USING PHYSIOLOGY AND BEHAVIOUR TO ASSESS ENRICHMENT STRATEGIES FOR THE WELFARE OF RAINBOW TROUT

Submitted by

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to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, February 2012

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.

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ACKNOWLEDGEMENTS

There are so many people that have made this PhD come together, but without funding no PhD, so thank you Great Western Research and AstraZeneca. Additionally, a thank you to The Society for Experimental Biology for the extra funding which financed my trip to Sweden.

A very grateful Thank You to: Dr. Rod Wilson (University of Exeter), my primary supervisor, for supplying constructive criticism, discussing fish welfare, and various aspects of life. Dr. Stewart Owen (AstraZeneca), my second supervisor who freely supported me with his 'golden nuggets' and repeatedly told me to keep going and to trust myself. Professor Svante Winberg (Uppsala University, Sweden) and his lab members Jossan, Hanna, Tobias, Dean, P-O, Houner, and David, for making me feel very welcome, and for helping me analyse 'my' brains. Dr. Katherine Sloman (University of the West of Scotland) and Dr. Lisa Leaver (University of Exeter) who both generously helped with brain storming, ELISA, statistical as well as general support. Thanks Gareth Readman and Alan Sharpe (AstraZeneca) for enrichment ideas and statistical help, and Tim Williams (AstraZeneca) for wider project support.

Many thanks for technical support and sampling help: Jan Shears, Michael Wetherell, Chris Cooper, Jonathan Whittamore, Luanne Wilkes, Charlie Hazlerigg, Jeff Murua, Tessa Scown, Anke Lange, Jenna Corcoran, Eliane De-Bastos, Marta Söffker, Tamsyn Uren-Webster, Sulayman Mourabit, with many many others offering to help. I hope I didn't forget anyone, if I did I'm so sorry, a great thanks to you too.

On a private note, you who always truly stand by me, you know who you are, Family and Friends, 'the special ones', what would I do without you? Tack!

My final thank you and love goes solely to Albin and Anton, världens bästa!

ABSTRACT

There is an increasing scientific acceptance that fish may feel some sort of fear, pain and distress, which in turn feeds a growing concern for their welfare. Humans impact the wellbeing of a large number of fish in various ways, one of them being through research. Welfare legislation in the UK demand welfare considerations for all animals used in scientific procedures. Furthermore, welfare and enrichment needs for fish are included in the Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes. As fish are extensively used in research, changing their housing and husbandry to improve welfare is of importance, since fish kept in laboratories are most likely subjected to impoverished environments. Although enrichment programs have been shown to improve health and welfare in various animal species, little is known of their potential for application to juvenile rainbow trout. How best to improve barren experimental tanks for female juvenile rainbow trout used in regulatory research was the broad aim of this PhD.

In this thesis, three enrichment strategies for rainbow trout have been examined, using physiological and behavioural welfare indicators. The first study assessed the effects of semitransparent shelters on trout welfare, and a clear message became evident; that shelters of this design should not be considered enrichment for rainbow trout as they had several significant negative impacts, indicating chronic stress in fish from shelter tanks relative to fish in a barren environment. The second study investigated impacts of reduced visual access to conspecifics in the same tank. Habitats with low visual contact between individuals have been suggested to reduce aggression for a range of species, and I have shown that visual barriers appeared to be beneficial to trout as well. The final experiment evaluated effects of high and low water currents on the wellbeing of rainbow trout, and results indicated increased fish welfare when water currents were supplied.

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LIST OF ABBREVIATIONS

3 R's	Replace, Reduce, Refine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
ANOVA	ANalysis Of VAriance
ASPA	Animals Scientific Procedures Act
BCA	Bicinchoninic acid
CF	Condition factor
DA	Dopamine
DBHA	Dihydroxybenzylamine
DOPAC	3, 4-dihydroxyphenylacetic acid
EDTA	Ethylenediamineteraacetic acid
ELISA	Enzyme linked immunosorbentassay
EU	European Union
HO	Home Office
HPA	Hypothalamus-pituitary-adrenal (axis)
HPI	Hypothalamus-pituitary-interrenal (axis)
HPLC	High Performance Liquid Chromatography
HSI	Hepatosomatic index
IASP	International Association for the Study of Pain
L-DOPA	3, 4-dihydroxyphenylalanin
MS222	Tricaine methane sulphonate
NA	Noradrenaline
OECD	Organisation for Economic Co-operation and Development
PCA	Perchloric acid
RIA	Radioimmuno assay

LIST OF ABBREVIATIONS

RSPCA	The Royal Society for the Prevention of Cruelty to Animals
SGR	Specific Growth Rate
UFAW	Universities Federation for Animal Welfare
UK	United Kingdom

LIST OF SPECIES NAMES

Species name	Common name
Aequidens pulcher	Blue acara
Anolis carolinensis	The Carolina anole arboreal lizard
Anolis aeneus	Bronze Anole lizard
Ameca splendens	Butterfly splitfin
Astatotilapia burtoni	African cichlid fish
Babesia microti	Protozoan causing ‘Texas fever’
Carassius auratus	Goldfish
Ceruus elaphus	Red deer
Clarias gariepinus Burchell	African catfish
Cyprinus carpio	Common carp
Danio rerio	Zebrafish
Epinephelus coioides	Grouper
Esox masquinongy	Muskellunge
Gadus morhua	Atlantic cod
Gasterosteus aculeatus	Three-spined stickleback
Geophagus brasiliensis	Pearl cichlid
Hippoglossus hippoglossus	Atlantic halibut
Ictalurus punctatus	Catfish
Oncorhynchus kisutch	Coho salmon
Oncorhynchus mykiss	Rainbow trout
Oryzias latipes	Japanese medaka
Pimephales promelas	Fathead minnow
Salmo salar	Atlantic salmon
Salmo trutta	Brown trout

LIST OF SPECIES NAMES

Species name	Common name
Salvelinus alpinus	Arctic charr
Sander vitreus	Walleye
Xiphophorus birchmanni	Swordtail fish

CHAPTER 1: General introduction

1.1 Animal behaviour

Ethology, the scientific study of animal behaviour (Monaghan and Wood-Gush, 1990) has made significant contributions to disciplines such as the study of human behaviour (Zetterström, 2007), neurosciences (van der Staay, 2006) and animal welfare (Millman et al., 2004). The first modern ethologist is believed to have been Charles Darwin who in 'The expression of the emotions in man and animals' (1872) lists feelings such as fear, anger, and affection, that he argued are expressed and experienced by animals (Dawkins, 2006a). Today his work is regarded as one of the starting points for modern behavioural studies (Zetterström, 2007).

In 1965 the Brambell report brought into focus the importance of studying behaviour as a tool to assess animal welfare (Appleby and Hughes, 2003). Behaviour is an animal's way to change in accordance with the environment to ultimately ensure survival. Hence, behavioural endpoints are important tools to identify needs, preferences, internal states, and to study the effects of exposure to stressors (Kane et al., 2004; Sherwin, 2007). Behavioural assays are some of the most easily observed indicators of animal welfare, they are non invasive, inexpensive and possibly more holistic than other methods (Peakall, 1996; Clotfelter et al., 2004). Additionally, the same general approach can be applied to many different species (Zala and Penn, 2004). However, environmental circumstances, species, and even strain will influence the expression of an animal's 'normal' behaviour, hence information regarding species specific, individual and social group behaviour is vital, when using it as a tool for assessing welfare (Appleby and Hughes, 2003).

1.2 Animal Welfare

The discipline of animal welfare is not new and it includes the study of ethology, physiology, genetics, evolution, neuroscience, cognitive science and consciousness (Dawkins, 2006b). In order to fully grasp this discipline it is essential to have an understanding of its history and

different definitions. Moreover, animal welfare cannot be discussed without considering the question ‘Can animals suffer?’ This topic has been covered at a later stage within this chapter, but to begin with I will review important milestones that significantly influence the discipline of animal welfare in today’s society.

In 1954 the founder of the Universities Federation for Animal Welfare (UFAW) advocated to scientifically investigate the use of humane techniques in laboratory animal experiments (Zurlo et al., 1996). Russell and Burch carried out the research and published ‘The Principals of Humane Experimental Technique’ in 1959 (Russell and Burch, 1959). In this publication they systematised practices known as the three R’s using the headings: Replacement, Reduction, and Refinement. Today the 3 R’s are the foundation of humane use of animals in research (Schluppi and Fraser, 2005), and anyone planning to use animals in science is required to prove there is no other alternative and that actions are in place to minimise suffering and numbers used. Though, to what degree this is implemented may vary between countries.

- Replace animals with alternative techniques to avoid use of animals all together if possible.
- Reduce number of animals used to a minimum.
- Refine experimental conduct to ensure as little suffering as possible, which includes better housing, improvement of procedures, and to improve animal welfare.

Furthermore, laws imposing replacement, reduction, and refinement alternatives in scientific research have been passed since the mid 80’s in the European Union as well as the United States (Zurlo et al., 1996).

Following Russell and Burch, Ruth Harrisson published the book ‘Animal Machines’ in 1964 in which she questioned the welfare of intensively farmed animals. This urged the British government to investigate the welfare of animals in intensive livestock husbandry and

consequently in 1965 the Brambell Committee was formed. The Brambell Committee produced a report that was refined into a framework called the 'Five Freedoms' of animal welfare which reads as follows (Webster, 2001; Appleby and Hughes, 2003):

An animal should experience

1) Freedom from thirst, hunger and malnutrition – by ready access to fresh water and a diet to maintain full health and vigour.

2) Freedom from discomfort – by providing a suitable environment including shelter and a comfortable resting area.

3) Freedom from pain, injury and disease – by prevention or rapid diagnosis and treatment.

4) Freedom to express normal behaviour – by providing sufficient space, proper facilities and company of the animal's own kind.

5) Freedom from fear and distress – by ensuring conditions which avoid mental suffering.

The aim of the Five Freedoms is to prevent suffering, and this framework strongly influences how we view animal welfare in today's society (Webster, 2001). The Five Freedoms is an acknowledged framework for assessing suffering in terrestrial animals, and it has been suggested that their objective can by the same token apply to farmed fish (Cooke, 2001). In the 1980s the European Union adopted Directives to protect animal welfare and issued recommendations such as, more opportunity for social contacts, balanced diet, and enriched environment (Veissier et al., 2008). Present animal welfare regulations in the United Kingdom demand welfare considerations for all animals used in scientific procedures (Animals Scientific Procedures Act, ASPA,1986), and legislations makes it an offence to cause or allow livestock to suffer unnecessary pain or distress (Ellis et al., 2002). Additionally, welfare as well as enrichment requirements for fish are included in Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (European Council, 2006).

Animal welfare is a very complex concept and much time and effort have gone into defining it (Fraser, 1995; Huntingford, 2006). Though there are several different views of animal welfare most definitions fall into one of the three following categories reviewed by Huntingford (2006):

1) *Feeling based* definitions with focus on subjective psychological states. Good welfare means the animal should feel well, and not be subjected to negative experiences, instead have positive ones.

2) *Function based* definitions have the basis in an animal's adaptability to its environment. Good welfare entails the animal to have good health and for its biological systems to function well.

3) *Nature based* definitions are founded upon the belief that every species has an inbuilt biological nature that it must express. Animals should therefore be able to express 'natural' behaviours and lead a 'natural' life in order for good welfare to take place.

Neither of these definitions is more right or wrong than the other, they solely convey different ideas about what we should pay attention to when dealing with the subject of animal welfare (Huntingford, 2006).

1.3 How to assess animal welfare

It is difficult in its own right to study the subjective state of another being, but this is even further complicated by animals having different senses and motivations to humans (Sherwin, 2007). Consequently, a major issue that animal welfare scientists face, is how to evaluate animal welfare (Dawkins, 2006b). Since animals cannot convey how they feel, indirect methods such as measurement of stress hormones or abnormal behaviour patterns are used to assess their wellbeing (Matheson, 2008). Researchers apply a wide range of

different methods to evaluate welfare and have generally opted for either of two approaches, take the sum of many different measures such as behavioural, physical, and physiological endpoints, or to focus on two questions ‘are the animals healthy?’ and ‘do they have what they want?’ (Dawkins, 2006b). In this PhD I have applied a range of physiological, physical and behavioural endpoints commonly used as welfare indicators, in order to establish enrichment criteria for juvenile rainbow trout (*Oncorhynchus mykiss*) used in regulatory research.

Poor physical health, such as injury, deformity, and diseases are reasonably easy to identify and quantify. Rushen (2003) advocates that there is an advantage of using health problems as tools to evaluate welfare since the relationship to suffering is obvious and more readily validated. On the other hand, Huntingford (2006) raised an important point that in the case of fish, the link between health and welfare is complex and it is crude to assume that disease is the outcome of poor living conditions or mismanagement since even fish well cared for may suffer from disease. Additionally, a healthy animal may have poor welfare due to boredom.

Physiological tools as well as behavioural indicators are used to assess an animal’s wellbeing. Autonomic responses such as heart rate and hormone levels (for example corticosteroids) are commonly used to measure animal welfare (Dawkins, 2006b). Cardiac change is frequently used as a welfare indicator in terrestrial farm animals, with the assumption that increased heart rate indicates increased stress levels (Maros et al., 2008). However, measures such as heart rate, heart rate variability, and respiration can be difficult to relate to welfare since changes in these variables can be induced by positive as well as negative activities (Dawkins, 2006b). Increased heart rate could be a sign of activity or expectation of punishing or rewarding stimuli (Paul, 2005). For example heart rate variability and heart rate in dogs significantly increase when the dog is oriented towards a favourite toy or petted by a stranger (Maros et al., 2008). Similar to cardiac measurements, cortisol

measurements are not easily interpreted since changes may occur in both fearful situations as well as for example a reaction to sexual activity (Paul, 2005).

Cortisol is the primary corticosteroid secreted by the adrenal system in teleost fish mainly in response to acute but also chronic stress (Mommsen et al., 1999). Cortisol concentration in blood, plasma, tissue, and holding water, has been used to monitor stress in fish (Ellis et al., 2004). It is important to do so as chronic stress may have important consequences upon animal welfare. In comparison to plasma cortisol, the activity of brain monoamines such as serotonin, change more slowly but are more permanent and not reversed under chronic stressful circumstances (Øverli et al., 2007b). Monoaminergic systems play a crucial role in linking behaviour and physiology (Sloman et al., 2005), hence brain monoamine levels is another tool to assess behavioural states and animal welfare. The monoamine neurotransmitter serotonin (5-Hydroxytryptamine, 5-HT) is essential for maintaining normal emotional and cognitive processes. One of the most characteristic effects of social stress in fish is a continuous increase in metabolism and release of 5-HT (Øverli et al., 2004a). However, a range of stressors may cause increased serotonin metabolism and release in teleost fish (Øverli et al., 2007b).

Exposure to chronic stress may compromise an animal's wellbeing, hence monitoring stress responses could give us vital information regarding animal welfare (Huntingford, 2006), and stress management is key in improving animal well being (Conte, 2004). However, it is important to acknowledge that physiological stress is not necessarily synonymous with suffering (Huntingford, 2006). For example, temporary physiological activation that prepares an animal for activity is not automatically detrimental to its welfare.

We are not able to comprehend animal emotions using only autonomic responses, therefore behavioural studies providing an indication of the animal's perspective in a non-invasive

manner are vital (Dawkins, 2006b). Behavioural studies are important in welfare research since altered behaviour is an early and easily observed response to poor conditions (Huntingford, 2006). Reduced food intake can for example indicate that the animal is not well before it develops clinical symptoms (Dawkins, 2006b). However, it is advisable not to consider behaviour in isolation, instead use it as a predictor and result of internal and external biological processes (Scott and Sloman, 2004). It is important to note that species, strain, test conditions, and the environment may influence animal behaviour (Paul et al., 2005). Information regarding behaviour of each species is therefore of great importance when using behaviour as a welfare indicator (Appleby and Hughes, 2003).

As presented, there are many methods available to assess animal welfare, but there are limitations to conclusions made by any one measure. Deductions regarding animal welfare are strengthened if based on more than one measurement. The use of a combination of measures, such as behavioural and physiological, is more likely to produce a more multifaceted representation of animal welfare (Paul et al., 2005)

1.4 Fish welfare

The concern for animals under human care has increased, and continues to grow (Broom, 2007; Dawkins, 2006b). However, people generally do not sympathise with fish as strongly as they do with other vertebrates, and as a result fish are not as well protected, particularly when compared to mammals and birds (Huntingford et al., 2006). This is a concern as humans' impact fish welfare on a large scale through research, aquaculture, sport fishing, or keeping them as pets. It is impossible to apply the same welfare guidelines for other animal species to fish, due to significant differences in their fundamental biology and environmental requirements. It is also unlikely that many welfare criteria can be applied from one fish species to another. Hence, it is of great importance to gain a deeper understanding of fish welfare and develop species specific guidelines for monitoring their wellbeing (Huntingford et al., 2006).

An apparently healthy fish in an adequate environment does not equal good fish welfare (Johansen et al., 2006). Johansen and colleagues (2006), thoroughly reviewed health and welfare guidelines for fish used in research, highlighted the fact that fish are often not included in national animal welfare laws. However, there is some published information upon this subject; the Fisheries Society of the British Isles issued a review with criteria for fish welfare, and care guidelines for fish used in research are available at http://oslovet.veths.no/fish (Johansen et al., 2006). The most recent and comprehensive framework covering care and use of fish in research, teaching and testing was presented by the Canadian Council on Animal Care. This is available at http://www.ccac.ca (Johansen et al., 2006). Nevertheless, recent knowledge regarding the functioning of the brain and nervous system has shown that the abilities and functioning of animals are more multifaceted than previously assumed. Hence, a reassessment of animal protection as well as care guidelines is needed (Broom, 2007).

As mentioned previously cortisol may have severe damaging effects upon fish as it has been reported to negatively affect growth, sexual maturation, reproduction, and immunological functioning, hence stress levels are important welfare indicators (Ellis et al., 2004; Wysocki et al., 2006. Fish react to stress with a sequence of adaptive neuroendocrine responses jointly termed as the stress response, and this has been extensively studied (Brown, 1993; Flik et al., 2002; Handy, 2003). There are two general responses to stress. The adrenergic response, in which stress induces the production of adrenaline and noradrenaline (catecholamines) in the chromaffin tissue (Broom, 2007). This prepares the animal for exercise by raised ventilation rate, cardiac output, blood flow to muscles, in addition to mobilising substrates for aerobic metabolism (Handy, 2003). The neuroendocrine stress response is the other stress response, mediated by the hypothalamic-pituitary-interrenal (HPI) axis (Huntingford et al., 2006). The HPI axis response is nearly identical to the mammalian hypothalamic–pituitary–adrenal axis (HPA) response (Broom, 2007). The hypothalamus discharge the corticotrophic releasing hormone, which results in the release of

adrenocorticotrophic hormone from the pituitary which in its turn affects the interrenal tissue and glucocorticosteroids (cortisol and corticosterone) are produced (Huntingford et al., 2006). Increased secretion of glucocorticosteroids from the hypothalamo-pituitary-adrenal axis into the blood and raised activity of the sympathetic branch of the autonomic nervous system are the most frequently examined physiological responses to acute stress (Appleby and Hughes, 2003).

Cortisol levels in fish have been proven to respond to a wide range of acute and chronic stressors, and it is an indicator of the primary physiological stress response of fish (Ellis et al., 2007). One of the functions of elevated cortisol is to mobilise energy to cope with stress (Barcellos, 2007). In the short term, increased cortisol levels are beneficial, stimulating the release of red cells and gill ion uptake (Handy, 2003). However, there are several harmful consequences of chronic activation of the stress response, such as immunosuppression, loss of appetite, impaired growth and wasting of muscles (Huntingford et al., 2006). Cortisol is therefore accepted as an important indicator of fish stress and has been included in fish welfare research (Turnbull et al., 2005; North et al., 2006). In stress research, measurements of cortisol are also important due to the fact that they require relatively easy techniques, and because cortisol shows a graduated response in relation to the stressor. A couple of hours after the exposure to a brief stressor cortisol concentrations may return to pre-stress levels (Waring et al., 1992), but elevated levels commonly persist for the duration of continuous stress (Pottinger et al., 1994). The physiological stress response will also depend upon the type, intensity and duration of the stressor (Appleby and Hughes, 2003).

Since stress can instigate behavioural changes and forced behavioural changes may cause stress, information regarding behaviour relevant to individual species is essential in fish welfare (Conte, 2004). Fish behaviours known to be influenced by stress comprise swimming performance, thermoregulation, orientation, avoidance, chemoreception, feeding, predator evasion and learning (Schreck et al., 1997). Conte (2004) argues that if fish change

behaviour, loses interest in feeding, or display lethargic or irregular swimming, it could indicate adverse conditions, stress, distress or pathogenic state. In addition to this there are a number of other fairly easily measured welfare indicators such as colour change, ventilation rate, skin and body condition, morphological abnormalities, injury, fin damage, reproductive performance, and heart rate that can be used when evaluating fish welfare (Huntingford et al., 2006; Johansen et al., 2006).

Given that animal welfare is concerned with the quality of life of a sentient being the subject of fish consciousness must be addressed (Chandroo et al., 2004b). The ability of fish to experience pain and distress is still being debated (Rose, 2002; Chandroo et al., 2004a; Chandroo et al., 2004b). Many researchers agree with Huntingford (2006) that even though fish are different from mammals and birds, they are still complex animals, and whether it is possible to support or dismiss their cognitive ability most researchers are in agreement with Johansen et al., (2006) that when information is lacking fish should be given the benefit of the doubt.

1.5 Can fish feel pain?

Investigating whether fish are conscious beings or if they can feel pain was not the purpose of this PhD. Nevertheless, it inevitably ties in with the subject of fish welfare since a key issue in any discussion regarding animal welfare is the potential for animal cognition (Yue et al., 2004; Broom, 2007). Hence, a brief review covering this subject is presented below.

There are a range of definitions of consciousness but researchers generally agree that consciousness refers to a mental state of awareness of internal and external stimuli (Chandroo et al., 2004b). Proof of animal cognition can be gathered through investigation of 1) brain function and anatomy, 2) flexible behaviour, 3) the ability to suffer (Dawkins, 2006b). Comparing brain physiology and functions of humans and animals appears to be the most

direct way of assessing animal cognition (Dawkins, 2006b). The neocortex which is important in generating subjective feelings in humans, is lacking in non-mammalian animals and fish, hence it is argued that fish cannot suffer (Rose, 2002). However, studies have proven that in fish the telencephalon contains the emotional system for conditioning of fear (Portavella et al., 2003) and avoidance learning (Overmier and Papini, 1986). This indicates that reactions to pain are not solely reflex but that higher brain centres are involved in learning, memory and emotion, which backs the argument that fish may have the capacity to perceive pain (Dunlop, 2006). Additionally, comparing brain function and anatomy might not be a conclusive way to investigate this issue since it has been shown that people with no cerebral cortex may have grand intellectual ability (Broom, 2007).

The second line of evidence for consciousness is based upon learning. If an animal is able to learn much information, and make few errors once performing learnt behaviours, it is more likely to be considered sentient (Broom, 2007). Fish have long been regarded as instinctive animals driven by reflex (Dunlop, 2006). However, it is acknowledged that the learning ability demonstrated in a range of different fish species indicates cognitive processes more complex than associative learning (Broom, 2007). There is a wide array of different examples proving complex behaviours and evidence of learning in fish, but to go into detail is beyond the scope of this review. Hence, just a few examples have been highlighted:

1. Fish have mental representations of their environment which they use for navigation (Burt de Perera and Guilford, 2008), and learning and memory in fish play key roles in complex behaviours such as foraging activities (Brown et al., 2006).

2. Many fish species interact in social groups and are able to recognise individual companions (Swaney et al., 2001; Spence and Smith 2007; Ward et al., 2007).

3. Dunlop et al., (2006) showed that fish learn to avoid a painful stimulus based on spatial cues and show evidence of spatial memory. Whilst Yue et al., (2004) suggest that trout can experience fear and are able to learn to avoid frightening stimuli.

The third line of evidence focus on pain and suffering (Dawkins, 2006b). Whether and to what extent animals can experience pain and suffering is an unresolved issue central to the study of animal welfare (Huntingford et al., 2006). Suffering includes a range of emotional conditions such as fear, boredom, and pain (Johansen et al., 2006). Suffering is a problematic issue since it is based upon an individual's subjective state which is inaccessible to anyone else, and for that reason it is advisable to apply the benefit of the doubt (Barnard, 2007). It may be even more difficult to assess pain and suffering in fish, since fish showing signs of weakness would potentially be attacked by predators, so the evolutionary process has favored behaviour that does not show illnesses and suffering (Johansen et al., 2006).

Pain and suffering in animals are likely to be different to the human perception of pain, but using definitions for human pain facilitates our understanding of animal pain (Kent and Molony, http://www.vet.ed.ac.uk/animalpain/). Even though pain is probably perceived differently, it most likely serves the same purpose in animals as it does for humans. Pain in humans is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, http://www.iasp-pain.org). Whether fish experience pain or not has been extensively debated (Rose, 2002; Braithwaite and Huntingford, 2004; Chandroo et al., 2004a; Broom, 2007). On one hand it is argued that due to the lack of neocortex in fish, the occurrence of fear and pain is impossible and responses to noxious stimuli are reflexive and not associated with a negative feeling (Rose, 2002). On the other hand it is believed that fish are able to suffer because in addition to showing avoidance behaviour to noxious stimuli they satisfy vital anatomical and physiological factors similar to mammals (Yue et al., 2004). Nerves that pass pain information in humans have

been found in rainbow trout (Sneddon, 2002; Sneddon and Gentle, 2002). Their receptors are located on the head, lips, and operculum and have the same physiological properties as mammalian nociceptors (Sneddon, 2003a). Hence, these findings support the notion of nociception and potentially detection of pain in rainbow trout. Moreover, Sneddon et al., (2003b) showed that when painful substances were injected into the lips of rainbow trout, behaviour and respiration rate changed in a way that could signify suffering, and the results of this painful stimulation were alleviated when fish were administered morphine. Learning research also suggests pain awareness in fish. Dunlop and co-workers (2006) showed that fish rapidly learn to avoid certain parts of a tank if an electric shock is delivered to the fish when it enters a specific part of the aquarium, and learning improved with stimulus intensity.

In 1987 The Institute of Medical Ethics put forward the following specific criteria to determine whether animals feel pain or not (Dunlop et al., 2006):

1) 'the animal responds to a noxious stimulus by avoidance or minimising damage'
2) 'the avoidance response is inelastic in that it always occurs'
3) 'the animal learns pain association'

According to these criteria fish qualify as capable of feeling pain. However, the key issue regarding pain in fish is whether pain perception involves an emotional experience, or if it is a sensory experience (Dunlop et al., 2006). Many researchers believe there is enough evidence to back fish as sentient beings. Still, even if we are unable to prove this we should give them the benefit of the doubt. We have been wrong before regarding the ability of animals and humans to perceive pain, so why not learn from this to avoid the possibility of it happening again.

1.6 Behaviour linked with physiology

Brain monoaminergic systems play essential roles linking behaviour and physiology (Sloman et al., 2005). There is such a tight linkage between neuroendocrine signal systems controlling physiological, emotional, and behavioural responses, that hardly any behaviours or emotions could occur without concurrent physiological activation (Øverli et al., 2006). Behaviours such as feeding (Øverli et al., 1998), locomotor activity (Genot et al., 1984) and social interactions (Winberg et al., 1996) are influenced by brain monoamines. Alterations in brain monoaminergic activity cause a range of behavioural outcomes by social interactions in fish (Winberg et al., 1991a).

Social defeat is a recognised powerful stressor for several fish species, and it may radically change an individual's physiology and behaviour (Øverli et al., 2004b), with serotonin noradrenaline and dopamine being key actors in regulating agonistic behaviour in animals. Subordinate fish commonly experience reduced feeding (Øverli et al., 1999) and aggression levels, as well as lower; growth rates (Pottinger and Pickering, 1992), body condition, hepatic energy reserves (Sloman et al., 2001b). Whilst having higher cortisol levels (Pottinger and Pickering, 1992) and metabolic rate (Sloman et al., 2000b). Dominant fish hold better positions in the environment, gain more food and generally exhibit aggression towards subordinate fish (Gilmour et al., 2005).

One of the most distinctive effects of social stress is a sustained increase in the metabolism and release of serotonin (Øverli et al., 2004a). Subordinate individuals experiencing continual stress demonstrate a chronic activation of the HPI axis as well as the brain serotonergic system (Winberg et al., 2007), whilst individuals that win fights for social dominance exhibit a fast down regulation of the serotonin response (Øverli et al., 1999). Thus, subordinate fish have significantly higher serotonin turnover in comparison to dominant individuals which is reflected by an increased concentration of its metabolite 5-

hydroxyindoleacetic acid (5-HIAA) and raised [5-HIAA]:[5-HT] ratios in the telencephalon, hypothalamus and brain stem (Winberg et al., 1997).

In contrast to serotonin, the catecholaminergic systems seem to be involved with increased aggression and dominant status (Winberg et al., 1992), but results are somewhat contrasting. McIntyre et al., (1979) showed that dominant individuals of rainbow trout had lower brain noradrenaline and higher dopamine concentrations than subordinate fish, and subordinate fish most frequently attacked had the greatest decrease in dopamine concentrations. Whilst Bell et al., (2007) found that both noradrenaline and the metabolite of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), were positively associated with aggressiveness in sticklebacks (*Gasterosteus aculeatus*). However, in Blue acara (*Aequidens pulcher*) and *Anolis carolinensis* (the Carolina anole arboreal lizard) dopamine systems have an inhibitory effect on agonistic behaviour (Höglund et al., 2005a).

Beside brain monoamines, cortisol and neuropeptides controlling the HPI axis also appear to vitally influence behaviours associated with social experience (Winberg et al., 2007a). For example, trout that react to stress with low plasma cortisol response are more successful in dyadic fights for social dominance when they encounter a conspecific who responds with a larger plasma cortisol increase (Winberg et al., 2007). It has also been shown that short term treatment with exogenous cortisol stimulates locomotor activity in rainbow trout, whilst the opposite holds true for long term treatment, which also inhibits aggressive behaviour (Øverli et al., 2007a).

1.7 Regulatory work

The usage of pharmaceuticals is a part of the western world which is not likely to change, hence it is essential to know their potential environmental effects. Concerns about effects of human pharmaceuticals (many non biodegradable) upon the environment was raised when their remains from treated sewage discharges were detected in surface waters (Laville et al.,

2004; Brown et al., 2007). Additionally, several fish and amphibian species have comparable target molecules that the pharmaceuticals were intended to interact with in humans (Brown et al., 2007), hence pharmacological interactions in non-target species is likely. This has been proven in different studies, with one of the most thoroughly documented being the feminization of male fish exposed to synthetic oral contraceptives through urban effluents (World Health Organization, 2002).

In the UK, environmental safety evaluation of chemicals is a regulatory requirement, and toxicity tests using fish are essential in order to estimate possible undesirable effects of discharged substances (Williams and Readman, 2009). As mentioned previously, all scientific procedures using animals are controlled under the Animals Scientific Procedures Act [1986], which covers key objectives concerned with improving the care and welfare of laboratory animals, and all establishments conducting scientific procedures are required to implement the 3 R's (Williams and Readman, 2009). Moreover, fish welfare as well as enrichment requirements are included in Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (European Council, 2006).

1.8 Environmental enrichment

Fish used in research are generally kept in barren tanks lacking in variation and possibly stimuli. A barren environment for free range hens has been identified as a key reason for poor use of the paddock, and aggressive behaviour is negatively correlated to foraging and exploratory behaviours (Nagle and Glatz, 2012). Hence, in addition to possibly increased aggressive behaviours, fish may also be subjected to several chronic stressors such as unfavourable water quality, high stocking densities, and handling, all of which could have detrimental effects on fish welfare (Huntingford et al., 2006). Obviously, there is a need for enriching laboratory environments.

Environmental enrichment has various definitions but the emphasis is generally on the provision of a stimulating environment (Williams and Readman, 2009). A range of enrichment alternatives have been reported to be available for application for aquaria in zoos (American Association of Zoo Keepers, 2008). It is important to acknowledge that fish welfare may be affected by conditions both above and below water, and that different species are very variable with respect to biological and ecological preferences. That is why enrichment strategies optimal for one species may not be beneficial for another. Additionally, environmental enrichment should not only meet the animal's needs but also be practical, inexpensive, and pose no risk to humans or animals, nor interfere with experiments. Thus the starting point should be adequate knowledge of a species natural history and for what purpose the fish are being used. Species information can then be used to select enrichment strategies that are feasible and behaviourally relevant for the animal under consideration. Some variables that could be considered as environmental enrichment have been reviewed:

1) Tank characteristics. There is a range of colours, materials and shapes that are utilised to construct fish tanks. Certain species of larval fish capture live food more efficiently when provided with a dark as opposed to a lighter background, and several studies have shown that the interior colour of tanks and the reflective nature of light often act as stressors affecting fish (Papoutsoglou et al., 2000; Rotllant et al., 2003). For example, a light environment compared to dark leads to higher levels of aggression in Arctic charr (*Salvelinus alpinus*) (Höglund et al., 2002).

2) Tank water flow also affect fish in various ways. Depending upon species and hydrodynamic conditions, fish may be attracted to or repelled by water currents (Liao, 2007). Water currents have been documented both to increase as well as decrease locomotory cost in different species (Liao, 2007). Water flow may also affect aggressive behaviour since studies have shown that salmonid fish, maintaining dominance hierarchies, show increased aggression and dominance in slow water (Williams and Readman, 2009). For example,

Arctic charr display less aggression when forced to swim at moderate speeds, i.e. 1-2 body lengths (Christiansen and Jobling, 1990; Adams et al., 1995). However, in contrast there are several fish species such as the fathead minnow (*Pimephales promelas*) that prefer static or low flowing waters (Williams and Readman, 2009).

In recirculating systems, which is a common practise, research has shown that aggression between fish is much higher in comparison to flow through systems, which possibly is due to an accumulation of olfactory cues from subordinate fish (Griffiths and Armstrong, 2000).

3) There is a wide range of feed and ways of distributing food that could be viewed as enrichment. The use of live feed may encourage fish foraging behaviour and hence activate the animal to a larger degree (Williams and Readman, 2009). Another aspect possibly viewed as enrichment is supplementing the feed, since it has been shown that dietary supplementation with L-tryptophan (serotonin precursor), suppressed aggression in juvenile rainbow trout (Winberg et al., 2001) and juvenile Atlantic cod (*Gadus morhua*) (Höglund et al., 2005b). It also reduces cannibalism in juvenile grouper (*Epinephelus coioides*) (Hseu et al., 2003).

4) Fish growth, physiology and potentially welfare may be affected by the light spectrum, which can be easily manipulated with little cost (Karakatsouli et al., 2008). Usage of light spectrum may be a useful tool to lower adverse effects of stress in fish. A study evaluating effects of coloured light on growth performance and stress response to confinement in rainbow trout showed that fish reared under blue light had smaller elevation of cortisol induced by acute stress, and mobilisation of liver lipids was absent in comparison to trout bred under white and red light (Karakatsouli et al., 2008). Light intensity may also cause stress and affect fish behaviour (Staffan, 2004; Strand et al., 2007). Almazan-Rueda et al., (2005) assigned higher activity displayed by the African catfish (*Clarias gariepinus Burchell*) in 150 lx compared to 15 lx, to be a stress reaction. In addition, Staffan (2004) found that

high light intensities (2200lx) in comparison to 16 and 200 lx increased the swimming activity of juvenile perch, which may be an indication of stress.

5) Equipment such as pumps, aerators, filtration systems etc. can produce underwater sounds and frequencies within the range of fish hearing (Davidson et al., 2007). Exposure to constant elevated noise could negatively affect fish welfare. Stress responses have been reported in goldfish (*Carassius auratus*) exposed to broadband white noise (Smith et al., 2004), and in common carp (*Cyprinus carpio*) experiencing boat noise (Wysocki et al., 2005).

6) For species that normally live in shoals social enrichment is of importance (Sloman and Armstrong, 2002). Harwood et al., (2002) showed that when brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) were held in sympatry the presence of another species reduced the intensity of aggressive interactions within a species.

Another aspect of social interaction is housing of single sexed stocks. Male brood stock of rainbow trout are more competitive if females are present (Conte, 2004) which could have welfare implications. Hence, in some aquaculture facilities males are located upstream of female pens, preventing female pheromones from initiating aggressive behaviour amongst male trout (Conte, 2004).

Environmental enrichment is widely believed to be beneficial to animal welfare, with evidence of welfare improvements from behavioural and physiological endpoints (Bateson and Matheson, 2007). Enrichment is supposed to enhance animal welfare, hence it is essential to evaluate whether it provides benefits or not, or if it even has negative effects. Not only is it of importance to determine that enrichment strategies do not have detrimental effects, but some of the proposed enrichment strategies are also inappropriate for certain experiments such as the addition of plastic plants which may release chemicals capable of

interfering with toxicology experiments. Hence, in order to overcome this problem all enrichments used in the studies presented in this thesis were made from chemically inert substances.

It is generally assumed that increased complexity and stimuli brings wellbeing to animals. However, Barnard and co-workers (1996) studied the effects of adding nest boxes and shelving on the testosterone/ immunity trade-off in male mice (*Mus musculus*), and found that mice in enriched cages were significantly more aggressive and less resistant to a *B. microti* infection than mice from barren environments. Additionally, Kelley et al., (2006) revealed that male butterfly splitfins (*Ameca splendens*) were more aggressive in structured environments than in unstructured ones. On the contrary, studies on brown trout (Sundbaum and Näslund, 1998), zebrafish (*Danio rerio*) (Basquill and Grant, 1998) and rainbow trout (Imre et al., 2002) have shown decreased aggression between individuals with increased habitat complexity. Environmental enrichment may not only reduce aggression but even promote co-habituation which was shown for the pearl cichlid (*Geophagus brasiliensis*) (Kadry and Barreto, 2010).

Increased habitat complexity may minimise visual contact between competitors as well as prey and predator fish held in neighbouring tanks, which in its turn may improve fish welfare. It has been shown that visual contact of a predator elicits typical neuroendocrine stress responses in zebrafish (Barcellos, 2007), goldfish, sticklebacks (Bell et al., 2007) and in swordtail fish (*Xiphophorus birchmanni*) (Coleman and Rosenthal, 2006). However, this may be species specific since walleye juveniles (*Sander vitreus*) showed no signs of stress after experiencing visual contact to its predator *Esox masquinongy* (Czesny et al., 2003). Increased habitat complexity has also been shown to improve habitat usage and animal activity. For example laboratory mice have been shown to be much less active and more restricted in their habitat use when they had little ground level structure and no overhead cover, while making much wider use of structurally complex habitats (Jensen et al., 2003),

which could imply better welfare. Clearly, more information is required on whether fish welfare can be improved by environmentally enriching stimuli and how to provide for the needs of fish (Broom, 2007).

1.9 Study species, rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792)

The focus of this thesis has been on rainbow trout since not only is it commonly used in regulatory research but it is also a recommended species in the guidelines by the Organisation for Economic Co-operation and Development (OECD) (http://www.oecd-ilibrary.org/environment/test-no-215-fish-juvenile-growth-test_9789264070202-en).

Additionally, rainbow trout are found in laboratories world wide as it is an established study species, and it is the dominant freshwater salmonid fish being farmed in Europe and North America (Ellis et al., 2002).

I do not intend to cover a description of the life cycle of rainbow trout, but I want to emphasise some important characteristics and traits particular to this species. Rainbow trout is a stream dwelling teleost species more solitary and territorial than social in its nature. However, when in groups they form strong dominance hierarchies both in the wild and when kept in small groups in the laboratory (Winberg and Lepage, 1998; Øverli et al., 1999; Larson et al., 2004), and aggression is a normal part of their behavioural repertoire which may cause serious welfare issues. Wild rainbow trout show aggressive behaviour to defend a territory, and although farmed rainbow trout generally are referred to as domesticated, behaviours that occur in the wild persist under culture conditions (Ellis et al., 2002). There is adequate support backing the fact that aggressive behaviour in experimental groups of rainbow trout can cause injury and even mortality. Although aggression is not the only source of stress to subordinate individuals, stressed trout can suppress normal immune function and hence increase susceptibility to diseases (Ellis et al., 2002). A confined laboratory environment where there are no means of escaping cohabitants may therefore be detrimental to individuals of this species.

1.10 Aim of the study

Since there is a continuous drive from regulatory sources (e.g. EU, UK Home Office inspectorate) and welfare organisations that enrichment for fish is the same as for mammals, and there is a wide range of variables that could be altered in order to improve fish welfare, it is vital to determine what type of environmental enrichment actually improve welfare. The aim of this PhD was therefore to investigate whether welfare can be improved in female juvenile rainbow trout kept in a laboratory environment, by the addition of enriching stimuli, and if so how to best provide this and objectively evaluate potential effects. I wish to highlight the fact that findings from these studies only apply to this species and more so to juvenile female rainbow trout.

Three different types of enrichment strategies were tested and all studies applied strictly to the 215-OECD guideline (http://www.oecd-ilibrary.org/environment/test-no-215-fish-juvenile-growth-test_9789264070202-en). The first study evaluated the effects of addition of semitransparent shelters to test tanks, upon physiological and behavioural welfare indicators in female juvenile rainbow trout. It is feasible to assume that the addition of overhead shelters could benefit fish welfare, as animals avoid open spaces due to predation risk. For example, foraging rodents have been showed to prefer areas with overhead cover when moving away from nest sites (Jensen et al., 2003), and the house mouse (*Mus domesticus*) prefer areas with even small amount of complexity to open space (Gray et al., 2000). Shelters were semitransparent in order to comply with Home Office code of practise, i.e. to have a clear view of experimental animals in test tanks (this does not apply to stock fish), which is applicable to all the three studies in this thesis, see the two following links for reference;

(http://tna.europarchive.org/20100408115128/http://scienceandresearch.homeoffice.gov.uk/animal-research/publications-and-reference/publications/code-ofpractice/code_of_practice_part1/index043d.html?view=Standard&pubID=428573)

(http://tna.europarchive.org/20100408120136/http://scienceandresearch.homeoffice.gov.uk/animal-research/publications-and-reference/publications/code-of-practice/code_of_practice_part2/hadcb312835.pdf?view=Binary)

It was hypothesised that the shelters could act as physical barriers and refuges for subordinates from aggressive individuals, and hence possibly increase overall tank welfare. Alternatively, cause increased aggression and potentially stress, if they were perceived as defendable resources by dominant individuals, i.e. decrease welfare through raised territorial behaviour.

In the second study visual barriers were added to test tanks and the same range of physiological and behavioural welfare indicators were studied. It was hypothesised that visual barriers could enable subordinate individuals to get out of sight from their aggressors, and potentially decrease overall tank aggression and increase fish welfare. The third study focused upon the application of water currents into test tanks. The addition of certain types of water flow could potentially affect welfare amongst rainbow trout in both a positive or negative way, as a pilot study showed that high water currents caused fish to exhibit significantly less overall aggression compared to both no and low current tanks. Additionally, there was a weak trend towards low water current tanks having higher numbers of aggressive acts than no water flow.

A range of stress indicators were analysed for all three studies, primary physiological stress responses were brain monoamines and plasma cortisol levels, secondary response was plasma ion concentrations, and tertiary responses; specific growth rates, condition factor, hepatosomatic index and fin damage. Behaviourally, aggression, swimming behaviour, as well as usage of enrichment were studied through extensive video footage.

CHAPTER 2: General material and methods

2.1 Source and maintenance of rainbow trout

Juvenile female rainbow trout of approximately 3 g in mass were supplied for the first two studies from Houghton Springs Fish Farm (Dorset), whilst fish in the third enrichment study were supplied by Exmoor Fisheries (Devon). All studies were undertaken at the Hatherly Laboratories, University of Exeter. Fish were kept indoors in holding tanks continuously supplied with dechlorinated tap water at + 10.5 ± 0.5 ˚C under a light dark regime of 13L:11D for 14 days before studies commenced. The light was switched on at 07.00 and off at 20.00. Trout were handfed with commercial trout pellets (Ecostart, BioFocus 17 by BioMar A/S) at 3 % of their body mass per day, which is a 215-OECD guideline request. The daily feed was divided into two portions and delivered at 10.00 and 17.00 hours and uneaten food was siphoned 30 minutes after each feeding occasion. These temperature, light, feeding and cleaning regimes were maintained throughout the holding and study periods, which was 28 days for all three experiments.

2.2 Experimental exposures of rainbow trout

Fish were quickly anaesthetised in a tricaine methane sulphonate solution (MS222, 200 mg/l, Pharmaq Ltd.) buffered with sodium bicarbonate ($NaHCO_3$), which had been aerated prior to use to ensure normal carbon dioxide and pH levels. On day 0 of the studies trout were freeze branded for individual recognition. This was viewed as the most humane way to brand fish due to their lack of cold nociceptors (personal communication, Lynne Sneddon, University of Chester and Liverpool). They were also measured for mass and fork length, and scored for fin damage before being randomly allocated to test tanks. No adverse effects of anaesthetic or marking were seen and all fish recovered quickly. Individuals entered tanks in succession, hence fish number one was first in and so forth. Each experimental tank contained eight juvenile rainbow trout visually isolated from other tanks by black laminated cards attached to tank sides, and from people entering the room by matt white plastic

curtains hanging in front of the tanks. The colour of the curtains matched the colour of the walls in the room. Tanks had no aeration, but biomass, loading and flow rates were set to ensure that dissolved oxygen concentration never fell below 80 % (measured minimum = 82.8 %), generally oxygen levels were above 90 %. Water flow through the test tanks was controlled by the use of a header tank which contained fully aerated water. The water level in the header tank was kept at constant level to ensure even and regular water pressure and flow rates to experimental tanks. The water inlet was at the front of the tanks, and over flow tubing was inserted at the rear feeding directly into the drain to avoid splashing of the tank, a factor which possibly could affect fish behaviour and space use. A flow rate of 6 l/g of fish/day was applied to the projected maximum final mass of the fish, and the initial loading rate was 1.0 g/l. To ensure sufficient water quality, measurements of temperature, pH, and oxygen were taken twice per week, and hardness once per week throughout the 28 experimental days.

On day 14, fish were measured for length and mass and the enrichment strategy was implemented in study one and two, study three had a slightly different set up. Information for each study has been supplied in detail in the Methods and Materials sections of respective study (section 3.2.2, 4.2.2, and 5.2.2).

On day 28 and 29, all fish were sampled and each tank was netted within seconds in one go from the experimental tank and transferred to an anaesthetic solution. Fish were terminated within 60 seconds by an overdose of buffered MS222 (240 mg/ l), and body measurements, blood and tissue samples were obtained. Blood samples were collected from the caudal vessels into syringes, fish were then killed by spinal section, weighed, and measured. Each syringe was pre-treated with sodium heparin 5,000 I.U./ ml as an anticoagulant (Monoparin, Wockhardt UK Ltd), and the time from netting to obtaining the final blood sample was less than 4 minutes. Syringes were kept on ice and blood samples were transferred to

microcentrifuge tubes and centrifuged at 4 ˚C, 16,000 *x g* for 5 minutes (Biofuge fresco, Heraeus, Hanau Germany). Following centrifugation, if plasma volumes allowed, 10 µl of plasma was diluted in 10 ml of ultrapure water with a quality of >18 MΩ (Maxima Ultrapure Water, ELGA) for ion analysis and stored at -20 ˚C, whilst remaining plasma was stored at -80 ˚C for cortisol analysis. Tissues such as the telencephalon, optic tectum, hypothalamus and brain stem as well as livers were dissected rapidly and transferred to labelled microcentrifuge tubes before being snap frozen in liquid nitrogen and stored at –80 ˚C. All work was carried out under a UK Home Office animal care license.

2.3 Behavioural endpoints

For all the three studies, tanks were filmed before the accustomed morning and afternoon feed and footage was analysed for three types of aggressive interactions according to Höglund et al., (2000) with slight modifications.

1) Approach/ attack: one fish approaches another with open mouth and flared operculum in high or low speed.
2) Nip/ bite: one fish nips or bites the other.
3) Chase: an approach brings about a flight response, and the attacking fish follows the escaping fish for a distance of more than three body lengths.

Behavioural scoring was validated by analysing the same footage repeatedly over time in a random fashion. For a large part of the footage it was not possible to establish whether or not the aggressor made physical contact with the receiver, hence all aggressive acts except chase were combined. In each 10 or 15 minute footage, the most dominant individual was noted on the basis of number of aggressive acts made and received, and position held in the social group. The initiator and recipient of each aggressive interaction were noted, as was time spent swimming by the main aggressor and receiver. Additionally, usage of ‘enrichment’

was logged. Fish were ranked as 1 (most dominant), 2 and 3 etc., from the number of aggressive acts performed and received. Previous behavioural studies on rainbow trout have shown that agonistic behaviour is suggested to be a reliable indicator of dominance (Johnsson and Björnsson, 1994; Johnsson and Åkerman, 1998). For study one and two, 40 hours of footage was gathered and analysed (4 hours of footage per tank for study 1, 2.2 hours of footage per tank in study 2), whilst the third study accumulated 45 hours of footage for analysis (1.89 hours of footage per tank). In total, approximately 18 months of full time work was dedicated to analyse behavioural endpoints.

2.4 Physiological endpoints

Out of physiological endpoints available as welfare indicators I analysed plasma cortisol and ion concentrations (section 3.3.5) as well as brain monoamine levels (section 3.3.7) for study 1, brain monoamines (section 4.3.6) for study 2, and plasma cortisol levels (section 5.3.5) for study 3.

2.4.1 Plasma cortisol and ion concentrations

Plasma cortisol levels were analysed as cortisol is a widely applied stress indicator. Plasma ion concentrations may also provide an insight into fish stress levels. Freshwater adapted rainbow trout, typically loses up to 25 % of the Na^+ and Cl^- content per day through branchial diffusion (Gonzalez and McDonald, 1992). Generally, ion loss is compensated for by active ion uptake (Postlethwaite and McDonald, 1995). However, when a range of circumstances challenge the physical homeostasis of freshwater fish (e.g. stress), it could cause increased loss of plasma ions.

Plasma cortisol concentrations were determined using a commercially available enzyme immunoassay kit (DRG Cortisol ELISA) supplied by Immunodiagnostic Systems Ltd. This is a solid phase enzyme linked immunosorbent assay which is based upon the principle of

competitive binding, and it has been used in other fish studies previously (Sloman et al., 2008). Plasma cortisol concentrations have been expressed in ng/ml. Plasma ion concentrations were determined by ionchromatography (Dionex ICS 1000; Camberley, UK) and values were corrected for dilution factor and reported in mmoles/l.

2.4.2 Brain monoamines

Brain monoamines play a crucial role in linking behaviour and physiology and they can be used in welfare evaluations since dopamine, noradrenaline and serotonin are linked to aggressive behaviour and stress. When examining existing literature with regards to brain parts and monoamines, researchers generally analyse the telencephalon, optic tectum, brain stem and hypothalamus. Different parts of the brain are used depending on the question at hand. With regards to serotonin and noradrenaline the cellbodies are mainly located in parts of the hindbrain (raphe, nucleus coerulus). Hence, analysing the hindbrain (e.g. brain stem) is key if you want to investigate these two systems. The telencephalon is highly interesting as this is the integrating part of the brain, and the centre for cognitive processes. Additionally, in fish, the telencephalon contains areas homologous to the hippocampus and amygdala in the mammalian brain. The hypothalamus is also of interest regarding endocrine regulation, but also for studies of aggressive behaviour (personal communication with Professor Svante Winberg, Uppsala University).

Frozen brain samples were flown to Uppsala University, Sweden where they were analysed in the following manner: brain parts were homogenised in 4 % (w/v) ice cold perchloric acid (PCA) containing 100 ng/ml of the internal standard 3, 4-dihydroxybenzylamine (DHBA) using a sonicator (Sonifer Cell Disruptor B-30, Branson Ultrasonics, DA 1 nbury, CT, USA), then centrifuged at 16,000 x *g* for 6 minutes at 4 ˚C and protein pellets were stored in –80 ˚C for analysis of protein content. The supernatant was analysed for serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), and noradrenaline (NA)

using high performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a solvent delivery system model 582 (ESA, Bedford, MA, USA), an autoinjector Midas type 830 (Spark Holland, Emmen, the Netherlands), a reverse phase column (Reprosil-Pur C18-AQ 3 µm, 100 mm × 4 mm column, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) and an ESA 5200 Coulochem II EC detector (ESA, Bedford, MA, USA) with two electrodes at reducing and oxidizing potentials of -40 mV and +320 mV. The mobile phase was deionised water containing 7 % acetonitrile with 75 mM sodium phosphate, 1.4 mM sodium octyl sulphate, and 10 µM EDTA with an adjusted pH of 3.1 by phosphoric acid. Samples were analysed immediately for monoamines and their metabolites or stored at –80 ˚C for no more than 3 days before analysis. Monoamines were quantified using standard solutions of known concentrations and corrected for recovery of the internal standard using the HPLC software ClarityTM 18 (DataApex Ltd, Prague, Czech Republic). To standardise monoamine concentrations, brain protein pellets were dissolved in EDTA buffer solution and analysed for protein content using a bicinchoninic acid BCA ™ Protein Assay Kit (PIERCE, USA) which applies colorimetric detection and quantisation of total protein content. Hence, concentrations of brain monoamines and metabolites have been expressed as ng/mg protein. Monoaminergic brain activity was estimated by the ratio between the concentration of monoamine metabolite and the concentration of parent monoamine ([5-HIAA]: [5-HT]) since it is suggested that monoamine levels on their own give an insufficient view of the monoaminergic activity (Shannon et al., 1986). [Metabolite]:[monoamine] ratios are less sensitive than metabolite levels on their own to changes in neural processes other than release rate, additionally this index is less sensitive to variance associated to tissue sampling and weight determination (Shannon et al., 1986). Moreover, concentrations of monoamine metabolites were also used since they provide a reliable index for monoaminergic activity (Shannon et al., 1986). I was unable to quantify DOPAC in several samples due to unidentified interfering peaks, hence it has been omitted from all samples.

2.5 Physical endpoints

There are a range of physical measurements used as welfare indicators, see section 1:3, out of which I analysed specific growth rate, condition factor, hepatosomatic index, and fin damage. Since continuous stress may have deleterious effects upon fish growth, mass and length specific growth rates were analysed, with the assumption that more stressed fish would have lower growth rates. Growth rates tie in with condition factor as this endpoint takes both mass and length into consideration, hence the same assumption as for growth rates was applied to the results of condition factors. Additionally, lower food intake (i.e. lower growth) and higher stress levels will influence the energy store of an individual. Since the hepatosomatic index is a measure of an individual's energy store, it was investigated in the following studies with the assumption that a more stressed fish would have a lower hepatosomatic index.

2.5.1 Specific growth rate (SGR)

Individual body mass and length specific growth rates were calculated as follows:

$$SGR = [\ln(BM_{final}) - \ln(BM_{initial})] / t*100$$

Where BM final and BM initial are the final and initial body mass respectively, and t the time elapsed (days) between body mass measurements. For length SGR the initial and final lengths in mm were exchanged for the body mass measures.

2.5.2 Condition factor (CF)

Condition factors allow us to quantitatively compare the condition of fish from different treatments, and it was calculated as (Nash et al., 2006):

$$CF = \text{Body mass} / (\text{length}^3)*100$$

Where length is the recorded fork length in cm and mass in g. Condition factors were calculated for day 0, 14 and 28 of the studies.

2.5.3 Hepatosomatic index (HSI)

The HSI was calculated as HSI:

$$M_{liver} / (M_{body} - M_{liver}) * 100$$

Where M_{liver} = liver mass in g, and M_{body} = body mass in g.

2.5.4 Fin damage

Fin damage is a sign of injury in a farmed animal and has therefore been identified to be a potential welfare indicator (Ellis et al., 2008). Every seven days assessments of dorsal and pectoral fin damage were made as fish were swimming freely, according to a photographic key used to assess fin damage in farmed rainbow trout. This key produced by Høyle et al., (2007), reflects the degree of fin tissue loss and it has scores from 0 to 5 with 0 being the reference point to a 'perfect' fin and 5 showing severe tissue loss, see figure 2.1.

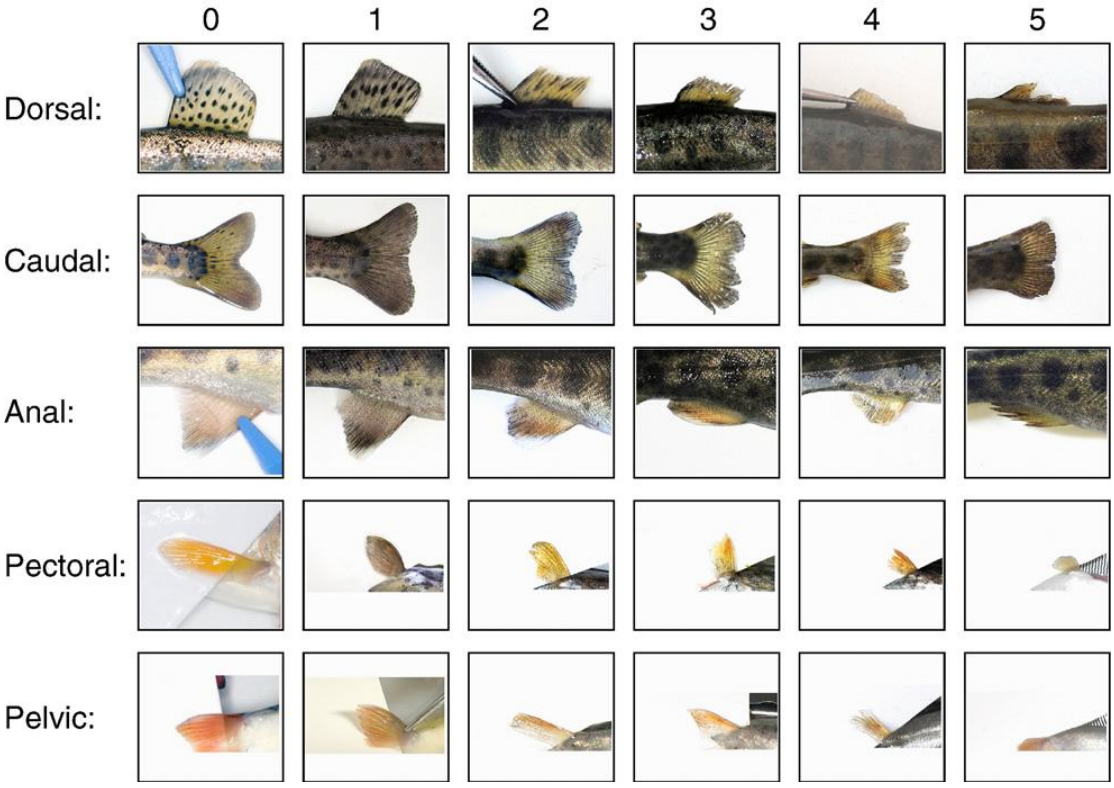

Figure 2.1. Photographic identification key of tissue loss from five different fins (dorsal, caudal, anal, pectoral and pelvic) in rainbow trout less than 50 g in mass (Høyle et al., 2007).

2.6 Statistical analysis

Parametric statistics, one-way ANOVA and ANOVA general linear models nested design, adjusting for rank, were used for data fulfilling normal distribution and homogeneity of variances. The model structure was as follows: endpoint = tank (treatment) rank treatment. Tank and rank were specified as random factors, except in initial explorations of the role of rank where it was defined as a fixed factor. Data that did not meet parametric assumptions were log or square root transformed before analysis. All values are presented as mean ± standard error of mean, and statistical analyses were performed using Minitab 15 Statistical Software. In all statistical analyses, differences at the 5 % level were considered significant.

To provide a fuller exploration of the data a more complex statistical approach was applied. Principal Component Analyses of brain monoamine variables, as well as the full dataset of chapter 3 were undertaken, in order to summarise the results of several different welfare indicators. This was only done for one results chapter, as the time scope for running multivariate analyses for all three chapters within deadline was not feasible. However, multivariate analyses will be executed in due course for the two remaining datasets, before any manuscripts are released.

With regards to the brain monoamine PCA results, it was found that in order to explain 80 % of the variation, six variables had to be used, and the first component only explained 19 % of the variation. Whilst for the entire data set, ten variables had to be used in order to explain 80 % of the variation and the first component only explained 14 % of the variation. Hence, any further exploration using this type of multivariate analysis was not carried out for this thesis, and the results have not been reported or discussed and further because of this.

2.7 Critical review of plasma cortisol levels

Generally problems and issues with experimental endpoints are not discussed in the present chapter of materials and methods, and within this thesis it has only been applied to plasma

cortisol. The reason being that there were serious problems analysing this endpoint, hence I have not drawn any conclusions from the cortisol results. Still, I wanted to highlight the fact that multiple attempts have been made to troubleshoot the plasma cortisol analyses during this PhD. Additionally, these problems apply to the discussion of both chapter three and five, hence it seemed more suitable to address this matter here instead of repeating it in two of the results chapters.

I found no significant differences in plasma cortisol concentrations between treatments for either study one or three (chapters three and five), which were the only two studies in which I tried to analyse plasma cortisol. For unknown reasons, plasma samples were of low quality and the ELISA standard curve appeared unreliable at the lowest concentrations (2.5 ng/ml). Hence, it was challenging to quantify plasma cortisol and no definitive conclusions have been drawn from these results. Cortisol levels in general appeared to be very low across treatments, but whether this was due to actual low levels in samples or poor plasma quality is hard to decipher. Why concentrations were this low remains unknown despite a major effort to resolve the problem. Radioimmuno assays (RIA) and ELISA assays were used and international researchers experienced in this field were contacted for advice (Dr. Katherine Sloman, University of the West of Scotland, UK, Dr. Fiona Mathews, University of Exeter, UK, Dr. Lisa Leaver, University of Exeter, UK, Professor Svante Winberg, Uppsala University, Sweden, Dr. Erik Höglund, Technical University of Denmark, Denmark, Professor Anne Brown, University of Exeter, UK, Dr. Thomas Pottinger, Centre for Ecology and Hydrology, UK, and Dr. Eduarda Santos, University of Exeter, UK) but similar problems had not been encountered previously. Hence, despite wide consultations with many researchers the issue remains unresolved.

In order to verify the suitability of the ELISA assay for trout samples in this PhD, reference plasma was taken from the same batch of fish used in the water current study (chapter five), applying exactly the same sampling procedures as in the enrichment studies. With the aim of

investigating what range of plasma cortisol to expect, six fish to be used as control/unstressed were sampled by one rapid netting attempt, and transferred to an overdose of buffered tricaine methane sulphonate (240 mg/l). Fish were terminated within 60 seconds, and blood was collected from the caudal vessels. Meanwhile five other trout were repeatedly stressed by being chased intermittently with a net in a bucket for 60 minutes prior to blood sampling. This was to provide an example of high levels of plasma cortisol. The ELISA assay worked well for the reference plasma, with a low mean ± SE of cortisol concentration for unstressed fish (2.73 ± 0.38 ng/ml; n = 6), whilst a 10-fold elevation was observed in the net-stressed fish (28.28 ± 4.13 ng/ml; n = 5). There was only one difference in treatment between reference plasma and plasma from the shelter and water current experiments in chapter three and five, reference plasma was not shipped to and returned from Sweden (plasma cortisol was supposed to be analysed by RIA at the same time as brain monoamines at Uppsala University, Sweden). Subsequently, I can only speculate that something may have gone wrong when samples were shipped, or perhaps this was a batch of low cortisol responding individuals as they came from a commercial trout farm which actively selects for traits such as high growth and low stress response.

It is not unusual for rainbow trout to have low levels of plasma cortisol. Recent studies showed low plasma cortisol concentrations in a range of salmonids. Pottinger (2010) reported mean plasma cortisol levels of 1.8 ± 0.4 SE ng/ml in unstressed *O. mykiss,* 3.4 ± 1.6 SE ng/ ml in *S. trutta* and for *S. alpinus* 5.8 ± 1.1 SE ng/ml. Whilst Cairns et al., (2008) found mean plasma cortisol levels in control rainbow trout ranging from 5.7 to 6.5 ng/ml, and values as low as 0.8 ng/ml were also observed. This may support low cortisol levels found in the present study being valid. It could also indicate that fish generally were relatively unstressed as by the time of sampling fish had spent 28 days together which may have provided ample time to settle group social structures. It has also been confirmed that rainbow trout used in these studies are from fast growing and docile individuals (personal communication with Hans Hoff, Houghton Springs Fish Farm, Dorset). The magnitude of

plasma corticosteroid concentrations induced by stress in rainbow trout is heritable (Fevolden et al., 1999), and the magnitude of the corticosteroid response is accompanied by certain behavioural traits (Pottinger, 2010), hence individuals can be characterized as high or low responding with regards to plasma cortisol levels (Øverli et al., 2007a). Selective breeding based upon low cortisol responding, fast growing individuals is common practise as rainbow trout is an economically important fish species, that may respond unsuitably to routine unavoidable events or conditions occurring in aquaculture (Huntingford, 2004). Hence selective breeding to improve economically important characteristics such as low stress responses and high growth rate is custom (Gjoen and Bentsen, 1997).

Cortisol is probably one of the most commonly used endpoints in animal stress and welfare research, but does this make it the best? How reliable are cortisol levels? It is open to question whether brain monoamines are perhaps better welfare indicators if individuals can be sacrificed, as they change more slowly over time and are more stable (Øverli et al., 2007b).

CHAPTER 3: Effects of semitransparent shelters on behavioural and physiological welfare indicators in juvenile rainbow trout

Abstract

In this study I evaluated the effects of semitransparent shelters upon female juvenile rainbow trout, utilising a range of different physiological and behavioural endpoints commonly used as welfare indicators in fish. The two different hypotheses were that shelters may act as refuges for subordinates from aggressive dominants and possibly increase fish welfare, or alternatively cause increased aggression and stress if perceived as defendable resources and possibly decrease welfare. I showed that structures of this design should not be viewed as enrichment with regards to juvenile rainbow trout, as serotonin metabolite results and serotonin turnover indicated higher chronic stress levels in trout from tanks with shelters. Additionally, both mass specific growth rate and the hepatosomatic index were significantly lower in these fish. Furthermore, positioning and features of tank structures appeared to be of significance.

3.1 Introduction

In a laboratory environment juvenile rainbow trout establish dominance hierarchies according to competitive ability through aggressive interactions (Adams et al., 1998), which cause individuals to experience wins and defeats. Social defeat is a recognised powerful stressor for several species (Øverli et al., 2004a).Subordinate fish commonly experience lower growth rates, disease resistance (Pottinger and Pickering, 1992), body condition, hepatic energy reserves (Sloman et al., 2001b), and higher cortisol levels. This could have serious welfare aspects in a species like rainbow trout by impinging upon the 'Five Freedoms' (see section 1.2 in the General Introduction). Sibling groups of juvenile salmonid fish held in laboratory environments are reported to be less aggressive than groups of non-kin (Brown and Brown 1993; Olsén et al., 1996) and female rainbow trout are less aggressive than males (Johnsson and Åkerman, 1998). However it is rarely possible to

house fish from a mixed sex stock as batches of single sex siblings and aggression between fish in experimental environments is a common occurrence. It is even feasible to suggest that a laboratory milieu may induce aggression for reasons reviewed below, especially as individuals are size matched, a requirement in the OECD guidelines see section 3.2.2.

1) Cost of risk taking. In the wild exposure to predation risk may increase with aggressive displays (Jakobsson et al., 1995), which is not the case in the laboratory. Hence, fish have fewer inhibitions for displaying agonistic behaviour (Höjesjö et al., 2002).

2) Metabolic cost. Intense aggressive behaviour could incur high metabolic costs in a natural environment, whilst under laboratory conditions food is abundant and metabolic costs are lower, hence it is more 'affordable' to display aggression (Höjesjö et al., 2002).

3) Stocking density. In general, the rate of aggression decreases as the number of fish per tank increases, since defending a favourable area by dominant individuals becomes increasingly difficult (Jørgensen et al., 1993). However, advisable stocking densities may not always be applied for pragmatic reasons. Additionally, the effect of stocking density upon aggressive behaviour may be species dependent since it has been shown that aggressive interactions can sometimes increase with greater fish density, for example amongst Atlantic salmon (Blanchet et al., 2006).

4) Constrained environment. Laboratory settings are generally more homogeneous and spatially constrained than wild environments and consequently subordinates have no way to escape aggressors (Höjesjö et al., 2002). Recent studies on cage trapped wild specimens of meadow voles (*Microtus pennsylvaicus*) (Lynn and Porter, 2008) and house sparrows (*Passer domesticus*) (Fletcher and Boonstra, 2006), showed significantly higher levels of stress hormones in cage trapped animals in comparison to directly sampled animals.

Studies on brown trout (Sundbaum and Näslund, 1998), zebrafish (Basquill and Grant, 1998), and rainbow trout (Imre et al., 2002) have shown decreased aggression between individuals with increased habitat complexity. Environmental enrichment such as the addition of semitransparent shelters may therefore decrease overall aggressive behaviour amongst rainbow trout and possibly increase their welfare. Additionally, providing a structured environment may not only reduce aggression but also lessen stress responses in fish (Woodley and Peterson, 2003; Höglund et al., 2005c). On the contrary, some fish species have been shown to be more aggressive in structured environments than unstructured ones (Kelley et al., 2006), thus this is a subject clearly in need of species specific attention.

Many salmonid species use caves and refuges to shelter (Cunjak, 1988; Fraser et al., 1993; Metcalfe et al., 1999). Hence, in the present study the effects of transparent shelters upon the following physiological and behavioural welfare indicators in female juvenile rainbow trout were examined; brain monoamines, plasma cortisol, plasma ion concentrations, specific growth rates, condition factor, hepatosomatic index, and fin damage. Aggression, swimming behaviour and shelter usage was studied through video footage. It was hypothesised that the added tanks structures would act as physical barriers and refuges for subordinates from aggressive individuals. Alternatively, it could cause increased aggression and add to existing social stress if they were perceived as defendable resources by dominant individuals.

3.2 Material and methods

See chapter 2 for a general overview.

3.2.1 Experimental animals

See section 2.1.

3.2.2 Experimental protocol

See section 2.2 for general experimental protocol.

Ten flow-through glass tanks of 40 litre volume were set up applying conditions of the 215-OECD guideline (http://www.oecd-ilibrary.org/environment/test-no-215-fish-juvenile-growth-test_9789264070202-en). Each tank contained eight fish and the initial mean mass was 4.93 ± SD 0.46 g (*N = 80*) which is a starting mass recommended by the 215-OECD guideline for a juvenile growth study, and fork length was 66 ± SD 2.16 mm (*N = 80*). All tanks were completely barren for the first half of the study, but on day 14 two dark grey tinted, semitransparent glass shelters were fixed to the bottom of five of the tanks, whilst five tanks had two transparent flat glass slides added to the bottom. This was done in order to apply the same disturbance to all tanks, as well as to make up for some of the added surface area, and the study continued for another two weeks. Shelters were built using dark gray tinted glass plates which were assembled to a rectangular structure see figure 3.1. One was placed near the front and right of the tank and the other near the rear and left with sufficient space between shelters and tank sides so fish could manoeuvre and swim freely in and around them see figure 3.2 and 3.3.

On day 14, when shelters were added, fish were also measured for mass and length and the food ration was recalculated to ensure a constant ration. Throughout the study four tanks were filmed for 15 minutes each before the morning and afternoon feed, during which times the aquarium room was closed to co-workers to avoid disturbances. On days 28 and 29 fish in five tanks were terminated respectively, and body measurements, blood and tissue samples were obtained as described in section 2.2

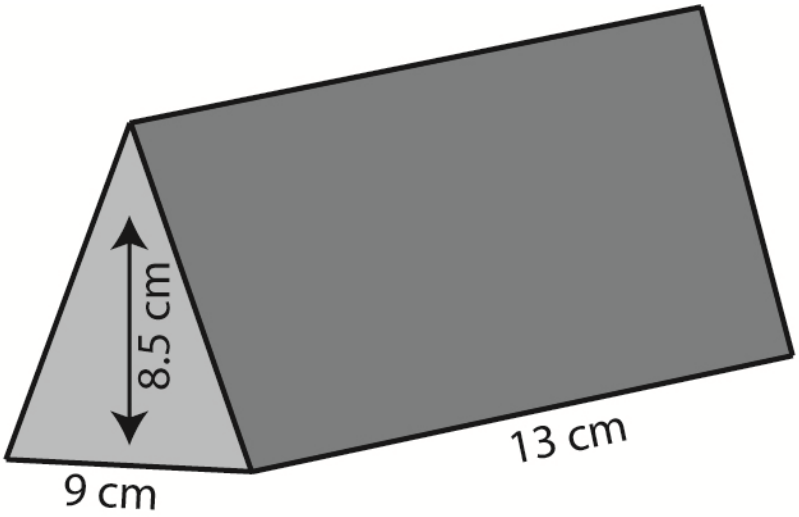

Figure 3.1. Schematic drawing of a semitransparent shelter including dimensions of length, width and height, not to scale.

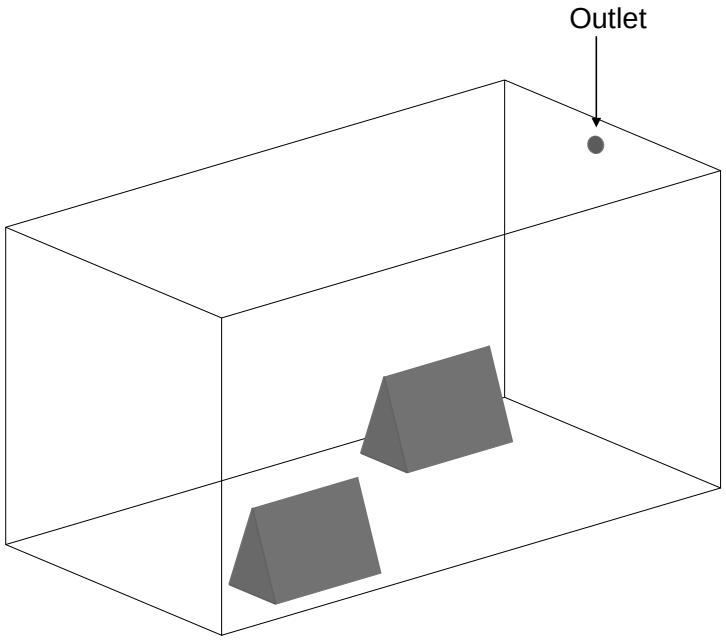

Figure 3.2. Side view of the layout of shelters in a test tank, not to scale.

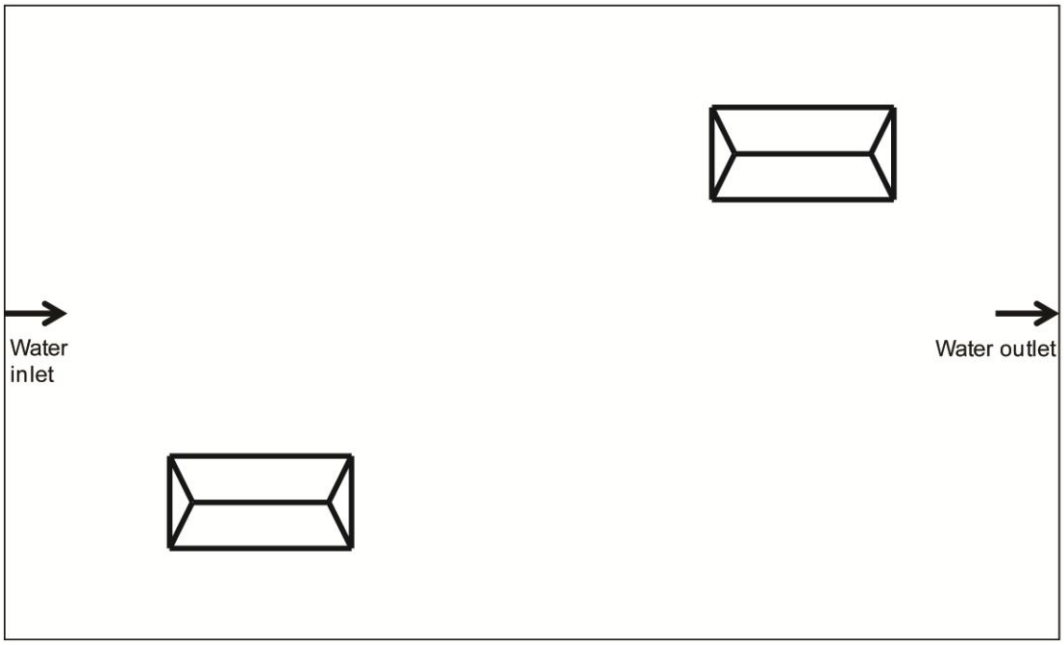

Figure 3.3. Top view of layout of shelters in a test tank, not to scale.

3.3 Endpoints and statistical analyses

Data was analysed using parametric analysis for differences between and within treatments, i.e. day 0 to 14 versus day 14 to 28. See section 2.6 for details of the nested ANOVA.

3.3.1 Mass and length specific growth rates

Mass and length specific growth rates for days 0 to 14 and days 14 to 28 were analysed within and between treatments using ANOVA general linear models nested design.

3.3.2 Condition factor

Condition factors were calculated for days 0 and 28, and ANOVA general linear models nested designs were executed to analyse differences between these days between and within treatments.

3.3.3 Hepatosomatic index

Differences in HSI between treatments were analysed using ANOVA general linear model nested design. Hepatosomatic index was also analysed for differences between ranks by using rank as a fixed factor.

3.3.4 Fin damage

Differences between and within treatments in dorsal and pectoral fin damage were analysed using ANOVA general linear model nested design. Fin damage day 28 was analysed between treatments, and day 0 versus day 28 within treatments.

3.3.5 Plasma ions and cortisol concentrations

Plasma ion content has been reported as mmoles/l, and chloride and sodium levels were analysed by ANOVA general linear model nested design. Plasma cortisol concentrations were transformed and analysed for differences between treatments using ANOVA general linear model nested design. Based on the fact that the quality of the plasma samples for some unknown reason was less than expected, and that the standard curve in the ELISA assay was not as reliable as desired at low concentrations, samples lower than 2.5 ng/ml were treated as below detectable limits. The analytical sensitivity of the kit was 2.5 ng/ml at its lowest concentration. For further details about cortisol analysis see section 2.4.1 and 2.7.

3.3.6 Behavioural endpoints

One-way ANOVAs were executed on frequency data of aggressive attacks for day 0 to 14 and day 14 to 28 within and between treatments, as well as usage of shelters.

3.3.7 Brain monoamines

Data for monoamine and metabolite concentrations as well as serotonin turnover in the telencephalon, optic tectum, brain stem and hypothalamus were log or square root transformed before analysed by ANOVA general linear models nested design.

3.4 Results

3.4.1 Mass and length specific growth rates

Fish in barren tanks had a 7 % greater mass specific growth rate day 14 to 28 in comparison to day 0 to 14, within treatment (*p = 0.001*, df = 1, 63). This trend was not found within the shelter treatment (*p = 0.437*, df = 1, 63). There was no significant difference in mass SGR between treatments day 0 to 14 (*p = 0.597*, df = 1, 63), but day 14 to 28 trout in barren tanks had a 9 % higher mass specific growth rate than trout from tanks with shelters which almost was a significant increase (*p = 0.059,* df = 1, 63), see figure 3.4.

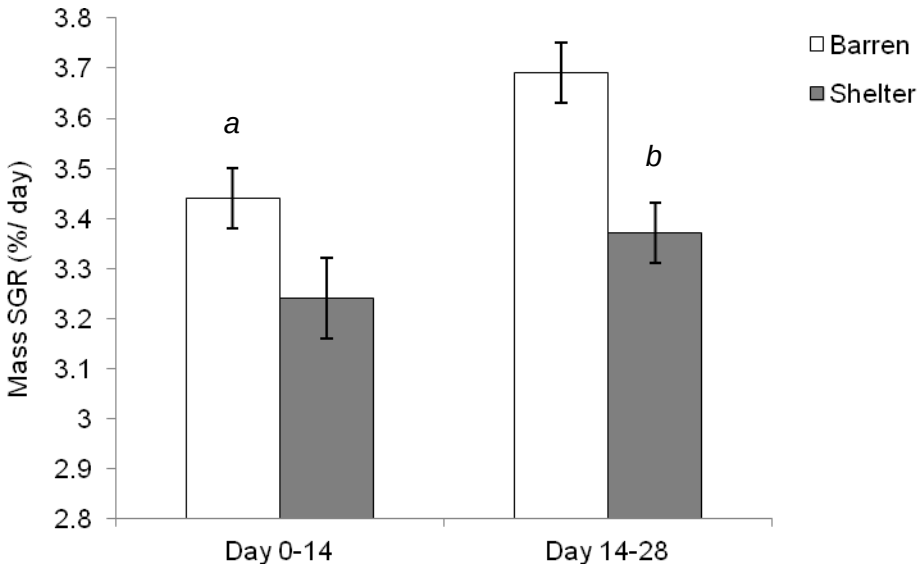

Figure 3.4. Mean mass specific growth rate expressed as percentage mass gain per day in grams ± SE, day 0 to 14 and 14 to 28 in barren and tanks with transparent shelters. With *a* indicating the significant difference within the barren treatment and *b* the difference in mass growth rate between barren and shelter tanks day 14 to 28.

There was no significant difference in length specific growth rate between treatments day 0 to 14 (*p = 0.376*, df = 1, 63) whilst day 14 to 28 rainbow trout in barren tanks had a significantly greater length SGR (*p = 0.042*, df = 1, 63), see figure 3.5. There was no

significant difference in length SGR within treatments between day 0 to 14 and day 14 to 28, barren tanks (*p = 0.461*, df = 1, 63) and shelter tanks (*p = 0.664*, df = 1, 63).

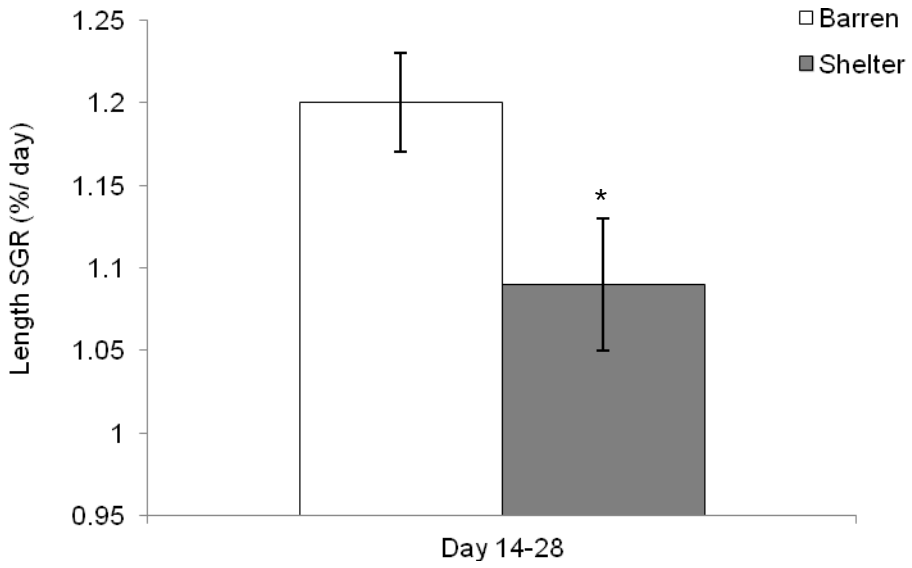

Figure 3.5. Mean length specific growth rate expressed as percentage length gain per day in mm ± SE, day 14 to 28 in barren and shelter tanks. Asterisk indicating the significantly greater length specific growth rate in trout from barren tanks.

3.4.2 Condition factor

There were no significant differences in condition factors between treatments day 0 (*p = 0.920*, df = 1, 63), or day 28 (*p = 0.837*, df = 1, 63). Nor was there a significant difference in condition within treatments day 0 versus day 28, tanks with shelters (*p = 0.975*, df = 1, 63), barren tanks (*p = 0.547*, df = 1,63), see table 3.1.

Table 3.1. Mean condition factor ± SE, (g/cm^3), of trout in semitransparent shelter and barren tanks day 0, 14 and 28.

	CF ± SE day 0 (g/cm^3)	CF ± SE day 14 (g/cm^3)	CF ± SE day 28 (g/cm^3)
Barren tanks	1.71 ± 0.02	1.69 ± 0.02	1.70 ± 0.02
Shelter tanks	1.71 ± 0.02	1.68 ± 0.02	1.71 ± 0.02

3.4.3 Hepatosomatic index

Fish in shelter tanks had a 10 % smaller hepatosomatic index in comparison to trout in barren tanks, which was almost significantly different (*p = 0.079,* df = 1, 63), see figure 3.6. Additionally, it was evident there was large tank variation within treatments as this was significant (*p = 0.047*, df = 1, 63), whilst there were no noteworthy differences between ranks with regards to HSI (*p = 0.0992*, df = 1, 63).

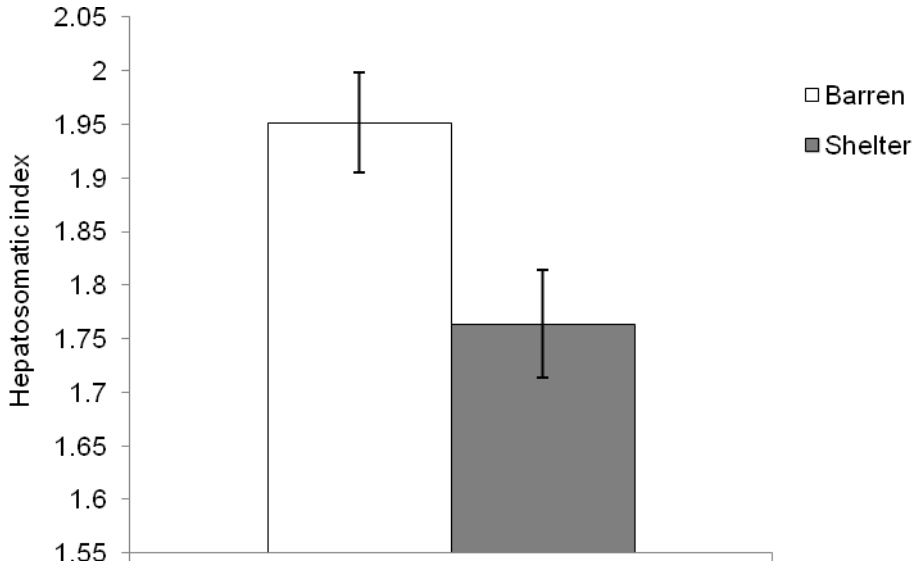

Figure 3.6. Mean hepatosomatic index ± SE of rainbow trout in barren and tanks with shelters.

3.4.4 Fin damage

There were no significant differences between dorsal ($p = 0.681$, df = 1, 63) or pectoral ($p = 0.274$, df = 1, 63) fin damage in barren versus shelter tanks day 28, see figure 3.7. However, dorsal scores in both barren and shelter tanks were 30 times higher by day 28 within treatments ($p = 0.003$, df = 1, 63 and $p = 0.001$, df = 1, 63, respectively), see figure 3.7.

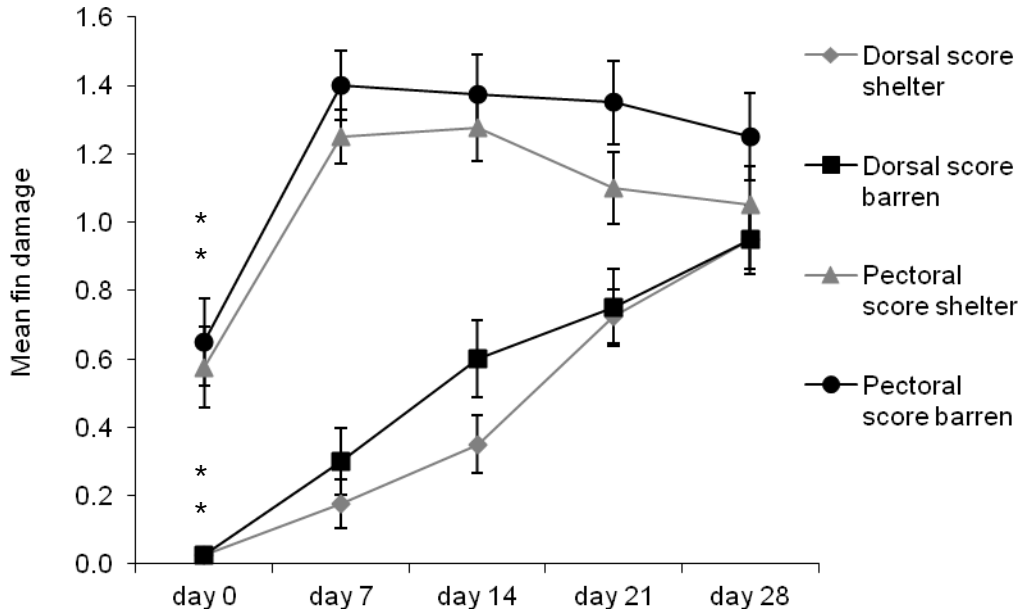

Figure 3.7. Mean fin damage ± SE for dorsal and pectoral fins, day 0, 7, 14, 21 and 28 in the two different treatments. Asterisks indicate significant differences in mean dorsal and pectoral fin damage within treatments, between day 0 and day 28.

With regards to pectoral fin scores the same trend as for dorsal scores was found. Fish in barren as well as shelter tanks had an almost two fold increase in pectoral damage over time ($p = 0.018$, df = 1, 63, and $p = 0.002$, df = 1, 63, respectively), see figure 3.7.

3.4.5 Plasma ion and cortisol concentrations

There were no significant differences in plasma sodium ($p = 0.092$, df = 1, 63), chloride ($p = 0.264$, df = 1, 63) or cortisol ($p = 0.514$, df = 1, 63) concentrations between treatments, see

table 3.2. However, there is a trend towards a significant relationship between sodium and treatment which may have been more evident with higher statistical power.

Table 3.2. Plasma variables, mean chloride and sodium levels reported in mmoles/l and cortisol in ng/ml ± SE for trout in barren and transparent shelter tanks.

	Chloride (mmoles/l)	Sodium (mmoles/l)	Cortisol (ng/ml)
Barren tanks	118.5 ± 1.5	153.1 ± 1.4	6.41 ± 0.65
Shelter tanks	119.5 ± 1.5	152.9 ± 1.5	10.9 ± 0.75

3.4.6 Behaviour

The majority of the fish generally spent most of the time motionless or just slightly moving tails or fins, except for the most aggressive trout or individuals receiving aggression. Due to low activity levels, fish were primarily located at or close to the bottom of the tank, which is in accordance with findings for juvenile brown trout by Johnsson et al., (2001) who concluded that this behaviour is typical for territorial stream dwelling trout held under laboratory conditions. In a 15 minute observation period dominant individuals in barren tanks swam for 92 % of the time, whilst in tanks with shelters the corresponding time was 86 %. Individuals receiving the most aggression in barren tanks swam for 62 % of a 15 minute observation period and the corresponding value in shelter tanks was 64 %. Dominant fish had a 33 % times higher activity level than subordinate fish receiving the most aggression which was significantly different (p= *0.001,* df = 1, 93), see figure 3.8.

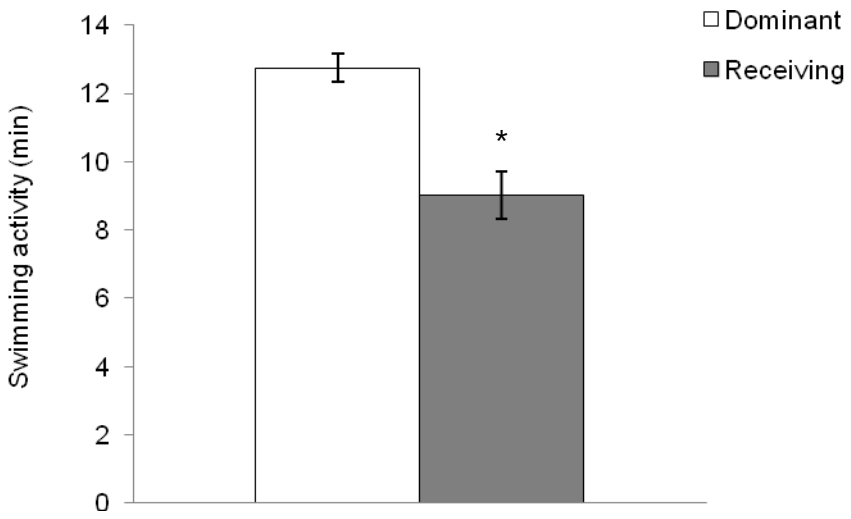

Figure 3.8. Mean swimming activity in minutes ± SE by dominant and most receiving trout during a 15 minute observation period. Open bars representing dominant trout and filled bars receiving individuals. Asterisk indicating the significant difference in swimming activity between dominant and subordinate trout.

The order of entering tanks did not influence which individual turned out to be the most dominant or subordinate, and the number of individuals displaying dominance varied between tanks. There were generally between two to six individuals per tank that were highly aggressive, showing dominance over at least some of the lower ranks throughout the 28 days. In only three out of the ten tanks did the fish with the greatest initial mass become dominant.

There was no significant difference in aggression between the first and second time period within or between treatments. Between treatments day 0 to 14 ($p = 0.204$, df = 1, 72) day 14 to 28 ($p = 0.210$, df = 1, 68), within treatment for barren tanks ($p = 0.393$, df = 1, 67) and shelter tanks ($p = 0.089$, df = 1, 73). The frequency of aggressive acts ranged from 0.3 to 11.1 per minute, and only in one out of the in total 144 observation periods, were there no aggressive acts taking place. Fish receiving aggression were never observed to seek refuge in a shelter. Still, shelters were used for 40 % of the total footage time, but only by one

individual at a time, with the rear shelter significantly more utilised than the front one ($p < 0.001$, df = 1, 54, One Way ANOVA).

3.4.7 Brain monoamines

Telencephalon

There were no significant differences between treatments in brain monoamine or metabolite concentrations or in serotonin turnover, in the telencephalon. DA ($p = 0.192$, df = 1, 61), NA ($p = 0.074$, df = 1, 61), 5-HIAA ($p = 0.213$, df = 1, 61), 5-HT ($p = 0.152$, df = 1, 61), and serotonin turnover ($p = 0.152$, df = 1, 61).

Optic tectum

Trout in tanks with shelters had a 35 % higher 5-HIAA concentration in the optic tectum in comparison to fish in barren tanks ($p = 0.011$, df = 1, 63), as well as a 44 % higher serotonin turnover ($p = 0.064$, df = 1, 63) see figure 3.9. There were no significant differences in other monoamine concentrations between treatments; DA ($p = 0.320$, df = 1, 63), NA ($p = 0.607$, df = 1, 63), 5-HT ($p = 0.918$, df = 1, 63).

Brain stem

Trout in tanks with shelters had a 47 % higher 5-HIAA concentration in the brain stem in comparison to fish in barren tanks ($p = 0.041$, df = 1, 63), see figure 3.9. There were no significant differences in other monoamine concentrations or serotonin turnover between treatments; DA ($p = 0.368$, df = 1, 63), NA ($p = 0.569$, df = 1, 63), 5-HT ($p = 0.456$, df = 1, 63), serotonin turnover ($p = 0.964$, df = 1, 63).

Hypothalamus

Fish in tanks with shelters had a 73 % higher hypothalamic 5-HIAA and 58 % higher dopamine concentration, which were significantly different in comparison to trout in barren

tanks ($p = 0.024$, df = 1, 58, and $p = 0.020$, df = 1, 58, respectively) see figure 3.9. There were no significant differences in other monoamine concentrations or serotonin turn over between treatments; NA ($p = 0.212$, df = 1, 58), 5-HT ($p = 0.575$, df = 1, 57), serotonin turnover ($p = 0.243$, df = 1, 58).

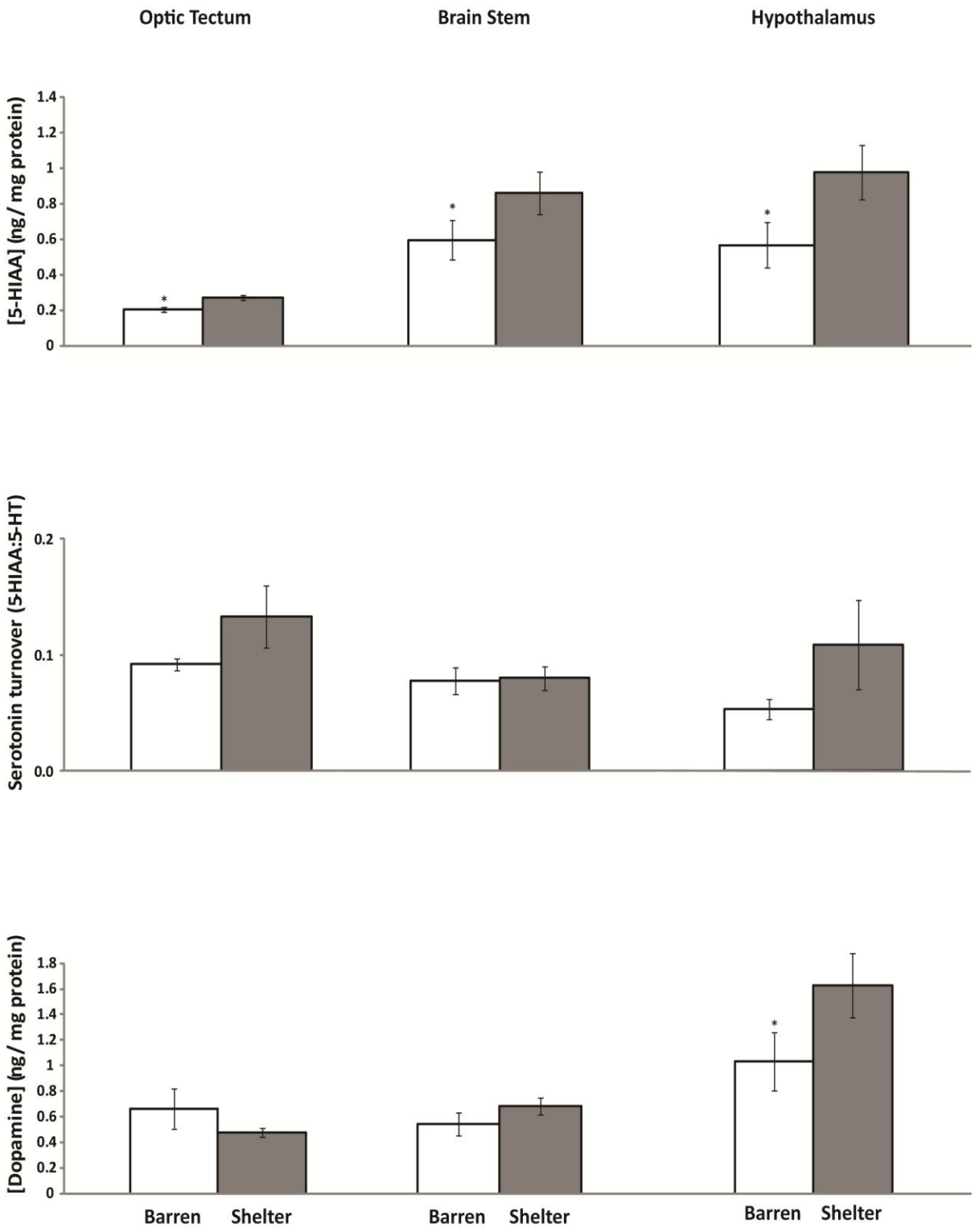

Figure 3.9. Mean 5-HIAA and DA concentrations, as well as serotonin turnover ± SE in the optic tectum, brain stem and hypothalamus of trout from barren and shelter tanks. As monoamine concentrations in the telencephalon were not significantly different between treatments this data has been excluded from this figure. Asterisks indicating significant differences between treatments.

3.5 Discussion

In the present study I evaluated the effects of semitransparent shelters upon female juvenile rainbow trout, utilising mass and length specific growth rates, condition factor, hepatosomatic index, fin damage, plasma ion and cortisol levels, aggressive behaviour, and brain monoamines as endpoints. Findings will be discussed in consecutive order below. Shelters of this design cannot be considered enrichment for juvenile rainbow trout as they have no positive influences but actually negative impacts on mass and length growth rate, hepatosomatic index, and brain monoamines, indicating chronic stress in trout from shelter tanks relative to fish in barren environments.

Mass and length specific growth rates and condition factor

Prior to the addition of shelters there were no significant differences between treatments in growth. Day 14 to 28 there were significantly lower specific growth rates for both body mass and length in tanks with shelters in comparison to barren habitats. The lower growth rates could reflect a lower food intake and/or increased metabolic rate, i.e. cost of living. There is anecdotal, but not quantitative, evidence for both of these playing a part in the reduced growth of fish in tanks with shelters, and these will be discussed below.

Decreased body mass is one of the most customary and distinct changes in animals during and following social stress. In rats, subordination is a strong stressor and whilst dominant rats often have little change of body mass, subordinates generally suffer slower growth rates or even weight loss due to social stress, which may continue for weeks after removal of the stressor (Dijkstra et al., 1992; Haller et al., 1999). The primary stress response in fish is the release of stress hormones; cortisol, adrenaline, and noradrenaline (Sumpter, 1997). Cortisol is released in the hypothalamo-pituitary-interrenal response and adrenaline and noradrenaline in what is referred to as the adrenergic response (Mazeaud and Mazeaud, 1981). Increased cortisol levels trigger physiological changes such as increased oxygen consumption, changes in gill morphology related to active ion transport (Sloman et al.,

2000a), changes in carbohydrate metabolism and tertiary physiological changes such as decreased growth and condition may also occur (Pickering and Pottinger, 1995).

In the present study the lower mass specific growth rate in tanks with shelters could indicate that adding this type of structure caused more stress within groups, which triggered a higher metabolic cost. If this was the case, raised stress levels were not expressed in increased frequency of aggressive behaviour or in rank versus mass specific growth rate or plasma cortisol levels. Another perhaps more plausible explanation is that a more complex environment made it harder for fish to see or access food pellets at the bottom of the tank. Fish in the present study readily fed from the bottom as well as water column and it was observed that some feed gathered between the tank side and shelters.

Trout with access to shelters also had a significantly lower length specific growth rate which may explain why there was no difference in the final condition factor between treatments. Condition factors are ratios between morphological and anatomical features (e.g. body mass divided by the cube of body length), and alterations of the condition factor reflect the nutritional or energy status of the fish. Hence, a decrease in condition factor is often symptomatic of a reduction of energy stores (Dennis and Bulger, 1995). As far as indicating fish welfare, condition factors showed no difference in the wellbeing of trout between the two treatments in the present study, whilst specific growth rates showed that this type of physical structure cannot not be viewed as enrichment for juvenile rainbow trout.

Hepatosomatic index

I found that compared to trout in tanks with shelters, trout in barren tanks had an almost significantly greater hepatosomatic index ($p = 0.079$, df = 1, 63). This may seem like an insignificant finding but taking into consideration that there was a significant tank variation with regards to HSI, it suggests that if the noise in this dataset could be eliminated by larger replication a significant relationship may have been more obvious.

HSI is a commonly used stress indicator (Pickering and Pottinger, 1995), as it has been shown that stressed juvenile rainbow trout have a decreased HSI (Barton et al., 1987), and that liver size decrease in catfish (*Ictalurus punctatus*) given supplementary cortisol (Davis et al., 1985). Sloman et al., (2001b) found a significant effect of social rank on liver conditions with higher liver conditions in dominant brown trout whilst subordinate fish had a significantly lower liver condition suggesting they had smaller livers and glycogen reserves. Here, the 'stressful' impact of the shelters was sufficient to produce an 11 % difference in the mean HSI of the treatment groups. Interestingly, I did not find any effects of the different stress levels between ranks with regards to the HSI or mass specific growth rate. This could be explained by social aggression being costly for both winners and losers as suggested by Neat et al., (1998), who found a significant depletion of total sugar reserves in the muscle and liver for both combatants. With regards to growth rates and hepatosomatic index it appears that fish in barren environments had a better welfare than trout in shelter tanks.

Fin damage

Aggressive attacks from a conspecific could be costly not just energetically but it may also result in injury such as fin damage. Nips to the fins and body are common in rainbow trout with dorsal and caudal fins receiving most of the attacks (Abbott and Dill, 1985). It is fairly clear that aggressive nipping is partially if not entirely the cause of fin damage, as rainbow trout reared solitarily have undamaged dorsal fins in contrast to fish held in groups (Ellis et al., 2002). Moreover, the position of the dorsal fin makes injuries unlikely to happen by abrasion of walls and floor (Abbott and Dill, 1985), which is the opposite to pectoral damage that may be caused by aggression as well as scraping (Ellis et al., 2002). Additionally, Moutou et al., (1998) showed that dorsal fin damage is linked to food supply and rank.

I found no significant differences in dorsal or pectoral fin damage between the treatments throughout the 28 days. This was expected as there were no differences in the amount of aggressive interactions between barren and shelter tanks, and fish in this study were never

food limited which has been shown to influence fin condition. Moutou et al., (1998) found that in groups of juvenile rainbow trout set on different feeding regimes, caudal fin damage developed with time in all groups whereas dorsal fin damage developed only under limited food rations. Moreover, the severity of fin damage was significantly dependent on the feed ration, with lower ration groups sustaining more fin damage. In the present study there was a significant increase in fin damage over time, for both treatments, and as aggression did not decrease throughout the study, fin quality was expected to deteriorate. With regards to fish welfare this endpoint showed no difference in animal wellbeing between treatments.

Plasma ions and cortisol

I found no differences in plasma chloride and sodium ion levels between the different treatments. A net loss of sodium and chloride ions is associated with acute stress in freshwater fish (Ashley, 2007). However, fish may actively compensate for the loss of ions. Gill epithelial chloride cells (mitochondria-rich cells) play a part in the absorptive transport of ions across the gills of freshwater fish and Sloman et al., (2000a) showed that gill epithelial chloride cell densities increase in stressed subdominant fish. On the other hand, the relationship between chloride cell proliferation and social stress remains unclear (Sloman et al., 2000a), hence the use of plasma ion content as a measure of welfare and effect of enrichment may not be straightforward. I found no significant differences in plasma ion or cortisol concentrations between treatments, but plasma samples were of low quality. Possible reasons for this have been thoroughly discussed in chapter two section 2.7. Hence, I have put no emphasis on this finding.

Aggressive behaviour and welfare

The welfare aspect of attempting to decrease aggressive behaviour amongst individuals by adding enrichment is obvious. Not only does aggression stress fish but aggressive interactions are also a major cause of injuries. It has been reported that in farmed Atlantic halibut (*Hippoglossus hippoglossus*), injuries from agonistic behaviour to eyes, tails and

pectoral fins cause secondary infections and mortality (Ashley, 2007). In the present study it was hypothesised that shelters would act as physical barriers and refuges for subordinates from aggressive individuals. Alternatively, shelters could cause increased aggression and stress and hence decrease welfare if they were viewed as defendable resources, since Gregory and Griffith (1996) showed that rainbow trout aggressively compete for shelters when seeking refuge. Behavioural results taken together in the present study, show that the addition of tank structures of this design did not lower aggression, and at no time did subordinate fish seek refuge in a shelter. This could possibly be explained by the fact that shelters were semitransparent, hence subordinate fish were not entirely out of sight even when inside it. However, in order to comply with UK legislation requirements for regulatory testing I could not use opaque shelters as experimental animals need to be visible from a top as well as front view, see links in section 1.10 for reference.

When shelters were used it was only by one individual at a time which is in accordance with Griffiths and Armstrong (2002) who showed that juvenile salmon prefer not to share a refuge. On the other hand, Armstrong and Griffiths (2001) showed that in salmon parr shelter usage is highly density dependent and when at high densities a smaller number of fish use available shelters. I also found a clear preference for usage of the rear shelter in comparison to the front one (with the front one hardly ever being used). Hence, positioning of tank structures may be of vital importance but this needs further investigation.

Dominance

It is believed that dominance hierarchies can be determined by fish size, with the biggest fish being dominant (Sabo and Pauley, 1997). Additional determinants of who becomes socially dominant include age, aggressiveness, HSI and prior residence (Harwood et al., 2003). This was not the case in our study. Our results did not support the theories that individuals becoming dominant are those that either arrive first in a new habitat, or have the largest initial mass or hepatosomatic index. Harwood et al., (2003) predicted that juvenile Atlantic

salmon arriving first obtain and defend the most profitable sites and they found that dominants gained advantage over subordinates with regards to time spent in high quality feeding sites. However, their experimental set up was fundamentally different to mine and may explain why I did not obtain results supporting this theory. Huntingford et al., (1990) proposed that the social hierarchy in salmonid fish may depend upon behavioural properties rather than just size, which is a theory that would support our findings. Resource holding power is an indicator of an individual's fighting ability, and in social interactions the size of a fish may seem to best signal the resource holding power (Huntingford et al., 1990). However body size may not be a dependable indicator if physiological states are also included (Beaugrand et al., 1996).

Animals often display their fighting ability using many different cues, such as visual, auditory and olfactory stimuli and physical contact (Höglund et al., 2000). Colour patterns play a part in controlling agonistic behaviour in fish, and rainbow trout and Atlantic salmon display social subordination by adopting a darker body colour which supposedly acts as a signal to reduce aggression from dominant fish (Abbot et al., 1985; O'Connor et al., 1999). However, it is worth noting that darkening of body and sclera appears to primarily be associated with the stress of subordination and secondarily act as an indicator of subordination (Eaton and Sloman, 2011). Metcalfe et al., (1995) found that standard metabolic rate indicates dominance in juvenile Atlantic salmon, whilst Guderley and Coutre (2005) showed that male sticklebacks assess the physiological status of their opponents by mainly the hepatosomatic index, and adjust their combat strategies accordingly. They suggested that fish assess each other's body shape or waterborne odours that reflect hepatic status. I did not find any results supporting this theory as there was no clear picture of what determined dominance in our trout groups. However, it has been suggested that factors determining social status remain unknown especially in size matched individuals (Sloman et al., 2001a), which is relevant to the present study.

Establishing and maintaining dominance in a group is stressful for both dominant and subordinate animals (Tamashiro et al., 2005). It is reasonable to assume that establishing a dominance hierarchy would involve a period of intense initial aggression and that aggression levels would decrease once dominance is established. The present study showed that there was no difference over time in aggression levels and that the frequency of aggression was not different between treatments. However, a different type of aggression was evident in the presence of shelters, which may have brought about a qualitative change rather than quantitative change in frequency. This change seems to have brought about more stress in subordinate fish in tanks with shelters, a matter further discussed in the following section.

Behaviour in relation to serotonin and dopamine

In the presence of shelters fish had significantly higher dopamine concentrations in the hypothalamus. Dopamine is produced in several different parts of the brain, and plays a role in modulating several different types of behaviours, though it has a central role in the modulation of aggressive behaviours (Seo et al., 2008). Rodent studies have shown elevated dopamine levels before, during, and following aggressive fights (Tidey and Miczek, 1996). Increased dopamine concentrations may suggest that trout in the shelter environments experienced higher levels of aggression in the present study. Furthermore, serotonin metabolite concentrations in the hypothalamus, brain stem and optic tectum, as well as serotonin turnover in the optic tectum were raised in fish from shelter tanks. In the fish brain, raphe nuclei neurons are the principal source of 5-HT release, and these neurons are distributed along the length of the brainstem. Findings in the present study indicate greater chronic social stress in fish from shelter tanks compared to trout in barren tanks, as serotonin activity changes more slowly than plasma cortisol but is more permanent and not reversed under chronic stressful circumstances (Øverli et al., 2007c). There are various possible explanations for this finding such as: 1) increased subtle social interactions due to competition for the shelters, 2) the social structure within the group changed when tank structures were introduced, or 3) a combination of points 1 and 2, i.e. an unstable social

hierarchy and competition for the shelters. Higher chronic stress levels were not expressed in increased aggressive behaviour, though serotonin is believed to bring calming control over aggressive behaviour (Summers et al., 2005a). Studies have shown that serotonin turnover is negatively related to aggression in lizards (Summers et al., 2005b), mammals and fish (Winberg et al., 1998). Indeed, dietary supplementation with the precursor of serotonin, L-tryptophan, suppresses aggression in juvenile rainbow trout (Winberg et al., 2001) and juvenile Atlantic cod (*Gadus morhua*) (Höglund et al., 2005).

It is possible that the hierarchy in lower ranked individuals became unstable during the latter part of the study period, or that interactions were too subtle to be identified as aggressive. There was a difference between tanks regarding how many individuals displayed dominance over time, suggesting that in some groups there were unstable hierarchies. Still, this was prominent throughout the 28 days and not just during the 'enrichment' period. However, something unexpected but very evident was that trout in tanks with shelters displayed a different spatial pattern to groups in barren tanks. The introduction of the shelters appeared to enable the most dominant individual to herd the rest of the group behind the rear shelter, where it repeatedly attacked them (personal observation). Unfortunately I have no scientific measure of this but in the presence of shelters fish appeared more stressed with increased ventilation rates and they displayed high levels of jerky body movements. However, it is important to highlight that this was my non quantified overall impression of the situation, as I have no measure of ventilation rates or jerky movements. Still, it is feasible to assume that it was a fairly stressful existence, to be trapped in a corner with no options to escape an aggravated dominant individual. It would be beneficial, to create an ethogram and score video footage yet again for these type of behaviours.

Resource rich territories provide many benefits such as shelters, food, nesting sites (Arnott and Elmwood, 2009). Therefore, if adding shelters was perceived as increased quality of the territory it could possibly have motivated dominant individuals to become more determined to

keep subordinates under control. As described by Ellis et al., (2002) fighting amongst rainbow trout entails fish circling each other, and nipping or biting the head, anterior body, and pectoral and dorsal fins of the other combatant. Fighting can persist for a long time period and at times (though rarely), combatants stop nipping and start to mouth fight by locking jaws instead (Ellis et al., 2002). Mouth wrestling was caught on film once in the present study, but excessive circling and nipping for extensive periods was recorded on several occasions (15 out of 160 footage periods).

Conclusions

I examined whether there were any physiological and/or behavioural differences between female juvenile rainbow trout in barren and tanks with shelters, indicating a better or worse welfare. I have shown that adding structures of this design should not be viewed as enrichment with regards to juvenile rainbow trout. Brain monoamine results indicate higher chronic stress levels in trout from tanks with shelters, and fish welfare was not improved with regards to mass specific growth rate or hepatosomatic index. Additionally, no other endpoints supported the semitransparent shelters as environmental enrichment. It also became apparent that positioning and features of tank structures may be of significance. Hence, the current practise within regulatory research of housing juvenile trout in barren experimental tanks, seem to be a better choice than adding transparent shelters of this particular design and layout.

CHAPTER 4: Effects of visual barriers on behavioural and physiological welfare indicators in juvenile rainbow trout

Abstract

In the present study the effects of reduced visual contact between female juvenile rainbow trout, through the addition of visual barriers to test tanks were examined. It was hypothesised that a reduced visibility habitat could allow subordinate trout to get out of sight from aggressive dominant individuals, thus possibly decrease overall tank aggression. I showed that visual barriers may be viewed as enrichment for juvenile rainbow trout as brain monoamine results indicated lower chronic stress levels and perhaps even decreased aggression in visual barrier tanks, in comparison to barren environments. Enrichment location may be of significance as it was apparent that dominant individuals quickly learnt where to position themselves to control other fish. Additionally, it became clear that most utilised endpoints in this study are not sensitive enough to pick up stress trends, except brain monoamines.

4.1 Introduction

Communication is a universal behaviour, fundamental to the social organisation of animals (Oliveira et al., 1998). Through social interactions, individuals receive numerous types of sensory information used for example to establish and maintain dominance hierarchies (Korzan and Summers, 2007; Rosenthal and Ryan, 2000; Summers et al., 2005a). The importance of olfactory communication has been well described in many vertebrate species (Lord et al., 2009), and it is established that olfactory cues influence social behaviour and aggression in salmonids (Griffiths and Armstrong, 2000). However, visual cues also play a crucial role in fish communication. Visual cues provide animals with the prospect of displaying their social status, aggressiveness and fighting ability to others (Höglund et al., 2000). Hence, several fish species use visual signals to maintain the social hierarchy

(Rosenthal and Ryan, 2000; Summers et al., 2005; Korzan and Summers, 2007). In salmonids, subordinate individuals indicate defeat through adopting a darker colouration which results in a reduced frequency of attacks from dominant fish, proving that visual cues bring about behavioural as well as physiological alterations in fish (Eaton and Sloman, 2011).

Changes in brain monoamine activity (Korzan et al., 2000; Korzan et al., 2007;), blood hormone concentrations (Oliveria et al., 2001; Höglund et al., 2002) and neuropeptide gene expression in response to visual cues amongst fish (Thompson and Walton, 2004) have been well studied. Chen and Fernald (2011) showed that amongst African cichlid fish (*Astatotilapia burtoni*) visual cues from conspecifics significantly contribute to regulate social behaviour (Chen and Fernald, 2011). Smaller males seeing a larger, threatening male through a transparent barrier suppressed their dominant behaviour for up to seven days. Also, the presence of a larger dominant male on the other side of a transparent barrier caused smaller subordinate males to have physiological changes for up to three days, including up-regulation of stress related gene expression. Furthermore, Höjesjö et al., (2007) found that both behaviour (assessment of fighting ability) and physiology (heart rate) were influenced for at least 24 hours in rainbow trout observing contests between size-matched conspecifics.

With salmonids such as rainbow trout being a visually orientated species it naturally follows that visual cover plays a major role in determining their spatial location (Eklöv and Greenberg, 1998). Dolinsek et al., (2007) showed a noticeable increase in the density of juvenile Atlantic salmon in streams with increased visual isolation, with the average territory size in low visibility habitats almost half of that in high visibility environments. This trend was also found for *Anolis aeneus* lizards (Eason and Stamps, 1992). The positive effect of cover on fish density is due to the visual isolation of individuals from one another, as this reduces the perception of conspecific competition or ‘threat’ (Mesick, 1988). However, cover type

preference and usage may vary between seasons and life stages, as Kemp et al., (2005) did not find a decreased territory size for Atlantic salmon in their second year of life in a low visibility environment. Vehanen et al., (2000) showed that juvenile brown trout display seasonal changes in both cover habitat and type preference, suggesting that cover availability is particularly important during different seasons.

Increased habitat heterogeneity generally results in reduced visibility which may affect aggression levels between visually orientated and territorial species. Studies with pigs (Waran and Broom, 1993), cattle, and goats (Aschwanden et al., 2009a) have shown that provision of structures for hiding have a positive effect on aggression levels (Aschwanden et al., 2009b). Sometimes drastically so, as Whittington and Chamove (1995) showed that the provision of visual cover consistently reduced aggressive behaviour by roughly 60 % for female red deer (*Ceruus elaphus*). Eklöv and Greenberg (1998) argue that visual cover may also reduce aggression amongst fish, as Mortensen (1977a) showed that removing visual cover resulted in increased aggression amongst brown trout which was followed by high mortality. Studies on zebrafish, Japanese medaka (*Oryzias latipes*), and pearl cichlid have produced similar findings with reduced levels of aggression and resource monopolisation in complex habitats in comparison to simple environments (Basquill and Grant, 1998; Kadry and Barreto, 2010). Höjesjö et al., (2004) also reported fewer attacks by dominant fish with less visual perception of opponents due to physical barriers, in comparison to when enrichment was absent.

This begs the question; could the use of visual shelters reduce aggression levels amongst juvenile rainbow trout sharing confined space in laboratory experimental tanks? As mentioned previously, rainbow trout is a visually orientated, aggressive, territorial species and in laboratory tanks spacing between individuals is more restricted and constant than in the wild. Therefore, any plausible and practical way of reducing aggression in a laboratory setting would be desirable. For this reason, in the present study the effects of visual barriers

upon physiological and behavioural welfare indicators were examined in female juvenile rainbow trout housed in tanks comparable to those used in environmental regulatory research. It was hypothesised that visual barriers would enable subordinate individuals to get out of the line of sight from their aggressors, and subsequently decrease overall tank aggression and possibly increase fish welfare. A range of stress indicators were examined; brain monoamines, specific growth rates, condition factor, hepatosomatic index and fin damage. Aggression and swimming behaviour was studied through video footage.

4.2 Material and methods

See chapter 2.

4.2.1 Experimental animals

See section 2.1

4.2.2 Experimental protocol

See section 2.2 for general methods and materials.

Eighteen flow-through glass tanks of 40 litre volume were set up applying the 215-OECD guideline, as mentioned in section 2.2. Initial mean mass was 4.18 ± SD 0.46 g (*N = 144*) and initial mean fork length was 71 ± SD 2.74 mm (*N = 144*). All tanks were completely barren for the first 14 days, but on day 14 six tanks received two vertically positioned opaque glass barriers. Another six tanks received two transparent barriers structurally identical to the visual barriers, and a further six tanks were kept barren, see figure 4.1. The study was continued for another two weeks. Each transparent barrier was made from a clear sheet of glass. The visual barriers were made from two glass sheets silicone sealed together but with a layer of grey paint applied to their inner sides. All glass barriers had the following dimensions; height 29 cm, length 21 cm, width 0.5 cm. On day 14 fish were also measured for mass and length and the food ration was recalculated to ensure a constant ration.

Throughout the experiment six tanks were filmed for 10 minutes each before the morning feed and equally before the afternoon feed, during which time the aquarium room was closed to co-workers. Day 28 and 29 nine tanks were terminated respectively and body measurements, blood and tissue samples were obtained, see section 2.2.

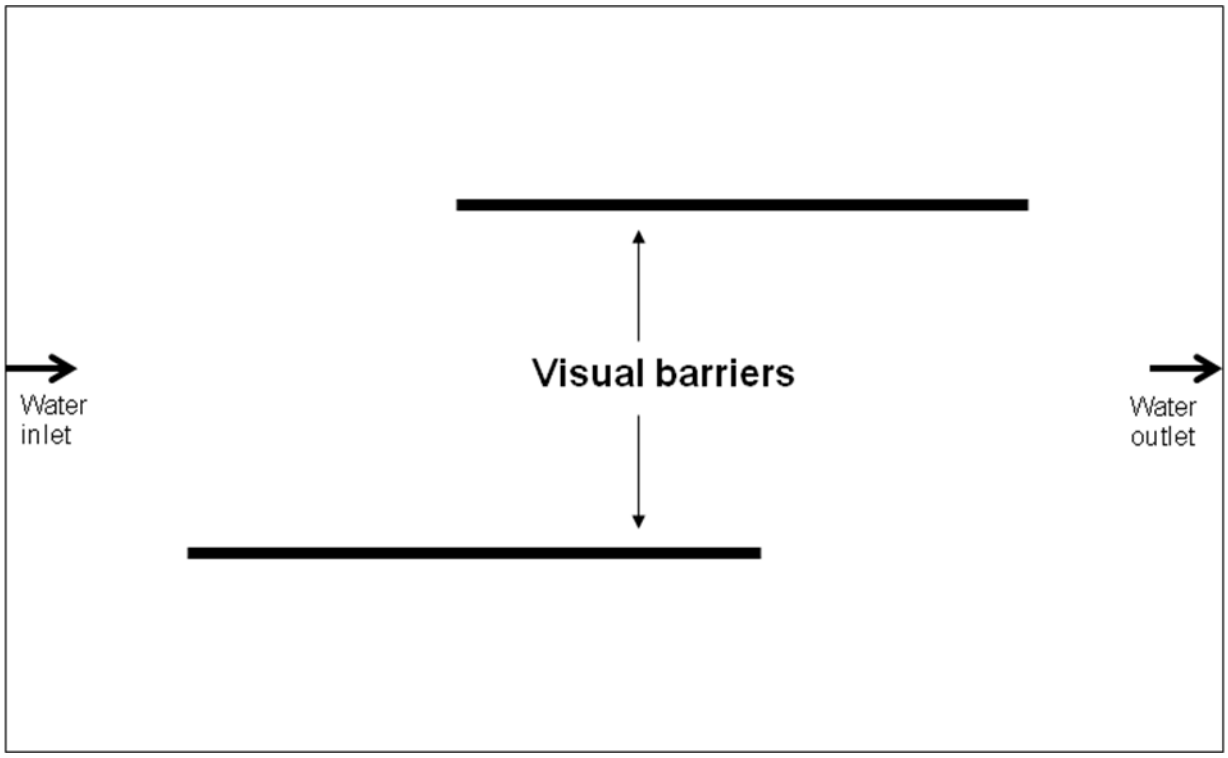

Figure 4.1. Schematic representation of tank design of visual barriers study viewed from above. Visual and transparent barriers were positioned in the same way, not by scale.

4.3 Endpoints and statistical analyses

4.3.1 Mass and length specific growth rates

For further details about calculations of indices see section 2.5.1 Mass specific growth rates were analysed for differences within and between treatments by ANOVA general linear models nested design, e.g. day 0 to 14 versus day 14 to 28 for barren, barrier, and visual barrier tanks kept separately. Length specific growth rates were log transformed before analysis in order to meet parametric assumptions. Additionally, mass specific growth rates were analysed for differences between ranks by keeping rank as a fixed factor in the general linear model analysis.

4.3.2 Condition factor

Condition factors were calculated for day 0 and 28 of the study, and analysed by ANOVA general linear models nested design for differences between and within treatments, e.g. CF day 0 versus CF day 28 for barren, barrier and visual barrier tanks kept separately.

4.3.3 Hepatosomatic index

Hepatosomatic index was calculated with liver mass as a percentage of body mass, and data was analysed for differences between treatments as well as between ranks by ANOVA general linear model nested design.

4.3.4 Fin damage

Pectoral and dorsal scores for day 0 and 28 were analysed by ANOVA general linear model nested design between as well as within treatments, e.g. day 0 versus day 28 for barren, barrier and visual barrier tanks kept separately.

4.3.5 Behavioural endpoints

For details about behavioural measurements see section 2.3. Frequency data of aggressive interactions for day 0 to 14 and day 14 to 28 within and between treatments was square root transformed and analysed by one way ANOVAs.

4.3.6 Brain monoamines

For details about the analysis of brain monoamine concentrations see section 2.4.2. Data for monoamine and metabolite concentrations as well as serotonin turnover in the telencephalon, optic tectum, brain stem and hypothalamus were log or square root transformed before analysed by ANOVA general linear models nested design. However, in this study brain monoamines have been standardised against wet mass.

4.4 Results

4.4.1 Mass and length specific growth rates

There were no significant differences in mass SGR between treatments day 0 to 14 or day 14 to 28 ($p = 0.261$, df = 2, 118 and $p = 0.891$, df = 2, 118 respectively). However, fish in all treatments had significantly lower mass SGR day 0 to 14 in comparison to day 14 to 28 within treatments; barren tanks ($p = 0.06$, df = 1,77), barrier tanks ($p = 0.018$, df = 1, 75), and visual barrier tanks ($p = 0.044$, df = 1, 77), see figure 4.2.

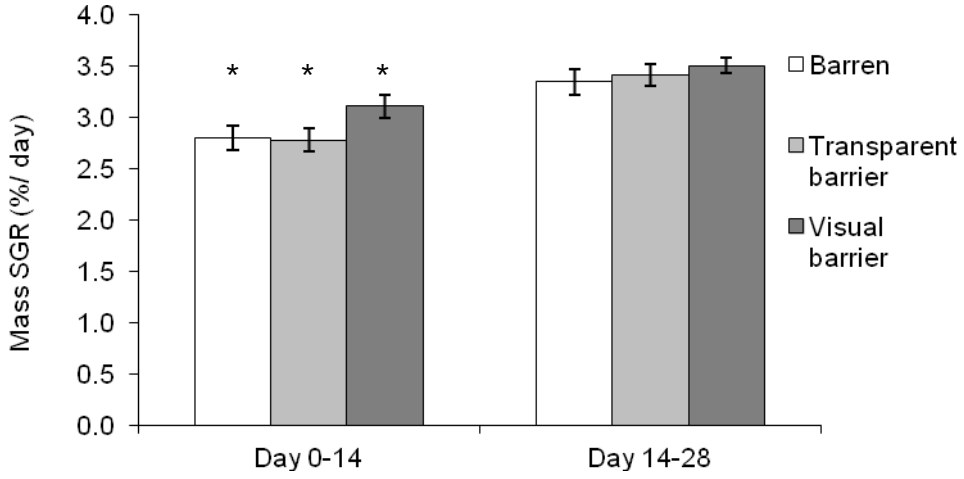

Figure 4.2. Mean mass specific growth rate expressed as percentage mass gain per day in grams ± SE, day 0 to 14 and 14 to 28 in barren, transparent barrier and visual barrier tanks. Asterisks indicating the significantly difference in mass SGR within treatments, i.e. between time period day 0 to 14 and day 14 to 28 for barren, barrier and visual barrier tanks kept separately.

There were no significant differences in length specific growth rates between treatments day 0 to 14 or day to 28 ($p = 0.529$, df = 2, 118 and $p = 0.979$, df = 2, 118, respectively). However, the same trend as for mass SGR was also evident for length SGR i.e. all treatments had a lower length SGR day 0 to 14 in comparison to day 14 to 28 within treatments. Barren ($p = 0.003$, df = 1, 77), barrier tanks ($p = 0.001$, df = 1, 75), visual barrier tanks ($p = 0.003$, df = 1, 77).

4.4.2 Condition factor

There were no significant differences in condition factors between treatments on day 0 ($p = 0.059$, df = 2, 118) or 28 ($p = 0.906$, df = 2, 118). Nevertheless, there was a significant increase in condition factors within all treatments between day 0 and 28. Trout in barren and visual barrier tanks had a 16 % greater condition factor day 28 ($p < 0.001$, df = 1, 77, and $p < 0.001$, df = 1, 77, respectively). Condition factors in fish in the barrier treatment followed suite with an 11 % increase in condition factor over time ($p < 0.001$, df = 1, 77).

4.4.3 Hepatosomatic index

Fish in transparent barrier tanks had a 9 % lower mean HSI than trout in barren tanks ($p = 0.202$, df = 2, 118), but not in comparison to individuals in visual barrier tanks, see figure 4.3. There were no significant differences in HSI with regards to rank ($p = 0.397$, df = 7, 188).

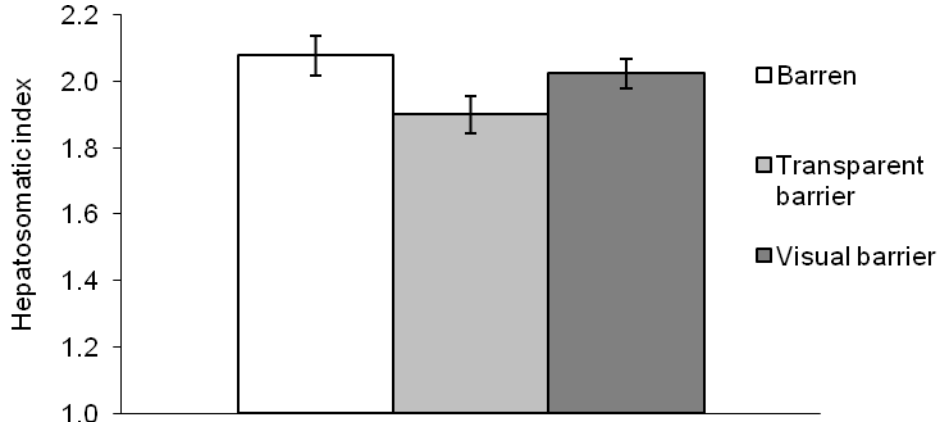

Figure 4.3. Mean HSI in percentage ± SE in trout from barren, transparent barrier, and visual barrier tanks.

4.4.4 Fin damage

There were no significant differences between treatments in dorsal fin score day 0 and day 28 ($p = 0.719$, df = 2, 118 and $p = 0.823$, df = 2, 118 respectively). The dorsal fins score remained stable over the first 14 days in all three treatments, only to increase more

drastically over the next fortnight. The mean dorsal score day 0 in barren tanks was 23 % higher day 28 in comparison to day 0 ($p = 0.090$, df = 1, 77). Trout in tanks with transparent barriers had a 17 % increase in dorsal fin deterioration ($p = 0.168$, df = 1, 75). Whilst fish in visual barrier tanks had a 21 % increase over time ($p = 0.169$, df = 1, 77), see figure 4.4. There were no significant differences in pectoral fin damage between treatments day 0 or day 28 ($p = 0.531$, df = 2, 118 and $p = 0.620$, df = 2, 118 respectively). Nor were there any differences within treatments in pectoral fin damage, day 0 versus day 28, barren ($p = 0.886$, df = 1, 77), barrier tanks ($p = 0.921$, df = 1, 77), and visual barrier tanks ($p = 0.787$, df = 1, 77).

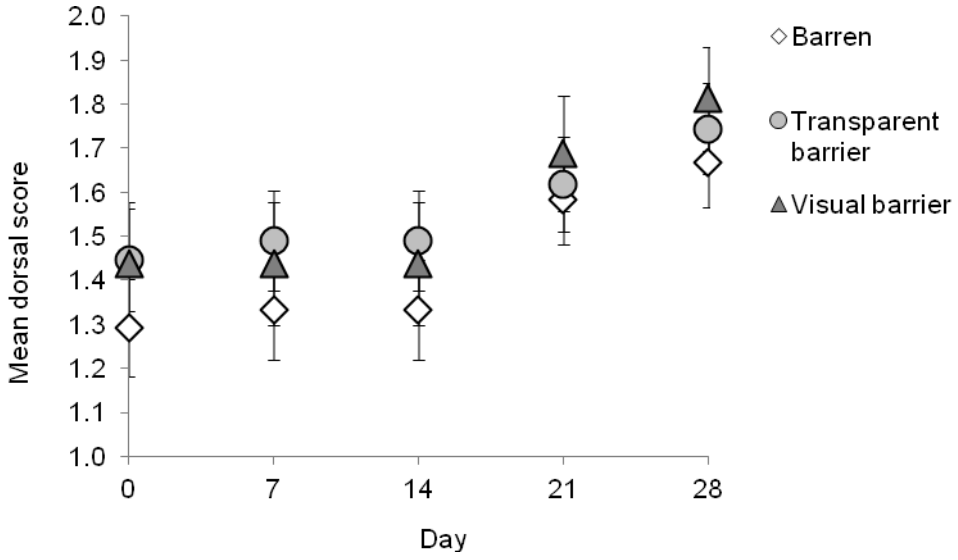

Figure 4.4. Mean dorsal score ± SE for barren, transparent barrier and visual barrier tanks day 0, 7, 14, 21 and 28.

4.4.5 Behaviour

Mean values for aggression frequency ranged from 3.5 to 5.5 aggressive acts per minute, across all the treatments and time periods.There were no significant differences between treatments day 0 to 14 ($p = 0.102$, df = 2, 117), or day 14 to 28 ($p = 0.622$, df = 2, 112). Aggression frequency within treatments was also analysed for day 0 to 14 versus day 14 to 28, barren tanks ($p = 0.311$, df = 1, 78), barrier tanks ($p = 0.589$, df = 1, 73), and visual barrier tanks ($p = 0.717$, df = 1, 78), see figure 4.5.

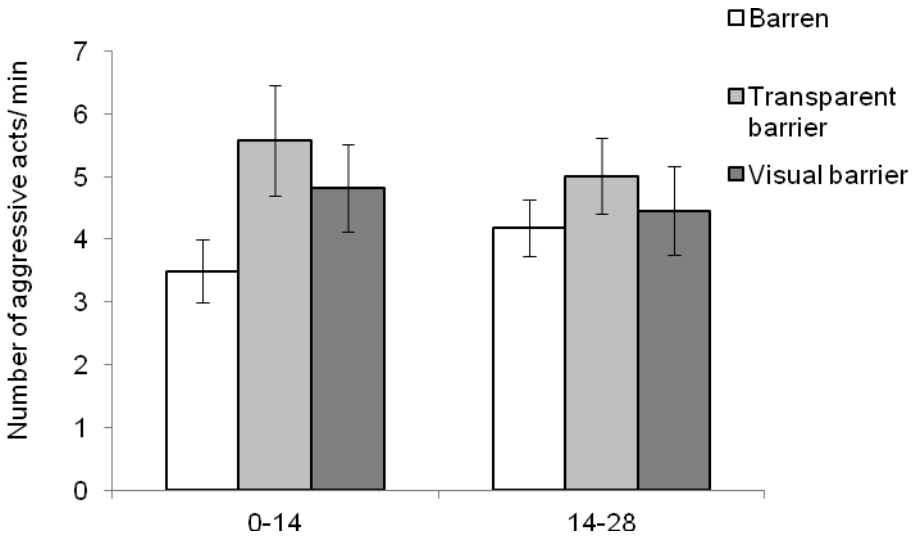

Figure 4.5. Mean frequency of aggressive acts ± SE day 0 to 14 and 14 to 28 in barren, transparent barrier, and visual barrier tanks.

Behaviour and growth

Since aggression is a reliable indicator of dominance in rainbow trout the numbers of aggressive acts made and received were taken into consideration when rank was assigned. It became apparent that the order of entering tanks did not influence which individual turned out to be the most dominant or subordinate. In contrast, in 14 out of the in total 18 tanks it was one of the two largest fish (based on their initial mass) that became dominant.

A closer look at mass growth rate in relation to rank showed that fish in all three treatments day 0 to 14 had a trend of decreasing mass specific growth rate with decreasing dominance status, see figure 4.6. Fish generally grew better regardless of rank, on day 14 to 28 than day 0 to 14, see figure 4.6 and 4.7. On day 14 to 28 there was a more even spread of mass growth between ranks for all treatments, see figure 4.7.

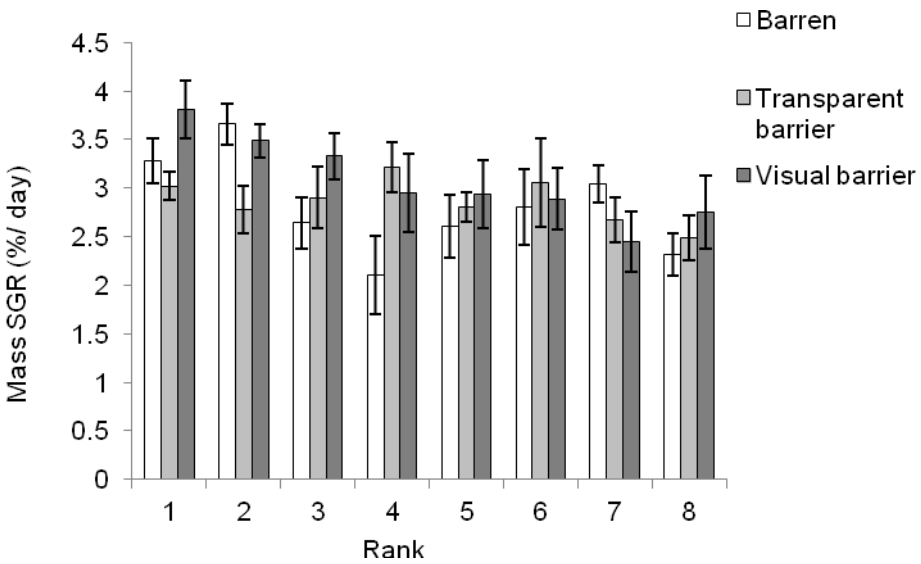

Figure 4.6. Mean mass specific growth rate day 0 to 14 ± SE expressed as % of body mass per day, versus dominance rank for all treatments.

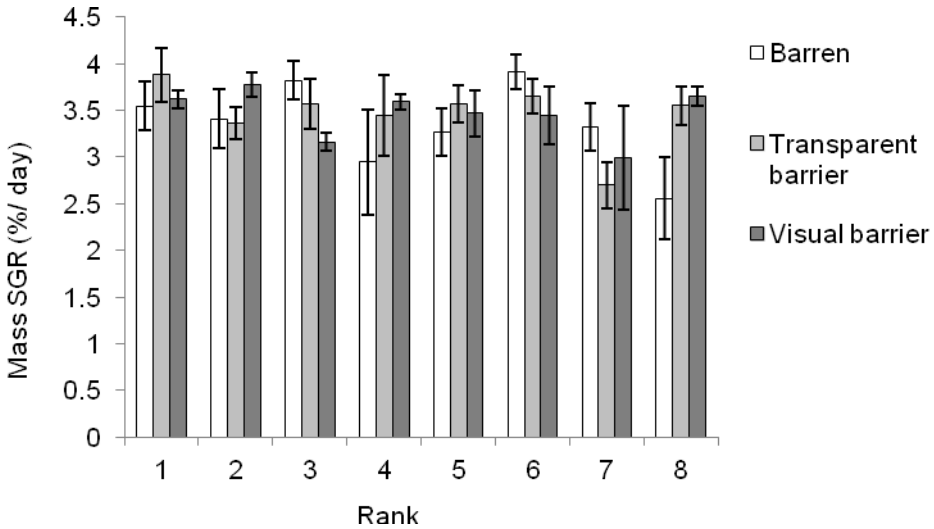

Figure 4.7. Mean total mass SGR day 14 to 28 expressed as % of body mass per day ± SE versus rank in all treatments.

4.4.6 Brain monoamines

Telencephalon

There were no significant differences in concentrations for any of the monoamines, nor serotonin turnover between any of the treatments. DA ($p = 0.371$, df = 2, 116), NA ($p = 0.752$, df = 2, 117), 5-HIAA ($p = 0.231$, df = 2, 117), 5-HT ($p = 0.571$, df = 2, 116), and serotonin turn over ($p = 0.472$, df = 2, 117). However, there was a weak trend towards fish in transparent barrier environments having higher brain monoamine concentrations than trout in barren and visual barrier tanks, see figure 4.8. For all four monoamines, fish from tanks with transparent barriers had the highest mean values, which on average was 10, 9, 30 and 22 % greater than that of the next highest group for dopamine, noradrenaline, 5-HIAA and serotonin respectively.

Optic tetctum and brain stem

There were no significant differences between treatments for any of the monoamine conentrations nor serotonin turn over in the optic tectum or brain stem. Optic tectum, DA ($p = 0.326$, df = 2, 115), NA ($p = 0.995$, df = 2, 118), 5-HIAA ($p = 0.898$, df = 2, 115), 5-HT ($p = 0.758$, df = 2, 115), and serotonin turn over ($p = 0.649$, df = 2, 115). Brain stem results, NA ($p = 0.699$, df = 2, 115), NA ($p = 0.678$, df = 2, 115), 5-HIAA ($p = 0.904$, df = 2, 115), 5-HT ($p = 0.806$, df = 2, 115), and serotonin ratio ($p = 0.458$, df = 2, 115). Although, as in the telencephalon there was a weak trend towards fish in transparent barrier environments having higher monoamine concentrations. For three of the measured monoamine concentrations, fish from tanks with transparent barriers had the highest mean values, which on average was 27, 6, and 5 % greater than that of the next highest group for dopamine, noradrenaline and serotonin respectively, see figure 4.8.

Hypothalamus

Fish in tanks with visual barriers had a 34 and 28 % lower hypothalamic dopamine concentration in comparison to trout in barren and transparent barrier tanks respectively ($p =$

0.003, df = 1, 70, and $p = 0.06$, df = 1, 71, respectively), see figure 4.8.They also had a 34 and 20 % lower noradrenaline concentration in the hypothalamus in comparison to individuals in barren and transparent barrier tanks respectively ($p = 0.031$, df = 1, 71 and $p = 0.116$, df = 1, 71), see figure 4.9. Serotonin concentrations followed suite with 39 % lower concentrations in fish from visual barrier tanks in comparison to trout from barren tanks ($p = 0.053$, df = 1, 70,) see figure 4.8. However, there were no significant differences between treatments for 5-HIAA concentrations ($p = 0.812$, df = 2, 109) or serotonin turn over ($p = 0.447$, df = 2, 106), but the trend was the same as for dopamine, noradrenaline and serotonin.

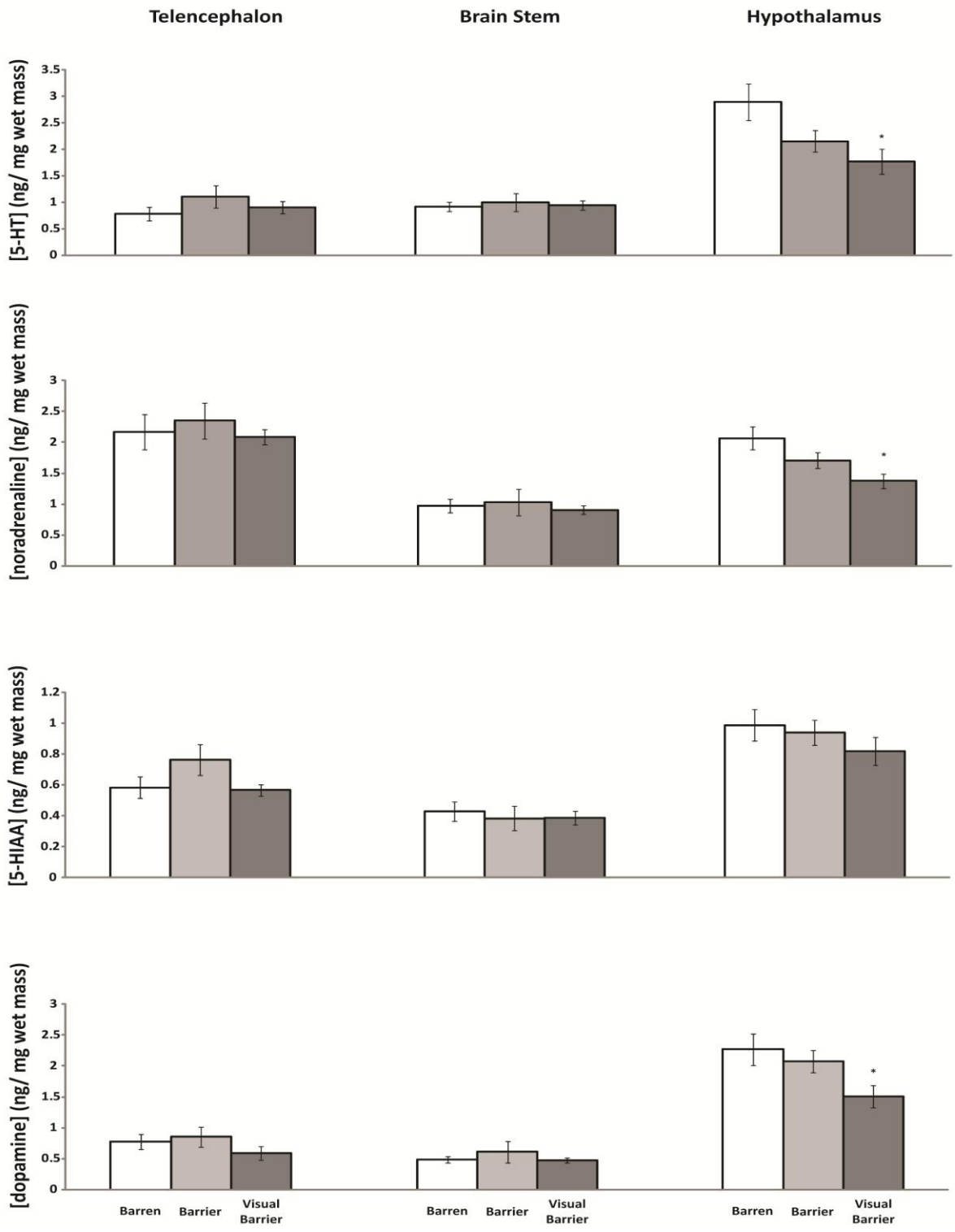

Figure 4.8. Mean dopamine (DA), noradrenaline (NA), 5-HIAA and serotonin (5-HT) concentrations in ng/mg wet weight ± SE in the telencephalon, brain stem and hypothalamus in trout from barren, transparent barrier and visual barrier tanks. Since monoamine concentrations in the optic tectum were not significantly different between treatments this data has been excluded. Asterisks signify significant differences in 5-HT, NA and DA levels between fish in barren and visual barrier tanks.

4.5 Discussion

In the present study, effects of reduced visual access to conspecifics in the same tank upon physiological and behavioural welfare indicators in juvenile rainbow trout were tested. Habitats with low visual contact between individuals have been suggested to reduce aggression for a range of various species and I have shown that this could potentially hold true for juvenile rainbow trout as well. I analysed mass and length specific growth rates, condition factors, hepatosomatic index, fin damage, aggressive behaviour and brain monoamines, and these endpoints will be discussed in order below, demonstrating that visual barriers possibly are beneficial to trout.

Specific growth rates and condition factors

Prior to the onset of enrichment there were no significant differences in mass or length specific growth rates between treatments, and neither was there a difference in mass or length specific growth rates during the enrichment period between barren, transparent barrier and visual barrier tanks. However, there was an apparent trend towards trout having a lower, mass specific growth rate day 0 to 14 in comparison to day 14 to 28 within treatments, which probably reflects fish acclimatising to the new environment.

As discussed previously the condition factor is body mass divided by the cube of body length, and changes in the condition factor reflect the fish's nutritional or energy status, hence a decrease in condition factor is generally considered to indicate a reduction of energy stores (Goede and Barton, 1990). In the present study there were no significant differences between treatments with regards to condition factors. Fish condition increased over time indicating an improvement in wellbeing as the study prolonged in all treatments. On the whole, specific growth rates and condition factors showed there was no difference in trout welfare between the three treatments. Either the environmental change did not affect fish to such an extent it impacted specific growth rates and or condition factor, or these endpoints are not sensitive enough measures for this particular enrichment strategy.

Hepatosomatic index

The hepatosomatic index is used as a stress indicator (Pickering and Pottinger, 1995), and Barton et al., (1987) showed that stressed juvenile rainbow trout have a decreased hepatosomatic index. I found that trout in transparent barrier tanks had a 9 % lower hepatosomatic index than trout in barren tanks, and 6 % lower HSI compared to fish in visual barrier tanks. This indicates that fish in tanks with transparent barriers experienced the highest stress levels. However, this finding was not supported by specific growth rates, condition factor, or any behavioural endpoints. On the other hand, brain monoamine levels in the telencephalon and brain stem showed weak trends that possibly supports this finding. Still, monoamine results were not significant and hypothalamic monoamine levels do not back this trend for the hepatosomatic index.

It was observed that in transparent barrier tanks, dominant individuals appeared to become more agitated when trying to attack others unsuccessfully through the barriers. Dominants did not give up as they could see the receiving fish, and when it finally navigated around the transparent barrier the impact appeared to be of higher intensity than the aggressive attacks observed in barren or visual barrier tanks (personal observation). It would be of interest to reanalyse the footage once more, with an ethogram aimed at aggression intensity.

Fin damage

Not only does aggressive behaviour stress fish but it is also a major cause of injuries. Injuries from agonistic behaviour to eyes and fins may cause secondary infections and mortality (Ashley, 2007), and attacks such as nips to the fins is common in rainbow trout with dorsal and caudal fins receiving most of the nipping (Abbott and Dill, 1985). I did not find any significant differences between dorsal or pectoral fin damage between treatments throughout the study, which probably reflects the fact that there were no differences in the amount of aggressive interactions. This is in accordance with the previous study. However, dorsal fin condition deteriorated significantly over time in all three treatments especially from day 14

onwards, which is expected as aggressive interactions did not decline enough over time for fin regrowth to occur.

Brain monoamines

I found no significant differences in brain monoamine concentrations or serotonin turnover between any of the treatments in the telencephalon, optic tectum or brain stem. However, in trout from tanks with visual barriers the hypothalamus had markedly lower dopamine and noradrenaline concentrations than that of fish in barren and transparent barrier tanks, and serotonin levels were also lower. Despite non significant results for hypothalamic serotonin turn over and 5-HIAA concentrations, they followed the same pattern as other monoamines.

These changes indicate that fish in tanks with visual barriers were exposed to lower chronic social stress levels in comparison to fish in barren tanks, as hypothesised based on subordinates being able to avoid visual contact from their aggressors. The social environment can be a considerable source of stress for a range of animals including fish (Fernandes-De-Castilho et al., 2008), and it is acknowledged that social stress may be even more multifaceted than stress from environmental variables (Zayan, 1991). Additionally, it appears as if trout kept in a low visibility environment may have experienced lower levels of aggression shown by the lower dopamine concentrations. I would normally predict that lower dopamine levels seen in trout in visual barrier tanks would be associated with lower aggression. However, this does not match the aggression frequency recorded in these fish, which was not significantly different from other treatments. This mismatch and the interplay between dopamine and other neurotransmitters will be discussed below.

Serotonin, dopamine and noradrenaline

In contrast to serotonin, dopamine is involved in behavioural activation, motivation and reward, but also has a central role in the modulation of aggressive behaviours (Seo et al., 2008). In humans, dopamine has been linked to aggression and recognition, and rodent

studies have shown elevated dopamine levels before, during, and following aggressive fights (Tidey and Miczek, 1996). In Arctic charr, dominant fish have elevated brain dopaminergic activity (Winberg et al., 1992b) and treatment with L-3,4-dihydroxyphenylalanine (L-DOPA), that elevates dopamine activity, increases the odds of fish becoming dominant in fights for social dominance between two size matched individuals (Winberg and Nilsson, 1992). Hence, elevated dopaminergic activity is generally an indication of greater aggressiveness and higher social status. Though, it is not a clear cut relationship as very high dopamine levels may even limit aggressive interactions (Höglund et al., 2005a).

Serotonin on the other hand brings calming control over aggressive behaviour (Summers et al., 2005a), and studies have shown that serotonin turnover is negatively related to aggression in lizards (Summers et al., 2005b), mammals and fish (Winberg and Lepage, 1998). The relationship between the calming influences of serotonin over aggression is not straight forward. Even though elevated serotonin levels in subordinate animals are likely to be key in mediating behavioural inhibition it is important to acknowledge that other neurotransmitter systems are also likely to be involved (Summers and Winberg, 2006).

Noradrenaline is released when a range of physiological changes are activated by a stressful event, which is caused by activation of an area of the brain stem (Ma, 1994). In mammals, it contains thousands of neurons, whilst in fish this nucleus contains few neurons (Ma, 1994). The significantly lower levels of noradrenaline in trout from a low visibility environment may also imply that this environment was less stressful, since stressed fish produce adrenaline and noradrenaline through the adrenergic response in the chromaffin tissue (Broom, 2007), which prepares it for exercise by raised ventilation rate, cardiac output, blood flow to gills and muscles, in addition to mobilising substrates for aerobic metabolism (Handy, 2003).

Monoamines and behaviour

If reduced dopamine and noradrenaline levels in fish from visual barrier tanks reflected lower chronic stress levels, then it was not expressed in decreased aggressive behaviour. This raises the question why there were no differences in aggressive interactions between treatments. Is it plausible that results are due to changes in the type rather than frequency of aggression? For example a decrease in subtle aggressive social interactions, communications too subtle to be recognised by utilised behavioural endpoints adopted in the present study. Aggressive behaviour amongst rainbow trout also entails signalling, as well as attack and fighting, and salmonids generally use a variety of cues to signal aggressive intent and social ranking. It is believed that aggressive intent is signalled through lateral display, fin erection and vigorous body movements (Berejikian et al., 1996; Keeley, 2000). McMahon and Hartman (1989) noted that Coho salmon (*Oncorhynchus kisutch*) defend sites primarily by erecting their dorsal fins.

As mentioned in the discussion of chapter three, when rainbow trout fight, fish circle each other and deliver nips and bites to the anterior part of the body of the other combatant (Ellis et al., 2002). If this behaviour escalates it may result in mouth wrestling, which was displayed in the semitransparent shelter study (chapter three). In the present experiment no mouth wrestling was observed but instead escalated aggressive behaviour occasionally resulted in dominant individuals pulling each other by the dorsal fins around the tank. This behaviour was observed once on video footage, but fish circling each other aggressively and disturbing the rest of the group was observed at several occasions in the laboratory as well as footage (12 out of ~230 film slots had fish aggressively circle each other).

Monoamines and enrichment

Results suggest that it was being out of view from conspecifics that produced the brain monoamine trends rather than the physical obstruction. This is based on the significant difference in dopamine and noradrenaline levels between fish in tanks with transparent

barriers and visual barrier tanks. Additionally, 5-HIAA and serotonin concentrations, as well as serotonin turnover followed the same pattern. This is highly plausible as animals often display their fighting ability using visual cues, and social interactions of this kind are able to significantly influence an animal's behaviour and physiology (Chen and Fernald, 2011). Colour patterns seem to play a particular part in controlling agonistic behaviour in fish, with salmonids such as rainbow trout and Atlantic salmon displaying social subordination by adopting a darker body colour which act as a signal to reduce aggression from dominant fish (Abbot et al., 1985; O'Connor et al,. 1999).

In the wild, territorial animals often use the complexity of their habitat to avoid social contact, which is something fish in a laboratory setting usually cannot do. Interactions between conspecifics are dynamic processes for many fish species, where dominant individuals have to maintain their status by physical attack or visual signs to subordinates that may be trying to achieve a higher rank in the hierarchy (Fernandes-de-Castilho et al., 2008). Hence, cover availability is an important feature of natural environments providing animals with the opportunity to hide from aggressive conspecifics by lowering visual contact and reducing animal communication (Estep and Baker, 1991; Cornetto et al., 2002). It seems reasonable to assume that in a low visibility environment, territory holders' i.e. dominant individuals may increase their activity levels or position themselves strategically in order to maintain visual contact and keep subordinates under control. In the present study dominant individuals quickly assessed which was the most favourable tank location with regards to keeping other fish under control by minimum effort involved (personal observation). Dominant fish positioned themselves at the front of the tank where by moving along the short end of the tank to the left and right, they would get an overview of other individuals. Nevertheless, they could still not see the entire tank at one single position, see figure 4.9. This behaviour was applied within a couple of hours of the addition of visual barriers to test tanks.

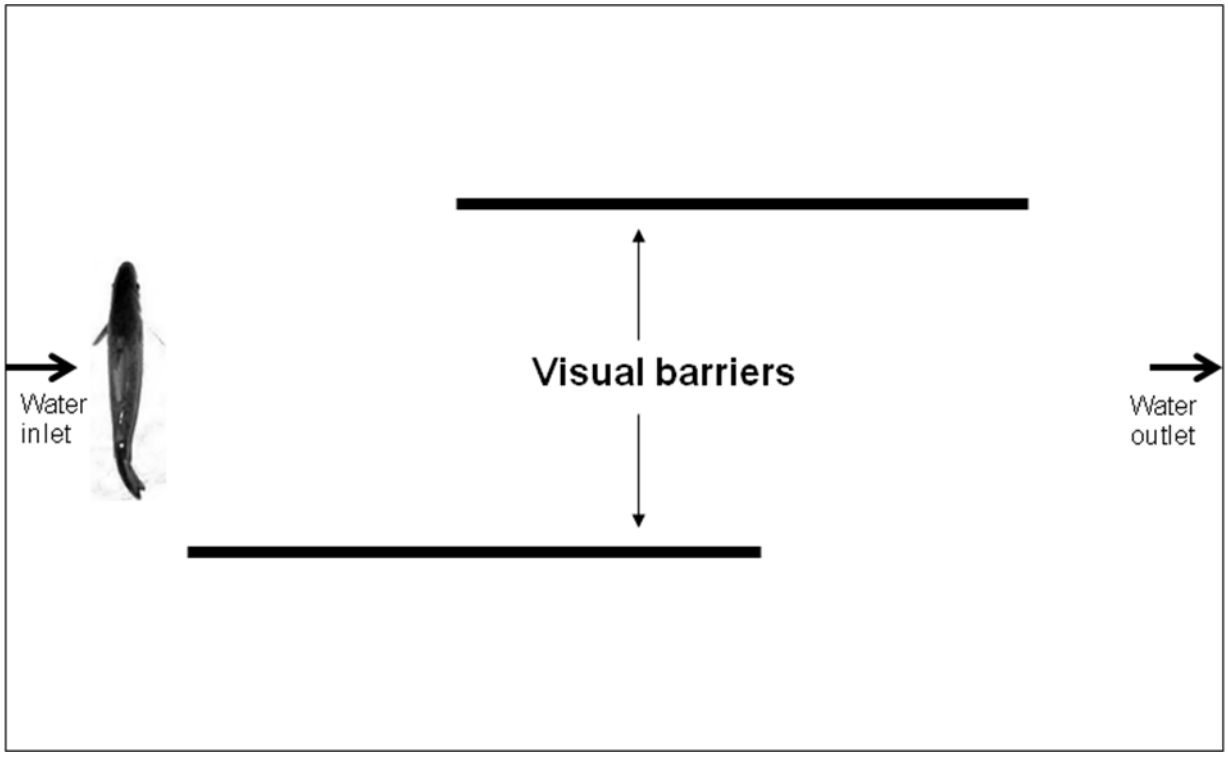

Figure 4.9. Top view of enrichment set up and position of dominant individuals in test tanks with visual barriers. Not to scale.

This phenomenon has been observed before as Kemp et al., (2005) reported that the addition of boulders did not affect territory size in salmon despite decreasing the visual field, because dominant fish positioned themselves on the top of boulders which aided them to maintain a good view of their habitat. Hence, positioning of visual barriers is a key aspect of the enrichment strategy. However, in the present study I did not have many options of where to locate visual barriers due to legislation within the UK for animal research that demands a free view of experimental animals in a test tank.

Dominance

It is feasible to assume that establishing a dominance hierarchy would entail an initial period of intense aggression, followed by reduced aggression over time once dominance is achieved. Findings from the present study back results from the previous one (chapter 3), in that there was no difference over time or between treatments with respect to the frequency

of aggression. Dominance was established based on the frequency of committed and received aggressive acts as aggression indices have been shown to be an effective measure of social rank (Bailey et al., 2000).

Alpha status (i.e. most dominant individual) was readily discernible and fairly stable whilst the status of subordinates was more difficult to define. Determinants of who becomes socially dominant have been suggested to include size, age, aggressiveness and prior residence (Harwood et al., 2003). It is believed that dominance hierarchies can be determined by fish size with the biggest fish being dominant (Sabo and Pauley, 1997). Results from the present study showed that the order of entering the tank did not determine who became dominant but the initial body mass did, as in 14 out of 18 tanks it was either of the two largest fish (in mass) that became dominant. Individuals of higher rank generally have a greater mass growth than fish of lower status. This trend was not prominent in the present study, which could suggest a more even spread of food.

Differences between top ranked fish and subordinates suggest that dominant trout consume larger amounts of food, implying a food monopoly which is well known amongst fish groups (Adams et al., 1995). It is generally accepted that feeding hierarchies correlate with dominance hierarchies in groups of fish, with uneven food access resulting in differential growth and size (Ellis et al., 2002). High ranked fish have greater food access and therefore grow faster than others (McCarthy et al., 1992). Also, social stress is believed to decrease foraging activity and aggression which naturally decreases food intake (Winberg, 1997). Reduced food intake in subordinate fish may also be due raised plasma cortisol levels (Gregory and Wood, 1999). It has been shown that dominance amongst brown trout is determined by body size, with dominant, aggressive, larger fish occupying the more favourable holding positions hence gaining more food than subordinates (Vehanen et al., 2000). However, as mentioned in previous discussion, Huntingford et al., (1990) proposed that the social hierarchy in salmonid fish may depend upon behavioural properties rather

than size. Hence, it is clear that what determines hierarchies in a fish group is not a straight forward subject. There are a range of endpoints that has been suggested to determine dominance such as resource holding power, hepatosomatic index (Guderley and Coutre, 2005), and standard metabolic rate (Metcalfe et al., 1995). However, in this case it appears as though the initial mass was at least part of the deciding factor determining dominance in our groups of juvenile rainbow trout.

Conclusions

In the present study I tested the effects of reduced visual access of conspecifics in the same tank, upon physiological and behavioural welfare indicators in juvenile rainbow trout. Habitats with less visual contact between individuals have been suggested to reduce aggression effectively, for a range of animal species. I have shown that adding vertical visual barriers could potentially be viewed as enrichment for juvenile rainbow trout, as brain monoamine results indicated lower chronic stress levels and potentially decreased aggressive interactions in trout from visual barrier tanks in comparison to barren environments. Interestingly, brain monoamine results may also point towards the fact that it is the visual barrier per se that produced the difference and not the physical obstruction. The location of enrichment may be of significance as it was apparent that dominant individuals quickly learnt how to best position themselves in order to control other fish in the tank. Furthermore, it became clear that brain monoamines were the only endpoints utilised in the present study that were sensitive enough to detect any stress trends associated with the proposed enrichment. I believe the addition of vertical visual barriers to test tanks warrants the extra cost and effort, as it appears to be the better choice with regards to fish welfare in comparison to current regulatory research, when trout are housed in completely barren tanks. Though, further studies on this subject are needed in order to refine the layout of the visual barriers. The addition of these visual barriers will increase tank area available for microbial growth, which is a factor that would have to be evaluated and accounted for in the OECD guidelines, as this could affect experimental results when testing chemicals.

CHAPTER 5 Effects of water currents induced by aeration on behavioural and physiological welfare indicators in juvenile rainbow trout

Abstract

Water currents are known to reduce aggression amongst fish. Hence, the present study focused on the effects of water currents as a potential form of enrichment upon welfare in juvenile rainbow trout. Water currents were produced in experimental tanks by airflow via airstones. High water current was produced using a large (95 mm long) airstone, and low water current by a small (25 mm long) airstone. Similar airflow rates (4.5-5 l/min) were applied to both sizes of airstone to minimise differences due to airflow per se, rather than the current created within the tank. Dissolved oxygen levels were not substantially influenced by aeration, as flow-through water replacement rates maintained >94 % oxygen even when aeration was absent. Since airflow was kept either on or off for a consecutive two week period within treatments, the period with no airflow acted as control. Findings suggest that water currents may be viewed as enrichment since enhanced mass specific growth rates indicated that presence of a water current (both low and high), may aid fish in coping with handling and new environment stress. The hepatosomatic index showed that fish with high water currents during the last fortnight of the study had greater energy reserves than fish with no current for the same time period. Additionally, it appeared as if currents possibly yielded an even food acquisition across the whole social hierarchy as middle and low ranked trout grew almost as well as dominants. However, this could potentially be explained by greater food assimilation or appetite as a result of fish undergoing more exercise, or that fish were kept on a fairly high food ration.

5.1 Introduction

Rainbow trout is a stream dwelling fish species, hence complex water movements are common in their natural environment. Water turbulence and altered flows, which are detected by fish through the lateral line, may have large effects upon swimming kinematics

and behaviours (Heggenes 2002, Liao et al., 2003). Water flow and turbulence affect fish by influencing swimming performance (Fish 1999, Liao et al., 2003a), habitat choice (Enders et al., 2003), as well as daily behavioural routines (Webb, 2002). Water flows with unpredictable or disorganised fluctuations in velocity may drive fish away whilst the opposite generally attracts them. The skill to manoeuvre water currents using body posture, fin orientation, and active movements is decisive to whether fish avoid turbulence or not (Liao, 2007). Turbulent flow is believed to break up swimming trajectories and possibly increases the cost of locomotion (Enders et al., 2003), hence it is self explanatory why fish would avoid this type of environment. On the other hand, it has been shown that fish are able to enhance their swimming performance by the ability to exploit unsteady flow conditions and thereby turning an environmental drawback into a benefit (Hinch and Rand, 2000), Studies upon trout swimming in experimentally generated vortices showed unique kinematics, termed the Kármán gait. This phenomenon is accompanied by a lower activity in red axial muscles (Liao, 2004), hence swimming in turbulent waters can sometimes be energetically less costly (Taguchi and Liao, 2011).

As mentioned previously a range of behaviours have been reported to be influenced by water currents, with one of them being habitat choice. For stream dwelling salmonids, water current velocity has been proposed to be the most important variable determining microhabitat selection (Fausch, 1993). Agonistic behaviour may also be affected as Vehanen et al., (2000) found that the frequency of aggression between juvenile rainbow trout decreased when they were exposed to high flows, since increased water velocity forced trout to swim instead of partaking in agonistic behaviour. Adams et al., (1995) came to a similar conclusion as they found that aggressive behaviour in Arctic charr (*Salvelinus alpinus* L.) was reduced by the introduction of water currents. However, studies have also reported low levels of aggression accompanied by low water flows. This is explained by the fact that juveniles of species such as the Atlantic salmon and brown trout are also found in still water (Erkinaro et al., 1998), where they display a different foraging tactic, i.e. they cruise for prey

instead of applying a sit and wait strategy and therefore have no territories to defend (Vehanen et al., 2000). Hence, water velocity and aggression may not simply have a negative linear correlation as this relationship probably depends upon the life history of an individual. However, it seems plausible that when fish are 'forced' to swim there is less time for fighting.

Considering existing literature, the addition of water currents to experimental test tanks was a plausible enrichment strategy for juvenile rainbow trout used in laboratories, in need of exploration. Additionally, it is challenging to create enrichment which does not interfere with either the rules of regulatory testing (e.g. OECD guidelines) or national policies regarding the use of animals in research (e.g. the Animals Scientific Procedures Act 1986 in the UK). For example, such rules include easy visibility of experimental animals in test tanks. The addition of different levels of airflow in test tanks could noticeably change the environment for the better as it resulted in different types of water currents within the tank, whilst maintaining a low impact upon local welfare related rules and regulatory testing requirements.

Aeration was only used for altering the physical water currents in the present study, and not for keeping 'normal' oxygen levels. Water oxygen levels were maintained at > 94 % without aeration by ensuring a flow-through of fully aerated water in excess of the requirements of the biomass within the tank. The aim of the present study was therefore to investigate aeration for its potential to produce localised water currents, and its practicality and effectiveness of improving fish welfare, since it would be an economical and straightforward method to apply for improving fish welfare by reducing aggression. Airstones and tubing are cheap consumable items and most laboratories will have easy access to compressed air or air pumps. Different experimental water currents were accomplished by the addition of either large or small airstones into test tanks. By applying a similar airflow to both sizes of airstone I generated treatments with nominally 'high' and 'low'water currents. Airflow was expected to produce not only water currents but also other types of stimuli which potentially could

improve welfare without interfering with scientific practice, such as vibrations and sound. Additionally, the introduction of currents and/or the air-bubbles themselves could also present an opportunity for 'play'.

It is fairly well established that water currents keep fish occupied by requiring more of an effort from the fish to remain stationary through swimming. Although swimming is energetically costly, studies have shown that when fish are exercised there is reduced aggression, enhanced food conversion efficiency, and improved growth rates (Houlihan and Laurent, 1987), as well as lower stress levels (Adams et al., 1995; Davison, 1997). Based on available literature and a pilot study within our laboratory (G. Milligan, J. Landin, R. Wilson, unpublished results) it was hypothesised that tanks with high water currents would have lower levels of aggression in comparison to tanks with low water currents, due to fish being occupied by water current induced activity, instead of agonistic behaviour. Indeed, the pilot study indicated markedly lower numbers of aggressive acts between rainbow trout in aquaria with high currents, whilst low currents produced a trend towards higher numbers of aggressive acts even in comparison to tanks with absence of any aeration. Thus, the present study investigated the effect of high and low water currents upon the welfare of juvenile rainbow trout, by analysing the following endpoints all considered useful when evaluating fish welfare: plasma cortisol concentrations, length and mass specific growth rates, condition factor, hepatosomatic index, fin damage, aggressive acts and interaction with enrichment.

5.2 Material and methods

See chapter 2.

5.2.1 Experimental animals

See section 2.1

5.2.2 Experimental protocol

See section 2.2 for general material and methods.

Twenty-four flow-through glass tanks of 40 litre volume were set up as mentioned in section 2.2, with a initial mean mass of 6.86 ± SD 1.36 g (*N = 192*), and a fork length of 83 ± SD 5.22mm (*N = 192*). Twelve tanks had large airstones (also referred to as high current treatment) attached to the bottom of the rear of the tank whilst remaining tanks had small airstones (low current treatment) attached the same way (only one airstone per tank). Airstones (Betta, J & K Aquatics Ltd, Taunton, UK) are porous 'stones' made of granular ceramics, that allow air to pass into fish tanks via a stream of bubbles. The strength of this stream of air-bubbles can be modified by adjusting the flow rate of the air supply. The different sized airstones were randomly allocated to test tanks and secured to the bottom rear of the tanks, using suction cups coated with silicone sealant. Hence there was a gap of approximately 1 cm between the tank bottom and the airstone. For low current tanks cylindrical airstones (15 mm diameter x 25 mm long) were used, while oblong airstones (95 mm long x 20 mm wide x 18 mm high) were used in high current tanks.

All airstones were connected to an air supply via individual valves to allow for precise control of air flow. The airflow through large and small airstones were similar, ranging from 4.5 to 5.0 l/min. Streams of air-bubbles emerged over the entire surface of the airstones with a strength that created subtle waves on the water surface, though mainly in the high current treatment. Despite airflow being very similar, water currents generated in the high and low treatments were visibly very different which were the result of the different lengths and the horizontal positioning of the airstones. Half of the large and small airstones were switched on for the first 14 days (labelled A-ON and C-ON; figure 5.1) whilst the other half remained switched off for the first 14 days (B-OFF and D-OFF; figure 5.1). On day 14, in tanks that had previously received currents, airstones were switched off (A-OFF and C-OFF; figure 5.1), and the other tanks were then switched on (B - ON and D – ON; figure 5.1), and the

study continued for another two weeks. Day 0 to 14 of the study will from now on be referred to as ‘first half’, and day 14 to 28 as the ‘second half’.

On day 14 fish were also removed from test tanks and measured for mass and length, and the food ration was recalculated to ensure a constant ration. Throughout the 28 days six tanks were filmed for 10 minutes each before the morning and afternoon feed, during which time the aquarium room was closed to co-workers. Fish from twelve tanks (i.e. half of the two treatments) were terminated on day 28, and fish from the other 12 tanks were terminated on day 29, and body measurements, blood and tissue samples were obtained, see section 2.2. The existing aeration regime was continued throughout day 28 and 29 for tanks sampled the last day.

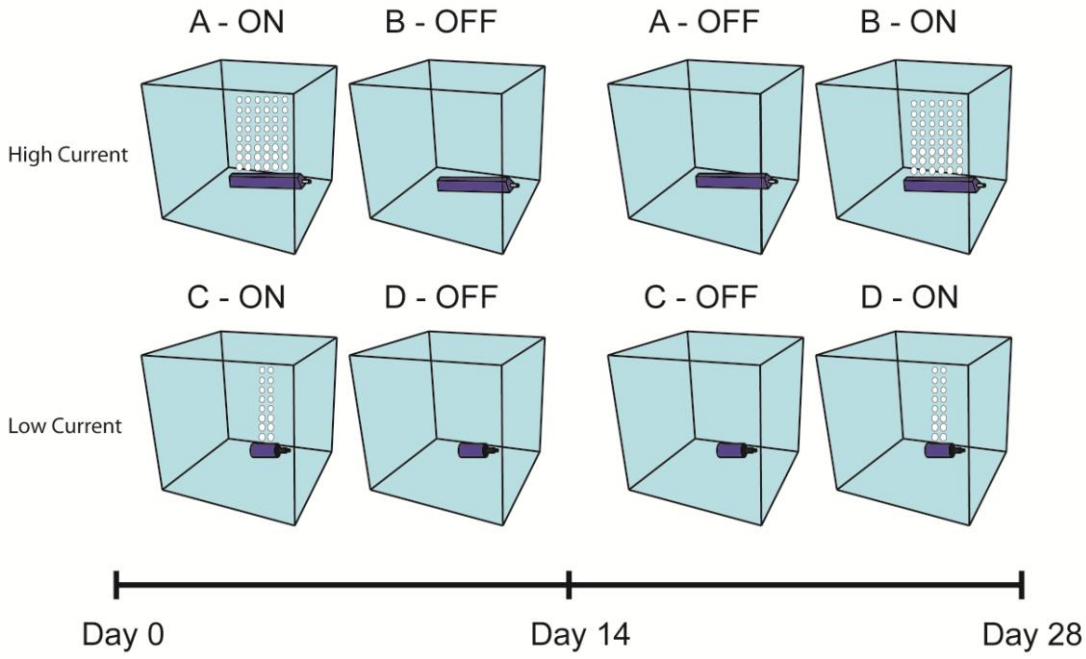

Figure 5.1. Schematic representation of enrichment strategy. Six tanks had large airstones switched on day 0 to 14 (A-ON) and in six tanks with large airstones the airflow was off day 0 to 14 (B-OFF). The same set up applied to the 12 tanks with small airstones (C-ON, D-OFF). On day 14 this was reversed and switched on airstones were turned off (A-OFF, C-OFF) and vice versa (B-ON, D-ON), and the study carried on for another 14 days.

Oxygen levels were measured by a Strathkelvin oxygen meter (Strathkelvin Instruments Limited, Glasgow) throughout all the three studies to ensure that sufficient levels were supplied. However, in the present study it also served to make sure there were no large differences in oxygen supply between treatments when airstones were on respectively off. Data representative of the entire set have been reported below in table 5.1.

Table 5.1 Mean oxygen levels ± SE in the different treatments when airstones were on respective off.

	Oxygen level day 0 ± SE	Oxygen level day 17 ± SE
Low current first half (day 0-14)	99.8 ± 0.2	94.3 ± 0.6
Low current second half (day 14-28)	97.2 ± 0.3	99.8 ± 0.2
High current first half (day 0-14)	100 ± 0.2	93.7 ± 0.6
High current second half (day 14-28)	97 ± 0.6	99 ± 0.2

5.3 Endpoints and statistical analyses

5.3.1 Mass and length specific growth rates

For further details about calculations of indices see section 2.5.1 Mass and length specific growth rates were analysed for differences within and between treatments by ANOVA general linear models nested design, e.g. day 0 to 14 versus day 14 to 28 for high and low flow tanks kept separately. The model structure was as follows: endpoint = tank (treatment) rank treatment. Tank and rank were specified as random factors, except in initial explorations of the role of rank where it was defined as a fixed factor.

5.3.2 Condition factor

Condition factors were calculated for day 0 and 28 of the study, see section 2.5.2 for calculation of indices. Data were square root transformed and analysed by ANOVA general linear models nested design for differences between and within treatments, e.g. CF day versus CF day 28 for low and high current tanks kept separately.

5.3.3 Hepatosomatic index

Hepatosomatic index was calculated with liver mass as a percentage of body mass, and data was square root transformed and analysed for differences between treatments as well as between ranks by ANOVA general linear model nested design.

5.3.4 Fin damage

Pectoral and dorsal scores for day 0 and 28 were analysed by ANOVA general linear model nested design, between as well as within treatments, e.g. day 0 versus day 28 for low and high currents kept separately.

5.3.5 Plasma cortisol

Cortisol concentrations were square root transformed and analysed for differences between treatments using ANOVA general linear models nested design. Based on the fact that the quality of the plasma samples for some unknown reason was not as good as expected, and that the standard curve for the ELISA assay was not as reliable as desired at low concentrations, samples lower than 2.5 ng/ml has been treated as below detectable limits. For further details about cortisol analysis see section 2.4.1.

5.3.6 Behavioural endpoints

For details about behavioural measurements see section 2.3. Frequency data of aggressive interactions for day 0 to 14 and day 14 to 28 within and between treatments was square root transformed and analysed by one way ANOVAs.

5.4 Results

5.4.1 Mass and length specific growth rates

There were no significant differences in mass specific growth rates between tanks with high or low water currents between the first ($p = 0.741$, df = 3, 154) or second half ($p = 0.709$, df = 3, 154). However, all treatments had lower mass specific growth rates the first half in comparison to the second half within treatments. Though this finding was only significant, or nearly so, for treatments with no current during the first half. Fish in tanks with low current during the second half had a 21 % greater mass specific growth rate when they were supplied with low water current ($p = 0.063$, df = 1, 63), see figure 5.2. In the treatment with high current during the second half fish had a 26 % greater mass specific growth rate when water current was supplied ($p = 0.020$, df = 1, 77), see figure 5.3. The increase in mass specific growth rate in the other two treatments between first and second half was 8 and 9 % in comparison. Low current first half ($p = 0.271$, df = 1, 77) and high current first half ($p = 0.526$, df = 1, 77).

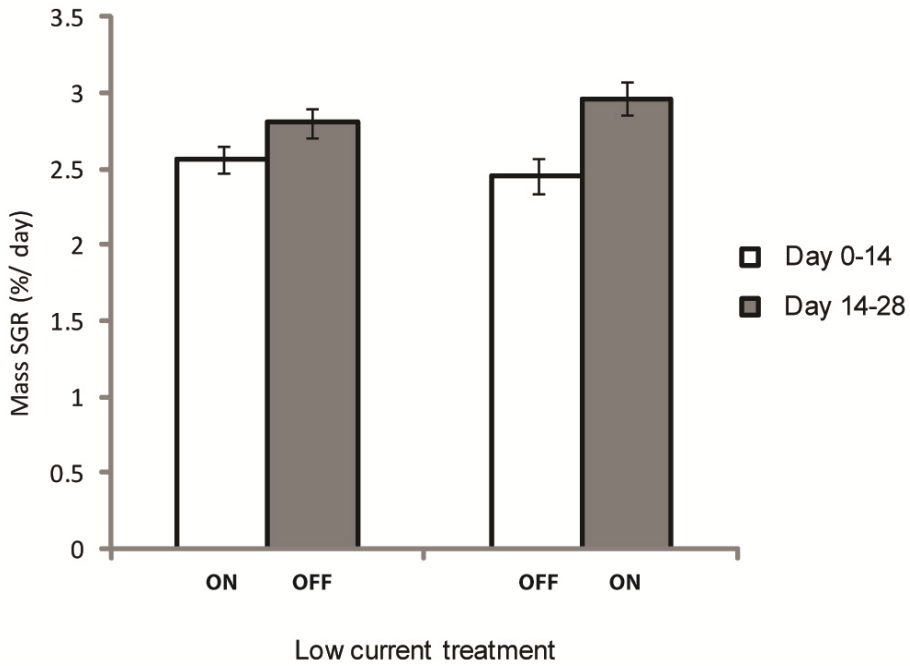

Figure 5.2. Mean mass specific growth rate (SGR) expressed as percentage body mass per day ± SE in tanks with low water current for the two different time periods.

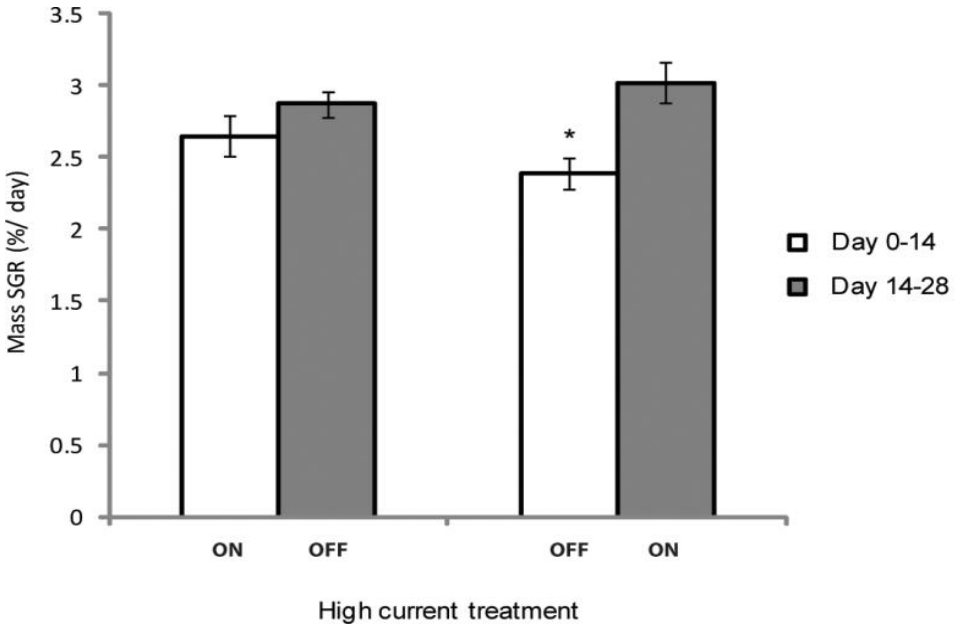

Figure 5.3. Mean mass specific growth rate (SGR) expressed as percentage body mass per day ± SE in tanks with high currents for the two different time periods. Asterisk indicates the significant difference in mass SGR in fish who had high current in the second half.

There were no significant differences in length specific growth rates between tanks with high or low water currents between the first ($p = 0.496$, df = 3, 154) or second half ($p = 0.500$, df = 3, 154). However, tanks with low current treatment had a 2.1 and 2.5 fold greater length growth rate during the second half ($p< 0.001$, df = 1, 77 and $p < 0.001$, df = 1, 63) see figure 5.4. Whilst high current tanks had a 1.9 and 2.8 fold greater length growth rate during the second half of the study ($p = 0.001$, df = 1, 77 and $p < 0.001$, df = 1, 77) see figure 5.5.

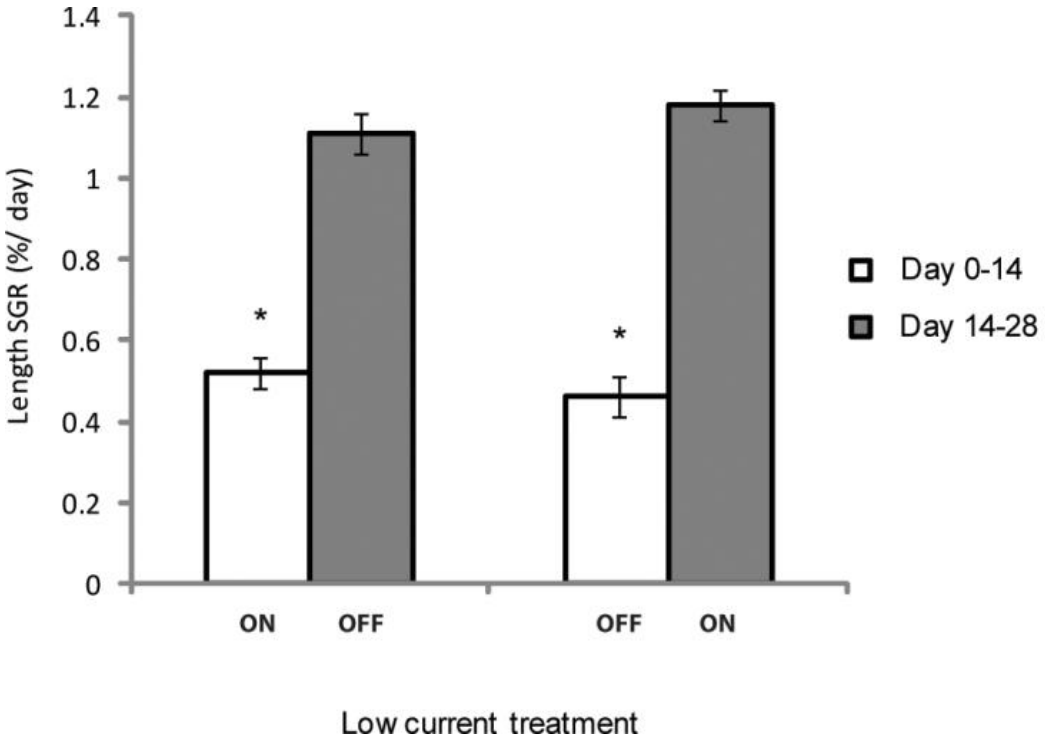

Figure 5.4. Mean length specific growth rate ± SE expressed as % length gain in mm/ day in tanks with low current during the two time periods. Asterisks indicate the significant difference within treatments between the first and second half (day 0 - 14 and day 14 - 28).

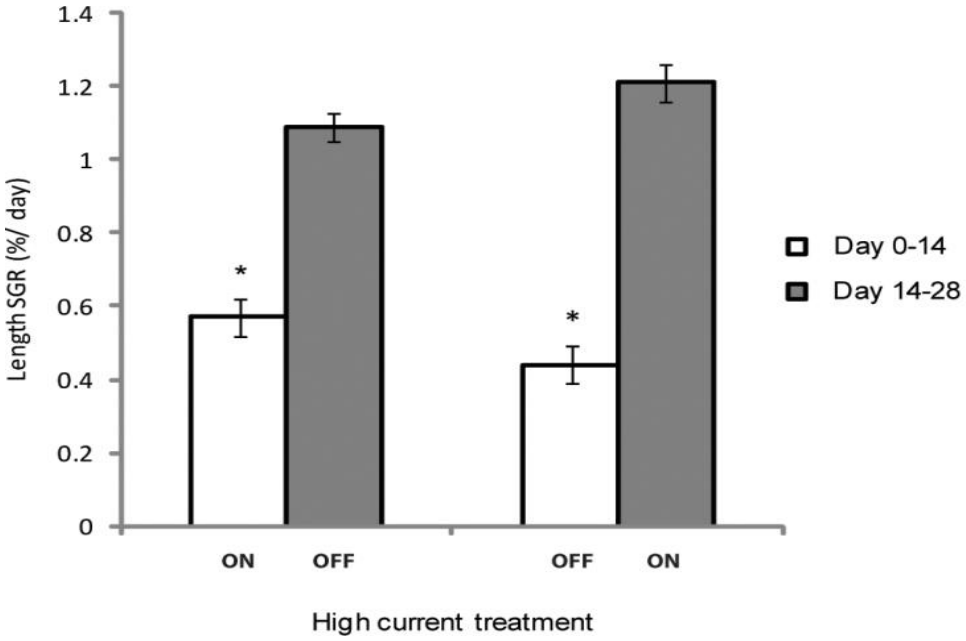

Figure 5.5. Mean length specific growth rate ± SE expressed as % of length gain in mm/ day in tanks with high currents for the two time periods. Asterisks indicate the significant difference within treatments between the first and second half (e.g. day 0-14 and day 14-28).

5.4.2 Condition factor

There were no significant differences in condition factors between tanks with high or low currents, or between tanks when currents were on or off, when compared on day 0 ($p = 0.649$, df = 3, 183) or on day 28 ($p = 0.898$, df = 3, 154). However, fish in all treatments had a significantly greater condition factor at the end of the study (day 28) in comparison to the start (day 0) within treatments. The average increase in condition index between the start and end ranged from 5 to 8 %, (see figure 5.6). Trout that were in tanks with a small airstone on day 0 to 14 ($p = 0.009$, df = 1, 77); trout in tanks with a small airstone on day 14 to 28 ($p = 0.040$, df = 1, 63). Trout that were in tanks with a large airstone on day 0 to 14 ($p = 0.007$, df = 1, 77); fish in tanks with a large airstone on day 14 to 28 ($p = 0.039$, df = 1, 77).

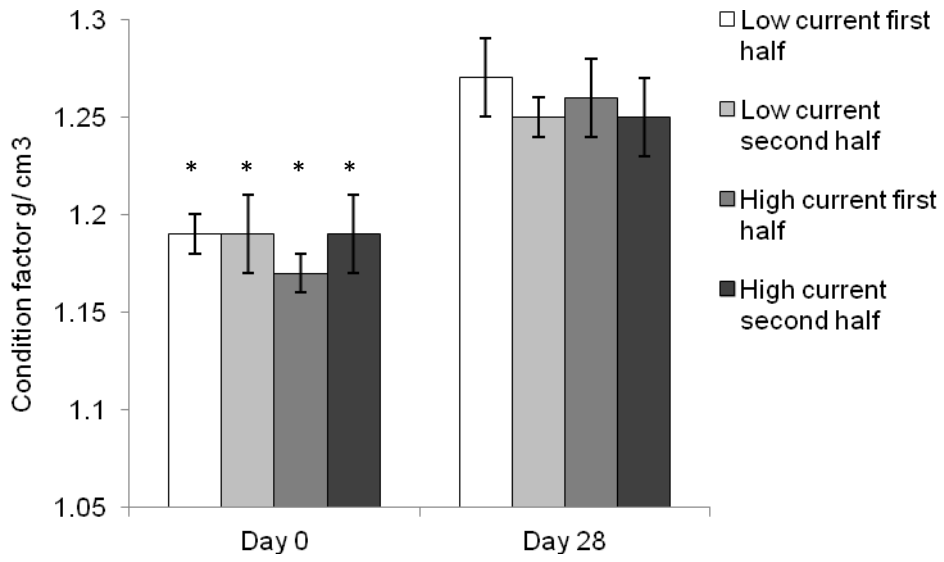

Figure 5.6. Mean condition factor in g/cm^3 ± SE on day 0 and 28 in tanks with low and high water currents at the two time periods. Asterisks indicate the significant difference in condition factor between day 0 and day 28 within treatments.

5.4.3 Hepatosomatic index

Trout in tanks with high current during the second half of the study had 9 and 11 % higher mean hepatosomatic index than trout in tanks with high and low current during the first half ($p = 0.112$, df = 1, 74 and $p = 0.045$, df = 1, 75 respectively) see figure 5.7.

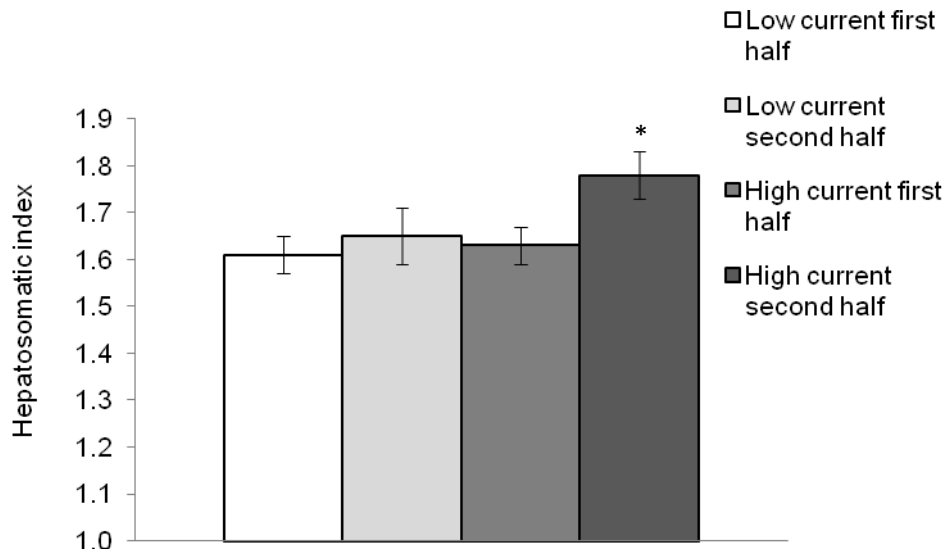

Figure 5.7. Mean HSI ± SE in tanks with low and high current during the two time periods. Asterisk indicates the significant difference in mean HSI between trout in high current second half tanks, and trout from tanks with low current during the first half of the study.

5.4.4 Fin damage

There were no significant differences between treatments in dorsal fin score day 0 ($p = 0.772$, df = 3, 154) or day 28 ($p = 0.226$, df = 2, 154). On the other hand, within all treatments there was a significant increase in mean dorsal fin score over time. In all treatments the dorsal fin damage increased at a fairly linear rate with time. On day 28 scores for all treatments were between 38 and 55 % higher than their respective values on day 0, see figure 5.7 and 5.8. Low current first half ($p < 0.001$, df = 1, 77); second half ($p < 0.001$, df = 1, 63). High current first half ($p < 0.001$, df = 1, 77); second half ($p < 0.001$, df = 1, 77). With regards to pectoral fin scores the exact same trend as for dorsal scores was found. There were no significant differences between treatments in pectoral fin score day 0 ($p = 0.596$, df = 3, 154) or day 28 ($p = 0.095$, df = 3, 154). However, within all treatments there was a marked increase in mean pectoral fin score over time. In all treatments the pectoral fin score increased at a fairly linear rate with time. On day 28 scores were all between 48 and 62 % higher than their respective values on day 0, see figure 5.8 and 5.9. Small airstones on day 0 to 14 ($p < 0.001$, df = 1, 77). Small airstones off day 0 to 14 ($p = 0.204$, df = 1, 63).

Large airstones on day 0 to 14 ($p < 0.001$, df = 1, 77). Large airstones off day 0 to 14 ($p < 0.001$, df = 1, 77).

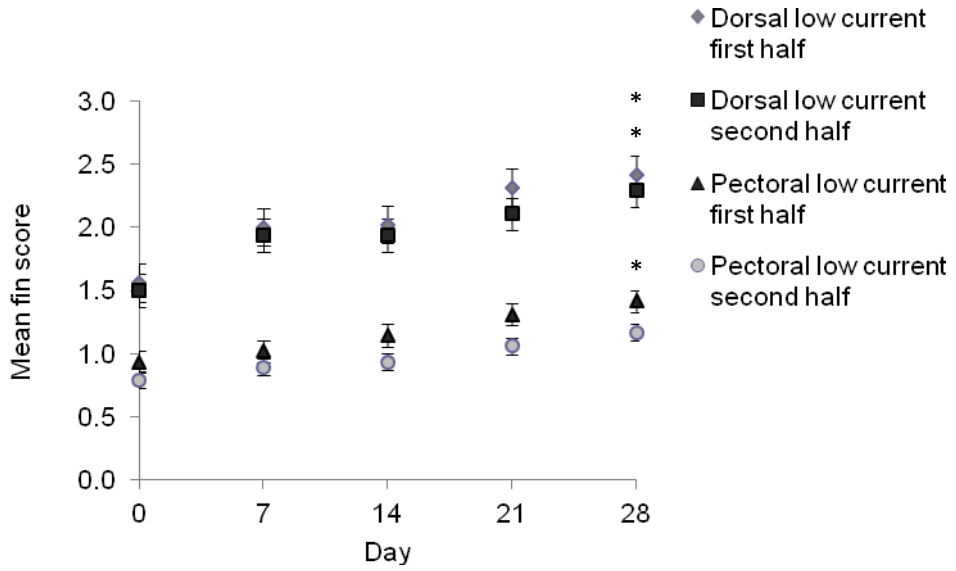

Figure 5.8. Mean dorsal and pectoral fin score ± SE on days 0, 7, 14, 21 and 28 in tanks with low currents. Asterisks indicate the significant increase in fin damage over time within treatments.

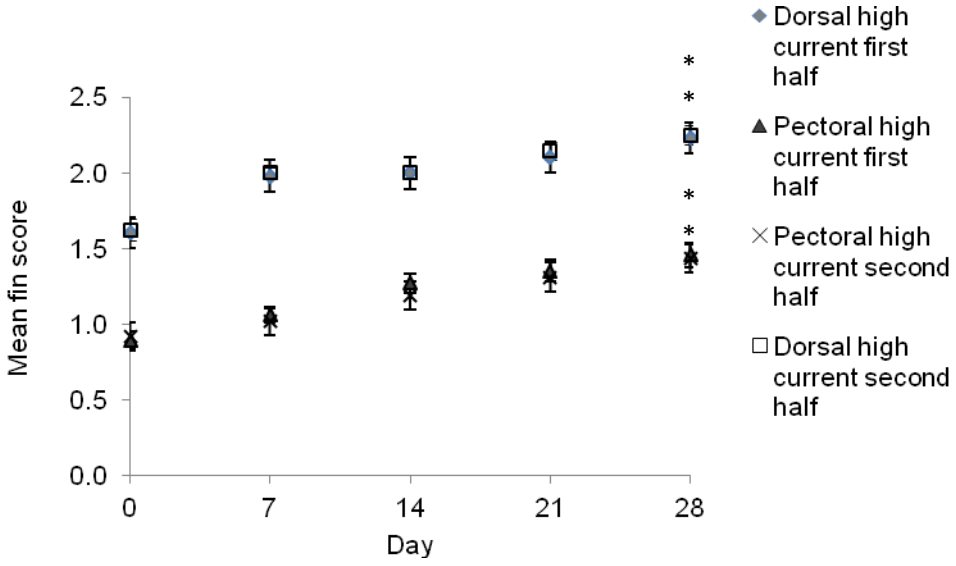

Figure 5.9. Mean dorsal and pectoral fin score ± SE day 0, 7, 14, 21 and 28 in tanks with high current treatment. Asterisks indicate the significant increase in fin damage over time within treatments.

5.4.5 Plasma cortisol

There were no significant differences in cortisol levels between treatments (p = 0.706, df = 3, 153). Mean values were relatively low in comparison to what is generally stated in litterature, 2 and 6 ng/ml (see figure 5.10).

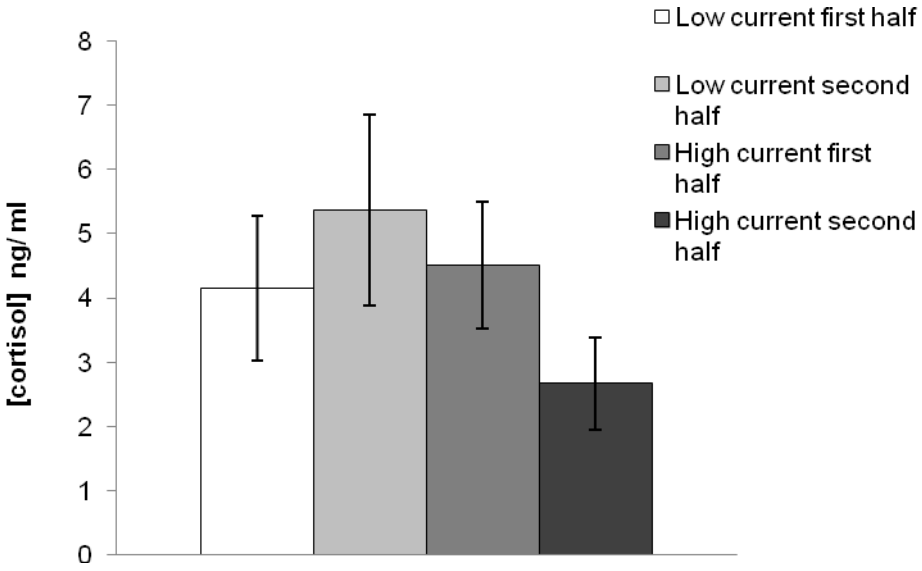

Figure 5.10. Mean plasma cortisol concentration (ng/ml) ± SE in fish from tanks with high or low water currents for the first or second half of the study period.

5.4.6 Behaviour and growth

There were no significant differences between treatments for high or low current with regards to the frequency of aggressive interactions during the first (*p = 0.791*, df = 3, 118) or second half (*p = 0.257*, df = 3, 113). Nor were there any large differences in aggression within treatments between the first and second time period. Low flow during the first period (*p = 0.189*, df = 1, 59), low flow second period (*p = 0.117*, df = 1, 55). High flow first period (*p = 0.516*, df = 1, 58), high flow second period (*p = 0.590*, df = 1, 59). See figure 5.11 a and b and figure 5.12 a and b.

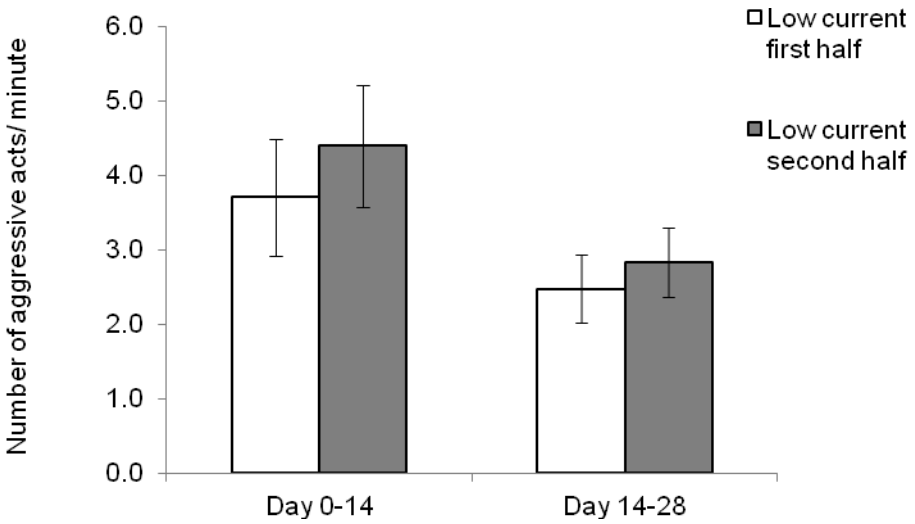

Figure 5.11 a.

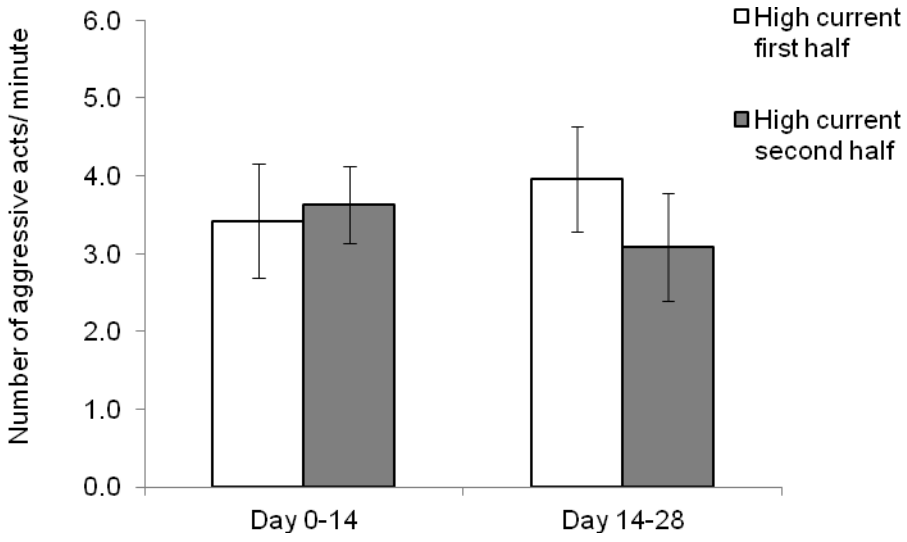

Figure 5.11 b. Mean frequency of aggressive acts ± SE for the first and second half in tanks with low (a) and high water current (b) switched on then off, or off then on.

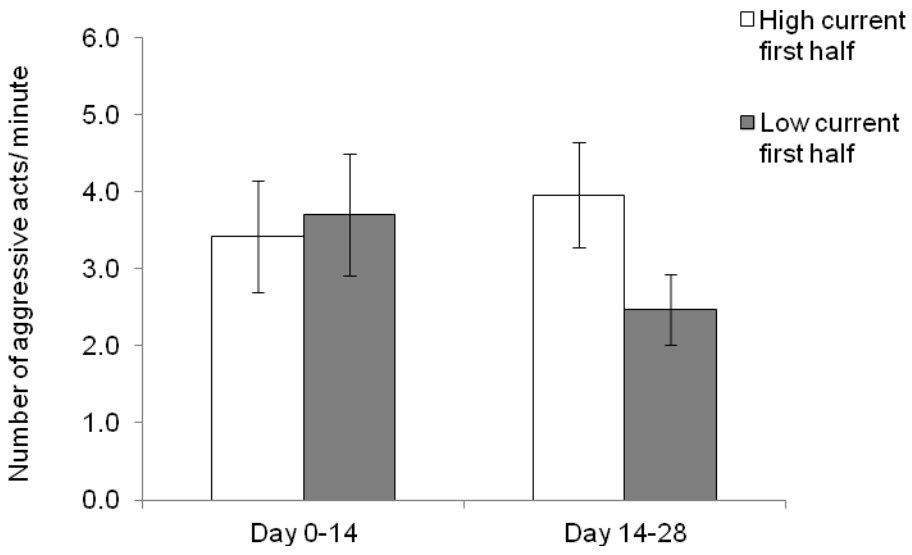

Figure 5.12 a.

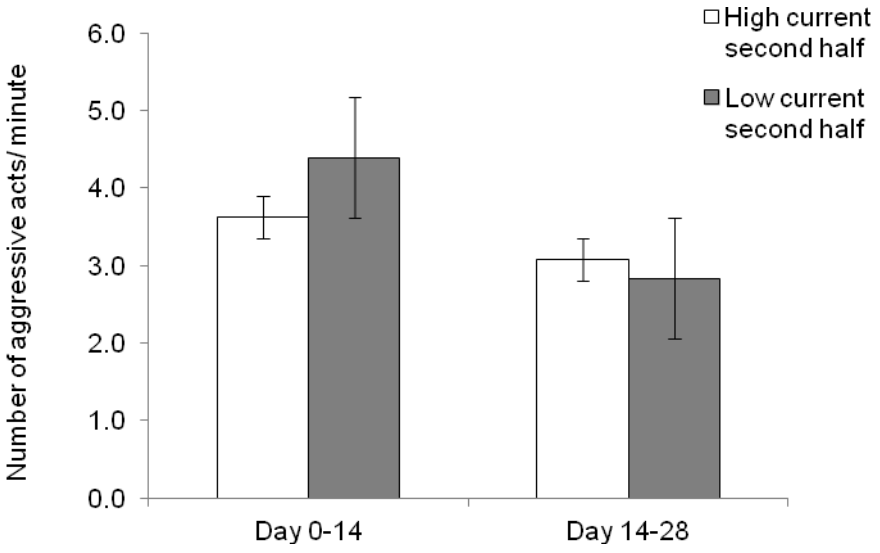

Figure 5.12 b. Mean frequency of aggressive acts ± SE for the first and second half in tanks with low and high current on to start with (a) and low and high current off to start with(b) which then was reversed.

The number of executed and received aggressive acts were taken into consideration when rank was assigned, and it became apparent that the order of entering tanks did not influence which individual turned out to be the most dominant or subordinate. Instead body size was the most reliable predictor of dominance with one of the two largest fish (based on initial mass) becoming dominant in 78 % of the fish groups. When mass growth rate was viewed in relation to rank it was obvious that fish in all treatments had a fairly even spread of mass growth between ranks for all treatments, see figure 5.13 and 5.14.

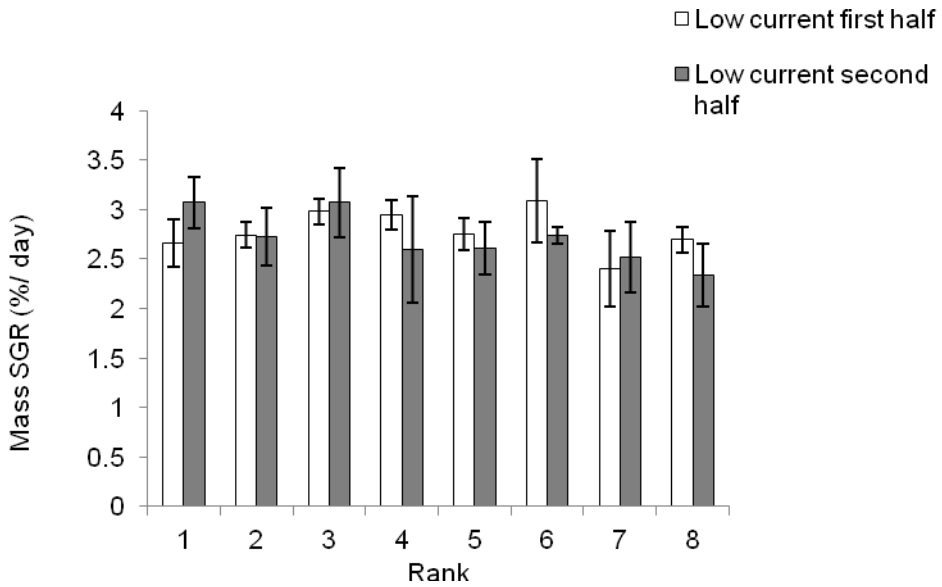

Figure 5.13. Mean mass specific growth rate for the entire time period ± SE expressed as % of body mass per day, versus dominance rank for trout in tanks with low current.

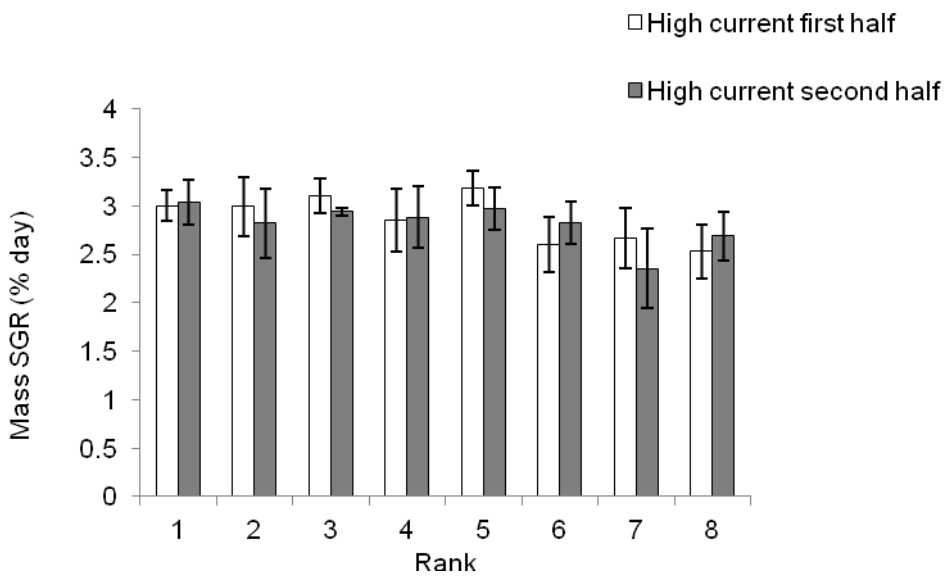

Figure 5.14. Mean mass specific growth rate for the entire time period ± SE expressed as % of body mass per day, versus dominance rank for trout in tanks with high current.

Regarding usage of enrichment, out of 20 hours of video footage with water currents, fish only spent 45 minutes in the streams of air-bubbles. Time spent interacting with enrichment was equally divided between high and low treatments, and at no instance did more than one fish at a time make use of the airbubbles. It was also observed that certain small subordinate individuals managed to fit themselves in the gap between the tank bottom and large airstone. This hiding behaviour was observed both when airstones were either on or off and it was the same individual who displayed this behaviour throughout the different tanks.

5.5 Discussion

In the present study I evaluated the effects of high and low water currents upon the welfare of female juvenile rainbow trout. The different levels of water current were achieved by the addition of tank aeration. However, added airflow did not serve the purpose of improving oxygen levels, as sufficient oxygen concentrations in test tanks were ensured by high flow rates of fully aerated water. Mass and length specific growth rates, condition factor, hepatosomatic index, fin damage, plasma cortisol levels, and aggressive behaviour were used as welfare indicators, and findings will be discussed in consecutive order below. On the whole, mass specific growth rates and hepatosomatic index point towards increased fish welfare when currents were supplied.

Mass and length specific growth rates

There were no differences in mass specific growth rates between treatments for either the first or second half. However, fish had a lower mass growth in the first half in comparison to the second half within each treatment. Though, this was only significant for fish in treatments with no current present during the first half (including both high and low currents). Hence, trout in tanks with no current during the first half had a significantly greater mass specific growth rate when currents were supplied in the second half (including both high and low currents). This may seem insignificant and expected as mass growth in the shelter and visual barrier study showed the same trend i.e. lower growth the first 14 days. However, this finding is interesting when taken in consideration of the lack of a marked difference between the two time periods for tanks that started with water currents. This indicates that fish were perhaps less stressed when currents were provided despite being recently handled and placed in a new environment, or it could be that fish fed or assimilated food better when water currents were provided. This would back previous findings that exercised fish enhance food conversion efficiency and improve growth rates (Houlihan and Laurent, 1987).

In previous studies as well as this one, fish grew better during the second half of the experiments (e.g. day 14 to 28), which has been explained by fish requiring time to acclimatise to a new environment. The non significant result for growth in fish that entered tanks with water currents to start with, regardless of its level, may point towards water currents alleviating stress in fish settling into a new environment after being handled. Additionally, tanks with currents during the second half increased the already expected better growth during this time period, to the extent of it being significant. Hence, not only do water currents perhaps allow fish to deal with stressors such as handling and a new environment, but it also appeared to improve growth in animals already acclimatised. It is worth highlighting that aeration of the stock tank was supplied. Hence, one could speculate that coming from an environment aerated with airstones, into one that is devoid of this, could potentially be more stressful and take longer to acclimatise to, in comparison to the aeration treatment for the first time period.

As for mass specific growth rates there were no significant differences for length gain per day between treatments. Although, within treatments both high and low current tanks had significantly greater length specific growth rates during the second part of the study in comparison to the first half, which is in accordance with the mass growth results. All in all, growth data seemed to indicate increased fish welfare when water currents were supplied, irrespective of it being high or low.

Condition factor and hepatosomatic index

There were no significant differences in condition factors either at the beginning or the end of the study between treatments, but within all treatments condition factors notably improved over time which paralleled the trend for specific growth rates. This indicates that fish from all four treatments were in very good health since condition factors for salmonids generally are within the range of 0.8 to 2.0, with 0.8 being a fish in poor condition and 1.6 an individual in excellent condition (Barnham and Baxter, 1998). Condition factors therefore indicate no

negative or significantly positive effect of the treatments in the present study with regards to fish welfare.

In contrast to the condition factor, the hepatosomatic index portrayed a perhaps alternative story. It became evident that fish in tanks with high water current during the second half of the study, had a significantly higher mean hepatosomatic index in comparison to trout from tanks with no current during the same time period. Interestingly, the difference to individuals in tanks supplied with low water currents during the latter half of the study was the smallest. Since the hepatosomatic index is indicative of energy reserves, it is used to interpret welfare (Barton et al., 1987; Pickering and Pottinger, 1995). Thus, elevated liver size in fish with the high water current shows that these fish had significantly higher energy storage which may be taken to indicate better welfare as a result of this treatment.

Fin damage

Fin damage is a sign of injury in farmed animals and it is therefore used as a welfare indicator (Ellis et al., 2008). Data for fin damage showed no effect of treatments, but there was a significant increase in fin damage over time for all treatments. As mentioned in the two previous results chapters, aggression did not decrease throughout the study period so fin quality can be expected to deteriorate due to cumulative damage, and especially dorsal fin condition as this generally is the first area to be attacked. With regards to fish welfare this endpoint showed no difference between treatments in animal wellbeing. However, similar to the two previous studies, there was a marked increase in fin deteriation over time. Even though fish did not seem to suffer from this, it is important to remember that with increasing tissue damage, it is possible that the likelihood of fish catching a disease increases.

Behaviour

In 25 % of the fish groups, a particular behaviour was observed when currents were supplied, where fish would stand head down in the stream of air-bubbles and occasionally

stop swimming and let the current carry them to the top of the tank. This behaviour which could be interpreted as 'play', was also reported in the previously mentioned pilot study. It was repeated throughout the 14 days of supplied water current, but was only displayed by fish with an intermediate rank. I can merely speculate that this was an exhibit of fish 'playing' and it is not clear why certain individuals did so, or if it had any benefits to their welfare. Though, it is worth highlighting that on no occasion when they displayed this behaviour did these fish receive aggression.

Then again, pose that this behaviour was not 'play', but perhaps a kind of fish stereotypy. Stereotypies are repetitive sequences of apparently purposeless behaviours. They are commonly displayed by zoo, farm and laboratory animals (Garner and Mason, 2002), and are commonly suggested to indicate poor welfare (Mason and Latham, 2004). The development of stereotypies is a way for the animal to handle the frustration of not being able to perform certain innate behaviours, and examples of stereotypies are pacing, rocking, and increased aggression (Edwards, 2009). It is suggested that animals develop stereotypies as a means of coping with a barren environment, hence indicating that the environment needs to change. Reducing stereotypy is one of the aims of environmental enrichment in zoos (Mason and Latham, 2004) as this would allow the animal to perform typical behaviours to return control over the environment and aid homeostasis (Garner, 2005). However, it has been shown that the performance of stereotypies are related to the release of endorphins which in one way provides the animal stress release form an unsuitable environment (Edwards, 2009). Hence, the prevention of a stereotypy may be a two way problem. Working with fish creates further problems in the sense that evaluating what is a stereotypy or not becomes more difficult as they lack facial expressions (to us humans). However, repeated aggressive behaviour when subordinates do not appear to be challenging the dominant could perhaps qualify as stereotypy, or the use of the air-bubbles by some individuals.

There were no significant differences in aggression levels between or within treatments, which on the one hand was not surprising as this was also the case in previous studies, where the same behavioural categories were used for scoring. On the other hand the lack of lower aggression levels are surprising, as existing literature suggest a noteworthy decrease in agonistic behaviour in juvenile rainbow trout (Vehanen et al., 2000) and Arctic charr (Adams et al., 1995) due to water currents. The lack of differences in aggression in the present study could either be due to the fact that:

1) there were no differences in aggression between tanks with or without water currents, or between high or low treatment.
2) the relatively high food ration of 3 % of body mass was sufficient provision for fish not to fight for food. It is important to mention that generally fish in growth studies are kept on 1 – 1.5 % of body mass rations. Which could be a key point, as this explains the lack of marked differences in aggression between treatments for all the three studies.
3) supplied water currents were not forceful enough to keep trout occupied by swimming.
4) expressed aggression was too subtle to be recognised by the scoring parameters. As suggested previously, a more subtle behavioural assessment may be informative.

To produce a new ethogram covering more subtle behaviours would most likely be fairly time consuming at least in the beginning, but once key behaviours are established and the observer has developed the skill to detect subtle body postures showing aggressive intent, it may prove to be a useful tool. This is far from an unrealistic goal, as it was easy to decipher dominant individuals from subordinate in most tanks over time. Moreover, I would like to highlight that in addition to the requirement of a thoroughly trained eye, it is probably equally important that the observer has a good understanding of rainbow trout behaviour.

Behaviour and growth

Analysing rank against total mass specific growth rate revealed that generally all trout had fairly similar growth rates. This was surprising as it is well known that dominant individuals tend to monopolise a food source, hence growth in a group of rainbow trout would be expected to be more heterogeneous. There are various possible reasons why the trend in mass specific growth rate with regards to rank was not more obvious:

1) exercise generated better food distribution and increased appetite, which has been reported by Totland (1987).

2) the food ration of 3 % of body mass was sufficient to feed all fish in the tank.

3) hierarchies were not strictly linear, hence food distribution was more evenly spread between individuals.

4) the advantages of being a dominant aggressive individual decreased when forced to swim, which was shown to hold true for Arctic charr (Brännäs, 2009). Brännäs (2009) also found that the lowest ranked fish gained less weight when exercised in comparison to resting fish, due to energetically costly efforts of keeping position in the current. Aggressive behaviour is believed to be energetically expensive and to distract individuals from feeding, which may explain why dominant fish can sometimes be exceeded by that of a subordinate fish in their growth (Brown et al., 1992)

From a welfare point of view a more homogenous growth amongst individuals would indicate a better welfare for the group as a whole as food has been more evenly spread within the group and not monopolised by dominant fish.

Plasma cortisol

I found no significant differences in plasma cortisol concentrations between treatments. As mentioned previously, it was challenging to quantify this endpoint and no firm conclusions have been made. All that can be concluded is that plasma cortisol levels appeared to be consistently low across treatments, but whether this was due to low levels in experimental animals or poor plasma quality is impossible to decipher. As discussed in section 2.7, a major effort to resolve the problem was made but the issue was not solved. Reasons why samples were either of poor quality (shipping problems), or cortisol levels were actually this low in this batch of trout have already been discussed. One highly feasible explanation is that this batch of fish came from a trout farm that breeds on trout with characteristics such as low responding cortisol levels when stressed.

Conclusions

In the present study, I tested the effects of high and low water currents upon physiological and behavioural welfare indicators in juvenile rainbow trout, since it is recognised that water currents may reduce time spent on aggressive interactions amongst fish. As rainbow trout is a stream dwelling species, one would expect that the introduction of water currents into test tanks would be beneficial. I have shown that the addition of water currents to test tanks, could potentially be viewed as enrichment for juvenile rainbow trout as it appeared to bring a better spread of food amongst individuals, since fish across the social hierarchy grew equally well. Though, this result could be due to enhanced food assimilation or appetite as a result of increased exercise. However, mass specific growth rates also indicated that water currents alleviated stress amongst fish that had experienced handling and entered a new environment, as well as improving growth in already acclimatised animals. On the whole, specific growth rates seemed to point towards increased fish welfare when water currents were supplied. The hepatosomatic index also backed this proposal, as it appeared as if fish with high currents during the latter part of the study had a greater energy reserve, than fish with no currents for the same time period. Additionally, there was a weak trend within the

HSI data towards high water current being superior to low. This was not reflected by behavioural data, hence it is of interest to investigate more subtle behavioural expressions. The addition of water currents into test tanks may be a superior option when housing juvenile rainbow trout in experimental regulatory test tanks, in comparison to the current practise, which is a totally barren environment. However, this is a matter that needs further investigation. Unfortunately, I have not yet had the opportunity to analyse brain monoamines for the present study, due to economical reasons, but hopefully this will be achieved within the near future.

CHAPTER 6: General discussion

6.1 Why is fish welfare important?

Whether fish can experience pain, distress and fear is still a matter of debate. The majority of the public is probably inclined to believe that fish do not feel pain, or at least have a lower ability to do so, because they are viewed as 'less' intelligent than birds and mammals. For clarities sake, I share the notion of many researchers who view fish as complex animals with intricate behaviours and abilities to experience pain and distress. Still, even if one views fish to be 'lower' standing animals due to the lack of cognition, it is worth considering that there is no logical reason to believe that greater cognitive ability makes the experience of pain worse. Perhaps even the opposite, pain may cause greater distress in animals with less or no ability to reason (Broom, 2001). As Professor John Webster (2005) claimed "you don't have to be clever to suffer". Hence, it is worth contemplating whether fear and distress could be similar or more intense for animals with lesser abilities for rationale.

Studies specifically aimed at examining pain and fear in fish, have produced important support for the ability of fish to experience both of these negative emotions (Sneddon et al., 2003; Sneddon, 2003a,b; Yue et al., 2004). As a result, there is an increasing scientific acceptance that fish can feel some sort of fear, pain and distress, which in turn feeds a growing concern for their welfare. I believe we owe fish the respect and moral considerations we give other sentient animals. Nevertheless, it is understandable that many perhaps prefer to view fish as non-feeling animals. Because, if we agree that fish feel pain and distress, then how would we cope with for example the fishing industry? This question could be extended to include 'sport fishing', as well as fish kept as pets, or in tanks at restaurants in order to be as fresh as possible for customers. It is beyond the scope of this thesis to dwell into this subject, but it is an interesting subject in need of questioning.

We impact the life and welfare of a large number of fish in various ways, one of them being through research. Currently fish are experiencing an increase in their use within research

due to the significantly lower cost of maintenance in comparison to mammals, and perhaps also because they are perceived to be less likely to feel pain and distress. In 2010 more than twice as many fish were used in comparison to rats in the UK. Fish represented 13 % of all vertebrate studies which was a 23 % increase on 2009, and this kind of increase has been the trend for the past ten years (Home Office, http://www.homeoffice.gov.uk/). Scientists using animals in their research are obliged to promote animal health and welfare. Not only is it in the interest of researchers to keep their animals under good conditions for ethical reasons, but the health of experimental animals may also significantly influence research results. Scientific data are only meaningful if the health of the research subjects and the conditions under which they are kept are good.

Fish are not as well protected legally as, for example mammals, at least outside of their use in research, e.g. domestic pets and farm animals. Still, welfare legislations in the UK makes it an offence to cause or allow livestock to suffer unnecessary pain or distress, and demand welfare considerations for all animals used in scientific procedures (Cooke, 2001). Furthermore, welfare as well as enrichment needs for fish are included in the Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes. Subsequently, whether we believe fish can suffer or not, it is highly important to implement good welfare practices if you want to have reliable research results, and abide by the law.

6.2 Animal welfare

As stated by Dawkins (2006b), animal welfare science ‘thinks big’, as it deals with imperative issues such as animal consciousness, health and emotions, and topics that affect millions of people and billions of animals.

There is a general agreement that good animal welfare should be an important part of maintaining animals in good health. Welfare assessment is based upon physiological and/or

mental states, with the latter being much harder to quantify in non-human animals. The inner experience of another being is not open to direct empirical research. To imagine that animals feel the way humans do could lead to flawed conclusions, but it is just as misleading to deny another species the potential of experiencing emotions such as pain, fear, and distress. It is more reasonable to accept it than risking neglecting important truths (Lehman, 1992).

However, we should not have to run that risk to such an extent, as today welfare scientists are equipped with various methods to understanding what makes good animal welfare. It is difficult in its own right to study the subjective state of animals but it is even further complicated by animals having different senses and motivations to humans. Since animals cannot convey how they feel, indirect methods are used to assess their wellbeing and a wide range of different methods to evaluate welfare are applied. Researchers generally opt for either of the following approaches, take the sum of many different measures such as behavioural and physiological endpoints, or focus on the questions ‘are the animals healthy?’ and ‘do they have what they need?’ Animal preference testing is a very useful tool in finding out what animal prefer with regards to enrichment, one can for example find out if fish prefer a certain light intensity, tank colour, or the company of conspecifics etc. It would have been of interest to have applied this type of experiment within this PhD, but it was not possible within the time frame. In this thesis I have applied a range of physiological, physical and behavioral endpoints commonly used as welfare indicators in fish in order to establish enrichment criteria for female juvenile rainbow trout used in regulatory research.

6.3 Environmental enrichment

There is a continued growth of public interest in animal welfare, especially within scientific research. Since fish are widely used in research, changing their housing and husbandry to improve welfare is valuable. Fish kept in laboratories are most likely subjected to environments less complex than their natural environment, and these artificial conditions may include for example restrictions in movement, sensory stimulation, and social

interactions. Since stress in fish may cause a much reduced ability to resist attack from disease and parasites, and reduce biological parameters such as growth and reproduction (Conte, 2004). Concerns have been raised that barren tanks not only lead to poor welfare but also result in laboratory animals being poor subjects in some scientific experiments. Perhaps even unrepresentative of how they may respond to experimental variables in nature. This is a particularly important point for regulatory research that seeks to understand how animals will respond to various anthropogenic stressors, including toxic chemicals. Ultimately this aims to set limits of safe levels of chemicals in the environment, hence it is important to know that test animals used in laboratory settings provide a relevant benchmark for responses to such toxicants. Thus, it is of outmost importance to work with 'healthy' animals.

Environmental enrichment is difficult to define, and it has been used to represent numerous different things. However, when used within animal welfare, it is only fitting if an enhancement in the welfare of an animal, as a result of changes to the environment has been observed. Today it is generally accepted that many types of enrichment have positive effects in animals. Actual effects of many enrichment strategies remain unclear, what we think ought to be enrichment may in actual fact have detrimental effects instead. Hence, it is becoming increasingly important to objectively assess whether proposed welfare improvements suggested by bodies that regulate the use of animals in research, such as the UK Home Office, are suitable for laboratory fish species as they tend to be based on mammalian research. How best to improve barren experimental tanks used in regulatory research was the broad aim of this PhD. With regards to studies undertaken in this thesis, I had to keep enrichment strategies within the regulations of toxicological testing. Obvious enrichment ideas such as the addition of gravel and or live plants are not feasible as they would not be applied in regulatory testing. Gravel and plants would increase the surface area available for microbial growth, and this could potentially interfere with chemicals being

tested. This explains the perhaps unorthodox enrichment strategies employed in the present studies.

6.4 The choice of study species - why rainbow trout?

Rainbow trout was a natural choice to work with as it is a well established model in research laboratories globally, both within the world of academia and industry, and it is also a suggested experimental species by the OECD-guidelines for relevance to cold water environments. Additionally, it displays behavioural traits that can be detrimental to welfare. Despite the fact that farmed rainbow trout commonly are referred to as domesticated, aggressive behaviours that occur in wild populations are still a part of their behavioural repertoire under culture conditions (Ellis et al., 2002). There is ample proof that aggression amongst rainbow trout kept in a confined space can cause injuries and even mortality, and since male rainbow trout are more aggressive than female it is advised that only female rainbow trout are utilised which is the reason why I only used female trout in our studies. Laboratory or farm bred experimental animals may not be as removed from their natural behaviour as one would like to think, which makes environmental enrichment an even more pressing matter.

However, with this in mind it begs the question whether using this species in a confined, barren laboratory set up is to recommend, or whether it is of interest to replace rainbow trout with another less aggressive species?

6.5 Overview of findings

For the two first studies I examined whether there were any differences physically, physiologically or behaviourally between female juvenile rainbow trout kept in tanks that were either barren or contained semitransparent shelters or visual barriers. These enrichment ideas came about because many salmonid species use refuges to shelter, and lower habitat visibility has been suggested to lower aggression. Endpoints commonly used in

fish welfare assessments were applied to indicate a better or worse welfare status between treatments.

In the discussion of chapter 3, I concluded that adding structures of a semitransparent shelters design should not be viewed as enrichment for juvenile rainbow trout. Trout welfare was not improved with regards to mass specific growth rates or hepatosomatic index. Moreover, brain monoamines actually indicated higher chronic stress levels in trout from tanks with shelters. It also became apparent that positioning and features of tank structures may be of significance as groups displayed different space usage when shelters were present. The shelters seemed to enable dominants to herd subordinates into the corner at the rear of the tank where they were regularly attacked. Aggressive acts also became more forceful, though the frequency of these did not increase.

In the second study testing visual barriers, I found some interesting results indicating that visual barriers potentially could be viewed as enrichment. Brain monoamine results showed potentially lower chronic stress levels and decreased aggressive interactions in fish, from tanks enriched with visual barriers. Furthermore, there were weak but possibly intriguing brain monoamine trends, showing that it was the visual barrier per se that produced the difference and not the physical obstruction. Yet again, the location of enrichment appeared to be of significance as dominant individuals quickly learnt where to position themselves in order to control other fish. I believe results showed that adding visual barriers of some sort to test tanks warrants the extra cost and effort. This matter is discussed in more detail in a later section.

In the third and final study the experimental design was slightly different in comparison to study one and two, as the effects of high or low water currents were tested. The difference being that no tanks were kept barren throughout the entire study period, but each treatment still had a no current period for 14 days as a control. Tank aeration strong enough to

produce water movements was a potential enrichment idea worth exploring, as it is probably the most simple and cheapest form of enrichment that most likely would have a broad acceptance. It is recognised that water currents may reduce aggressive interactions amongst fish. However, I did not find any differences in aggression within or between treatments. Still, it was apparent that water currents (without substantially altering the oxygen availability) could possibly be viewed as enrichment, as indicated by mass specific growth rates and the hepatosomatic index. It appeared to facilitate a more even feed spread amongst individuals within the hierarchy, since middle and low ranked fish grew as well as dominant trout. This finding could also be due to enhanced food assimilation or appetite as a result of increased exercise. Mass growth rates may also indicate that water currents alleviated stress amongst fish that had experienced handling and entered a new environment. As well as improving growth in already acclimatised animals. Though, these are only loose hypotheses, in need of further studies in order to draw any firm conclusions about it. Still, it was an interesting trend, too important not to mention. All in all, mass growth rates viewed either between treatments or with regards to rank seem to point towards increased fish welfare when water currents were supplied. The hepatosomatic index backed this finding since trout with high airflow during the latter part of the study, had a greater energy reserve in comparison to fish with no airflow for the same time period. What is more, there was a weak trend towards high current being superior to low. This was not reflected in behavioural results, which yet again prompted the investigation of more subtle behaviours.

There were two subjects that became apparent in all the three studies. Firstly, a measure of more subtle behaviours may prove fruitful in future work. In hindsight it is clear that scoring behaviours such as lateral display and fin erection, as well as position in the water column would have been beneficial, but due to time limitations it was not possible to re-analyse footage within the frame of this PhD. Secondly, it is evident that measurement of brain monoamine levels are a useful way forward to analyse fish welfare and possible future enrichment ideas, if experimental animals can be sacrificed in the end. Unfortunately brain

monoamines and their metabolites have not yet been analysed for the aeration study but I hope this will be done in the near future. This would most likely shine some light on existing trends.

Additionally, it is important to mention here that the lack in quantitative aggressive behaviour between treatments for all three studies, could perhaps also be explained by the high feeding ration set by the 215-OECD guideline, and not just the need for an evaluation of more subtle behaviours. Fish were fed 3 % of the body mass per day in all three studies, which is high in comparison to existing literature. Another point worth highlighting, is that generally when dominance and social structures are studied, is appears as if a lower number of individuals are kept together in a group, which makes it more readily to evaluate which individuals are dominant and subordinate. I had eight fish per tank which most likely complicated the study of the social structure. However, the number of fish per tank was due to the set loading rate of the OECD guideline, as this PhD set out to analyse enrichment strategies for fish used in regulatory research and not fish hierarchies.

These studies confirmed the difficulty in determining what is beneficial to fish welfare and not. The addition of shelters were expected to be beneficial, though I did hypothesise that if they were perceived as areas worth protecting, it could cause stress within a fish group. On the other hand, it also became evident how important this subject is.

6.6 Applications of findings in this thesis

I would like to explore the idea of whether regulatory researchers would change how they currently run their research based on findings in this thesis. In order to weigh up options, a cost analysis was made of the addition of visual barriers used in study number two. To supply one tank with this type of visual barriers the cost in total would be approximately £75 (materials ~ £20, cost for time to make and maintain it for a period of 14 days ~ £75). I believe that when a positive impact of environmental change is found, it is worth exploring its

potential. However, data backing the trend of less chronic stress in that particular study would probably not convince many to change the way its run today, especially since there were no evidence of other more tangible improvements (e.g. SGR, fin damage). It would probably be difficult to convince the scientific community to start using such 'enrichments' based on this evidence alone. However, in chapter four I have objectively shown that some physical manipulation of test tank environments can result in measurable welfare changes, hence this should inspire further work to find even better and perhaps cheaper options for enrichment for trout that could be implemented. As for the third study it would be of interest to analyse brain monoamines as well as suggested previously further analyse the footage for more subtle behaviours as the application of airstones to create water currents would be an easily applied and cheap way of enriching trout environment. In the final study, fish came from a stock environment where aeration was supplied, into experimental tanks with or without aeration, which begs the question; if experimental tanks are enriched, should not stock tanks be enriched too, and vice versa? It is feasible to assume that fish coming from an enriched stock tank to a barren environment ought to be a stressful experience.

I would also like to raise the question of having legislation and guidelines that require visibility of experimental animals from above as well as in front of tanks. Is this good animal welfare? I believe it is feasible to assume that most fish are prey at least at some stage of their lives, and that it would go against their instinct to be highly visible in an open area, generally against a white background. It seems fair to assume that fish would benefit from visual as well as perhaps physical shelter in their tanks.

6.7 Shortcomings of enrichment studies

One important aspect to emphasise is that I worked only with female groups of trout which may influence results. This was the case as no male rainbow trout are used in regulatory research due to high aggression levels. If applying the findings from presented studies to results from other laboratory studies that do not follow such guidelines, it is important to

highlight it would most likely not translate to male rainbow trout, as there are some definite differences between sexes in this species. For example, Øverli et al., (2006) reported that juvenile female rainbow trout resumed feeding faster than males, following disturbance. Moreover, they also found that females settled down and ceased panic behaviour faster than males when subjected to confinement. Johnson and Åkerman (1998) showed that male juvenile rainbow trout are more aggressive than females within interspecific dyadic encounters, and immature male brown trout have also been shown to be more aggressive and bolder than females (Johnsson et al., 2001). However, this may be a species specific issue since Bakker (1994) found similar levels of aggressive behaviour in male and female juvenile sticklebacks. Fish species are very variable with respect to morphology, habitat, diet, ecology and social organisation, and for that reason they differ in their response to environmental change. An enrichment strategy optimal for one species might not be beneficial for another. Thus, it is important to highlight that findings reported in this thesis only apply to female juvenile rainbow trout. Additionally, caution should be taken when interpreting stress physiology as well as behavioural measures in relation to welfare, since stress measures may portray little about potential suffering (Ashley, 2007). Still, a better understanding of behaviour and physiology tied in with a given situation will aid animal welfare scientists to more reliably examine welfare questions (Ashley, 2007).

Despite a thorough exploration of existing literature and undertaking PCA statistical tests, it was impossible to determine which endpoints are the most meaningful with regards to fish welfare. Hence, multiple statistical tests were executed within each results chapter, which is a potential shortcoming. When an association between two variables is rejected on the grounds of P being greater than a critical value, it is still possible that the association is due to chance. In this thesis I used a critical P value of 0.05, which means that in 100 statistical tests five would be significant due to chance e.g. false positives. Multiple comparisons are an issue under active research, but to date there is no generally accepted approach for dealing with this problem (McDonald, 2009). There are various ways to potentially solve this

problem, one could either use a lower P value than 0.05, or apply a Bonferroni correction. However, the use of Bonferroni corrections has been strongly contested as it reduces the probability of Type I error at the cost of increasing the possibility of a Type II error e.g. false negative (Rothman, 1990; Nakagowa, 2004; Brooks et al., 2011). If you make important decisions based on a false positive it could potentially be a costly error with regards to both time and money. On the other hand, a false negative could result in missing an important discovery. Hence, one need to be cautious when interpreting results in order not to fall into either of the false positive or negative traps. However, with regards to findings in this thesis, before anything would be concluded as a potential enrichment strategy, it would have to pass a ring test, e.g. the suggested enrichment strategy would be rigorously tested in a way that statistical certainty would be assured.

Another important issue I would like to raise is the one of statistical power. An underpowered study may find no significant differences even in the presence of a real difference between treatments. Since I was limited by space, time and money in all three experiments, the statistical power of the reported studies are not as good as could be desired. This should be kept in mind when interpreting results. Hence, a P value larger than but close to 0.05 could be significant with higher replication. An ideal study design would apply both sample size calculations and hypothesis generation in the experiments (Kaufmann and Kallmes, 2007), and if I had the possibility to run the studies again with unlimited resources this would be the route to follow. Nevertheless, I would like to reiterate that even if results were not always significant, careful consideration should be given to results which approached, but did not meet the conventional threshold of $P < 0.05$ as they may indicate the presence of biologically important relationships.

6.8 Future work

Literature searches on animal welfare for 2012 reveal that this field is still dominated by land living mammals and birds. Generally, enrichment for mammals and birds is fairly different to what would be applicable to fish as fish inhabit a different physical world, and hence are physiologically different. Additionally, it is important to note that when we discuss 'fish' we are referring to > 30 000 species, with greater species diversity than any other vertebrate group. Hence, what suits one species will most likely not be applicable to another. Many countries in the developed world have come relatively far regarding welfare issues for mammals and birds in comparison to fish. Consequently, results presented in this thesis are novel as there has been little previous research measuring the welfare of rainbow trout using either behavioural or physiological approaches in response to tank enrichment. I have undertaken a careful set of experiments investigating original enrichment strategies for juvenile rainbow trout used in regulatory research, measuring a wide range of parameters, and the results provide new information relevant to the improvement of fish welfare.

However, the welfare of farmed fish has attracted attention in recent years, resulting in noteworthy changes within the aquaculture industry (Berrill et al., 2012). Meetings with stakeholders and opposing ethical views have been arranged to identify current and future priorities for farmed fish welfare in the UK. From these discussions, seven specific areas in need of development for future improvements in farmed fish welfare were identified. The top two priority areas are: "*establish a better understanding of what good fish welfare is*" and "*the need for welfare monitoring and documentation systems*" (Berrill et al., 2012). On the list is also "*a need for integration and application of behavioural and physiological measures*". This is also what is needed for fish used in research and any progress in this area, despite it being farmed fish, would most certainly benefit welfare issues within regulatory science as well. Hence, an open channel of communication between different fields of interests is most important.

I would like to take the opportunity to address the question of whether the tight size matching within mass, in the 215-OECD guideline, potentially sets the stage for enhanced aggression, and I also want to highlight an interesting observation that may be an artefact of this requirement. From all the footage for the three studies, it seemed as if there are three different 'types' of social structures, specifically in relation to the type of dominant individuals, that have emerged; 'the aggressive dominant', 'the calm dominant', and 'the gladiators'. In 'the aggressive dominant' category, there is one obvious dominant fish which attacks others repeatedly no matter their behaviour, whilst 'the calm dominant' is the opposite. In the latter social set up, the dominant displays aggression especially to start with, but after the initial period of aggression it generally keeps a fairly 'low' aggression rate. The worst case scenario is 'the gladiator' set up, in which there are several individuals that view themselves as dominant and fight each other without showing behaviours of defeat, hence fuelling further aggression. I would assume that the latter set up is a result of the tight size matching prescribed by the guideline, and that depending on what type of social structure there is, and how many individuals that view themselves as an alpha fish in a group, will influence the overall wellbeing of others. Correct or not, this is a question for further work to resolve, but a closer look at the size data for these tanks in relation to aggression and monoamine data should reveal any patterns worth investigating. I believe an easy way of enriching rainbow trout environments, or improve the 215-OECD guideline by decreasing aggression, would be to have one obvious larger fish in the group. This individual should not be that big it would pose a predatory threat, but large enough to keep others under control and have no need to establish itself as dominant in a too aggressive way.

Regarding future work within the field of animal welfare, I believe it is essential to consider 'critical anthropomorphism', a concept coined by Burghardt, (1985). As described by Burghardt (2004) critical anthropomorphism entails careful replicable observation, as well as a sound knowledge of natural history, ecology, sensory and neural systems of animals. I also believe it is a mistake not to utilise resources available at most likely every research

place, the animal caretakers. Experienced animal carers habitually filter information about an animal, reaching a holistic assessment of its wellbeing, and identifying subtle behavioural changes. There is emerging support that qualitatively assessed aspects linked to welfare can be assessed consistently in a range of species. This in collaboration with physiological and physical measures would most likely be beneficial to this field of science.

Not only are more behavioural and physiological studies essential in order to build a solid framework for animal welfare policy, but of equal importance is that those results are made available (Barnard, 2007). Otherwise it is plausible that inappropriate legislation is founded upon biased ideas of what is good welfare and practice (Barnard, 2007). There is still much to learn before well justified enrichment guidelines tailored to species needs can be put in place, but it is a step in the right direction that funding is supplied for exploring this field, and that we are beginning to embrace the concept that fish are sentient animals that can feel pain.

APPENDIX - Statistical output for results chapter 3

Mass SGR day 0-14 between treatments

General Linear Model: mass sgr day 0-14

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for mass sgr day 0-14, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   8   1.4690   1.4690  0.1836  0.81  0.597
rank              7   1.4588   1.4588  0.2084  0.92  0.499
treatment         1   0.7745   0.7745  0.7745  4.22  0.074
Error            63  14.3040  14.3040  0.2270
Total            79  18.0062

S = 0.476495   R-Sq = 20.56%   R-Sq(adj) = 0.39%
```

Mass SGR day 14-28 between treatments

General Linear Model: mass sgr day14-28

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for mass sgr day14-28, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   8   3.3185   3.3185  0.4148  2.05  0.054
rank              7   1.0414   1.0414  0.1488  0.74  0.643
treatment         1   2.0094   2.0094  2.0094  4.84  0.059
Error            63  12.7404  12.7404  0.2022
Total            79  19.1096

S = 0.449698   R-Sq = 33.33%   R-Sq(adj) = 16.40%
```

Mass SGR within barren tanks, day 0-14 versus day 14-28

General Linear Model: mass sgr

```
Factor           Type    Levels  Values
tank(treatment)  random      10  3, 5, 6, 9, 10, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  1, 2


Analysis of Variance for mass sgr day14-28, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   8   4.3235   4.3235  0.5404  2.18  0.041
rank              7   2.9704   2.9704  0.4243  1.71  0.122
treatment         1   0.3621   0.3621  0.3621  0.67  0.437
Error            63  15.5933  15.5933  0.2475
Total            79  23.2493

S = 0.497506   R-Sq = 32.93%   R-Sq(adj) = 15.90%
```

Mass SGR within shelter tanks, day 0-14 versus day 14-28

General Linear Model: mass sgr day

```
Factor                Type    Levels  Values
tank_1(treatment_1)   random      10  1, 2, 4, 7, 8, 1, 2, 4, 7, 8
rank_1                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_1           fixed        2  1, 2


Analysis of Variance for mass sgr day14-28_1, using Adjusted SS for Tests

Source                DF   Seq SS  Adj SS  Adj MS      F      P
tank_1(treatment_1)    8   0.4640  0.4640  0.0580   0.39  0.925
rank_1                 7   1.5010  1.5010  0.2144   1.43  0.211
treatment_1            1   1.2978  1.2978  1.2978  22.38  0.001
Error                 63   9.4797  9.4797  0.1505
Total                 79  12.7425

S = 0.387907   R-Sq = 25.61%   R-Sq(adj) = 6.71%
```

Length SGR 0-14 between treatments

General Linear Model: length sgr day 0-14

```
Factor            Type    Levels  Values
tank(treatment)   random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank              random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment         fixed        2  0, 1


Analysis of Variance for length sgr day 0-14, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS   Adj MS     F      P
tank(treatment)    8  0.50948  0.50948  0.06368  1.65  0.129
rank               7  0.64031  0.64031  0.09147  2.37  0.033
treatment          1  0.05604  0.05604  0.05604  0.88  0.376
Error             63  2.43474  2.43474  0.03865
Total             79  3.64056

S = 0.196588   R-Sq = 33.12%   R-Sq(adj) = 16.14%
```

Length SGR day 14-28 between treatments

General Linear Model: length sgr day14-28

```
Factor            Type    Levels  Values
tank(treatment)   random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank              random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment         fixed        2  0, 1


Analysis of Variance for length sgr day14-28, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS   Adj MS     F      P
tank(treatment)    8  0.36923  0.36923  0.04615  0.79  0.615
rank               7  0.28546  0.28546  0.04078  0.70  0.675
treatment          1  0.26823  0.26823  0.26823  5.81  0.042
Error             63  3.69214  3.69214  0.05861
Total             79  4.61507

S = 0.242086   R-Sq = 20.00%   R-Sq(adj) = 0.00%
```

Length SGR within barren tanks, day 0-14 versus day 14-28

General Linear Model: length sgr day14-28

```
Factor           Type    Levels  Values
tank(treatment)  random      10  3, 5, 6, 9, 10, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  1, 2


Analysis of Variance for length sgr day14-28, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS   Adj MS     F      P
tank(treatment)   8  0.37700  0.37700  0.04712  0.77  0.634
rank              7  0.31397  0.31397  0.04485  0.73  0.648
treatment         1  0.02832  0.02832  0.02832  0.60  0.461
Error            63  3.87691  3.87691  0.06154
Total            79  4.59619

S = 0.248069   R-Sq = 15.65%   R-Sq(adj) = 0.00%
```

Length SGR within shelter tanks, day 0-14 versus day 14-28

General Linear Model: length sgr

```
Factor               Type    Levels  Values
tank_1(treatment_1)  random      10  1, 2, 4, 7, 8, 1, 2, 4, 7, 8
rank_1               random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_1          fixed        2  1, 2


Analysis of Variance for length sgr day14-28_1, using Adjusted SS for Tests

Source               DF   Seq SS   Adj SS   Adj MS     F      P
tank_1(treatment_1)   8  0.50171  0.50171  0.06271  1.41  0.211
rank_1                7  0.05641  0.05641  0.00806  0.18  0.988
treatment_1           1  0.01275  0.01275  0.01275  0.20  0.664
Error                63  2.80537  2.80537  0.04453
Total                79  3.37623

S = 0.211021   R-Sq = 16.91%   R-Sq(adj) = 0.00%
```

HSI between treatements

General Linear Model: HSI

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for HSI, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS   Adj MS     F      P
tank(treatment)   8  1.39636  1.39636  0.17455  2.12  0.047
rank              7  0.09151  0.09151  0.01307  0.16  0.992
treatment         1  0.70709  0.70709  0.70709  4.05  0.079
Error            63  5.19863  5.19863  0.08252
Total            79  7.39359

S = 0.287259   R-Sq = 29.69%   R-Sq(adj) = 11.83%
```

Condition factors between treatments day 0

General Linear Model: cf day0

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for cf0, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS   Adj MS     F      P
tank(treatment)   8  0.03504  0.03504  0.00438  0.29  0.968
rank              7  0.13659  0.13659  0.01951  1.28  0.277
treatment         1  0.00005  0.00005  0.00005  0.01  0.920
Error            63  0.96351  0.96351  0.01529
Total            79  1.13519


S = 0.123668   R-Sq = 15.12%   R-Sq(adj) = 0.00%
```

Condition factors between treatments day 28

General Linear Model: cf day28

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for cf28, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS   Adj MS     F      P
tank(treatment)   8  0.12608  0.12608  0.01576  1.01  0.436
rank              7  0.08393  0.08393  0.01199  0.77  0.614
treatment         1  0.00072  0.00072  0.00072  0.05  0.837
Error            63  0.98044  0.98044  0.01556
Total            79  1.19117


S = 0.124750   R-Sq = 17.69%   R-Sq(adj) = 0.00%
```

Condition factors within barren treatment day 0 versus day 28

General Linear Model: cf0 and 28

```
Factor           Type    Levels  Values
tank(time)       random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
time             fixed        2  0, 1


Analysis of Variance for cf0and28barren, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS   Adj MS     F      P
tank(time)        8  0.02898  0.02898  0.00362  0.24  0.981
rank              7  0.12354  0.12354  0.01765  1.19  0.324
time              1  0.00143  0.00143  0.00143  0.40  0.547
Error            63  0.93723  0.93723  0.01488
Total            79  1.09118

S = 0.121970   R-Sq = 14.11%   R-Sq(adj) = 0.00%
```

Condition factors within shelter treatment day 0 versus day 28

General Linear Model: cf0 and 28

```
Factor           Type    Levels  Values
tank(time)       random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
time             fixed        2  0, 1


Analysis of Variance for cf0and28enr, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS   Adj MS     F      P
tank(time)        8  0.13214  0.13214  0.01652  0.97  0.464
rank              7  0.03608  0.03608  0.00515  0.30  0.949
time              1  0.00002  0.00002  0.00002  0.00  0.975
Error            63  1.06764  1.06764  0.01695
Total            79  1.23587


S = 0.130180   R-Sq = 13.61%   R-Sq(adj) = 0.00%
```

Dorsal fin damage between treatments day 28

General Linear Model: dorsal28

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for dorsal28, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   8   8.8000   8.8000  1.1000  3.12  0.545
rank              7   4.8000   4.8000  0.6857  1.95  0.077
treatment         1   0.2000   0.2000  0.2000  0.18  0.681
Error            63  22.2000  22.2000  0.3524
Total            79  36.0000


S = 0.593617   R-Sq = 38.33%   R-Sq(adj) = 22.67%
```

Dorsal fin damage within barren treatment day 0 versus day 28

General Linear Model: dorsal0and28

```
Factor            Type    Levels  Values
tank2(time)       random      10  1, 2, 4, 7, 8, 1, 2, 4, 7, 8
rank2             random       8  1, 2, 3, 4, 5, 6, 7, 8
time              fixed        2  0, 1


Analysis of Variance for dorsal0and28barren, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS   Adj MS      F      P
tank2(time)       8   8.0000   8.0000   1.0000   8.30  0.000
rank2              7   1.2875   1.2875   0.1839   1.53  0.174
time               1  17.1125  17.1125  17.1125  17.11  0.003
Error             63   7.5875   7.5875   0.1204
Total             79  33.9875

S = 0.347040   R-Sq = 77.68%   R-Sq(adj) = 72.01%
```

Dorsal fin damage within shelter treatment day 0 versus day 28

General Linear Model: dorsal0and28

```
Factor            Type    Levels  Values
tank3(time)       random      10  3, 5, 6, 9, 10, 3, 5, 6, 9, 10
rank3             random       8  1, 2, 3, 4, 5, 6, 7, 8
time              fixed        2  0, 1


Analysis of Variance for dorsal0and28enr, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS   Adj MS       F      P
tank3(time)        8   1.0000   1.0000   0.1250    0.47  0.875
rank3              7   2.9875   2.9875   0.4268    1.59  0.154
time               1  21.0125  21.0125  21.0125  168.10  0.000
Error             63  16.8875  16.8875   0.2681
Total             79  41.8875


S = 0.517741   R-Sq = 59.68%   R-Sq(adj) = 49.44%
```

Pectoral fin damage between treatments day 28

General Linear Model: pectoral28

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for pectoral28, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   8   4.6500   4.6500  0.5812  1.10  0.372
rank              7   7.6000   7.6000  1.0857  2.06  0.061
treatment         1   0.8000   0.8000  0.8000  1.38  0.274
Error            63  33.1500  33.1500  0.5262
Total            79  46.2000


S = 0.725390   R-Sq = 28.25%   R-Sq(adj) = 10.02%
```

Pectoral fin damage within barren treatment day 0 versus day 28

General Linear Model: pectoral0and28

```
Factor            Type    Levels  Values
tank2(time)       random      10  1, 2, 4, 7, 8, 1, 2, 4, 7, 8
rank2             random       8  1, 2, 3, 4, 5, 6, 7, 8
time              fixed        2  0, 1


Analysis of Variance for pectoral0and28barren, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS     F      P
tank2(time)        8   6.6000   6.6000  0.8250  1.50  0.175
rank2              7   9.4000   9.4000  1.3429  2.45  0.028
time               1   7.2000   7.2000  7.2000  8.73  0.018
Error             63  34.6000  34.6000  0.5492
Total             79  57.8000


S = 0.741085   R-Sq = 40.14%   R-Sq(adj) = 24.94%
```

Pectoral fin damage within shelter treatment day 0 versus day 28

General Linear Model: pectoral0and28

```
Factor            Type    Levels  Values
tank3(time)       random      10  3, 5, 6, 9, 10, 3, 5, 6, 9, 10
rank3             random       8  1, 2, 3, 4, 5, 6, 7, 8
time              fixed        2  0, 1


Analysis of Variance for pectoral0and28enr, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS      F      P
tank3(time)        8   1.8000   1.8000  0.2250   0.53  0.827
rank3              7  13.2875  13.2875  1.8982   4.50  0.000
time               1   4.5125   4.5125  4.5125  20.06  0.002
Error             63  26.5875  26.5875  0.4220
Total             79  46.1875


S = 0.649634   R-Sq = 42.44%   R-Sq(adj) = 27.82%
```

Plasma cortisol levels between treatments

General Linear Model: cortisol

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for cortisol, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   8    90.93    90.93   11.37  0.49  0.861
rank              7   109.08   109.08   15.58  0.67  0.698
treatment         1     5.31     5.31    5.31  0.47  0.514
Error            63  1470.46  1470.46   23.34
Total            79  1675.78


S = 4.83121   R-Sq = 12.25%   R-Sq(adj) = 0.00%
```

Plasma sodium and chloride levels between treatments

General Linear Model: Na ion

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for na ion, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   8   62.978   62.978   7.872  1.43  0.202
rank              7   73.372   73.372  10.482  1.90  0.084
treatment         1   28.930   28.930  28.930  3.67  0.092
Error            63  347.106  347.106   5.510
Total            79  512.386

S = 2.34726   R-Sq = 32.26%   R-Sq(adj) = 15.05%
```

General Linear Model: Cl ion

```
Factor           Type    Levels  Values
tank(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  0, 1


Analysis of Variance for cl ion, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   8   5368.8   5368.8   671.1  1.68  0.121
rank              7   2450.6   2450.6   350.1  0.88  0.530
treatment         1    970.6    970.6   970.6  1.45  0.264
Error            63  25174.8  25174.8   399.6
Total            79  33964.7


S = 19.9900   R-Sq = 25.88%   R-Sq(adj) = 7.06%
```

Time spent swimming by dominant versus most receiving individual

One-way ANOVA: swim versus dominance

```
Source   DF       SS       MS      F      P
hirarki   1  1154330  1154330  20.51  0.000
Error    93  5233653    56276
Total    94  6387983

S = 237.2   R-Sq = 18.07%   R-Sq(adj) = 17.19%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level   N   Mean  StDev  ---+---------+---------+---------+------
0      42  542.3  281.1  (------*------)
1      53  764.3  195.9                       (-----*------)
                         ---+---------+---------+---------+------
                          500       600       700       800
```

Frequency of aggression between treatments day 0-14

One-way ANOVA: agro0-14barren versus trt

```
Source  DF      SS     MS     F      P
trt      1   11.33  11.33  1.64  0.204
Error   72  497.29   6.91
Total   73  508.62

S = 2.628   R-Sq = 2.23%   R-Sq(adj) = 0.87%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level   N   Mean  StDev  --+---------+---------+---------+-------
0      36  4.298  2.591              (-----------*------------)
1      38  3.516  2.663  (-----------*-----------)
                         --+---------+---------+---------+-------
                         2.80      3.50      4.20      4.90
```

Frequency of aggression between treatments day 14-28

One-way ANOVA: agro14-28barren versus trt

```
Source  DF      SS     MS     F      P
trt      1   10.24  10.24  1.59  0.212
Error   68  438.10   6.44
Total   69  448.34

S = 2.538   R-Sq = 2.28%   R-Sq(adj) = 0.85%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  ----+---------+---------+---------+-----
0      39  3.300  2.424  (----------*----------)
1      31  4.070  2.676            (------------*------------)
                          ----+---------+---------+---------+-----
                            2.80      3.50      4.20      4.90
```

Frequency of aggression within barren treatment day 0-14 versus day 14-28

One-way ANOVA: agro0-14barren versus trt

```
Source  DF      SS    MS     F      P
trt      1    5.25  5.25  0.74  0.393
Error   67  477.09  7.12
Total   68  482.34

S = 2.668   R-Sq = 1.09%   R-Sq(adj) = 0.00%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  ------+---------+---------+---------+---
0      31  4.070  2.676           (---------------*---------------)
1      38  3.516  2.663  (--------------*-------------)
                          ------+---------+---------+---------+---
                              3.00      3.60      4.20      4.80
```

Frequency of aggression within shelter treatment day 0-14 versus day 14-28

One-way ANOVA: agro0-14enr versus trt

```
Source  DF      SS     MS     F      P
trt      1   18.65  18.65  2.97  0.089
Error   73  458.30   6.28
Total   74  476.95

S = 2.506   R-Sq = 3.91%   R-Sq(adj) = 2.59%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  ----+---------+---------+---------+-----
1      36  4.298  2.591               (----------*----------)
2      39  3.300  2.424  (----------*----------)
                          ----+---------+---------+---------+-----
                            2.80      3.50      4.20      4.90
```

Time spent in front and back hide

One-way ANOVA: time spent versus position

```
Source    DF       SS       MS      F      P
position   1  1892993  1892993  33.42  0.000
Error     54  3058952    56647
Total     55  4951945

S = 238.0   R-Sq = 38.23%   R-Sq(adj) = 37.08%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  ---+---------+---------+---------+------
1      28  413.1  302.6                          (-----*-----)
2      28   45.4  147.4  (-----*-----)
                          ---+---------+---------+---------+------
                             0       150       300       450

Pooled StDev = 238.0
```

Brain monoamine and metabolite levels between treatments

Telencephalon
General Linear Model: DA

```
Factor              Type    Levels  Values
Tank nr(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment           fixed        2  0, 1


Analysis of Variance for DA (ng/ mg protein), using Adjusted SS for Tests

Source              DF    Seq SS    Adj SS   Adj MS     F      P
Tank nr(treatment)   8   9508187   9287615  1160952  1.35  0.236
rank                 7   6808923   6541051   934436  1.09  0.382
treatment            1   2356234   2356234  2356234  2.03  0.192
Error               61  52371596  52371596   858551
Total               77  71044939


S = 926.580   R-Sq = 26.28%   R-Sq(adj) = 6.95%
```

General Linear Model: NA

```
Factor              Type    Levels  Values
Tank nr(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment           fixed        2  0, 1


Analysis of Variance for NE (ng/ mg protein), using Adjusted SS for Tests

Source              DF     Seq SS     Adj SS   Adj MS     F      P
Tank nr(treatment)   8   11937543   11959569  1494946  0.89  0.532
rank                 7   13402717   12887154  1841022  1.09  0.378
treatment            1    6283228    6283228  6283228  4.20  0.074
Error               61  102631977  102631977  1682491
Total               77  134255465

S = 1297.11   R-Sq = 23.55%   R-Sq(adj) = 3.50%
```

General Linear Model: 5-HIAA

```
Factor              Type    Levels  Values
Tank nr(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment           fixed        2  0, 1


Analysis of Variance for 5-HIAA (ng/ mg protein), using Adjusted SS for Tests

Source              DF    Seq SS   Adj SS  Adj MS     F      P
Tank nr(treatment)   8   2920452  2915290  364411  2.70  0.013
rank                 7    763867   763538  109077  0.81  0.583
treatment            1    665474   665474  665474  1.83  0.213
Error               61   8221848  8221848  134784
Total               77  12571641


S = 367.130   R-Sq = 34.60%   R-Sq(adj) = 17.45%
```

General Linear Model: 5-HT

```
Factor              Type    Levels  Values
Tank nr(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment           fixed        2  0, 1


Analysis of Variance for 5-HT (ng/ mg protein), using Adjusted SS for Tests

Source              DF     Seq SS     Adj SS   Adj MS     F      P
Tank nr(treatment)   8   31123705   31601522  3950190  1.81  0.093
rank                 7   26386490   26022306  3717472  1.70  0.125
treatment            1    9884886    9884886  9884886  2.51  0.152
Error               61  133247229  133247229  2184381
Total               77  200642310

S = 1477.97   R-Sq = 33.59%   R-Sq(adj) = 16.17%
```

General Linear Model: RATIO

```
Factor              Type    Levels  Values
Tank nr(treatment)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment           fixed        2  0, 1


Analysis of Variance for RATIO, using Adjusted SS for Tests

Source              DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(treatment)   8   2.2945   2.3139  0.2892  1.17  0.333
rank                 7   1.6040   1.6029  0.2290  0.92  0.494
treatment            1   0.3712   0.3712  0.3712  1.28  0.290
Error               61  15.1087  15.1087  0.2477
Total               77  19.3783
S = 0.497678   R-Sq = 22.03%   R-Sq(adj) = 1.58%
```

Hypothalamus

General Linear Model: DA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
Rank          fixed        8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        2  1, 2


Analysis of Variance for DA (ng/ mg protein), using Adjusted SS for Tests

Source        DF      Seq SS      Adj SS      Adj MS     F      P
Tank nr(TRT)   8  2298948663  2247199264   280899908  4.30  0.000
Rank           7   884260354   777312339   111044620  1.70  0.127
TRT            1  2352159401  2352159401  2352159401  8.38  0.020
Error         58  3791360731  3791360731    65368288
Total         74  9326729149


S = 8085.07   R-Sq = 59.35%   R-Sq(adj) = 48.14%
```

General Linear Model: NA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
Rank          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        2  1, 2


Analysis of Variance for NE (ng/ mg protein), using Adjusted SS for Tests

Source        DF      Seq SS      Adj SS     Adj MS     F      P
Tank nr(TRT)   8  1408091291  1372800736  171600092  3.98  0.001
Rank           7   388009151   364240756   52034394  1.21  0.314
TRT            1   315654136   315654136  315654136  1.84  0.212
Error         58  2503327477  2503327477   43160819
Total         74  4615082055
S = 6569.69   R-Sq = 45.76%   R-Sq(adj) = 30.79%
```

General Linear Model: 5-HIAA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
Rank          fixed        8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        2  1, 2


Analysis of Variance for 5-HIAA (ng/ mg protein), using Adjusted SS for Tests

Source        DF    Seq SS    Adj SS   Adj MS     F      P
Tank nr(TRT)   8  10576669  10182541  1272818  3.01  0.007
Rank           7   4791555   4654877   664982  1.57  0.161
TRT            1   9836606   9836606  9836606  7.74  0.024
Error         58  24488811  24488811   422221
Total         74  49693640

S = 649.785   R-Sq = 50.72%   R-Sq(adj) = 37.13%
```

General Linear Model: 5-HT

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
Rank          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        2  1, 2

Analysis of Variance for 5-HT (ng/ mg protein), using Adjusted SS for Tests

Source        DF       Seq SS       Adj SS      Adj MS     F      P
Tank nr(TRT)   8   9118292518   9401639439  1175204930  4.00  0.001
Rank           7   2149345342   2150636169   307233738  1.05  0.410
TRT            1    399940930    399940930   399940930  0.34  0.575
Error         57  16731325977  16731325977   293532035
Total         73  28398904766
S = 17132.8   R-Sq = 41.08%   R-Sq(adj) = 24.55%
```

General Linear Model: RATIO

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
Rank          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        2  1, 2

Analysis of Variance for RATIO, using Adjusted SS for Tests

Source        DF   Seq SS   Adj SS   Adj MS     F      P
Tank nr(TRT)   8  0.27043  0.27308  0.03414  1.29  0.267
Rank           7  0.20206  0.19236  0.02748  1.04  0.415
TRT            1  0.05417  0.05417  0.05417  1.59  0.243
Error         58  1.53462  1.53462  0.02646
Total         74  2.06129
S = 0.162662   R-Sq = 25.55%   R-Sq(adj) = 5.01%
```

Brain stem

General Linear Model: 5-HT

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for 5ht, using Adjusted SS for Tests

Source     DF      Seq SS      Adj SS     Adj MS     F      P
tank(TRT)   8  1182798225  1182798225  147849778  2.34  0.029
rank        7   433772461   433772461   61967494  0.98  0.453
TRT         1    90823475    90823475   90823475  0.61  0.456
Error      63  3980090755  3980090755   63176044
Total      79  5687484915
S = 7948.34   R-Sq = 30.02%   R-Sq(adj) = 12.25%
```

General Linear Model: 5-HIAA

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for 5hiaa, using Adjusted SS for Tests

Source     DF    Seq SS    Adj SS   Adj MS     F      P
tank(TRT)   8   4430116   4430116   553764  1.20  0.315
rank        7   2011668   2011668   287381  0.62  0.736
TRT         1   3295915   3295915  3295915  5.95  0.041
Error      63  29129141  29129141   462367
Total      79  38866839
S = 679.976   R-Sq = 25.05%   R-Sq(adj) = 6.02
```

General Linear Model: DA

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for da, using Adjusted SS for Tests

Source     DF      Seq SS      Adj SS    Adj MS     F      P
tank(TRT)   8   341453069   341453069  42681634  2.00  0.061
rank        7   132180848   132180848  18882978  0.88  0.525
TRT         1    38892483    38892483  38892483  0.91  0.368
Error      63  1346670032  1346670032  21375715
Total      79  1859196431
S = 4623.39   R-Sq = 27.57%   R-Sq(adj) = 9.17%
```

General Linear Model: NA

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for ne, using Adjusted SS for Tests

Source     DF      Seq SS      Adj SS     Adj MS     F      P
tank(TRT)   8  1123803978  1123803978  140475497  2.37  0.027
rank        7   325792824   325792824   46541832  0.79  0.601
TRT         1    49459489    49459489   49459489  0.35  0.569
Error      63  3728980150  3728980150   59190161
Total      79  5228036441
S = 7693.51   R-Sq = 28.67%   R-Sq(adj) = 10.56%
```

General Linear Model: RATIO

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for ratio, using Adjusted SS for Tests

Source     DF    Seq SS    Adj SS    Adj MS     F      P
tank(TRT)   8  0.079285  0.079285  0.009911  2.69  0.013
rank        7  0.034569  0.034569  0.004938  1.34  0.246
TRT         1  0.000021  0.000021  0.000021  0.00  0.964
Error      63  0.232050  0.232050  0.003683
Total      79  0.345925
S = 0.0606904   R-Sq = 32.92%   R-Sq(adj) = 15.88%
```

Optic tectum

General Linear Model: DA

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for da, using Adjusted SS for Tests

Source     DF    Seq SS    Adj SS  Adj MS     F      P
tank(TRT)   8   4854386   4854386  606798  1.12  0.364
rank        7   2629913   2629913  375702  0.69  0.678
TRT         1    682104    682104  682104  1.12  0.320
Error      63  34199373  34199373  542847
Total      79  42365775
S = 736.782   R-Sq = 19.28%   R-Sq(adj) = 0.00%
```

General Linear Model: NA

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for ne, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS   Adj MS     F      P
tank(TRT)   8    9635061    9635061  1204383  0.67  0.717
rank        7    4672968    4672968   667567  0.37  0.916
TRT         1     344276     344276   344276  0.29  0.607
Error      63  113478219  113478219  1801242
Total      79  128130524
S = 1342.10   R-Sq = 11.44%   R-Sq(adj) = 0.00%
```

General Linear Model: 5-HT

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for 5ht, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS   Adj MS     F      P
tank(TRT)   8   17152971   17152971  2144121  1.12  0.365
rank        7    3764305    3764305   537758  0.28  0.960
TRT         1      24400      24400    24400  0.01  0.918
Error      63  121018320  121018320  1920926
Total      79  141959996
S = 1385.97   R-Sq = 14.75%   R-Sq(adj) = 0.00%
```

General Linear Model: 5-HIAA

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for 5hiaa, using Adjusted SS for Tests

Source     DF  Seq SS  Adj SS  Adj MS      F      P
tank(TRT)   8   65969   65969    8246   0.80  0.606
rank        7   47421   47421    6774   0.66  0.708
TRT         1   88024   88024   88024  10.67  0.011
Error      63  650335  650335   10323
Total      79  851748


S = 101.601   R-Sq = 23.65%   R-Sq(adj) = 4.26%
```

General Linear Model: RATIO

```
Factor     Type    Levels  Values
tank(TRT)  random      10  1, 2, 4, 7, 8, 3, 5, 6, 9, 10
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT        fixed        2  0, 1


Analysis of Variance for ratio, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS     F      P
tank(TRT)   8  0.0091257  0.0091257  0.0011407  1.24  0.293
rank        7  0.0038444  0.0038444  0.0005492  0.60  0.757
TRT         1  0.0052505  0.0052505  0.0052505  4.60  0.064
Error      63  0.0580862  0.0580862  0.0009220
Total      79  0.0763069


S = 0.0303645   R-Sq = 23.88%   R-Sq(adj) = 4.55%
```

APPENDIX - Statistical output for results chapter 4

Mass SGR day 0-14 between treatments

General Linear Model: MASSsgr 0-14

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for MASSsgr 0-14, using Adjusted SS for Tests

Source      DF   Seq SS   Adj SS  Adj MS     F      P
tank(trt)   15   9.5957   9.4839  0.6323  1.21  0.275
rank         7  10.5692  10.6093  1.5156  2.90  0.008
trt          2   1.8624   1.8624  0.9312  1.47  0.261
Error      118  61.7552  61.7552  0.5233
Total      142  83.7825

S = 0.723429   R-Sq = 26.29%   R-Sq(adj) = 11.30%
```

Mass SGR day 14-28 between treatments

General Linear Model: MASSsgr 14-28

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for MASSsgr 14-28, using Adjusted SS for Tests

Source      DF   Seq SS   Adj SS  Adj MS     F      P
tank(trt)   15  22.0977  22.4340  1.4956  3.88  0.000
rank         7   6.8758   6.8631  0.9804  2.54  0.018
trt          2   0.3482   0.3482  0.1741  0.12  0.891
Error      118  45.4967  45.4967  0.3856
Total      142  74.8184

S = 0.620939   R-Sq = 39.19%   R-Sq(adj) = 26.82%
```

Mass SGR within barren treatment day 0-14 versus 14-28

General Linear Model: MASSsgr 0-14

```
Factor     Type    Levels  Values
tank(trt)  random      12  3, 6, 7, 11, 15, 16, 3, 6, 7, 11, 15, 16
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        2  0, 1


Analysis of Variance for MASSsgr 0-14, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS     F      P
tank(trt)  10  15.5634  15.5634  1.5563  3.31  0.001
rank        7  13.9632  13.9632  1.9947  4.24  0.001
trt         1   6.9937   6.9937  6.9937  4.49  0.060


Error      77  36.2167  36.2167  0.4703
Total      95  72.7371

S = 0.685819   R-Sq = 50.21%   R-Sq(adj) = 38.57%
```

Mass SGR within barrier treatment day 0-14 versus 14-28

General Linear Model: sgr barrier

```
Factor          Type    Levels  Values
tank_1(trt_1)  random      12  2, 4, 9, 10, 13, 18, 2, 4, 9, 10, 13, 18
rank_1         random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1          fixed        2  1, 2


Analysis of Variance for sgr barrier, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
tank_1(trt_1)  10   9.8732   9.5348  0.9535  2.78  0.006
rank_1          7   5.1584   5.1584  0.7369  2.15  0.048
trt_1           1   7.5302   7.5302  7.5302  7.90  0.018
Error          75  25.7029  25.7029  0.3427
Total          93  48.2648

S = 0.585411   R-Sq = 46.75%   R-Sq(adj) = 33.97%
```

Mass SGR within visual barrier treatment day 0-14 versus 14-28

General Linear Model: sgr

```
Factor          Type    Levels  Values
tank_2(trt_2)  random      12  1, 5, 8, 12, 14, 17, 1, 5, 8, 12, 14, 17
rank_2         random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_2          fixed        2  2, 3


Analysis of Variance for sgr ob, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
tank_2(trt_2)  10   6.8195   6.8195  0.6819  1.47  0.169
rank_2          7   7.8571   7.8571  1.1224  2.41  0.027
trt_2           1   3.6168   3.6168  3.6168  5.30  0.044
Error          77  35.8260  35.8260  0.4653
Total          95  54.1194

S = 0.682109   R-Sq = 33.80%   R-Sq(adj) = 18.33%
```

Length SGR 0-14 between treatments

General Linear Model: LENGTHsgr

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for LENGTHsgr 0-14, using Adjusted SS for Tests

Source      DF    Seq SS   Adj SS   Adj MS     F      P
tank(trt)   15   1.76671  1.77491  0.11833  1.53  0.104
rank         7   0.71357  0.71840  0.10263  1.33  0.242


trt          2   0.15732  0.15732  0.07866  0.66  0.529
Error      118   9.10180  9.10180  0.07713
Total      142  11.73940

S = 0.277730   R-Sq = 22.47%   R-Sq(adj) = 6.70%
```

Length SGR day 14-28 between treatments

General Linear Model: LENGTHsgr 14-28

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for LENGTHsgr 14-28, using Adjusted SS for Tests

Source      DF    Seq SS   Adj SS   Adj MS     F      P
tank(trt)   15   2.63001  2.69511  0.17967  2.26  0.008
rank         7   1.69532  1.69484  0.24212  3.04  0.006
trt          2   0.00754  0.00754  0.00377  0.02  0.979 x
Error      118   9.38329  9.38329  0.07952
Total      142  13.71616
```

Length SGR within visual barriers day 0-14 versus 14-28

General Linear Model: length sgr_2 versus rank_2, trt_2, tank_2

```
Factor         Type    Levels  Values
tank_2(trt_2)  random      12  1, 5, 8, 12, 14, 17, 1, 5, 8, 12, 14, 17
rank_2         random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_2          fixed        2  2, 3


Analysis of Variance for length sgr_2, using Adjusted SS for Tests

Source         DF    Seq SS   Adj SS   Adj MS      F      P
tank_2(trt_2)  10   1.54073  1.54073  0.15407   1.77  0.080
rank_2          7   0.99141  0.99141  0.14163   1.63  0.139
trt_2           1   2.33447  2.33447  2.33447  15.15  0.003
Error          77   6.68733  6.68733  0.08685
Total          95  11.55394

S = 0.294701   R-Sq = 42.12%   R-Sq(adj) = 28.59%
```

Length SGR within barrier tanks day 0-14 versus 14-28

General Linear Model: length sgr_

```
Factor         Type    Levels  Values
tank_1(trt_1)  random      12  2, 4, 9, 10, 13, 18, 2, 4, 9, 10, 13, 18
rank_1         random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1          fixed        2  1, 2


Analysis of Variance for length sgr_1, using Adjusted SS for Tests

Source         DF    Seq SS   Adj SS   Adj MS      F      P
tank_1(trt_1)  10   1.33806  1.27241  0.12724   1.64  0.112


rank_1          7   0.56997  0.56997  0.08142   1.05  0.404
trt_1           1   3.45933  3.45933  3.45933  27.19  0.000 x
Error          75   5.81734  5.81734  0.07756
Total          93  11.18471
```

Length SGR within barren tanks day 0-14 versus 14-28

General Linear Model: length sgr

```
Factor     Type    Levels  Values
tank(trt)  random      12  3, 6, 7, 11, 15, 16, 3, 6, 7, 11, 15, 16
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        2  0, 1


Analysis of Variance for length sgr, using Adjusted SS for Tests

Source     DF    Seq SS   Adj SS   Adj MS      F      P
tank(trt)  10   1.61957  1.61957  0.16196   2.19  0.027
rank        7   1.14274  1.14274  0.16325   2.21  0.042
trt         1   2.35317  2.35317  2.35317  14.53  0.003
Error      77   5.68954  5.68954  0.07389
Total      95  10.80502

S = 0.271827   R-Sq = 47.34%   R-Sq(adj) = 35.03%
```

HSI between treatments

General Linear Model: HSI

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for HSI, using Adjusted SS for Tests

Source      DF   Seq SS   Adj SS  Adj MS     F      P
tank(trt)   15   3.9638   4.0644  0.2710  2.21  0.009
rank         7   0.8803   0.9054  0.1293  1.05  0.397
trt          2   0.9671   0.9671  0.4836  1.79  0.202
Error      118  14.4765  14.4765  0.1227
Total      142  20.2877

S = 0.350260   R-Sq = 28.64%   R-Sq(adj) = 14.13%
```

CF between treatments day 0 and day 28

General Linear Model: cf0

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for cf0, using Adjusted SS for Tests

Source       DF    Seq SS    Adj SS    Adj MS     F      P
MASSday 28    1  0.000343  0.003720  0.003720  0.45  0.504


tank(trt)    15  0.151196  0.147165  0.009811  1.18  0.295
rank          7  0.014889  0.013555  0.001936  0.23  0.976
trt           2  0.080143  0.080143  0.040071  4.08  0.039
Error       117  0.970831  0.970831  0.008298
Total       142  1.217402

S = 0.0910917   R-Sq = 20.25%   R-Sq(adj) = 3.21%
```

General Linear Model: cf28

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for cf28, using Adjusted SS for Tests

Source      DF   Seq SS   Adj SS   Adj MS     F      P
tank(trt)   15  0.14456  0.14539  0.00969  0.85  0.626
rank         7  0.02674  0.02678  0.00383  0.33  0.937
trt          2  0.00194  0.00194  0.00097  0.10  0.906
Error      118  1.35304  1.35304  0.01147
Total      142  1.52628

S = 0.107081   R-Sq = 11.35%   R-Sq(adj) = 0.00%
```

CF within barren treatment day 0 versus 28

General Linear Model: cf0 AND 28

```
Factor     Type    Levels  Values
tank(trt)  random      12  3, 6, 7, 11, 15, 16, 3, 6, 7, 11, 15, 16
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        2  0, 1


Analysis of Variance for cf0 AND 28, using Adjusted SS for Tests

Source     DF    Seq SS    Adj SS    Adj MS      F      P
tank(trt)  10  0.101759  0.101759  0.010176   1.11  0.367
rank        7  0.171093  0.171093  0.024442   2.66  0.016
trt         1  0.451409  0.451409  0.451409  44.36  0.000
Error      77  0.707418  0.707418  0.009187
Total      95  1.431679

S = 0.0958501   R-Sq = 50.59%   R-Sq(adj) = 39.04%
```

CF within barrier treatment day 0 versus 28

General Linear Model: cf0AND28

```
Factor         Type    Levels  Values
tank_1(trt_1)  random      12  2, 4, 9, 10, 13, 18, 2, 4, 9, 10, 13, 18
rank_1         random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1          fixed        2  1, 2


Analysis of Variance for cf0AND28_1, using Adjusted SS for Tests

Source         DF   Seq SS   Adj SS   Adj MS      F      P
tank_1(trt_1)  10  0.08182  0.08576  0.00858   0.71  0.708
rank_1          7  0.05809  0.05809  0.00830   0.69  0.679
trt_1           1  0.81926  0.81926  0.81926  95.53  0.000


Error          75  0.90017  0.90017  0.01200
Total          93  1.85934

S = 0.109555   R-Sq = 51.59%   R-Sq(adj) = 39.97%
```

CF within barren treatment day 0 versus 28

General Linear Model: cf0AND28

```
Factor             Type    Levels  Values
tank_1_1(trt_1_1)  random      12  1, 5, 8, 12, 14, 17, 1, 5, 8, 12, 14, 17
rank_1_1           random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1_1            fixed        2  2, 3


Analysis of Variance for cf0AND28_1_1, using Adjusted SS for Tests

Source             DF    Seq SS    Adj SS    Adj MS      F      P
tank_1_1(trt_1_1)  10  0.100660  0.100660  0.010066   1.55  0.139
rank_1_1            7  0.034428  0.034428  0.004918   0.76  0.625
trt_1_1             1  0.764286  0.764286  0.764286  75.93  0.000
Error              77  0.500526  0.500526  0.006500
Total              95  1.399899

S = 0.0806246   R-Sq = 64.25%   R-Sq(adj) = 55.89%
```

Dorsal fin damage between treatments day 0 and 28

General Linear Model: Dfin 0

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for Dfin 0, using Adjusted SS for Tests

Source      DF    Seq SS   Adj SS  Adj MS     F      P
tank(trt)   15   15.4350  15.4505  1.0300  1.44  0.139
rank         7    1.7418   1.7297  0.2471  0.35  0.931
trt          2    0.6942   0.6942  0.3471  0.34  0.719
Error      118   84.1989  84.1989  0.7135
Total      142  102.0699

S = 0.844719   R-Sq = 17.51%   R-Sq(adj) = 0.73%
```

General Linear Model: Dfin 28

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for Dfin 28, using Adjusted SS for Tests

Source      DF   Seq SS   Adj SS  Adj MS     F      P
tank(trt)   15  19.5377  19.4410  1.2961  2.81  0.001


rank         7   3.0380   3.0356  0.4337  0.94  0.477
trt          2   0.5114   0.5114  0.2557  0.20  0.823
Error      118  54.3394  54.3394  0.4605
Total      142  77.4266

S = 0.678604   R-Sq = 29.82%   R-Sq(adj) = 15.54%
```

Pectoral fin damage between treatments day 0 and 28

General Linear Model: Pfin 0

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for Pfin 0, using Adjusted SS for Tests

Source      DF   Seq SS   Adj SS  Adj MS     F      P
tank(trt)   15   14.137   14.167   0.944  0.93  0.538
rank         7    3.500    3.496   0.499  0.49  0.841
trt          2    1.246    1.246   0.623  0.66  0.531
Error      118  120.361  120.361   1.020
Total      142  139.245

S = 1.00995   R-Sq = 13.56%   R-Sq(adj) = 0.00%
```

General Linear Model: Pfin 28

```
Factor     Type    Levels  Values
tank(trt)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5, 8,
                           12, 14, 17
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        3  0, 1, 2


Analysis of Variance for Pfin 28, using Adjusted SS for Tests

Source      DF    Seq SS    Adj SS  Adj MS     F      P
tank(trt)   15   12.5093   12.5288  0.8353  0.94  0.525
rank         7    4.3528    4.3437  0.6205  0.70  0.675
trt          2    0.8247    0.8247  0.4123  0.49  0.620
Error      118  105.1384  105.1384  0.8910
Total      142  122.8252

S = 0.943930   R-Sq = 14.40%   R-Sq(adj) = 0.00%
```

Dorsal fin damage within barren treatment day 0 versus 28

General Linear Model: Dfin 0 AND 28

```
Factor     Type    Levels  Values
tank(trt)  random      12  3, 6, 7, 11, 15, 16, 3, 6, 7, 11, 15, 16
rank       random       8  1, 2, 3, 4, 5, 6, 7, 8
trt        fixed        2  0, 1


Analysis of Variance for Dfin 0 AND 28, using Adjusted SS for Tests

Source     DF   Seq SS   Adj SS  Adj MS     F      P
tank(trt)  10   9.5833   9.5833  0.9583  1.92  0.054
rank        7   2.6250   2.6250  0.3750  0.75  0.629
trt         1   3.3750   3.3750  3.3750  3.52  0.090
Error      77  38.3750  38.3750  0.4984
Total      95  53.9583

S = 0.705958   R-Sq = 28.88%   R-Sq(adj) = 12.25%
```

Dorsal fin damage within barrier treatment day 0 versus 28

General Linear Model: Dfin 0AND28

```
Factor          Type    Levels  Values
tank_1(trt_1)   random      12  2, 4, 9, 10, 13, 18, 2, 4, 9, 10, 13, 18
rank_1          random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1           fixed        2  1, 2


Analysis of Variance for Dfin 0AND28_1, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
tank_1(trt_1)   10   9.8819   9.9726  0.9973  1.92  0.056
rank_1           7   3.5446   3.5446  0.5064  0.97  0.457
trt_1            1   2.2029   2.2029  2.2029  2.21  0.168
Error           75  39.0089  39.0089  0.5201
Total           93  54.6383

S = 0.721193   R-Sq = 28.61%   R-Sq(adj) = 11.47%
```

Dorsal fin damage within visual barrier treatment day 0 versus 28

General Linear Model: Dfin 0AND28

```
Factor              Type    Levels  Values
tank_1_1(trt_1_1)   random      12  1, 5, 8, 12, 14, 17, 1, 5, 8, 12, 14, 17
rank_1_1            random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1_1             fixed        2  2, 3


Analysis of Variance for Dfin 0AND28_1_1, using Adjusted SS for Tests

Source              DF   Seq SS   Adj SS  Adj MS     F      P
tank_1_1(trt_1_1)   10  15.3750  15.3750  1.5375  2.13  0.032
rank_1_1             7   4.1667   4.1667  0.5952  0.82  0.570
trt_1_1              1   3.3750   3.3750  3.3750  2.20  0.169
Error               77  55.5833  55.5833  0.7219
Total               95  78.5000

S = 0.849624   R-Sq = 29.19%   R-Sq(adj) = 12.64%
```

Pectoral fin damage within barren treatment day 0 versus 28

General Linear Model: Pfin 0 AND 28

```
Factor      Type    Levels  Values
tank(trt)   random      12  3, 6, 7, 11, 15, 16, 3, 6, 7, 11, 15, 16
rank        random       8  1, 2, 3, 4, 5, 6, 7, 8
trt         fixed        2  0, 1


Analysis of Variance for Pfin 0 AND 28, using Adjusted SS for Tests

Source      DF   Seq SS   Adj SS  Adj MS     F      P
tank(trt)   10   4.8542   4.8542  0.4854  0.57  0.833
rank         7   9.0729   9.0729  1.2961  1.52  0.172
trt          1   0.0104   0.0104  0.0104  0.02  0.886
Error       77  65.5521  65.5521  0.8513
Total       95  79.4896

S = 0.922673   R-Sq = 17.53%   R-Sq(adj) = 0.00%
```

Pectoral fin damage within barrier treatment day 0 versus 28

General Linear Model: Pfin 0AND28

```
Factor          Type    Levels  Values
tank_1(trt_1)   random      12  2, 4, 9, 10, 13, 18, 2, 4, 9, 10, 13, 18
rank_1          random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1           fixed        2  1, 2


Analysis of Variance for Pfin 0AND28_1, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
tank_1(trt_1)   10   9.6771   9.7900  0.9790  1.23  0.287
rank_1           7   7.3732   7.3732  1.0533  1.32  0.251
trt_1            1   0.0102   0.0102  0.0102  0.01  0.921
Error           75  59.7161  59.7161  0.7962
Total           93  76.7766

S = 0.892308   R-Sq = 22.22%   R-Sq(adj) = 3.55%
```

Pectoral fin damage within visual barrier treatment day 0 versus 28

General Linear Model: Pfin 0AND28

```
Factor              Type    Levels  Values
tank_1_1(trt_1_1)   random      12  1, 5, 8, 12, 14, 17, 1, 5, 8, 12, 14, 17
rank_1_1            random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1_1             fixed        2  2, 3


Analysis of Variance for Pfin 0AND28_1_1, using Adjusted SS for Tests

Source              DF   Seq SS  Adj SS  Adj MS     F      P
tank_1_1(trt_1_1)   10   12.187  12.188   1.219  1.20  0.307
rank_1_1             7   13.156  13.156   1.879  1.84  0.091
trt_1_1              1    0.094   0.094   0.094  0.08  0.787
Error               77   78.469  78.469   1.019
Total               95  103.906

S = 1.00949   R-Sq = 24.48%   R-Sq(adj) = 6.83%
```

Brain monoamines and metabolite levels between treatments

Telencephalon

General Linear Model: DA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                              8, 12, 14, 17
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        3  1, 2, 3


Analysis of Variance for DA, using Adjusted SS for Tests

Source         DF    Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)   15   12.1449  12.8384  0.8559  1.02  0.442
RANK            7    4.9217   4.8951  0.6993  0.83  0.563
TRT             2    1.8168   1.8168  0.9084  1.06  0.371
Error         116   97.5137  97.5137  0.8406
Total         140  116.3970

S = 0.916862   R-Sq = 16.22%   R-Sq(adj) = 0.00%
```

General Linear Model: NA

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                               8, 12, 14, 17
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for NE, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    15   45.444   45.306   3.020  1.08  0.386
RANK             7   13.660   13.762   1.966  0.70  0.671
TRT              2    1.752    1.752   0.876  0.29  0.752
Error          117  328.316  328.316   2.806
Total          141  389.172

S = 1.67515   R-Sq = 15.64%   R-Sq(adj) = 0.00%
```

General Linear Model: 5-HIAA

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                               8, 12, 14, 17
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for 5-HIAA, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    15   4.9429   5.1819  0.3455  1.40  0.157
RANK             7   1.1394   1.1837  0.1691  0.69  0.683
TRT              2   1.1205   1.1205  0.5603  1.62  0.231
Error          117  28.7966  28.7966  0.2461
Total          141  35.9994

S = 0.496110   R-Sq = 20.01%   R-Sq(adj) = 3.60%
```

General Linear Model: 5-HT

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                               8, 12, 14, 17
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for 5-HT, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    15   19.280   19.795   1.320  1.29  0.218
RANK             7    7.787    7.804   1.115  1.09  0.373
TRT              2    1.538    1.538   0.769  0.58  0.571
Error          116  118.470  118.470   1.021
Total          140  147.075

S = 1.01059   R-Sq = 19.45%   R-Sq(adj) = 2.78%
```

General Linear Model: RATIO

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                               8, 12, 14, 17
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for RATIO, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    15  139.262  141.028   9.402  1.88  0.032
RANK             7   75.574   75.492  10.785  2.16  0.043
TRT              2   14.890   14.890   7.445  0.79  0.472
Error          117  585.121  585.121   5.001
Total          141  814.847

S = 2.23630   R-Sq = 28.19%   R-Sq(adj) = 13.46%
```

Hypothalamus

General Linear Model: DA

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                               8, 12, 14, 17
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for DA, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    15   47.187   46.859   3.124  2.23  0.009
RANK             7   19.374   18.702   2.672  1.91  0.075
TRT              2   22.684   22.684  11.342  3.63  0.052
Error          109  152.586  152.586   1.400
Total          133  241.832

S = 1.18316   R-Sq = 36.90%   R-Sq(adj) = 23.01%
```

DA for visual barriers versus barren tanks

General Linear Model: DA

```
Factor             Type    Levels  Values
Tank nr_1(TRT_1)   random      12  2, 4, 9, 10, 13, 18, 1, 5, 8, 12, 14, 17
RANK_1             random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT_1              fixed        2  2, 3


Analysis of Variance for DA_1, using Adjusted SS for Tests

Source             DF   Seq SS  Adj SS  Adj MS      F      P
Tank nr_1(TRT_1)   10   10.101   9.489   0.949   0.85  0.584
RANK_1              7   12.468  12.294   1.756   1.57  0.159
TRT_1               1   14.800  14.800  14.800  15.61  0.003
Error              70   78.291  78.291   1.118
Total              88  115.661

S = 1.05757   R-Sq = 32.31%   R-Sq(adj) = 14.90%
```

DA for visual barriers versus barrier tanks

General Linear Model: DA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      12  3, 6, 7, 11, 15, 16, 1, 5, 8, 12, 14, 17
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        2  1, 3


Analysis of Variance for DA, using Adjusted SS for Tests

Source        DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)  10   42.741   41.868   4.187  2.69  0.007
RANK           7   22.908   21.975   3.139  2.02  0.064
TRT            1   18.735   18.735  18.735  4.48  0.060
Error         71  110.327  110.327   1.554
Total         89  194.711

S = 1.24655   R-Sq = 43.34%   R-Sq(adj) = 28.97%
```

General Linear Model: NA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                              8, 12, 14, 17
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        3  1, 2, 3


Analysis of Variance for NE, using Adjusted SS for Tests

Source         DF    Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)   15   31.0107  32.3641  2.1576  3.31  0.000
RANK            7    6.4401   6.1145  0.8735  1.34  0.238
TRT             2   15.6291  15.6291  7.8146  3.63  0.052
Error         109   71.0282  71.0282  0.6516
Total         133  124.1082

S = 0.807239   R-Sq = 42.77%   R-Sq(adj) = 30.17%
```

NA visual barrier versus barren tanks

General Linear Model: NA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      12  3, 6, 7, 11, 15, 16, 1, 5, 8, 12, 14, 17
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        2  1, 3


Analysis of Variance for NE, using Adjusted SS for Tests

Source        DF   Seq SS   Adj SS   Adj MS     F      P
Tank nr(TRT)  10  24.3388  24.7128   2.4713  3.52  0.001
RANK           7   9.8118   9.3521   1.3360  1.90  0.082
TRT            1  15.4947  15.4947  15.4947  6.27  0.031
Error         71  49.8639  49.8639   0.7023
Total         89  99.5092

S = 0.838038   R-Sq = 49.89%   R-Sq(adj) = 37.19%
```

NA visual barrier versus barrier tanks

General Linear Model: NA

```
Factor            Type    Levels  Values
Tank nr_1(TRT_1)  random      12  2, 4, 9, 10, 13, 18, 1, 5, 8, 12, 14, 17
RANK_1            random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT_1             fixed        2  2, 3


Analysis of Variance for NE_1, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr_1(TRT_1)  10  13.8293  13.5413  1.3541  2.63  0.009
RANK_1             7   1.3625   1.4030  0.2004  0.39  0.905
TRT_1              1   4.0283   4.0283  4.0283  2.97  0.116
Error             70  36.0168  36.0168  0.5145
Total             88  55.2369

S = 0.717304   R-Sq = 34.80%   R-Sq(adj) = 18.03%
```

General Linear Model: 5-HIAA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                              8, 12, 14, 17
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        3  1, 2, 3


Analysis of Variance for 5-HIAA, using Adjusted SS for Tests

Source         DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)   15  25.7179  25.5955  1.7064  8.61  0.000
RANK            7   1.5029   1.5338  0.2191  1.11  0.365
TRT             2   0.7182   0.7182  0.3591  0.21  0.812
Error         109  21.6121  21.6121  0.1983
Total         133  49.5511

S = 0.445282   R-Sq = 56.38%   R-Sq(adj) = 46.78%
```

General Linear Model: 5-HT

```
Factor            Type    Levels  Values
Tank nr_1(TRT_1)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1,
                                  5, 8, 12, 14, 17
RANK_1            random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT_1             fixed        3  1, 2, 3


Analysis of Variance for 5-HT, using Adjusted SS for Tests

Source             DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr_1(TRT_1)   15   71.451   70.866   4.724  1.58  0.092
RANK_1              7   16.290   15.304   2.186  0.73  0.647
TRT_1               2   31.196   31.196  15.598  3.31  0.064
Error             108  323.461  323.461   2.995
Total             132  442.397

S = 1.73061   R-Sq = 26.88%   R-Sq(adj) = 10.64%
```

5-HT visual barrier versus barren tanks

General Linear Model: 5-HT

```
Factor                Type    Levels  Values
Tank nr_1_1(TRT_1_1)  random      12  3, 6, 7, 11, 15, 16, 1, 5, 8, 12, 14, 17
RANK_1_1              random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT_1_1               fixed        2  1, 3


Analysis of Variance for 5-HT_1_1, using Adjusted SS for Tests

Source                DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr_1_1(TRT_1_1)  10   67.749   61.048   6.105  1.82  0.072
RANK_1_1               7   32.097   31.180   4.454  1.33  0.250
TRT_1_1                1   29.417   29.417  29.417  4.83  0.053
Error                 70  234.551  234.551   3.351
Total                 88  363.814

S = 1.83050   R-Sq = 35.53%   R-Sq(adj) = 18.95%
```

5-HT visual barrier versus barrier tanks

General Linear Model: 5-HT_

```
Factor                    Type    Levels  Values
Tank nr_1_1_1(TRT_1_1_1)  random      12  2, 4, 9, 10, 13, 18, 1, 5, 8, 12, 14,
                                          17
RANK_1_1_1                random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT_1_1_1                 fixed        2  2, 3


Analysis of Variance for 5-HT_1_1_1, using Adjusted SS for Tests

Source                    DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr_1_1_1(TRT_1_1_1)  10   23.887   23.999   2.400  1.09  0.381
RANK_1_1_1                 7    7.107    7.936   1.134  0.52  0.820
TRT_1_1_1                  1    3.178    3.178   3.178  1.32  0.277
Error                     70  153.900  153.900   2.199
Total                     88  188.072

S = 1.48276   R-Sq = 18.17%   R-Sq(adj) = 0.00%
```

General Linear Model: RATIO

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      18  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 18, 1, 5,
                              8, 12, 14, 17
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        3  1, 2, 3


Analysis of Variance for RATIO, using Adjusted SS for Tests

Source         DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)   15  26.0485  28.8503  1.9234  3.39  0.000
RANK            7   5.5121   5.6350  0.8050  1.42  0.205
TRT             2   3.2470   3.2470  1.6235  0.85  0.447
Error         106  60.0825  60.0825  0.5668
Total         130  94.8900

S = 0.752872   R-Sq = 36.68%   R-Sq(adj) = 22.35%
```

Brain stem

General Linear Model: DA

```
Factor          Type    Levels  Values
Tank nr(TRT)    random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                                5, 8, 12, 14, 17, 18
RANK            random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT             fixed        3  1, 2, 3


Analysis of Variance for DA, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    17   7.8377   8.0293  0.4723  1.01  0.449
RANK             7   2.0972   2.2942  0.3277  0.70  0.669
TRT              2   0.3407   0.3407  0.1703  0.36  0.699
Error          115  53.5935  53.5935  0.4660
Total          141  63.8690

S = 0.682664   R-Sq = 16.09%   R-Sq(adj) = 0.00%
```

General Linear Model: NA

```
Factor          Type    Levels  Values
Tank nr(TRT)    random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                                5, 8, 12, 14, 17, 18
RANK            random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT             fixed        3  1, 2, 3


Analysis of Variance for NE, using Adjusted SS for Tests

Source          DF    Seq SS    Adj SS  Adj MS     F      P
Tank nr(TRT)    17   14.2610   14.8327  0.8725  0.95  0.513
RANK             7    3.6661    3.9662  0.5666  0.62  0.738
TRT              2    0.6968    0.6968  0.3484  0.39  0.678
Error          115  105.0756  105.0756  0.9137
Total          141  123.6995

S = 0.955877   R-Sq = 15.06%   R-Sq(adj) = 0.00%
```

General Linear Model: 5-HIAA

```
Factor          Type    Levels  Values
Tank nr(TRT)    random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                                5, 8, 12, 14, 17, 18
RANK            random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT             fixed        3  1, 2, 3


Analysis of Variance for 5-HIAA, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    17   4.1746   4.0757  0.2397  1.46  0.122
RANK             7   0.8457   0.8488  0.1213  0.74  0.639
TRT              2   0.0441   0.0441  0.0220  0.10  0.904
Error          115  18.8623  18.8623  0.1640
Total          141  23.9267

S = 0.404993   R-Sq = 21.17%   R-Sq(adj) = 3.34%
```

General Linear Model: 5-HT

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                               5, 8, 12, 14, 17, 18
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for 5-HT, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    17  11.1900  11.8492  0.6970  1.06  0.399
RANK             7   2.6911   2.8561  0.4080  0.62  0.737
TRT              2   0.2978   0.2978  0.1489  0.22  0.806
Error          115  75.4297  75.4297  0.6559
Total          141  89.6085

S = 0.809883   R-Sq = 15.82%   R-Sq(adj) = 0.00%
```

General Linear Model: RATIO

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                               5, 8, 12, 14, 17, 18
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for RATIO, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    17   8.6716   8.8214  0.5189  2.94  0.000
RANK             7   0.5910   0.6511  0.0930  0.53  0.813
TRT              2   0.6728   0.6728  0.3364  0.81  0.458
Error          115  20.3053  20.3053  0.1766
Total          141  30.2407

S = 0.420199   R-Sq = 32.85%   R-Sq(adj) = 17.67%
```

Optic tectum

General Linear Model: DA

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                               5, 8, 12, 14, 17, 18
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for DA, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS   Adj MS     F      P
Tank nr(TRT)    17  0.73065  0.65930  0.03878  1.13  0.336
RANK             7  0.41706  0.42638  0.06091  1.77  0.100
TRT              2  0.08706  0.08706  0.04353  1.16  0.326
Error          115  3.95349  3.95349  0.03438
Total          141  5.18825

S = 0.185414   R-Sq = 23.80%   R-Sq(adj) = 6.57%
```

General Linear Model: NA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                              5, 8, 12, 14, 17, 18
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        3  1, 2, 3


Analysis of Variance for NE, using Adjusted SS for Tests

Source         DF    Seq SS   Adj SS   Adj MS     F      P
Tank nr(TRT)   17   1.96116  1.88685  0.11099  1.57  0.082
RANK            7   0.33103  0.32591  0.04656  0.66  0.705
TRT             2   0.00091  0.00091  0.00045  0.00  0.995
Error         115   8.10732  8.10732  0.07050
Total         141  10.40041

S = 0.265515   R-Sq = 22.05%   R-Sq(adj) = 4.42%
```

General Linear Model: 5-HIAA

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                              5, 8, 12, 14, 17, 18
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        3  1, 2, 3


Analysis of Variance for 5-HIAA, using Adjusted SS for Tests

Source         DF   Seq SS   Adj SS   Adj MS     F      P
Tank nr(TRT)   17  1.50659  1.52034  0.08943  2.18  0.008
RANK            7  0.53451  0.51370  0.07339  1.79  0.095
TRT             2  0.01620  0.01620  0.00810  0.11  0.898
Error         115  4.70897  4.70897  0.04095
Total         141  6.76626

S = 0.202355   R-Sq = 30.41%   R-Sq(adj) = 14.67%
```

General Linear Model: 5-HT

```
Factor        Type    Levels  Values
Tank nr(TRT)  random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                              5, 8, 12, 14, 17, 18
RANK          random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT           fixed        3  1, 2, 3


Analysis of Variance for 5-HT, using Adjusted SS for Tests

Source         DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)   17   1.9345   1.8340  0.1079  1.00  0.468
RANK            7   0.6403   0.6400  0.0914  0.84  0.553
TRT             2   0.0603   0.0603  0.0302  0.28  0.758
Error         115  12.4575  12.4575  0.1083
Total         141  15.0926

S = 0.329129   R-Sq = 17.46%   R-Sq(adj) = 0.00%
```

General Linear Model: RATIO

```
Factor         Type    Levels  Values
Tank nr(TRT)   random      20  3, 6, 7, 11, 15, 16, 2, 4, 9, 10, 13, 16, 18, 1,
                               5, 8, 12, 14, 17, 18
RANK           random       8  1, 2, 3, 4, 5, 6, 7, 8
TRT            fixed        3  1, 2, 3


Analysis of Variance for RATIO, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Tank nr(TRT)    17   6.2377   6.6844  0.3932  1.63  0.066
RANK             7   3.5738   3.5746  0.5107  2.12  0.046
TRT              2   0.3051   0.3051  0.1525  0.44  0.649
Error          115  27.6611  27.6611  0.2405
Total          141  37.7777

S = 0.490440   R-Sq = 26.78%   R-Sq(adj) = 10.22%
```

Frequency of aggression between treatments day 0-14

One-way ANOVA: Frequency of aggressive versus trt

```
Source   DF      SS    MS     F      P
trt       2    88.8  44.4  2.33  0.102
Error   117  2232.9  19.1
Total   119  2321.7

S = 4.369   R-Sq = 3.83%   R-Sq(adj) = 2.18%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  -----+---------+---------+---------+----
0      43  3.495  3.266  (-------*--------)
1      36  5.578  5.330               (--------*---------)
2      41  4.822  4.446          (--------*--------)
                          -----+---------+---------+---------+----
                             3.0       4.5       6.0       7.5

Pooled StDev = 4.369
```

Frequency of aggression between treatments day 14-28

One-way ANOVA: Frequency of aggressive versus trt

```
Source   DF      SS    MS     F      P
trt_3     2    13.3   6.6  0.48  0.622
Error   112  1559.7  13.9
Total   114  1573.0

S = 3.732   R-Sq = 0.84%   R-Sq(adj) = 0.00%

                          Individual 95% CIs For Mean Based on Pooled StDev
Level   N   Mean  StDev    +---------+---------+---------+---------
0      37  4.184  2.685    (-----------*-----------)
1      39  5.003  3.776              (-----------*-----------)
2      39  4.459  4.467       (-----------*----------)
                            +---------+---------+---------+---------
                          3.0       4.0       5.0       6.0

Pooled StDev = 3.732
```

Frequency of aggression within barren treatment day 0-14 versus 14-28

One-way ANOVA: Frequency of aggressive versus trt

```
Source  DF      SS    MS     F      P
trt      1    9.43  9.43  1.04  0.311
Error   78  707.47  9.07
Total   79  716.89

S = 3.012   R-Sq = 1.31%   R-Sq(adj) = 0.05%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level   N   Mean  StDev  ---+---------+---------+---------+------
1      43  3.495  3.266  (------------*------------)
2      37  4.184  2.685           (-------------*-------------)
                         ---+---------+---------+---------+------
                          2.80      3.50      4.20      4.90

Pooled StDev = 3.012
```

Frequency of aggression within barrier treatment day 0-14 versus 14-28

One-way ANOVA: Frequency of aggressive_1 versus trt_1

```
Source  DF      SS    MS     F      P
trt_1    1     6.2   6.2  0.29  0.589
Error   73  1536.2  21.0
Total   74  1542.4

S = 4.587   R-Sq = 0.40%   R-Sq(adj) = 0.00%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level   N   Mean  StDev  -----+---------+---------+---------+----
1      36  5.578  5.330        (--------------*--------------)
2      39  5.003  3.776  (--------------*--------------)
                         -----+---------+---------+---------+----
                            4.0       5.0       6.0       7.0

Pooled StDev = 4.587
```

Frequency of aggression within visual barrier treatment day 0-14 versus 14-28

One-way ANOVA: Frequency of aggressive_2 versus trt_2

```
Source  DF      SS    MS     F      P
trt_2    1     2.6   2.6  0.13  0.717
Error   78  1548.9  19.9
Total   79  1551.6

S = 4.456   R-Sq = 0.17%   R-Sq(adj) = 0.00%


                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level   N   Mean  StDev  --+---------+---------+---------+-------
1      41  4.822  4.446       (-----------------*-----------------)
2      39  4.459  4.467  (-----------------*-----------------)
                         --+---------+---------+---------+-------
                         3.20      4.00      4.80      5.60

Pooled StDev = 4.456
```

APPENDIX - Statistical output for results chapter 5

Mass SGR day 0-14 between treatments

General Linear Model: mass sgr day 0-14

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for mass sgr day 0-14, using Adjusted SS for Tests

Source            DF    Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   19   17.0778  17.0778  0.8988  1.58  0.068
rank               7    8.0994   8.0994  1.1571  2.03  0.054
treatment          3    1.1313   1.1313  0.3771  0.42  0.741
Error            154   87.6409  87.6409  0.5691
Total            183  113.9494

S = 0.754385   R-Sq = 23.09%   R-Sq(adj) = 8.60%
```

Mass SGR day 14-28 between treatments

General Linear Model: mass sgr day14-28

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for mass sgr day14-28, using Adjusted SS for Tests

Source            DF    Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   19   42.9859  42.9859  2.2624  5.31  0.000
rank               7    7.7223   7.7223  1.1032  2.59  0.015
treatment          3    3.1648   3.1648  1.0549  0.47  0.709
Error            154   65.5801  65.5801  0.4258
Total            183  119.4531

S = 0.652568   R-Sq = 45.10%   R-Sq(adj) = 34.76%
```

Mass SGR within small airstone on day 0-14 treatment day 0-14 versus 14-28

General Linear Model: mass SGR

```
Factor           Type    Levels  Values
tank(treatment)  random      12  2, 5, 9, 13, 16, 18, 2, 5, 9, 13, 16, 18
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  1, 2


Analysis of Variance for mass SGR, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)  10  10.3574  10.3574  1.0357  2.48  0.012
rank              7   3.5083   3.5083  0.5012  1.20  0.312
treatment         1   1.4049   1.4049  1.4049  1.36  0.271
Error            77  32.1300  32.1300  0.4173
Total            95  47.4006

S = 0.645967   R-Sq = 32.22%   R-Sq(adj) = 16.37%
```

Mass SGR within small airstone off day 0-14 treatment day 0-14 versus 14-28

General Linear Model: mass SGR

```
Factor                Type    Levels  Values
tank_1(treatment_1)   random      10  4, 7, 10, 19, 22, 4, 7, 10, 19, 22
rank_1                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_1           fixed        2  2, 3


Analysis of Variance for mass SGR_1, using Adjusted SS for Tests

Source                DF   Seq SS   Adj SS  Adj MS     F      P
tank_1(treatment_1)    8  11.5814  11.5814  1.4477  2.81  0.010
rank_1                 7   4.2351   4.2351  0.6050  1.18  0.330
treatment_1            1   6.7585   6.7585  6.7585  4.67  0.063
Error                 63  32.4320  32.4320  0.5148
Total                 79  55.0069

S = 0.717492   R-Sq = 41.04%   R-Sq(adj) = 26.07%
```

Mass SGR within large airstone on day 0-14 treatment day 0-14 versus 14-28

General Linear Model: mass sgr

```
Factor                Type    Levels  Values
tank_2(treatment_2)   random      12  3, 8, 11, 14, 20, 24, 3, 8, 11, 14, 20, 24
rank_2                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_2           fixed        2  3, 4


Analysis of Variance for mass sgr_2, using Adjusted SS for Tests

Source                DF   Seq SS   Adj SS  Adj MS     F      P
tank_2(treatment_2)   10  26.1139  26.1139  2.6114  6.05  0.000
rank_2                 7   4.6998   4.6998  0.6714  1.55  0.162
treatment_2            1   1.1248   1.1248  1.1248  0.43  0.526
Error                 77  33.2471  33.2471  0.4318
Total                 95  65.1856

S = 0.657100   R-Sq = 49.00%   R-Sq(adj) = 37.07%
```

Mass SGR within large airstone off day 0-14 treatment day 0-14 versus 14-28

General Linear Model: mass sgr

```
Factor                Type    Levels  Values
tank_3(treatment_3)   random      12  1, 6, 12, 15, 17, 21, 1, 6, 12, 15, 17, 21
rank_3                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_3           fixed        2  4, 5


Analysis of Variance for mass sgr_3, using Adjusted SS for Tests

Source                DF   Seq SS   Adj SS  Adj MS     F      P
tank_3(treatment_3)   10  12.0111  12.0111  1.2011  1.67  0.102
rank_3                 7   3.5228   3.5228  0.5033  0.70  0.671
treatment_3            1   9.2612   9.2612  9.2612  7.71  0.020
Error                 77  55.2675  55.2675  0.7178
Total                 95  80.0625

S = 0.847207   R-Sq = 30.97%   R-Sq(adj) = 14.83%
```

Length SGR day 0-14 between treatments

General Linear Model: length sgr day 0-14

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for length sgr day 0-14, using Adjusted SS for Tests

Source            DF    Seq SS    Adj SS   Adj MS     F      P
tank(treatment)   19   3.38408   3.38408  0.17811  2.14  0.006
rank               7   0.88108   0.88108  0.12587  1.52  0.166
treatment          3   0.44139   0.44139  0.14713  0.83  0.496
Error            154  12.78986  12.78986  0.08305
Total            183  17.49641

S = 0.288186   R-Sq = 26.90%   R-Sq(adj) = 13.13%
```

Length SGR day 14-28 between treatments

General Linear Model: length sgr day14-28

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for length sgr day14-28, using Adjusted SS for Tests

Source            DF    Seq SS    Adj SS   Adj MS     F      P
tank(treatment)   19   4.97716   4.97716  0.26196  2.96  0.000
rank               7   0.85494   0.85494  0.12213  1.38  0.218
treatment          3   0.64261   0.64261  0.21420  0.82  0.500
Error            154  13.64263  13.64263  0.08859
Total            183  20.11735

S = 0.297638   R-Sq = 32.18%   R-Sq(adj) = 19.41%
```

Length SGR within small airstone on day 0-14 treatment day 0-14 versus 14-28

General Linear Model: length SGR

```
Factor           Type    Levels  Values
tank(treatment)  random      12  2, 5, 9, 13, 16, 18, 2, 5, 9, 13, 16, 18
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  1, 2


Analysis of Variance for length SGR, using Adjusted SS for Tests

Source           DF    Seq SS   Adj SS   Adj MS      F      P
tank(treatment)  10   1.93950  1.93950  0.19395   2.02  0.042
rank              7   0.34564  0.34564  0.04938   0.51  0.821
treatment         1   8.26210  8.26210  8.26210  42.60  0.000
Error            77   7.39291  7.39291  0.09601
Total            95  17.94015

S = 0.309858   R-Sq = 58.79%   R-Sq(adj) = 49.16%
```

Length SGR within small airstone off day 0-14 treatment day 0-14 versus 14-28

General Linear Model: length sgr

```
Factor                Type    Levels  Values
tank_1(treatment_1)   random      10  4, 7, 10, 19, 22, 4, 7, 10, 19, 22
rank_1                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_1           fixed        2  2, 3


Analysis of Variance for length sgr_1, using Adjusted SS for Tests

Source                DF    Seq SS    Adj SS    Adj MS      F      P
tank_1(treatment_1)    8   1.79279   1.79279   0.22410   2.99  0.007
rank_1                 7   0.29746   0.29746   0.04249   0.57  0.780
treatment_1            1  11.60533  11.60533  11.60533  51.79  0.000
Error                 63   4.72542   4.72542   0.07501
Total                 79  18.42101

S = 0.273874   R-Sq = 74.35%   R-Sq(adj) = 67.83%
```

Length SGR within large airstone on day 0-14 treatment day 0-14 versus 14-28

General Linear Model: length sgr

```
Factor                Type    Levels  Values
tank_2(treatment_2)   random      12  3, 8, 11, 14, 20, 24, 3, 8, 11, 14, 20, 24
rank_2                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_2           fixed        2  3, 4


Analysis of Variance for length sgr_2, using Adjusted SS for Tests

Source                DF    Seq SS   Adj SS   Adj MS      F      P
tank_2(treatment_2)   10   3.12130  3.12130  0.31213   3.56  0.001
rank_2                 7   0.53056  0.53056  0.07579   0.87  0.538
treatment_2            1   6.57032  6.57032  6.57032  21.05  0.001
Error                 77   6.74531  6.74531  0.08760
Total                 95  16.96748

S = 0.295975   R-Sq = 60.25%   R-Sq(adj) = 50.95%
```

Length SGR within large airstone off day 0-14 treatment day 0-14 versus 14-28

General Linear Model: length sgr

```
Factor                Type    Levels  Values
tank_3(treatment_3)   random      12  1, 6, 12, 15, 17, 21, 1, 6, 12, 15, 17, 21
rank_3                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_3           fixed        2  4, 5


Analysis of Variance for length sgr_3, using Adjusted SS for Tests

Source                DF    Seq SS    Adj SS    Adj MS      F      P
tank_3(treatment_3)   10   1.50765   1.50765   0.15077   1.56  0.134
rank_3                 7   0.69790   0.69790   0.09970   1.03  0.415
treatment_3            1  13.99160  13.99160  13.99160  92.80  0.000
Error                 77   7.43330   7.43330   0.09654
Total                 95  23.63046


S = 0.310703   R-Sq = 68.54%   R-Sq(adj) = 61.19%
```

HSI large airstone off day 0-14 versus small airstone on day 0-14 treatments

General Linear Model: HSI

```
Factor          Type    Levels  Values
TANK_1(trt_1)   random      12  2, 5, 9, 13, 16, 18, 1, 6, 12, 15, 17, 21
rank_1          random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1           fixed        2  0, 3


Analysis of Variance for HSI_1, using Adjusted SS for Tests

Source          DF    Seq SS   Adj SS   Adj MS     F      P
TANK_1(trt_1)   10   1.63964  1.50052  0.15005  1.56  0.135
rank_1           7   0.66249  0.66026  0.09432  0.98  0.451
trt_1            1   0.79447  0.79447  0.79447  5.26  0.045
Error           75   7.21095  7.21095  0.09615
Total           93  10.30755

S = 0.310074   R-Sq = 30.04%   R-Sq(adj) = 13.25%
```

HSI large airstone off day 0-14 versus large airstone on day 0-14 treatments

General Linear Model: HSI

```
Factor              Type    Levels  Values
TANK_1_1(trt_1_1)   random      12  3, 8, 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank_1_1            random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1_1             fixed        2  2, 3


Analysis of Variance for HSI_1_1, using Adjusted SS for Tests

Source              DF   Seq SS  Adj SS  Adj MS     F      P
TANK_1_1(trt_1_1)   10   1.7006  1.8362  0.1836  1.83  0.070
rank_1_1             7   0.9728  0.8982  0.1283  1.28  0.274
trt_1_1              1   0.5667  0.5667  0.5667  3.05  0.112
Error               74   7.4394  7.4394  0.1005
Total               92  10.6794

S = 0.317068   R-Sq = 30.34%   R-Sq(adj) = 13.39%
```

HSI large airstone off day 0-14 versus small airstone off day 0-14 treatments

General Linear Model: HSI

```
Factor                  Type    Levels  Values
TANK_1_1_1(trt_1_1_1)   random      11  4, 7, 10, 19, 22, 1, 6, 12, 15, 17, 21
rank_1_1_1              random       8  1, 2, 3, 4, 5, 6, 7, 8
trt_1_1_1               fixed        2  1, 3


Analysis of Variance for HSI_1_1_1, using Adjusted SS for Tests

Source                  DF   Seq SS   Adj SS  Adj MS     F      P
TANK_1_1_1(trt_1_1_1)    9   1.6145   1.4621  0.1625  1.01  0.439
rank_1_1_1               7   1.1293   1.0919  0.1560  0.97  0.458
trt_1_1_1                1   0.5049   0.5049  0.5049  3.11  0.114
Error                   69  11.0680  11.0680  0.1604
Total                   86  14.3167

S = 0.400507   R-Sq = 22.69%   R-Sq(adj) = 3.65%
```

Plasma cortisol levels between treatments

General Linear Model: cortisol

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for cortisol, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   19  1301.55  1301.76   68.51  1.73  0.037
rank               7    87.03    87.28   12.47  0.31  0.946
treatment          3    96.94    96.94   32.31  0.47  0.706
Error            153  6058.34  6058.34   39.60
Total            182  7543.86

S = 6.29261   R-Sq = 19.69%   R-Sq(adj) = 4.47%
```

CF between treatments day 0 and 28

General Linear Model: cf0

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for cf0, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS   Adj MS     F      P
tank(treatment)   19  0.22064  0.22064  0.01161  1.10  0.359
rank               7  0.05843  0.05843  0.00835  0.79  0.598
treatment          3  0.01943  0.01943  0.00648  0.56  0.649
Error            154  1.63054  1.63054  0.01059
Total            183  1.92905

S = 0.102898   R-Sq = 15.47%   R-Sq(adj) = 0.00%
```

General Linear Model: cf28

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for cf28, using Adjusted SS for Tests

Source            DF    Seq SS    Adj SS    Adj MS     F      P
tank(treatment)   19  0.433507  0.433507  0.022816  2.39  0.002
rank               7  0.053453  0.053453  0.007636  0.80  0.589
treatment          3  0.013418  0.013418  0.004473  0.20  0.898
Error            154  1.472181  1.472181  0.009560
Total            183  1.972560

S = 0.0977733   R-Sq = 25.37%   R-Sq(adj) = 11.31%
```

CF within treatments day 0 versus day 28

Small airstone on day 0-14 treatment

General Linear Model: cf0 vs 28

```
Factor           Type    Levels  Values
tank(treatment)  random      12  2, 5, 9, 13, 16, 18, 2, 5, 9, 13, 16, 18
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  1, 2


Analysis of Variance for cf0 vs 28, using Adjusted SS for Tests

Source           DF    Seq SS    Adj SS    Adj MS      F      P
tank(treatment)  10  0.148177  0.148177  0.014818   1.49  0.160
rank              7  0.198896  0.198896  0.028414   2.86  0.011
treatment         1  0.156504  0.156504  0.156504  10.56  0.009
Error            77  0.766105  0.766105  0.009949
Total            95  1.269682

S = 0.0997467   R-Sq = 39.66%   R-Sq(adj) = 25.56%
```

Small airstone off day 0-14 treatment

General Linear Model: cf0 vs 28

```
Factor                Type    Levels  Values
tank_1(treatment_1)   random      10  4, 7, 10, 19, 22, 4, 7, 10, 19, 22
rank_1                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_1           fixed        2  2, 3


Analysis of Variance for cf0 vs 28_1, using Adjusted SS for Tests

Source                DF   Seq SS   Adj SS   Adj MS     F      P
tank_1(treatment_1)    8  0.09802  0.09802  0.01225  0.98  0.463
rank_1                 7  0.04576  0.04576  0.00654  0.52  0.816
treatment_1            1  0.07308  0.07308  0.07308  5.96  0.040
Error                 63  0.79084  0.79084  0.01255
Total                 79  1.00771

S = 0.112040   R-Sq = 21.52%   R-Sq(adj) = 1.59%
```

Large airstone on day 0-14 treatment

General Linear Model: cf0 vs 28

```
Factor                Type    Levels  Values
tank_2(treatment_2)   random      12  3, 8, 11, 14, 20, 24, 3, 8, 11, 14, 20, 24
rank_2                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_2           fixed        2  3, 4


Analysis of Variance for cf0 vs 28_2, using Adjusted SS for Tests

Source                DF    Seq SS    Adj SS    Adj MS      F      P
tank_2(treatment_2)   10  0.185838  0.185838  0.018584   2.43  0.014
rank_2                 7  0.055574  0.055574  0.007939   1.04  0.412
treatment_2            1  0.208532  0.208532  0.208532  11.22  0.007
Error                 77  0.588651  0.588651  0.007645
Total                 95  1.038595

S = 0.0874347   R-Sq = 43.32%   R-Sq(adj) = 30.07%
```

Large airstone off day 0-14 treatment

General Linear Model: cf0 vs 28

```
Factor               Type    Levels  Values
tank_3(treatment_3)  random      12  1, 6, 12, 15, 17, 21, 1, 6, 12, 15, 17, 21
rank_3               random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_3          fixed        2  4, 5


Analysis of Variance for cf0 vs 28_3, using Adjusted SS for Tests

Source               DF    Seq SS    Adj SS    Adj MS     F      P
tank_3(treatment_3)  10  0.222113  0.222113  0.022211  2.46  0.013
rank_3                7  0.074294  0.074294  0.010613  1.18  0.326
treatment_3           1  0.124814  0.124814  0.124814  5.62  0.039
Error                77  0.694489  0.694489  0.009019
Total                95  1.115710

S = 0.0949702   R-Sq = 37.75%   R-Sq(adj) = 23.20%
```

Dorsal fin damage between treatments day 0 and 28

General Linear Model: Dfin 0

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for Dfin 0, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   19   4.3917   4.3917  0.2311  1.03  0.433
rank               7   5.4728   5.4728  0.7818  3.47  0.002
treatment          3   0.2605   0.2605  0.0868  0.38  0.772
Error            154  34.6522  34.6522  0.2250
Total            183  44.7772

S = 0.474357   R-Sq = 22.61%   R-Sq(adj) = 8.04%
```

General Linear Model: Dfin 28

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for Dfin 28, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   19   4.1208   4.1208  0.2169  0.91  0.568
rank               7   0.4130   0.4130  0.0590  0.25  0.972
treatment          3   1.0313   1.0313  0.3438  1.59  0.226
Error            154  36.5870  36.5870  0.2376
Total            183  42.1522

S = 0.487419   R-Sq = 13.20%   R-Sq(adj) = 0.00%
```

Dorsal fin damage within treatments day 0 versus day 28

Small airstone on day 0-14 treatment

General Linear Model: Dfin 0 vs 28

```
Factor           Type    Levels  Values
tank(treatment)  random      12  2, 5, 9, 13, 16, 18, 2, 5, 9, 13, 16, 18
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  1, 2


Analysis of Variance for Dfin 0 vs 28, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS   Adj MS      F      P
tank(treatment)  10   3.6042   3.6042   0.3604   1.28  0.255
rank              7   0.2396   0.2396   0.0342   0.12  0.997
treatment         1  17.5104  17.5104  17.5104  48.58  0.000
Error            77  21.6354  21.6354   0.2810
Total            95  42.9896

S = 0.530075   R-Sq = 49.67%   R-Sq(adj) = 37.91%
```

Small airstone off day 0-14 treatment

General Linear Model: Dfin 0 vs 28

```
Factor                Type    Levels  Values
tank_1(treatment_1)   random      10  4, 7, 10, 19, 22, 4, 7, 10, 19, 22
rank_1                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_1           fixed        2  2, 3


Analysis of Variance for Dfin 0 vs 28_1, using Adjusted SS for Tests

Source                DF   Seq SS   Adj SS   Adj MS      F      P
tank_1(treatment_1)    8   1.4500   1.4500   0.1812   0.65  0.731
rank_1                 7   1.0000   1.0000   0.1429   0.51  0.820
treatment_1            1  11.2500  11.2500  11.2500  62.07  0.000
Error                 63  17.5000  17.5000   0.2778
Total                 79  31.2000

S = 0.527046   R-Sq = 43.91%   R-Sq(adj) = 29.67%
```

Large airstone on da 0-14 treatment

General Linear Model: Dfin 0 vs 28

```
Factor                Type    Levels  Values
tank_2(treatment_2)   random      12  3, 8, 11, 14, 20, 24, 3, 8, 11, 14, 20, 24
rank_2                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_2           fixed        2  3, 4


Analysis of Variance for Dfin 0 vs 28_2, using Adjusted SS for Tests

Source                DF   Seq SS   Adj SS  Adj MS      F      P
tank_2(treatment_2)   10   2.2083   2.2083  0.2208   1.09  0.380
rank_2                 7   2.1667   2.1667  0.3095   1.53  0.170
treatment_2            1   9.3750   9.3750  9.3750  42.45  0.000
Error                 77  15.5833  15.5833  0.2024
Total                 95  29.3333

S = 0.449868   R-Sq = 46.87%   R-Sq(adj) = 34.46%
```

Large airstone off day 0-14 treatment

General Linear Model: Dfin 0 vs 28

```
Factor               Type    Levels  Values
tank_3(treatment_3)  random      12  1, 6, 12, 15, 17, 21, 1, 6, 12, 15, 17, 21
rank_3               random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_3          fixed        2  4, 5


Analysis of Variance for Dfin 0 vs 28_3, using Adjusted SS for Tests

Source               DF   Seq SS   Adj SS  Adj MS      F      P
tank_3(treatment_3)  10   1.2500   1.2500  0.1250   0.61  0.804
rank_3                7   3.1250   3.1250  0.4464   2.17  0.047
treatment_3           1   9.3750   9.3750  9.3750  75.00  0.000
Error                77  15.8750  15.8750  0.2062
Total                95  29.6250

S = 0.454058   R-Sq = 46.41%   R-Sq(adj) = 33.89%
```

Pectoral fin damage between treatments day 0 and 28

General Linear Model: Pfin 0

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for Pfin 0, using Adjusted SS for Tests

Source            DF    Seq SS    Adj SS   Adj MS     F      P
tank(treatment)   19   8.95833   8.95833  0.47149  6.82  0.000
rank               7   0.84783   0.84783  0.12112  1.75  0.101
treatment          3   0.91123   0.91123  0.30374  0.64  0.596
Error            154  10.65217  10.65217  0.06917
Total            183  21.36957

S = 0.263002   R-Sq = 50.15%   R-Sq(adj) = 40.77%
```

General Linear Model: Pfin 28

```
Factor           Type    Levels  Values
tank(treatment)  random      23  2, 5, 9, 13, 16, 18, 4, 7, 10, 19, 22, 3, 8,
                                 11, 14, 20, 24, 1, 6, 12, 15, 17, 21
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        4  1, 2, 3, 4


Analysis of Variance for Pfin 28, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS     F      P
tank(treatment)   19   8.0208   8.0208  0.4221  1.54  0.080
rank               7   1.4783   1.4783  0.2112  0.77  0.614
treatment          3   3.0987   3.0987  1.0329  2.45  0.095
Error            154  42.2717  42.2717  0.2745
Total            183  54.8696

S = 0.523920   R-Sq = 22.96%   R-Sq(adj) = 8.45%
```

Pectoral fin damage within treatments day 0 versus day 28

Small airstone on day 0-14

General Linear Model: Pfin 0 vs 28

```
Factor           Type    Levels  Values
tank(treatment)  random      12  2, 5, 9, 13, 16, 18, 2, 5, 9, 13, 16, 18
rank             random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment        fixed        2  1, 2


Analysis of Variance for Pfin 0 vs 28, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS  Adj MS      F      P
tank(treatment)  10   2.1042   2.1042  0.2104   1.37  0.209
rank              7   0.5729   0.5729  0.0818   0.53  0.806
treatment         1   5.5104   5.5104  5.5104  26.19  0.000
Error            77  11.8021  11.8021  0.1533
Total            95  19.9896

S = 0.391502   R-Sq = 40.96%   R-Sq(adj) = 27.16%
```

Small airstone off day 0-14

General Linear Model: Pfin 0 vs 28

```
Factor               Type    Levels  Values
tank_1(treatment_1)  random      10  4, 7, 10, 19, 22, 4, 7, 10, 19, 22
rank_1               random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_1          fixed        2  2, 3


Analysis of Variance for Pfin 0 vs 28_1, using Adjusted SS for Tests

Source               DF   Seq SS   Adj SS  Adj MS     F      P
tank_1(treatment_1)   8  11.7500  11.7500  1.4687  9.91  0.000
rank_1                7   0.7875   0.7875  0.1125  0.76  0.623
treatment_1           1   2.8125   2.8125  2.8125  1.91  0.204
Error                63   9.3375   9.3375  0.1482
Total                79  24.6875

S = 0.384986   R-Sq = 62.18%   R-Sq(adj) = 52.57%
```

Large airstone on day 0-14

General Linear Model: Pfin 0 vs 28

```
Factor               Type    Levels  Values
tank_2(treatment_2)  random      12  3, 8, 11, 14, 20, 24, 3, 8, 11, 14, 20, 24
rank_2               random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_2          fixed        2  3, 4


Analysis of Variance for Pfin 0 vs 28_2, using Adjusted SS for Tests

Source               DF   Seq SS   Adj SS  Adj MS      F      P
tank_2(treatment_2)  10   2.0208   2.0208  0.2021   0.94  0.506
rank_2                7   1.7396   1.7396  0.2485   1.15  0.341
treatment_2           1   7.5938   7.5938  7.5938  37.58  0.000
Error                77  16.6354  16.6354  0.2160
Total                95  27.9896

S = 0.464806   R-Sq = 40.57%   R-Sq(adj) = 26.67%
```

Large airstone off day 0-14

General Linear Model: Pfin 0 vs 28

```
Factor                Type    Levels  Values
tank_3(treatment_3)   random      12  1, 6, 12, 15, 17, 21, 1, 6, 12, 15, 17, 21
rank_3                random       8  1, 2, 3, 4, 5, 6, 7, 8
treatment_3           fixed        2  4, 5


Analysis of Variance for Pfin 0 vs 28_3, using Adjusted SS for Tests

Source                DF   Seq SS   Adj SS  Adj MS      F      P
tank_3(treatment_3)   10   1.1042   1.1042  0.1104   0.71  0.712
rank_3                 7   2.4063   2.4063  0.3438   2.21  0.042
treatment_3            1   6.5104   6.5104  6.5104  58.96  0.000
Error                 77  11.9688  11.9688  0.1554
Total                 95  21.9896

S = 0.394257   R-Sq = 45.57%   R-Sq(adj) = 32.85%
```

Frequency of aggression between treatments day 0-14

One-way ANOVA: agro versus trt

```
Source   DF      SS    MS     F      P
trt       3    16.3   5.4  0.35  0.791
Error   118  1844.3  15.6
Total   121  1860.6

S = 3.953   R-Sq = 0.88%   R-Sq(adj) = 0.00%


                          Individual 95% CIs For Mean Based on Pooled StDev
Level   N   Mean  StDev   +---------+---------+---------+---------
1      31  3.700  4.410      (-------------*-------------)
2      28  4.386  4.349              (--------------*--------------)
3      31  3.419  4.087    (-------------*-------------)
4      32  3.531  2.834     (-------------*-------------)
                          +---------+---------+---------+---------
                          2.0       3.0       4.0       5.0

Pooled StDev = 3.953
```

Frequency of aggression between treatments day 14-28

One-way ANOVA: agro versus trt

```
Source   DF       SS     MS     F      P
trt2      3    40.67  13.56  1.36  0.257
Error   113  1123.26   9.94
Total   116  1163.93

S = 3.153   R-Sq = 3.49%   R-Sq(adj) = 0.93%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  -------+---------+---------+---------+--
1      30  2.470  2.536  (-----------*----------)
2      29  2.897  2.505      (-----------*-----------)
3      29  4.076  3.670                  (-----------*----------)
4      29  3.076  3.703        (-----------*----------)
                          -------+---------+---------+---------+--
                               2.0       3.0       4.0       5.0

Pooled StDev = 3.153
```

Frequency of aggression within treatments, day 0-14 versus 14-28

Small airstone on day 0-14

One-way ANOVA: agro versus time

```
Source  DF     SS    MS     F      P
time     1   23.1  23.1  1.77  0.189
Error   59  770.0  13.1
Total   60  793.1

S = 3.613   R-Sq = 2.91%   R-Sq(adj) = 1.26%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  --------+---------+---------+---------+-
1      31  3.700  4.410             (------------*------------)
2      30  2.470  2.536  (------------*------------)
                          --------+---------+---------+---------+-
                                2.0       3.0       4.0       5.0

Pooled StDev = 3.613
```

Small airstone off day 0-14

One-way ANOVA: agro_versus time_

```
Source  DF     SS    MS     F      P
time_1   1   31.6  31.6  2.53  0.117
Error   55  686.4  12.5
Total   56  718.0

S = 3.533   R-Sq = 4.40%   R-Sq(adj) = 2.66%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  -------+---------+---------+---------+--
1      28  4.386  4.349             (-----------*----------)
2      29  2.897  2.505  (----------*----------)
                          -------+---------+---------+---------+--
                               2.4       3.6       4.8       6.0

Pooled StDev = 3.533
```

Large airstone on day 0-14

One-way ANOVA: agro versus time

```
Source    DF     SS    MS     F      P
time_1_1   1    6.5   6.5  0.43  0.516
Error     58  878.2  15.1
Total     59  884.6

S = 3.891   R-Sq = 0.73%   R-Sq(adj) = 0.00%


                          Individual 95% CIs For Mean Based on Pooled StDev
Level   N   Mean  StDev    +---------+---------+---------+---------
1      31  3.419  4.087    (-------------*-------------)
2      29  4.076  3.670          (--------------*-------------)
                            +---------+---------+---------+---------
                          2.0       3.0       4.0       5.0

Pooled StDev = 3.891
```

Large airstone off day 0-14

One-way ANOVA: agro versus time

```
Source    DF     SS    MS     F      P
time_1_2   1    3.2   3.2  0.29  0.590
Error     59  633.0  10.7
Total     60  636.2

S = 3.276   R-Sq = 0.50%   R-Sq(adj) = 0.00%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  -------+---------+---------+---------+--
1      32  3.531  2.834         (-------------*--------------)
2      29  3.076  3.703  (--------------*---------------)
                          -------+---------+---------+---------+--
                               2.40      3.20      4.00      4.80

Pooled StDev = 3.276
```

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