing another over a distance of greater than two body lengths. If a chase continued

after the retreating animal changed direction this was counted as an additional aggressive interaction. To quantify use of space within the tank the nine 2-dimensional areas created by the 3 x 3 grid (viewed from the top of the tank, using the mirror) were numbered 1-9. In tanks containing glass structures, long, medium and short rods were located in grids 1, 5 and 9 respectively. The position of each fish was recorded every 30 seconds for five minutes and a total calculated for each grid.

2.2.2 Whole-body cortisol assay

The cortisol extraction procedure was modified from Ramsay et al. (2006). Briefly, whole zebrafish were thawed on ice, weighed and placed in individual 15 ml falcon tubes. Each fish was homogenised in 0.5 ml of cold phosphate buffered saline (PBS) for one minute using a bench-top homogeniser (Ultra-Turrax T25, F.T. Scientific Instruments Ltd., Bredon, U.K.) and the probe rinsed into the sample tube with a further 0.5 ml of PBS. Homogenised samples were vortexed briefly and maintained on ice. Diethyl ether (3 ml) was added to each sample, briefly vortexed then centrifuged at 500 × *g* (Sanyo Mistral 3000i) for 2 minutes to separate aqueous and diethyl ether layers. Samples were then placed in a -80 °C freezer for 15 minutes to freeze the aqueous layer, and the liquid diethyl ether layer was then poured into a clean test tube. This process was repeated with a further 3 ml diethyl ether, and then combined diethyl ether portions were dried under a gentle stream of nitrogen gas at room temperature for 2 hours. These were stored at -20 °C for a maximum of 48 hours. After thawing on ice, samples were reconstituted in 250 µl of assay buffer and

vortexed thoroughly before use. Cortisol concentrations were measured using a commercial enzyme-linked immunoassay (ELISA) kit (Assay Designs, U.K. 900-071). Cortisol levels were normalised based on the mass of the whole-body sample and reported as ng/g. Prior to use the kit was validated for use with zebrafish whole-body samples, with average linearity and recovery of spiked samples of 96 and 81% respectively.

2.3 Data analysis

All statistical analyses were performed using Minitab V15 (Minitab Inc.). Activity and shoaling density data were log transformed and data regarding time spent in areas containing structures was square root transformed to meet the assumptions of normality. Repeated measures analysis of variance (ANOVA) was used to detect any differences in activity level, shoaling density, time spent in the bottom third of the tank and total aggressive interactions between control and structured tanks over the four observation days. A Tukey post hoc test was used to determine where significant differences lay. One-way ANOVA was used to compare time spent in areas containing enrichment (or corresponding areas in control tank) between control and enriched tanks. For analysis of whole-body cortisol values ANOVA was used to detect any differences in cortisol between fish from structured and control tanks. In all cases, statistical significance was accepted at $p < 0.05$.

3.0 Results

3.1 Time-dependent behavioural responses to tank structures

Results of all behavioural endpoints are summarized in Table 1. There was no effect of treatment ($F_{1,68}$ = 0.24, p = 0.625) or observation day ($F_{3,68}$ = 0.98, p = 0.409) on activity, but there was a significant interaction effect ($F_{3,68}$ = 3.13, p < 0.031). There was also no effect of either treatment ($F_{1,68}$ = 0.44, p = 0.507) or observation day ($F_{3,68}$ = 1.54, p = 0.213) on the shoaling density. Typical shoaling size throughout the study was a maximum of 2 fish per cell (*i.e.*, one of the 27, 3-dimensional cells would typically contain two fish).

The observation day had a strong effect on the time spent by fish in the bottom third of the tank with fish from both treatments spending significantly less time in this lower zone on day 1 than on any of the following days ($F_{3,72}$ = 45.71, p < 0.001). On day 1, fish in structured tanks spent significantly more time (three times longer) in the bottom third than fish from control tanks that spent less than 8 % of their time there ($F_{1,18}$ = 28.03, p < 0.05). In contrast, after day 1, fish in both treatments spent the majority (50-60 %) of their time in this lower third of the tanks (Table 1).

There was a significant interaction effect of time and treatment on the level of aggression observed within tanks ($F_{3,72}$ = 2.37, p = 0.048). Aggression in control tanks was sustained at 5-7 acts per tank, per minute on days 1 and 3, but then dropped significantly to less than 3 acts per minute on days 5 and 7. Aggression in structured tanks was similarly high at the start compared to

controls (6-7 acts per minute) and remained at this high level on days 3 and 5 only dropping to a significantly lower level (<2 per minute) on day 7 (Fig. 2).

Neither treatment ($F_{1,68}$ = 0.07, p = 0.796) nor observation day ($F_{3,68}$ = 1.22, p = 0.308) had a significant effect on the amount of time spent in areas containing structures (or the corresponding areas within control tanks).

3.2 Whole Body Cortisol

Whole body cortisol concentrations were significantly higher on day one than any of the following sampling days ($F_{3, 68}$ = 6.12, p = 0.001). However, there was no effect of treatment at any time point ($F_{1, 68}$ = 2.28, p = 0.136) (Fig. 3).

4.0 Discussion

Within this study, we did not observe any differences in locomotory activity induced by inclusion of our hypothesised complex environmental enrichment or on different observation days. Increased activity level or locomotion is a behaviour that is associated with an anxiety response in fish (Gerlai et al., 2000). This has been observed in zebrafish exposed to drugs noted for their anxiogenic or anxiolytic properties, and indeed is a key behavioural endpoint in regulatory studies, as well as in wider research. For

example, zebrafish subjected to cocaine withdrawal, a documented source of anxiety both in fish as well as humans, show increased locomotion (López-Patiño et al., 2008). Zebrafish exposed to nicotine, which is commonly observed to attenuate anxiety in a variety of subjects, show reduced levels of activity (Levin et al., 2007). Locomotory activity has also been shown to change in response to other stressors. Blaser and Gerlai (2006) found that zebrafish introduced to a novel tank showed increased activity, and the same response was also seen for fish that were monitored for aggression towards a mirror image. Both of these situations were expected to cause the fish a degree of stress. Similarly, McFarlane et al. (2004) showed increased locomotion in rainbow trout that were subjected to a crowding stress, and anecdotal observations of other species of laboratory fish suggest that this is a typical fish response to stressors generally.

There are several scenarios that might explain why we observed no differences in activity level on the days of observation. Firstly, it is possible that the physical structures simply had no effect on this behaviour. The primary intention of our treatment design was to provide a refuge and therefore a less "stressful" or "risky" environment than the control (barren) tanks. However, this does not imply that control tanks were necessarily stressful or anxiogenic, and definitely not to the same extent as the toxicant exposures or crowding reported by others (discussed above). The addition of a proposed enrichment, therefore, may not have changed the anxiety levels of fish at all, or at least not sufficiently that it was reflected in their locomotion rate. Another option we must consider relates to the way we analysed our data. To avoid pseudoreplication, activity levels of all fish within one tank were averaged. Several studies have observed that both innate activity levels and anxiety-related responses of zebrafish vary

greatly between individuals, with some showing “freezing” and others more erratic movements (López-Patiño et al., 2008). Similarly, natural variations in behaviours relating to dominance and subordinance are common (Larson et al., 2006). Focusing on the average of all activity levels within one tank would, therefore, explicitly miss this level of information and allow us to test the effect of the treatment. However, there were no significant differences in either standard deviation ($F_{(7,29)} = 0.746$, $p = 0.51$) or maximum range ($F_{(7,29)} = 0.910$, $p = 0.512$) of individual activity levels between tanks (data not shown).

In our study, shoaling cohesion did not vary between treatments or with time. Other laboratory studies have observed that “tightness” of a shoal varies in response to perceived risk and anxiety (Egan et al., 2009; Rehnberg and Smith, 1988). This is an adaptive response which, in the wild, would confer increased protection from predators (Speedie and Gerlai, 2008). When transferred to a new environment, we would expect fish to swim closer together and that this increase in cohesion would diminish over time (Miller and Gerlai, 2007). Our study did not confirm this hypothesis, indeed we show that shoal density did not change over the seven day period. However, it may be that the initial cohesion could be present over very short time periods and if this lasted less than 24 hours then it would not have been detected by our experimental design. Further, we predicted that if the presence of glass structures did provide a refuge, this could decrease the amount of time taken for shoaling density to return to former levels. Similar to our results reported here, Miller and Gerlai (2007) found that shoal cohesion in zebrafish was constant, both upon introduction to a novel environment and following a period of habituation. They suggested the possibility that shoaling density is insensitive to novelty (as opposed to more significant and overtly life-threatening stressors such as

predation), or that longer periods of exposure to a novel environment would be required to see an effect. In that study the observation tank they used, being white and devoid of hiding places, probably remained aversive to the fish throughout the experiment. Within our study it is possible that the size of the tanks did not allow us to observe patterns of shoaling as effectively as we would have been able with bigger tanks, we can only speculate on effects at higher densities. However these tanks were four times larger, and a stock density 12 times lower than typically required for an OECD study, but were chosen to allow effective observation and fish interaction.

Despite being regarded as a shoaling fish, zebrafish also show high levels of aggression, both in males and females (Moretz et al., 2007). Upon initial introduction a new group of fish will typically show high levels of aggression which gradually decline over several days as stable dominant/subordinate relationships are formed (Larson et al., 2006). This pattern was supported by the results of the present study, but contrary to our expectations and hypothesis, it appeared that the presence of the physical structures increased the amount of time taken for aggression to decline (Fig 2). There have been conflicting results found regarding the effects of tank complexity on aggression in fish. In a study by Kelley and co workers (2006) butterfly splitfins (*Ameca splendens*) demonstrated higher levels of aggression in structured tanks, where dominant fish used enrichment to establish territorial boundaries. A similar finding was reported by Mikheev et al. (2005) in a study providing sections of pipe to juvenile perch (*Perca fluviatilis*). Both of these studies highlight the need for enrichment to be provided on a species specific and scientifically valid basis. Conversely, Basquill and Grant (1998) found that aggression was reduced in zebrafish in a complex habitat containing simulated

vegetation compared to a simple one. This was thought to be related to the difficulty of defending such a habitat, and the affect of the vegetation on visibility, a hypothesis supported by Höjesjö and Johnsson (2004) in a study looking at the growth of dominant zebrafish in complex and simple environments. These studies provided the basis for our hypothesis of providing an enriching complex environment for zebrafish, but seem a little at odds with our findings. Such differences between studies may be due to the design and placement of proposed physical enrichments. The simulated vegetation used by Basquill and Grant (1998) allowed visual isolation of individuals. The design and location of enrichment used by Kelley et al. (2006) and Mikheev et al. (2005), however, allowed no such visual isolation but instead permitted monopolisation by dominants. This highlights an important consideration concerning the design of enrichment, and shows that different types of tank structure can have vastly different effects on the behaviours of different species. Our glass rods were intended to provide a refuge for the shoal (Fig 1). However, they may have allowed subordinate fish to escape from dominant individuals more easily and it is possible that this increased the amount of aggressive interactions required for dominant/subordinate relationships to be established (Fig 2). It is also possible that dominant fish did use structures as a way of establishing territories. However, these explanations can only be speculative, as analysis of the social hierarchy and relationship between specific individuals was not possible within the present study.

Within this study, the artificial structures had an affect on the time spent in the bottom of the tank on day one only, where fish from enriched tanks spent more time in the bottom third than those in barren tanks (Table 1). However, in all tanks fish spent a much lower proportion of time in the bottom third on the

first observation day in comparison to all other days. The amount of time away from the surface of the tank (or nearer to the bottom) is another frequently cited measure of fear and anxiety in zebrafish (Blaser and Gerlai, 2006; Egan et al., 2009; Gerlai et al., 2000) and is reduced significantly by exposure to either alcohol and nicotine, both of which have anxiety-reducing effects in many species, including zebrafish (Gerlai et al., 2000 and Levin et al., 2007 respectively). Similarly, López-Patiño et al. (2008) found that fish showing stereotypies also move closer to the bottom of the tank, similar to the manner reported as anxiety-like behaviour in zebrafish. Blaser and Gerlai (2006) found that zebrafish introduced to a novel tank initially spent a large proportion of time in the bottom third of the water column and over time, use of the upper water increased. If the physical structures used in the present study were providing a refuge, and thus reduced the perceived risk of the novel environment, we would have predicted fish to utilise the upper water column at an earlier point than fish in barren tanks. Our results do not support this hypothesis and in fact show the reverse of that reported by other studies, i.e. they spent more time in the bottom third as time progressed, and more time near the bottom during on the first day in the structured tanks compared to barren tanks. At this point we are unable to say why this result is different from that observed previously. A possible explanation is that fish were initially displaying exploratory behaviour of the novel environment and this behaviour then decreased in the following days. Another hypothesis might be that fish were responding to their own reflection, which was clearly visible on the bottom of the tank, typical of the laboratory senario.

Time spent by fish in the areas of tank containing glass rods, and the corresponding areas within control tanks did not differ significantly between

treatments or observation days (Table 1). Again, there are several possible explanations for this result, the first being simply that the fish had no preference for the areas containing the rods. It is also possible that the size of the tank meant that fish were relatively near to the structures at any time, regardless of their position in the tank. The standard lengths of fish averaged 24 ± 4 mm, and so all individuals were within a maximum of 2 body lengths of the nearest rod structure at all times. Finally, the rods may not have been "realistic" enough. In a study by Delaney et al. (2002), zebrafish spent 99 % of their time in areas containing plastic vegetation which, in anthropogenic terms, more closely resembled natural plant matter than did our glass rods. In the Delaney study the aquarium was an attempt to provide a mesocosm-type environment to closely match wild habitat. Our intention was to increase the environmental complexity using materials compatible with regulatory toxicology studies, rather than mimic plants, hence our design and colour choice, with the overall aim to address the hypothesis that increased environmental complexity reduces stress.

The concentration of cortisol measured in fish within the present study is within the same range as recorded in other studies with zebrafish (Egan et al., 2009; Pottinger and Calder, 1995) although it is lower than that cited in some studies (Barcellos et al., 2007; Ramsay et al., 2006). Cortisol was significantly higher on day one than on any of the subsequent days (Fig. 3). This was most likely because fish were stressed from the transfer to experimental tanks, which has been documented as a significant source of stress affecting laboratory fish (Pottinger and Calder, 1995). On days 2, 4 and 7, sufficient time had passed for the cortisol concentration to return to a lower level (Fig. 3). Importantly there was no difference in whole-body cortisol concentration between fish in control

and enriched tanks at any of the time points measured, indicating that the presence of the enrichment rods did not appear to either reduce or increase the stress levels of the fish via this simplistic measurement.

Although it is widely accepted that increased environmental complexity can improve the welfare of captive animals, we have not found this to be reflected in the behaviours or whole-body cortisol levels of juvenile zebrafish provided with simple physical structures. The presence of the vertical glass rods had no affect on activity level or shoaling density and fish did not spend a greater amount of time in areas of tanks containing these physical features. It did, however, increase the amount of time taken for dominant/subordinate relationships to be established and for levels of aggression to decrease. This in itself may be viewed as a negative consequence. It is generally deemed to be undesirable to have a situation where subordinate individuals have limited means of escape, and hence one of the main drivers for enrichment is the anthropogenic view that we should provide a place of refuge. However, provision of these structures (as refuge) resulted in prolonged aggression, which was the opposite of the intended effect. Our enrichment also had no effect on whole-body cortisol levels which, aside from day one when levels were increased following transfer to experimental tanks, remained low throughout all days and treatments. Therefore there is a little tension between the simple physiological measure of stress (cortisol) and the behavioural measures. In this case it appears that behaviour is the more sensitive endpoint.

A limitation of the conclusions from our present study is that all behavioural measurements and terminal sampling occurred at times when disturbance of the fish was kept to a minimum. As the nature of the

hypothesised enrichment was to provide fish with a form of refuge, it is possible that potential benefits may only be apparent when fish are stimulated or feel threatened by external factors (i.e., not just within the context of a normal social hierarchy between con-specifics). We would suggest, therefore, that further studies look at the effect of enrichment on the response of fish to typical laboratory stressors. It would also be of benefit to look at a wider variety of enrichment types before the inclusion of anthropomorphically perceived enrichment is applied without scientifically robust evidence of benefit.

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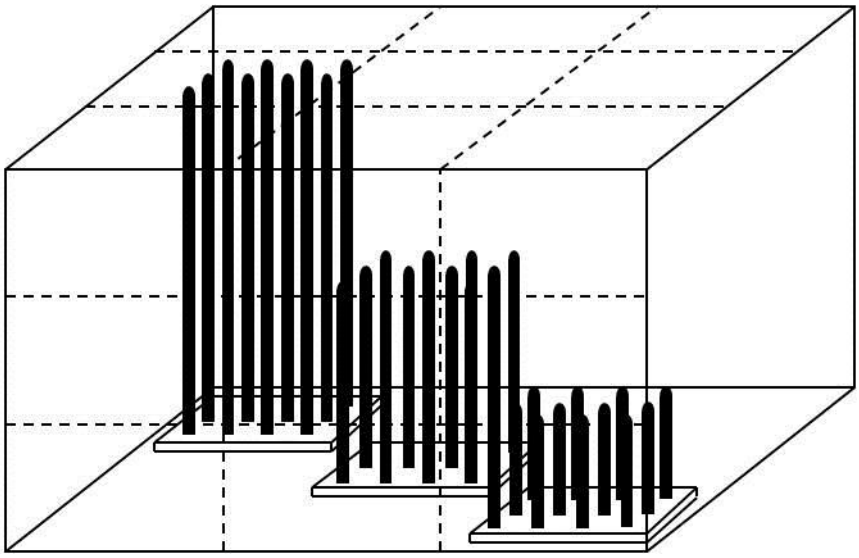

Figure 1. Front view of the experimental tanks containing three glass rod structures of different heights. Dotted lines indicate division of tank for behavioural analysis.

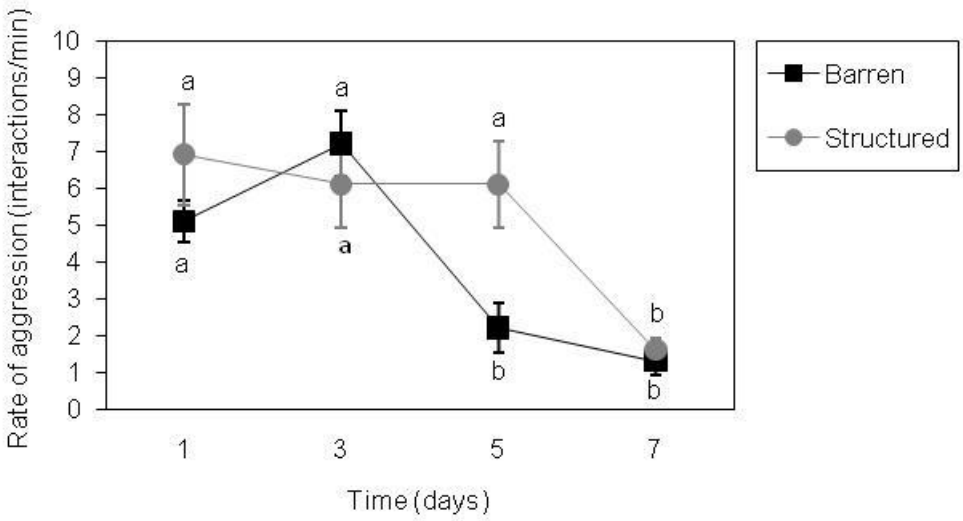

Figure 2. Mean number of aggressive interactions per minute in tanks of fish at four time periods (n = 10). Error bars represent one S.E. and different letters indicate a significant difference of $P < 0.05$.

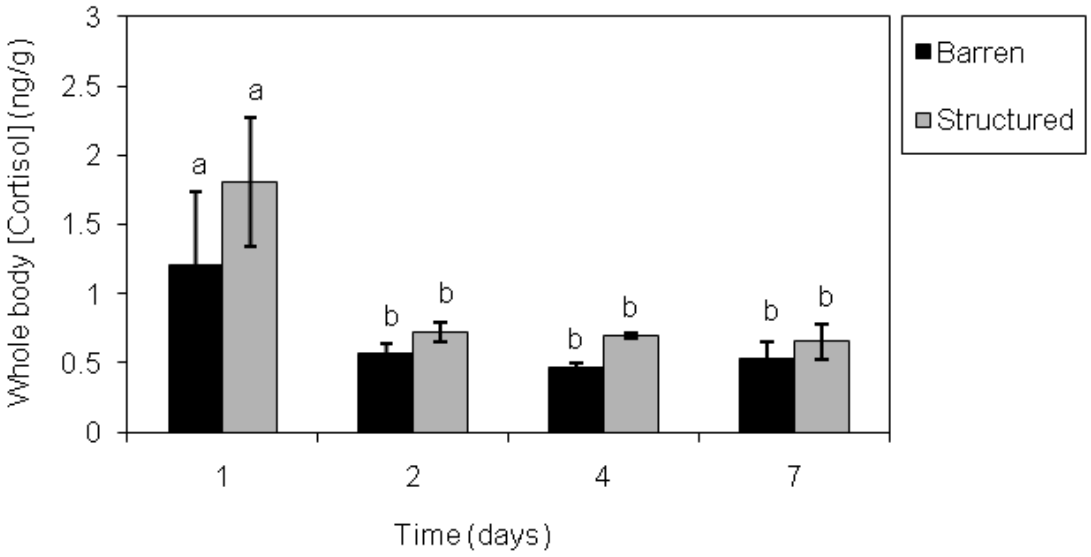

Figure 3. Mean whole-body cortisol concentrations of fish at four time periods. Error bars represent one S.E. and different letters indicate a significant difference between treatments of $P < 0.05$.

Table 1. Effects of glass rod structures on zebrafish behaviours. Data are presented as mean ± S.E.

	Day 1		Day 3		Day 5		Day 7	
	Control	Structured	Control	Structured	Control	Structured	Control	Structured
Activity	18.35 ± 0.48	17.39 ± 0.52	20.33 ± 0.93	16.90 ± 1.47	17.21 ± 0.63	20.90 ± 1.40	17.04 ± 0.93	17.71 ± 2.15
Shoaling density	2.21 ± 0.08	2.27 ± 0.07	2.12 ± 0.10	2.04 ± 0.10	2.30 ± 0.18	2.14 ± 0.10	2.38 ± 0.18	2.30 ± 0.13
% time in bottom third of tank	7.73 ± 1.08	24.14 ± 2.90	55.88 ± 1.58	59.07 ± 4.75	53.18 ± 3.79	52.16 ± 5.41	53.18 ± 6.46	60.52 ± 5.32
% time in grid containing short rods	12.63 ± 2.16	14.54 ± 2.51	11.96 ± 2.36	14.09 ± 3.15	9.94 ± 3.04	14.24 ± 3.44	11.46 ± 3.63	12.27 ± 2.91
% time in grid containing medium rods	9.60 ± 1.56	9.24 ± 2.28	11.30 ± 1.78	10.90 ± 1.94	9.60 ± 1.92	8.93 ± 2.04	9.27 ± 1.62	6.50 ± 1.60
% time in grid containing long rods	10.28 ± 1.91	15.00 ± 3.05	11.46 ± 1.74	11.37 ± 2.15	15.32 ± 1.74	11.37 ± 2.15	12.30 ± 1.48	9.39 ± 2.78