EFFECTS OF STOCKING DENSITY ON THE WELFARE OF FARMED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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Abstract

The welfare of farmed fish is a subject of growing public, commercial and governmental interest. The Farm Animal Welfare Council's report on the welfare of farmed fish (Anon., 1996a) highlighted stocking densities used in intensive fish production as a major welfare concern. This thesis investigates links between stocking density and welfare of rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1972).

The effects of stocking density and water quality deterioration on the welfare of rainbow trout were assessed in two controlled experiments. Fish welfare was assessed by measuring a range of morphometric (growth, fin condition), physiological (haematocrit, plasma cortisol and glucose) and immune response (lysozyme activity) indicators. Principal Components Analysis (PCA) was used to generate welfare indices based on coherence that existed between the individual welfare indicators.

The first experiment stocked different numbers of fish into the tanks at the same inflow rate (60 l min^{-1}) to achieve stocking densities of 10, 40, and 80 kg m^{-3}. Results suggested that, provided good water quality was maintained, stocking densities around 80 kg m^{-3} did not produce consistent negative effects on growth rate, stress response or immunological indicators of welfare. Fin erosion increased with increasing density, although the exact cause of the erosion remains unclear. Increased size variation and elevated cortisol levels in the 10 kg m^{-3} treatment, possibly linked to dominance hierarchies, indicated that low, as well as high, stocking densities may have a detrimental effect on trout welfare.

The second experiment assessed the effect of water quality deterioration by adjusting inflow rate (20, 40 or 60 l min^{-1}) in tanks containing the same initial stocking density of fish (16 kg m^{-3}). Inflow rate affected growth during the 3 month summer period when water temperatures were highest (≈14°C), with significantly better growth observed in the 60 l min^{-1} treatment. There was no significant effect of inflow rate on cortisol, haematocrit or fin erosion, but higher mortality and poorer body condition were observed in the 20 l min^{-1} treatment.

A questionnaire survey of stocking density practices on UK trout farms found marked differences in the stocking practices and the perception of a high SD when comparing farms producing trout exclusively for the table market with fisheries and restocking farms. A lack of accurate flow rate data from respondents highlighted the difficulties of trying to apply alternative methods of quantifying stocking density rather than the conventional unit of kg m^{-3}. On-farm work successfully applied the system of welfare assessment in a range of selected commercial systems and confirmed some of the findings from the tank based experiments and questionnaire survey.

In summary, increased stocking density resulted in increased fin erosion although there was also the suggestion that there are welfare implications at low as well as high SD. Systems applying high SD or loading rates may run an increased risk of mass mortality in the event of system failure, necessitating the need for increased supervision and appropriate back-up systems. A universally applied SD limit will not guarantee good fish welfare and it is suggested that defining limits of key water quality parameters may prove to be more effective. Further work is required to establish thresholds of water quality parameters to safeguard fish welfare.

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Chapter 1: General Introduction

The welfare of farmed fish is a subject of growing public, commercial and governmental concern (Lymbery, 1992, 2002; Kestin, 1994; Anon., 1996a; FSBI, 2002; Lines *et al.*, 2003). As a relatively new major agricultural sector, aquaculture was largely overlooked by both pressure organisations and welfare legislators until the last two decades. Aquaculture now represents the world's fastest growing sector of food production and there are increasing concerns surrounding the ethics and the environmental impact of fish farming (Lymbery, 2002).

The stocking densities used in intensive fish production are seen as a welfare concern and specific references to this issue have been made by pressure groups (Lymbery 1992; 2002) and also by the Farm Animal Welfare Council (FAWC; Anon., 1996a). Similar to concerns surrounding the intensity of terrestrial livestock *e.g.* poultry (Anon., 1992a; 1995; 1997) and pig farming industries (Anon., 1996b; Council of Europe Directives 2001/88/EC; 2001/93/EC), the densities at which fish are farmed has been targeted as a potential welfare issue. Unlike terrestrial farm animals, where minimum spatial areas are stipulated to provide for an animal's behavioural needs (Anon., 1996c), there are currently no regulations regarding the densities at which fish can be farmed.

The FAWC report on the welfare of farmed fish (Anon., 1996a) made 121 recommendations relating to the farming of Atlantic salmon (*Salmo salar* Linnaeus, 1758), brown and rainbow trout (*Salmo trutta* Linnaeus, 1758; *Oncorhynchus mykiss*, Walbaum, 1792). The FAWC report made 43 specific recommendations for trout, four of which related specifically to stocking density (Table 1.1).

Table 1.1. Recommendations relating to the stocking density of farmed trout (Anon., 1996a)

No.	Recommendation
69	The stocking densities in hatcheries should allow for adequate oxygen provision for each fish.
70	The stocking density must allow fish to show most normal behaviour with minimal pain, stress and fear. Scientific research is needed on the effect of stocking density on fish welfare but it seems that 30-40 kg m^{-3} is too high a stocking rate for trout. Higher densities may be acceptable for short periods prior to slaughter and during treatment for diseases and parasites.
71	Research should be undertaken urgently to determine acceptable maximum stocking densities taking account of factors referred to in paragraph 143 of the report and include objective measures of the welfare of fish. These results should be available within five years, at which point we expect to recommend the introduction of legislation to limit stocking densities.
80	Stocking density should be kept within manageable levels for holding facilities.

The government published a response to the FAWC report in which stocking density was identified as an issue for inclusion in a proposed 'welfare code' for farmed fish production, stating that "stocking density employed at any particular time should pay proper regard to the need to allow fish to show normal behaviour with minimal pain, stress and fear" (Anon., 1998).

This introduction will discuss what is meant by welfare, the existing legislation relating to fish welfare, the different measures of stocking density and the ways in which stocking density can potentially affect fish welfare. An outline of the topics covered in the remainder of the thesis is also provided.

1.1. What is welfare?

Although there is no universally accepted definition for welfare, welfare is commonly seen to represent an assortment of notions relating to health, well-being and quality of life *i.e.* the physical and mental state of an animal in relation to its environment (Appleby & Hughes, 1997; Duncan & Fraser, 1997). The lack of a concise definition is arguably one of the reasons that welfare is often criticised by other fields of science as being vague and subjective. One of the main difficulties in defining welfare is the fact that suffering is a central theme to animal welfare, and suffering is inherently difficult to assess. Assessing suffering is hindered by subjective interpretations of an animal's feelings. With fish, this is further complicated due to the lack of vocalisations and difficulties associated with observing behavioural patterns underwater.

A concise definition covering all aspects of welfare may not be possible and a recent briefing paper from the Fisheries Society of the British Isles (FSBI) categorised definitions of welfare into three main areas (FSBI, 2002):

1. Feelings-based definitions: *e.g.* mental state, well being.
2. Function-based definitions: *e.g.* animal's health status; biological function.
3. Nature-based definitions: *e.g.* animal can live natural life and display natural behaviour.

In light of the difficulties associated with defining welfare, an alternative approach has been to instead define conditions that compromise welfare *i.e.* lay down the conditions that must be met to ensure acceptable welfare. One such approach is The 'Five Freedoms' (Anon., 1992b; Table 1.2), which is now one of the most widely

accepted frameworks for animal welfare policy (Appleby & Hughes, 1997; Cooke, 2001). The Five Freedoms were originally developed with terrestrial animals in mind and some of the points are not relevant to fish welfare *e.g.* freedom from thirst and ready access to freshwater. However, the principals of the Five Freedoms are recognised to be equally applicable to fish (Cooke, 2001; Anon., 2002; Ellis *et al.*, 2002; FSBI, 2002).

Table 1.2. The five freedoms of animal welfare (Anon., 1992b)

Welfare Freedom
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 suitable environment including shelter and a comfortable resting area*
3. Freedom from pain, injury and disease *by prevention or by rapid diagnosis and treatment*
4. Freedom of normal behaviour *by providing sufficient space, proper facilities and company of animal's own kind*
5. Freedom from fear and distress *by ensuring conditions which avoid mental suffering*

Using the same concept as the Five Freedoms, the use of Five Domains has also been suggested based on the recognition of five main areas in which welfare may be compromised (Table 1.3).

Table 1.3. The Five Domains in which welfare may be compromised (Mellor & Stafford 2001: cited in FSBI, 2002)

Domain of welfare infringement	
Domain 1	Water and food deprivation, malnutrition
Domain 2	Environmental challenge
Domain 3	Disease injury and functional impairment
Domain 4	Behavioural/interactive impairment
Domain 5	Mental and physical suffering

Lymbery (2002) suggested that welfare should be separated into two components that focus on production-related welfare (*i.e.* keeping the animal alive and growing) and factors associated with quality of life (*i.e.* preventing animal from suffering as a result of behavioural or environmental deprivation). By separating welfare into these components Lymbery suggested that traditional production-based indicators alone do not sufficiently represent all aspects of welfare, and that good performance in terms of growth and feed conversion, is not necessarily implicit to good welfare.

Perhaps the most succinct way of capturing both the physical and mental aspects of animal welfare is presented by Dawkins (2004) in the form of two questions;

1) Are animals healthy?
2) Do they have what they want?

Dawkins stated that these two questions avoid the ambiguity of using the word 'need' *e.g.* animals may be highly motivated to obtain something, even though they

do not need it, if need is viewed in the context of being something required for survival or good health. Dawkins (2004) described an experiment that used preference testing to determine what farmed chickens wanted by connecting boxes containing food, water and a nesting box (everything the chickens needed) to either an enriched (wood-shavings with a box of sprouting wheat) or barren environment (bare wire floor). The chickens preferred the enriched environment, but faecal cortisol levels were higher and there was a greater loss of egg shell thickness associated with the enriched environment (cited in Dawkins, 2004). This example demonstrates some of the difficulties in assessing animal welfare, as use of the objective measures alone (cortisol; egg shell thickness) could have been interpreted as being indicative of poor welfare, though the fact that the chickens spent more time in the enriched environment suggests that this is what they wanted.

Finally, a less conventional interpretation of animal welfare focuses on the importance of consciousness and what is "in the mind's eye" of an animal (Kirkwood, 2004). The idea that animals are capable of sentient experience (having the power of perception by the senses) and capable of conscious thoughts akin to humans remains a subject of great debate. Probably the first detailed acknowledgement that animals are capable of emotional expression dates back to 1872 when Charles Darwin published 'The expression of the emotions in man and animals' (Darwin, 1872). Before this time it was largely believed that man was the only animal capable of inner reflection, largely attributed to a connection with an omnipotent God. Using a series of illustrated examples, Darwin demonstrated the wide array of emotions that animals display, such as fear (trembling and tucking of the tail between the legs of a dog), wellbeing and contentment (whinnying in horses, purring in cats) and aggression (erection of dermal appendages and vocalisations; growling, roaring, snarling *etc.*).

Fish have long been known to display complex behavioural patterns such as migratory behaviour (*e.g.* Atlantic salmon), intricate nest building and courtship rituals (*e.g.* Siamese fighting fish *Betta splendens*) and there are also examples of con-specific signalling to indicate social rank and aggressive intent (Abbot & Dill, 1985; O'Connor *et al.*, 1999). However, it is largely dependent on personal perception to decide if such behaviour equates to sentience.

The subject of cognition in fish is an area of growing interest and research has shown that fish are capable of complex, flexible behaviours and of forming mental representation (reviewed by Braithwaite & Huntingford, 2004). To date, most salmonid cognition work has focused on aggressive behavioural interactions. Ellis *et al.* (2002) summarised aggressive behavioural interactions in salmonids as consisting of signalling (body colouration, posture and erecting of depressing fins), attacking (displacement, charging and chasing) and fighting (non-reciprocated nips or mouth-fighting). In the wild, stream dwelling salmonids are reported to be territorial (Hartman, 1965; Berejikian *et al.*, 1996), but under culture conditions they have been shown to form social hierarchies (Johnsson *et al.*, 1996; Adams *et al.*, 2000; Sloman *et al.*, 2000). Position in the hierarchy is largely dictated by body size (Abbott *et al.*, 1985; Adams *et al.*, 2000), with uneven food acquisition as larger fish monopolise the food source further enforcing the hierarchy (McCarthy *et al.*, 1992). There is also evidence of endocrine mediation of aggression, through hormones such as cortisol (Pottinger & Carrick, 2001) and growth hormone (Johnsson *et al.*, 1996). Further evidence suggests that learning also plays a role in dominance in salmonids, with examples of changes in aggressive behaviour depending on previous experience (Abbot *et al.* 1985) and previewing of an opponents fighting ability (Johnsson & Åkerman, 1998).

Fish are receiving more recognition for the complex animals that they are, but a question that remains unresolved is the capacity of fish to suffer. Suffering is central to the welfare concept and the necessity of safeguarding fish welfare will always be questioned without evidence that fish are capable of experiencing suffering akin to that of humans. Rose (2002) argued that the experience of pain and emotion in fish is untenable as fish lack any functional equivalent to the mammalian neocortex. Rose acknowledged the ability of fish to display a wide array of non-conscious, neuroendocrine and stress responses, but remained largely dismissive of evidence of learning behaviour and suggested any such evidence was merely associated or implicit learning *i.e.* learning relationships between a stimulus or stimuli and a behavioural response. Rose concluded that behavioural responses to noxious stimuli are separate from the physiological experience of pain, and that awareness of pain in humans is dependent on functions of specific regions of the cerebral cortex and the absence of such a region or functional equivalent in fish means that they are incapable of awareness of fear, or experiencing pain.

Rose's review generated much debate and the idea that humans and higher primates are alone in their capacity to suffering has been viewed as extreme. Braithwaite and Huntingford (2004) recently reviewed research outlining the nociceptive and cognitive capacities of fish. Using Rose's own rationale it was suggested that if fish have the capacity for mental representation, then they should also have the capacity to experience suffering. Though acknowledging the fact that fish are unlikely to experience pain and suffering in the same way as humans, it was concluded that fish possess both the apparatus and powers of perception necessary to experience pain (Braithwaite & Huntingford, 2004).

Recent work that has generated much media interest has focused on nociception in the rainbow trout (Sneddon 2002; 2003a; 2003b; Sneddon *et al.*, 2003). Physiological (electrophysiology) and anatomical findings showed that rainbow trout possess A-delta and C fibres in the trigeminal nerve ganglion akin to those responsible for nociception in higher vertebrates (Sneddon, 2002). Braithwaite and Huntingford (2004) discussed some unpublished work in which the natural tendency to avoid a novel object was reduced in groups of fish that were exposed to noxious stimuli (Sneddon *et al.* in press). There was also evidence to suggest that morphine acted as an analgesic, as the avoidance of the novel object by fish treated with morphine and acetic acid was similar to control fish, suggesting that fish treated with the acid alone may have been distracted with the pain of the stimulus. Another experiment that used the same noxious stimuli demonstrated that following exposure to the acid, rainbow trout displayed anomalous behaviour (rocking form side to side on pectoral fins and rubbing snouts in the gravel), increased opercular beat and a took a longer period of time to resume feeding (Sneddon, 2003). The anomalous behaviour and the time taken to resume feeding were reduced in groups treated with acid and morphine compared with acid alone. Sneddon (2003) concluded that they had fulfilled all of the criteria for animal pain as proposed by Zimmerman (1986), where pain in animals is defined as an adverse sensory experience that is caused by a stimulus that can or potentially could cause tissue damage; this experience should elicit protective motor (move away from) and vegetative reactions (*e.g.* inflammation and cardiovascular responses) and should also have an adverse effect on the animal's general behaviour (*e.g.* cessation of normal behaviours).

There are strong arguments both for and against the ability of fish to experience pain, though scientists representing both sides of the argument agree there

is a need for further research into the subject. In light of such uncertainty, an approach often advocated by researchers working in animal welfare is to take the stance that pain should be considered without an emotional element and that a distinction should be drawn between human and animal pain (*e.g.* Bateson, 1991). A more simplistic approach is to give the benefit of any doubt that exists regarding an animal's ability to suffer in favour of the animal, and take necessary measures to minimise any suffering that might occur as a result of contact with man.

1.2. Summary of existing fish welfare legislation

The welfare of fish farmed for food is presently covered by the Agriculture Act 1968, which makes it an offence to cause unnecessary pain or unnecessary distress (Anon., 1996c). This legislation only covers farms on agricultural land, but protection on other sites is offered by the Protection of Animals Act 1911, which protects against general offences of cruelty against any domestic or captive animal (including fish). Other legislation and regulations that are relevant to fish welfare include:

- The Registration of Fish Farming and Shellfish Farming Business Order 1985; requires all fish farms to be registered and specifies the requirement for all registered farms to keep records of stock movements and mortality.
- Welfare of Animals (Transport) Order 1997; requires animals to be transported in a way that does not, and is not likely to, cause injury or unnecessary suffering.
- The Welfare of Animals Regulations (Slaughter and Killing) 1995; makes it an offence for anyone engaged in the movement, restraint and stunning of fish to cause or permit any fish to sustain any avoidable excitement, pain or suffering.

- Fish Health Regulations, 1997; granted powers of inspection to The Fish Health Inspectorate for the collection of samples for the purposes of monitoring and control of certain diseases.

As animals farmed for food, fish are afforded a degree of protection by the statutory regulations relating to cruelty, but such legislation was designed with terrestrial animals rather than fish in mind. The only legislation with specific references and conditions relating to fish are the Fish Health Regulations, although their main focus is controlling the introduction and spread of diseases rather than promoting fish welfare. However, this situation is soon likely to change with the proposed introduction of new laws relating specifically to fish welfare by the Council of Europe (Council of Europe, 2002). The Standing Committee of the European Convention for the Protection of Animals Kept for Farming Purposes first started drafting fish welfare conditions in 1998 and at the time of writing, the draft was undergoing its 13^{th} revision.

The introduction of pan-European legislation is understandably a slow and complicated process, but there are already several quality schemes and codes of practice that make specific references to the safeguarding of fish welfare *e.g.* the British Trout Association Code of Practice, (Anon., 2002) and the Federation of European Aquaculture Producers (Anon., 2000). However, as codes of practice (CoP) are not legally binding, they are often viewed by pressure groups as being inadequate and there remains a demand for the introduction of legally binding legislation specifically aimed at safeguarding fish welfare (Lymbery, 2002).

1.3. What is stocking density?

This section will define what is meant by stocking density (SD) and describe some of the different ways in which SD can be expressed. Despite the widespread use of the term SD, there are very few definitions in the literature, although it is commonly used to refer to the density of fish at any point in time (Wedemeyer, 1996). Strictly speaking, SD should refer to the concentration that fish are originally stocked into a system and Ellis *et al.*, (2001) suggested that the term SD is actually a misnomer and that 'stock density' or just 'density' would be more appropriate terms to reflect the changes in fish concentration during the commercial production cycle (SD increases due to somatic growth and is reduced by thinning and grading). However, due to its prevalent use, the term 'stocking density' has been used in this thesis, where SD describes the weight of fish per unit volume of water at a given point in time, using the metric unit of kg m^{-3}. For improved clarity, reviewed literature using alternative expressions of SD have been converted into kg m^{-3}.

There are many alternative expressions of stocking density (reviewed by Ellis *et al.*, 2001) that incorporate different measurements to represent the spatial and physiological needs of the fish (Table 1.4). Haskell (1955) first proposed the idea 'carrying capacity' *i.e.* the animal load that a farming system can support, based on physiological, rather than spatial requirements. Haskell's carrying capacity was based on the assumption that food consumption will be proportional to both oxygen consumption and waste production and therefore suggested that carrying capacity would be limited by the amount of feed fed per day per unit volume of water.

Table 1.4. Expressions of stocking density and a summary of how well they account for fish's spatial and physiological requirements and environmental factors; reproduced from Ellis *et al.* (2001)

Author's term	Unit (metric equivalent)	Relation to requirements		Inclusion of environmental factors					Example reference
		Spatial	Physiological	Inflow rate	Fish size	Feed Ration	Temperature	Oxygen availability	
Stocking rate	no. m^{-2}	√	×	×	×	×	×	×	Soderberg *et al.*, 1983
Stocking density	kg m^{-2}	√	√	×	×	×	×	×	Sedgwick, 1985
Rearing density	no. m^{-3}	√√		×	×	×	×	×	Papoutsoglou *et al.*, 1989
Stocking density/ Static loading density	kg m^{-3}	√	√	×	×	×	×	×	Iwamoto *et al.*, 1986
Density Index	kg m^{-3} cm^{-1}	√	√	×	×	×	×	×	Piper *et al.* 1982
Carrying capacity/ Loading factor/rate Metabolic loading density	kg l^{-1} min^{-1}	×	√√	√	×	×	×	×	Iwamoto *et al.*, 1986
Carrying capacity	Kg food d^{-1} m^{-3}	×	√	×	√	√	√	×	Haskell, 1955
Carrying capacity	Kg food O_2^{-1} l^{-1} min^{-1}	×	√√	√	√	√	√	√	Willloughby, 1968
Flow Index/ Loading factor	kg l^{-1} min^{-1} cm^{-1}	×	√√	√	√	√	√	×	Piper *et al.*, 1982

Haskell's concept was further developed through the late 1960's and 70's by other researchers in the United States to incorporate factors such as flow rates (Willoughby, 1968) and fish size (Westers, 1970; Piper, 1970). Westers (one of the World's most well respected sources on production considerations relating to intensive fish farming) has proposed many different ways to calculate carrying capacity that recognise the importance of limiting factors such as oxygen availability, ammonia production, water exchange rate and water reuse (Westers, 2001).

Loading Factor is an alternative expression of density that has been used in several studies (Piper *et al.*, 1970; Larmoyeux & Piper, 1973). A maximum permissible Loading Factor of 1.5 is recommended for trout, which can be used in the following equation to determine the permissible weight of fish, at a given water inflow for a given size of fish (Piper, 1970):

$$\textbf{Loading Factor} = \frac{\mathbf{W}}{\mathbf{L \times I}}$$

W = weight of fish (lbs.)
L = fish length (inches)
I = Inflow rate (gallons per minute)

Piper *et al.* (1982) later renamed Loading Factor to 'Flow Index' (FI) and provided a table to correct for altitude and water temperature, where the maximum recommended FI decreased with increasing water temperature and altitude *e.g.* the recommended FI at 4.5°C at sea level is 2.7, but at 17.8°C and 9,000 ft. the recommended FI is 0.83. Piper *et al.* (1982) also recognised that although FI accounted for the physiological requirements (oxygen consumption and ammonia production) and environmental factors (temperature and altitude) to allow good

survival and growth, the system was not necessarily optimal for disease control and fish quality, so also proposed the Density Index:

$$\textbf{Density Index} = \frac{W}{L \times V}$$

W = weight of fish (lbs.)
L = fish length (inches)
V = System volume (cubic feet)

Density Index (DI) also takes spatial requirements into consideration, for which Piper *et al.* (1982) suggested a maximum value of 0.5 for trout, *i.e.* the density in lbs. ft^{-3} should not exceed half the fish length in inches (equates to a metric ratio of 3.2, between fish length in cm and density in kg m^{-3}).

Loading Rate (LR; also referred to as 'Loading Density') is another expression of density that has been applied in several studies (*e.g.* Brauhn *et al.* 1976; Iwamoto *et al.* 1986) and was also previously used in the BTA CoP (Anon., 1996d). Loading Rate is calculated as the weight of fish per unit of water flow (kg l min^{-1}) and similar to carrying capacity, LR recognises the physiological requirements for oxygen provision and the removal of metabolic waste (*e.g.* ammonia, carbon dioxide, faecal waste).

1.4. How can stocking density affect fish welfare?

The different expressions of stocking density were designed to take account of the various spatial and physiological requirements of the fish, and latterly, to also incorporate other factors such as fish size and environmental parameters (Table 1.4). The expressions of density can be grouped into those that focus on spatial requirements (SD and DI), and those that focus on physiological requirements

(carrying capacity, FI *etc.*). These groupings represent the two main routes by which SD can potentially affect fish welfare:

1) Behavioural routes of welfare infringement
2) Physiological routes of welfare infringement

A summary of the hypothetical ways in which SD could infringe welfare in regard to the Five Domains of animal welfare is shown in Table 1.5.

Table 1.5. Hypothetical pathways by which stocking density could compromise welfare in regard to the Five Domains of welfare infringement

Domain of welfare infringement and causative mechanisms by which stocking density may potentially infringe welfare
Domain 1 *Water and food deprivation, malnutrition*
o Uneven food acquisition (low and high SD)
o Physical obstruction preventing visual location and access to food (high SD)
o Aggressive behaviour preventing the access of subordinate fish to food (low SD)
o Increased energy requirements due to increased activities at higher SD
o Poor feeding response (low SD)
Domain 2 *Environmental challenge*
o Water quality deterioration:
• Limited oxygen availability due to increased biomass (high SD)
• Accumulation of harmful metabolic waste *e.g.* ammonia, carbon dioxide, and faecal waste (high SD)
Domain 3 *Disease, injury and functional impairment*
o Increased transmission of disease and parasites (high SD)
o Increased fin and body damage due to aggressive nipping (low & high SD)
o Increased fin and body damage due to abrasion against tanks surfaces (high SD)
o Impaired immune function due to chronic physiological stress (low & high SD)
Domain 4 *Behavioural/interactive impairment*
o Formation of dominance hierarchies (low SD)
o Aggressive behaviour restricting access of subordinates to food (low & high SD)
Domain 5 *Mental and physical suffering*
o Pain caused by fin and body damage (low & high SD)
o Fear in subordinate fish (low & high SD)

1.5. Recommended maximum stocking densities for rainbow trout

The FAWC report (Anon. 1996a) suggested that stocking densities of 30 – 40 kg m^{-3} were potentially detrimental to rainbow trout welfare and called for research to be carried out to determine an acceptable maximum limit of SD to safeguard welfare. However, the feasibility of specifying a maximum SD has since been questioned (Ellis *et al.* 2001) and the value of SD (kg m^{-3}) as a unit has also been suggested to be of little or no value for safeguarding fish welfare (Ellis *et al.*, 2002). There are obvious differences that complicate the process of determining acceptable stocking densities for fish compared with terrestrial farm animals:

- Fish live in a three-dimensional medium.
- Fish are dependent on water not only for their spatial requirements, but also for the physiological requirements just as terrestrial animals need air; Westers (2001) drew comparisons with the high rates of water exchange used in flow-through aquaculture systems and the high air turn over rates used in intensive production of broilers.
- Fish culture systems are usually not static, especially in the case of intensive rainbow trout farming, so the rate of water exchange will also affect the acceptable SD.
- Fish are poikilothermic, so water temperature will have a much greater effect on their metabolism than air temperature will for terrestrial animals.
- Water chemistry is subject to regional variation and factors such as pH and alkalinity have profound effects on the tolerance of fish to toxic waste products such as ammonia and carbon dioxide (discussed in greater depth in Chapter 5).

These factors mean that adopting an approach similar to that used in the UK to prescribe the spatial requirements of calves, pigs and battery hens (Anon., 1996c), may not take account of the physiological requirements of the fish. The summary of the various expressions of density (Table 1.4) shows that no single measurement takes account of all of the physiological and environmental factors that could potentially affect fish welfare. The prevalent use of the SD (kg m^{-3}) is likely to reflect the ease with which it can be calculated (the only information required is the volume of the rearing system and the weight of fish inside). The idea of a maximum SD was probably more relevant before the intensification of trout farming and the introduction of modern fish farming systems (*e.g.* liquid oxygen injection). Different farming systems have different capacities for the amount of fish that they can support and providing for spatial need alone is not sufficient to safeguard fish welfare.

Wedemeyer (1996) wrote of the elusive nature of quantifying density tolerance and suggested that estimates that are too conservative waste space, whereas densities that are too high may cause stress, disease and problems such as fin erosion. Similarly, Westers suggested that unlike measurements of carrying capacity (physiological requirements), it is more difficult to determine safe, optimal spatial requirements and stated that there is a lack of understanding with regard to optimum densities for particular sizes and species of fish (Westers, 2001).

The difficulty of specifying a maximum SD is reflected in the wide range of recommendations in the literature (Table 1.6).

Table 1.6. Published recommendations for maximum stocking density of rainbow trout; reproduced from Ellis *et al.* (2002)

System	Author	Recommendation (kg m^{-3})
Cages	Boydstun & Hopelain, 1977	≤40
	Collins, 1972	≥55
	Kilambi *et al.*, 1977	>45
	Sahin *et al.*, 1999	20-25
	Teskeredzic *et al.*, 1986	20
	Wojno, 1976	4-18
Tanks	Kebus *et al.*, 1992	≥267
	Kincaid *et al.*, 1976	40-80
	Kindschi *et al.*, 1991a	196-261/<147 depending on strain
	Mäkinen & Ruohonen, 1990	>50
	Rigolino *et al.*, 1989	43
Raceways	Laks & Godfriaux, 1981	160
	Papoutsoglou *et al.*, 1980	40-50
	Papoutsoglou *et al.*, 1987	≥88.5
	Piper, 1970	90
	Wedemeyer, 1996	8-35 (for fish of 0.5 to 30g)

The wide variation in recommendations for maximum SD may be partly explained by the variety of experimental systems used in studies, but even when recommendations are grouped into generic system types (*e.g.* cage; tank; raceway) there is still a large degree of variability.

In the past, recommendations have focused primarily on optimising growth and maximising profitability rather than safeguarding fish welfare (Piper *et al.*, 1980; Wedemeyer, 1996). Previous studies have demonstrated that it is possible to grow rainbow trout at very high stocking densities with no negative effects on growth or mortality; Buss *et al.* (1970) achieved densities of 545 kg m^{-3} in small-scale units with no effect on mortality, and Bagley *et al.* (1994) showed that growth of young rainbow trout was unaffected by densities of up to 500 kg m^{-3}. However, it is generally accepted that good welfare encompasses more than just optimum growth and survival (FSBI, 2002).

1.6. Stocking density policy for other farmed animals

The space allowances for other types of farmed animals have already been considered in some depth (Table 1.7).

Table 1.7. Recommendations for space allowances for terrestrial livestock extracted from codes of recommendations for the welfare of livestock (Defra, 2004).

Animal	Holding System (age/sex/size)	Space Allowance
Cattle	All types	None specified*
Ducks	Slatted/mesh floors (10 days to 3 weeks)	25 ducklings m^{-2}
	Slatted/mesh floors (3 to 8 weeks)	8 ducklings m^{-2}
	Solid Floors (10 days to 3 weeks)	14 ducklings m^{-2}
	Solid Floors (3- 8 weeks)	7 ducklings m^{-2}
	Grass runs (3-8 weeks)	2,500 ducklings $hectare^{-1}$**
Pigs	Pens (Weaners/rearing pigs up to 10kg)	0.15 m^2 per pig
	Pens (Weaners/rearing pigs >10-20kg)	0.20 m^2 per pig
	Pens (Weaners/rearing pigs >20-30kg)	0.30 m^2 per pig
	Pens (Weaners/rearing pigs >30-50kg)	0.40 m^2 per pig
	Pens (Weaners /rearing pigs >50-85kg)	0.55 m^2 per pig
	Pens (Weaners/ rearing pigs >85-110kg)	0.65 m^2 per pig
	Pens (Weaners/ rearing pigs >110kg)	1.00 m^2 per pig
	Pens (Pregnant sows)	2.25 m^2 per pig***
	Pens (Boars)	6 m^2 per boar
Chickens	All types (Laying Hens)	12 hens m^{-2}
	All types (Broilers of slaughter weight of 1.8-3.0 kg)	34 kg m^{-2}
Rabbits	Cages (5-12 weeks)	0.07m^2 per rabbit
	Cages (>12 weeks)	0.18 m^2 per rabbit
	Hutches (5-12 weeks)	0.09 m^2 per rabbit
Sheep	Housed (Lowland Ewes 60-90 kg)	1.2-1.4 m^2 per ewe
	Housed (Lowland Ewes lambing)	2.0-2.2 m^2 per ewe
	Housed (Hill Ewes 45-65 kg)	1.0-1.2 m^2 per ewe
	Housed (Hill Ewes lambing)	1.8-2.0 m^2 per ewe
	Housed (Lambs up to 12 weeks)	0.5-0.6 m^2 per ewe
	Housed (Lambs 12 weeks -12 months)	0.8-0.9 m^2 per ewe
	Housed (Rams)	1.5-2.0 m^2 per ewe
Turkeys	Broiler-type housing	0.260 m^2 per kg
	Tier brooders	0.515 m^2 per kg
	Pole barns	0.410 m^2 per kg
	Enclosed range area	10 m^2 per bird

* the code states that space allowances for cattle should be based on the age, sex, individual size, herd size, behavioural needs and also whether or not the cattle have horns.

** may be increased to 5,000 ducklings per hectare if the run is well-grassed

***1.3m^2 of which must be continuous solid floor

For some animals the spatial requirements of some animals are stipulated very specifically depending on factors such as size (*e.g.* pigs), type of production (*e.g.* broiler chickens vs. laying hens), or type of housing (*e.g.* turkeys). In contrast, the spatial recommendations for cattle are very broad, and focusing on specific welfare requirements that should be met rather than stipulating an actual maximum stocking density.

1.7. Outline of the thesis

The work carried out in this thesis was funded by Defra (Department of Environment, Fisheries and Rural Affairs) as a direct result of the recommendations made in the FAWC report on the welfare of farmed fish (Anon., 1996a). As such, the research brief was aimed specifically at addressing the issues outlined by FAWC with regard to stocking density of farmed rainbow trout (Table 1.1).

This introductory chapter has addressed the theme of fish welfare and defined stocking density and some of the practicalities/impracticalities of applying different expressions of SD to fish welfare. Chapter 3 will discuss ways in which fish welfare can be assessed and provides experimental evidence for the effects of chronic and acute stress responses in rainbow trout. Chapters 4 and 5 form the main body of experimental work, with objective measures of fish welfare applied to assess the effects of stocking density (Chapter 4) and water quality deterioration (Chapter 5) in controlled studies. Chapter 6 provides an overview of stocking density practices on UK trout farms based on the results of a postal questionnaire. Chapter 7 will cover the on-farm application of welfare assessment in a longitudinal survey of selected commercial rainbow trout farms, and the thesis concludes with a general discussion.

Chapter 2: General Materials and Methods

This chapter describes the materials and methods applicable to more than one experiment. Procedures that are specific to a particular chapter are described in the materials and methods section at the start of relevant chapters.

2.1. Fish husbandry

2.1.1. Experimental Animals

Stocks of all female, farm-reared rainbow trout were used in all but one experiment where fish of mixed sex were used. Where possible fish of the same strain were used but details of the origin, source, age and size of fish are detailed in the materials and methods section of each chapter.

2.1.2. Experimental sites

All experimental work discussed in chapters 3, 4 and 5 was carried out at the University of Stirling's Niall Bromage Freshwater Research Facility (previously named Buckieburn) located near to Stirling (56:03°N; 3:59°W). All experimental tank systems received first use water from a reservoir located approximately 1 km to the North of the site. The characteristics of the water from this reservoir were 'soft' with a low alkalinity (around 25 mg l^{-1}), pH 6.5 – 7, and had an annual temperature range of 0 – 17 °C. The water had brown discolouration due to the high peat (humic acid) content of the soil in the catchment area.

2.1.3. Fish maintenance

The fish in Chapters 3, 4 and 5 were all held in fibreglass tanks under flow-through water conditions.

The system used for the majority of the experimental work (Chapters 4 and 5) comprised 10 x 2 m diameter circular fibreglass tanks with an external standpipe adjusted to maintain a water depth of approximately 60 cm and a volume of 1.8 m^3. During the interim between experiments discussed in chapters 4 and 5, the 2 m system was upgraded with the addition of fibreglass lids, fitted with two 16 watt lights (RS Components Ltd.; Northants, UK) creating approximately 500 lux at the water surface and 300 lux at the tank floor. The lights were controlled by clockwork timers (Kingshield timer, Powerbreaker PLC; Essex, UK), which were adjusted in accordance with sunrise and sunset in a Simulated Natural Photoperiod (SNP).

2.2. Anaesthesia

Fish were anaesthetised in a 1:10,000 bath of 2-phenoxy ethanol (Sigma; Dorset, UK) made up in farm water. Anaesthesia typically took approximately 3 min. Recovery from anaesthesia was achieved using a bath of fresh, aerated farm water. No mortalities were recorded following anaesthesia.

2.3. Fish euthanasia

For the removal of blood for analysis in Chapters 3, 5 and 7 the fish were euthased prior to the removal of the sample in accordance with Home Office regulations for a Schedule I kill. Fish were placed in an anaesthetic bath with a 1:5,000 solution of 2-phenoxy ethanol and following anaesthesia fish were killed with a strong single blow to the head such that loss of sensibility was instantaneous.

2.4. Growth measurements

Fish were weighed to the nearest 0.1 g on an electronic balance (Model QC7DCE-S, Sartorius AG; Germany). Length measurements were made using a customised fish measuring board with all or some of the following measurements used in various trials; Standard Length, Fork Length, Total Length (Figure 2.1).

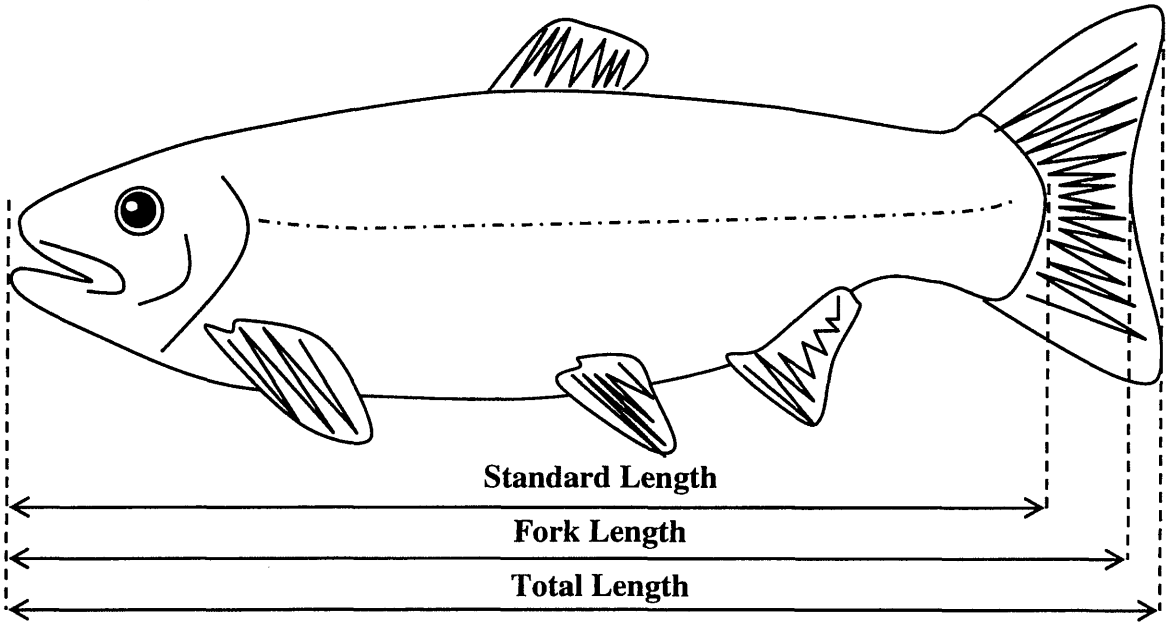


Figure 2.1. Schematic diagram showing the position of the measurements for standard, fork and total length

2.5. Blood sampling

All blood samples were taken from the dorsal-caudal aorta of anaesthetised or dead fish. Either 1 or 2 ml syringes (Terumo Europe N.V.; Leuven, Belgium) were used depending on the amount of blood required. Generally, for fish of less than 250 g a 23G sterile hypodermic needle (Terumo Europe N.V.; Leuven, Belgium) and for fish over 250 g a larger gauge 21 G needle was used. Syringes were rinsed with a 4 mg ml^{-1} solution of porcine intestinal heparin (Sigma; Dorset, UK) to allow the collection of plasma. The blood was then emptied from the syringe into 1.5 ml micro-centrifuge

tubes and centrifuged at a relative centrifugal force (RCF) of 1200 G (2500 rpm) for 15 min at 4°C. Plasma was then transferred to another ependorf tube and stored at -70°C until analysis were performed.

2.6. Welfare Indicators

2.6.1. Cortisol radioimmunoassay

Concentrations of plasma cortisol were determined using a radioimmunoassay adapted from Pickering *et al.* (1987).

Assay Buffer

The following constituents were dissolved in 100 ml of nanopure water in a volumetric flask with the aid of a magnetic stirrer and heated plate:

Sodium dihydrogen orthophosphate	0.74 g
Disodium hydrogen orthophosphate	2.88 g
BSA	1.00 g
Sodium Chloride	4.00 g
EDTA	0.15 g
Sodium Azide	0.05 g

Once dissolved, the volume was made up to 500 ml with nanopure water, mixed, and cooled to 4 °C. Buffer was generally made up the day before each assay and stored at 4 °C, but the addition of sodium azide permitted the buffer to be used for up to 7 days. All the chemicals used were of Analar grade and supplied by either Sigma or BDH Chemicals Ltd.

Charcoal Buffer

On the morning of day 2 of the assay, the following constituents were dissolved in a conical flask in 100 ml of nanopure water with the aid of a heated plate with magnetic stirrer.

Sodium dihydrogen orthophosphate	0.37 g
Disodium hydrogen orthophosphate	1.44 g
Gelatine	0.25 g

Once the gelatine was in solution the charcoal and dextran was added in the following quantities and the buffer was made up to 250 ml with a further 150 ml of nanopure water. The buffer was then left to stir on ice for at least 1 h before use.

Activated Charcoal	1.25 g
Dextran	0.25 g

Radiolabel

[1,2,6,7-^{3}H]Cortisol radiolabel was supplied by Amersham Pharmacia Biotech UK Ltd. in quantities of 9.25 MBq (250 μCi). The radiochemical was supplied in 0.25 ml of a toluene:ethanol (9:1 v/v) solution at an initial purity of 99.8%. An intermediate stock solution was prepared by diluting 20 μl of stock solution in 2 ml of absolute ethanol and from this a working solution of approximately 5000 disintegrations per minute (dpm) per 100 μl was made (approximately 75 μl in 25 ml of assay buffer). The radiolabel and intermediate stock were stored at –20 °C.

Antibody

Freeze-dried sheep anti-cortisol serum was supplied by Diagnostics Scotland (1g per vial), hydrated with 20 ml of fresh assay buffer (1:20 dilution) and then frozen in 1 ml

aliquots. One aliquot was diluted with a further 20 ml of assay buffer to achieve a 1:400 dilution as required (enough for an assay of 90 samples in duplicate).

Cortisol Standard

A standard was prepared from 1.0 g Hydrocortisone in hydrolysed powdered form (Sigma, UK). The following stock standards were prepared:

Stock 1 (50 μg ml^{-1}): 10 μg cortisol in 20 ml absolute ethanol
Stock 2 (5 μg ml^{-1}): 100 μl Stock 1 in 10 ml absolute ethanol
Stock 3 (50 ng ml^{-1}): 100 μl Stock 2 in 10 ml absolute ethanol store at -20 °C.

A working standard of 4 ng ml^{-1} was made by diluting 400 μl of Stock 3 in 4.6 ml ethyl acetate.

Cortisol Assay Protocol

Sample Extraction

1. For each sample, 200 μl of plasma was added to a separate polypropylene tube (LP3P: Thermo Life Science, Hampshire, UK). If plasma was limited, as was often the case for small fish, 100 μl plasma was extracted instead.
2. In the fume cupboard, 1 ml ethyl acetate (BDH Chemicals Ltd) was added to each sample before capping.
3. Samples were spun on a rotary mixer for 1 h at room temperature and then centrifuged at 4 °C for 10 min at a RCF of 430 g (1500 rpm).

Samples were stored at 4 °C until assayed (samples were assayed in duplicate within 6 months of the extraction date.

Day 1

1. A serial dilution of cortisol working standard (4 ng ml^{-1}) was prepared with 200 µl of ethyl acetate (12.5-800 pg $tube^{-1}$) in LP3P polypropylene tubes.
2. 200 µl ethyl acetate was added to a further 4 tubes that act as the zero standard and the non-specific binding (NSB).
3. 200 µl of each extracted sample was transferred into a separate LP3P tube (if high levels of cortisol were expected, a dilution factor was be used).
4. The standards and sample extracts were dried down in a vacuum oven at less than 35 °C. Tubes were then covered and cooled to 4 °C for at least 1 h.
5. 100 µl of assay buffer was added to all tubes.
6. 100 µl of anti-cortisol was added to all tubes except the NSBs, to which 100µl of assay buffer was added instead.
7. 100 µl of tritiated cortisol was added to all tubes.
8. All tubes were vortexed, covered and left to incubate at 4 °C for 18 h.

Day 2

1. Charcoal buffer was made up and stirred on ice for 1 h before adding 1 ml to each tube.
2. Tubes were vortexed and left to incubate at 4°C for 30 min.
3. Tubes were centrifuged at RCF of 1270 G (2500 RPM) for 12 min at 4°C.
4. For each standard and sample, 1000µl of supernatant was transferred into a scintillation vial (Canberra Packard Ltd.) before adding 4 ml of scintillation fluid (Ultima Gold, Canberra Packard Ltd.).

5. To provide an estimation of the total amount of radioactivity added to each sample 100µl of tritiated cortisol was added to two further vials containing 4 ml of scintillation fluid (these were referred to as the total counts).
6. The blank comprised of a vial containing 4 ml of scintillation fluid to correct for background radioactivity.
7. All vials were capped, labelled and until the pellet was homogenised with the scintillation fluid using a vortex mixer.
8. The scintillation activity (dpm) of each vial in was measured in a scintillation counter (Tri-Carb 2500TR, Canberra Packard, Ltd.) for 5 min; the blank vial was placed first to allow automatic subtraction of background radioactivity.

Calculations

1. To correct for the difference between the total reagent volume per tube (1300 µl) and the volume of supernatant added to each vial (1000 µl), it was necessary to multiply the average dpm for each vial by 1/1.3.
2. The average dpm of the non-specific binding was subtracted from all of the standards and samples.
3. The percentage binding (percentage of radiolabel bound to antibody) of the standards and samples was calculated relative to the total counts [% binding = (standard or sample dpm / total dpm) x100]
4. The percentage binding of the standards was plotted against cortisol concentration on log-linear paper or with the use of a graphics package. The pharmacology function on SigmaPlot 8 (SPSS Inc., USA) was used to draw the standard curve and calculate the cortisol concentration for unknown samples (Figure 2.2)
5. The concentration of cortisol in each tube was read from the graph and then multiplied by 0.03 to correct for the volume of extract assayed (200 µl from 1.2

ml; x 6), volume of plasma extracted (200 µl; x 5 ml), and finally converted to ng ml^{-1} (x 1/1000).

Quality Control

The sensitivity of the assay (*i.e.* the minimum amount of cortisol that is statistically distinguishable from zero) was 12.5 pg $tube^{-1}$. Two pooled samples of rainbow trout plasma were obtained from 'unstressed' fish, and from fish 1 h after being subjected to standardised handling stress (confined in a net for 1 min) were used as quality controls (QCs) to check the reproducibility of the measurements between each assay. The cortisol concentrations of the 'unstressed' and 'stressed' samples were approximately 4 and 30 ng ml^{-1} respectively. The intra-assay coefficient of variation was 2.4% (determined by comparing the concentration of an aliquot of the 'stressed' QC at the start and end of each assay), and the inter-assay coefficient variation was 11.2%. Assays were rejected if the difference between the cortisol concentration measured from the QCs was greater than 2 standard deviations from the average cortisol concentration of the QCs obtained from previous assays.

Validation

To ascertain that the cortisol in the standard was immunologically similar to that in the rainbow trout plasma, serial dilutions (1:2) of the extracted rainbow trout plasma were used to create an inhibition curve (Figure 2.3).

When plotted against a serial dilution of cortisol standard, no statistically significant difference ($P<0.05$) was observed between the slope of the inhibition plot and the standard curve regression lines using an 'in house' programme on Microsoft Excel 2000 (courtesy of Dr Iain Berrill).

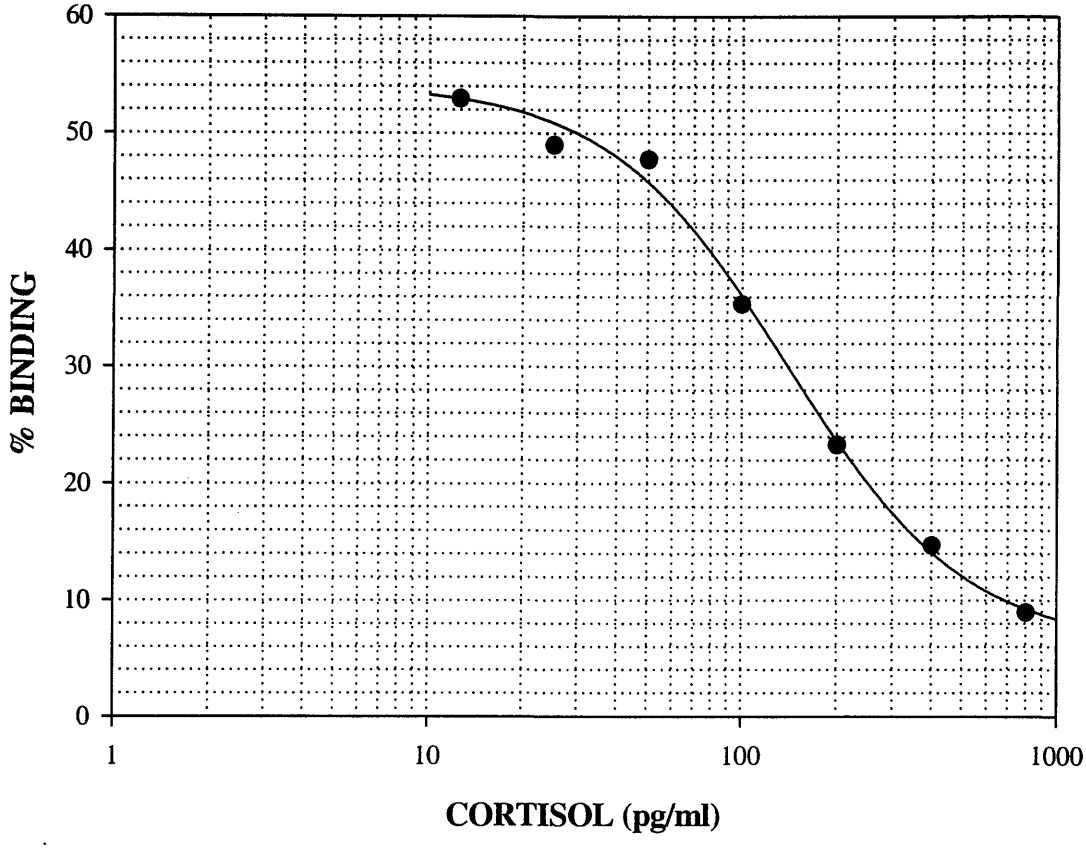


Figure 2.2. A typical standard curve from a cortisol radioimmunoassay; the concentration of cortisol in a sample was determined by intersecting the standard curve at the point corresponding to the percentage binding in the sample.

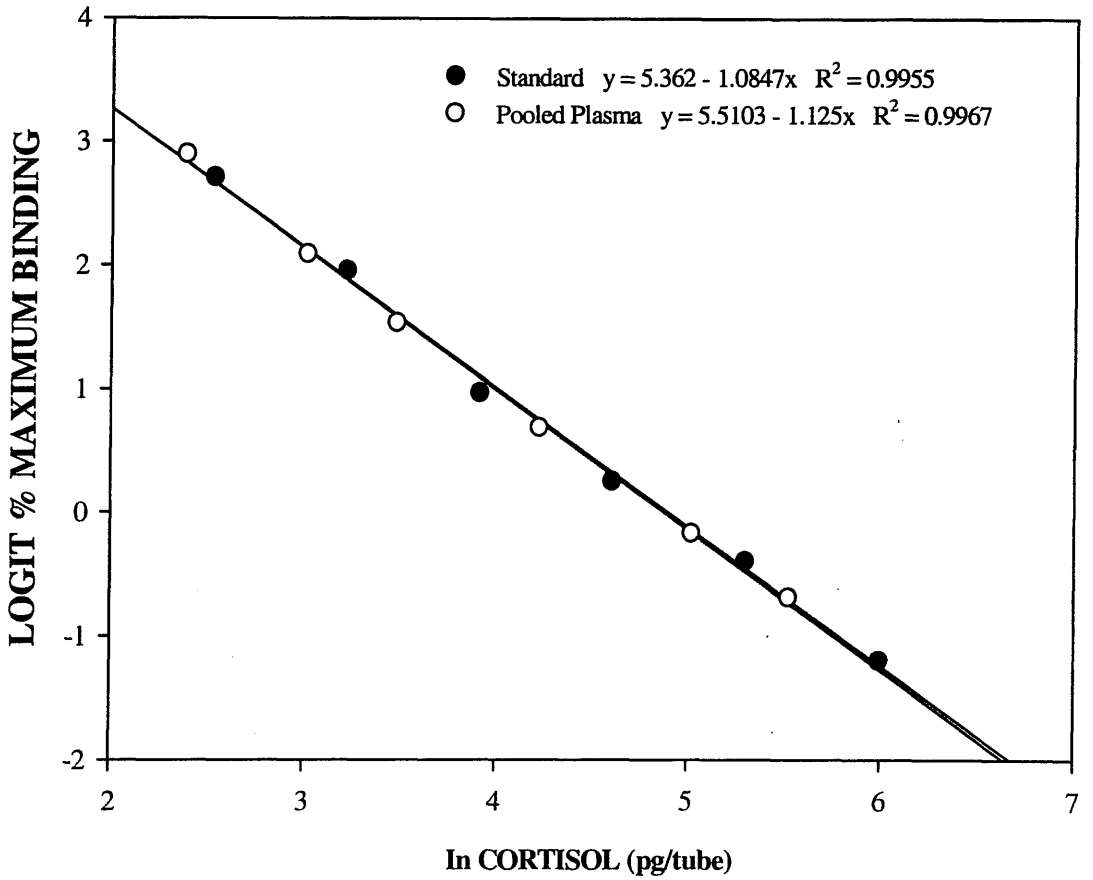


Figure 2.3. Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 200μl aliquots of pooled rainbow trout plasma extract (200μl plasma in 1 ml ethyl acetate) with the cortisol standard curve. Each point represents the mean of duplicate measurement; the X-axis denotes the natural log of the cortisol content in the standards. The two curves have been transferred to a linear relationship using the logit transformation outlined in Randall 1992: logit b = ln (b/100-b) where b is the proportion of radiolabel bound to antibody expressed as a percentage of that of the zero standard (% maximum binding).

2.6.2. Lysozyme activity

Lysozyme activity was measured tubimetrically using a modified 96 well plate method (Lygren *et al.* 1999) adapted from the tubimetric method described by Ellis (1990). This assay was based on the decrease in absorbance of a cell suspension of *Micrococcus leisodeikticus* that occurs when the cells are lysed by lysozyme within the plasma samples.

Assay Buffer

A 0.04M pH 5.8 Sodium phosphate buffer (SPB) from the following stocks:

Stock A: 0.2M solution of NaH_2PO_4; 31.20 g in 1L Distilled water

Stock B: 0.2M solution of Na_2HPO_4; 35.59 g in 1L Distilled water

1. The stock buffers were mixed together to achieve pH 5.8 (for 100 ml approximately 8 ml of stock B was added to 92 ml of Stock A).
2. The 0.2 M SPB buffer was diluted with 100 ml distilled water to give a 0.1 M SPB solution (final volume 200 ml).
3. A 2:5 dilution of the 0.1 M solution was carried out to achieve a 0.04 M SPB (*i.e.* dilute 40 ml buffer with 60 ml distilled water)

Assay

1. 10 µl sample was added to 4 wells of a 96 well micro-plate (Nunc, Hampshire, UK)
2. 190 µl of 0.2 µg ml^{-1} *Micrococcus lysodeiticus* was (0.02 g per 100 ml 0.04 M SPB) was added to each well using a multi-channel pipette.

3. Absorbance at 540 nm was measured using an ELISA micro-plate reader (MRX, Dynex labsystems, UK) at 1 and 5 min after adding the *Micrococcus lysodeiticus*.
4. Lysozyme activity was calculated using the following calculation:

$$\left(\frac{A_1 - A_2}{t} \right) / 0.001 \times 100 = U\ min^{-1}ml^{-1}$$

A_1 = absorbance at time 1

A_2 = absorbance at time 2

T = time (4 min)

2.6.3. Plasma Glucose

The assay used to determine the glucose concentration in plasma or serum samples was supplied by Sigma Diagnostics and adapted from the method outlined by Trinder (1969). The assay is based on the principle of a two step enzymatic reaction resulting in a change of absorbance. Glucose in the sample is oxidised into gluconic acid and hydrogen peroxide, which then reacts with the 4-aminoantipyrine and p-hydroxybenzene sulfonate in the presence of peroxidase to form quinoneimine dye, with a maximum absorbance at 505 nm:

1. **Glucose + $H_2O + O_2$** $\xrightarrow{\text{Glucose Oxidase}}$ **Gluconic Acid + H_2O_2**

2. **H_2O_2 + 4-Aminoantipyrine + p-Hydroxybenzene Sulphonate** $\xrightarrow{\text{Peroxidase}}$ **Quinoneimine Dye + H_2O**

Sample collection and Storage

Plasma samples were centrifuged and separated within 30 min of collection using a micro-centrifuge (Sigma, Philip Harris 1-15).

Methodology

1. A 96 well micro-plate was loaded in the following way (Figure 2.4):
   - 1.75 μl of plasma was added to 4 wells of columns 1 to 10 of a 96 well micro-plate (one plate was used to assay 20 samples in quadruplicate).
   - 1.75 μl of 300 mg dL^{-1} glucose standard (Glucose/Urea Nitrogen combined standard, SIGMA diagnostics) was added to all 8 wells of column 11.
   - 1.75 μl of deionised water was added to all 8 wells of column 12.
2. 350 μl of glucose (Trinder) reagent was added to all wells using a multi-channel pipette.
3. The plate was loaded into an ELISA micro-plate reader (MRX, Dynex labsystems, UK) and the absorbance was measured at 505 nm (the programme used included a 10 seconds 'shake' period of to remove any air bubbles).
4. Samples were placed into an incubator set to 25 °C for 18 min.
5. After the 18 min the absorbance at 505 nm was measured for a second time.
6. The glucose concentration was determined as follows:

$$\frac{\text{ASAMPLE - ABLANK}}{\text{ASTANDARD - ABLANK}} \times \textbf{Concentration of Standard (mg dL}^{-1}\textbf{)}$$

e.g.

ABLANK = 0.017
ASAMPLE = 0.230
ASTANDARD = 0.419

$$\frac{0.230 - 0.017}{0.419 - 0.017} \times 300 = 159$$

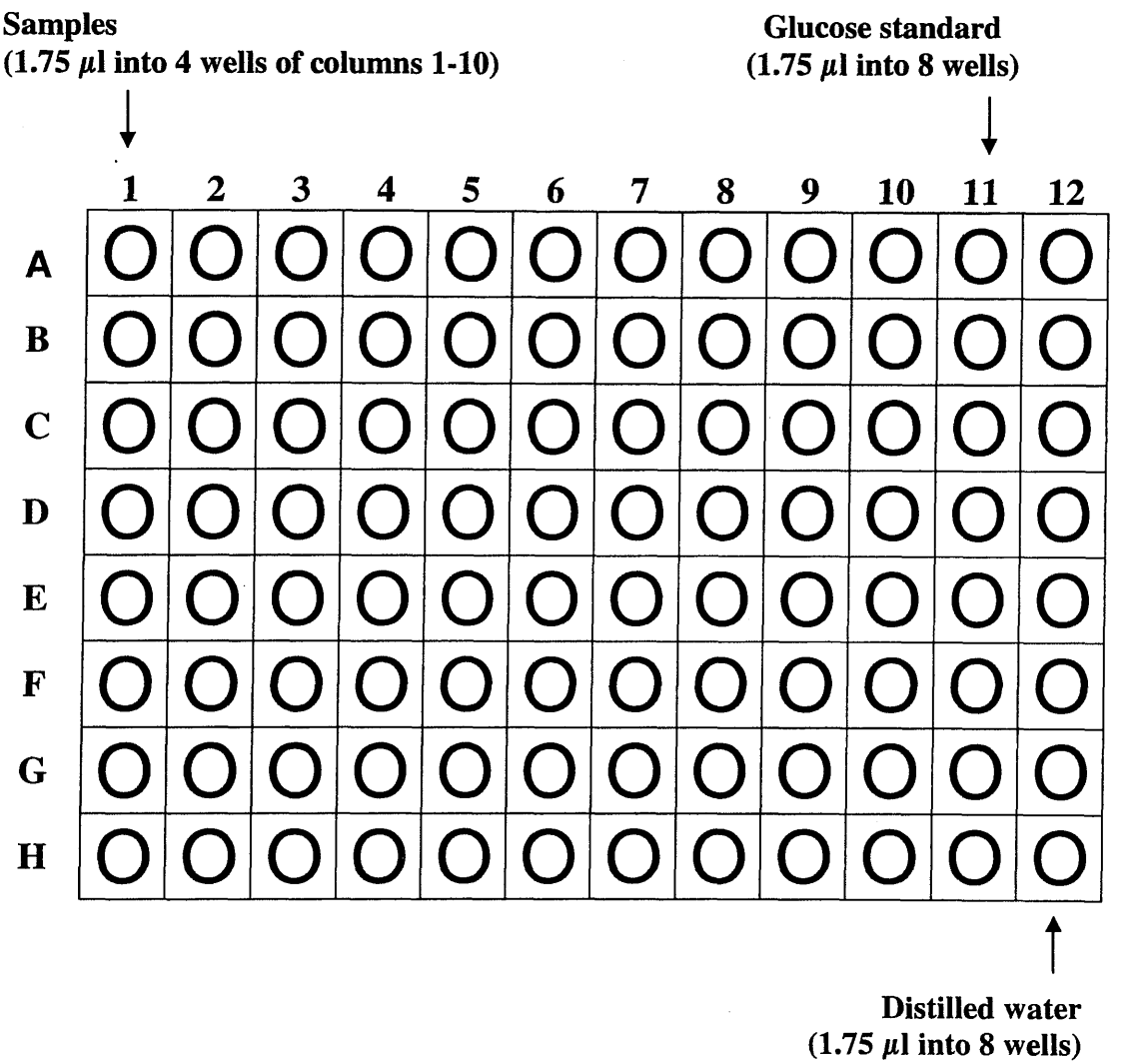


Figure 2.4. The layout of a 96 well microplate for a glucose assay

2.6.4. Haematocrit

Haematocrit is the volume percentage of the red blood cells in blood and varies depending on the health and physiological condition of a fish. Samples were taken in duplicate *i.e.* two capillary tubes filled per blood sample using the following procedure:

1. The tip of a pre-heparinised capillary tube was inserted into a collected blood sample and allowed to fill by capillary action. Once about 80% full, the flow of blood was stopped by covering the opposite end of the capillary tube before sealing with sealant (Critaseal; BDH).
2. Capillary tubes were placed into a Hawksley Micro-haematocrit centrifuge (Hawksley & Son, UK) with the sealed ends facing the outside wall of the rotor, flush to the rubber seal. The tubes were then spun for 3 min at RCF of 14,000 G.
3. The packed cell volume was then determined by measuring the length of the packed red blood cells from the top of the sealant to the bottom of the 'buffy' layer of white blood cells (Length 1). The total length of the fraction of the blood sample was then measured *i.e.* from the top of the sealant to the top of the clear plasma (Length 2); see Figure 2.5.

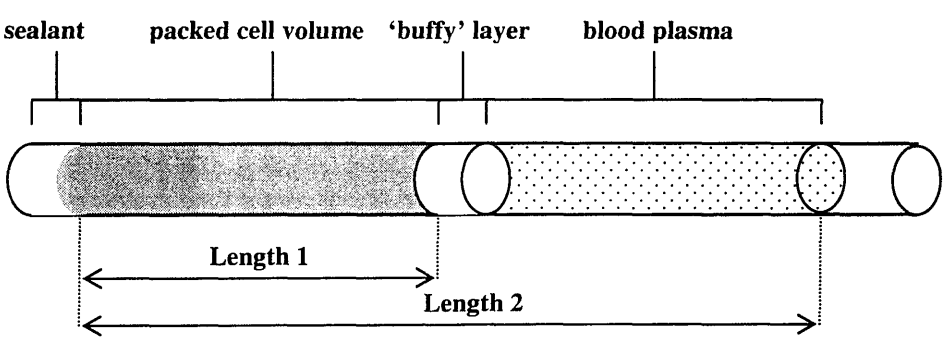


Figure 2.5. Schematic diagram of a haematocrit sample after centrifugation

4. The percentage packed cell volume was then calculated by:

[Length 1 (mm) / Length 2 (mm)] x 100

2.6.5. Somatic indices

Ratios of organ to body weights have previously been suggested for use in assessment of fish health and condition (Goede & Barton, 1990).

2.6.5.1. Hepatosomatic index (HSI)

Hepatosomatic index is an expression of the relative weight of the liver as a percentage of total body weight:

(Liver weight / Total body weight) x 100

The liver was removed from dead fish by making two perpendicular cuts with a scalpel, one of which was ran vertically adjacent to the gill operculum, and the other horizontally along the underside of the fish between the pectoral and pelvic fins along to the anus. The body wall was then pulled back to allow access to the internal organs of the fish. The connective tissue around the oesophagus was cut using scissors allowing the viscera to be pulled out and separated from the liver using forceps and a scalpel. The gall bladder was separated from each liver as it was sometimes burst during separation from the viscera, resulting in the contents being emptied. The liver was blotted on tissue paper and weighed to the nearest 0.1 g.

2.6.5.2. Splenosomatic index (SSI)

The spleen was separated from the connective tissue around the gut using forceps and a scalpel. Once removed, the spleen was blotted on tissue paper before weighing to the nearest 0.1g allowing SSI index to be calculated as follows:

Splenosomatic Index = (Spleen weight / Total body weight) x 100

2.6.5.3. Condition factor

Condition factor (CF) is often used as an indicator of body conformation for salmonids (Herbinger & Friars, 1991). CF was calculated from fork length and total weight of individual fish using the following equation:

Condition factor = [Weight (g) x 100] / length $(cm)^3$

2.6.6. Fin measurements

2.6.6.1. Fin Index

A qualitative scale was used to score to the dorsal and caudal fins from 0 – 3 based on the perceived degree of erosion; where 0 = minimal visible damage (<5% of fin missing), 1 = minor damage (5-30% of fin missing), 2 = Moderate damage (between 30 and 70% missing) and 3 = Severe damage (>70% missing); see Figures 2.6 and 2.7. This scoring system was modified from the one proposed by Moutou *et al.* (1998) where a score of 0 indicated a fin with no visible damage from populations of wild and hatchery reared trout. However, all of the fish used in this thesis were farmed and had some degree of damage was apparent on even the best fins, so it was considered more appropriate to use the score of 0 to indicate minimal visible damage (<5%).

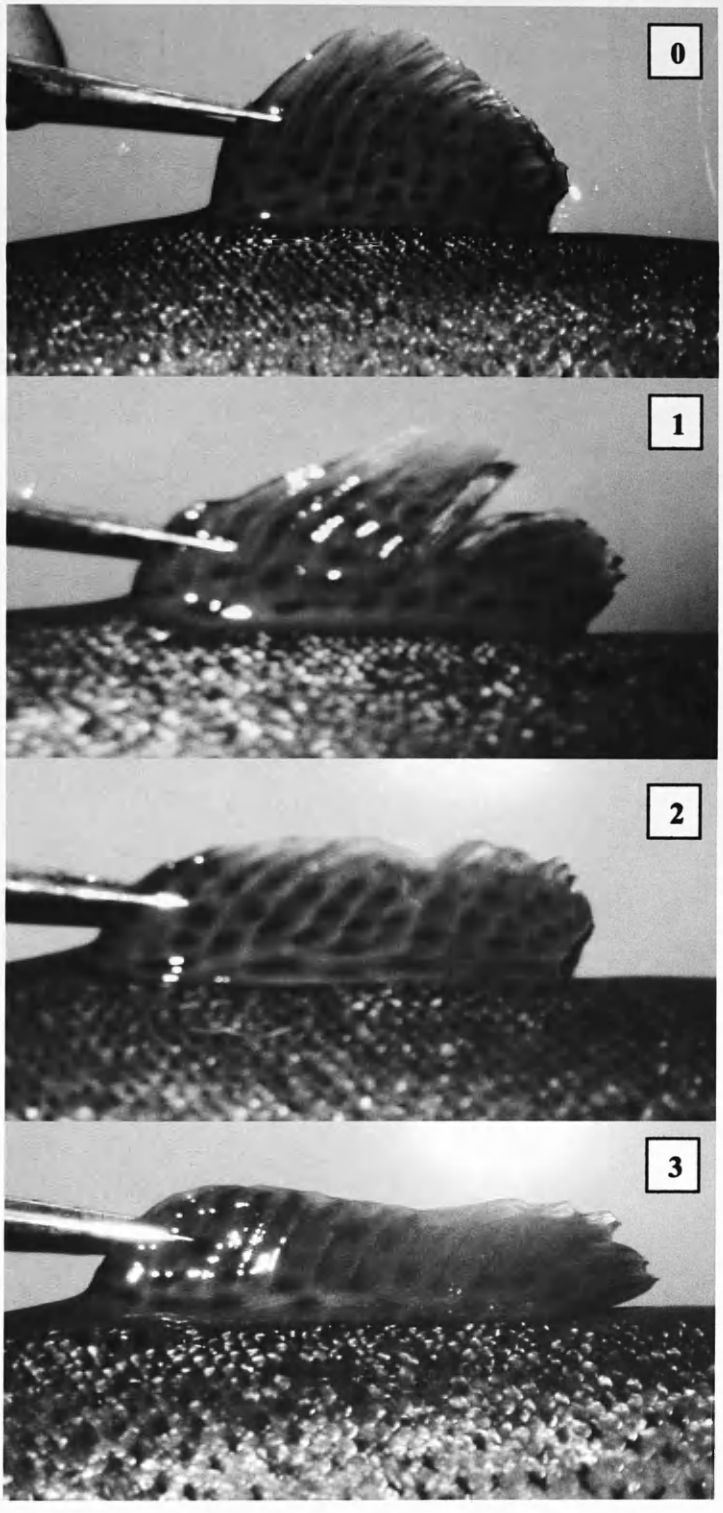


Figure 2.6. Dorsal fin erosion index photographic scale; 0 = minimal visible damage (<5% missing), 1 = minor damage (5-30% of fin missing), 2 = moderate damage (30-70% missing), 3 = Severe damage (>70% missing) (modified from Moutou *et al.*, 1998)

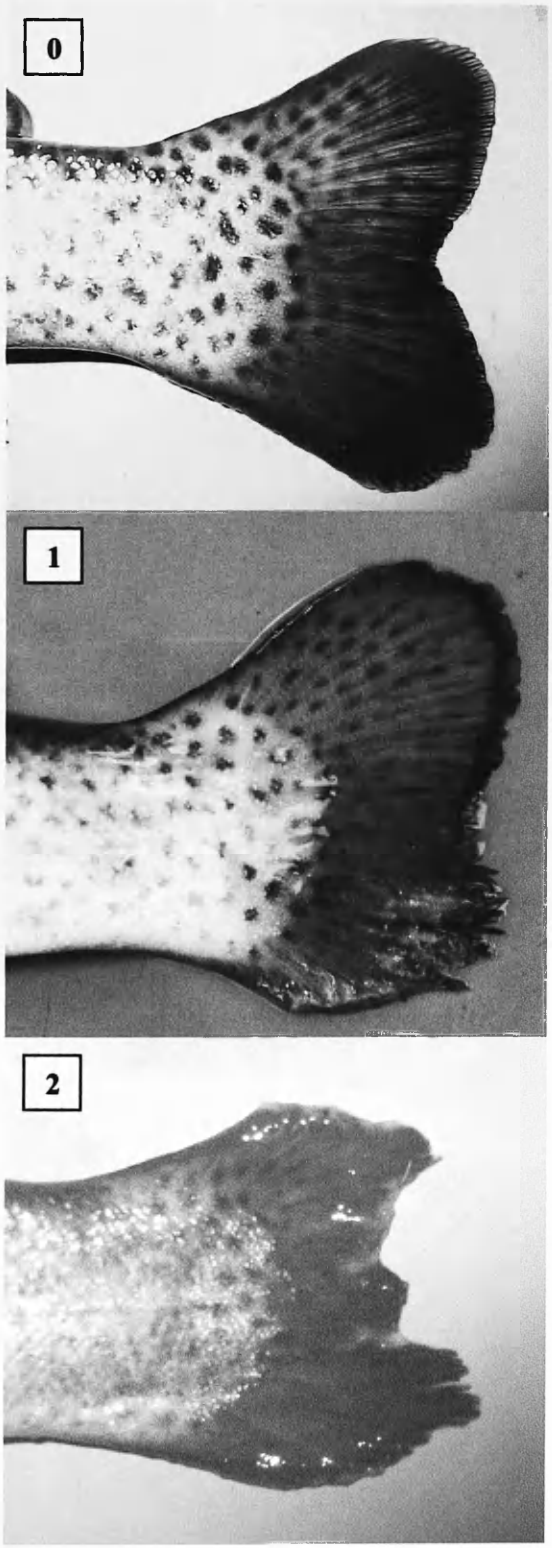


Figure 2.7. Caudal fin erosion index photographic scale; 0 = minimal visible damage (<5%), 1 = minor damage (5-30% of fin missing), 2 = moderate damage (30-70% missing) (modified from Moutou *et al.*, 1998)

2.6.6.2 Relative Fin Length (RFL)

Measurements of the rayed fins were made using callipers (Batty; Switzerland). The measurements were taken parallel to fin rays from the base to the outside edge at the longest point of each fin. The approximate positioning of the measurements is shown in Figure 2.8. The total length of the fish was also recorded (see Figure 2.4 for details) allowing the length of the fins to be expressed in relation to the size of the fish to allow the relative fin length (RFL) to be calculated:

Relative fin length = (fin length x 100)/(total length)

Several previous studies have used the RFI measurement for rainbow trout (Kindschi, 1987; Bosakowski & Wagner, 1994a) and the system is seen to be a less subjective form of assessing fin erosion than scoring systems.

2.6.7. Performance based indicators of welfare

2.6.7.1. Specific growth rate (SGR)

Specific growth rate was calculated based on changes in weight over a known time using the following calculation:

$$\textbf{Specific growth rate (SGR)} = [\text{Ln } Wt_2 - \text{Ln } Wt_1] / (t_2 - t_1)$$

Wt_1 = fish weight (g) at time t_1
Wt_2 = fish weight (g) at time t_2

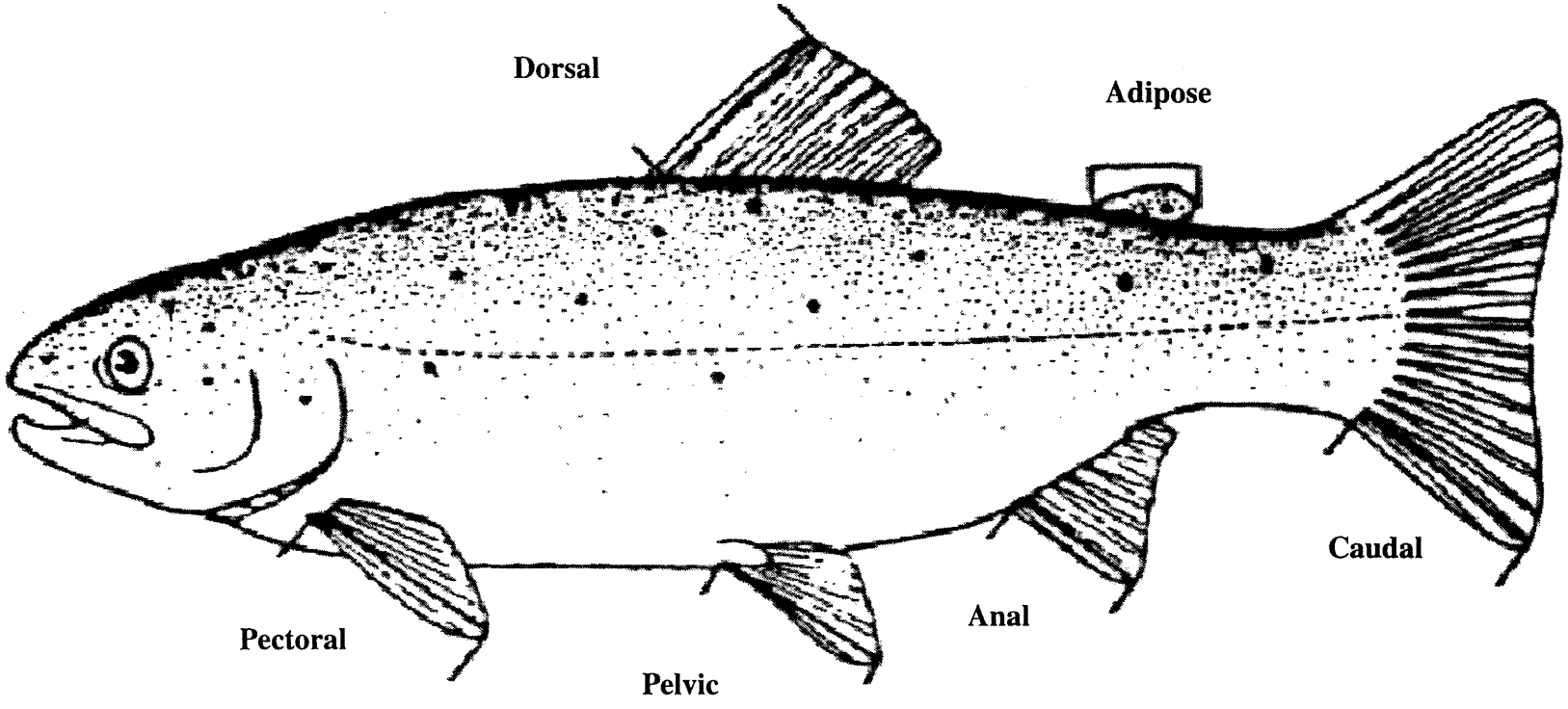


Figure 2.4. Location of measurement of fins for calculation of the Relative Fin Length; reproduced from Bosakowski and Wagner (1994a)

2.6.7.2. Feed conversion ratio (FCR)

Feed conversion ratio provides a crude estimate of how efficiently the food that is presented to the fish is converted into somatic growth over a specified period of time. FCR was calculated using the following formula where the lower the FCR, the more efficiently food is being converted into somatic growth:

FCR = Feed fed (kg) / Fish biomass increase (kg)

2.7. Water Quality Analysis

2.7.1. Water Sample Collection

Water samples were collected in 500 ml polypropylene bottles (Arco, UK). Bottles were completely filled so that no air bubbles were present. In the tank based studies the sample was taken directly above the outflow screen and in the farm based studies samples were taken from the inflow and outflow of culture systems at approximately half of the total water depth. Alkalinity and pH were determined before filtration and ammonia was determined on filtered samples. If not analysed immediately, water samples were stored in a cool box.

2.7.2. Determination of Water Quality Parameters

2.7.2.1. pH

Determination of pH in was carried out in the field using a portable Jenway 3150 pH meter (Jenway Limited, UK) and in the laboratory with a Philips PW9409 pH meter (Philips, UK). Both meters were calibrated before use with sachets of pH 4 and pH 7 'perpHect™' buffers (Orion Research Inc., USA). Approximately 100 ml of unfiltered sample was decanted into a clean glass beaker from a 500 ml water sample.

The pH probe was then immersed in the water sample and the reading was allowed to stabilise before being recorded. The probe and beaker were rinsed with distilled water between each sample.

2.7.2.2. Dissolved oxygen (DO)

For all of the experiments carried out at the Niall Bromage Freshwater Research Facility the oxygen was measured using OxyGuard® probes (OxyGuard International A/S). The static probes (located above the outflow) of each of the tanks were calibrated against the hand-held probes on a regular basis.

DO was measured on-farm directly from the inflow and outflow of culture systems using a Oxi 197 portable DO meter (WTM, UK) by positioning the probe directly into the water column at approximately half of the total water depth.

2.7.2.3. Total Alkalinity

Total alkalinity is due almost entirely to hydroxides, carbonates and the total dissolved solids. A volume of 100 ml of unfiltered farm water of was measured into a clean conical flask and a few drops of BDH 4.5 indicator were added before titrating against hydrochloric acid (HCl). The endpoint of the titration (pH 4.5) resulted in a colour change from turquoise to a peachy/pink colour (detection of endpoint was aided by carrying out the procedure above sheet of white waterproof paper). The molarity of the HCl was usually 0.01 M, although for samples collected from farms with hard water (SE of England) it was necessary to use 0.1 M HCl.

Alkalinity was determined using the following equation from the Standard Methods for the examination of water and waste water (Standard Methods, 1975).

$$\textbf{Total Alkalinity (m eq}^{-1}\textbf{)} = \frac{\mathbf{N \times V_1 \times 1000}}{\mathbf{V_2}}$$

V_1 = volume of acid to achieve endpoint

V_2 = volume of sample (100ml)

N = concentration of acid (normally 0.01M)

2.7.2.4. Ammonia

Total ammonia nitrogen (TAN) was measured in the field using the salicylate method and a Hach® field kit (Hach®, USA).

1. Duplicate 25 ml samples of water samples were measured into graduated cylinders.
2. 25 ml of deionised water was measured into another cylinder to act as a blank.
3. The contents of one Salicylate Reagent Powder Pillow were added to each cylinder. Each cylinder was capped and shaken until reagents were dissolved.
4. The reagents were left to react with the water samples for 3 min.
5. Following the 3 min period, the contents of one Alkaline Cyanurate Powder Pillow were added to each cylinder.
6. Cylinders were capped and shaken until the reagent dissolved and allowed 15 min to react (green colour developed if ammonia nitrogen was present).
7. The reader was zeroed by pouring the blank into the sample cell and measuring absorbance at 655 nm.
8. Subsequent samples were compared with the blank by measuring absorbance at 655 nm, resulting in a reading [total ammonium nitrogen (TAN) mg l^{-1}].

Results were expressed as ammonia (NH_3) or ammonium (NH_4^+) concentration (mg l^{-1}) by multiplying by 1.22 or 1.29 respectively. The proportion of unionised ammonia was calculated by referring to ionisation tables (Piper *et al.*, 1982) after determining the temperature and pH of the sample. For proof of accuracy a standard (0.20 mg l^{-1} $N\text{-}NH_3$) was used in place of a sample. If the concentration of $N\text{-}NH_3$ in a 25 ml sample exceeded 0.50 mg. l^{-1}, a dilution factor was applied.

2.8. Statistical Analysis

Unless otherwise stated, the principals of most of the statistical methods used in this thesis are described in Zarr (1996).

2.8.1. Basic calculations

Throughout this thesis the arithmetic or sample mean was used to provide an estimate of the population mean along with the standard error of the mean (SEM) to represent the sample distribution. The coefficient of variation (CV) was presented on occasion as a measure of relative variability to allow the comparison of variation in populations with different means. All basic statistics were calculated with the aid of Microsoft Excel 2000.

2.8.2. Parametric assumptions

Prior to detailed statistical analysis, all data were first analysed to confirm normality and homogeneity of variance. This process was necessary to conform with the fundamental assumptions of parametric techniques *i.e.* that observations must be derived randomly, be independently distributed, display homogeneity of variance and display a normal Gaussian distribution.

2.8.3. Testing for normality and homogeneity of variance

Normality of distribution was tested throughout this thesis using the Kolmogorov-Smirnov test to quantify the discrepancy between sample distributions from the ideal Gaussian distribution. The *F*-test was used for the comparison of two sample variances and Bartlett's test was used to compare the homogeneity of more than two sample variances. In both cases the tests were carried out using the Instat statistical package (Instat version 3.0, Graphpad Software Inc.) and variance was considered homogeneous if the calculated value was lower than the tabulated value, at the 5% level.

2.8.4. Comparison of two samples

Student's *T*-test was used to compare the means of two samples using the Instat statistical package (Instat version 3.0, Graphpad Software Inc.). If the variance of the two samples were homogenous the means were compared using Student's t-test utilising a pooled estimate of the variance. If the variances were heterogeneous, the means were compared using Student's *T*-test utilising estimates of each variance and reduced degrees of freedom. If the calculated value for T was greater than the tabulated value for T at $P = 0.05$ (5%) or less, the difference between means was concluded to be statistically significant. If data failed parametric assumptions of the *T*-test a non-parametric Mann-Whitney *U*-test was used (Instat version 3.0, Graphpad Software Inc.).

2.8.5. Parametric comparison of multiple samples

Provided samples passed parametric assumptions, a one-way analysis of variance (ANOVA) was used for the preliminary comparison of the means of three or more

samples using the Instat statistical package (Instat version 3.0, Graphpad Software Inc.). Tukey's test was used for all post-hoc comparisons for parametric tests where a statistical difference was observed ($P<0.05$). Tukey's test allowed the pair-wise comparison of sample means to identify where the significant variation existed.

2.8.6. Non-parametric comparison of multiple samples

If the prerequisite assumptions for parametric analysis were not met, Kruskal-Wallis' non-parametric multi-comparison test was performed in place of a one-way ANOVA using the Instat statistical package (Instat version 3.0, Graphpad Software Inc.). If a significant difference was observed ($P<0.05$), Dunn's post-hoc test was used for pair-wise comparison of samples.

2.8.7. Multivariate analysis

2.8.7.1. General Linear Models

Multivariate analysis was carried out through construction of General Linear Models (GLMs) using the Statistica software package (Staistica version 6.0, Statsoft, Inc.). A GLM allows numerous factor levels to be incorporated into the model allowing the robustness of each test to be increased beyond that of the conventional ANOVA and multiple regressions. Furthermore, GLMs can also account for random factors and replicate effects when presenting the statistical significance levels. Where GLMs were carried out, the sample size (n) was generally large enough to allow normality and homogeneity of variance to be confirmed by the examination of residual plots.

2.8.7.2. Generalised Linear Models

The Generalised Linear Model (GLZ) function on Statistica 6.0 was used if the residual plots of a GLM did not display normal distribution or homogeneity of variance, even following subsequent transformation of data. The GLZ approach is more robust than the GLM and allows non-linear as well as linear effects to be tested for variables that may not be normally distributed (*e.g.* gamma, Poisson, binomial *etc.*)

2.8.7.3. Principal Components Analysis

Numerous variables that were used in this thesis to assess fish welfare and analysis of these variables in isolation, and even pair-wise regression of variables, could only provide limited information. Furthermore, the relationships between the individual variables were not always clear, the direction of change in relation to welfare was not always the same, and some indictors were subject to a large degree of intraspecific variation. These factors presented difficulties in the interpretation of the data and the determination of its structure.

In order to better understand interaction between variables, Principle Components Analysis (PCA) was used to generate a 'score' that was representative of coherent trends within the data set. By transforming the original variables to a smaller number of uncorrelated variables based upon coherence that existed between in the data, PCA aided the job of identifying patterns among the different variables. Using PCA also avoided the subjectivity of allocating a 'weighting' to variables based upon their perceived importance in terms of fish welfare.

PCA is an evolution of the concept of correlation between two variables *i.e.* a line of best fit that passes through a 2-dimensional scattering or cloud of data. The

computation of factors in PCA consists of converting a symmetric correlation matrix of all of the variables into a multidimensional space, determined by the number of variables and number of cases (fish). By the creation of a new set of axes, called factor axes, obtained in a lower dimensional space, PCA allows a line or lines (factor axes) to be plotted through the centroid of the cloud of points. PCA results in the generation of a new set of uncorrelated variables called principal components (PCs) that are linear combinations of the original variables based on the straight lines that best fit the clouds of points in the multi-dimensional vector space (see Jambu, 1991).

The resulting PCs account for the inherent variation of the data to the maximum possible extent. Each PC has an Eigenvalue, which is a representation of how much of the observed variability is accounted for by a particular PC (*i.e.* the higher the Eigenvalue the more variability it is accounted for). A simple line graph called a Scree plot that shows the Eigenvalues for each of the PCs and is used to aid the selection meaningful PCs; it is generally accepted that only the PCs situated to the left of this point where the graph levels off (the 'elbow') and with an Eigenvalue of greater than 1 should be included in further analysis (Jambu, 1991).

The relative contribution of each of the variables within the different PCs was obtained along with Factor Scores for each of the PCs for all of the individual fish. This process effectively resulted in the generation of one or two new variables for each fish, which can be seen to represent welfare status or other trends in the data. The factor scores for the PCs were included as independent variables in GLMs with a range of categorical and continuous predictors in an effort to establish the effect of the environmental parameters on rainbow trout welfare.

Chapter 3: Assessment of fish welfare

3.1. Introduction

This chapter of the thesis focuses on some of the different parameters that can be used to assess fish welfare. A brief overview of the origins and evolution of welfare assessment will be given before leading on to discuss specific examples of parameters that can be used to assess welfare in rainbow trout. This section also presents data from three short experiments that measured a range of welfare indicators in situations of acute and chronic stress.

The concept of stress in biological systems has been, and still is, the subject of much discussion and disagreement (see Pickering, 1981; Barton & Iwama, 1991). The stress concept is intrinsic to the approach of welfare assessment used in this thesis and this is a fitting point at which to define the context in which the term stress is used throughout this work. The use of the term stress in this thesis loosely refers to the physiological responses resulting from a stimulus, which will be referred to as the stressor. This definition is based on one of the earliest definitions of stress, where stress was defined as the '"the sum of all the physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of a physical or chemical force" (Selye, 1950). There are many different definitions and interpretations of the term stress in the literature, but in an eloquent introduction to the book 'Stress in fish', Pickering pointed out that all definitions of stress share the common principle of a stimulus acting on a biological system and the subsequent reaction of the system (Pickering, 1981).

There can be no mention of the stress concept without referring back to the work of Professor Hans Selye, who in a series of publications dating back to 1936

provided the foundation of the present day understanding of stress (reviewed in Selye 1973). Selye points out that an important step in scientific understanding of the stress concept is the distinction between stress and stressor, whereby 'stress' is the reaction exhibited by an animal and 'stressor' is the factor producing the stress (Selye 1973).

Central to the stress concept is the need for biological systems to maintain balance, and fish, in common with all with all animals, must maintain a stable internal environment in order to develop, grow and reproduce normally (Pottinger, 2000). One of the more recent definitions described stress as "the change in biological condition beyond the normal resting state that challenges homeostasis and thus, presents a threat to health" (Barton & Iwama, 1991). This immediately leads us to one of the pitfalls associated with attempting to define stress *i.e.* what constitutes the 'normal' resting state of an animal, and the argument that the stress response is merely a component of normal metabolism.

Selye's definition of stress assumed that stress was part of normal metabolism and proposed the theory of the general adaptation system (GAS), where the stress response is separated into three phases: an initial period of alarm generalised as a "call to arms" of the body's defences, followed by a period of resistance whereby an animal may display an adaptive response that results in a degree of tolerance as a result of repeated exposure to a stressor, which, if the stressor is severe or prolonged enough, is followed by a stage of exhaustion (Selye, 1950). As our understanding of the different components of the stress response has become clearer the applicability of the GAS-concept has been questioned and it has since been argued that the GAS is flawed as it assumes an identical response to all forms of stressors (Schreck 1982; Morberg, 2000).

One way in which the concept of stress has evolved is in the distinction of the different components of the stress response and the separation of aspects that are adaptive *i.e.* help the fish to overcome the stressor, from those that are maladaptive effects *i.e.* reduce the animals fitness (Figure 3.1; reproduced from Barton & Iwama, 1991).

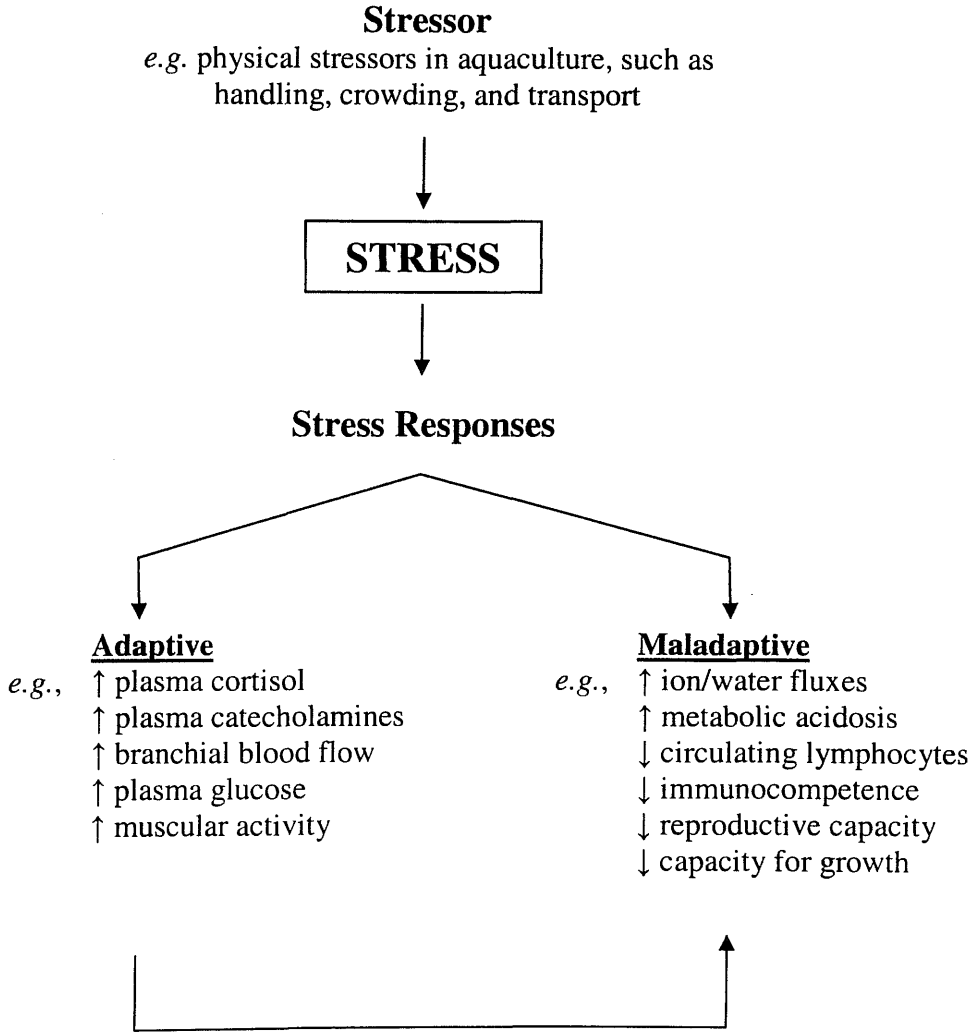


Figure 3.1. Simple representation of the relationship among the stressor, stress and representative adaptive and maladaptive responses in fish; reproduced from Barton & Iwama, (1991); ↑ indicates an increase in a factor (stress response) and ↓ indicates a decrease.

3.1.1. Measuring stress in fish

The rapid growth of the aquaculture industry over the last few decades has resulted in great interest in the stress physiology of fish, particularly for species of commercial importance. The study of stress and the quantification of its effects is complex, as stress responses can be observed at different levels of biological organisation (from molecular up to ecosystem level), and over different periods of time *i.e.* chronic and acute stressors. One approach used to simplify the process has been to separate the stress response into primary, secondary and tertiary responses (Wedemeyer, 1996):

(i) Primary Response

Neuroendocrine responses:

a. Activation of the sympathetico-chromaffin system resulting in the release of catecholamines (primarily adrenaline) from chromaffin cells located mainly in the head-kidney (adrenal medulla equivalent).

b. Stimulation of the hypothalamic-pituitary-interrenal axis (HPI-axis), resulting in the release of adrenocorticotropic hormone (ACTH) from the hypothalamus and the rapid up-regulation and release of corticosteroids from interrenal tissue.

(ii) Secondary Response

Including physiological alterations such as changes in metabolic rate and blood chemistry.

(iii) Tertiary Response

Effects observed are on a whole-animal scale (*e.g.* impaired growth and reproductive success).

Quaternary responses for population scale effects of stress (*e.g.* decreased recruitment or prevalence of a species) are also sometimes reported, but have been excluded since they are not relevant to aquaculture. A schematic representation of some of the stress responses in fish is presented overleaf in Figure 3.2.

Primary stress responses (neuroendocrine)

HPI-axis

- Corticotrophin releasing hormone, (CRH) released by hypothalamus.
- Anterior pituitary stimulated by CRH to release Adrenocorticotrophic Hormone (ACTH).
- ACTH stimulates synthesis and secretion of cortisol by the interrenal tissue of the head kidney.

Sympathetico-chromaffin system

- Catecholamines released from chromaffin cells located mainly in the head-kidney.

Secondary stress responses

Physiological changes

↑ Blood glucose
↑ Blood lactate
↓ Liver glycogen
↓ Muscle protein (catabolism)
↑ Heart rate
↑ Water content (in FW)
↑ Diuresis

Immunosupression

↓ Leukocyte count (lymphocytopaenia)
↓ Antibody production in lymphocytes
↓ Serum bactericidal activity (lysozyme, complement)
↓ Phagocytosis (macrophages, lymphocytes)

Tertiary stress responses

↓Growth
↓Body condition
↓ Reproductive success
↑ Disease

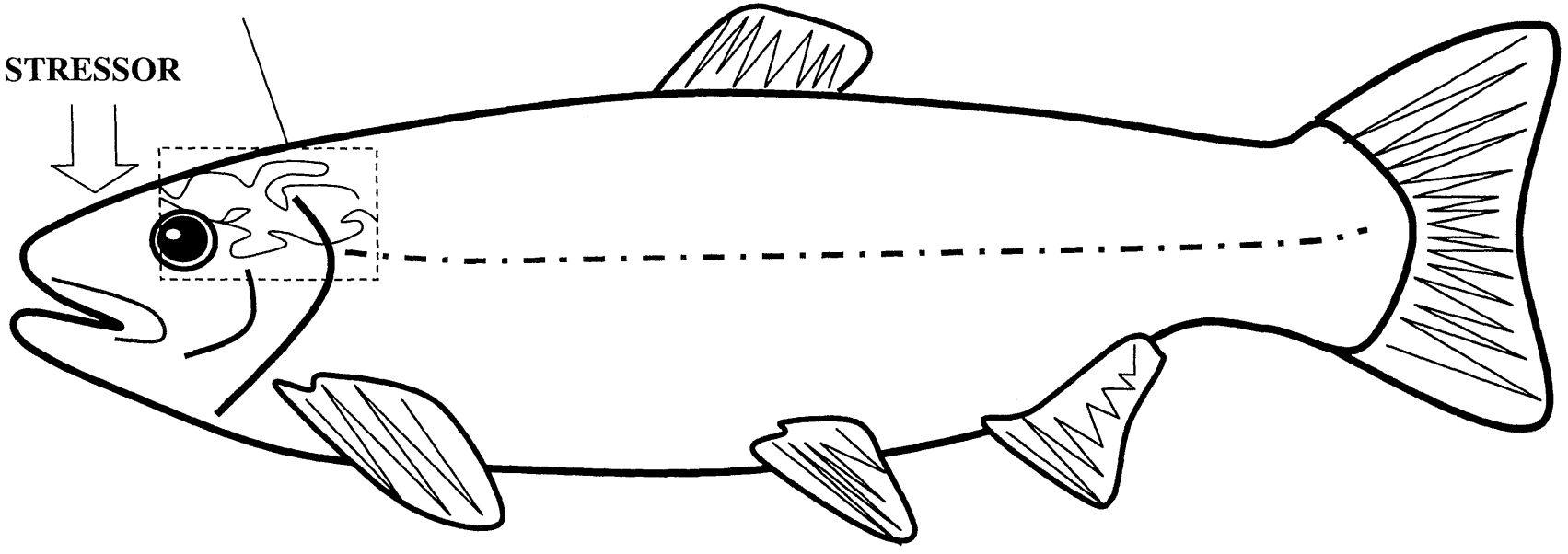


Figure 3.2. Schematic representation of the responses in fish following perception of a stressor; based on commonly reported for responses from the following sources: Donaldson, 1981; Barton & Iwama, 1991; Pottinger *et al.*, 1994; Wedemeyer, 1996.

3.1.2. Primary stress responses in fish

3.1.2.1. The hypothalmic-pituitary-interrenal axis (HPI-axis)

Cortisol is the principle corticosteroid in fish and is frequently used to assess the level of stress in fish (Donaldson, 1981). Cortisol levels rise rapidly in response to almost any kind of external disturbance that triggers the hormonal 'cascade' known as the HPI-axis, or acute stress response in fish (Pottinger & Moran, 1993). The magnitude and duration of the cortisol response following exposure to an acute stressor has been shown to vary considerably between different species of salmonid (Donaldson, 1981). There is also wide variation in the cortisol response between strains of the same species (Pickering & Pottinger 1989) and individuals of the same strain (Pottinger & Carrick, 1999a). In rainbow trout, pre-stress levels of plasma cortisol are generally acknowledged to be less than 5 ng ml^{-1} (Barton, 1980; Pickering & Pottinger, 1989). Following stimulation of the HPI-axis plasma cortisol concentrations increase rapidly to reach peak concentrations of between 40-200 ng ml^{-1}, at 45 min to 3 h following an acute stressor (usually handling). Plasma cortisol will gradually return to pre-stress baseline concentrations between 48 and 72 h post-stress (Barton, 1980; Barton *et al.*, 1987; Pickering & Pottinger, 1989).

The HPI-axis represents a non-specific stress response and can be initiated by a wide range of stimuli (Donaldson, 1981; Barton & Iwama, 1991). A common means of assessing the activity of the HPI-axis of fish is to subject groups of fish to a standardised stress and measure plasma cortisol concentrations over the following 48 to 72 h period (Table 3.1).

Table 3.1. Summary of documented changes in plasma cortisol concentration in rainbow trout subjected to variety of stressors (adapted from Barton & Iwama, 1991)

Plasma cortisol conc. (ng ml^{-1})		Stressor and conditions	Reference
Pre-stress	Post-stress		
<2	43	Handling and 90s confinement	Barton *et al.* (1980)
<2	70	1 h agitation	
<2	213	Continuous confinement	
<2	50	6 h transportation	
9	140	30 s handling, pH6.6	Barton *et al.* (1985)
23	340	30 s handling, pH 5.5	
5	100	Brief handling and confinement	Sumpter *et al.* (1986)
7	53	5 min restraint and confinement	
11	32	10 wk habituation, 30 s handling	Barton *et al.* (1987)
8	65	No habituation, 30 s handling	
<5	80	Handling plus 1 h confinement	Pickering & Pottinger (1989)
<5	40	30 s handling	
3	9	6 wk confinement	
2	8	21 d at 120 kg m^{-3}	

3.1.2.2. Sympathetic hormone response

The sympathetic hormone response, also known as the adrenergic response and sympathetico-chromaffin system, is less well studied in fish than the HPI-axis, partly as a result of difficulties in measuring the response due to rapid onset (Barton & Iwama, 1991). The sympathetic system has fewer stages than the HPI-axis and upon perception of a stressor, levels of catecholamines [primarily adrenalin (epinephrine) or noradrenalin, depending on species] are secreted directly from chromaffin cells. In mammals the main location of chromaffin tissue is the adrenal medulla, but lacking a corresponding structure the main location of chromaffin tissue in teleost fish is the head kidney (Mazeaud & Mazeaud, 1981). Similar to the cortisol response, levels of adrenalin in rainbow trout rise from low basal levels, normally <5 nmoles l^{-1} to reach peak levels that are reported to range from as low as 5.5 nmoles l^{-1} up to 720 nmoles l^{-1} (reviewed in Barton & Iwama, 1991). Also in common with the cortisol response,

catecholamines are released into the blood in response to stimulation regardless of the nature of the stress. This presents difficulties in separating the effects of cortisol and catecholamines on secondary stress responses, although the main actions of catecholamines are understood to be modulation of cardiovascular and respiratory functions and mobilisation of energy stores to help meet the extra demands associated with stressful situations (see reviews by Mazeaud & Mazeaud, 1981; Reid *et al.* 1998).

3.1.2.3. Selection for stress resistance based on primary stress responsiveness

There has been considerable interest in the potential for selecting stress resistant strains of rainbow trout (Pottinger, 2000). The concept of selecting for animals with low stress responsiveness has been widely applied in the poultry farming industries for turkeys (reviewed by Freeman, 1976) and chickens (Gross & Siegel, 1985). A similar approach has also been adopted for rainbow trout by teams of scientists working with Fevolden and Røed at the Institute of Aquaculture Research in Norway, and Pottinger at the Windermere Laboratory of the Institute of Freshwater Ecology, UK. These research groups have published the results from numerous studies suggesting a genetic basis for the cortisol responsiveness of rainbow trout (Fevolden *et al.*, 1991; 1992; 1999; 2002; 2003; Fevolden & Røed, 1993; Pottinger & Moran 1993; Pottinger *et al.*, 1994; Pottinger & Carrick, 1999a; 1999b; Trenzado *et al.*, 2003).

The approach taken by these two laboratories was very similar, whereby individuals were selected based on the magnitude of their peak cortisol response following exposure to a standardised stressor (Fevolden *et al.*, 1991; Pottinger *et al.*, 1994). Fish were separated into high cortisol responding (HC) and low cortisol

responding (LC) families and the progeny of these families were subsequently crossed to produce families with divergent cortisol responses.

The cortisol response has been shown to be consistent (Pottinger *et al.*, 1992) and display a relatively high heritability (h^2) of 0.50 (Fevolden *et al.*, 2002), although the benefits of selecting for lower stress responsiveness remain unclear. Pottinger suggested that in theory, reducing the responsiveness of an individual to stressors should provide a broad range of benefits with the potential for improved growth, reproductive performance and disease resistance (Pottinger, 2000). However, initial attempts to assess the benefits of selecting for stress responsiveness have proved ambiguous. Fevolden *et al.* (2002) reported significant negative phenotypic correlations between individual-specific growth rate and post-stress levels of cortisol, suggesting that lower stress responsiveness had a growth promoting effect. However, Pottinger and Carrick (1999a) found the opposite, with fish selected for high stress responsiveness for glucose and cortisol growing more rapidly than those selected for low responsiveness. Fevolden *et al.* (2003) recently reported a beneficial effect of low cortisol response on survival of rainbow trout subjected to a combined salt and confinement stress and suggested that fish with higher responsiveness to stress were less able to cope with multiple stressors.

3.1.3. Secondary stress responses in fish

3.1.3.1. Energy mobilisation

The mobilisation of energy reserves during the acute stress response is one of the most widely reported effects of elevated levels of corticosteroids and is one of the responses that forms the basis of the 'fight or flight' response in fish (Pickering & Pottinger, 1989; Trenzado *et al.*, 2003). Cortisol and catecholamines are both reported

to play an important role in the up-regulation of gluconeogenesis by stimulating the liver to convert fat and protein to intermediate metabolites (Mazeaud & Mazeaud, 1981; Matteri *et al.*, 2000). Cortisol is known to carry out many important 'house-keeping' functions in maintaining homeostasis (Mommsen *et al.*, 1999), but in situations of chronic stress, its metabolic actions can become maladaptive resulting in protein catabolism and elevated plasma glucose concentration (hyperglycaemia) (Matteri *et al.*, 2000).

Hyperglycaemia is one of the most well reported secondary stress response in fish, with reports dating back to 1921 (Scott, 1921: *in* Mazeaud & Mazeaud, 1981). In rainbow trout, glucose levels will normally show an approximate two or three-fold increase to peak levels following exposure to a stressful stimulus (Pottinger & Carrick 1999a; Trenzado *et al.*, 2003). The natural range for clinically healthy rainbow trout is reported to be in the range of 41-151 mg dL^{-1} (Wedemeyer, 1996), although the basal and peak levels of glucose will be affected by diet and nutritional status as well as stress *per se* (Vijayan & Moon, 1992).

A reduction in liver glycogen levels is often reported to accompany stress induced hyperglycaemia (Trenzado *et al.*, 2003). Hepatosomatic index and liver glycogen have been shown to be reduced in rainbow trout that were fed cortisol or subjected to a daily handling stress (Barton *et al.*, 1987). Pottinger and Carrick (1999a) selected rainbow trout based on high or low glucose stress response (HG or LG) following exposure to acute handling stress in the same way as described earlier for cortisol response. Interestingly, there was no link between the cortisol and glucose responsiveness, but there were significant differences in size between the low and high responding fish for each trait, with the HC fish significantly larger than the LC fish, and the same true of the HG fish compared with the LG fish. There was no size

mediated effect within either the low or high responding groups for glucose or cortisol, and the authors suggested that size differences between high and low responding groups were a result of stress responsiveness, possibly a result of behavioural differences.

The lack of association between the peak glucose and cortisol response suggests that the actions of the HPI-axis and sypathetico-chromaffin system in rainbow trout may not be closely coupled. Pottinger and Carrick (1999a) suggested that mobilisation of glucose during the stress response in rainbow trout may be primarily related to the catecholamine pulse following tissue stimulation of the sympathetic nervous system. Barton and Iwama (1991) emphasised the need for work to establish whether cortisol is genuinely gluconeogenic, or just acts to sustain glucose levels following an initial response to catecholamines by influencing the action of other metabolic hormones such as insulin, or thyroid hormone.

3.1.3.2. Haematological Changes

The main haematological changes that take place during the stress response include changes in haematocrit, leucocrit, erythrocyte numbers, leucocyte numbers, lymphocyte:red blood cell (RBC) ratio, thromobocyte numbers and blood clotting time (Barton & Iwama, 1991). One of the most commonly used haematological measurements is haematocrit, which provides a rapid and simple measurement of the relative fraction of blood made up of erythrocytes, referred to as the % packed cell volume (PCV). The range of haematocrit reported for clinically healthy rainbow trout is between 24-43% PCV (Wedemeyer, 1996). Increased haematocrit can be interpreted as an indication of an acute stress response, though it is unclear if this is mediated by corticostreroids or a transient response to acute disturbances (Barton *et*

al., 1987). High haematocrit can also indicate haemoconcentration through gill damage, while low haematocrit can be indicative of anaemia and haemodilution (Wedemeyer, 1996).

Barton *et al.* (1987) observed an increase in haematocrit in rainbow trout that were fed cortisol and suggested that the increase may have been due to a cortisol-mediated decrease in extracellular fluid relative to blood cell volume. Swelling of erythrocytes, accompanied by a redistribution of body fluids has been suggested as a possible cause of increased haematocrit in the acute stress response of rainbow trout (Milligan & Wood, 1982), although the only evidence that this is cortisol mediated in rainbow trout is provided by Barton *et al.* (1987).

3.1.4. Tertiary stress responses in fish

Growth is probably the most well studied aspect of fish biology and the nature of aquaculture as a food production industry means that this is normally driven by economic interest in commercially important species aimed at maximising profits. Growth integrates all of the biotic and abiotic variables acting on an organism making it an ideal indicator of tertiary effects of environmental stressors (Goede & Barton, 1990). There is a vast volume of published work relating to the effects of stress on growth of fish (see reviews by Schreck 1981; 1982; Pickering, 1990) and it is generally recognised that stress has an overall negative on growth (Schreck 1981; Pickering, 1990; FSBI, 2002).

Growth in fish is indeterminate and flexible, depending on the intake and the utilisation of energy; with stress having direct and indirect effects on both of these processes resulting in a negative effect on energy balance (FSBI, 2002). Energy used during the stress response will be directed away from somatic growth and can be seen

as a representation of the metabolic cost of dealing with the disturbance and regaining homeostasis following a stressful episode (Schreck, 1981). This is illustrated in Schreck's model for growth and the concepts of potential and realised growth (Figure 3.3), where the maximum potential for growth is genetically predisposed, and the realised growth is that observed after the effect of limiting factors of environment (*e.g.* temperature, nutrition) and stress (Schreck, 1981).

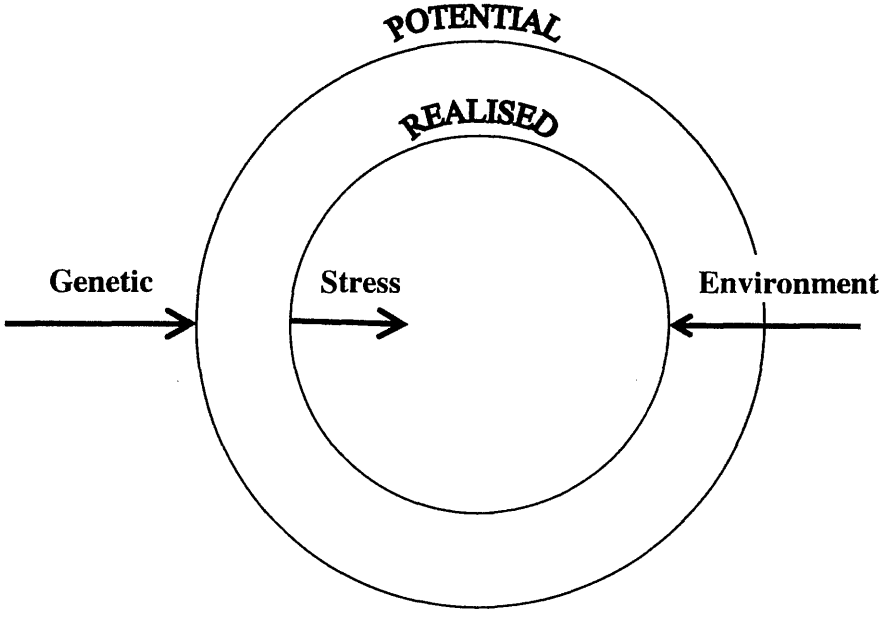


Figure 3.3. The relationship between the potential and realised performance capacities and the influence of ultimate (genetic) and proximate (environment and stress) limiting factors (reproduced from Schreck, 1981).

A little-explored avenue of research is the effect of stress on the appetite of a fish. It may be that reduced growth commonly associated with the metabolic cost of stress is partly due to a reduction in the consumption of feed bought about by hormonal regulation of appetite. This subject remains relatively unstudied though

Jørgensen *et al.*, (1993) attributed a reduction in growth of Arctic charr (*Salvelinus alpinus* Linnaeus 1758) held at low stocking densities to reduced feed intake.

Numerous methods of assessing the performance fish have been developed for the aquaculture industry, some of which are potentially useful as indictors of tertiary stress responses. Measurements commonly applied as performance indicators in salmonid farming include:

Specific growth rate (SGR; see section 2.6.7.1 for calculation, page 42)

SGR reflects the rate of fish growth over a specified period of time, with higher SGR indicating more rapid growth. SGR can be estimated for groups of fish based on the mean weight or length at time 1 and 2, or for individual fish if fish have been tagged *e.g.* PIT-tagging

Feed conversion ratio (FCR; see section 2.6.7.2 for calculation, page 44)

FCR provides an indication of how efficiently food is converted into somatic growth. Though FCR is an intrinsic function of the balance between carbohydrate, protein, and lipid in the feed, it is strongly affected by the rearing environment, including factors such as feed wastage, temperature, water quality, behaviour, and stressful husbandry procedures such as grading (Westers, 2001). The lower the value for FCR, the more efficiently food is turned into flesh, with optimum FCR in salmon and trout in the region of 1.1:1 *i.e.* 1.1kg of food will produce 1 kg of fish tissue (Wedemeyer, 1996).

Condition factor (CF; see section 2.6.5.3 for calculation, page 39)

CF is a crude indicator of the nutritional status of fish providing an indication of the energy reserves or fatness of a fish (Goede & Barton, 1990). CF of a well fed fish will be higher than for a poorly fed fish of the same length. There are differences in the values depending on the units of measurement of length and weight (*i.e.* imperial or metric measurements) though the most commonly reported values for salmonids are based on the British system (inches, pounds) where CF for rainbow trout is normally >1 (Westers, 2001).

3.1.5. Stress and the immune system

In common with other vertebrates, the immune system in fish has a specific and non-specific component. Although the two components of the immune response are inherently linked, non-specific responses are generally directed towards micro-organisms and foreign material, while the specific response involves the production of antibodies and activated T-lymphocytes that target and bind to antigens on invading viruses (Anderson, 1990). The association between stress and an increased susceptibility to disease has been recognised for some time and forms the basis for the stage of exhaustion in Selye's GAS concept (Selye, 1936). While immunosuppression is a commonly recognised maladaptive response to stress (Figure 3.1.) the mechanisms involved in this relationship are poorly understood (reviewed by Ellis, 1981).

The immunosuppressive effects of corticosteroids form the basis of the relationship between increased susceptibility to disease and stress, although Ellis emphasised that stress responses can also be immunostimulating and suggested that 'immunoregulation' is a better term than 'immunosuppression' regarding the effects

of stress response on defence systems (Ellis, 1981). Despite gaps in the understanding of immune function of fish, stress is known to increase susceptibility of fish to infectious disease (Wedemeyer, 1976; Snieszko, 1976). Wedemeyer (1996) listed some of the mechanisms by which immune function is affected by stress:

- Decreased serum bactericidal activity (complement, lysozyme)
- Impaired phagocytosis (macrophages, lymphocytes)
- Decreased leukocyte count (lymphocytopaenia)
- Decreased antibody production by lymphocytes

Specific examples of most of these mechanisms can be found in the literature for rainbow trout. Möck and Peters (1990) observed reduced lysozyme activity in rainbow trout for at least 24 h following 2 h transportation, and Fevolden *et al.* (2002) demonstrated a significant negative phenotypic correlation between cortisol and lysozyme activity in rainbow trout selected for low and high stress responsiveness. Nanaware *et al.* (1994) observed reduced macrophage phagocytosis in rainbow trout subjected to a range of environmental stressors.

Despite observing interrenal acclimation (*i.e.* cortisol levels retuned to basal levels) in rainbow trout subjected to chronic crowding stress (172 kg m^{-3}), Pickering and Pottinger (1987a) observed a consistent reduction in numbers of circulating lymphocytes over a 3 week period. A decrease in numbers of circulating lymphocytes was observed in rainbow trout (Barton *et al.*, 1987) and brown trout (Pickering, 1984) treated with cortisol. Pickering and Pottinger (1989) also reported a dose-dependent increase in mortality from secondary bacterial and fungal diseases in brown trout that were given intraperitoneal cortisol implants.

3.1.6. Summary

This introduction provided a brief overview of the stress response in fish and with the aim of providing an appreciation of some of the physiological changes that can be measured to quantify stress. The subject of stress in fish is well studied and most aspects of stress have been the subject of comprehensive reviews (*e.g.* Pickering, 1981; Barton & Iwama, 1991). The main points of these reviews have been summarised with the inclusion of key references that provide specific examples for quantifying stress responses in rainbow trout. The main focus of this introduction has been on the variables that were subsequently applied to assess fish welfare in the following experimental chapters.

The indicators discussed in this introduction represent just some of the more common measurements that can be used to assess welfare and are by no means exhaustive. Alternative approaches of assessing welfare such as observing behavioural responses are equally valid (Dawkins, 2004) and welfare assessment should ideally take account of behavioural processes as well as indicators of condition and physiology (FSBI, 2002). In situations of commercial aquaculture detailed behavioural studies are practically difficult due to the vast numbers of fish involved and the fact that fish are not always visible from surface or sub-surface observation. However, recent developments in the field of hydro-acoustics have given rise to novel techniques for assessment of shoaling and feeding behaviour in Atlantic salmon (Juell *et al.*, 2003; Bron *et al.* in preparation).

Behavioural observations are non-invasive and allow animals to be observed in their normal farm habitat. Such observations are intrinsic to good fish husbandry and an experienced stock worker would be expected to be familiar with the way that

their stock behaves. Any radical deviation from the 'normal' behaviour could be used as an early warning system for more serious problems.

Although difficult to quantify, such behavioural clues may alert farmers to other problems *e.g.* reduced feeding may be a result of a water quality problem, or certain behaviour may signal an outbreak of disease. Subjective indices of welfare are gaining credibility to the extent that the validity of welfare assessment based solely on objective indicators has been questioned (Wemelsfelder, 1997).

The selection of welfare indicators involves striking a balance between practicalities, resources (time, experience and economics) and scientific justification. The approach taken to assess fish welfare in this thesis was based upon objective quantification of stress responses at different levels of organisation, measuring components of the primary (cortisol) and secondary responses to stress (glucose and haematocrit) in conjunction with an indicator of immune function (lysozyme activity) and measurements of growth, body condition and mortality. In addition, water quality was monitored to provide an indication of the quality of the environment in which the fish lived. As previously discussed, due to the relationship between increased stocking density and water quality deterioration, the measurement of key water quality parameters was essential to distinguish the effects of water quality on welfare from the effects of stocking density *per se*.

3.2. Experimental assessment of the acute stress response of rainbow trout

In order to establish the acute stress response of the different strains of rainbow trout used in this thesis, two short trials were conducted in which fish were exposed to a standardised handling stress. Various parameters were measured from the plasma of fish before the exposure (baseline or residual levels), and at timed intervals following the exposure (post-stress), to establish the timing and magnitude of peaks and also the time taken to return to baseline levels. The first experiment was carried out with the same batch of fish used in Chapter 4, which will be referred to as Strain 1. Experiment 2 used fish from the same batch as Chapter 5, which will be referred to as Strain 2.

3.2.1. Experiment 1: The acute stress response of rainbow trout Strain 1

3.2.1.1. Materials and Method

Experiment 1 took place from 26th and 27th July 2000. During this period water temperature ranged between 14.4 and 14.9°C and photoperiod was 17 h of daylight and 7 h of darkness. The water level in a 5 m diameter stock tank was lowered and 100 female rainbow trout (529g ± 96g SD) of Strain 1 were randomly netted and moved into a 2 m diameter tank where they were allowed 10 days to acclimatise.

At 8.30 am on the morning of sampling, 10 fish were removed, killed and blood sampled to provide 'unstressed', baseline plasma levels of stress indicators. Concurrently, the standpipe of the 2 m tank was dropped and the tank drained until the remaining 90 fish were emersed. The fish were rapidly netted into buckets following a total period of no more than 2 min exposure to air and were distributed in groups of 10 into 9 x 1 m^2 tanks with external standpipes adjusted to create a water depth of 30 cm (volume 0.3 m^3). Each tank was fitted with an opaque fibreglass cover

fitted with light a 16 W drum fitting light (RS Components Ltd.; Northants, UK). During the natural photoperiod regime light was controlled using a photosensitive switch (RS components Ltd., Northants, UK) adapted by Alex Brewsters electrical contractors (Stirling, UK)

One tank of fish was killed and blood sampled at each of the following times: 15 min, 30 min, 40 min, 60 min, 3 h, 6 h, 24 h and 48 h post-stress. Plasma was extracted from the blood samples and frozen at –70°C. Plasma concentrations of cortisol and lysozyme activity were measured from each of the samples.

Statistical analysis

If data were normally distributed (Kolmogorov-Smirnov test) and with equal standard deviation (according to Bartlett's test) a one-way parametric ANOVA was carried out with a Tukey-Kramer multiple comparisons post-hoc test. If data were not normally distributed and/or there were significant differences in the standard deviation between samples, data were transformed to result in a normal distribution. If the transformed data still failed the assumptions required for ANOVA, a Kruskal-Wallis non-parametric ANOVA was carried out with a Dunn's multiple comparison post-hoc test.

3.1.1.2. Results

There was a marked change in plasma cortisol following exposure to the handling stress, with levels rising from a baseline level of 6.7 ng l^{-1} to a peak of 163.0 ng l^{-1} at 30 min post-exposure (Table 3.2). The concentration of cortisol dropped steadily over the following 6 h, but there was an unexpected increase 24 h post-exposure when cortisol levels of 80.9 ng l^{-1} were measured (Figure 3.4a).

Table 3.2. Cortisol and lysozyme activity response in rainbow trout Strain 1 following a standardised handling stress; mean values of fish at each time point with SEM in italics

Time Post-Stress (min / h)	Plasma Cortisol ($ng\ l^{-1}$)	Lysozyme Activity ($U\ min^{-1}\ ml^{-1}$)	Number of fish sampled
0	6.7 ± *6.8*	2491 ± *1007*	10
15 min	30.9 ± *10.8*	2218 ± *559*	10
30 min	163.0 ± *66.2*	2272 ± *466*	11
45 min	144.5 ± *70.6*	1458 ± *272*	10
60 min	88.9 ± *46.7*	1708 ± *155*	11
3 h	47.3 ± *21.4*	1567 ± *477*	9
6 h	23.9 ± *17.7*	1600 ± *363*	12
12 h	48.5 ± *17.9*	1816 ± *227*	11
24 h	80.9 ± *30.5*	1696 ± *392*	8
48 h	32.7 ± *33.5*	2018 ± *241*	10

The pattern of change in lysozyme activity following the handling stress was not as well defined as the cortisol response, although there appeared to be a decrease from the post-stress level of 2491 U min^{-1} ml^{-1} to a minimum of 1458 U min^{-1} ml^{-1} occurring at 45 min post-stress (Table 3.2, Figure 3.4b).

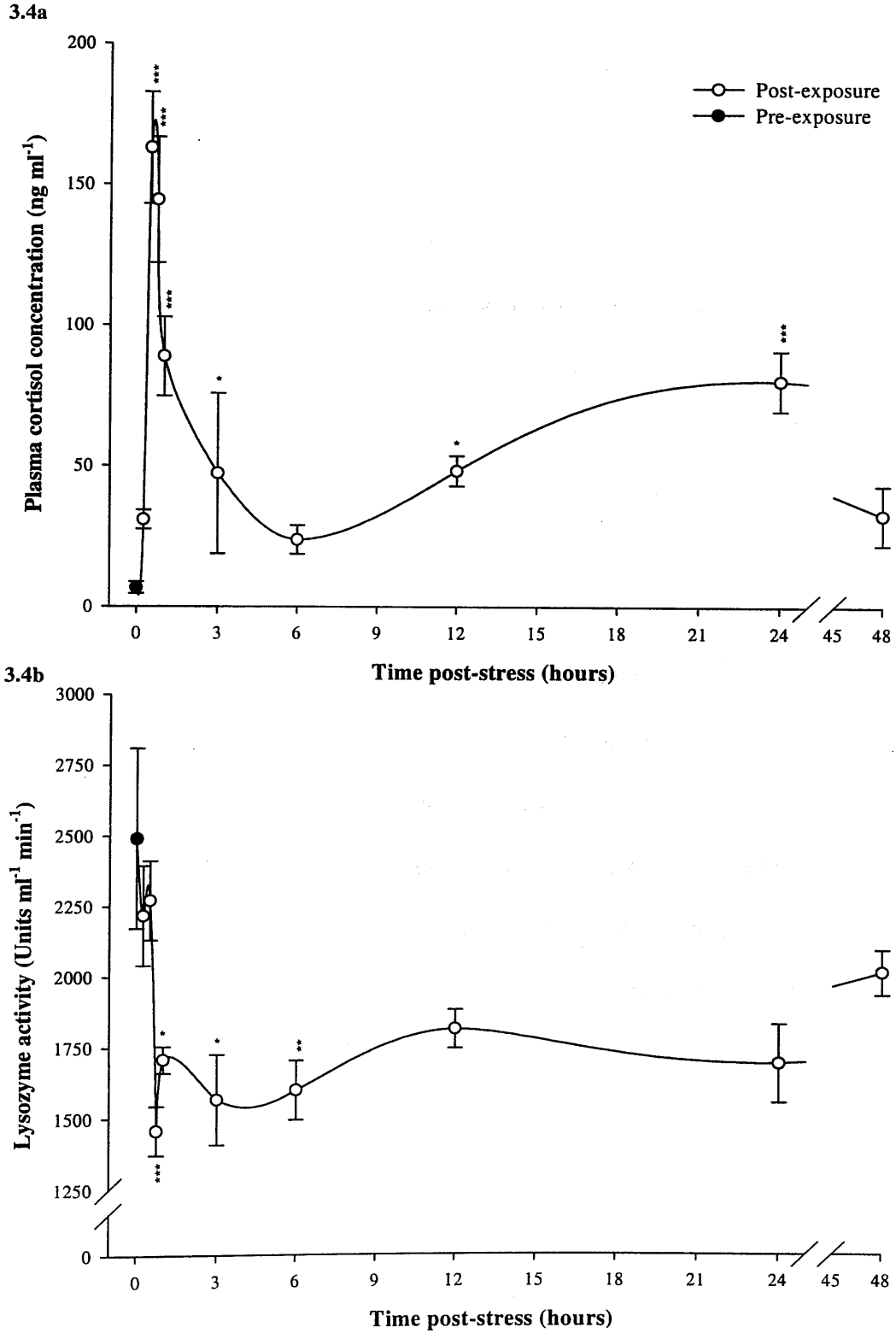


Figure 3.4. Changes in plasma cortisol concentration (a) and lysozyme activity (b) in rainbow trout following exposure to a standardised handling stress in experiment 1; each point represents the mean ± SEM (n=10)with significant differences compared with pre-stress levels denoted by asterisks; ***P<0.001, **P<0.01, *P<0.05.

3.2.2. Experiment 2: The acute stress response of rainbow trout Strain 2

3.2.2.1. Materials and Method

Experiment 2 was carried out between 26th and 28th January 2002. Water temperature ranged between 2.8 and 3.4°C and photoperiod was 8 h of daylight and 16 of darkness.

220 rainbow trout of Strain 2 were randomly removed from a 5 m stock tank containing approximately 7250 fish into a 2 m diameter tank and allowed 5 days to acclimatise. The fish were mixed sex with a mean weight 132g (±43g SEM) Any fish showing signs of precocious maturation were not included in the experiment.

The methodology used was the same as in experiment 1, but with the following changes.

- 20 fish were sampled at each of the time points instead of 10.
- The timing of samples was adjusted to include sample points at 2 h and 72 h post-stress, with the omission of the 15 and 45 min samples.
- Instead of simulating the photoperiod with artificial light as in experiment 1, the feeding hatches on the lids were left open to allow natural daylight to enter tanks.
- Haematocrit and glucose were also measured in addition to cortisol and lysozyme activity.

3.2.2.2. Results

The values for the blood parameters measured at each of the sample points are shown in Table 3.3. There was a significant increase in plasma cortisol following exposure to the handling stressor, with concentrations rising from a pre-stress level of 12.4 ng ml^{-1} to around 35 ng ml^{-1} at 1, 2 and 3 h post-stress time points (Figure 3.5a). At the 6 h post-stress time point plasma cortisol concentration was no longer significantly

different from pre-stress levels and by 24 h post-stress cortisol had dropped below the pre-stress concentrations. Low levels of cortisol were observed at 24, 48 and 72 h post-stress and though not statistically different from pre-stress concentrations (P>0.05), mean cortisol concentrations were less than half the pre-stress at all of these time points.

Table 3.3. Changes in blood parameters of rainbow trout Strain 2 following a standardised handling stress; mean ± SEM with number of samples shown in parenthesis.

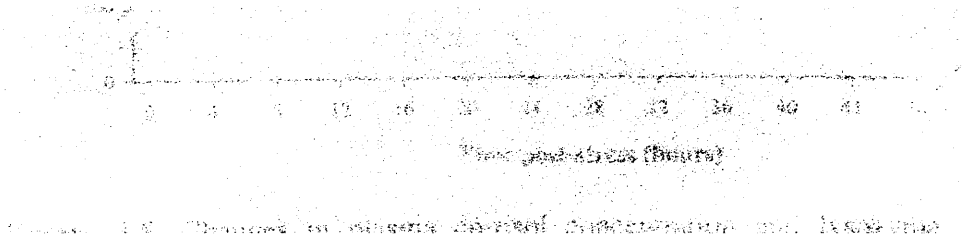
Time post-stress (h)	Plasma cortisol (ng l^{-1})	Lysozyme activity (U min^{-1} ml^{-1})	Haematocrit (%)	Glucose (mg dL^{-1})
0	12.4 ± 2.1 (10)	866 ± 72 (15)	39 ± 6 (20)	56 ± 19 (7)
0.5 h	22.9 ± 2.9 (10)	787 ± 63 (20)	53 ± 5 (20)	55 ± 11 (10)
1 h	36.3 ± 3.2 (10)	1084 ± 83 (19)	53 ± 5 (19)	56 ± 12 (11)
2 h	37.5 ± 5.2 (10)	799 ± 62 (18)	52 ± 6 (19)	69 ± 12 (9)
3 h	33.0 ± 4.4 (10)	884 ± 41 (19)	55 ± 4 (19)	57 ± 9 (11)
6 h	24.3 ± 6.2 (10)	853 ± 65 (20)	54 ± 7 (20)	68 ± 10 (11)
12 h	27.5 ± 5.4 (10)	934 ± 91 (20)	54 ± 6 (20)	97 ± 10 (13)
24 h	3.8 ± 0.6 (10)	1000 ± 62 (18)	50 ± 5 (20)	108 ± 7 (13)
48 h	6.2 ± 1.5 (10)	820 ± 58 (20)	45 ± 4 (20)	105 ± 11 (13)
72 h	3.1 ± 0.9 (10)	1111 ± 88 (20)	49 ± 6 (20)	72 ± 6 (13)

It was unclear if the handling stress resulted in elevation or suppression of lysozyme activity in Strain 2. There were significant differences between the lysozyme levels at the different post-stress time points, but when compared with the pre-stress levels there were no significant differences. Lysozyme activity appeared to be elevated 1 h post-stress when activity was 1084 U min^{-1} ml^{-1} compared with a pre-stress level of 866 U min^{-1} ml^{-1}, although over the 72 h period there was no clear trend

(Figure 3.5b). The levels of lysozyme activity were generally much lower than those observed in Experiment 1, possibly due to the low water temperature.

Pre-stress levels of haematocrit were around 40% PCV and there was a rapid increase following the handling stress, after which haematocrit remained significantly higher than pre-stress levels until 48 h post-stress (Figure 3.6a). Haematocrit values at the 72 h time point were again significantly higher than pre-stress levels, but the level of significance was lower ($P<0.05$) than the earlier time points within 12 h of the handling stress ($P<0.001$).

Plasma glucose concentration displayed a relatively slow but clear response to the handling stress. For the first 6 h post-stress, levels of glucose remained similar to pre-stress concentrations of 56 mg dL^{-1}. By 12 h post-stress, plasma glucose appeared to be elevated at a concentration of 96 mg dL^{-1}, though the difference compared with pre-stress levels was not significant ($P>0.05$). Plasma glucose continued to increase and was significantly higher than pre-stress levels at 24 and 48 h post-stress, with concentrations of 108 and 105 dL^{-1} respectively. At 72 h post-stress plasma glucose concentration was no longer significantly increased from pre-stress levels suggesting that the peak of glucose had passed (Figure 3.6b).



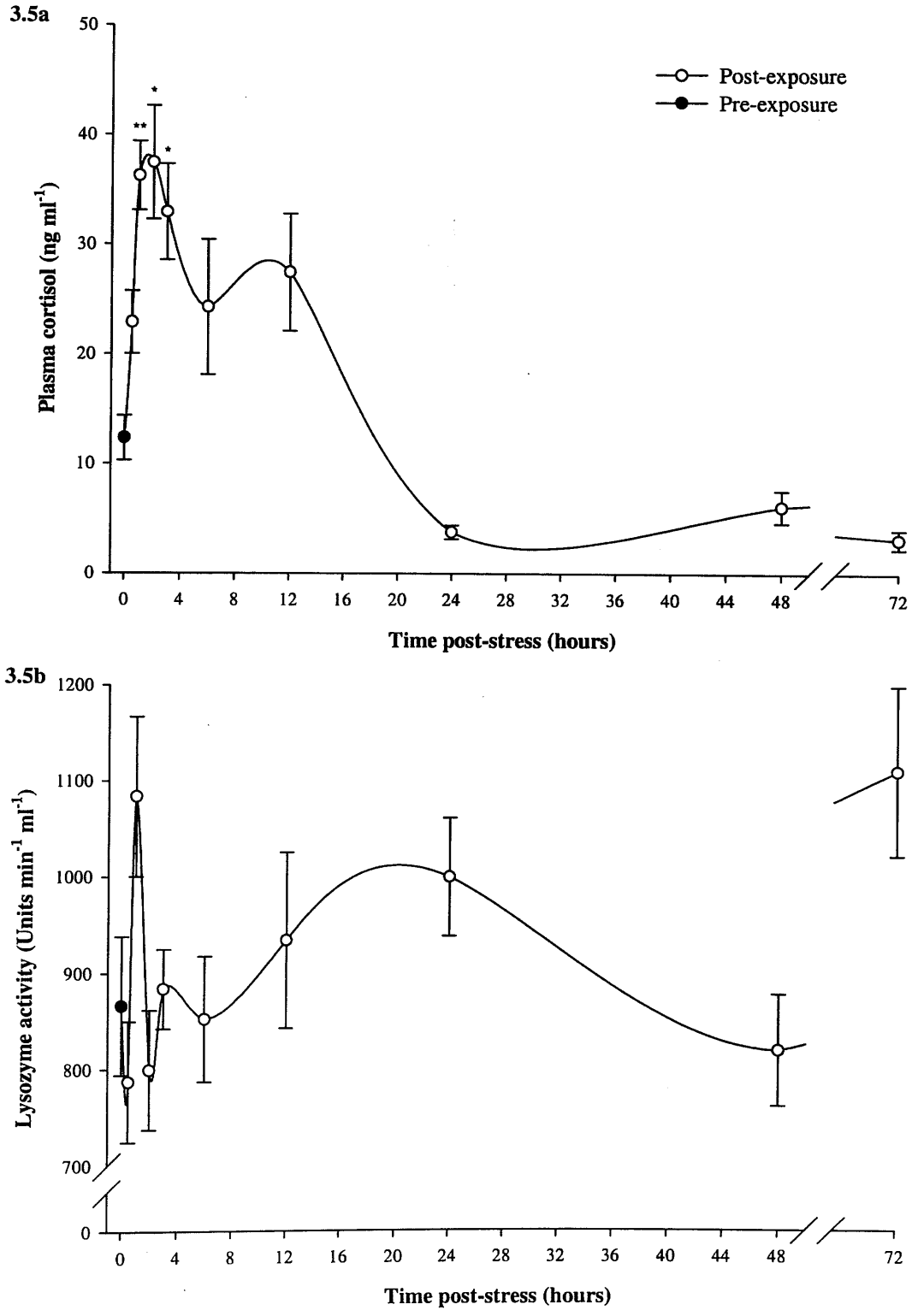


Figure 3.5. Changes in plasma cortisol concentration and lysozyme activity in rainbow trout following exposure to a standardised handling stress in experiment 2; each point represents the mean ± SEM (n=20), with significant differences compared with pre-stress levels denoted by asterisks; $^{**}P<0.01$, $^{*}P<0.05$.

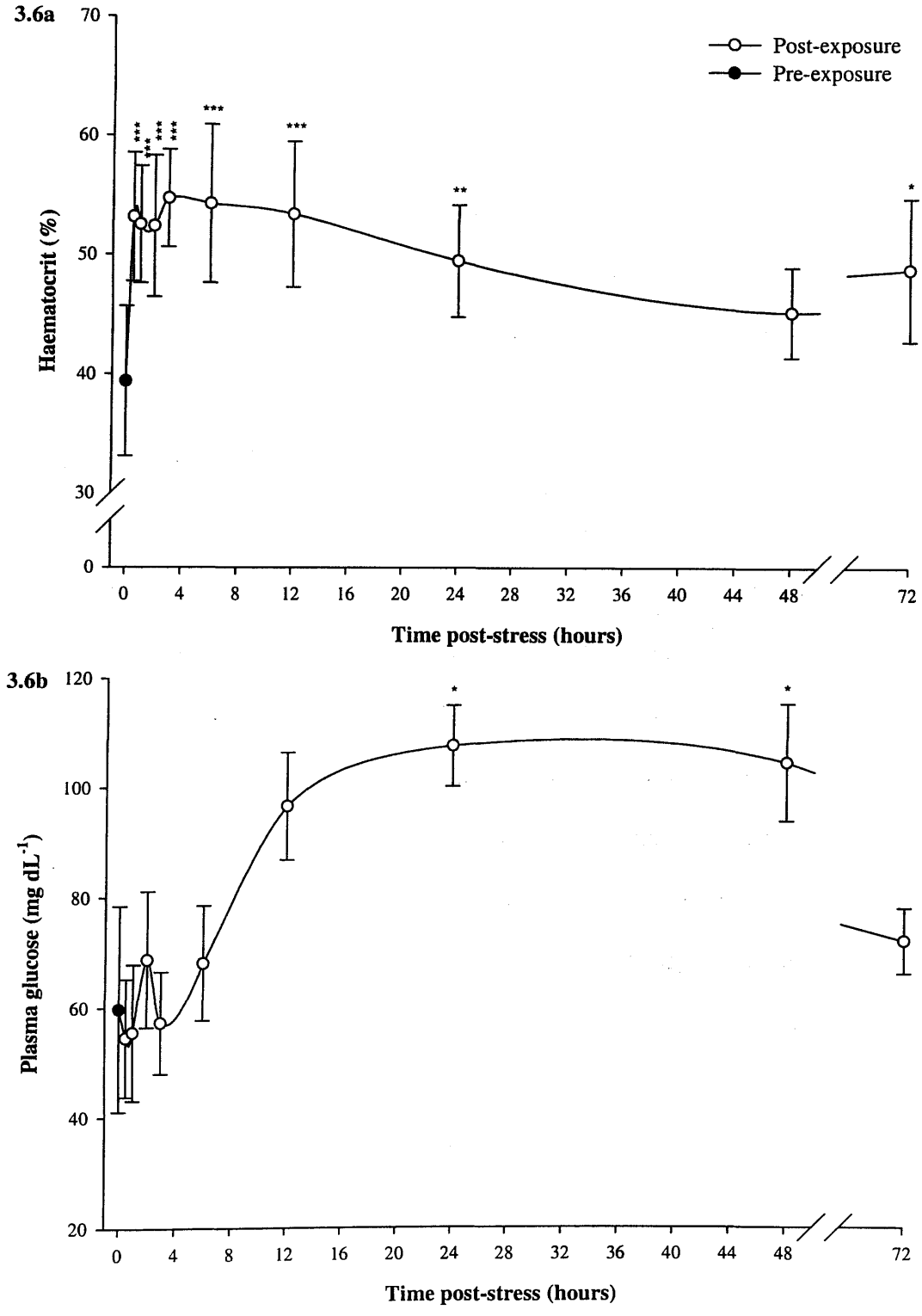


Figure 3.6. Changes in haematocrit and plasma glucose concentration in rainbow trout following exposure to a standardised handling stress; each point represents the mean ± SEM (n=20), with significant differences compared with pre-stress levels denoted by asterisks; ***P<0.001, **P<0.01, *P<0.05.

3.2.3. Summary of results for experiments 1 and 2

Cortisol response

Plasma cortisol increased significantly in both strains of rainbow trout in response to the handling stress. Peak cortisol levels occurred at 30 min in experiment and 2 h in experiment 2. There was a large difference in the magnitude of the peaks, with the mean post-stress peak in cortisol much higher in experiment 1 (163 vs. 37 ng ml^{-1}). The pre-stress levels of cortisol were higher in experiment 2 with a mean concentration of 12.4 ng ml^{-1} compared with 6.7 ng ml^{-1} in experiment 1. The low levels of cortisol observed at the 24, 48 and 72 h post-stress sample points in experiment 2 suggested that the pre-stress concentration of 12.4 ng ml^{-1} may not have been a true basal level, and that there may still have been the remnants of a previous stress response, possibly due to the shorter period of acclimation in experiment 2 (5 days acclimation compared with 10 days in experiment 1), and/or lower water temperatures during the period of acclimation. Basal levels of plasma cortisol in unstressed rainbow trout are normally in the range of 0-5 ng ml^{-1} (Barton *et al.*, 1980; Pickering & Pottinger, 1989), so this would again suggest that the pre-stress levels observed in experiment 2 were not true basal levels. The magnitude and duration of the peak cortisol response observed in experiments 1 and 2 were around the range of 40-200 ng ml^{-1} reported for other strains of rainbow trout exposed to similar standardised stressors (Pickering & Pottinger, 1989).

The time taken for plasma cortisol to return to pre-stress baseline levels was 6 h for both strains, although the comparatively high pre-stress level and low peak concentration of cortisol in experiment 2 meant that the differences at 6 and 12 h were not statistically significant from pre-stress levels even though plasma cortisol concentrations continued to decrease at the 24, 36 and 72 h time points. The

prolonged cortisol response and slower on-set of the peak levels of cortisol in experiment 2 was possibly due to the low water temperatures during the time of experiment 2 (water temperature ranged between 14.4 and 14.9°C during experiment 1 and 2.8 and 3.4°C for experiment 2).

Levels of cortisol in Strain 1 were elevated again at 12 and 48 h post-stress compared with the pre-stress sample, but at 72 h post-stress the difference was no longer significant. It may have been that the automated switching on and off of lights caused the apparent increase in cortisol at 12 and 48 h post-stress, as it would have been the first time that these fish would have been exposed to artificial light.

To summarise, the cortisol response observed in experiment 1 had a considerably higher peak than in experiment 2 and was also more rapid, in terms of both the time taken to peak and the time taken to return to pre-stress levels. Although there was a difference in cortisol response observed in the two exposure experiments, direct comparison was hindered by differences in the age and size of fish (mean weight of Strain 1 was 529 g compared with 130 g for Strain 2), and also differences in water temperature. It was therefore not possible to determine if the difference in response was due to a strain difference as there were likely to have been confounding influences of other factors.

Lysozyme activity

There was a marked change in lysozyme activity following the handling stress in experiment 1, with significant reductions in lysozyme activity observed from 45 min to 6 h post-stress, after which levels began to increase and return to the pre-stress levels. The pattern of response was almost a mirror image of the cortisol response,

although the relatively high lysozyme activity at 15 and 30 min post-stress suggested that the change in lysozyme activity was slower than the cortisol response.

The lysozyme response in experiment 2 was difficult to interpret and there were no significant differences between pre-stress and post-stress lysozyme activity. Levels of lysozyme activity were considerably lower in experiment 2, but this was probably due to the lower water temperatures at the time of experiment 2. This is biologically valid, as enzyme activity would be expected to be reduced at lower ambient temperatures in poikilothermic animals; this is discussed later in more detail in Chapters 4 and 5.

Based on the pattern of response in experiment 1, the pre-stress level of 866 U min^{-1} ml^{-1} observed in experiment 2 was lower than expected. This again suggested that the pre-stress sample point for Strain 2 may not have been a true basal level for 'unstressed' fish. The lowest level of lysozyme activity observed in experiment 2 occurred at 30 min (787 U min^{-1} ml^{-1}), which again did not fit the pattern of response observed in experiment 1. However, apart from the pre-stress and 30 min time points, the pattern of response would be the same as that shown for Strain 2 *i.e.* a reduction in activity corresponding with high levels of cortisol, followed by a return to high levels when cortisol returned to baseline levels.

Haematocrit

Haematocrit was only measured in experiment 2, but there appeared to be a clear response, with levels increasing from pre-stress levels of around 40% to a peak of 55%, 3 h post-stress. The levels of haematocrit were in agreement with those observed by Benfrey and Biron (2000) following exposure of diploid and triploid rainbow trout to an acute confinement stress. Haematocrit began to decrease after 24 h

and at 48 h levels were no longer significantly elevated compared with pre-stress levels, though a slight increase in haematocrit at 72 h post-stress meant that this point was also significantly higher than pre-stress levels. The haematocrit response was very rapid in its onset and also prolonged. The increase in haematocrit was observed before the increase in cortisol and levels remained elevated after cortisol had dropped below pre-stress levels. This suggested that changes in haematocrit may not be mediated by corticosteroids. The relative ease with which haematocrit can be measured and the prolonged period of response suggested that haematocrit is potentially a very useful welfare indicator.

Glucose

The handling stress resulted in a significant increase in plasma glucose concentration at 24 and 48 h post-stress, but the increase in plasma glucose concentrations appeared to be initiated earlier, between 2 and 6 h post-stress. The pattern of glucose response of Strain 2 was in general agreement with previously published responses to similar handling stressors. Trenzado *et al.* (2003) showed plasma glucose in rainbow trout to peak at around 24 h post-stress and Barton *et al.* (1987) found a peak of glucose at around 6 h post-stress in response to a confinement stress. Benfrey and Biron (2000) suggested a more rapid rise in glucose elevation with significant increases observed just 30 min post-stress in rainbow trout and brook trout (*Salvelinus fontinalis* Mitchell, 1815). The apparent slower on-set of response in the present study may have been due to the low water temperatures for the duration of experiment 2 (3.4°C max compared with 9.2 °C for Benfrey & Biron, 2000).

3.3. Assessing the chronic stress response of rainbow trout

Several different approaches have previously been used to assess the effect of chronic stress on rainbow trout. One such method has been the repeated exposure to a standardised stress over a period of time (*e.g.* Barton *et al.*, 1986; 1987). Another approach involved simulating the effects of chronic stress by implanting (Pickering & Pottinger, 1989) or feeding (Barton *et al.*, 1987) exogenous cortisol. A third approach was to maintain groups of fish in conditions that are perceived to be chronically stressful as demonstrated by Pickering and Pottinger (1989) and Pickering *et al.* (1991), who used different stocking densities (between 20 and 120 kg m^{-3}) to simulate what they termed 'chronic crowding stress'.

3.3.1. Experiment 3 - The effect of chronic elevation of plasma cortisol on the stress physiology of rainbow trout

This trial was conducted between August - September 2000 by Dr. Clive Randall as part of a National Environmental Research Council (NERC, ROPA GR3/R9827) grant to investigate endocrine, growth and reproductive interactions in rainbow trout. The original aim of this experiment was to attempt to detect and measure levels of leptin, a hormone that has been shown to regulate appetite and energy expenditure in mammals (Johnson *et al.*, 2000). Based on the hypothesis that appetite is reduced in stressful situations, exogenous cortisol was implanted at different concentrations in an attempt to detect differentiated levels of a leptin-like peptide in rainbow trout plasma. Following the departure of Dr Randall from the Institute of Aquaculture in September 2000 and several unsuccessful attempts to detect and measure leptin using commercially available radioimmuno assay kits, the plasma samples were used in this study to provide a model for the effect of chronic stress on indicators of welfare. The

original design and set up of this experiment was the work of Dr Randall, but all subsequent sampling and analysis was the work of the author.

3.3.3.1. Materials and Methods

50 juvenile female rainbow trout of Strain 1 were randomly selected from a 5 m diameter stock tank containing approximately 2000 fish of mean weight 140 g (± 3.6g SEM). Ten fish were randomly distributed into 5 x 1 m^2 diameter square tanks (as described in experiment 1) and allowed 2 weeks to acclimate. Each of the tanks and treatments was allocated a random number using the random number generator on a calculator; the random numbers were sorted in ascending order to pair the tanks and treatment (Table 3.4).

Table 3.4. Distribution of tanks and treatments in experiment 3

Tank Number	Implant size (mm)	Cortisol concentration (mg)
1	3.0	Placebo
2	3.0	2.5
3	3.0	5
4	4.5	15
5	4.5	Placebo

21-day release implants were purchased from Innovative Research (Sarasota, USA). The implants were of two different sizes (3 or 4.5 mm) and contained 2.5 – 15 mg of hydrocortisone; placebo implants of each size were also purchased containing an inert pellet.

The fish were anaesthetised as described in section 2.2 and a 1 cm incision was made slightly above and behind the pelvic fins with a scalpel. Muscle layers were eased apart and the implant was introduced into the peritoneal cavity. The incision

area was sealed with a 3:1 mixture of Orahesive powder (Squibb and Sons Ltd.; Middlesex, UK) and Cicatrin antibiotic (The Welcome Foundation Ltd.; Middlesex, UK) to prevent infection.

Blood sampling took place at 10, 20 and 30 days post-implantation and extracted plasma was frozen at –70°C. A daily ration of 1.42 % body weight per day was fed to the fish for the duration the experiment.

Statistics

Non-parametric Kruskal-Wallis, ANOVA was used to compare the levels of cortisol, glucose and haematocrit at each of the time points. Dunn's test was used to compare means of implant treatments relative to the respective placebos if $P<0.05$. A one-way ANOVA was carried out for lysozyme activity

3.3.3.2. Results

Water temperature at the start of the trial was 14.5°C and this decreased as the trial progressed to a low of 10.7°C on the final day of the trial.

Mortality

Mortality was very low, with 100% survival in all but the 4.5 mm placebo treatment, where one fish was euthased on day 10 due to infection of the implant incision.

Growth

A problem with the weighing balance meant that weight data was only recorded on day 30 of the trial. Length data was recorded at days 20 and 30 (Figure 3.7a) and from this it was possible to calculate the length specific growth rate (L-SGR) for each of

the treatments. The change in length between days 20 and 30 of the trial showed that highest L-SGR occurred in the 4.5 mm placebo treatment and lowest L-SGR occurred in the 4.5 mm 15 mg treatment (0.23 vs. 0.12; Figure 3.7b). As fish were not PIT-tagged it was not possible to carry out statistical analysis for individual L-SGR and was only possible to estimate the mean L-SGR of each treatment.

Cortisol

There was a significant effect of the implants on plasma cortisol levels (Kruskal-Wallis, non-parametric ANOVA; $P<0.001$); the plasma cortisol levels at days 10, 20 and 30 of the experiment are shown in Figure 3.8a. The 4.5 mm, 15 mg implant resulted in comparatively high levels of cortisol and a highly significant post-hoc difference was observed at all of the sample points compared with the 4.5 mm placebo implant ($P<0.001$; Dunn's). There was just one significant post-hoc difference in cortisol levels of the 3 mm implant treatments observed on day 30 when cortisol was significantly higher in the 3 mm, 5 mg treatment compared with the 3 mm placebo. There were no significant differences in cortisol concentrations between the 3 and 4.5 mm placebo treatments suggesting that there was no effect of implant size. The levels of cortisol that were observed appeared to decrease with time and this was well illustrated in the concentration observed in the 15 mg implant treatment (Figure 3.8a).

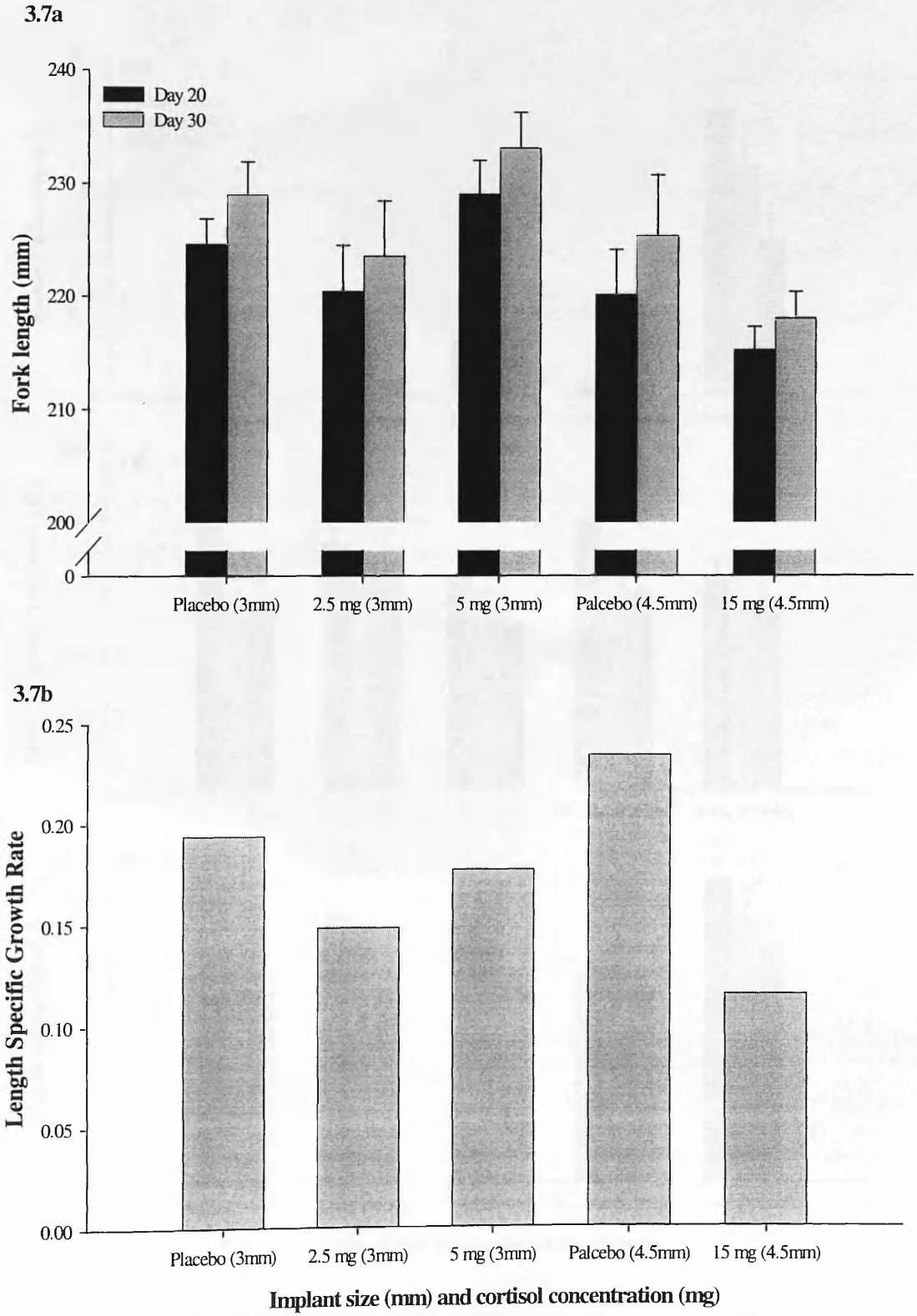


Figure 3.7. Changes in length of rainbow trout given implants containing different concentrations of cortisol. Figure 3.7a shows mean (±SEM) fork length at days 20 and 30 for each treatment (n=10) and Figure 3.7b shows the length specific growth rate for this period.

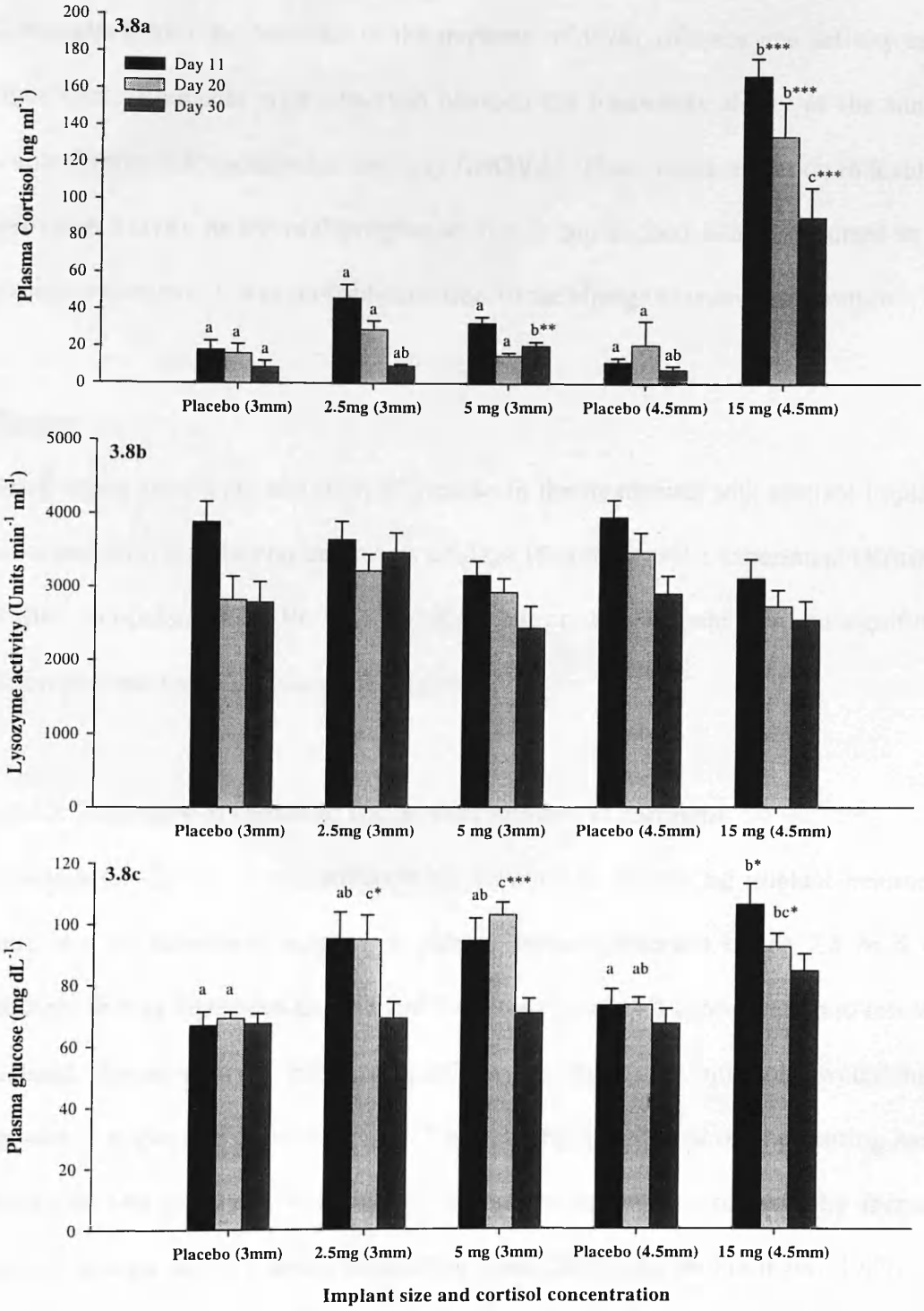


Figure 3.8. Changes in blood parameters of rainbow trout given implants containing different concentrations of cortisol; bars represent the mean (±SEM) for each treatment (n=10), with significant differences at each time point denoted by columns not sharing the same letter. Levels of significance compared to the placebo implant of the same size are indicated by asterisks; ***P<0.001, **P<0.01, *P<0.05.

Lysozyme activity

There appeared to be no effect of the implants on levels of lysozyme activity as no significant differences were observed between the treatments at any of the sample points (Figure 3.8b; parametric one-way ANOVA). There was a reduction in levels of lysozyme activity as the trial progressed, but as this reduction also occurred in the placebo treatments, it was probably an effect of the change in water temperature.

Glucose

There was a significant elevation of glucose in the treatments with cortisol implants compared with the placebo treatments on days 10 and 20 of the experiment (Kruskal-Wallis, non-parametric ANOVA; $P<0.001$), but on day 30 there were no significant differences between the treatments (Figure 3.5c).

3.3.3.3. Summary of results of the cortisol implant experiment

Although the cortisol levels were clearly elevated in the 15 mg implant treatment, there was no consistent increase in plasma cortisol observed in the 2.5 or 5 mg implants. It may have been that 2.5 and 5 mg were too low a concentration to result in elevated plasma cortisol, but this is unlikely as these concentrations would have equated to respective doses of around 7 and 35 mg kg^{-1} (based on the starting mean weight of 140 g), which were within the ranges reported to successfully increase cortisol plasma cortisol levels in rainbow trout (Pickering & Pottinger, 1989). As there were no differences in plasma cortisol levels between the different concentrations of 3 mm implants and just one significant difference compared with the placebo, it may have been that the 3 mm implants were not functioning properly. However, despite the lack of plasma cortisol increases in the 3 mm cortisol implants

there appeared to be an elevation of plasma glucose concentration relative to placebos in both the 3 and 4.5mm implant treatments on days 10 and 20 of the experiment. There were no differences in glucose observed on day 30 between any of the treatments and it may be that there was acclimation or exhaustion of the energy mobilising effect of the cortisol. However, this is purely speculative as no examples of glucose measurement in response to cortisol administration were found in the literature for a period of greater than 24 h following feeding of cortisol carried out by Barton *et al.* (1987).

There were no significant differences in levels of lysozyme activity between any of the groups, but lysozyme appeared to decrease in all treatments as the trial progressed. As lysozyme activity also appeared to decrease in placebo treatments as well as implant treatments, it is unlikely this was due to exogenous cortisol and more likely to have reflected the change in water temperature between the start and end of the trial (approximately 4°C higher at the start of the trial). Although not statistically significant, levels of lysozyme activity were consistently lower in the 4.5mm 15mg treatment compared with the 4.5mm placebo. As the 15mg implant was the only treatment in which plasma cortisol was consistently elevated it may be that there was an effect of chronically elevated cortisol on lysozyme, but this was masked by the reduction in lysozyme activity in all treatments bought about by the drop in temperature and the inconsistent effect of the 3mm implants.

Growth data collected during this experiment was limited, but the changes in length measured between days 20 and 30 suggested that there may have been a suppressive effect of the cortisol implants on growth (Figure 3.4b). Lowest growth was observed in the 15 mg treatment, with the L-SGR in this treatment almost half that of the 4.5 mm placebo treatment (0.12 vs. 0.23). A growth suppressing effect of

exogenous cortisol was reported by Barton *et al.* (1987) who demonstrated clear suppression of somatic growth in rainbow fed cortisol. In addition to the catabolic, energy mobilising effects of corticosteroids and their catecholamine pre-cursors, other suggestions for the cause of reduced growth include the cortisol-mediated control of levels of pituitary growth hormone and reduced feed intake (Pickering *et al.*, 1991).

3.4. Discussion

The experiments in this chapter have demonstrated some of the changes that occur in rainbow trout in situations of acute and chronic stress. Experiments 1 and 2 showed that acute stress response to be characterised by a rapid elevation in cortisol from basal levels of around 5 ng ml^{-1} to peak levels occurring around 1 h post-stress. These experiments also demonstrated differences in the magnitude and duration of cortisol response, but it was unclear whether this was due to strain-specific differences in cortisol responsiveness, or due to other factors such as differences in fish size and water temperature.

The increase in cortisol appeared to be accompanied by a corresponding decrease in lysozyme activity during the acute stress response, but experiment 3 found no significant reduction in lysozyme activity following chronic elevation of cortisol. There is some evidence in the literature for a negative correlation between cortisol responsiveness and lysozyme activity (Feveolden *et al.*, 2002), and also a report of reduced lysozyme activity following exposure to an acute stress episode (Möck & Peters, 1990). However, lysozyme activity is not yet a commonly measured parameter in fish and differences in methodology and working units make interpretation of results difficult. The rapid increase in haematocrit and slower increase in glucose

concentration following the acute handling stressor (experiment 2) was in general agreement with previous reports of the acute stress response of rainbow trout.

The results of the cortisol implant experiment were on the whole inconclusive, partly due to experimental design and partly due to a lack of confidence in the action of the 3 mm implants. There appeared to be increased plasma glucose in the cortisol implant treatments relative to placebo treatments, but the effects of the exogenous cortisol on lysozyme activity were inconclusive. There are many unanswered questions arising from experiment 3 that certainly warrant further investigation, although any such study would need to be replicated with individually PIT-tagged fish and would also benefit from temperature control. Feed intake should also be measured to determine if the apparent reduction in growth observed in this study and by Barton *et al.* (1987) was a result of a reduction in feed intake, or due to increased energy utilisation triggered by the exogenous cortisol. The original aim of experiment 3 was to measure leptin in fish, with the hypothesis that increased cortisol concentrations would result in appetite suppression. Unfortunately, attempts made at measuring leptin were unsuccessful due to lack of specificity to the commercially available antibodies (mouse and human), but this promises to be an exciting area of future research.

Chapter 4: The effects of stocking density on the welfare of farmed rainbow trout

4.1 Introduction

The FAWC report (Anon., 1996a) suggested that stocking densities of above 30 – 40 kg m^{-3} may be detrimental to rainbow trout welfare and that research was required to determine an upper SD limit that would safeguard fish welfare. Although the concept of fish welfare may be relatively new and complex (Lymbery, 1992; Kestin, 1994; Anon., 1996a; FSBI, 2002), many of the principles of good welfare are inherent to the general aim of trout growers *i.e.* to produce good quality, healthy fish. Considerable research effort has been invested in the investigation of the effects of SD on productivity, health, and stress physiology of rainbow trout, although until recently there have been few specific references to fish welfare. A summary of the main findings of these studies will now follow.

In a recent review of the relationships between SD and welfare in rainbow trout, Ellis *et al.* (2002) summarised the findings of 43 studies. A summary of study conditions and the authors' conclusions of the effects of stocking density on mortality, growth and fin condition from the reviewed studies that measured these parameters are presented in Table 4.2, with the addition of a further 2 studies (Bebak *et al.*, 2002; Boujard *et al.*, 2002) that were not included in the original review. A more detailed table summarising the effects of stocking density on all of the welfare indicators that were measured in these studies can be viewed in Appendix 1.

Table 4.1. Summary of studies examining the effects of stocking density on indicators of trout welfare, with authors' conclusions concerning the effect of increasing density indicated by ' +, -, 0' signifying beneficial, adverse, and no/inconclusive effect; NS = not specified

Study	Stocking Density ($kg\ m^{-3}$)		Study System	Duration (days)	Main Effects		
	Start	End			Mortality	Growth	Fin Condition
Alanärä & Brännäs, 1996	2-64	NS	Tanks	30		-	
Atay *et al.*, 1986	0.8-4.8	10-38.3	Raceways	182	0	-	
Bagley *et al.*, 1994	0.4-7.0	22-478	Tanks	99	+	0	
Baker & Ayles, 1990	10-85 maintained		Tanks	42		-	
Bircan, 1997	10.8-18.7	20-35	Cages		0	0	
Boydstun & Hopelain, 1977	5-15	16-39	Cages	125	0	-	-
Boujard *et al.* 2002	2.6-13	28-100	Tanks	125	0	-	
Brauhn *et al.*, 1976			Tanks	175		-	
Collins, 1972	14-29	41-83	Cages	115	0	0	
Holm *et al.*, 1990	107-219	249-455	Tanks	129	-	-	
Iwamoto *et al.*, 1990	3-16	8-32	Raceways	190		-	
Kebus *et al.*, 1992	50-232	50-232	Cages			0	
Kilambi *et al.*, 1977	32-98	73-171	Cages	138	0	-	
Kincaid *et al.*, 1976	14-124	27-170	Tanks	84-133	-	-	
Kindschi *et al.*, 1991a	8-67	30-295	Tanks	126	-	+/- strain dependent	0
Leatherland, 1993	25-70	62-148	Tanks	84		-	
Leatherland & Cho, 1985	60-120	134-299	Tanks	224		-	
Li & Brocksen, 1977	0.07-0.37		Raceways			-	
Mäkinen & Ruohonen, 1990	5-62	15-147	Tanks	182	-		-
Miller *et al.*, 1995	2.5-10	19-68	Raceways	203-215	0	0/-	
Murai & Andrews, 1972	7.2-14.4	24-42.6	Tanks	112	-	-	
Papoutsoglou *et al.*, 1979	0.4-1.7	7.5-15.8	Tanks	52	-	-	
Papoutsoglou *et al.*, 1980	1.9-18.8	7.7-38.5	Raceways	181		-	
Papoutsoglou *et al.*, 1987	0.03-0.26	14-89	Raceways	365	+	-	
Pickering & Pottinger, 1987a	24-172		Tanks	21		-	
Piper, 1970	14 -94 (simulated)		Tanks	300		-	

Table 4.1 continued.

Study	Stocking Density (kg m^{-3})	Study System	Duration (days)	Main Effects	Study	Stocking Density (kg m^{-3})	Study System
Procarione *et al.*, 1999	40-120		Tanks	28		-	
Purser & Hart, 1991	0.15-1.2	52-85	Tanks	224	-	-	-
Refstie, 1977	1.26-7.3	58-74	Tanks	42 - 180	+/-	-	
Rigolino *et al.*, 1989	5.8-12.2	21-50	Tanks	245	0	0	
Roell *et al.*, 1986	0.94-1.88	0.7-4.5	Cages	64	0	0	
Rosenthal *et al.*, 1984	20-140 (simulated)		Raceways	69		-	
Sahin *et al.*, 1999	0.35-0.72	7.4-16	Cages	181	0	0	
	2.9-7.0	18.9-40.2		182	0	-	
Soderberg *et al.*, 1983	0.16-0.32	0.26-0.71	Ponds	122	0	0	
Teskeredžić *et al.*, 1986	0.8-4.5	4.6-20.6	Cages	170	-	-	
Trzebiatowski *et al.*, 1981	3.3-19.8	35.4-170.2	Cages	147	+	-	
Tsintsadze, 1981			Tanks			-	
Unlu & Baran, 1992	1.9-3.4		Tanks	142		-	
Wagner *et al.*, 1996b		15-32	Raceways	109		0	0
Winfree *et al.*, 1998			Tanks	154	-	0	-
Wonjo, 1976	1.9-4.2	11.8-18.9	Cages	143	0	-	

The range of stocking densities, types of system, sizes and strains of fish used in these 48 studies was understandably diverse. Studies were carried out in tanks, raceways, ponds and cages, with final experimental densities ranging from less than 1 kg m^{-3} (Soderberg *et al.*, 1983) to more than 450 kg m^{-3} (Holm *et al.*, 1990). The duration of the trials also varied considerably, ranging from less than a month (Wedemeyer, 1976; Pickering & Pottinger, 1987) to one year (Papoutsoglou *et al.*, 1987). The experimental design of most of the reviewed studies involved monitoring fish from an initial SD, which increased through the course of the trial as the fish grew, with the exceptions of Kincaid *et al.* (1976) who regularly adjusted SD, and Baker and Ayles (1990) who maintained experimental SD through the course of their experiment.

A wide range of parameters have been used to assess the effects of SD on rainbow trout and a summary of some of density effects on some of the more commonly measured indicators is presented in table 4.2.

Table 4.2. Summary of the effects of stocking density on indicators of rainbow trout welfare; see Appendix 1 for details of individual studies

Welfare Indicator	No. of studies in which parameter was measured	No. of studies reporting an adverse effect of increasing SD	No. of studies reporting no/inconclusive effect of increasing SD	No. of studies reporting a beneficial effect of increasing SD
Mortality	26	11	12	4
Food Intake	4	4	0	0
Food Conversion Efficiency	24	13	9	2
Body Condition Index	15	9	5	1
Hepatosomatic Index	2	2	0	0
Growth	43	32	13	0
Size Variation	4	1	3	0
Haematocrit/erythrocyte count	7	1	6	0
Leucocrit	3	0	3	0
Fin Condition	7	5	2	0
Gill Condition	1	1	0	0
Plasma Cortisol	7	2	4	2
Plasma Glucose	5	0	5	0
Oxygen Consumption	3	0	3	0

4.1.1 Mortality

Mortality is the ultimate endpoint of any experiment and was measured in 26 studies that assessed the effects of stocking density on rainbow trout. An adverse effect of increasing SD on mortality was reported in 11 studies, a beneficial effect was reported in 4 studies, and 12 studies found an inconclusive effect; Refstie (1977) observed both

negative and beneficial effects of increased SD depending on genetic strain. The exact cause of mortality was often not reported and the only references to specific causes of mortality at high stocking densities were: injury (Collins, 1972), ectoparisites (Soderberg *et al.*, 1983), increased transmission of infectious pancreatic necrosis virus (Bebak *et al.*, 2002), severe aggressive interactions (Laidley & Leatherland, 1988; Pottinger & Pickering, 1992), and cannibalism due to uneven growth (Kindischi *et al.*, 1991a).

Kindischi *et al.* (1991a) maintained wild and domesticated strains of rainbow trout at stocking densities of up to 295 kg m^{-3} with oxygenated water. Interestingly, cannibalism was only observed in the wild strain of rainbow trout, and had it not been for mass mortality events due to a series of systems failures in the tanks containing the domesticated strain, the authors imply there would have been no significant effect of SD on mortality. This highlights some important considerations that must be made with regard to SD studies, firstly that strain differences in the tolerance of rainbow trout to SD are likely to exist, and also that systems operating at high stocking densities run an increased risk of mass mortality episodes due to system failure, a point which was also made by other authors (Piper, 1970; Miller *et al.*, 1995).

Mortality has been associated with low as well as high SD, with both Laidley and Leatherland (1988), and Pottinger and Pickering (1992) observing increased mortality in rainbow trout kept in pairs compared with groups containing larger numbers of individuals. Both studies attributed the mortality to aggressive social interactions caused by one fish in the pair becoming dominant over the other. Soderberg *et al.* (1983) undertook a study that followed growth and mortality of rainbow trout stocked in static ponds. Although no significant effect of SD on mortality of rainbow trout was observed, differences in mortality between ponds

displayed a negative correlation with average daily maximum un-ionised ammonia exposure (R^2 = -0.75). Mortality was attributed to ectoparasites, *Costia spp.*, *Trichodina spp.*, and *Tricophrya spp.* and the authors suggested that fish subjected to high levels of unionised ammonia might have been more susceptible to parasitic infection (Soderberg *et al.*, 1983).

Increased disease transmission is commonly assumed to occur at higher stocking densities (Lymbery, 1992; 2002). Possible mechanisms by which this may occur include: assisted horizontal transmission of disease due to closer proximity of fish, deterioration of water quality predisposing fish to infections and impaired immune function caused by crowding stress (Shepherd & Bromage, 1988; Noble & Summerfelt, 1996; Wedemeyer, 1996). Despite logical rationale, increased disease transmission at higher SD was only recently demonstrated experimentally for rainbow trout, where an increase in the peak death rate and reduced overall chance of survival was observed in fish challenged with IPN at higher SD (Bebak *et al.*, 2002)

4.1.2 Growth, feeding and nutritional status

Somatic growth is said to integrate all of the biotic and abiotic variables acting on an organism (Goede & Barton, 1990), thus making it an ideal indicator of tertiary effects of environmental stressors. Measuring growth is also simple and inexpensive and it is therefore a very popular parameter to study. The effect of SD on growth was measured in 43 of the 48 previous studies, and increased SD was found to have a beneficial effect in just one study. Kindshi *et al.* (1991a) observed poorest growth at the lowest SD for a wild strain of rainbow trout, which the authors attributed to a poor feeding response. A domesticated strain of trout were used in the same study and subjected to the same ranges of SD, but showed a step-wise decrease in growth with

increasing SD. Apart from this one exception, the majority of experiments (74%) reported negative effects of increasing SD on growth of rainbow trout, with a further 13 studies reporting no or inconclusive effects.

In addition to measuring growth as changes in weight/length, the effect of increasing SD on feed conversion efficiency (FCR) was also measured in 24 studies. The majority of these studies found a negative effect of increased SD on FCR, with 13 studies reporting a negative effect of increasing SD on FCR, 2 reporting a beneficial effect, and a further 9 studies that were inconclusive. It is often suggested that a poor feeding response can occur if SD is too low (Purser & Hart, 1991; Winfree *et al.*, 1998; Kindchi *et al.*, 1991a), but an adverse effect of increasing SD on feed intake was observed in all 4 of the studies in which it was measured (Papoutsoglou *et al.*, 1979; Leatherland, 1993; Alanärä & Brännäs, 1996; Boujard *et al.* 2002).

The summary of the studies in which growth and FCR were measured indicates a strong association between increasing SD and reduced growth. Pickering and Stewart (1984) interpreted reduced growth at higher SD in a number of salmonid studies to be a result of either reduced food intake or poorer food conversion efficiency. Numerous authors proposed mechanisms by which increasing SD might reduce growth, most of which focused on either the physiological effects of water quality deterioration or behavioural changes as a result of increased social interaction. Ellis *et al.* (2002) collated the potential mechanisms by which suppressed growth could occur, and this is reproduced in Table 4.3. Although feasible, there is little experimental evidence to support the suggested mechanisms for reduced growth in rainbow trout as a result of increased SD.

Table 4.3. Compilation of suggested mechanisms associated with increasing SD and reduced food intake and poorer FCR of rainbow trout (reproduced from Ellis *et al.* 2002).

Cause	Process affected	Mechanism
Water quality deterioration	Food intake	Metabolite concentration reducing feeding activity.
	Conversion efficiency	Metabolite concentration affecting energy expenditure.
Social interaction	Food intake	Physical obstruction preventing visual location of food.
		Physical obstruction preventing access to food.
		Aggressive behaviour preventing access of subordinates to food.
		Aggressive behaviour reducing appetite of subordinates.
	Conversion efficiency	Reduced gut absorption efficiency.
		Increasing energy expenditure due to higher planes of excitation due to presence of conspecifics with visual range.
		Increasing energy expenditure due to occupation of suboptimal environmental conditions.
		Decreasing growth due to increased cell atrophy or decreased cell proliferation.
		Decreasing growth due to increased protein and lipid catabolism as a result of increased anabolic metabolism.
		Increasing energy expenditure due to increased activity levels.
Innate survival strategy	Conversion efficiency	Limitation of growth potential when exposed to overcrowded conditions.

Condition factor (CF) is commonly used in salmonids to give an indication of the energy reserves or fatness (see section 2.6.5.3) and was measured in 15 studies that assessed the effects of SD on rainbow trout. An adverse effect of increased SD was observed in 9 studies (60%), with 6 reporting no effect, suggesting that high SD has the potential to negatively impact on nutritional status (Table 4.2).

The liver plays a major role in carbohydrate metabolism in fish, storing around one-eighth of total glycogen and it is also the main site for oxidation of lactate to pyruvate (Smith, 1982). Two studies measured the effects of SD on the liver of rainbow trout (Leatherland & Cho, 1985; Leatherland, 1993). Hepatosomatic index (HSI) was found to decrease with increasing SD in both studies, but no effect of SD was observed on hepatic glycogen or hepatic lipid. Leatherland and Cho (1985) found

no decrease in liver glycogen at increased SD, but suggested that all other indicators were suggestive of food-deprived animals (reduced thyroid hormone levels, plasma protein, growth and liver size). Levels of glycogen are relatively unaffected by exercise, but rapidly become depleted during fasting, resulting in a rapid decrease of HSI (Smith, 1982; Barton *et al.*, 1988). Although not clear from the experimental protocols, it may have been that fish were fasted before sampling and this may have masked treatment differences in glycogen that existed prior to fasting (Leatherland & Cho, 1985; Leatherland, 1993). Other indicators of nutritional status based upon the composition of the body have also been measured, but results have been less conclusive. No effect of SD was found on protein or ash content of rainbow trout (Leatherland & Cho, 1985; Winfree *et al.*, 1998), and from 5 studies that measured lipid content, a reduction with increased SD was observed in just one study (Papoutsoglou *et al.*, 1987), with the rest observing no effect.

4.1.3 Health and condition profile

As well as indications of nutritional status, condition indices provide a relatively simple and rapid indication of how fish cope with their environments (Goede & Barton, 1990). An autopsy-based assessment of health and condition profile (HCP) of fish was proposed by Goede and Barton (1990), which comprised of the following measurements: weight, length, CF, blood constituents, damage to external extremities (fin and tail damage, scale loss) eye damage, gill condition, pseudobranch, thymus appearance, mesenteric (visceral) fat deposits, spleen (size and appearance), hind gut inflammation, kidney appearance, colouration of liver and bile and state of sexual maturity. The HCP has been used to assess the effects of SD on rainbow trout in

several studies (Kindschi *et al.*, 1991a, Miller *et al.*, 1995; Wagner *et al.*, 1996a), but components of the HCP are commonplace in most studies.

4.1.3.1. Blood composition

Haematocrit, is perhaps the most commonly measured blood parameter and was measured in 7 studies (Table 4.2), although just one study found an effect of SD, where an elevated level of haematocrit was interpreted as an effect of stress (Wagner *et al.*, 1996a). Precise interpretation of haematocrit results is complicated by a large 'normal' range (24-43% for rainbow trout) and due to the fact that both elevated and lowered levels can be taken as indications of poor health (Wedemeyer, 1996). The abundance (leucocrit) and type of blood cells (erthrocytes, neutrophils, lymphocytes and thrombocytes) was measured in 3 studies (Appendix 1) with one study observing an adverse effect of increased SD. Pickering and Pottinger (1997a) observed a significant reduction of lymphocyte and thrombocyte numbers in crowded (172 kg m^{-3}) compared with uncrowded (24 kg m^{-3}) treatments, but found no effect of SD on numbers of circulating erythrocytes or neutrophils.

4.1.3.2. Fin condition

Fin condition was measured in 7 studies, 5 of which (71%) found an adverse effect of increased SD. Fins were either measured quantitatively, by measurement of fins relative to body length (Kindschi *et al.*, 1991a; Miller *et al.*, 1995), or qualitatively using scoring or ranking systems (Boydstun & Hopelain, 1977; Mäkinen & Ruohonen, 1990). Although the exact cause of fin erosion was unknown, several plausible causes were suggested by authors, which included abrasion against surfaces of rearing units, nipping by conspecifics, infection, and water quality deterioration.

4.1.3.3. Other indicators of health status

The thymus is a lymphoid organ that is located just under the epithelium at the posterior margin of the opercular cavity. Goede and Barton (1990) proposed a 3-point thymus index based upon the degree of haemorrhaging and the general appearance of the thymus. Wagner *et al.* (1996a) found that increasing SD adversely affected thymus index, but Miller *et al.* (1995) observed no effect of SD, despite operating at higher maximum SD (maximum SD used by Miller *et al.* was 68 kg m^{-3} compared with a maximum of 32 kg m^{-3} in Wagner's study). Rosenthal *et al.* (1984) examined gill and spleen condition and found the length of gill lamellae and spleen size decreased with increasing SD in a system that simulated the effects of increasing SD on water quality deterioration (this study will be discussed in greater depth in Chapter 5).

4.1.4 Stress indicators

High SD is generally assumed to be stressful to fish (Shepherd & Bromage, 1988; Lymbery, 1992; 2002; Anon., 1996a) and several authors have used the term 'crowding stress' to association with increased SD (Pickering & Stewart, 1984; Pickering & Pottinger, 1987a; Wedemeyer, 1996). However, in the 7 studies that attempted to measure the effects of SD on circulating levels of cortisol, only 2 concluded that increasing SD had an adverse effect *i.e.* cortisol levels increased with SD (Pickering & Pottinger, 1987a; Pickering *et al.*, 1991). A study comparing cortisol levels in 'crowded' and 'uncrowded' groups of rainbow trout (24 and 100 kg m^{-3} respectively) initially found significantly higher levels of cortisol in the fish in the crowded treatments (≈8 and 7 vs. 4 and 1 ng ml^{-1} in crowded and uncrowded

treatments and 2 and 6 days respectively), but subsequent samples at 10, 14 and 21 days found no such differences (Pickering & Pottinger, 1987a). Pickering *et al.* (1991) also reported higher plasma cortisol levels in rainbow trout reared at 100 compared with 25 kg m^{-3} during a period of increasing water temperature and decreasing dissolved oxygen, but later in the trial when both treatments were provided with additional aeration this difference was no longer apparent.

In contrast, Leatherland and Cho (1985) and Procarione *et al.* (1999) both found highest levels of cortisol in the lowest SD treatments. Two other studies observed significantly increased levels of cortisol in rainbow trout that were kept in pairs, which the authors attributed to severe behavioural interaction due to one fish dominating the other (Laidley & Leatherland, 1988; Pottinger & Pickering, 1992). Laidley and Leatherland (1988) demonstrated this interaction with a comparison of the high and low plasma cortisol concentrations from the paired fish; the mean 'high' level from 6 replicates was 160 nmol l^{-1} compared with a mean 'low' cortisol level of around 10 nmol l^{-1}. All other studies that used cortisol as an indicator of stress found an inconclusive effect of SD. Interrenal cell diameter can also be measured to provide an indication of interrenal activity (Donaldson, 1981), but in the 2 studies in which it was measured, no effect of SD was observed (Leatherland & Cho 1985; Kebus *et al.*, 1992).

In addition to measurement of plasma cortisol levels and other indicators of HPI-axis responsiveness, secondary responses such as increased plasma glucose, haematocrit and metabolic rate, decreases in plasma chloride and white blood cells, and atrophy of gastric mucosa have also been assessed (Kebus *et al.*, 1992). There is, however, very limited evidence to suggest that increasing SD has an effect on any of these factors (Appendix 1).

4.1.5 Summary

Wide discrepancies exist in the data from previous studies investigating the effects of SD on indicators of welfare in rainbow trout. Growth was measured in most studies and the majority (74%) found an adverse effect of increasing SD, but even this is not as clear-cut as it may appear. Ellis *et al.* (2002) illustrated this by a comparison of studies that reported depressed growth at relatively low (<40 kg m^{-3}) SD (Murai & Andrews, 1972; Wojno, 1976; Boydstun & Hopelain, 1977; Alanärä & Brännäs, 1996; Sahin *et al.*, 1999), with studies that found no effect at densities in excess of 100 kg m^{-3} (Kebus *et al.*, 1992; Bagley *et al.*, 1994).

A more consistent effect of increasing SD was observed on fin erosion. Adverse effects of SD were observed at <40 kg m^{-3} (Boydstun & Hopelain, 1977) and none of the studies reported a beneficial effect of increasing SD on fin erosion. The studies measured fin erosion over a wide range of SD and different systems (tanks, troughs, raceways and cages).

The evidence for 'crowding stress' occurring at increased SD in rainbow trout is poor and there was little substantiation for adverse effects of increasing SD on either primary or secondary indicators of stress. There are difficulties in interpretation of the results for stress indicators as the most commonly applied indicators (cortisol, glucose, haematocrit) are perhaps more appropriate indicators of acute stress, and SD is more likely to be a chronic stressor. Inter-study comparisons of observed levels of stress indicators are very difficult as most of the commonly used indicators are subject to wide ranges of intraspecific and environmental variation. The use of such indicators poses an additional problem in regard to the selection of the most appropriate means of control, as maintaining fish in experimental systems or under aquaculture conditions is arguably stressful in its self.

The potential for water quality to act as a root cause of infringed welfare will be discussed in greater detail in Chapter 5, but it is possible that lack of attention paid to the confounding interaction of water quality parameters at increased SD may have contributed to the inconsistency of results from previous studies. Water quality is inherently difficult to measure as parameters are continuously fluctuating and in the absence of expensive monitoring equipment each parameter would be subject to separate manual analysis. Even in studies where authors have argued that water quality deterioration was not the cause of adverse effects attributed to high stocking densities, Ellis *et al.* (2002) have suggested that such statements may be flawed because there is a poor understanding of critical thresholds for key water quality parameters such as dissolved oxygen and the various forms of ammonia, and also that point samples for water quality fail to take into account temporal fluctuations.

The findings of trials investigating the effects of stocking density on the growth and stress response of rainbow trout are often conflicting and this is reflected by the large variations in author's recommendations for maximum stocking densities and loading rates (Table 1.1 authors' recommendations). The main focus of the majority of previous studies concentrates on the effect of SD on performance of the fish using end-points of specific growth rate and mortality rather than quantifying welfare *per se*. Anon. (1996a) recognised the lack of appreciation and the inconsistent interpretation of the effects of SD on fish welfare and called for scientific work to investigate the issue.

4.2. Experimental investigation of the effects of stocking density on the welfare of rainbow trout

The aim of this experiment was to investigate the effects of SD on the welfare of rainbow trout. Under controlled conditions, this experiment applied stocking densities reflective of low, medium and high-density commercial farming operations (10, 40 & 80 kg m^{-3}) during the on-growing stages of trout production (≈150-500g). A number of physiological, morphological and performance-based indicators were measured in an effort to assess the affects of stocking density on rainbow trout welfare. A major consideration of this experiment was the minimisation of the confounding effects of water quality deterioration. This was achieved through the application of high rates of water exchange and provision of additional aeration. The experiment was carried out under ambient water temperature and photoperiod in order to investigate the contribution of seasonal environmental fluctuations on fish welfare.

4.2.1. Materials and Methods

4.2.1.1. Experimental fish

This trial used 3800 female rainbow trout (181.0 ± 3.5 g) obtained from a South African stock purchased from Selcoth fisheries (Dumfrieshire, Scotland). The fish were certified disease free on arrival and acclimated at ambient temperature and photoperiod for 3 weeks prior to the start of the experiment in a 5 m diameter tank (≈40 m^3) at a SD of 17.5 kg m^{-3}.

4.2.1.2. Tanks and flow regimes

During the experimental trial period, fish were maintained in 2 m (volume 1.82 m^3) diameter fibreglass tanks at ambient temperature and photoperiod, with inflow rates

set to 60 l min^{-1} (2 tank volume exchanges per hour). A theoretical disturbance level (TDL) was attributed to each tank within the system depending on its position and the likelihood of the tank being subject to disturbance i.e. tanks adjacent to the main walkway leading from one tank house through to the other (tanks 3, 4, 8 and 9) were the assumed to be the most likely to be disturbed and were assigned a TDL of 3, while tanks that backed onto the wall (tanks 1, 2 and 5) were least likely to be disturbed and were assigned a TDL of 1. One replicate from each of the density treatments was allocated into each group of tanks according to the TDL (Figure 4.1).

4.2.1.3. Water quality and temperature

Inflow rates were set high to ensure that water quality parameters remained within the limits suggested by Wedemeyer (1996). Water temperature of the farm inflow was monitored twice daily throughout the course of the trial. Dissolved oxygen (DO) concentration was monitored twice daily from the outflow of each tank up until 6th November 2000, after which dissolved oxygen remained above 10 mg l^{-1} at all times. A multi-channel Oxyguard® system (A06DC230, Oxyguard®, Denmark) was installed on 17th January 2001, which recorded DO and temperature 5 min intervals via probes located above the outflow of each tank. Alarms were triggered and additional aeration supplied via an air pump and diffusion stone if DO in the outflow dropped below 6 mg l^{-1}.

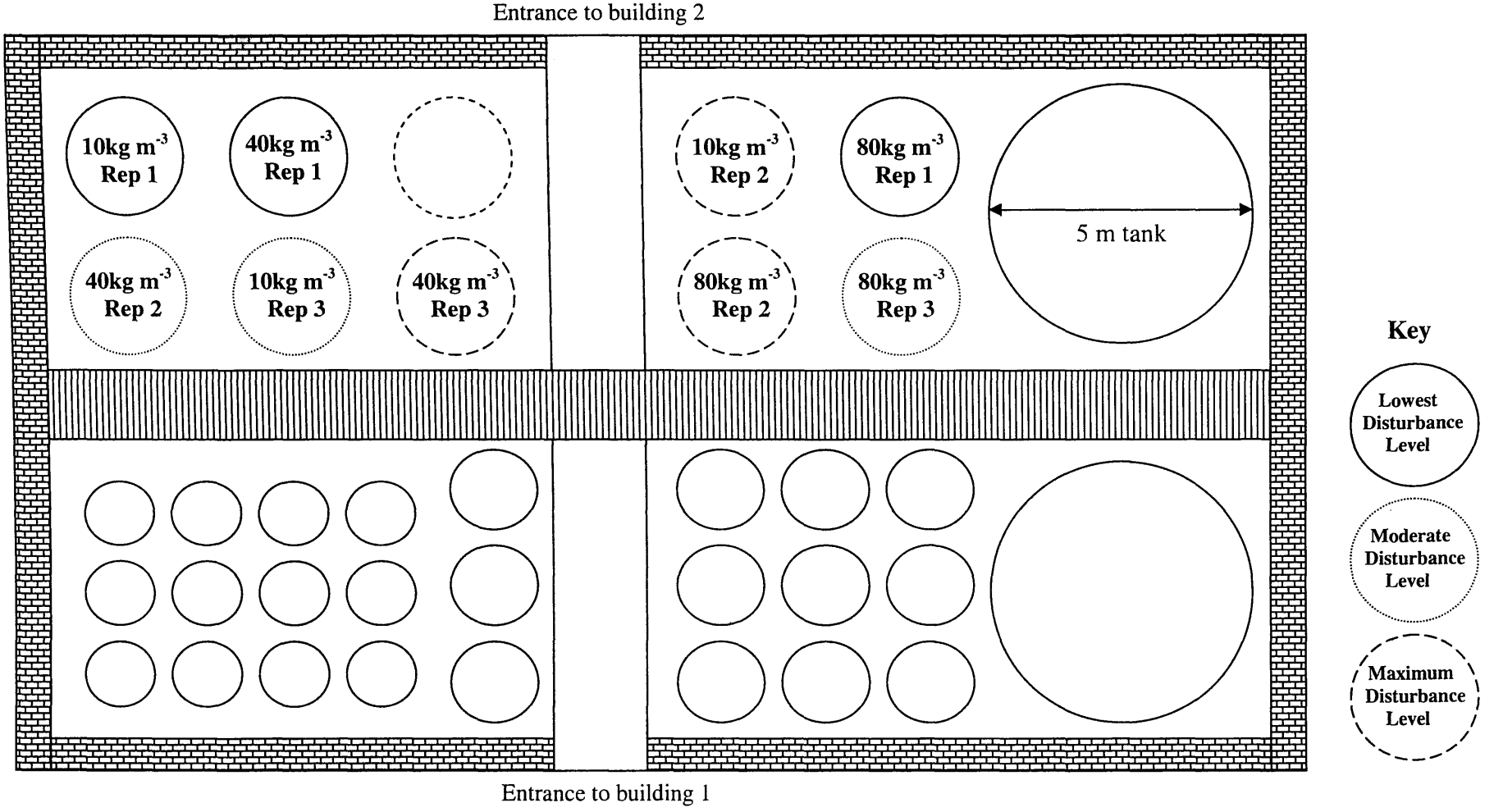


Figure 4.1. Tank house layout showing distribution of treatments and theoretical disturbance level of tank positions.

4.2.1.4. Feed and feeding

Throughout the experiment, fish were hand-fed a quality commercial finishing feed (AminoBalance™ - Trouw Aquaculture) at a daily ration (% body weight/day) calculated from the manufacturer's tables as a function of water temperature, mean weight and fish numbers in each tank.

4.2.1.5. Experimental Protocol

On September 14th 2000, fish were randomly distributed into 2 m diameter tanks to achieve stocking densities of 10, 40, and 80 kg m^{-3} in triplicate. An initial sample of 100 fish was weighed, measured and allocated fin condition scores for dorsal and caudal fins as described in section 2.6.6.1 (Table 4.4). A further 15 fish were sacrificed and taken back to the laboratory where each of the fins was measured using a pair of callipers allowing the relative fin index to be calculated as described in section 2.6.6.2.

The fish were sampled after 2 weeks, and thereafter at approximately monthly intervals (minimum sampling interval was 19 days, maximum was 41 days). Sampling procedures were as follows:

- 60 fish were removed from each tank and anaesthetised in 2-phenoxyethanol (Sigma) at a dose of 1:20,000 (0.5 ml per 10 l of tank water).
- Each fish was weighed to the nearest gram, allocated a score for dorsal and caudal fin erosion, and fork length was measured to the nearest mm.
- The first 10 fish from each tank were individually blood sampled within 5 minutes of capture.

- Haematocrit levels were calculated in duplicate from each of the blood samples on site (see section 2.6.4 for details).
- Plasma was extracted and stored as described in section 2.5; see additional sections in Chapter 2 for individual protocols for measuring lysozyme activity, glucose and cortisol.

The biomass and stocking density in each tank was re-calculated following monthly sampling and the appropriate number of fish were removed to maintain the stocking densities at the desired levels.

4.2.1.6. Statistical Analysis

All of the statistical methods applied in this Chapter are described in section 2.8 of Chapter 2. The majority of the analysis was carried out with GLMs to assess the effect of time and stocking density with each of the welfare indicators included as dependent variables. All percentage data was arcsine transformed before statistical analysis and details of any other data transformations that were carried out to conform to parametric assumptions are provided in appropriate sections.

The statistical analysis concluded with the application of Principal Components Analysis (PCA) to generate welfare indices based on coherence in the data for the individual welfare parameters (see section 2.8.7.3 for details).

4.2.2. Results

The mean measurements for weight, length, CF and fin scores for the initial sample of 100 fish taken on September 14th 2000 are shown below in Table 4.4.

Table 4.4. Morphometric measurements collected on 14/9/00 (n=100).

Weight (g)		Length (cm)		Condition Factor		Dorsal Fin Score		Caudal Fin Score	
Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
180.5	3.5	238.2	1.3	1.3	0.01	0.72	0.06	0.68	0.05

4.2.2.1. Stocking Density Regulation

Stocking density increased between sample points as fish grew, resulting in maximum SD in excess of 100 kg m^{-3} in the 80 kg m^{-3} treatment. Fish were removed following monthly sample points to re-establish the desired stocking densities (Figure 4.2).

4.2.2.2. Water Quality

Water temperature ranged from a maximum of 13.2°C in August to a low of 0.1°C, recorded in February (Figure 4.3). Oxygen remained above 6 mg l^{-1} at all times although additional aeration was required to achieve this in the 40 and 80 kg m^{-3}. Following the installation of the Oxyguard® system in January 2001, it was possible to log the DO from each of the experimental tanks (Figure 4.4).

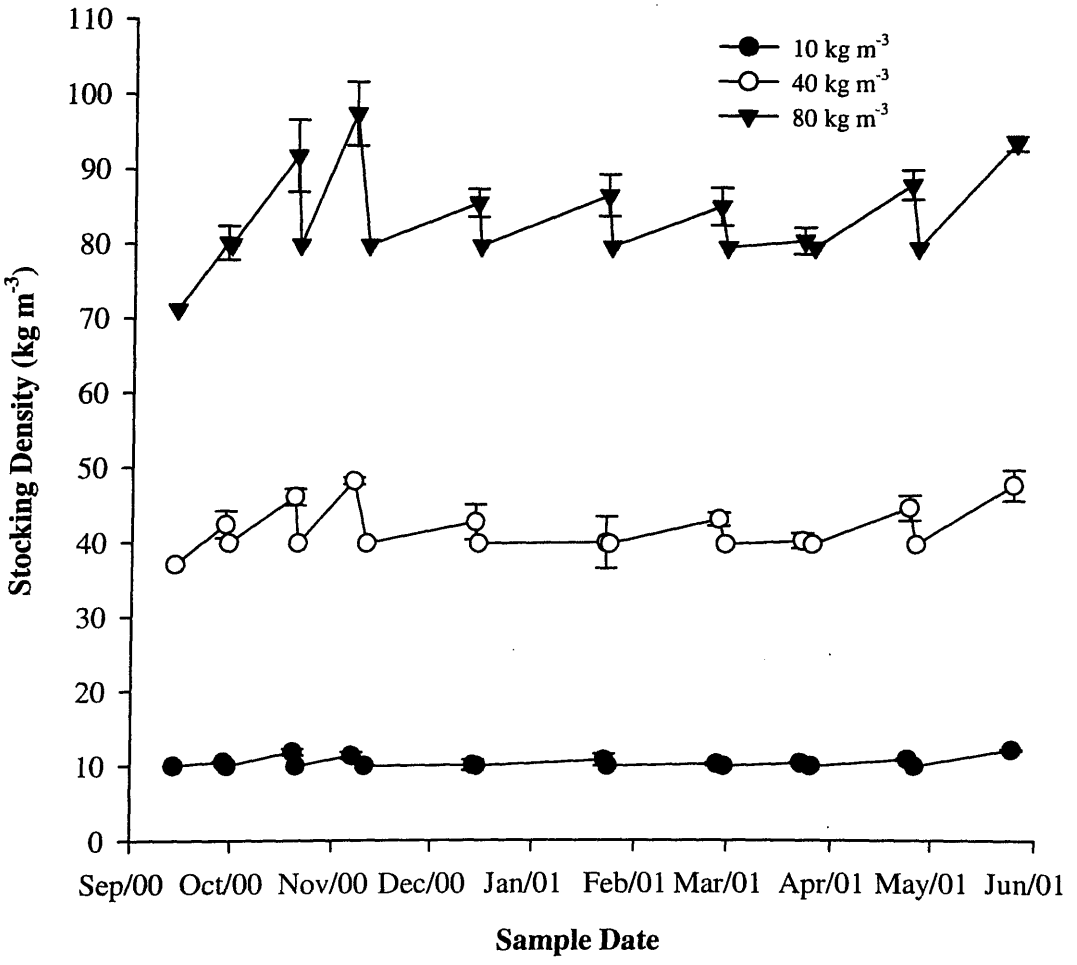


Figure 4.2. Biomass regulation in tanks of rainbow trout cultured at different stocking densities; mean ± SEM of 3 replicates, *n*= 180.

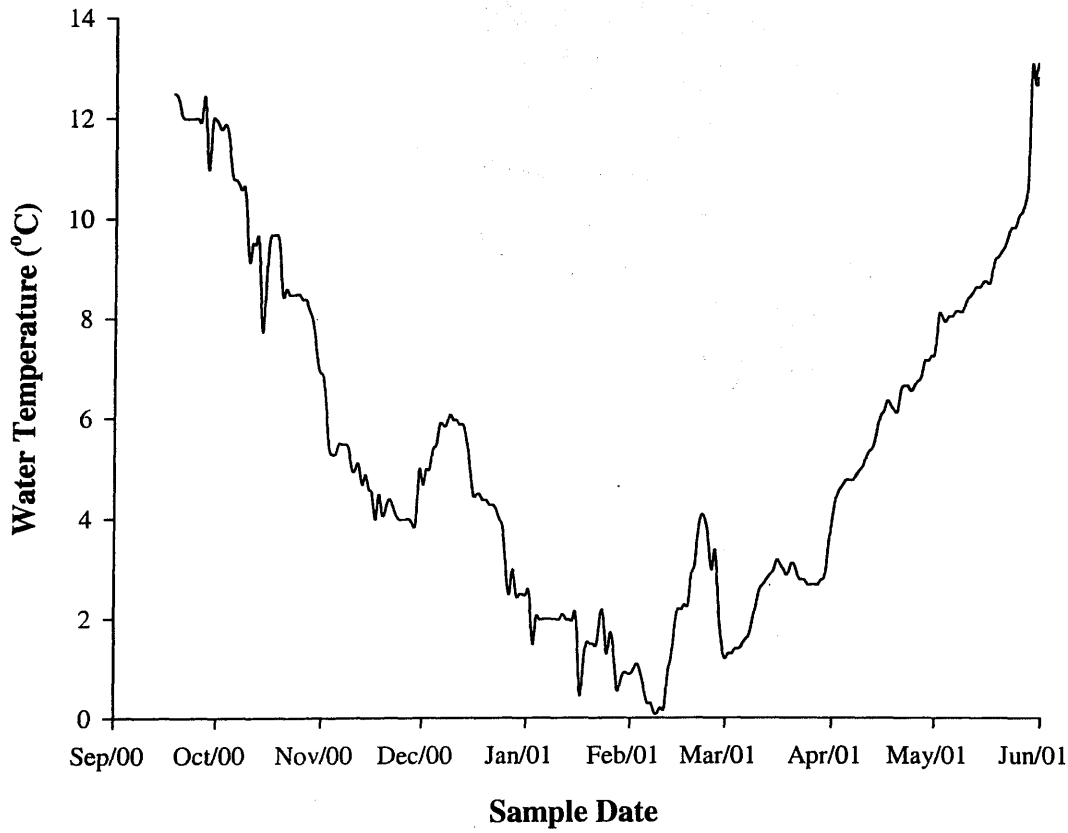


Figure 4.3 Farm inflow water temperature profile.

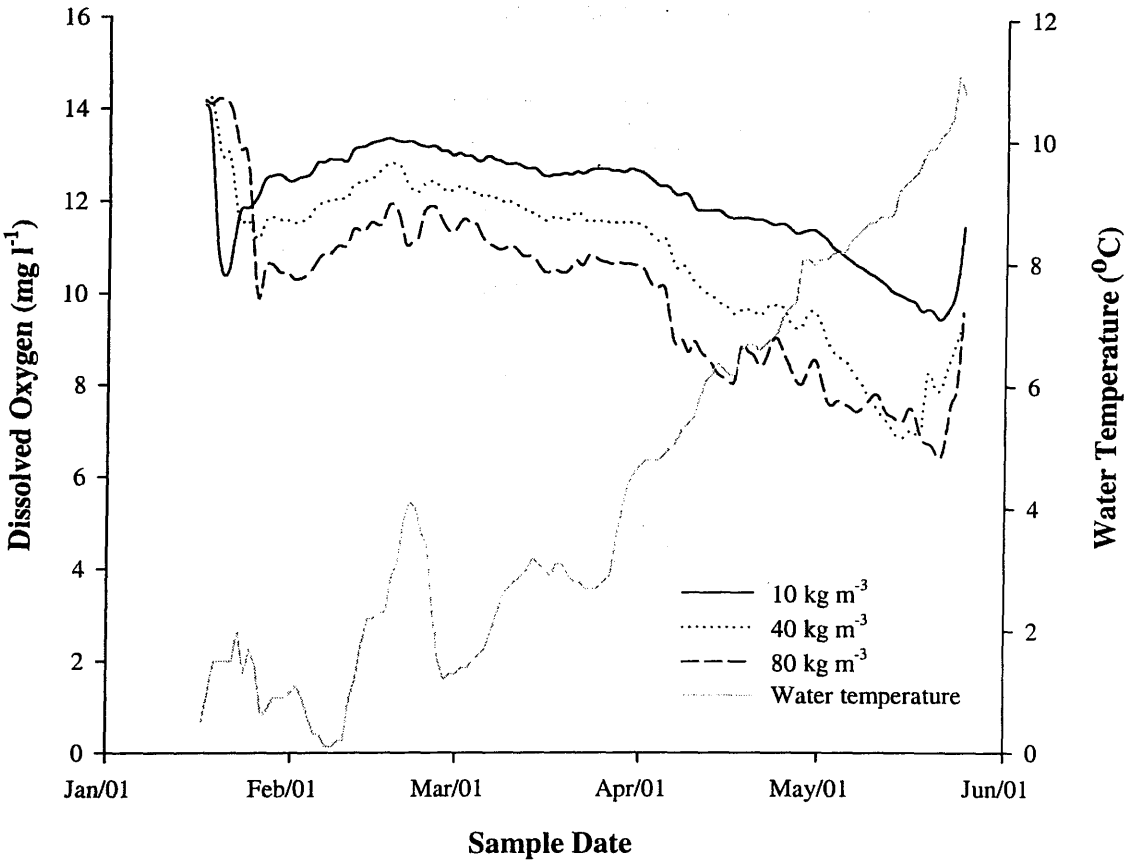


Figure 4.4. Dissolved oxygen in tanks of rainbow trout cultured at different stocking densities; each line represents the mean value for each treatment calculated from the daily average of 3 replicates (error bars emitted for clarity).

There were clear differences in the levels of ammonia between the treatments with peak levels measured during June (Table 4.5). The pH ranged between pH 6.5 and 6.9, so even around the time of highest water temperatures, only 0.15% of TAN existed as NH_3 (calculated from ammonia ionisation tables; Piper *et al.* 1982).

Table 4.5. Water quality parameters for individual replicate tanks at peak water temperature (12.6°C) and feeding rate

Water Quality Parameter	Stocking Density								
	10 kg m^{-3}			40 kg m^{-3}			80 kg m^{-3}		
pH	6.68	6.72	6.75	6.72	6.66	6.59	6.59	6.60	6.54
Total ammonia nitrogen (NH_3-N; mg l^{-1})	0.08	0.12	0.05	0.54	0.46	0.69	0.84	0.89	0.96
Ammonia (NH_3; mg l^{-1})	0.10	0.15	0.06	0.66	0.56	0.84	1.03	1.09	1.17
Conversion factor (%)	0.09	0.11	0.15	0.12	0.12	0.12	0.09	0.09	0.09
Un-ionised ammonia (NH_3; mg l^{-1})	<0.00015			<0.0009			<0.00100		

4.2.2.3. Mortality

Mortality remained low in all tanks (Table 4.6) and there was no trend relating to the time at which mortality occurred (Figure 4.5).

Table 4.6. Cumulative mortality for groups of rainbow trout held at different stocking densities for 10 months.

Stocking density	Replicate	Mortality	
		Replicate Mortality (%)	Treatment Mean (%)
10 kg m^{-3}	1	4.00	2.33
	2	0.00	
	3	3.00	
40 kg m^{-3}	1	1.89	1.27
	2	1.10	
	3	0.81	
80 kg m^{-3}	1	1.69	1.41
	2	1.41	
	3	1.13	

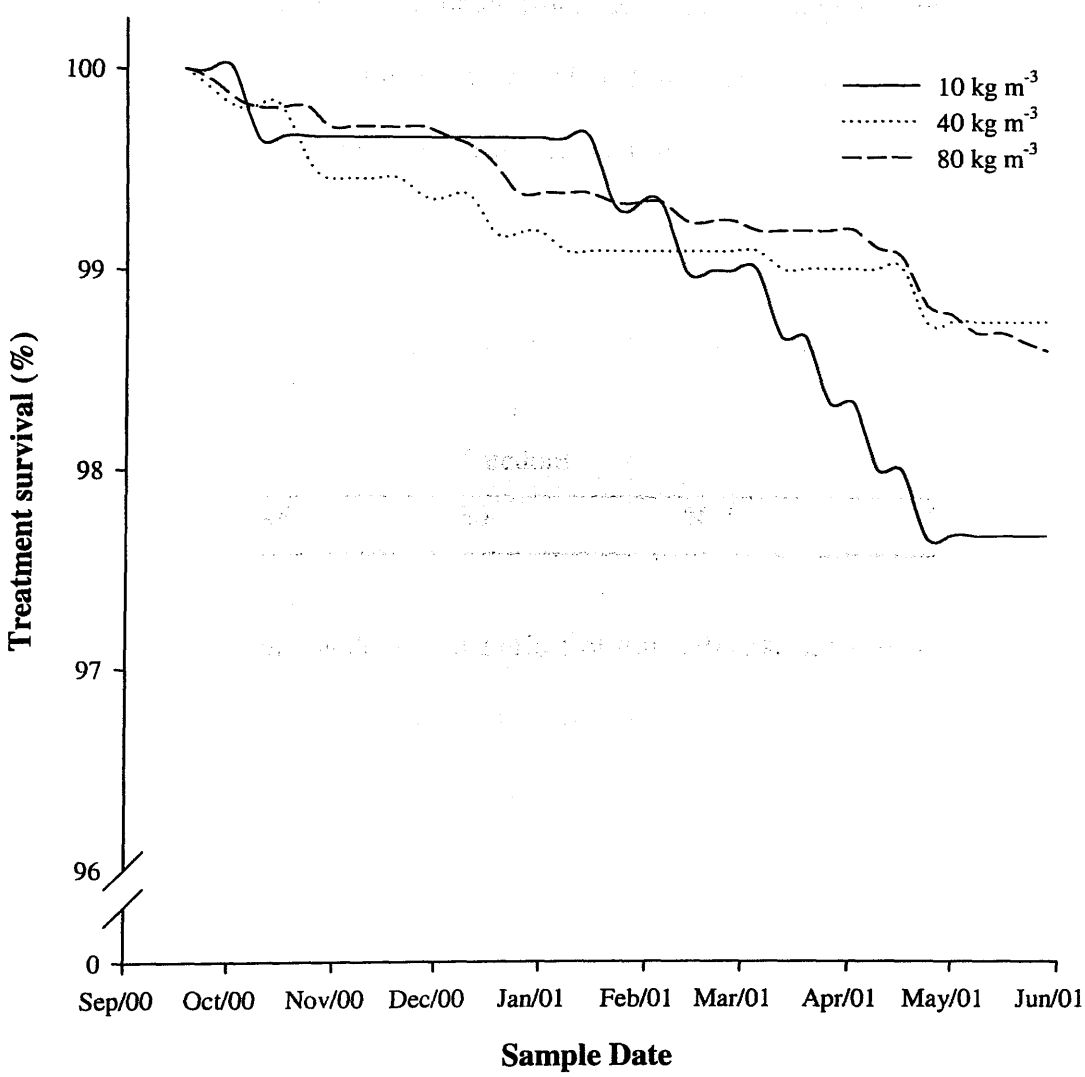


Figure 4.5. Total treatment survival for rainbow trout cultured at different stocking densities.

4.2.2.4. Growth

Throughout the course of the trial there was little difference in growth observed between the treatments; after a 9 month period of culture the maximum difference between mean weight in treatments was just 28 g (40 vs. 80 kg m^{-3}; Figure 4.6). The individual weights of the fish recorded from each monthly sampling point (n=60 fish per tank; 180 fish per treatment) were included as a dependent variable in a GLM with time and SD as categorical predictors, and replicate as a random factor (Table 4.7).

Table 4.7. Whole model effects for GLM using weight as a dependent variable.

Dependent Variable	Adjusted R^2	Degrees of Freedom	F	P
Weight	0.48	80	56.3	0.00

The main effect of the model was the effect of time (P<0.001), likely to be reflecting fish growth as the trial progressed. However there was no significant effect of SD (P=0.31) or replicate (P=0.697); see Table 4.8 for univariate effects of the GLM.

Table 4.8. Univariate tests of significance for GLM using fish weight as a dependent variable.

Dependent Variable	Effect	Effect	Degrees of Freedom	F	P
Weight	Intercept	Fixed	1	23263.50	0.000
	Time	Fixed	8	813.08	0.000
	Treatment	Fixed	2	1.58	0.312
	Replicate	Random	2	0.40	0.697
	Time*Treatment	Fixed	16	1.02	0.465
	Time*Replicate	Random	16	0.92	0.559
	Treatment*Replicate	Random	4	15.69	0.000
	Time*Treatment*Rep.	Random	32	0.73	0.867
	Error		4691		

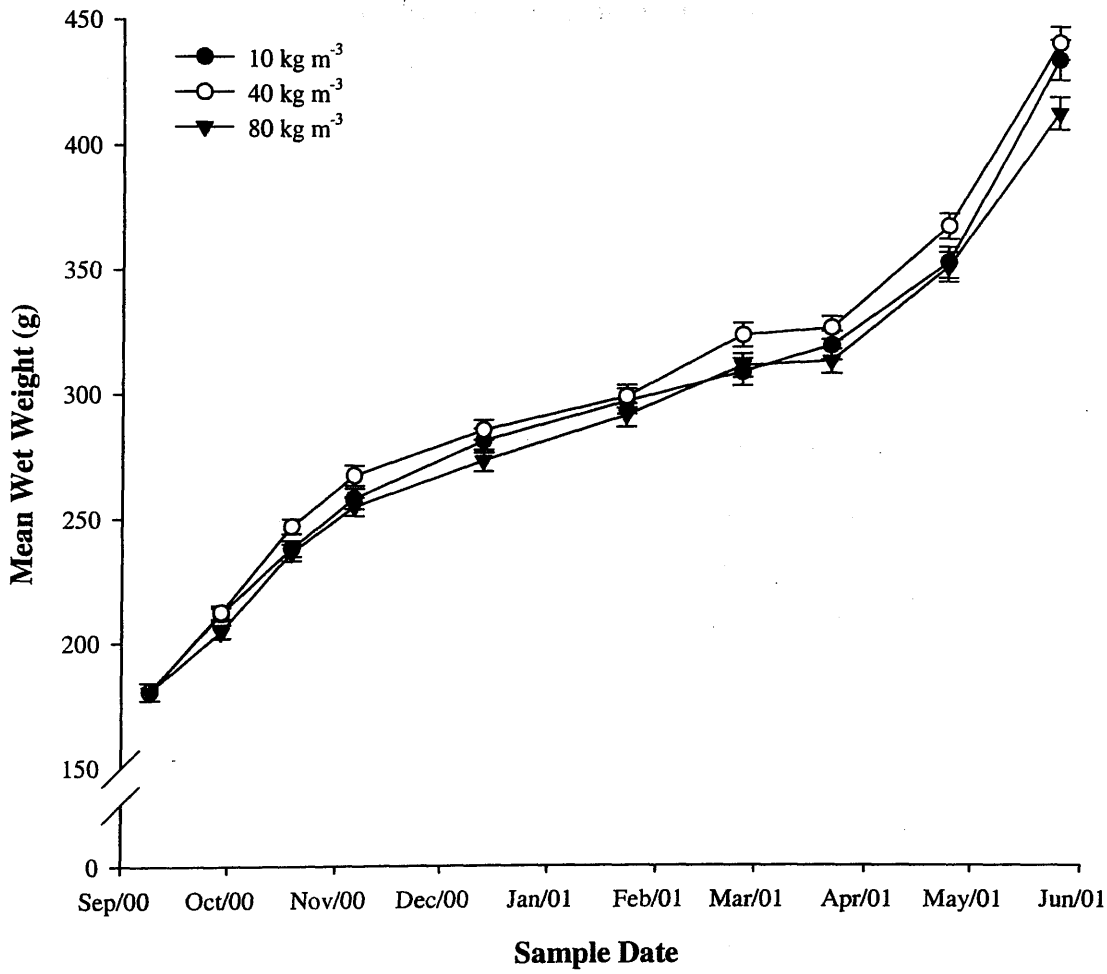


Figure 4.6. Growth of rainbow trout reared at different stocking densities; mean ± SEM of 3 replicates, n=180.

There was a significant interaction between treatment and replicate ($P<0.001$), and when the model was repeated individually for each of the treatments there were significant replicate differences within the 10 ($P<0.001$) and 40 kg m^{-3} treatments ($P<0.01$). However, in both cases there was no significant interaction with time ($P=0.98$ and 0.40 respectively for the 10 and 40 kg m^{-3} treatments). A plot of the changes in mean weight of replicates of each of the density treatments is presented in Figure 4.7.

Specific Growth Rate (SGR)

Fluctuations in SGR mirrored water temperature as would be expected (Figure 4.8). During warmer temperatures, SGR was comparable to those that might be expected under commercial conditions (>1% body weight per day) (Shepherd & Bromage, 1988; Westers, 2001). Between the February and March sample points SGR was very low in the 40 and 80 kg m^{-3} treatments with negative growth recorded in several of the replicates. Although little or no growth is routinely experienced during winter months at the Niall Bromage Freshwater Research Facility due to low water temperatures, it is possible that the negative SGR was a reflection of sampling error *i.e.* 60 fish were sampled from tanks containing approximately 250 and 500 fish for the 40 and 80 kg m^{-3} treatments compared with the 10 kg m^{-3} treatment where nearly all fish within each tank were sampled.

The GLM was repeated using the estimated specific growth rate (SGR) between sample points for each replicate within the different SD treatments as a dependent variable. There was no effect of SD ($P=0.22$) or replicate ($P=0.22$) and time was the only significant effect on the model ($P<0.001$).

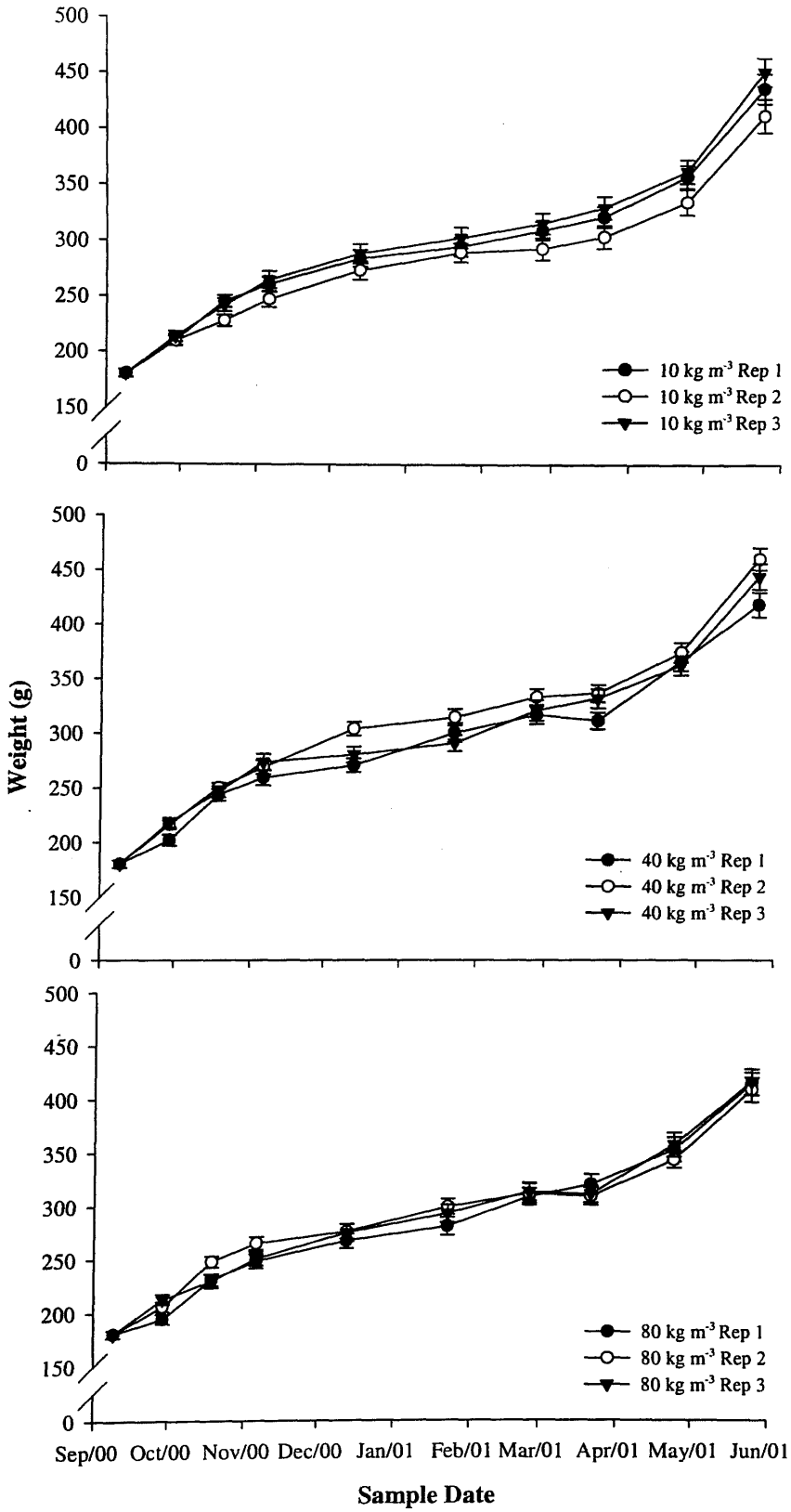


Figure 4.7. Replicate differences in growth of rainbow trout cultured at different stocking densities; mean ± SEM, n= 60.

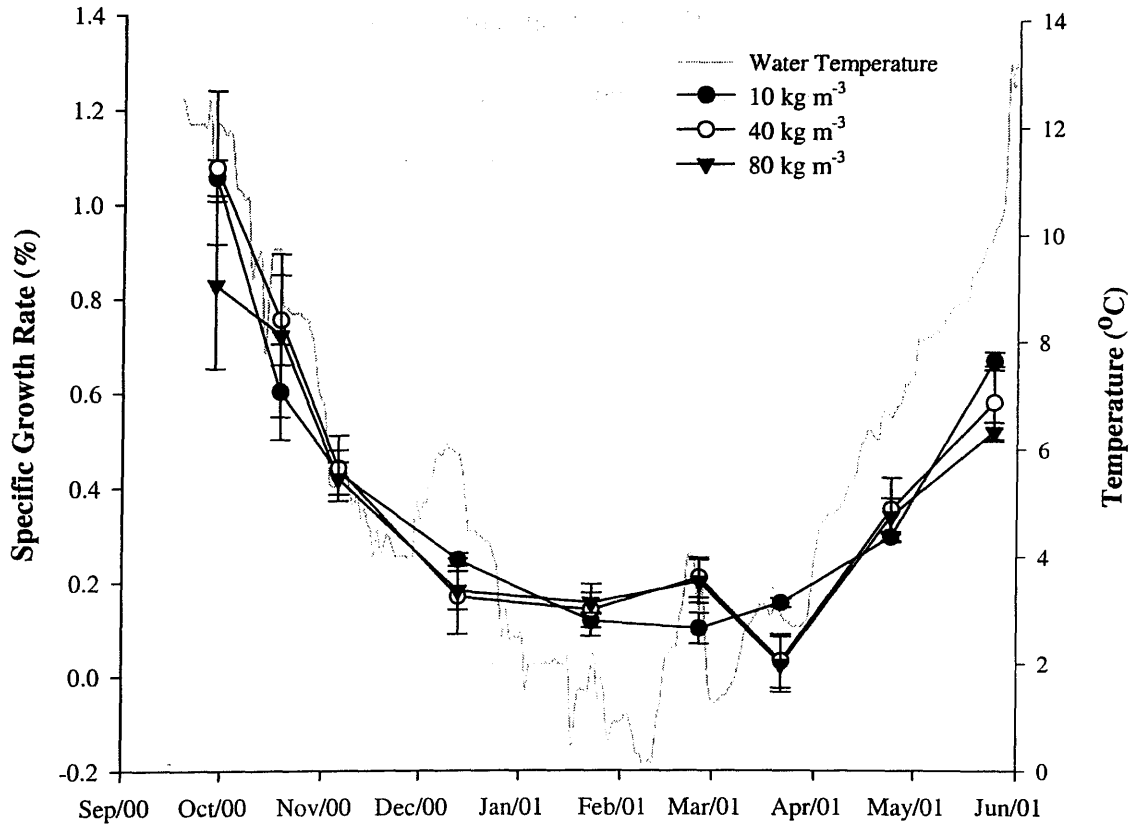


Figure 4.8. Specific Growth Rate (estimated) of rainbow trout cultured at 3 different stocking densities; mean ± SEM of 3 replicates, *n*=60 per replicate.

Feed Conversion Ratio (FCR)

Similarly to SGR, feed conversion ratio (FCR) was around industry norms (Westers, 2001) at the warmer water temperatures with FCR's approaching 1:1 in all treatments (Figure 4.9). The poorest (highest) FCR occurred in the period between January and February for the 10 kg m^3 treatment (mean FCR 4.3:1) and between February and March for the 40 and 80 kg m^{-3} treatments (mean FCR = 3.8 and 4.9:1 respectively). The apparent delay between lowest water temperature and poorest FCR and SGR in the 40 and 80 kg m^{-3} treatments compared with the 10 kg m^{-3} treatment is again possibly an artefact of sampling rather than a reflection of any underlying physiological mechanism.

4.2.2.5 Condition Factor (CF)

The mean CF remained steady for the duration of the trial with values for the most part between 1.30 and 1.35 for all of the density treatments (Figure 4.10). There were no obvious seasonal fluctuations, but there was a significant effect of time ($P<0.001$) when the log transformed CF data for the monthly samples of 60 fish from each tank ($n=180$ per treatment) was included as a dependent variable in the GLM.

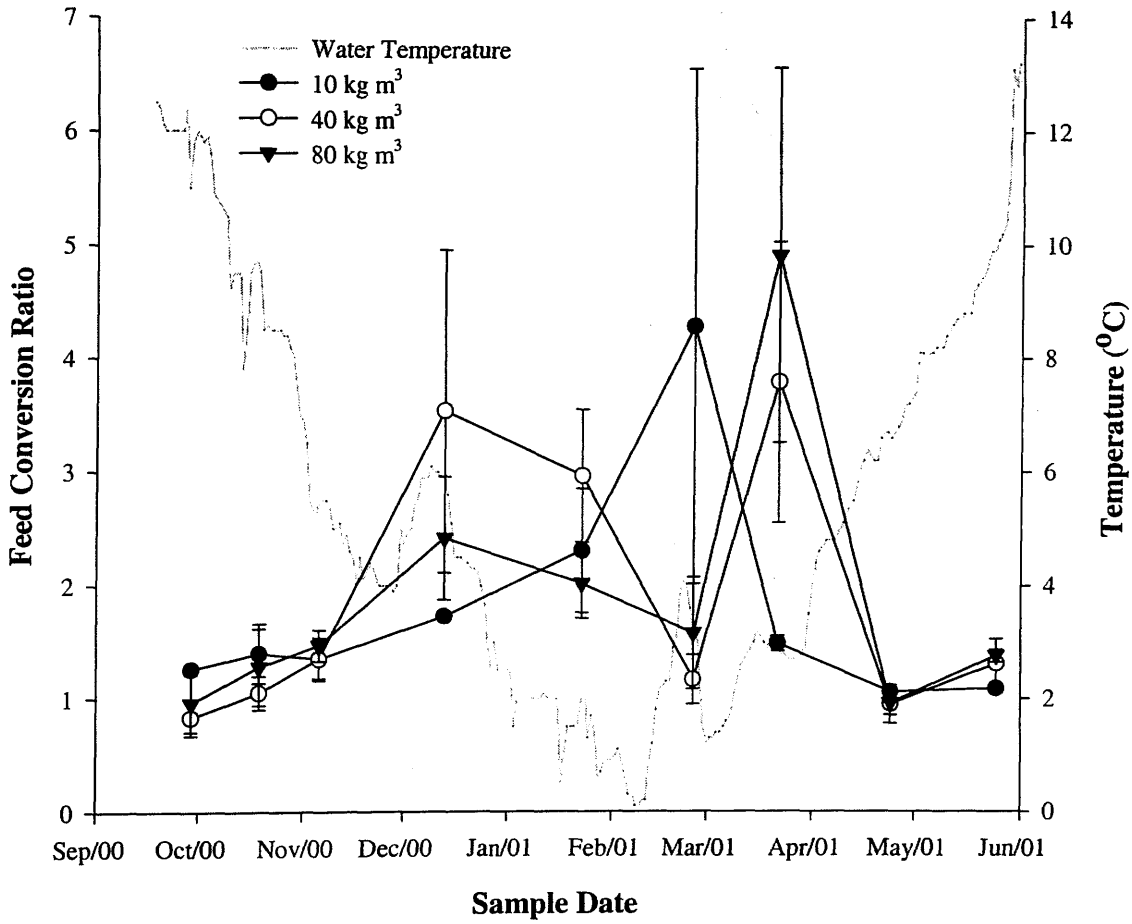


Figure 4.9. Feed Conversion Ratio (estimated) of rainbow trout reared at different stocking densities; mean ± SEM of 3 replicates based on sample of 60 fish per tank.

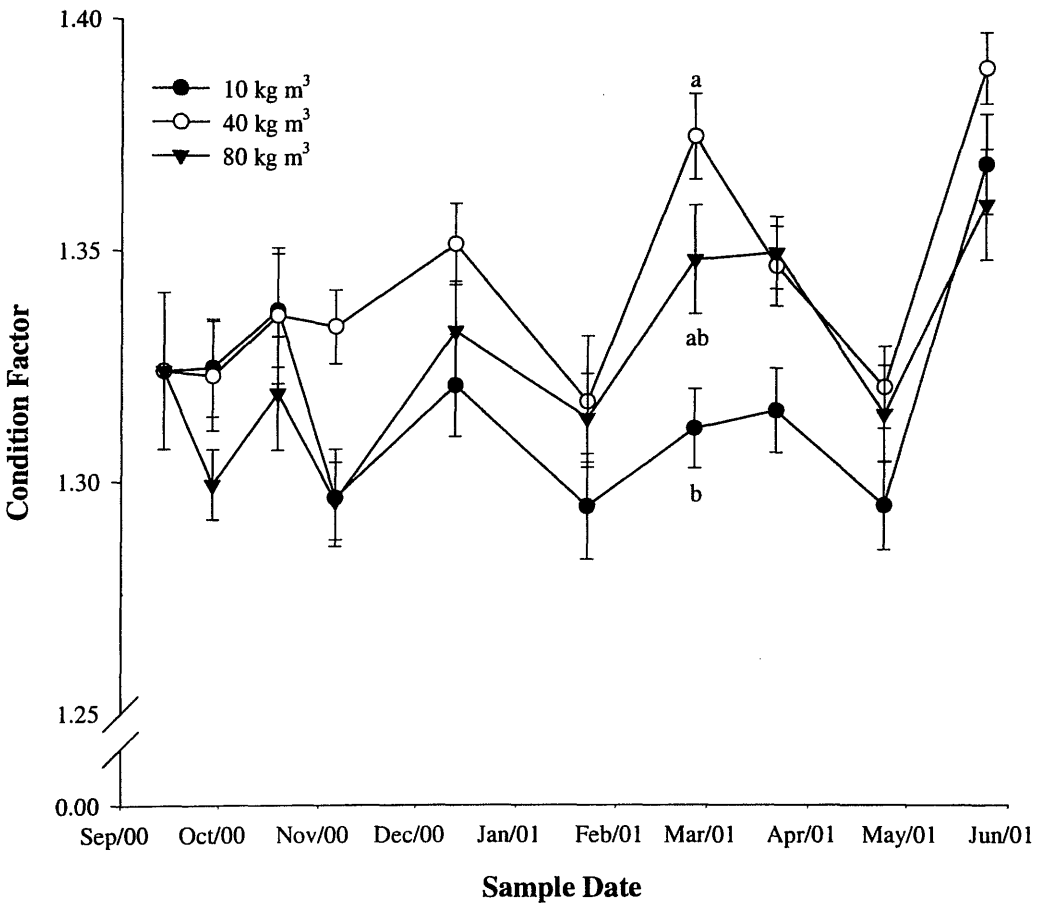


Figure 4.10. Condition Factor of rainbow trout reared at different stocking densities; mean ± SEM of 3 replicates based on sample of 60 fish per tank. The presence of the same letter indicates no significant difference between treatments at the sample point (P>0.05).

There was no effect of SD or replicate on CF, but there were significant interactions between SD and time (P=0.023) and SD and replicate (P=0.004; Table 4.9). Post-hoc analysis found a significant difference in CF between the 40 and 10 kg m^{-3} treatments in March (Tukey's; P<0.05), and also confirmed significant differences between replicates in the 10 (Replicate 1 v Replicate 2; P<0.001) and 40 kg m^{-3} treatments (Replicate 1 v Replicates 2 and 3; P<0.01).

Table 4.9. Univariate tests of significance for GLM using condition factor as a dependent variable.

Dependent Variable	Effect	Effect	Degrees of Freedom	*F*	*P*
Weight	Intercept	Fixed	1	316621.7	0.000
	Time	Fixed	8	6.5	0.001
	Treatment	Fixed	2	1.5	0.326
	Replicate	Random	2	0.0	0.988
	Time*Treatment	Fixed	16	2.3	0.023
	Time*Replicate	Random	16	3.0	0.004
	Treatment*Replicate	Random	4	13.4	0.000
	Time*Treatment*Rep	Random	32		
	Error		4691		0.000

4.2.2.6 Haematocrit

There appeared to be a marked effect of water temperature on haematocrit values, with packed cell volume increasing in colder temperature and then dropping again as water temperature increased (Figure 4.11). There was a general trend for the 10 kg m^{-3} treatment to have the highest haematocrit, and the 80 kg m^{-3} to have the lowest values (5 of the 8 sample points where haematocrit was measured). With the exception of the December and January sample points, the mean haematocrit levels were inside the range of 24-43% reported for clinically healthy rainbow trout (Wedemeyer, 1996).

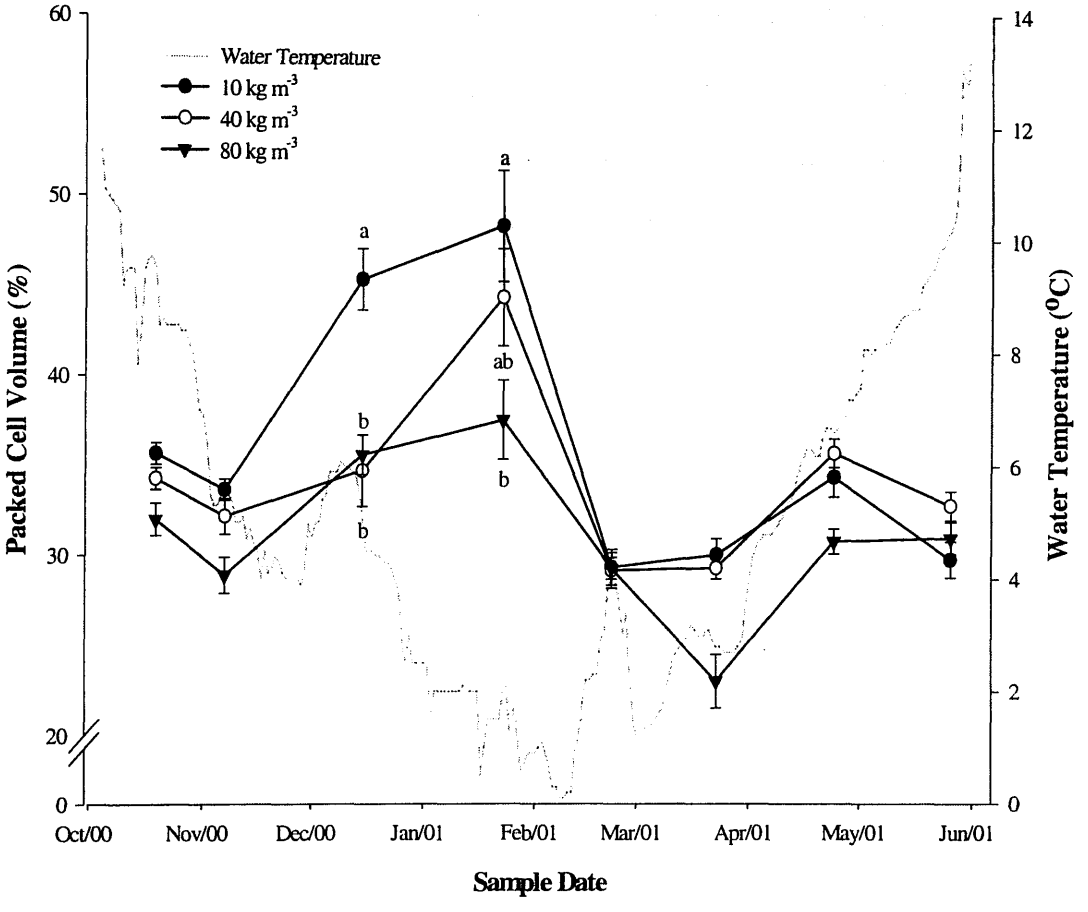


Figure 4.11. Haematocrit values for rainbow trout reared at different stocking densities; mean ± SEM of 3 replicates (10 fish per replicate). The presence of the same letter indicates no significant difference between treatments at the sample point (P>0.05).

The arcsine transformed haematocrit data from the monthly blood samples (n=10 fish per tank; 30 fish per treatment) was included in a GLM as a dependent variable with time, SD and replicate. The model was significant $P<0.001$ with an adjusted R^2 of 0.52. Univariate analysis found a significant effect of stocking density on haematocrit (P=0.010), and also a significant interaction (P=0.048) between SD and time (Table 4.10). Post-hoc significant differences were detected in December and January, when levels in the 10 kg m^{-3} treatment were significantly higher compared with the 40 and 80 kg m^{-3} treatments in December, and the 80 kg m^{-3} treatment in January (Tukey's, $P<0.01$).

Table 4.10. Univariate tests of significance for GLM using haematocrit as a dependent variable.

Dependent Variable	Effect	Effect	Degrees of Freedom	*F*	*P*
Haematocrit	Intercept	Fixed	1	135932.8	0.000
	Time	Fixed	7	63.1	0.000
	Treatment	Fixed	2	18.3	0.010
	Replicate	Random	2	11.4	0.472
	Time*Treatment	Fixed	14	2.1	0.048
	Time*Replicate	Random	14	0.5	0.896
	Treatment*Replicate	Random	4	0.7	0.576
	Time*Treatment*Rep	Random	28	2.5	0.000
	Error		626		

4.2.2.7 Lysozyme Activity

Lysozyme activity mirrored water temperature with levels decreasing through winter and increasing again as water temperature increased (Figure 4.12). The results for the GLM using lysozyme activity as a dependent variable are shown in Table 4.11. There was a significant effect of SD on lysozyme activity (P=0.014) and there was also a significant interaction between SD and time (P=0.045). Post-hoc analysis (Tukey's)

found lysozyme activity in the 40 kg m^{-3} treatment to be significantly lower than the 10 and 80 kg m^{-3} treatments in November ($P<0.05$). November was the only month in which a significant difference was detected, but there was a general trend for the 80 kg m^{-3} treatments to have the highest lysozyme activity and the 10 kg m^{-3} the lowest (six of the nine sample points).

Table 4.11. Univariate tests of significance for GLM using lysozyme as a dependent variable.

Dependent Variable	Effect	Effect	Degrees of Freedom	*F*	*P*
Lysozyme activity	Intercept	Fixed	1	13074.56	0.000
	Time	Fixed	8	50.86	0.000
	Treatment	Fixed	2	14.82	0.014
	Replicate	Random	2	0.34	0.725
	Time*Treatment	Fixed	16	2.01	0.045
	Time*Replicate	Random	16	2.02	0.045
	Treatment*Replicate	Random	4	0.60	0.666
	Time*Treatment*Rep	Random	32	1.31	0.121
	Error		710		

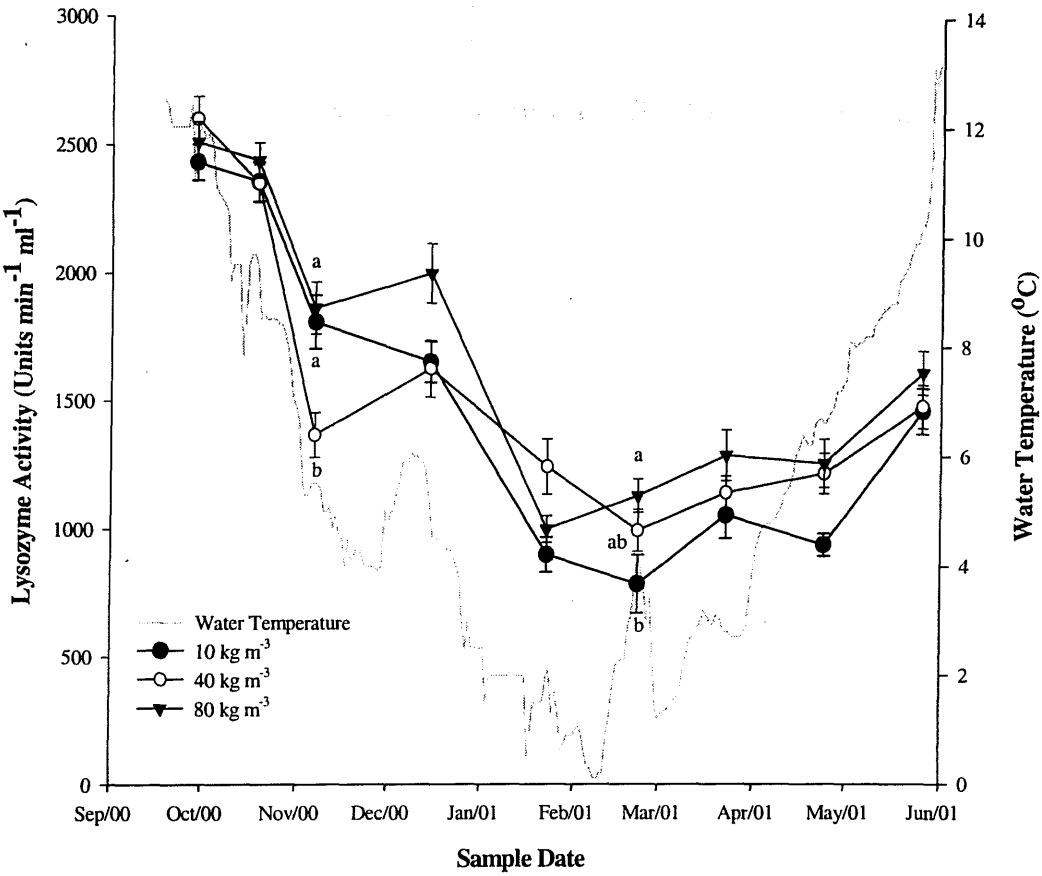


Figure 4.12. Lysozyme activity in the plasma of rainbow trout reared at different stocking densities; mean ± SEM of 3 replicates (10 fish per replicate). The presence of the same letter indicates no significant difference between treatments at the sample point (P>0.05).

4.2.2.8 Cortisol

There were marked differences in the levels of cortisol observed between the SD treatments at different points throughout the experiment (Figure 4.13). The cortisol data were log transformed and included as a dependent variable in a GLM with time, SD and replicate (Table 4.12).

Table 4.12. Univariate tests of significance for GLM using cortisol as a dependent variable.

Dependent Variable	Effect	Effect	Degrees of Freedom	*F*	*P*
Cortisol	Intercept	Fixed	1	672.34	0.001
	Time	Fixed	8	11.52	0.000
	Treatment	Fixed	2	10.28	0.027
	Replicate	Random	2	2.13	0.217
	Time*Treatment	Fixed	16	2.60	0.010
	Time*Replicate	Random	16	1.39	0.210
	Treatment*Replicate	Random	4	1.07	0.388
	Time*Treatment*Rep	Random	32	4.91	0.000
	Error		711		

The GLM detected significant effects of both time ($P<0.001$), SD ($P=0.027$) and also a significant interaction between the effects of time and SD ($P=0.010$). Post-hoc analysis found levels of cortisol in fish from the 10 kg m^{-3} treatment to be significantly higher than the other SD treatments at five of the nine sample points (Tukeys, $P<0.01$). Cortisol levels in the 40 and 80 kg m^{-3} treatments were generally very similar except for in October and March, when cortisol in the 40 kg m^{-3} treatment was significantly higher than the 80 kg m^{-3} (Tukeys, $P<0.01$).

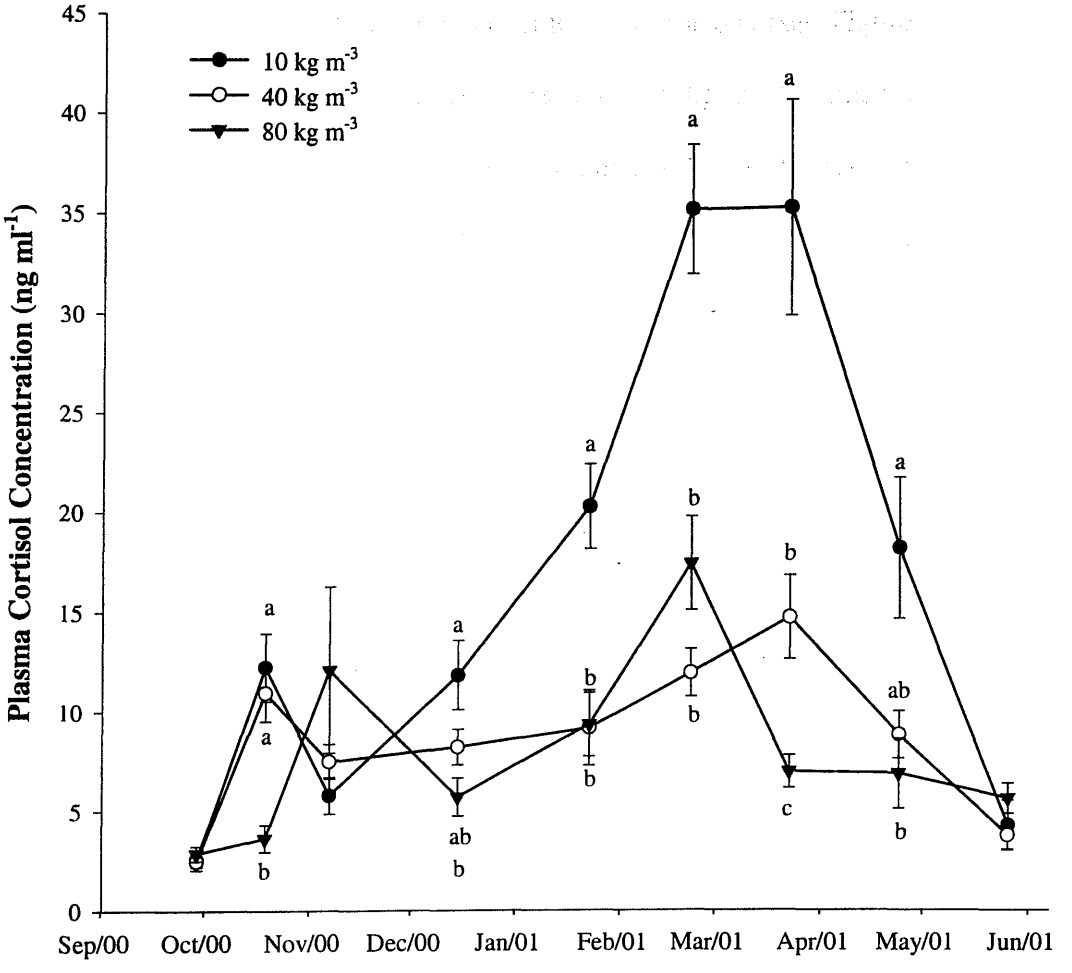


Figure 4.13. Plasma cortisol in rainbow trout reared at different stocking densities; mean ± SEM of 3 replicates per treatment (10 fish per replicate). The presence of the same letter indicates no significant difference between treatments at the sample point (P>0.05).

The levels of cortisol observed in the first and final sample points (September and May) were low in all treatments ($\approx$ 5 ng ml^{-1}), with cortisol levels consistent with reported baseline levels in unstressed rainbow trout (Donaldson, 1981). However, the levels observed in the 10 kg m^{-3} treatment in February and March are representative of peak levels of cortisol following an acute stress episode (see Figure 3.4; page 79, Chapter 3). In February all of the replicates in the 10 kg m^{-3} treatment exhibited high levels of cortisol (40, 36 and 30 ng ml^{-1} in replicates 1, 2 and 3 respectively), but the high levels of cortisol observed in March were principally due to one of the replicates in the 10 kg m^{-3} (replicate 1) where a mean cortisol level of 71 ng ml^{-1} was observed (Figure 4.14).

Before considering the implications of the high levels of cortisol in the 10 kg m^{-3} treatment in-depth, it was first necessary to identify possible confounding effect/s of factors other than stocking density. Plasma cortisol concentrations are highly sensitive to a range of stressors and even the slightest manipulation (deliberate or accidental) can potentially alter cortisol through stimulation of the HPI-axis (Mommsen *et al.*, 1990). However, examination of some of the more obvious potential confounders in this experiment failed to provide a logical explanation.

The experimental design endeavoured to take into account the positioning of tanks and the associated level of disturbance (Figure 4.1). Comparison of mean cortisol levels from tanks grouped together on the basis of TDL found no significant differences (P=0.41) between the TDL groupings (Kruskal-Wallis ANOVA; Figure 4.15).

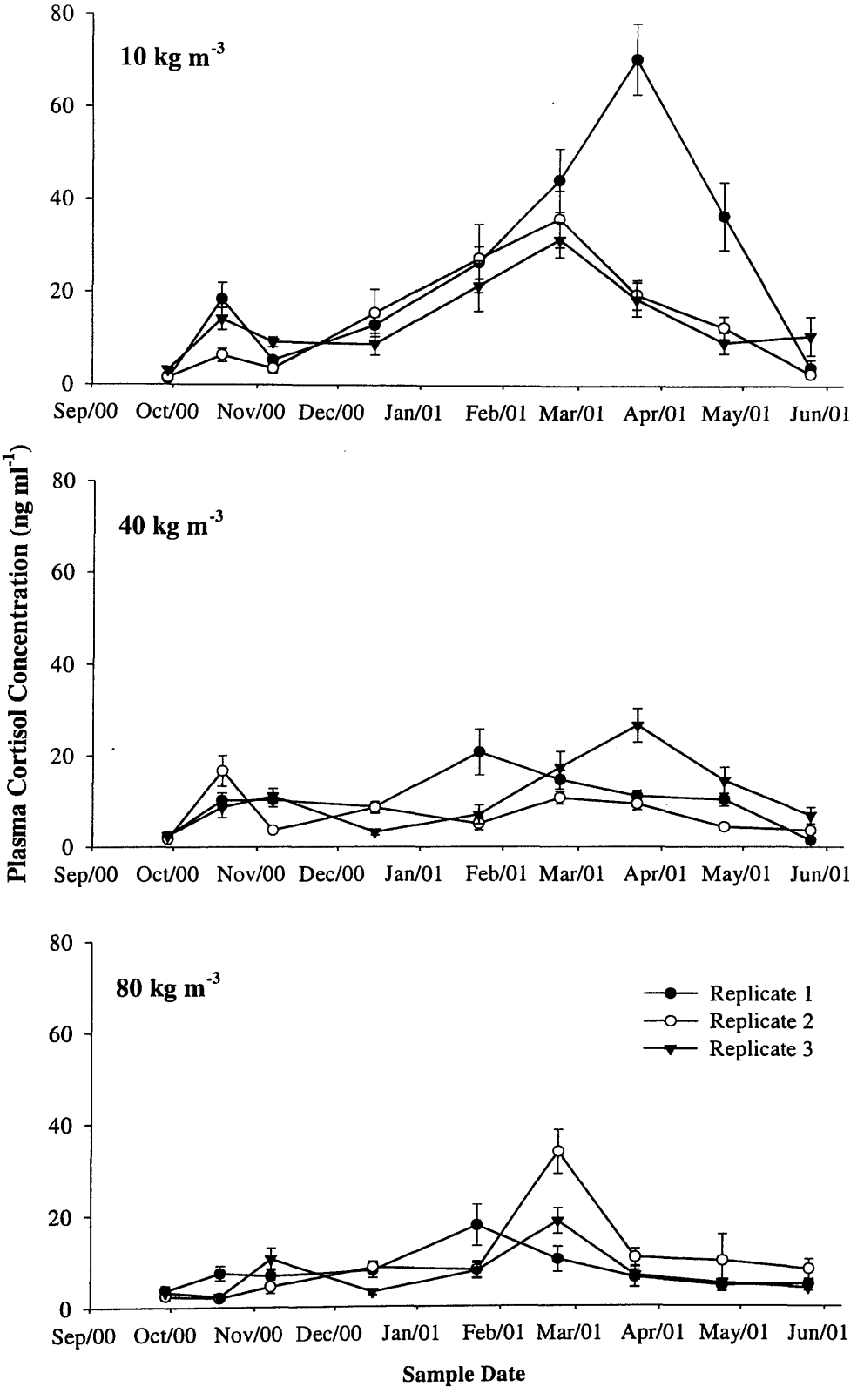


Figure 4.14. Replicate differences in plasma cortisol levels in rainbow trout reared at different stocking densities.

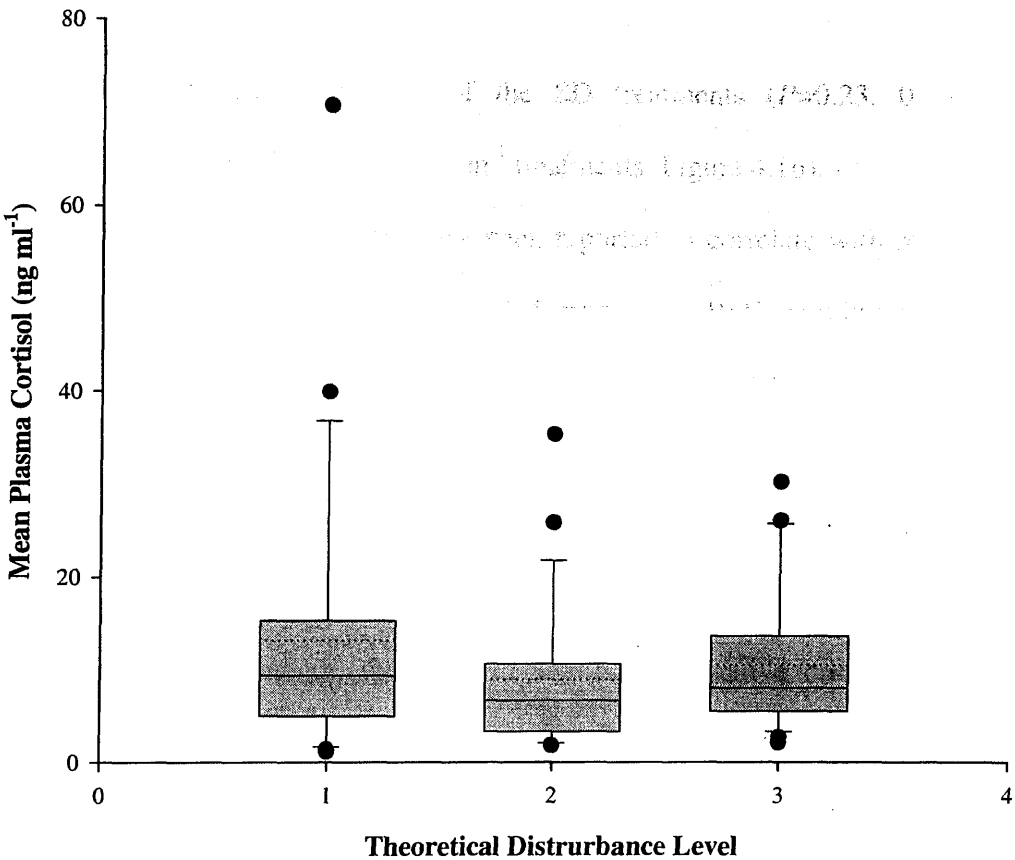


Figure 4.15. Effect of theoretical disturbance level on mean plasma cortisol concentrations in rainbow trout reared at different stocking densities. Boxes represent 25th to 75th percentiles, with dashed and solid lines representing the mean and median respectively; error bars denote 10th and 90th percentiles with dots representing outliers (>95% confidence interval). Disturbance levels were estimated on a scale of 1 to 3 where 1 represents tanks with the least chance of disturbance and 3 the highest (see figure 4.1 for plan of tank house).

Another possible explanation for the higher cortisol in the 10 kg m^{-3} treatment could have been the fact that it took longer to catch and sample the fish in this treatment as there were fewer fish in these tanks making it more difficult to net the fish. However, a regression of the sequential sampling order against plasma cortisol showed no correlation in any of the SD treatments (P=0.23, 0.66 and 0.89 respectively in the 10, 40 and 80 kg m^{-3} treatments; Figure 4.16).

Elevated cortisol levels have been reported to correlate with reduced growth (Barton *et al.*, 1987; Pickering, 1990; Pickering *et al.*, 1991) and immunosuppression (Ellis, 1981; Pickering & Pottinger, 1989; Nanaware *et al.* 1994). Further analysis was carried out to investigate the relationships between plasma cortisol levels and growth rate and lysozyme activity. When the monthly SGR estimated from each of tanks for the duration of the experiment was correlated with the mean cortisol concentration for the corresponding month, there was a significant negative correlation coefficient (P=0.03, R = -0.43, Figure 4.17). A runs test suggested that the relationship between increased cortisol and reduced SGR was not linear (P<0.001). A linear regression of cortisol concentration against lysozyme activity in fish from all of the SD treatments found there to be a significant negative correlation (R = -0.3112, R^2 = 0.49). A runs test suggested that the relationship was not linear, with significantly fewer runs than would be expected (P<0.001; Figure 4.18).

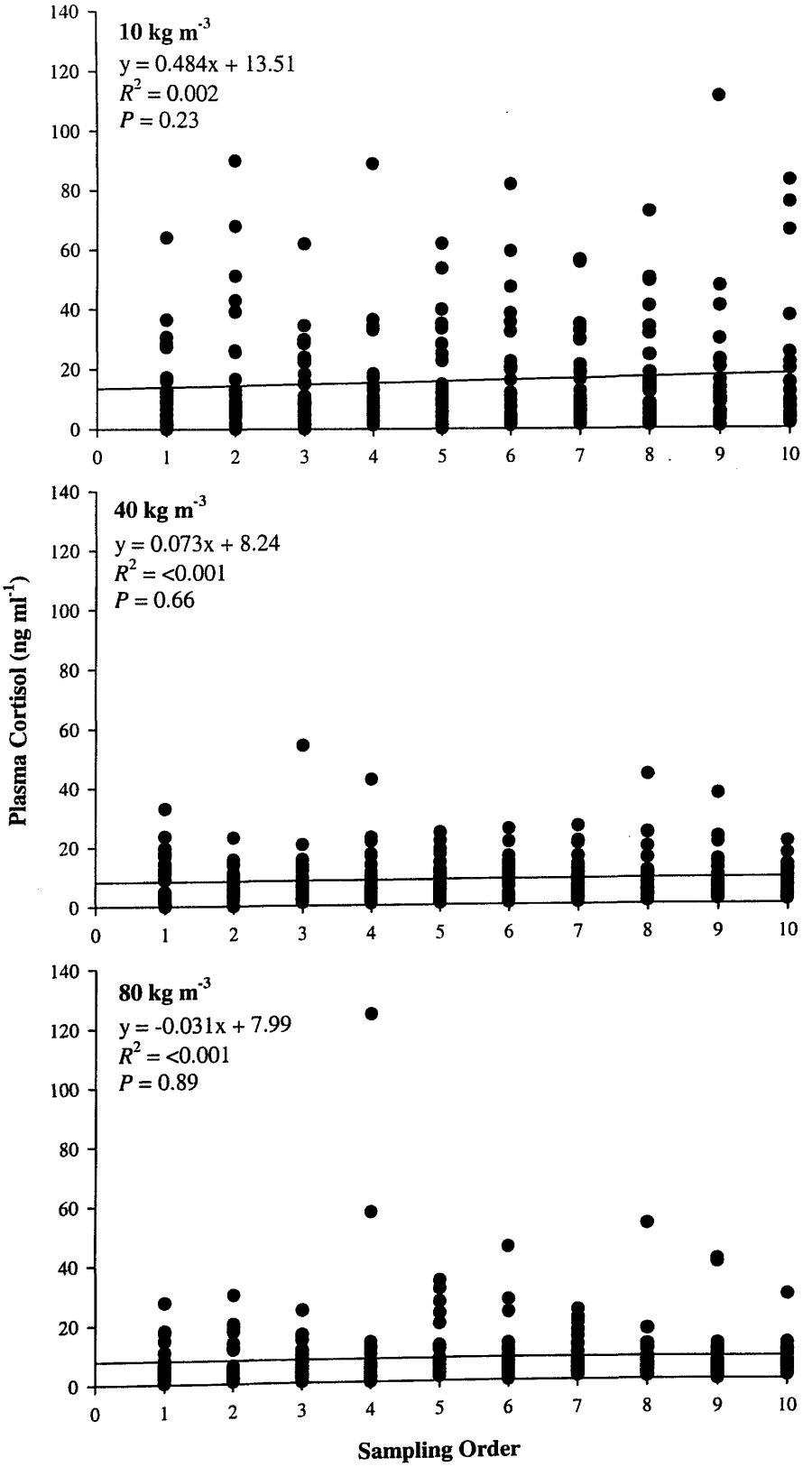


Figure 4.16. Correlation between sampling order and plasma cortisol concentration in rainbow trout reared at different stocking densities.

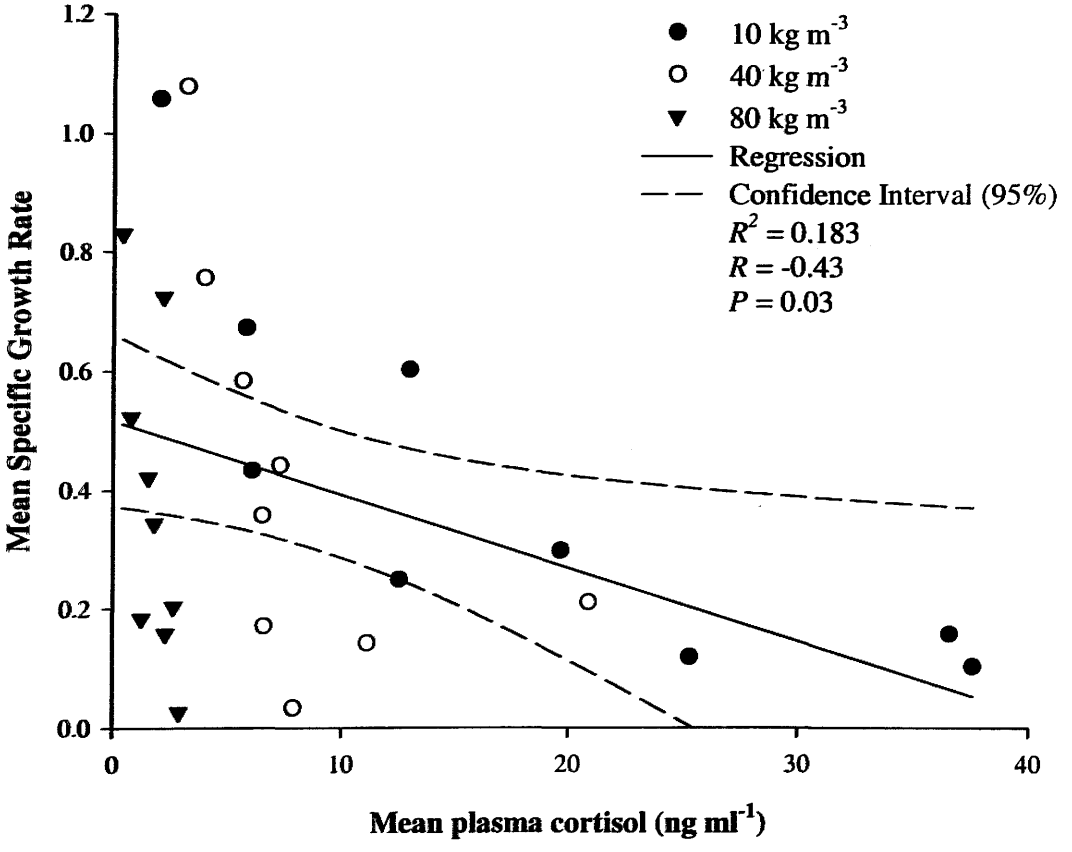


Figure 4.17. Correlation between mean plasma cortisol concentration and mean specific growth rate in rainbow trout reared at different stocking densities

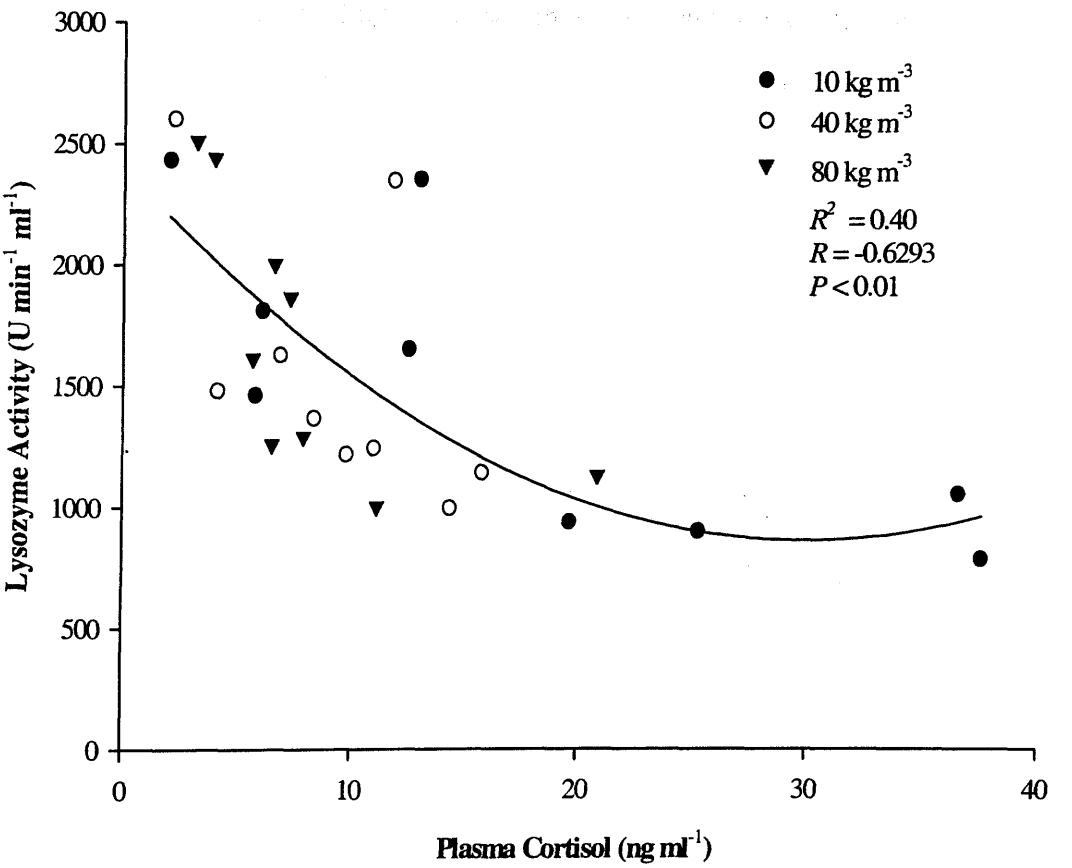


Figure 4.18. Correlation between mean plasma cortisol and mean lysozyme activity concentration in rainbow trout reared at different stocking densities.

4.2.2.9 Fin Erosion

Fin Indices

The individual scores for the dorsal and caudal fin indices were transformed [Log(fin score +2)] and included as dependent variables in a GLM with time, SD treatment and replicate (Table 4.13). There was a significant effect of time (P=0.001) on both fin indices, and a post-hoc analysis detected significant differences between the fin indices to be significantly higher (worse fins) at the end of the experiment compared with the start (Tukey's, P<0.01). Changes in mean dorsal and caudal fin index in the SD treatments through the course of the experiment are presented in Figure 4.19.

Table 4.13. Univariate tests of significance for GLM using fin index scores for dorsal and caudal fins as dependent variables

Dependent Variable	Effect	Effect	Degrees of Freedom	F	*P*
Dorsal Index	Fin Intercept	Fixed	1	147283.46	0.000
	Time	Fixed	7	7.89	0.001
	Treatment	Fixed	2	5.67	0.068
	Replicate	Random	2	0.28	0.767
	Time*Treatment	Fixed	14	2.55	0.017
	Time*Replicate	Random	14	1.88	0.076
	Treatment*Replicate	Random	4	5.61	0.002
	Time*Treatment*Rep.	Random	28	0.98	0.496
	Error		4190		
Caudal Index	Fin Intercept	Fixed	1	2127095.72	0.000
	Time	Fixed	7	7.27	0.001
	Treatment	Fixed	2	1.15	0.402
	Replicate	Random	2	0.03	0.970
	Time*Treatment	Fixed	14	1.17	0.351
	Time*Replicate	Random	14	1.17	0.347
	Treatment*Replicate	Random	4	4.42	0.007
	Time*Treatment*Rep.	Random	28		
	Error		4190		

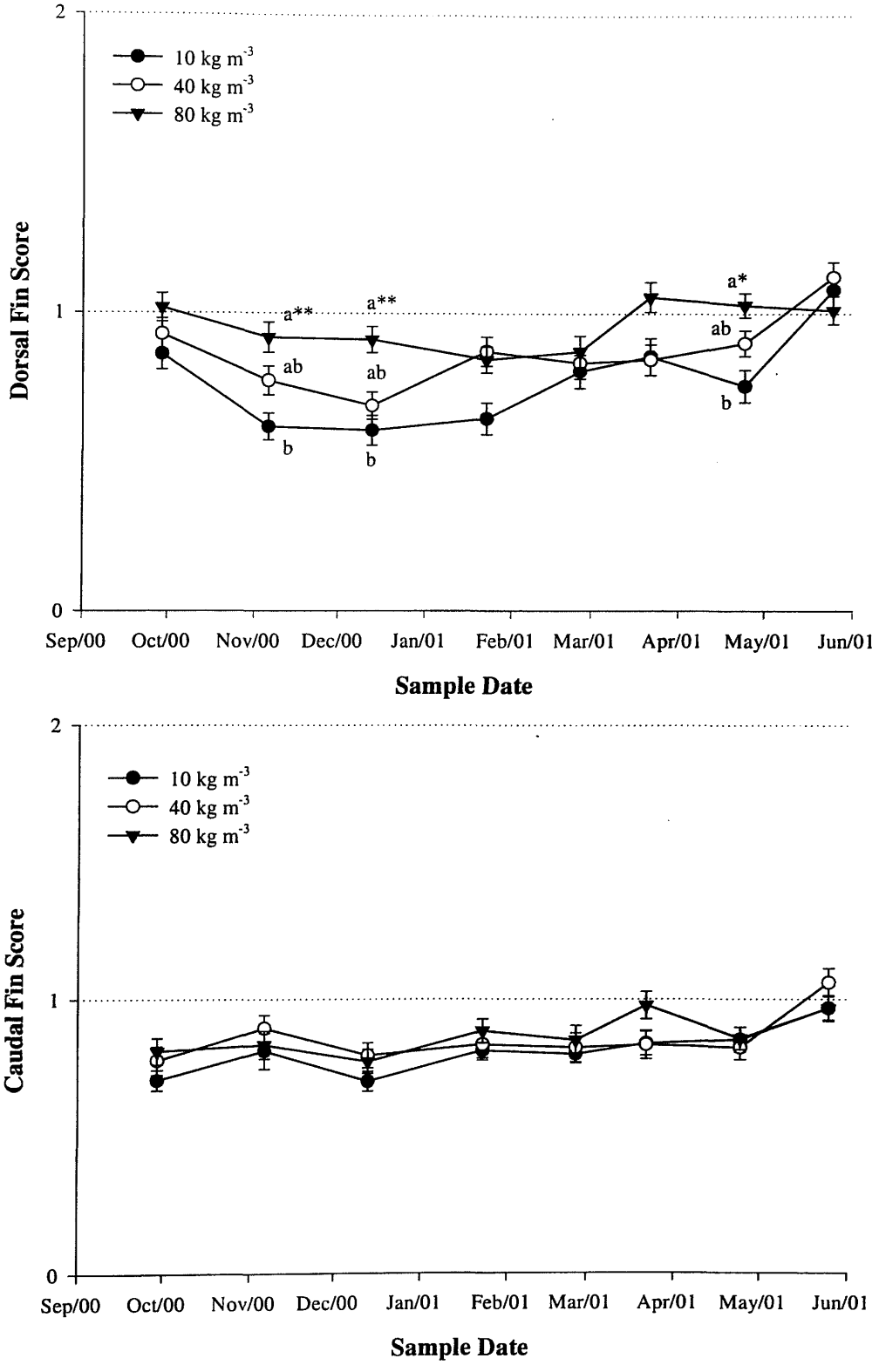


Figure 4.19. Dorsal (a) and caudal (b) fin index of rainbow trout reared at different stocking densities; mean ± SEM of 3 replicates per treatment (n=60 fish per replicate). Points within the same column that do not share a common letter are significantly different (Tukey's, *P<0.01, **P<0.001).

There was a significant effect of SD on dorsal fin index (DFI) and a significant interaction between the effects of SD and time (P=0.017), with significant post-hoc differences observed between the 10 and 80 kg m^{-3} treatments in November, December (P<0.001), and April (P<0.01). However, there were no significant differences in DFI between the treatments at the end of the trial (Figure 4.19a).

Throughout the course of the experiment, caudal fin index (CFI) score ranged between 0 and 2, and damage to the caudal fin was generally less severe in appearance than the dorsal fin, where DFI scores of 3 were common. There was a significant effect of time on CFI (P=0.001), and by the end of the experiment CFI was significantly higher than at the start (P<0.001). There was no significant effect of stocking density on CFI (P=0.402), nor any interaction between the effects of SD and time (P=0.347).

Relative Fin Length (RFL)

Comparison of the RFL values for the SD treatments at the end of the experiment showed a significant effect of SD on the dorsal, caudal, and left and right pectoral fins (ANOVA, P<0.001). Post-hoc analysis showed the 40 and 80 kg m^{-3} treatments to have significantly lower RFL than the 10 kg m^{-3} treatment for dorsal and caudal fins (Tukey's, P<0.001), but no differences were observed between the 40 and 80 kg m^{-3} treatments (P>0.05). However, analysis of the RFL for the pectoral fins showed a step-wise significant decrease in RFL with increasing SD (Tukey's, P<0.001; Figure 4.20). Significant differences were observed between the left and right pectoral fins in the 40 and 80 kg m^{-3} treatments (Paired T-test; P<0.01) suggesting that the left sided pectoral fins suffered more severe erosion than those on the right.

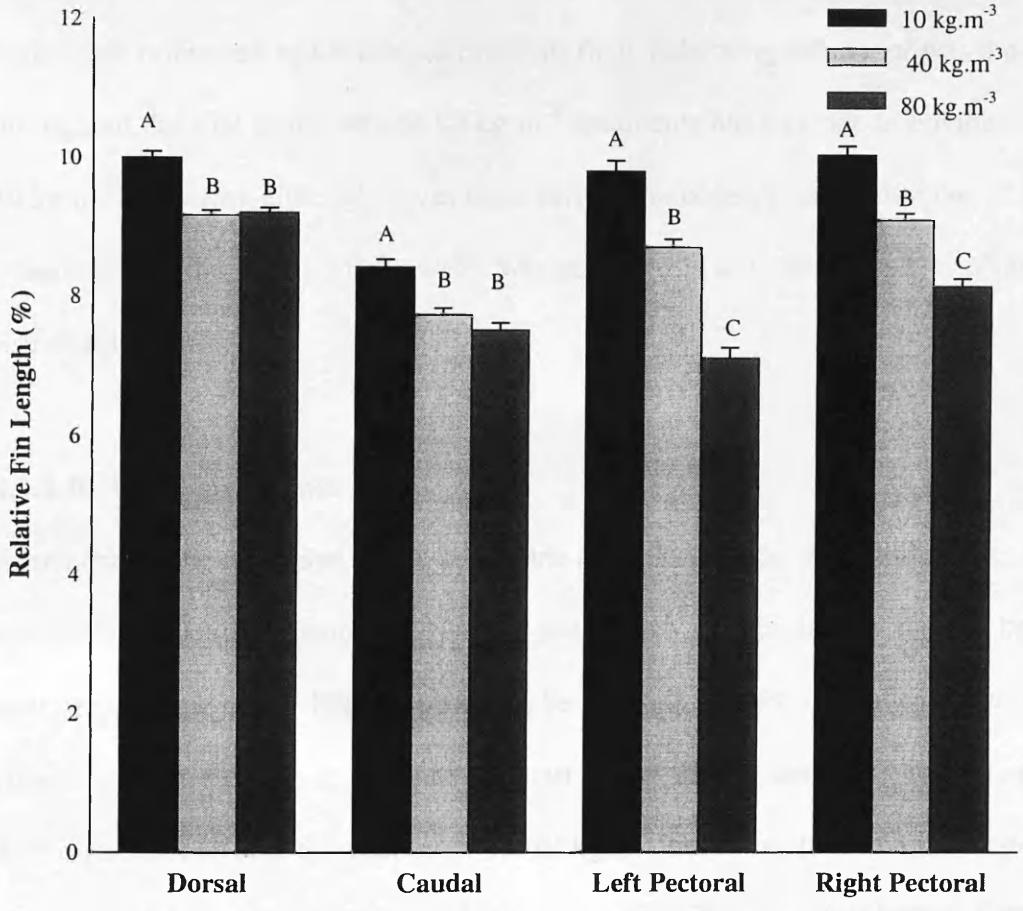


Figure 4.20. Relative Fin Length of rainbow trout reared at different stocking densities. Treatment means and SEM with different letters denoting significant differences for each fin ($P<0.01$)

An example of asymmetric damage to the pectoral and pelvic fins of a fish from one of the 80 kg m^{-3} replicates is shown in Figure 4.21. This observation was consistent through all of the replicates and was likely to have been a result of the water current direction within the tanks. The inflow pipe was angled to result in clockwise water current into which the fish would generally school *i.e.* hold station with heads orientated against the direction of flow. Schooling behaviour was the norm throughout the trial in the 40 and 80 kg m^{-3} treatments but was not so obvious in the 10 kg m^{-3} treatment, although it was more difficult to observe fish within the 10 kg m^{-3} treatment as the clarity of the water was poor and it was only possible to observe fish near the surface.

4.2.2.10 Size Distribution

At the end of the trial there was a noticeable difference in the population structure of the different density treatments, with a wider size distribution in the 10 kg m^{-3} treatment (Figure 4.22). There appeared to be a higher number of smaller fish that had grown very little or not at all since the start of the trial in the 10 kg m^{-3} treatment. Although the final size distribution in the 10 kg m^{-3} treatments passed a Kolmogorov-Smirnov normality test, suggesting that it followed a Gaussian distribution, Bartlett's test for homogeneity of variance suggested that there were significant differences in the variance between the different populations ($P<0.01$). A comparison of the change in coefficient of variation for weight (CV_W) also demonstrated the difference in size distribution between the SD treatments, with the 10 kg m^{-3} treatment showing a steady increase in CV_W whilst the 40 and 80 kg m^{-3} treatments remained lower (Figure 4.23).

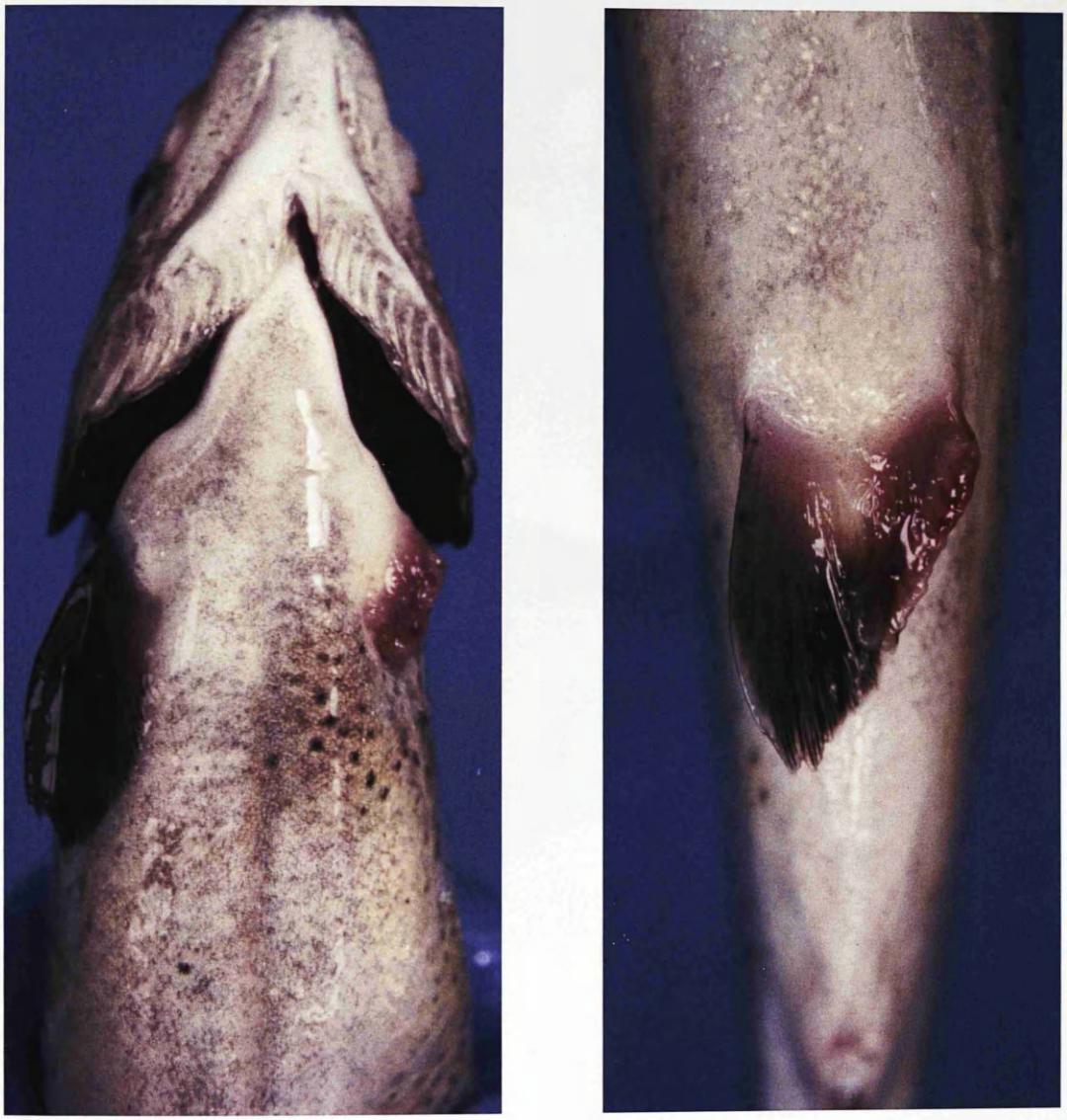


Figure 4.21. Examples of asymmetric erosion of the pectoral and pelvic fins from a fish in the 80 kg m^{-3} treatment.

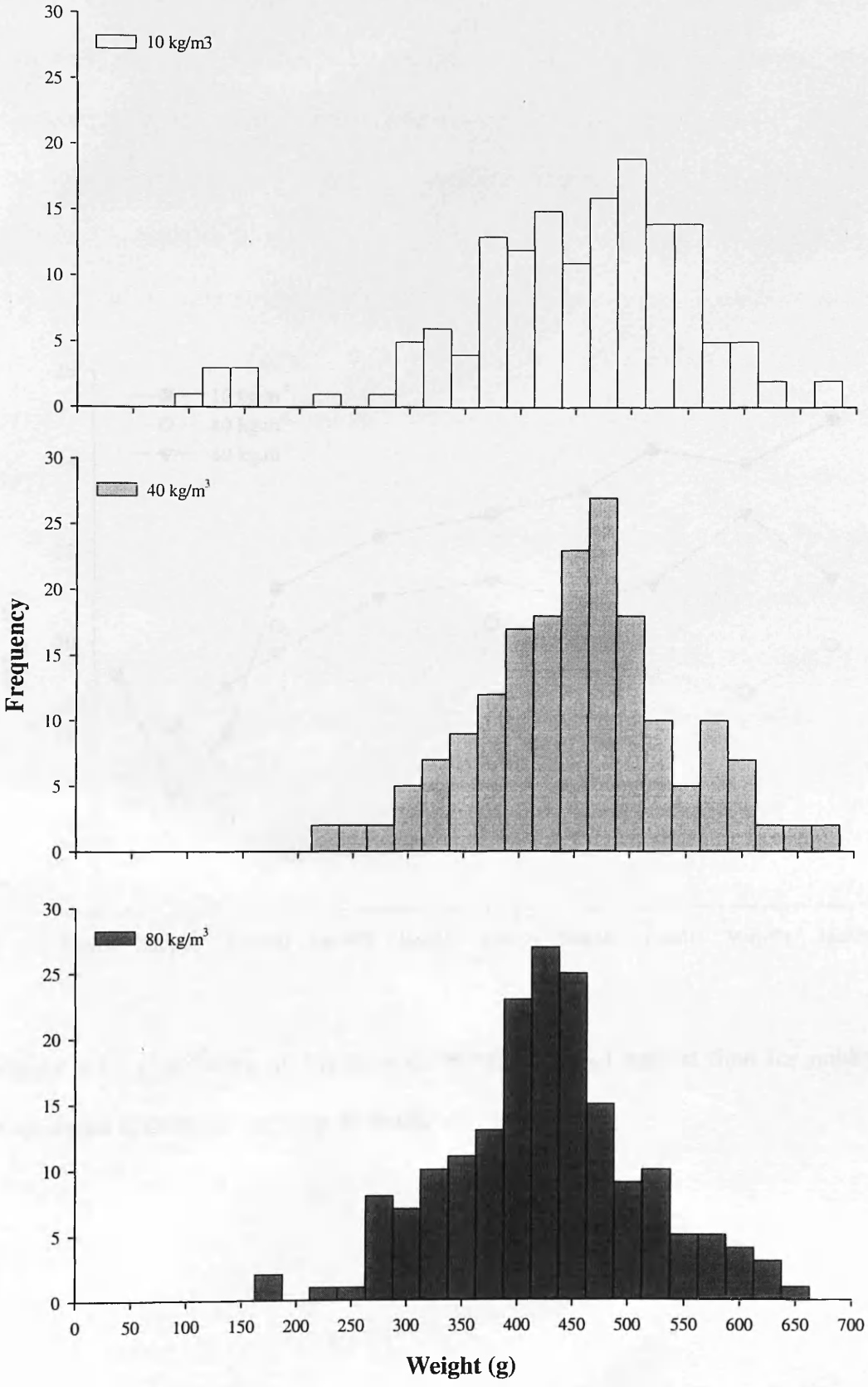


Figure 4.22. Size distribution of rainbow trout reared at different stocking densities.

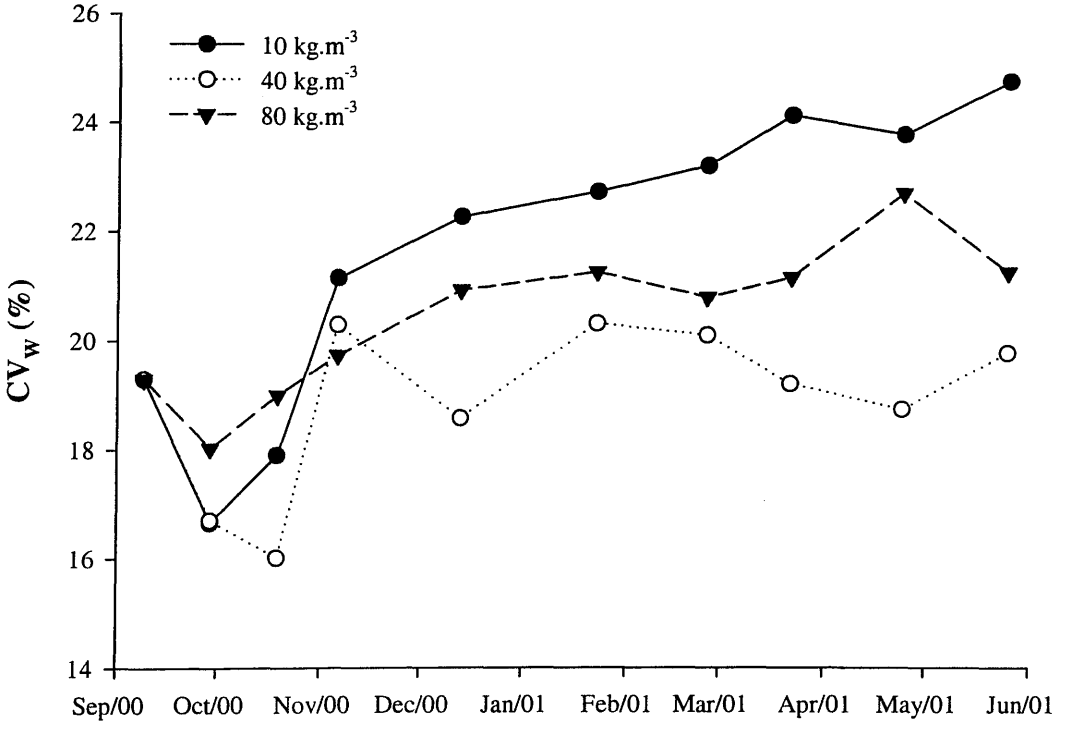


Figure 4.23. Coefficient of Variation of Weight (CoV_W) against time for rainbow trout reared at different stocking densities.

4.2.2.11 Principal Components Analysis

This study applied numerous indicators in an effort to assess fish welfare. These indicators were subject to a considerable amount of intraspecific variation and were also influenced by factors other than welfare. This meant that analysis of the individual parameters in isolation and even pair-wise regression of variables could only provide limited information. In order to better understand the interaction between the various welfare indicators, PCA was used as a data reduction technique to generate 'welfare indices' based upon the statistical relationships observed between the simultaneously measured individual indicators of welfare. The following variables were used to generate Principal Components (PCs); CF, plasma cortisol, lysozyme activity, haematocrit, dorsal and caudal fin scores; data from September and October were not included in the analysis as data for some of the variables was missing for these months. Two PCs were selected based on Eigenvalues of the correlation matrix and quality of representation of the data. Both of the PCs had Eigenvalues of greater than 1 and when combined, they explained 60% of the total variance observed between the welfare indicators (Figure 4.24). The bearing of the welfare indicator variables contributing to each PC is shown in Table 4.14.

Table 4.14. Factor coordinates of the variables included in the PCs, based on correlations of the variables and factor axes from the correlation matrix.

Variable	PC1		PC2	
	Factor coordinates	Contribution	Factor Coordinates	Contribution
Condition Factor	-0.20	0.03	-0.62	0.31
Dorsal Fin Index	0.02	0.00	-0.43	0.15
Caudal Fin Index	0.20	0.03	-0.56	0.25
Plasma cortisol	0.75	0.42	-0.14	0.02
Haematocrit	0.28	0.06	0.59	0.26
Lysozyme activity	-0.78	0.46	0.08	0.01

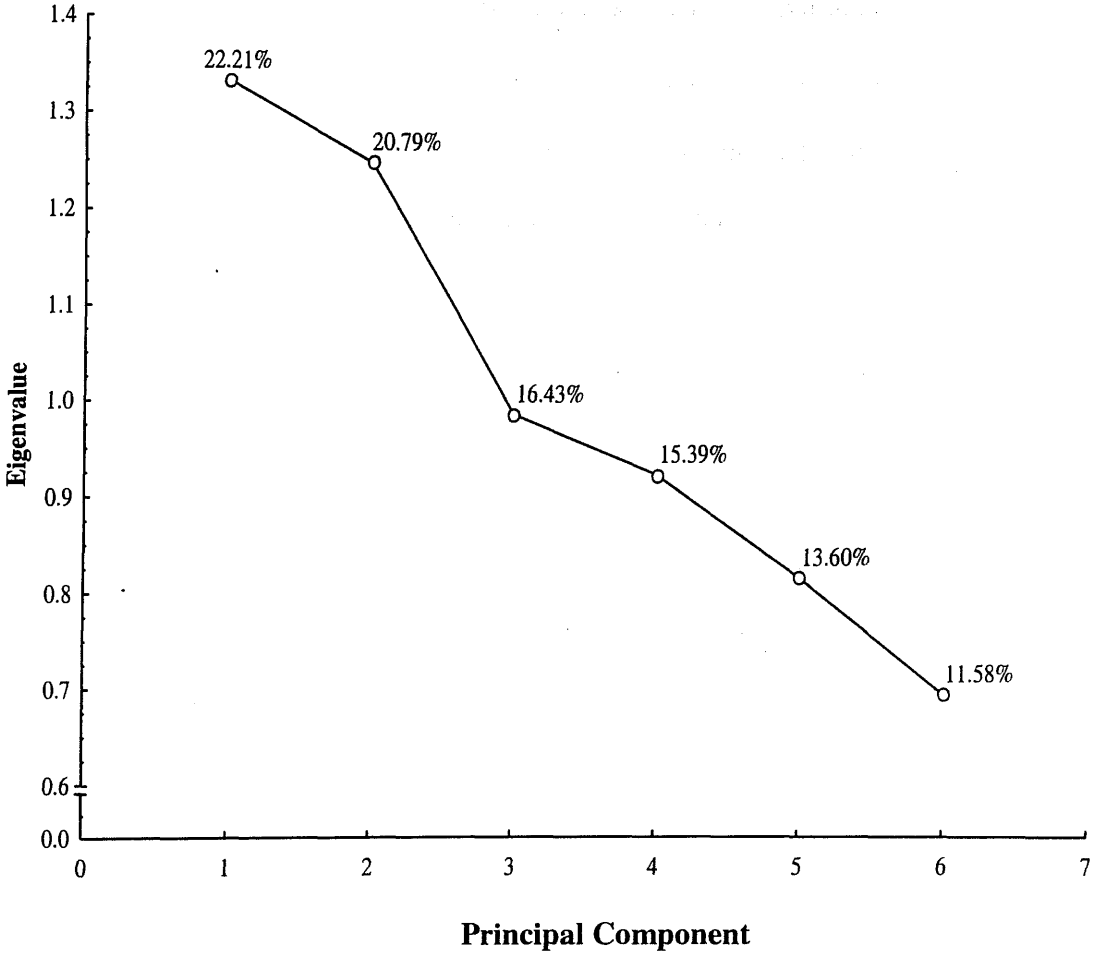


Figure 4.24. Eigenvalues of the correlation matrix and percentage of explained variance for principal components generated from indicators used to assess rainbow trout welfare.

The main variables contributing to PC1 were cortisol and lysozyme and the factor coordinates of these variables meant that a fish with high plasma cortisol, and low lysozyme activity would have relatively high coordinates, and this was interpreted as poor welfare status. Interpreting the factor coordinates for PC2 is more difficult, with strong, negative factor coordinates of CF, and the dorsal and caudal fin score variables, and positive coordinates for haematocrit. This would indicate that a fish with low dorsal and caudal fin scores (good fins) combined with low CF and high haematocrit would have a high factor score for PC2. Intact fins would intuitively be associated with good welfare, but so would a high CF, so in the case of PC2 it was difficult to interpret whether high factor coordinates should be associated with good or poor welfare. The possible implications of PC2 will be examined in greater depth in the discussion.

The relative contribution of the variables in each of the PCs is illustrated in Figure 4.25, based upon their factor coordinates. The position of a variable within the correlation circle indicated the bearing of its contribution on the observed factor score for each PC based upon the direction (+ve of –ve) and distance from the origin of the circle *e.g.* a fish with high cortisol and low lysozyme activity would have had a high factor score for PC1.

The individual factor scores for each of the PCs were used as dependent variables in GLMs that used stocking density and time as categorical predictors and dissolved oxygen as a continuous predictor; in all cases this was shown to be the model that most effectively described the data based on the adjusted R^2 values and distribution of residuals (Table 4.15).

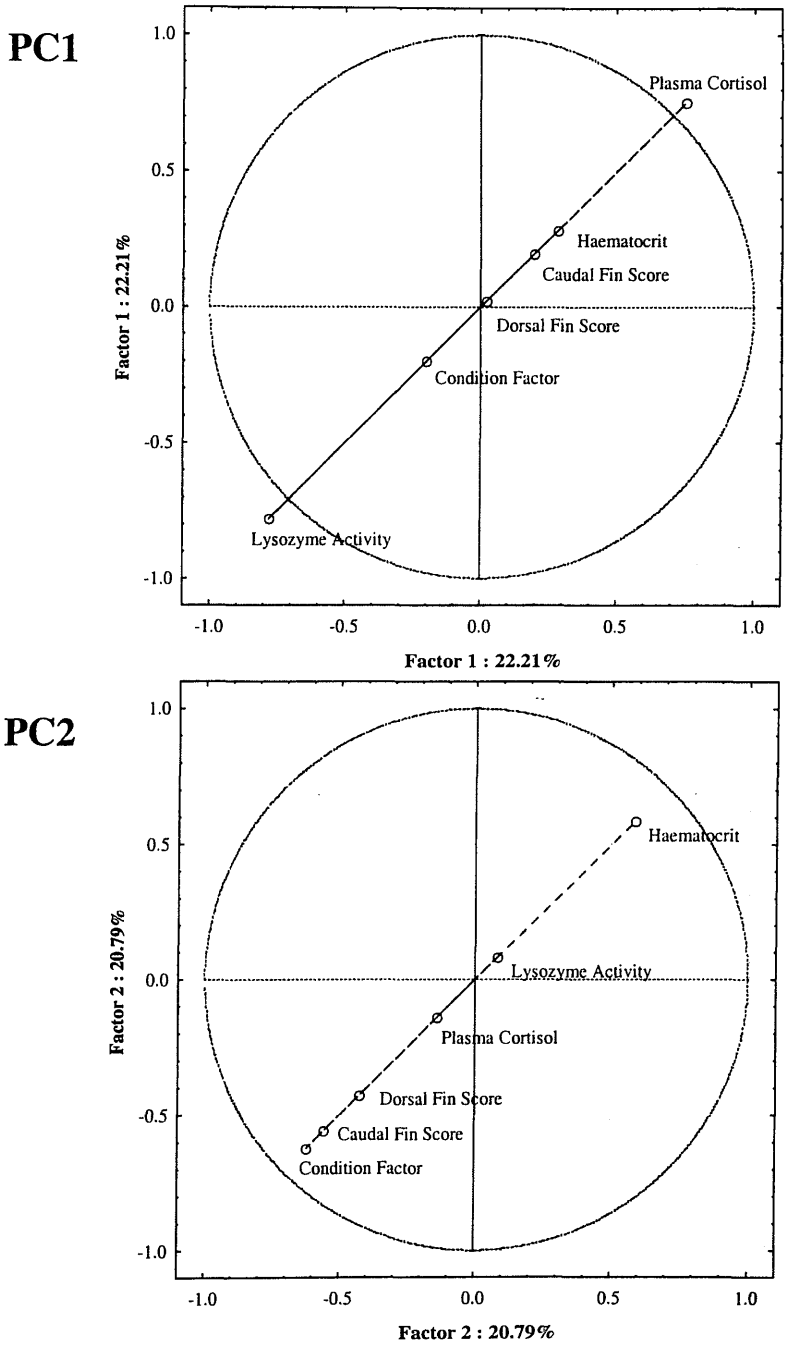


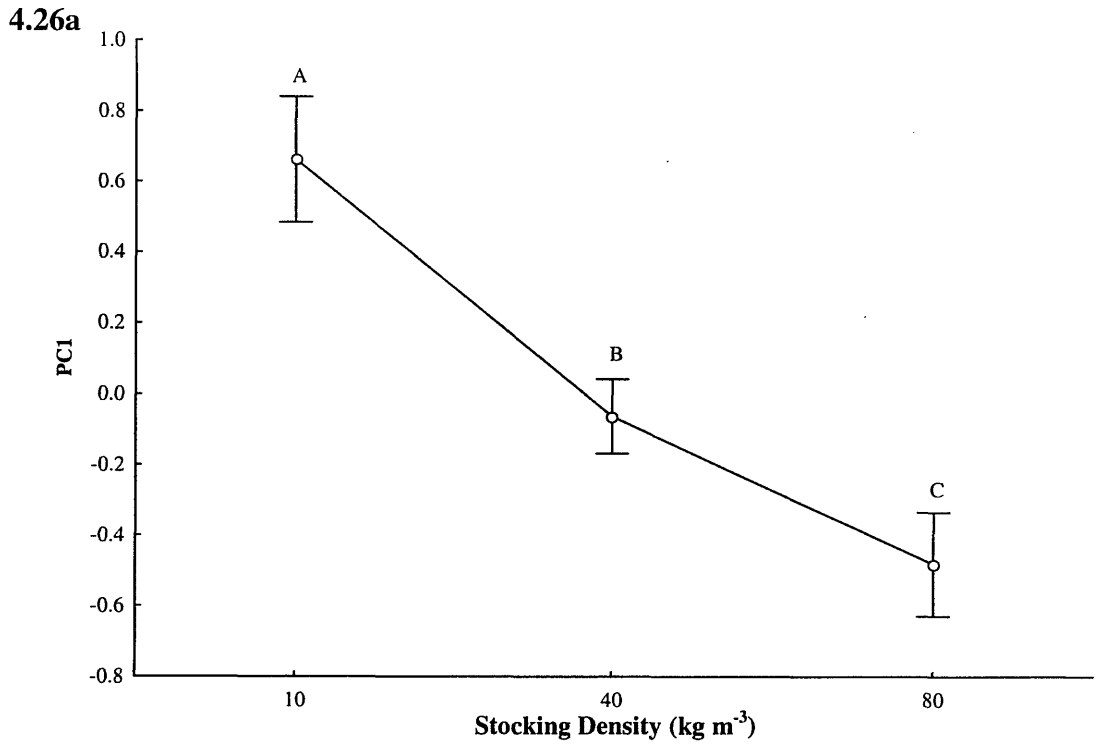
Figure 4.25. Relative contribution of welfare indicators to PC1 and PC2 illustrated through their positioning within the correlation matrix.

Table 4.15. Effects of time, stocking density and dissolved oxygen for GLMs using Principal Components of trout welfare as dependent variables.

	PC1	PC2
Whole model adjusted R^2	0.464	0.271
Whole model *P*	0.000*	0.000*
Dissolved oxygen	0.009*	0.092
Time	0.000*	0.000*
SD	0.000*	0.312
Time x SD	0.000*	0.141

PC1

The GLM detected a significant effect of SD on PC1, with an inversely proportional relationship between factor scores for PC1 and increasing SD (Figure 4.26a; $P<0.01$). The major variables contributing to PC1 were plasma cortisol (+ve) and lysozyme activity (-ve), and increasing factor coordinates of PC1 were interpreted as being indicative of poor welfare status. The decrease in PC1 with increasing SD confirmed the patterns observed earlier in the analysis of plasma cortisol and lysozyme activity. This further confirmed that fish at the lowest SD treatment were displaying signs of acute stress and possible suppression of the non-specific immune response. There was also a significant interaction between the effects of SD and time ($P<0.01$), with significant post-hoc differences detected between the 10 kg m^{-3} compared with the 40 and 80 kg m^{-3} treatments on 5 of the 7 months included in the PCA (Tukey's, $P<0.01$; Figure 4.26b). The GLM also detected a significant effect of DO on PC1 ($P<0.01$). However, it was unclear how DO contributed towards the higher factor score for PC1 at lower stocking density as DO was generally highest at low SD (Figure 4.4).



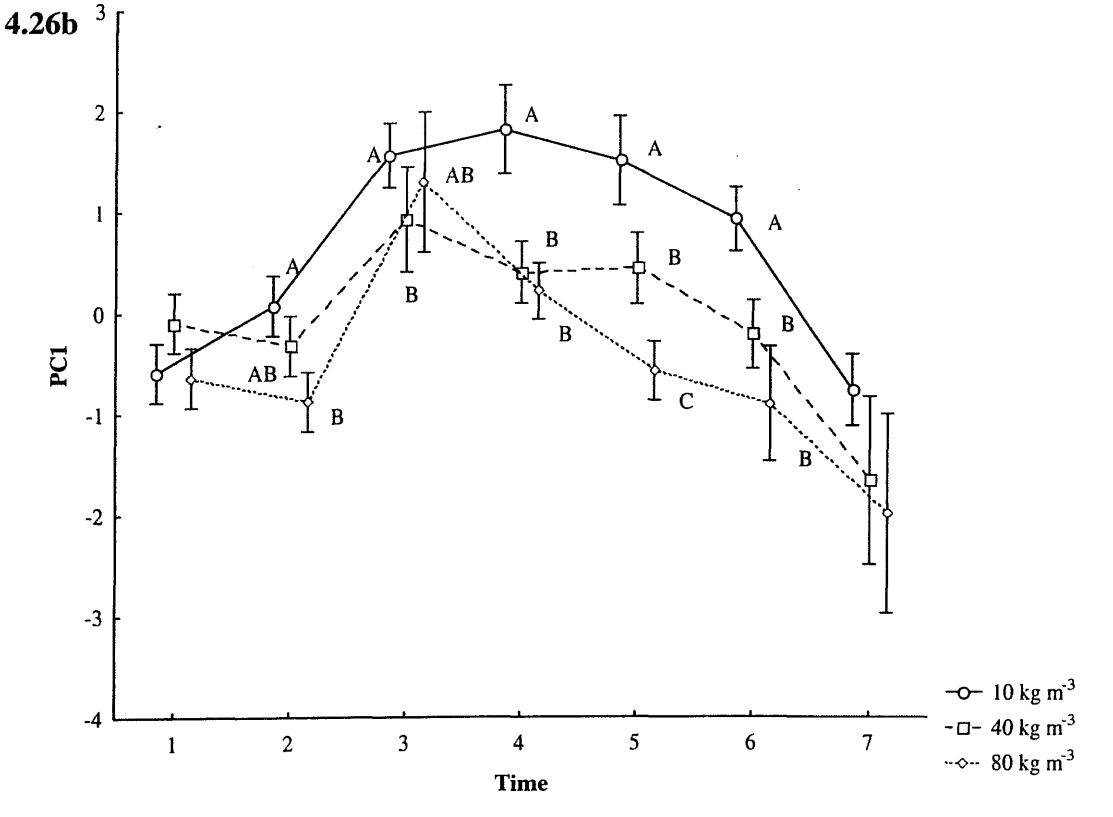


Figure 4.26. General linear model results for PC1; univariate results for stocking density (a), and stocking density when corrected for time (b). Columns not sharing a common letter are significantly different at that sample point (Tukey's, $P<0.01$)

PC2

There was no significant effect of SD on PC2 (Figure 4.27a), nor was there a significant interaction between the effects of SD and time (Figure 4.27b). The nature of variables contributing to PC2 showed a degree of conflict, with negative contributions from factors that could be associated with both good (*e.g.* CF) and poor welfare (*e.g.* fin erosion). Although the relationship was not significant, PC2 appeared to be lower in the 80 kg m^{-3} treatment compared with the 10 and 40 kg m^{-3}, treatments, and this may have been due to the increased fin erosion that was evident the highest density treatment. Additionally, it was likely that the lower CF observed in the 10 kg m^{-3} also contributed to this pattern.

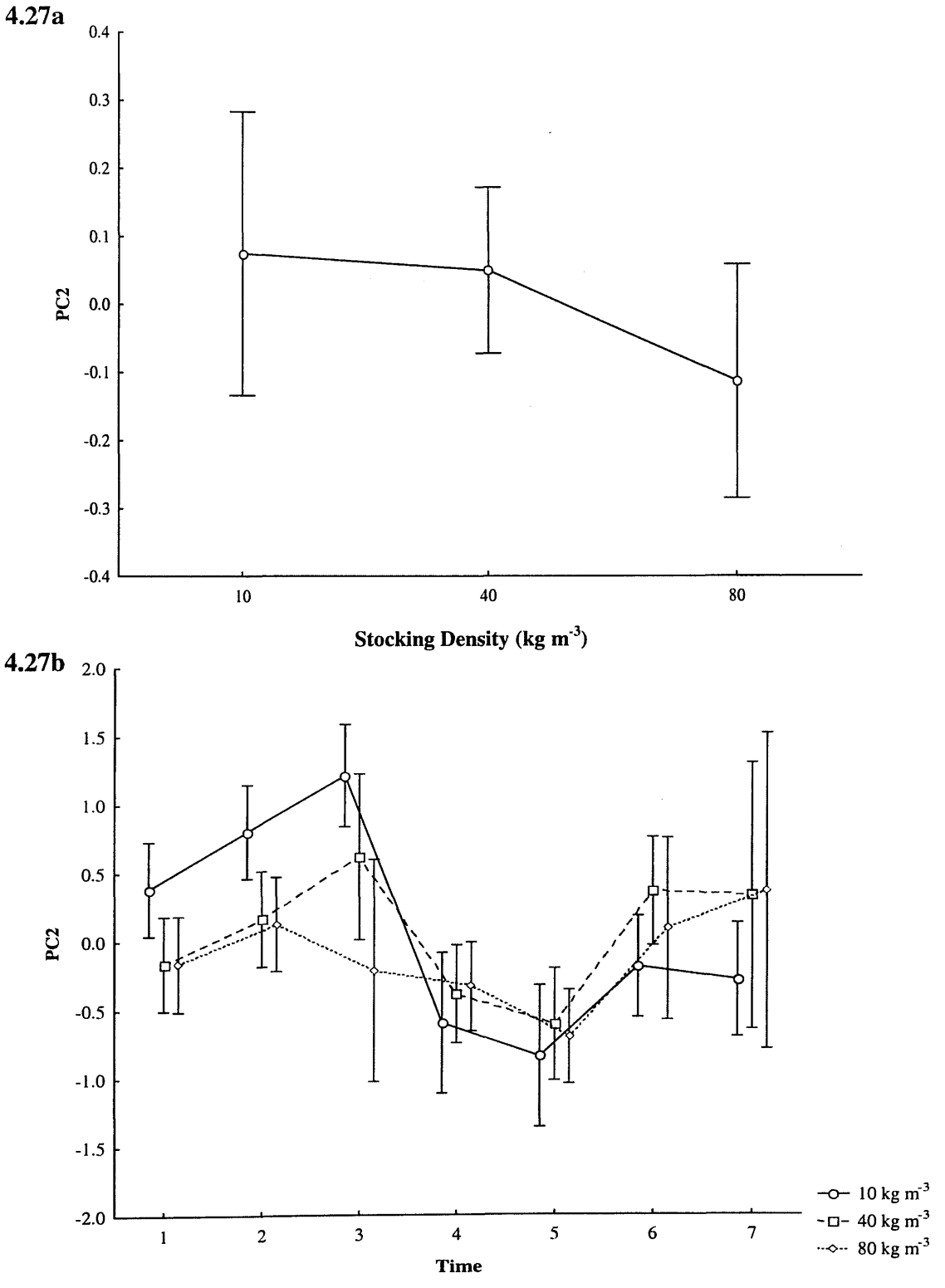


Figure 4.27. General linear model results for PC2; univariate results for stocking density (a), and stocking density when corrected for time (b).

4.2.3. Discussion

This experiment sought to investigate the effect of stocking density on rainbow trout welfare by applying a range of stocking densities reflective of low, medium and high-density commercial operations. The experimental design aimed to be representative of typical farming practices, but under controlled conditions. Through the course of this experiment mortality remained low and the performance of the fish in terms of FCR and SGR was reflective of industry norms (Westers 2001; Piper *et al.*, 1982).

Production

The majority of studies that have investigated the effects of increased SD on growth of rainbow trout have found a negative effect of increasing SD (Table 4.1). Similarly to the present study, 13 of the 43 studies that assessed the effect of SD on growth of rainbow trout were inconclusive (Piper, 1970; Collins, 1972; Soderberg *et al.*, 1983; Roell *et al.*, 1986; Pickering & Pottinger, 1987a; Rigolino *et al.*, 1989; Kebus *et al.*, 1992; Bagley *et al.*, 1994; Miller *et al.*, 1995; Bircan, 1997; Wagner *et al.*, 1996a; Winfree *et al.*, 1998; Sahin *et al.*, 1999), and just 1 study showed an adverse effect of low density on growth (Kindschi *et al.*, 1991a).

Some aspects of experimental design could explain the lack of association between SD and growth in some of the studies that observed no effect of increasing SD on growth. The experimental densities used in some of the studies were very low and it may have been that they were too low to elicit an effect on growth *e.g.* the highest SD treatments used by Roell *et al.* (1986) and Soderberg *et al.* (1983) were 4.5 kg m^{-3} and 0.71 kg m^{-3} respectively. Three of the studies that observed no effect of SD on growth were conducted in cages (Collins, 1972; Kebus *et al.*, 1992; Bircan, 1997; Sahin *et al.*, 1999), where the effect of water quality deterioration associated

with increasing SD may not have remained localised to cages with high SD. Some of the trials may have been too short for a significant effect of increased SD on growth to occur *e.g.* in a trial that lasted just 21 days, Pickering and Pottinger (1987a) suggested that a significant reduction in growth would have been observed in their highest SD treatment if the trial had progressed longer. Other studies specified that high levels of water quality were maintained through high inflow rates *e.g.* Miller *et al.* (1995) used inflow rates of 299-333 l min^{-1} in 4 m^{-3} raceway system and Bagley *et al.* (1994) achieved very high SD (478 kg m^{-3}) without observing an effect of SD on growth by adjusting inflow rates to ensure that DO remained above 8 mg l^{-1}. In the remaining studies there was either insufficient information provided regarding study conditions (Winfree *et al.*, 1998), or there was no clear explanation for the fact that no reduction in growth was observed (Piper, 1970; Rigolino *et al.*, 1989; Wagner, *et al.*, 1996a). The only study that reported reduced growth of rainbow trout at low SD was Kindschi *et al.* (1991a), in which growth was lower in a wild strain of rainbow trout in the lowest SD treatment. The authors attributed the reduced growth at the lowest SD to a poorer feeding response. The same study also reported the findings of a trial that was carried out concurrently with a domesticated strain of trout over the same range of SD, which observed reduced growth with increasing SD. The differential response to SD exhibited by the wild and domesticated strains of rainbow trout, suggests that mechanisms other than water quality deterioration could be responsible for observed differences in growth between studies.

In the present experiment there was no effect of SD on the mean weight within the treatments, but there appeared to be differences in the variance of weight observed within the SD treatments, with greatest variance observed in the 10 kg m^{-3}.

Size variation has been measured in 4 previous studies that investigated the effect of SD on rainbow trout (Kilambi *et al.*, 1977; Kindschi *et al.*, 1991a; Purser & Hart, 1991; Bagley *et al.*, 1994), but none of these studies reported reduced size variation at higher SD. Increased size heterogeneity (*e.g.* larger coefficient of variation of weight) has been suggested to indicate of the presence of dominance hierarchies (Jobling, 1995), where a hierarchy can be defined as comprising of a group of dominant individuals at the top of the hierarchy, followed by a number of subordinants and, thereafter, a number of subordinates with low rank positions (Symons, 1970). The greater size variation in the 10 kg m^{-3} treatment was possibly due to the presence of a hierarchy.

Bagley *et al.* (1994) suggested that aggression is generally highest at intermediate SD, and that the formation and maintenance of hierarchies must become exceedingly difficult at high stocking densities; possibly the situation in the present study in the 40 and 80 kg m^{-3} treatments. This has important implications for production, and it may be that increasing SD could be used as method to reduce variation arising from hierarchical interactions. The implications of the hierarchy are discussed further in relation to cortisol levels, behaviour and the PCA.

Cortisol

The high levels of cortisol observed in the 10 kg m^{-3} treatment were perhaps the most unexpected result of this experiment and also the most difficult to interpret. Examination of potential confounding effect/s of factors other than stocking density failed to provide alternative explanation. Seven previously published studies have used plasma cortisol as an indicator of stress in experiments investigating the effects of stocking density on rainbow trout, but just two of these found higher levels of

cortisol at increased SD (Leatherland & Cho, 1985; Procarione *et al.*, 1999). Leatherland & Cho (1985) found significantly higher levels of plasma cortisol in rainbow trout reared at 134 kg m^{-3} compared with 210, and 277 kg m^{-3} (approximately 65 vs. 18 and 9 ng ml^{-1} respectively measured 4h after last feeding). If the increased cortisol in the lowest SD treatment was a genuine treatment effect rather than an artefact of experimental design (the study was not replicated, carried out in 60 l aquaria, and just 10 fish were sampled for cortisol per treatment), it was unlikely that it was caused by the same mechanism as the present study as the lowest final stocking density in Leatherland and Cho's study was 134 kg m^{-3}.

In a 24 h sampling regime of cortisol in rainbow trout reared at different densities, Procarione *et al.* (1999) observed highest levels of cortisol in fish kept at the lowest density. The authors expressed surprise at these results as the lowest density treatment displayed the best growth and FCR, but was the only treatment that showed signs of stress (elevated glucose and reduced chloride levels in addition to the elevated cortisol).

Further evidence for increased cortisol at low SD is provided from studies where significant increases in cortisol were observed in trout that were kept in pairs (Laidley & Leatherland, 1988; Pottinger & Pickering, 1992). The authors suggested that this was likely to have been a result of extreme behavioural interaction, with one fish becoming dominant over the other. The majority of studies measuring cortisol as an indicator of stress in rainbow trout have found no effect of stocking density (Table 4.2).

There is little evidence in the literature to support a negative effect of increased SD on plasma cortisol in rainbow trout, or to provide an explanation for the high levels of cortisol observed in 10 kg m^{-3} treatment in this study. There are,

however, reports of similar observations for other salmonid species. A study that investigated the effects of stocking density on growth and stress response in brook charr at a similar range densities to those used in this study (30, 60 & 120 kg m^{-3}) found an inverse linear relationship between cortisol and density (Vijayan & Leatherland, 1988). The authors suggested that the lower levels of cortisol at higher densities may have been an adaptive response to a chronic stressor and cited Pickering and Pottinger's (1987a) observations of initial transient increases in cortisol followed by a return to 'baseline' levels within several days of exposure to sustained high density. Another explanation offered by Vijayan and Leatherland was that the differences in observed levels of cortisol were reflective of the metabolic needs of the fish, and they suggested that the highest levels of cortisol were linearly correlated with the fastest growing fish. However, this theory is not supported by the results of this trial, as there was a negative correlation between mean cortisol and SGR (R = -3.80, R^2 = 0.144, P<0.001; Figure 4.17).

Vijayan and Leatherland (1990a) also found that preparations of the head kidney of brook charr that were acclimated to high stocking density (120 kg m^{-3}) had higher levels of resting cortisol secretion than fish acclimated to 30 kg m^{-3}. A similar result has also been reported for coho salmon (*Oncorhynchus kisutch* Walbaum, 1792), where the same methodology showed higher resting cortisol secretion from interrenal tissue from fish held at high stocking density (Patiño *et al.*, 1986). These *in vitro* findings suggested that interrenal cells of salmonids maintained at high stocking density are spontaneously active and that the liver may play a role in the metabolic clearance rate (MCR) of cortisol, resulting in the apparent acclimation of plasma cortisol levels from tissue measurements of fish maintained at high stocking densities (Vijayan & Leatherland, 1990a). Further evidence to support the theory of apparent

acclimation of the cortisol response to continued exposure to a stressor comes from a study that investigated the effects of chronic cortisol administration and daily exposure to acute handling stress (Barton *et al.*, 1987), where the authors suggested that the capacity of fish to elicit an interrenal response to additional stressors is reduced through continuous negative feedback of cortisol on the HPI-axis.

There is some evidence for reduced plasma cortisol in fish reared at high stocking densities, but it is unclear if this is a result of behavioural acclimation, interrenal exhaustion, increased MCR, or through regulation of the HPI-axis as a result of down-regulation of ACTH receptors or modification of ACTH levels. In a comprehensive review of cortisol in teleost fish, Mommsen *et al.* (1999) discussed some of these points and also questioned the validity of quantifying cortisol and its effects based solely on measurement of plasma cortisol concentrations. The authors noted that plasma cortisol concentrations reflect the net effect of production and clearance of the hormone and that this is dependent upon binding proteins, target tissue receptors and catabolism of cortisol and highlight the importance of understanding the regulatory factors that modulate cortisol and the physiological responses that it elicits.

The present study observed peaks in cortisol during the winter months, with the cortisol concentrations peaking in the 80 kg m^{-3} treatment in February, and the 10 and 40 kg m^{-3} treatments in March (Figure 4.13). No such seasonal pattern of cortisol levels is previously reported for rainbow trout. A similar seasonal change has been reported in juvenile Atlantic salmon (Thorpe *et al.*, 1987), but the authors suggested that the peak levels (46 ng ml^{-1}) observed in March were likely to have been due to behavioural and physiological responses to the smoltification process.

Haematocrit

Haematocrit was significantly higher in the 10 kg m^{-3} treatment in December and January, but at all other times no significant differences were observed. The nature of the effect of SD on haematocrit was unclear *i.e.* haematocrit may have been elevated in the 10 kg m^{-3} treatment, indicating an acute stress response, or reduced in the other treatments, possibly suggesting anaemia. Just one of the seven studies that measured the effect of SD on haematocrit in rainbow trout found an effect of increased SD (Table 4.2), where Wagner *et al.* (1996a) concluded that haematocrit was elevated in the high SD treatment. Cortisol was also significantly elevated in the 10 kg m^{-3} treatment in January ($P<0.001$), which supports the suggestion for a stress mediated increase in haematocrit. However, if this was the case it remains unclear why there was no corresponding elevation of haematocrit in February, March or April when cortisol was also significantly elevated in the 10 kg m^{-3} treatment.

Lysozyme activity

Although there are no previous reports of lysozyme activity being used as an indicator of crowding stress, it has been measured in conjunction with cortisol in numerous previous studies (Demers & Bayne, 1997; Fevolden *et al.*, 1991, 1992, 1994, 1999, 2002; Fevolden & Røed, 1993; Muona & Soivio, 1992). Direct comparisons with previously published data are hindered by differences in methodology and the lack of a universally recognised unit, although it is possible to make general comparisons of the direction of change in response to a stressor.

The present study found significant effects of SD on lysozyme activity at certain time points during the trial, but these were not consistent and interpretation was complicated by a strong effect of water temperature. Although not always

statistically significant, there was a trend for lysozyme activity to be highest in the 80 and lowest in the 10 kg m^{-3} treatment. Previously published data is slightly conflicting, but there is evidence to suggest an inversely proportional relationship between lysozyme activity and plasma cortisol concentrations (Fevolden *et al.*, 2002). A linear regression of cortisol concentration against lysozyme activity in the present study showed there to be a significant negative correlation ($P<0.01$; Figure 4.18). This relationship was further confirmed in the PCA, where the main factors contributing to PC1 were cortisol (positive factor coordinates) and lysozyme activity (negative factor coordinates), so that a fish with high cortisol and low lysozyme activity had a high factor score for PC1. Corticosteroids are known to be potent immunosuppressants (Pickering, 1984; Barton *et al.*, 1987; Wedemeyer, 1996) and this could explain the negative correlation observed between cortisol and lysozyme activity.

An alternative explanation for the differences in lysozyme activity between the SD treatments could be that lysozyme activity was elevated in the higher SD treatments as a result of stimulation of the immune system in response to the higher levels of fin erosion observed with increased SD. The fin index scores for the dorsal and caudal fins of the 30 fish that were blood sampled each month from the treatments was insufficient to confirm this relationship; if RFL had been calculated for these fish it may have been possible to confirm a relationship between fin damage and lysozyme activity.

Fin Condition

Perhaps the most consistently reported effect of stocking density on rainbow trout is an increased prevalence of fin erosion at higher densities. The lower RFL in the 40 and 80 kg m^{-3} treatments observed in this study is in accord with previously published

studies (Boydstun & Hopelain, 1977; Mäkinen & Ruohonen, 1990; Purser & Hart, 1991; Bosakowski & Wagner, 1994a; Miller *et al.*, 1995; Winfree *et al.*, 1998). Although fin damage is generally accepted to increase with increasing SD, the exact cause is unknown, although suggestions include abrasion against the sides of rearing units or conspecifics, aggressive nipping, accidental nipping during feeding, handling, poor water quality, and pathogen infection (Abbot & Dill, 1985; Kindschi, 1987; Bosakowski & Wagner 1994a, 1994b).

Two different methods were used to quantify fin damage in this study and both methods of fin assessment found increased fin damage with increased SD. Both systems found the dorsal and caudal fins in the 40 and 80 kg m^{-3} treatments to be significantly smaller than those in the 10 kg m^{-3}. Neither method found any difference between caudal fins in the 40 and 80 kg m^{-3} treatments, perhaps suggesting that a threshold density was passed somewhere between the 10 and 40 kg m^{-3}, but that after passing this threshold density, the level of damage plateaued.

The two methods of fin assessment yielded slightly different results for dorsal fin score, with no treatment effect apparent at the end of the trial using the fin index system, but significant differences between treatments using the RFL measurements; this is likely to be a reflection of the increased statistical power of continuous vs. categorical data. The RFL system was also used to assess pectoral fin damage and found a cumulative effect of stocking density resulting in reduced RFL of pectoral fins. There was also the unexpected result of lower RFL scores for the left pectoral fins compared with the right pectoral fins in the 40 and 80 kg m^{-3} treatments.

These results suggest that different fins may be differentially prone to distinct types of physical damage due to their position. The different methods of fin damage assessment produced different results, with the RFL measurements appearing to be

the more sensitive of the two methods; a conclusion also drawn by Bosakowski & Wagner (1994a). However, the fin index system did have an advantage over the RFL system in that it permitted observations of large numbers of fish to be made through the course of the trial, something that would not have been possible using the more laborious RFL method.

Abbot and Dill (1985) reported that dorsal fin damage in juvenile steelhead was primarily a function of aggressive nipping, and the same observation has been reported for juvenile Atlantic salmon (Turnbull *et al.*, 1998). This is logical, as the positioning of the dorsal fin would make damage as a result of abrasion against tank surfaces unlikely. The increased dorsal fin damage at 40 and 80 kg m^{-3} compared with 10 kg m^{-3} could have been due to the higher numbers of fish, resulting in an increased potential for aggressive or accidental damage through nipping, though this does not explain why there was not an increase in dorsal fin damage at 80 compared with 40 kg m^{-3} when there were twice as many fish present. A possible explanation could come from a previously published study that showed size heterogeneity reduced aggression in Atlantic salmon parr (Adams *et al.*, 2000). The greater size variation in the 10 kg m^{-3} treatment (Figures 4.22 & 4.23) may have resulted in a similar situation where the presence of a few larger dominant fish resulted in a mean reduction of aggressive interactions within the population.

The positioning of the pectoral fins would suggest that they would be susceptible to contact with tank surfaces, and this is supported by findings of Turnbull *et al.* (1998), who inferred abrasion against tank surfaces as the cause of observed damage to pectoral fins of Atlantic salmon reared in isolation. The fact that there appeared to be an additional effect of water current direction on the extent of the damage to the pectoral fins in this study may offer clues to the cause of the damage.

The left pectoral fin was significantly smaller than the right pectoral in the 40 and 80 kg m^{-3}; if the fish predominantly swam against the clockwise water current, this corresponded to the inside fin, ruling out perhaps the most obvious explanation of abrasion against the outside wall of the tank.

There are no similar reports of such fin erosion in the literature so the cause of this damage is purely conjecture. If we assume that the fish spent majority of their time schooling into the direction of water current, the motion would be coming from the oscillation of the fish's body with the caudal fin providing the propulsion. The pectoral fins would be used to provide direction and as the tanks are round it might also be assumed that the outside fin would be held close to the body while the inside fin would be extended to provide the turning circle while, making it a obvious target for aggressive or accidental nipping or more likely to come into contact with the bottom surface of the tank or other fish. The asymmetric damage was only evident in the 40 and 80 kg m^{-3} treatments, suggesting that both direction of flow and stocking density contributed to the damage. The apparent contribution of these two factors suggested that the erosion may be a function of sub-optimal positioning of the fish within the tank *i.e.* more fish were forced to occupy space close to the bottom of the tank resulting in abrasion against the surface of the tank floor at higher stocking densities.

Behavioural interactions

Although it was not possible to carry out detailed behavioural observations of the fish in this study, there are several previously published studies that have attempted to assess the effects of stocking density on behaviour of salmonids. It has been suggested that Arctic charr grow better at higher SD as a result of decreased social interactions

(Wallace *et al.*, 1988; Baker & Ayles, 1990; Brown *et al.*, 1992). At high SD, Arctic charr have been shown to spend more time shoaling, with fewer aggressive interactions (Brown *et al.*, 1992). Alanära and Brännäs (1996) demonstrated reduced bite activity (triggering of demand feeders) of top-ranking fish in both rainbow trout and Arctic charr with increased SD and suggested that the ability of dominant fish to monopolise demand feeders was reduced at higher SD.

Jobling (1985) proposed that short-term bouts of aggression associated with feeding can lead to reduced feed intake and subsequent reduced growth by certain fish. An alternative suggestion for reduced growth at lower SD in Arctic charr is increased energy expenditures as a result of increased aggressive behavioural interactions (Brown *et al.*, 1992).

Although dominance hierarchies are relatively well studied in salmonids, the implications of hierarchies in terms of fish welfare are rarely considered beyond the aspect of reduced growth. One of the few exceptions is a study that assessed the physiological effects of dominance hierarchies on brown trout (Sloman *et al.*, 2000). The authors suggested that second-ranked individuals occupied the least beneficial position within the hierarchy, based on a reduced CF compared with increased CF in the first-ranked (dominant) and third-ranked (subordinate) fish over the same duration.

Hierarchies are not just associated with poor welfare and there may also be some beneficial aspects implications to fish welfare. Increased size heterogeneity was shown to be beneficial in Atlantic salmon parr, whereby the introduction of a few larger individuals resulted in reduced aggression and significantly higher growth rate (Adams *et al.*, 2000). Similarly, Brännäs *et al.* (2002) observed lower incidences of

aggressive interactions in unsorted groups of Arctic charr compared with groups of size-sorted large and intermediate sized individuals.

The Five Freedoms makes specific reference to freedom from hunger and malnutrition, and in the case of a subordinate fish, access to food may be restricted by dominant fish. Other potential welfare freedoms that could be affected by dominance hierarchies include freedom from pain, injury and disease (fin nipping) and hypothetically, freedom from fear and distress.

Principal Components Analysis

The PCA was used as a final stage of the data analysis to identify underlying coherence that existed within the dataset. Two PCs were identified from the PCA. The first PC (PC1) confirmed the negative correlation that was observed between cortisol and lysozyme activity (PC1). When the factor scores for PC1 were include in a GLM with time, SD and DO, there were significant differences between the 10 kg m^{-3} treatment and the other SD treatments.

The bearing of contribution of the variables in PC2 was initially unclear, with seemingly contradictory contributions of low scores for dorsal and caudal fin indices (good fins) and low condition factor and high haematocrit. However, during the experiment small fish that with visibly poor body condition, but almost perfectly intact fins were occasionally sampled. It may have been that PC2 was reflecting these individuals, which represented subordinate fish within the experimental populations. If these subordinate individuals were not actively competing for food, nor attempting to occupy a premium position within the tanks, it may have been that they were able to avoid fin damage. These fish were generally more prevalent in the 10 kg m^{-3}

treatment, but when the factor scores for PC2 were include in the GLM, the only significant effect was of time.

A final discussion point of the GLM results for the PCs was the significant effect of time on both models. There was a clear seasonal effect of time, with PC1 peaking between January and March (time points 4, 5 and 6), while PC2 was lowest during February and March. High factor coordinates for PC1 were interpreted as indicating poor welfare status and the same was true for low factor scores for PC2, suggesting a consistent effect of reduced welfare status during the winter months.

Problems were encountered when attempts were made to incorporate water temperature into the GLMs, due to the lack of variation that existed within the temperature data between the treatments, but it is probable that some of the significance that was attributed to time in the models was a result of temperature fluctuations that may not have been present if the trial had been carried out at a constant water temperature. As poikilotherms, the physiology of rainbow would be expected to be influenced by water temperature, especially in temperate regions where large seasonal fluctuations exist. In the present study, fluctuations in ambient water temperature exacerbated the observed variation of the welfare indicators, which were already subject to considerable intra-specific variation. This highlights the difficulty of specifying acceptable (or even 'normal') levels of welfare indicators.

In summary, the present study found no effect of increased SD on growth, possibly because good water quality was maintained throughout the trial and that critical thresholds for DO and/or NH_3 were not exceeded. The increased size variation, coupled with elevated cortisol in the 10 kg m^{-3} treatment suggested that there may be welfare implications of low as well as high SD. There an increased level of fin damage observed with increasing SD.

Chapter 5: The effects of water exchange rate on the growth and welfare of rainbow trout

5.1 Introduction

In addition to their spatial needs, fish are also dependent upon water for both provision of their oxygen and removal of the waste products of their metabolism (ammonia, CO_2, suspended solids). Stocking density and water quality deterioration are interrelated, and in a given volume of water the supportable biomass of fish will be proportional to the requirements for oxygen and the products of metabolism. Ellis *et al.* (2002) concluded that some of the studies that attempted to examine the effects of SD may have overlooked or underestimated the confounding influence of water quality deterioration.

This introduction will review aspects of the water quality that are of particular importance when considering SD and rainbow trout culture. Topics covered include a summary of the recommended 'safe' limits for key water quality parameters for salmonid culture, the physical, biological and chemical interactions that occur between these parameters, and the mechanisms by which exceeding these limits can result in poor fish welfare. This introduction will also review key studies that have taken alternative experimental approaches to simulate or eradicate deterioration of water quality caused by increased SD.

Managing water quality is acknowledged to be one of the most important ways of reducing stress and susceptibility to disease in fish husbandry (Wedemeyer, 1996). A summary of thresholds for the key water quality parameters and the generally accepted requirements for salmonid culture are presented in Table 5.1. Although these

levels provide a useful guideline, it is worth noting that they have been derived mainly from acute and chronic toxicity testing and do not necessarily reflect the optimum conditions for safeguarding fish welfare.

Table 5.1 Water chemistry limits recommended to protect the health of salmonids in intensive fish culture (abridged from Wedemeyer, 1996).

Parameter	Recommended limits
pH	pH 6-9
Alkalinity	>20 mg l^{-1} (as $CaCO_3$)
Ammonia (un-ionised)	<0.02 mg l^{-1}
Gas supersaturation	<110% total gas pressure (103% salmonid eggs/fry)
Nitrate (NO_3^-)	<1.0 mg l^{-1}
Nitrite (NO_2^-)	<0.1 mg l^{-1}
Oxygen	6 mg l^{-1}
Total dissolved solids	<200 mg l^{-1}
Total suspended solids	<80 mg l^{-1}

5.1.1 Oxygen

Dissolved oxygen (DO) is perhaps the most important limiting factor associated with fish production. DO is typically expressed as either mg l^{-1} or as percent of saturation, where saturation refers to the amount of a gas dissolved when the aqueous and atmospheric phases are in equilibrium (Piper *et al.*, 1982). At higher altitudes and, more importantly, higher temperatures, the amount of oxygen that can be dissolved in a given volume of water decreases and DO can become limiting. Low DO levels are also often attributed to increased oxygen consumption as a result of increased activity in anticipation of, during, and in the h following feeding (Kindschi *et al.* 1991b).

The minimum recommended DO concentration of for rainbow trout culture is typically in the range of 5-6 mg l^{-1} (Brett, 1979; Piper *et al.*, 1982; Colt & Watten,

1988; Wedeyemer, 1996). Failure to maintain DO above these lower limits can lead to the fish having a reduced tolerance to ammonia (Thurston *et al.*, 1981), increased probability of disease (Sniesko, 1974), and adverse effects on growth and overall survival rates (Kindschi *et al.*, 1991a). These problems associated with low DO can be alleviated by increasing the oxygen concentration in the water. Increasing water exchange rate is usually the simplest way to increase DO, but if this is not a viable option, pure oxygen can be injected directly into the water, or water can be aerated using equipment such as paddle wheels or diffusers.

Several recent studies have used supplemental oxygen to rear trout at high densities (Duolos & Kindschi 1990; Kindschi *et al.*, 1991a; Kindschi *et al.*, 1991b; Miller *et al.*, 1995). These studies have demonstrated the ability to achieve high stocking densities (294 kg m^{-3} in Kindschi *et al.*, 1991a), increasing the overall carrying capacity of a system. Laks and Godfriaux (1981) went so far as to suggest that trout benefited in terms of growth when reared in oxygen-supersaturated water.

Miller *et al.* (1995) found no significant differences in health or condition indices between fish reared at differing densities with oxygen supplementation, though the authors cited slightly decreased growth, increased fin erosion and the need for increased supervision as possible drawbacks for high density culture. Doulos and Kindschi (1990) highlight a further potential drawback of rearing salmonids in water supersaturated with oxygen, namely, gas bubble disease. However, despite observing gas bubble disease in 94% of cutthroat trout (*Oncorhynchus clarki* Richardson, 1836) reared in raceways with oxygen supersaturated water (172%), no cases of gas bubble disease were observed in rainbow trout cultured with slightly lower supersaturation (150%). Comparison of fin quality, growth and feed conversion between supersaturated groups with control fish held in water at or below saturation, found no

differences for either species (Doulos & Kindschi, 1990). There are mixed reports regarding the effect of SD on oxygen consumption (usually calculated in terms of grams consumed per kg of fish). Medland and Beamish (1985) and Miller *et al.* (1995) both observed increased oxygen consumption (mg kg^{-1} h^{-1}) at higher stocking densities compared with controls, which they attributed to increased activity levels and energy expenditure at higher SD. However, no density-related differences in oxygen consumption were reported by Kindschi *et al.* (1991a). Oxygen consumption, per unit body weight, is reported to decrease with increased size of fish (Piper *et al.*, 1982; Kindschi *et al.*, 1991a), although Miller *et al.* (1995) observed no such correlation. There is limited information regarding strain affects on oxygen consumption in rainbow trout, although Kindschi *et al.* (1991b) found no differences in oxygen consumption between wild and domesticated strains over periods longer than 24 h.

Culturing rainbow trout at high stocking densities with supplemental oxygen has been shown to be both possible and economically viable, but doing so increases the risk of mortality due to equipment failure and requires the provision of increased supervision and appropriate backup equipment (Kindschi 1991a; Miller *et al.*, 1995). It is possible that the decreased growth that has generally been observed in some studies at higher stocking densities with supplemental oxygen was a reflection of other factors such as accumulation of ammonia or carbon dioxide and/or behavioural interactions rather than a deleterious effect of the oxygen *per se*.

5.1.2. Carbon Dioxide

Satisfying the need for oxygen has lead to the identification of other limiting factors for production that were previously considered less important e.g. carbon dioxide

accumulation (Summerfelt, 2000). High concentrations of CO_2 may result in a reduced capacity of the haemoglobin to transport oxygen, formation of calcareous deposits in the kidneys (nephrocalcinosis), and blood acidosis (Colt & Watten, 1988; Wedemeyer, 1996; Summerfelt, 2000). Dissolved CO_2 of 20 mg l^{-1} will begin to impair blood oxygen transport and at concentrations of 30-40 mg l^{-1}, oxygen carrying capacity may become reduced to a point where even high environmental dissolved oxygen concentrations may be insufficient to prevent decreased blood oxygen levels (Wedemeyer, 1996).

Colt and Watten (1988) and Wedemeyer (1996) both suggested that CO_2 concentrations should be maintained below 10-20 mg l^{-1} in culture systems for salmon and trout. However, these estimates may be conservative, as Heinen *et al.* (1996) suggested a safe upper limit for chronic exposure to CO_2 of 30 mg l^{-1}, and Smart *et al.* (1979, *cited in* Smart, 1981) found no significant effect on fish growth or feed conversion until CO_2 concentrations approached 55 mg l^{-1}.

There is a lack of information in specific regard to CO_2 and SD though logic would dictate that CO_2 production mirrors O_2 consumption, so there will be an increased risk of accumulating harmful levels of CO_2 at higher stocking densities, especially at low rates of water exchange. The potential for CO_2 levels to increase will be substantially increased in multi-pass commercial facilities, where strategically positioned oxygen injection units enable water to be re-used through numerous holding systems; this will be discussed in greater detail in Chapter 6.

The toxicity of CO_2 is affected by environmental factors such as DO, temperature and pH. Low DO increases the toxicity of CO_2, but increasing water temperature decreases the solubility of CO_2 (Wedemeyer, 1996). The effect of increased pH on toxicity of carbon dioxide is illustrated in the following equation,

whereby, below pH 5 most dissolved carbon dioxide exists as CO_2; between pH 7-9 as non-toxic HCO_3^-; and above pH 11, as the CO_3^{-2} ion (Wedemeyer, 1996):

$$CO_2 + H_2O \leftrightarrow H_2CO_2 \leftrightarrow H^+ + HCO_3^- \leftrightarrow H^+ + CO_3^{-2}$$

5.1.3. Nitrogenous Waste

5.1.3.1. Ammonia

In aqueous solution, ammonia exists as either un-ionised (NH_3) or ionised (NH_4^+) ammonium ions. The proportion of ammonium existing as NH_3 increases with pH and water temperature and can be interpreted from ionisation tables (e.g. Piper *et al.*, 1982).

$$NH_3 + H^+ + OH^- \quad \rightleftharpoons \quad NH_4^+ + OH^-$$

Ammonia is the end product of protein catabolism and is the primary nitrogenous metabolite excreted by fish. The main pathway for ammonia excretion in fish is across the gills; by passive diffusion for NH_3, or active NH_4^+/Na^+ exchange (Randall & Wright, 1987). Un-ionised ammonia is much more toxic to fish than NH_4^+ (Alabaster & Lloyd, 1980; Smart, 1981); Thurston *et al.* (1981) speculated that NH_3 is 300-400 times as toxic as NH_4^+. It is suggested that the difference in toxicity may be due to the fact that NH_3 will readily diffuse across the gill membrane, whereas the ionised form will not; this is especially relevant in situations of acute toxicity (Smart, 1975). Levels of NH_4^+ in the blood of the fish have been shown to increase if the levels in surrounding water are elevated and it is thought that excretion may become inhibited (Fromm & Gillette, 1968). Even though NH_3 is more toxic than NH_4^+, under water quality conditions suitable for trout farming, the proportion of the total ammonia nitrogen (TAN) in solution that exists as NH_4^+ is always much greater than

NH_3. For example, even at the upper limits of temperature and pH recommended for salmonid culture (16°C and pH 8.5; Council of Europe, 2002) only 11% of TAN will exist as NH_3.

The recommended upper limit of NH_3 for fish culture is around 0.02 mg l^{-1} (Wedemeyer, 1996), and the 96 h LC50 (lethal concentration resulting in 50% mortality of exposed fish over a 96 h period) for rainbow trout is 0.60 mg l^{-1} (Thurston & Russo, 1983). One of the most common sublethal effects of ammonia is gill damage (Rosenthal *et al.*, 1984; Soderberg *et al.*, 1984). Other sublethal toxic effects of ammonia on rainbow trout include kidney damage (Thurston *et al.*, 1984), behavioural changes indicative of neurological dysfunction (Daoust & Ferguson, 1984), deleterious effects on survival and development of eggs and fry (Burkhalter & Kaya, 1977), increased fin erosion (Bosakowski & Wagner, 1994b), increased ventilation frequency (Lang *et al.*, 1987), and decreased growth and feed conversion efficiency (Brauhn *et al.*, 1976).

In addition to the effect of pH and temperature on the relative proportion of un-ionised ammonia, other factors such as salinity, free CO_2, and dissolved oxygen can also affect ammonia toxicity (Wedemeyer, 1996). Ammonia production is highly variable over a 24 h period and is affected by factors such as the time of day, feeding and water temperature. Wagner *et al.* (1995) observed fluctuations of peak NH_3 concentration up to 490% higher than baseline concentrations. Another effect of elevated ammonia on rainbow trout is reported by Möck & Peters (1990), who observed a decreased level of lysozyme activity at 36 h following a chance pollution incident in which two rearing ponds were contaminated with liquid fertiliser. Levels of NH_3 increased from 0.005 mg l^{-1} up to 0.229 mg l^{-1} in one pond and 0.450 mg l^{-1} in

another; the respective decrease in lysozyme activity was 73.4% and 54.5% of pre-exposure levels.

Rainbow trout have been shown to display an adaptation to experimentally elevated NH_3, whereby initial responses of greatly increased ventilation frequency and reduced food intake returned to levels only slightly above and below pre-exposure and controls (Lang *et al.*, 1987). In an experiment that investigated the effects of water reuse on growth of rainbow trout, Larmoyeux and Piper (1973) moved fish from the first trough (single-use water) of a cascade system into the last trough system (7^{th} use water) and noted that within 30 mins the fish displayed signs of distress. The authors concluded that the fish in the lower levels of the cascade had adapted to environmental stresses associated with elevated levels of ammonia and reduced DO and that the fish from higher in the cascade, which were accustomed to better water quality, could not tolerate such conditions without acclimatisation.

In specific regard to SD, Wagner *et al.* (1995) found effect of SD on NH_3 production (on a per fish basis) in rainbow trout cultured in raceways, with ratios of NH_3 production highly correlated with biomass. Soderberg *et al.* (1983) found no effect of SD on the growth or mortality of rainbow trout cultured in static water ponds, though growth and mortality both displayed significant negative correlation with the average daily maximum levels of un-ionised ammonia.

At higher SD the increased biomass of fish respiring in a given volume of water will invariably result in increased levels of ammonia. If high SD or loading rates are coupled with high water temperatures and alkaline waters (>pH7), the potential for ammonia levels to result in poor welfare will be greatly increased. For more complete reviews of ammonia toxicity, see reviews by Alabaster & Lloyd, (1982); Meade, (1985); Randall & Wright (1987).

5.1.3.2. Nitrite

Nitrite (NO_2^-) is an intermediate product of ammonia oxidation by the nitrifying bacteria such as *Nitrosomonas* spp. in the simplified equation:

$$2\,NH_4 + 3\,O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$$

Nitrite can be further reduced into the less toxic nitrate by *Nitrobacter* spp.

$$NO_2^- + NH_4 + 6O_2 \rightarrow 2H_2O + 2NO_3^-$$

Nitrite is toxic to freshwater vertebrates through oxidation of haemoglobin to met-haemoglobin, a form incapable of carrying oxygen to tissues (Piper *et al.*, 1982; Vedel *et al.*, 1998). The recommended upper limit for nitrite is <0.1 mg l^{-1} in soft water and 0.2 mg l^{-1} in hard water (Wedeymer, 1996). There is evidence in the literature for increased susceptibility to parasitic infection of juvenile rainbow trout exposed to sublethal concentrations of nitrite (Carballo & Munoz, 1991). However, there is limited information relating specifically to SD and nitrite.

Recirculation systems are not common in commercial trout production in the UK (this will be discussed in greater detail in Chapter 6), so the potential for nitrite levels to accumulate is very low, even at high SD. However, if water is recirculated there is a far greater potential for harmful levels of nitrite level to accumulate. This can be exacerbated at low water temperatures when the reproduction of *Nitrobacter* spp. in the biofilter of recirculating water systems can be reduced (Noble & Summerfelt, 1996).

5.1.3.3. Nitrate

Nitrate (NO_3) is less toxic to fish than nitrite although there is less understanding of the mode of toxicity of nitrate on fish. The recommended upper limit for salmonid culture varies but appears to have been lowered with the passage of time; Kincheloe *et al.* (1979) found nitrate concentrations of 5-10 mg l^{-1} to be mildly toxic to developing eggs and early fry of rainbow trout, Larsen (cited in Piper *et al.*, 1982) then recommended an upper limit of 3.0 mg l^{-1}, and most recently, Wedeymer (1996) recommended an upper limit of 1.0 mg l^{-1}. Similarly to nitrite, unless water is recirculated, levels of nitrate normally remain well below recommended safe limits, even at high SD.

5.1.4. Limitations of recommended water quality thresholds

Although recommended limits for salmonids exist for key water quality parameters, there are substantial contradictions in the recommendations. Such contradictions are a function of numerous biological and environmental factors that affect both the toxicity of the individual parameters and the fish's tolerance to them. The size, strain and previous exposure of a fish are recognised to affect tolerance to water quality parameters (Piper *et al.*, 1982; Colt & Watten, 1988). Most of these recommendations originate from information defining chronic and acute toxicity levels (e.g. 96 h LC_{50}) for each parameter in isolation and are not necessarily considered in the context of fish welfare, nor do they account of the interaction between the different water quality parameters.

Feeding plays a key role in water quality fluctuations, with peaks and troughs of DO and ammonia common features in fish farms following feeding. Unfortunately toxicity test data are frequently generated with starved fish and therefore of little

relevance to farmed fish, this is especially true for ammonia since there is evidence that fasting exacerbates ammonia toxicity (Wicks & Randall, 2002). The variation in recommended safe water quality limits and the questionable relevance of such data makes it difficult for farmers and legislators to identify practical guidelines highlighting the need for the review of water quality recommendations.

5.1.5. Effect of environmental factors on water quality deterioration

5.1.5.1. Temperature

Water temperature is of fundamental importance to all aspects of fish culture and due to the vast volumes of water that are required for trout farming, temperature is also one of the most difficult variables to control. The recommended range of water temperature for salmonid culture is 7-18°C, and 8-10°C for eggs and fry (Council of Europe, 2002). Temperature will ultimately determine the availability of oxygen, rate of metabolism of fish, and also has profound effects on the toxicity of ammonia and free carbon dioxide.

There is evidence for acclimation of rainbow trout to temperatures of up to 26.3°C (Charlton *et al.*, 1970: cited in Alabaster & Lloyd, 1982), though higher temperatures (>16°C) are generally considered problematic to the trout as a result of the reduced solubility of dissolved oxygen and increased toxicity of ammonia. This is well illustrated by Klontz (1993), who showed that increasing water temperature from 9°C to 15°C decreased availability of DO by approximately 13% while the metabolic rate and ammonia excretion of a 100g rainbow trout living in this increased by 68% and 99% respectively. If this example is put into a commercial perspective where there may be in excess of 20,000 fish in a single holding unit, it is clear that there is a far greater potential for DO and ammonia to reach critical limits at higher water

temperatures. It is common for feeding to be restricted or suspended at water temperatures of 16°C or above (Anon., 2002). Similar to oxygen, the solubility of CO_2 also decreases at higher temperatures (Alabaster & Lloyd, 1982).

5.1.5.2. Acidity

The range of pH recommended for rainbow trout culture is pH 6.0 – 8.5 in fresh water and pH 7.0 – 8.5 in seawater (Council of Europe, 2002). As mentioned earlier, due to acid base interactions, fluctuations in pH will affect the toxicity of ammonia and carbon dioxide. Wagner *et al.* (1997) observed an increased stress response in rainbow trout kept in water at pH 9.0 and above; laboratory and field tests also showed increased mortality in at pH levels greater than 9.3–9.4. Bosakowski and Wagner (1994b) found fin erosion in a number of species of hatchery reared trout to be correlated with lower alkalinities (lower capacity of water to neutralise acid). For reviews on the effects of pH on freshwater fish see reviews by Fromm (1980), and Alabaster and Lloyd (1982).

5.1.5.3. Gas supersaturation

The main problem associated with rearing fish in gas-supersaturated water is gas bubble disease. When water is supersaturated with a gas, a fish's blood may also become supersaturated and subsequent changes in temperature or pressure can bring supersaturated gases out of solution forming bubbles in blood vessels or tissues. This may result in possible restriction of respiratory circulation, and subsequent death by asphyxiation (Piper *et al.*, 1982). Wedeymer (1996) recommended an upper limit of 110% total partial pressure of dissolved gases for salmonid culture (103% for salmonid eggs/fry; 102% for lake trout). However, Piper *et al.* (1982) report that

nitrogen gas concentrations in excess of 105% cannot be tolerated by rainbow trout fingerlings.

5.1.5.4. Suspended solids

Wedemeyer (1996) recommended an upper limit of 80 mg l^{-1} suspended solids for intensive fish culture. Suspended solid matter occurs naturally in nearly all rivers and lakes, although the actual concentration of matter is subject to large temporal and spatial fluctuations. Suspended solids may be either organic or inorganic and the threat of the solids to fish health will be highly dependent on the nature of the material. Suspended solids may pose a threat to fish health by:

1. Direct physical damage to respiratory structures (abrasion),
2. Indirect physical damage caused by gill fusion to basic salts and toxic metals, or,
3. Environmental degradation resulting from reduced oxygen levels caused by the respiration of micro- organisms during the oxidation of organic matter.

There is evidence to suggest that rainbow trout can survive short periods of exposure to very high levels of suspended solids (5-300 g l^{-1}), although subsequent observations of gill epithelium showed thickening and proliferation often resulting in death (Herbert & Merkens, 1961). Reduced growth and an increased susceptibility to 'fin-rot' are also reported as deleterious effects of suspended solids on rainbow trout (Herbert & Richards, 1963). Piper *et al.* (1982) commented that 'turbidity' based on light penetration may affect the ability of fish to find food. For a more complete coverage of the effects of suspended solids on freshwater fish, the reader is referred to a comprehensive review by Alabaster & Lloyd (1982).

5.1.5.5. Water exchange rate

As the rate of water exchange in a system increases, so too will the provision of DO and the removal of toxic metabolites such as ammonia and CO_2, and therefore the biomass of fish that a system can support. This is often referred to as the carrying capacity, a concept that was first proposed by Haskell (1955). In fish culture, carrying capacity depends upon water flow, volume, exchange rate, temperature, oxygen content, pH, size and species (Piper *et al.*, 1982). Several authors have built upon Haskell's ideas and further developed methods of calculating the maximum biomass of fish that a system can safely support (Willoughby, 1968; Piper, 1970; Westers, 1970).

5.1.6 Effects of water quality on trout welfare

5.1.6.1 Water quality and stress response

Stocking rainbow trout at high levels has been shown to cause deterioration in water quality (Rosenthal *et al.*, 1984; Larmoyeux & Piper, 1973), and this may act as a chronic environmental stressor. Pickering *et al.* (1991) found that chronic stress caused by low DO resulted in elevated plasma cortisol. Pickering and Pottinger (1987b) demonstrated how combinations of reduced oxygen and low pH significantly increased plasma cortisol levels in rainbow and brown trout. This experiment also found the unexpected result of a suppressed cortisol response following a combined exposure to elevated CO_2 and ammonia; the authors suggested that a possible anaesthetic effect of the increased level of CO_2 may have been responsible for the decrease in stress response (Pickering & Pottinger, 1987b). Swift (1981) and Donaldson (1981) provided further evidence of increased cortisol levels in rainbow trout following acute exposure to NH_3 and low DO.

5.1.6.2 Water quality and disease

It is generally accepted that poor water quality will result in an increased prevalence of disease, although such statements are not often supported by evidence for causal mechanisms. It has been shown in the previous sections that poor water quality can result in elevated plasma cortisol levels in fish, and chronic elevation of plasma cortisol is associated with deleterious effects on immunocompetence (Pickering & Pottinger, 1989).

There are several reports of increased susceptibility to opportunistic infections as a result of exposure to poor water quality. Noble and Summerfelt (1996) described outbreaks of bacterial gill disease in rainbow trout occurring in response to high levels of ammonia caused by overloaded recirculating systems (Density Index > 0.5; Piper *et al.*, 1982) during the grow-out phase. Similarly, Soderberg *et al.* (1983) correlated increased mortality due to ectoparasitic protozoa with increased ammonia, suggesting that fish exposed to ammonia were more susceptible to infection. Bosakowski and Wagner (1994b) correlated NH_3 levels with increased fin erosion, but suggested that the association may have also been due to confounding influences of other water quality parameters such as metabolic wastes, microbes, and suspended solids. Similarly, observations of increased susceptibility to 'fin-rot' in rainbow trout exposed to increased levels of suspended solids made by Herbert and Richards (1963) may have been a confounding effect of increased bacterial loading.

5.1.7 Experimental evidence for the effect of SD related deterioration in water quality on trout welfare

Several studies have attempted to separate the effects of water quality deterioration from behavioural or physiological interactions that may occur at higher stocking

densities as a result of increased numbers of fish in a given space. One such approach has been to simulate the effects of water quality deterioration caused at higher SD by either altering the loading rates of tanks through manipulation of inflow rate, or by passing water successively through compartments containing a known biomass of fish. These approaches have allowed the effects of water quality deterioration to be separated from any behavioural or physiological effects.

Three previous studies have manipulated inflow rate to investigate the effects of water exchange rate on rainbow trout (Brauhn *et al.* 1976; Baker & Ayles, 1990; Ross *et al.*, 1995). In a large-scale study that investigated the effects of loading rate on the growth of rainbow trout in circular tanks, increased loading rate (achieved by either increasing biomass or reducing inflow rate), was shown to display consistent and predictable impairment of growth and feed conversion (Brauhn *et al*,. 1976). Brauhn *et al.* (1976) demonstrated that critical thresholds of TAN could be exceeded in advance of DO becoming limiting.

Baker and Ayles (1990) manipulated inflow rates in tanks of rainbow trout reared at a constant SD (25 kg m^{-3}) and found the optimum loading level to be 1.0 kg l^{-1} min^{-1}, with growth rate decreasing when this at loading levels greater than 1.0 kg l^{-1} min^{-1}. The authors attributed this reduced growth to deterioration in water quality, though interestingly growth was also reduced at lower loading rates (0.38, 0.52 & 0.75 kg l^{-1} min^{-1}) where water quality was better. The authors suggested that higher current speeds caused by the higher inflow rates at the lower loading levels could have resulted in increased swimming speeds, and also may have resulted in food pellets being more quickly washed down the drain.

The experimental design used by Ross *et al.* (1995) meant that it was not possible to make direct comparisons between the effects of different inflow rates, as

the different flow regimes were not carried out concurrently. The study did, however, produce some interesting findings regarding the influence of system design and the effect of speed and direction of water current on growth and behaviour.

An alternative approach to altering inflow rates to simulate water quality deterioration caused by increased SD has been to maintain rainbow trout in a series of holding units at the same density and cascade water successively through each unit in series. Several studies were carried out in such a system at the Bozeman Fish Culture Development Centre (Montana, USA), where water was passed through a duplicate series of 7 aluminium troughs that were stocked with the same biomass of rainbow trout (Piper, 1970; Larmoyeux & Piper, 1973; Mayer & Kramer, 1973). The findings reported by Piper (1970), and Larmoyeux and Piper (1973) were effectively from the same experiment, though the latter provided a more detailed account of the study. Larmoyeux and Piper (1973) found that water quality deteriorated through the series with oxygen dropping from 7.7 mg l^{-1} in the first trough down to 3.3 in the 7^{th}, and TAN increased from 0.1 mg l^{-1} to 0.8 mg l^{-1}. Growth remained uniform through the first 4^{th} troughs, after which there was a significant reduction in growth and increase in FCR. The reduced growth occurred when TAN exceeded 5 mg l^{-1} and DO dropped below 5 mg l^{-1}, although low DO was suggested as the critical factor, since another experiment (unpublished) at the same facility kept fish for 6 weeks in an environment with DO >7 mg l^{-1} and TAN concentration of 0.8-1.0 mg l^{-1} with no negative observed effects. In a study that used the same system and similar experimental design, Mayer and Kramer (1973) also found growth to deteriorate at the same position (trough 4) within the cascade of troughs, though no information regarding water quality was provided in this study.

Similar to the cascade approach, Rosenthal *et al.* (1984) simulated SD related deterioration in water quality by using screens in an experimental channel to create seven compartments, each stocked with 20 kg m^{-3} of rainbow trout to create a cumulated equivalent of 140 kg m^{-3}. The average levels of TAN increased progressively from an average of 0.02 mg l^{-1} to 0.35 mg l^{-1} from the first compartment through to the last, while DO dropped from an average of around 9 mg l^{-1} in the first compartment down to 5 mg l^{-1} in the last. A significant reduction in growth was observed after the fourth compartment where average TAN and DO were around 0.2 and 6.8 mg l^{-1} respectively.

Instead of simulating the effect of SD on water quality deterioration, a divergent experimental approach has been to effectively eliminate any differences in water quality, flow and loading rates between density treatments and instead focus specifically on effects resulting from differences in the actual numbers of fish. This has been achieved in several studies by suspending netted compartments stocked with different numbers of fish within mutual holding systems (Soderberg & Krise, 1986; Kebus *et al.*, 1992; Procarione *et al.*, 1999). Kebus *et al.* (1992) grew rainbow trout at densities of 50 and 232 kg m^{-3} in rectangular nylon net cages suspended in round tanks. The authors found no differences in growth, condition, haematocrit, interrenal cell diameter, or peak serum cortisol levels following exposure to a standardised stressor. The authors concluded that providing good water quality was maintained, it is possible to rear juvenile rainbow trout at densities of as high as 232 kg m^{-3} (DI =11.1 g l^{-1} cm fish length) without impaired growth or chronic stress. Similarly, Soderberg and Krise (1986) found no differences in growth rates of lake trout (*Salvelinus namaycush*) reared at density indices (DI) of 0.8, 1.6, 3.2 and 6.4 g l^{-1} cm^{-1} (published in imperial units of 0.25, 0.50, 1.0 and 2.0 lbs ft^3 inch) in nets suspended in

2 m diameter circular tanks, but the authors did observe increased mortality at the highest DI.

Contrary to the findings of Soderberg and Krise (1986) and Kebus *et al.* (1992), Procarione *et al.* (1999) reported differences in the growth rate of juvenile rainbow trout reared at loading rates of 0.5 or 0.75 kg l^{-1} min^{-1}. Furthermore, there was a significant effect of DI, with groups of fish cultured at 5.6 and 8.4 g l^{-1} cm gaining significantly less weight than those at the lowest DI of 2.8 g l^{-1} cm. Procarione *et al.* (1999) acknowledged the fact that these findings were in discord with similar studies and suggested that growth might not be impaired until a critical limit of temperature or loading is exceeded, after which the differences become apparent.

5.1.8. Summary

There are mixed reports on the effect of SD on oxygen consumption, with Kindschi *et al.* (1991b) finding no effect of SD, but Medland and Beamish (1985) and Miller *et al.* (1995) both observed increased oxygen consumption (mg kg^{-1} h^{-1}) at higher SD. However, at higher SD there will invariably be a greater biomass of fish respiring and metabolising in a given volume of water, so the risk of DO becoming limiting, or metabolites accumulating will increase proportionally.

It has been shown that by paying close attention to water quality parameters it is possible to rear fish successfully at increased stocking densities (Kindschi *et al.*, 1991a; Kebus *et al.*, 1992; Miller *et al.*, 1995). However, it must be added that culturing fish at such densities runs an increased risk of mass mortality in the event of equipment failure and that high density culture requires the increased supervision and appropriate backup equipment.

The dynamic and interactive nature of the various water quality parameters are reflected in the wide and often contradictory ranges of published recommended critical limits. The complexity of these interactions is reflected in Smart's recommendation that no single aspect of water quality should ever be considered in isolation from the influence of other water quality parameters (Smart, 1981). Determining 'safe' upper limits is further complicated by the fact that no two culture systems are the same and the prevailing environmental factors should also be taken into consideration for each production facility in regard to tolerance limits. Experiments designed to minimise the effects of other factors except for water quality (Larmoyeux & Piper, 1973; Rosenthal *et al.*, 1984; Baker & Ayles, 1990) provide strong evidence to suggest that deterioration in water quality is the root cause of reduced growth and other problems associated with high stocking densities. This is further supported by experiments that have found no effect of SD on growth when the influence of water quality has been removed (Soderberg & Krise, 1986; Kebus *et al.*, 1992).

5.2. Experimental investigation into the effects of water quality deterioration on the welfare of rainbow trout

The overall aim of this experiment was to investigate the effect of water quality deterioration associated with increased loading levels on the welfare of rainbow trout.

5.2.1. Materials and Methods

5.2.1.1. System specifications

The same 2 m diameter tank system used in Chapter 4 was also used in this experiment, however, between the two experiments the system was upgraded (Figure 5.1). Upgrades to the system included:

- In-line Dataflow indicators (DFM900; Parker Filtration, Norfolk, UK) were fitted to the inflow pipe of each tank allowing the flow rate to be measured (± 1 l min^{-1}) using the hand-held Dataflow monitor (DFM950; Parker Filtration, Norfolk, UK). The system worked on the principle of measuring the speed of rotation of a turbine that was driven by the inflowing water.
- The pipe work was altered to add a down-pipe into each of the tanks to reduce the affect of the different rates of inflow on surface disturbance and water current.
- Opaque fibreglass covers were added to each tank fitted with 2 x 100 W drum bulkhead lights with prismatic covers and 4-pin, 2D tungsten lamps (RS, Northampton, UK). Lighting was regulated using analogue clock timers (RS electric) that were adjusted on a weekly basis to simulate natural photoperiod (SNP).
- An oxygen injection system was installed that delivered oxygen to each tank via a DAD3 ceramic diffuser (Dryden Aquaculture, Edinburgh).

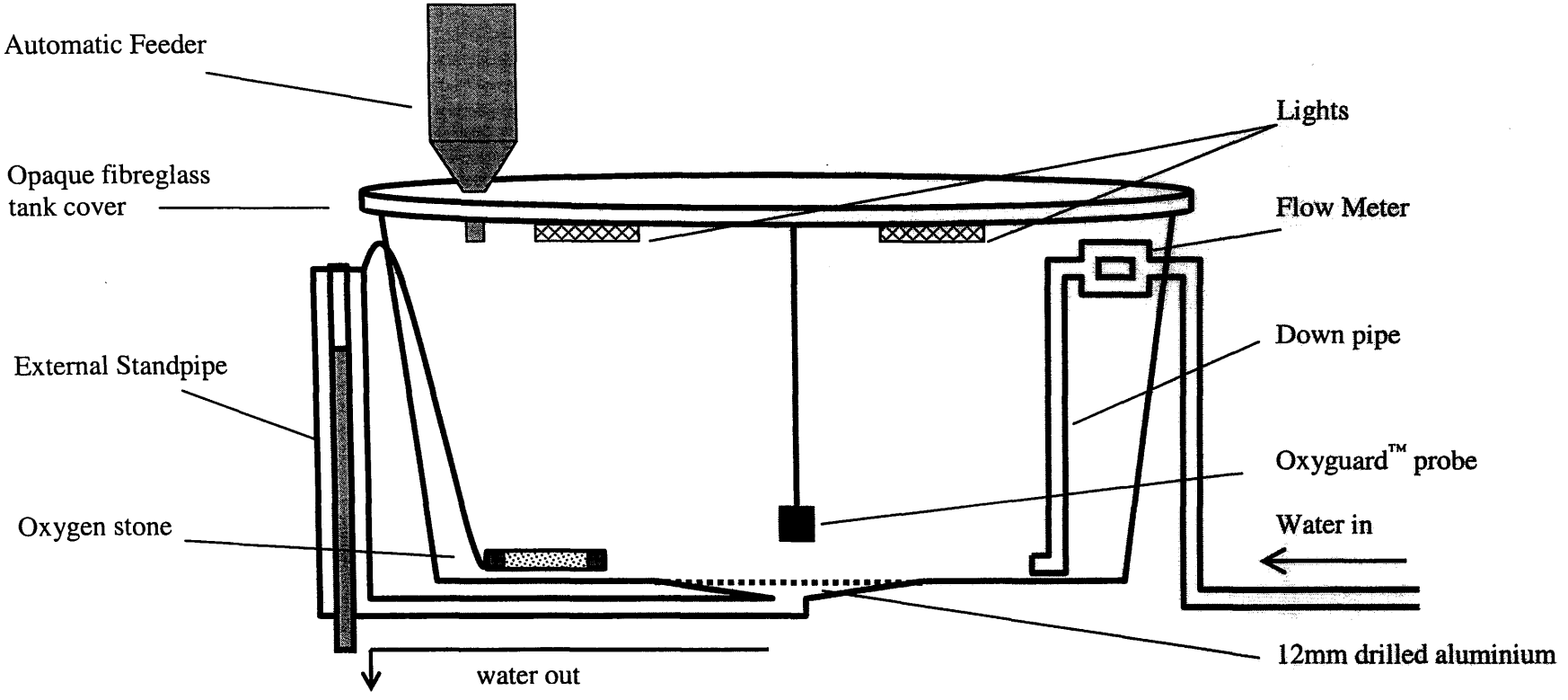


Figure 5.1. Schematic diagram of the upgraded tank set-up used in the water exchange experiment.

5.2.1.2. Water quality monitoring

An Oxyguard® monitoring system was used throughout the trial taking temperature and dissolved oxygen readings at 5 min intervals from a probe situated directly above the outflow of each of the tanks (Figure 5.1). Alarms were triggered if dissolved oxygen dropped below 5 mg l^{-1} and additional oxygen was added to tanks as necessary to ensure that levels were maintained above this level. Data was relayed through the Oxyguard® system into a desktop computer.

Ammonia and pH were measured once a month, approximately 1 h before feeding and again at approximately 3 h after first feeding. A 24 h water quality profile of DO and ammonia was also carried out carried out towards the end the experiment when loading rates were at their highest.

5.2.1.3. Feeding

Fish were fed a percentage body weight ration of a commercial diet (Skretting). The ration and pellet size of the diet varied with water temperature and fish size in accordance to manufacturer's tables. All tanks of fish were fed the same amount of feed throughout the course of the trial, which was calculated from the mean weight of all 9 tanks of fish (estimated from the 40 PIT-tagged fish in each tank) and numbers of fish in each tank. Food was distributed via automatic feeders that were set to deliver a 3 second burst of feed to each tank at 15-30 min intervals. Feed interval was adjusted to ensure that all feed was delivered during daylight hours of a simulated natural photoperiod. Feeding commenced from approximately 8.30 am each morning, until the feed hopper was empty, which normally occurred between 3 and 6 pm.

5.2.1.4. Experimental fish

Mixed-sex rainbow trout fingerlings were used in this experiment. The fish were hatched on-site from virgin-spawning female brood stock of Danish origin, crossed with male fish from an established domesticated stock at the Niall Bromage Freshwater Research Facility, also of Danish origin.

5.2.1.5. PIT tagging

Passive integrated transponder (PIT) tags (Avid tags, Norco; Ca, USA) were used to permit identification of individuals within the study population. Small, cylindrical PIT tags (12 mm) were inserted into the peritoneal cavity by making a small incision in the posterior, ventral surface of the fish and injecting the tag into the cavity. A 3:1 mixture of Orahesive powder (Squibb and Sons Ltd.; Middlesex, UK) and cicatrin antibiotic (The Welcome Foundation Ltd.; Middlesex, UK) was applied to the incision area to prevent infection. The adipose fin of tagged fish was removed during the tagging procedure to permit visual identification of tagged individuals within the non-tagged population.

5.2.1.6 Experimental set-up

The trial was set up on 12th February 2002 by hand-grading 1800 rainbow trout of mean weight 134.9 g (SE ± 1.1 g) from a 5 m stock tank containing approximately 7000 fish. The fish were graded by length (200–250 mm) under a light dose of anaesthetic. Any fish that were showing signs of precocious maturation were not included in the study.

- 200 fish were randomly distributed into 9 of the 2 m diameter tanks.

- 40 fish in each tank were PIT-tagged and the adipose fin was removed from these fish to make them easily identifiable. Weight, length (total and fork) and measurements of all fins were recorded for each PIT-tagged fish at the start of the trial.

Flow Regimes

In-flow rates were set using the Dataflow indicators and monitor as follows:

- 60 l min^{-1} (30 min tank volume water exchange)
- 40 l min^{-1} (45 min tank volume water exchange)
- 20 l min^{-1} (90 min tank volume water exchange)

5.2.1.7. Sampling protocol

Subsequent sampling took place on a monthly basis over the course of two days as follows:

Day 1

Each month 10 untagged fish were randomly selected from each tank and sacrificed. The following measurements were taken from each fish: total and fork length (± 1 mm) (total and fork), weight (± 1 g), fin length of all rayed fins (± 1 mm). Each fish was blood sampled to allow haematocrit and plasma lysozyme activity cortisol, and glucose concentration to be measured. The liver and spleen were also weighed (± 0.1 g) to allow calculation of HSI and SSI. Examination of gonads took place to allow the sex and maturation status of the fish to be determined.

Day 2

The weight and fork length were measured from the 40 PIT-tagged fish in each tank. The empty tank in the system permitted the fish within each tank to be sorted into the adjacent tank in an anti-clockwise direction. This permitted the cleaning and maintenance of tanks with minimal handling of the fish. The trial lasted for 10 months so by the end of the trial the fish had experienced one month in each tank within the system, removing any effect that position of the tank may have had (Table 5.2).

Table 5.2 Distribution of treatments through the course of the water exchange experiment.

Tank No.	Feb	March	April	May	June	July	Aug	Sep	Oct	Nov
1	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1	Control
2	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1	Control	40 l min^{-1} Rep 3
3	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1	Control	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1
4	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1	Control	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1
5	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1	Control	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2
6	Control	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1
7	20 l min^{-1} Rep 1	Control	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3
8	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1	Control	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2
9	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1	Control	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3	20 l min^{-1} Rep 2
10	20 l min^{-1} Rep 2	60 l min^{-1} Rep 2	60 l min^{-1} Rep 3	20 l min^{-1} Rep 1	Control	40 l min^{-1} Rep 3	60 l min^{-1} Rep 1	40 l min^{-1} Rep 1	40 l min^{-1} Rep 2	20 l min^{-1} Rep 3

Additional Measurements

The fins of all PIT-tagged fish were measured at the August sample point and again at the conclusion of the experiment. At the conclusion of the trial the HSI and SSI of each PIT-tagged fish was calculated. Additionally, the gonads of these PIT-tagged fish were removed, allowing the sex and gonadosomatic index (GSI) of each fish to be calculated [GSI= (gonad weight/total weight) x 100].

5.2.1.8. Statistical Analysis

Most of the statistical analysis was carried out as described previously in sections 2.8 and 4.2.1.3. A repeated measures ANOVA was used to compare the fins of PIT-tagged fish within each treatment at the start, middle and end of the experiment (Instat version 3.0, Graphpad Software Inc.). The individual welfare parameters were included as dependent variables in GLMs with time, inflow rate and replicate as a random factor. The collection of water quality data from individual tanks also allowed the GLMs to be extended, to include temperature, DO, and ammonia as continuous predictors. A more detailed description if the modelling for specific welfare parameters is presented in section 5.3.7

The statistical analysis concluded with the application of Principal Components Analysis (PCA) as an exploratory and data reduction tool to generate welfare indices based on coherence in the data for the individual welfare parameters. The factor scores for the most appropriate principal components (PCs) were then included as dependent variables in GLMs as (see section 2.8.7.3 for details).

5.3 Results

5.3.1 Water Quality

5.3.1.1. Temperature

The water temperature profile for the course of the experiment is shown in Figure 5.2. The lowest water temperature of 2.6°C was observed in February near the start of the trial, and water temperature peaked at 16°C during August. A rapid drop in water temperature occurred towards the end of October, when water temperature plummeted from around 12°C down to 5.5°C in the space of 10 days. The rapid drop in water temperature was likely to have been due to mixing of water in the reservoir following a period of heavy rain in the last week of October 2002, which was preceded by a long period of below-average rainfall.

5.3.1.2. Dissolved Oxygen

There were two periods during the trial when it was not possible to log the DO data collected from the individual tanks. This was initially was due to a problem with the desktop PC into which the data was logged, and in the second instance it was a result of software failure of the Oxyguard® package. Although the dissolved oxygen data were not logged during these two periods, the monitoring and alarm system continued to function normally.

The average daily DO for each of the treatments is shown in Figure 5.3. It was necessary to supplement the oxygen in the tanks of the 20 l min^{-1} treatment from early in the trial to maintain DO above 5 mg l^{-1}. By April 9th, when water temperatures reached 6.7°C, all tanks in the 20 l min^{-1} treatment required supplementary oxygen and did so for the remainder of the trial.

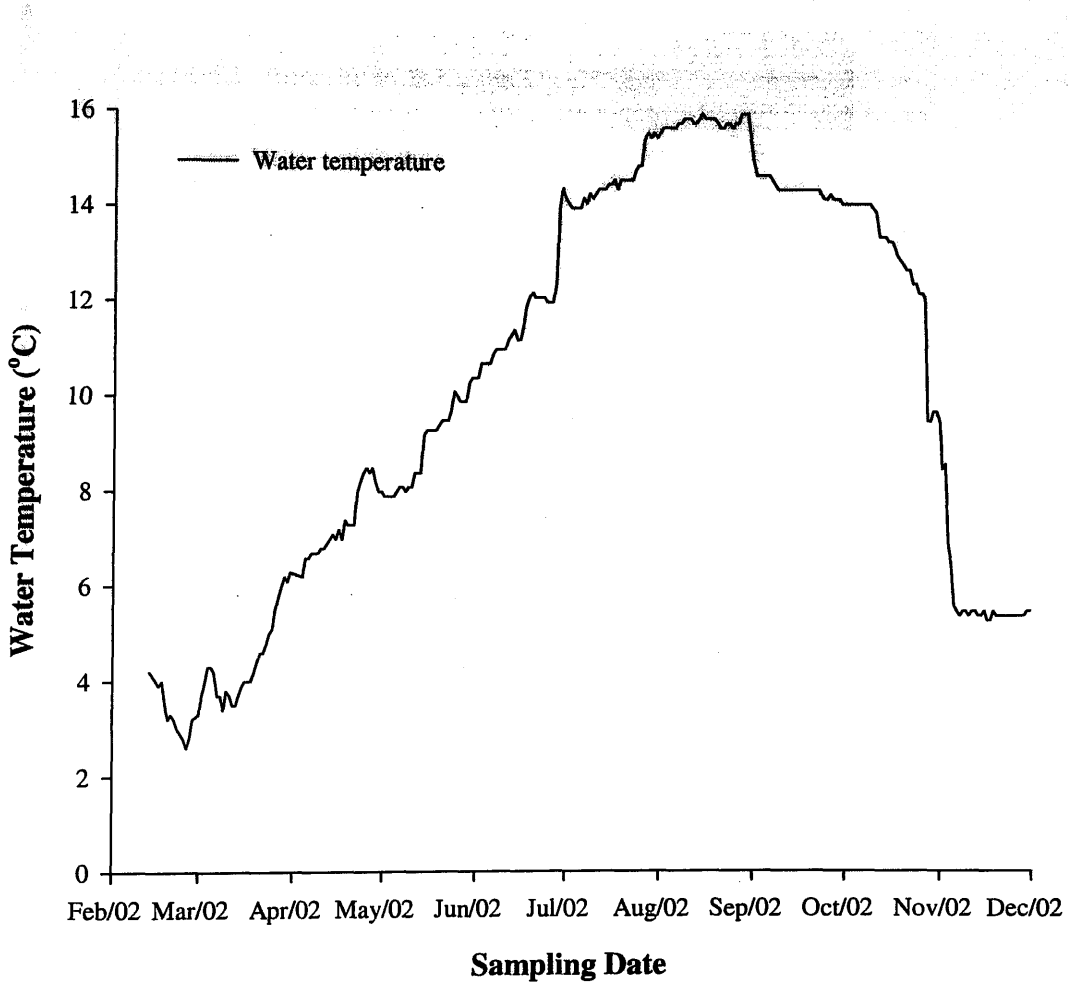


Figure 5.2. Seasonal change of water temperature through the course of the water exchange experiment.

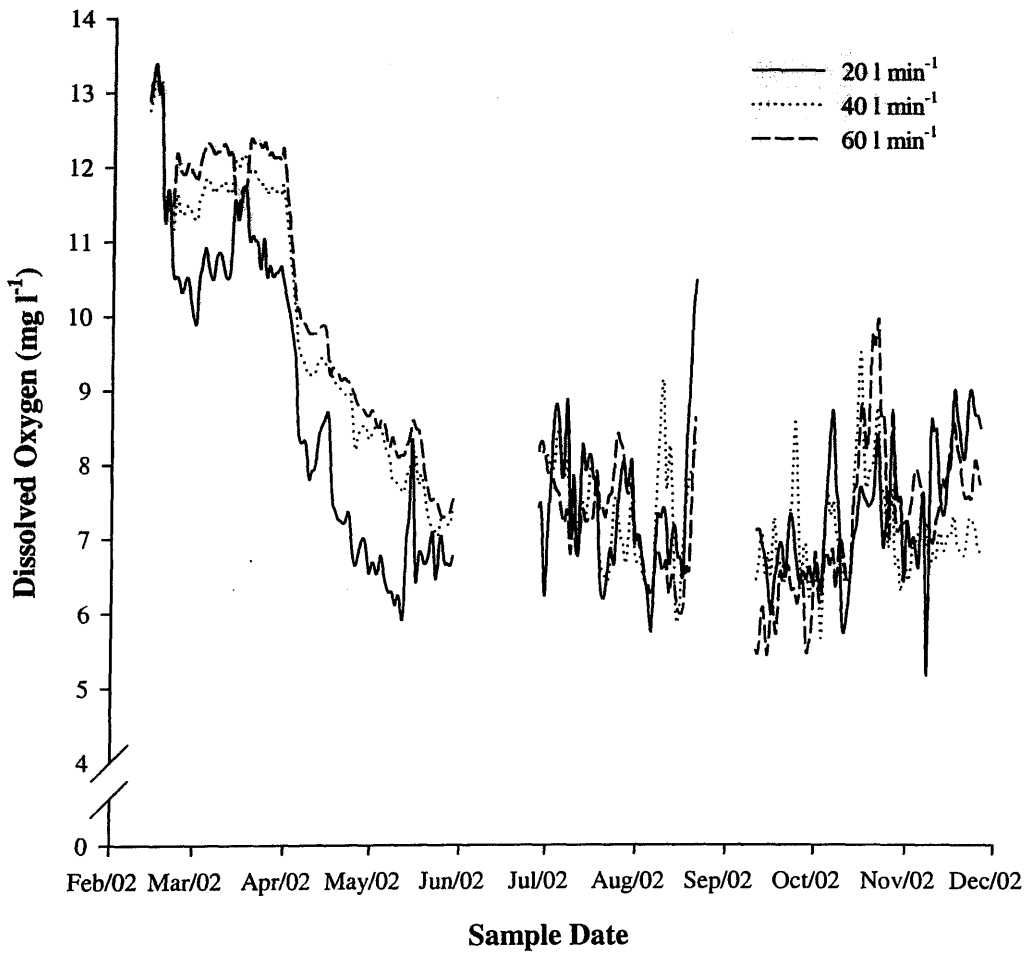


Figure 5.3. Dissolved oxygen in tanks of rainbow trout cultured in tanks with different inflow rates; each line represents the mean value for each treatment, calculated from the daily average of 3 replicates (error bars are omitted for clarity).

All tanks within the 40 l min^{-1} treatment received supplementary oxygen by the end of May, and as water temperatures began to peak and biomass increase through August, it was also necessary to supplement oxygen in the 60 l min^{-1} treatment. When supplementary oxygen was added to tanks, the initial differences in DO that were apparent between the treatments were no longer evident (Figure 5.3). When the monthly mean DO for individual tanks was included as a dependent variable in a GLM with time and inflow rate as categorical predictors, there was no significant effect of inflow rate on DO. There was a significant effect of time on DO ($P<0.001$), which was likely to have been due to the combined effects of changes in water temperature, fish biomass and feed rates at different stages in the experiment.

Although the supplementary oxygen enabled DO to be maintained above the threshold of 5 mg l^{-1}, the system used did not permit DO to be automatically maintained within a pre-set range. Manual adjustment of the valves controlling the oxygen supply to each tank was carried out to prevent the levels becoming excessively high, but this could only be carried out during the working hours of the staff at the facility. A 24 h profile of DO was measured in November (1 h intervals), when the biomass within each of the tanks was highest. Figure 5.4 shows that during the 24 h sampling period, the highest mean DO was observed in the 40 l min^{-1} treatment, suggesting that during this period the additional oxygenation added to the tanks outweighed the effect of differences in inflow rate.

5.3.1.3. pH

The pH of the water remained stable for the duration of the experiment at between pH 6 and 7.

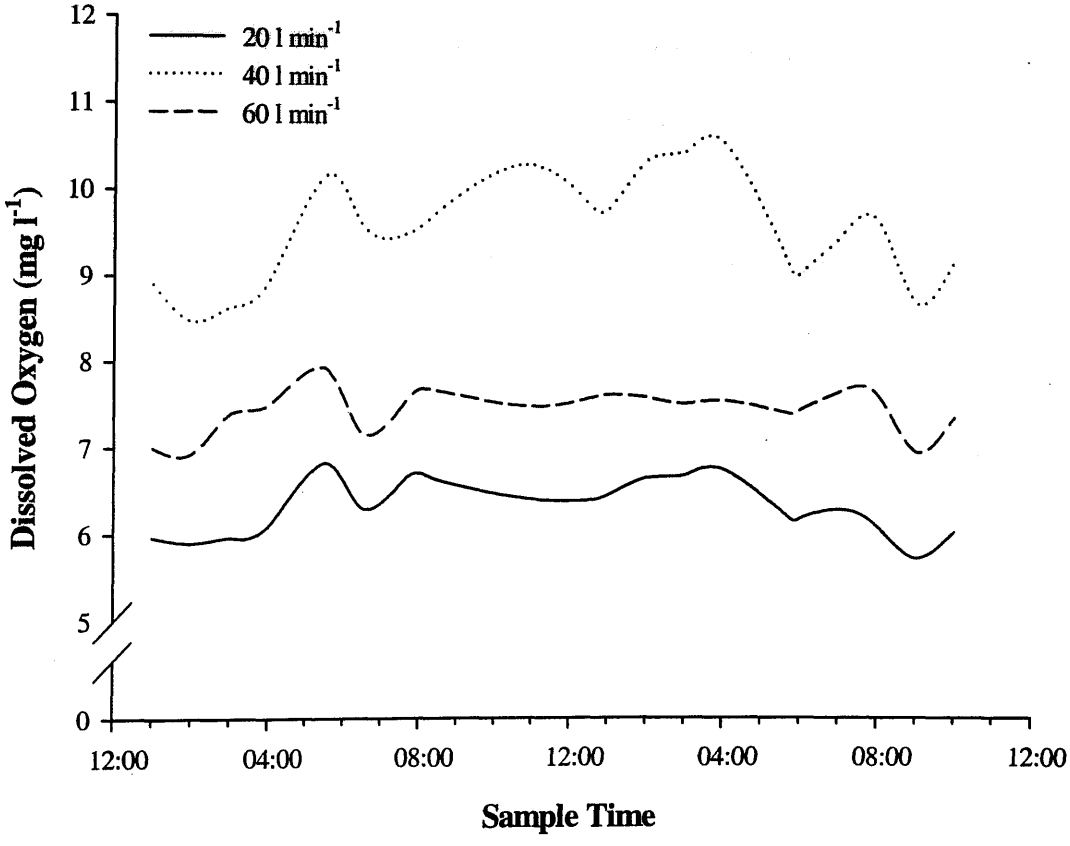


Figure 5.4. Dissolved oxygen profile through a 24 h period in tanks of rainbow trout cultured in tanks with different inflow rates; sampling interval was 1 h, each line represents the mean value for each treatment (error bars omitted for clarity).

5.3.1.4. Ammonia

At approximately monthly intervals between the sampling of the fish, total ammonia nitrogen (TAN) was measured from the outflow of each of the tank. Samples took place 1 h prior to the first daily of feed of the fish, and again between 3-4 h after first feed during. This was true of all months except for October, when a shortage of reagents meant that it was only possible to carryout the post-feed measurements (Figure 5.5). There was a highly significant treatment effect of inflow rate on TAN ($P>0.001$) when post-feed TAN concentrations were included in a GLM as a dependent variable. There was again a significant effect of time on TAN concentrations ($P<0.001$) and a whole model effect of time and inflow rate with replicate included as a random factor ($P<0.01$). Levels of TAN were generally highest in the post-feed water samples, although the difference was not always significant. Paired *T*-test comparison of the pre and post-feed TAN found significant differences ($P>0.05$) during all months except June, July and September. In November, a 24 h profile of TAN measurements was carried out and provided the unexpected result of a peak in TAN during the middle of the night (Figure 5.6).

The pH and temperature of water samples were also measured to allow the un-ionised ammonia concentration to be calculated from ionisation tables (Piper *et al.*, 1982). The pH remained between 6 and 7 throughout the trial, which resulted in the levels of un-ionised ammonia (NH_3) being very low, with the maximum level of NH_3 of 0.0007 mg l^{-1}, well below the 0.01 mg l^{-1} that is generally accepted to be the maximum safe limit for salmonids (Wedemeyer, 1996).

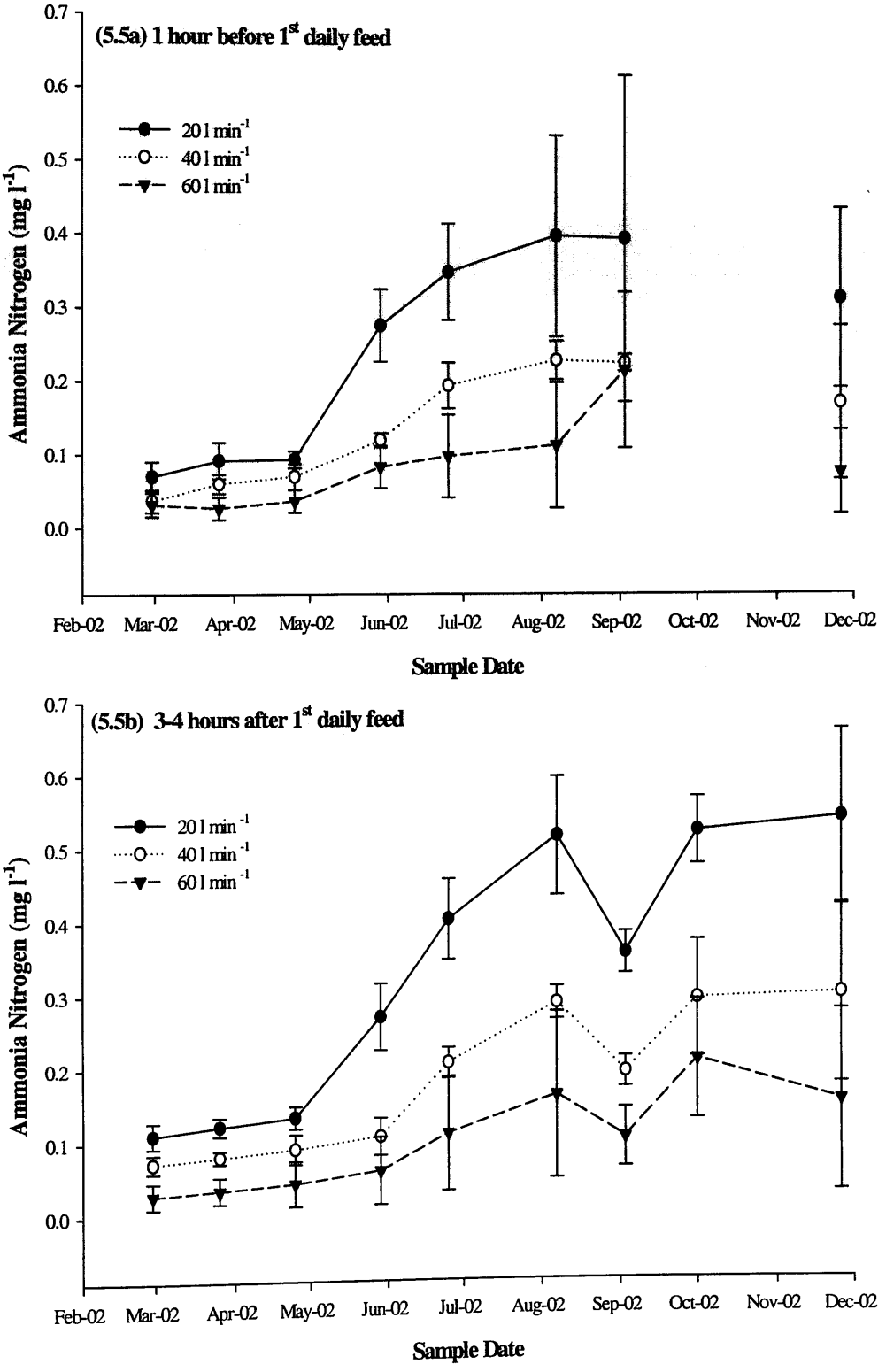


Figure 5.5. Total ammonia nitrogen profile in tanks of rainbow trout cultured in tanks with different inflow rates 1 h before (5.5a), and 3-4 h following (5.5b) the first daily feed; each point represents the treatment mean ± SEM.

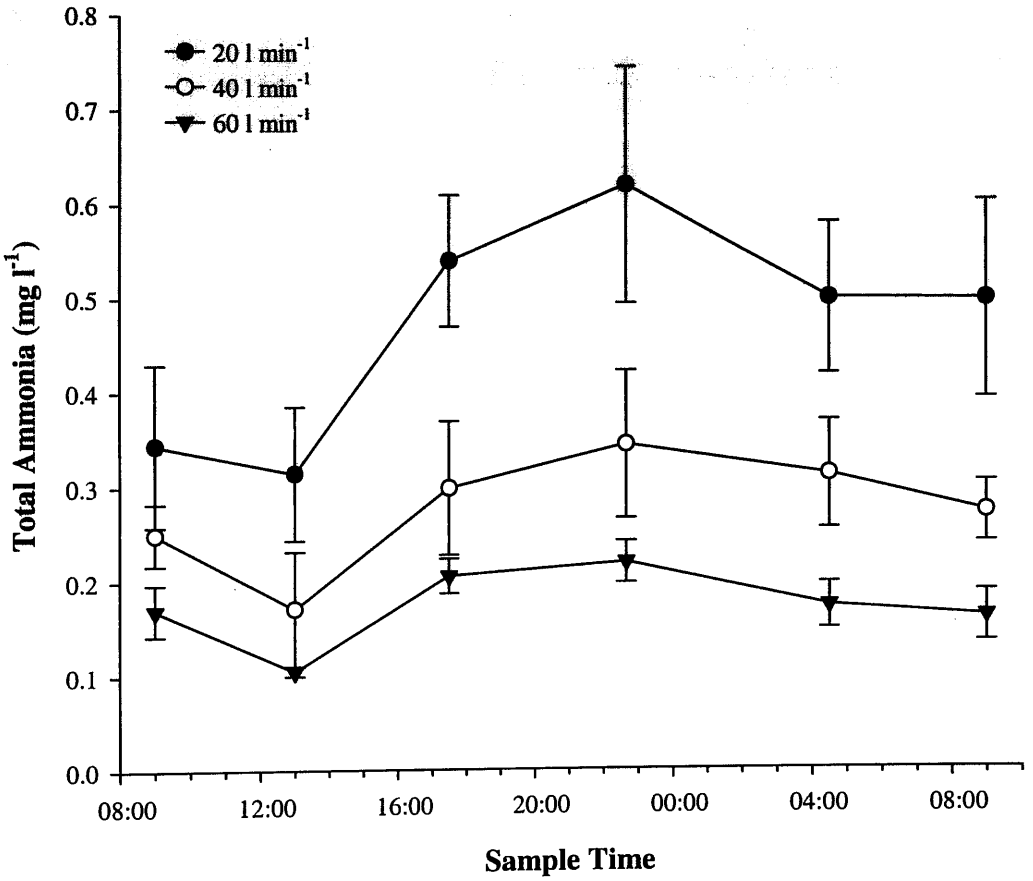


Figure 5.6. Total ammonia nitrogen profile through a 24 h period in tanks of rainbow trout cultured in tanks with different inflow rates; sampling interval was 4 h, each point represents the treatment mean ± SEM.

5.3.2 Stocking density and loading rate

At the start of the experiment, the SD was 16 kg m^{-3} in all tanks. Growth was poor at the start of the trial and SD remained almost static in for the first 4 months as the small increase in biomass due to growth was offset by the monthly removal of 10 fish for sampling. From June onwards there was a steady increase in SD and by the end of the trial SD exceeded 40 l min^{-1} in all treatments (Figure 5.7). At the end of experiment there was no significant difference between the mean SD in each of the treatments, which were 40.1, 41.2 and 45.5 kg m^{-3} respectively for the 20, 40 and 60 l min^{-1} treatments. There were clear differences in Flow Index (FI) between the tanks from the start of the trial and these remained through until the end. The highest FI was observed in the 20 l min^{-1} treatment, and by October FI exceeded 2 in all replicates of this treatment (Figure 5.8).

5.3.3 Mortality

A mass mortality event occurred in the 20 l min^{-1} treatment on 4th April caused by a plumbing failure that resulted in the loss of 118 fish. A damaged standpipe seal resulted in the level of the tank gradually dropping over the course of the night and this was not detected until the following morning when only a few centimetres of water remained in the tank. The dead fish in this replicate were replaced with stockfish of the same age and origin and sampling continued as normal for the remainder of the trial, although data from this replicate was not included in any statistical analysis. A blockage to the inflow of another replicate of the 20 l min^{-1} treatment in the final week of the trial resulted in the loss of 5 fish, but the alarms were triggered in time to prevent major mortalities. Apart from these two events, mortality remained very low (Table 5.3).

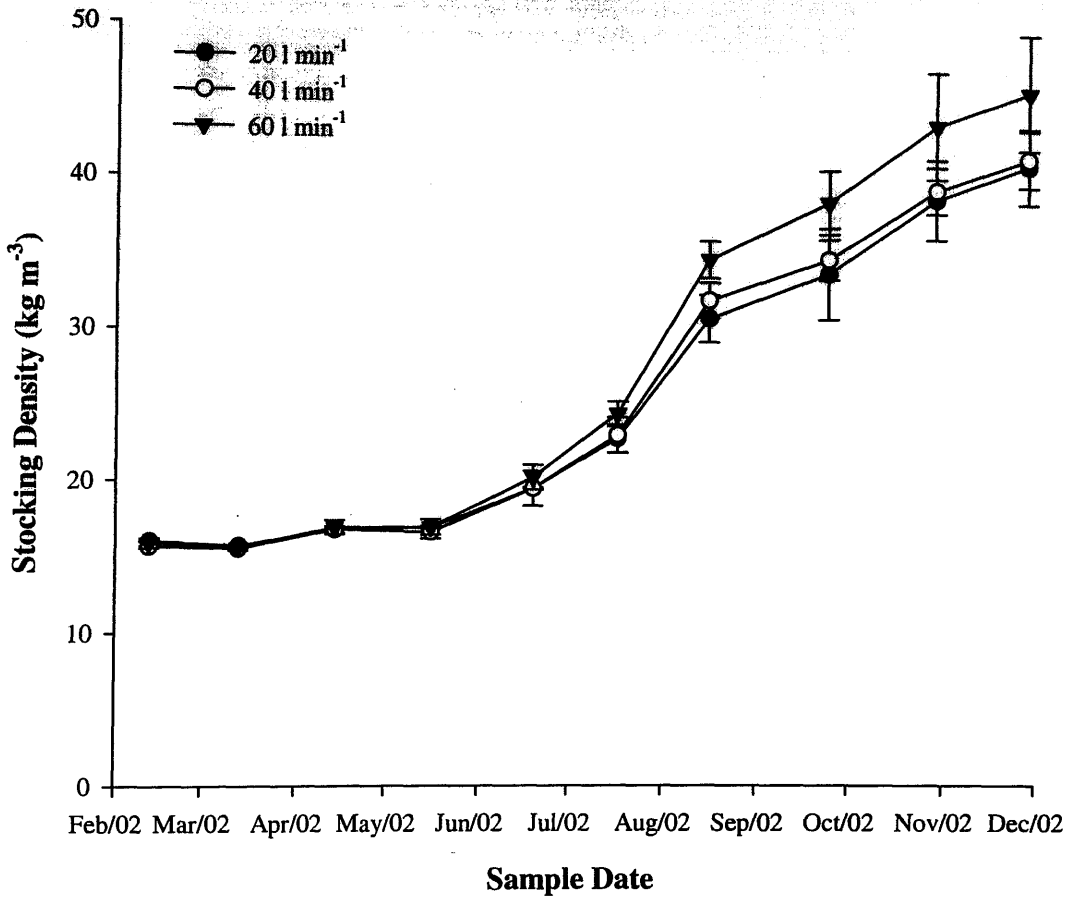


Figure 5.7. Stocking Density of rainbow trout cultured in tanks with different inflow rates; Mean ± SEM of 3 replicates (only 2 replicates for 20 l min^{-1} treatment following the loss of one replicate).

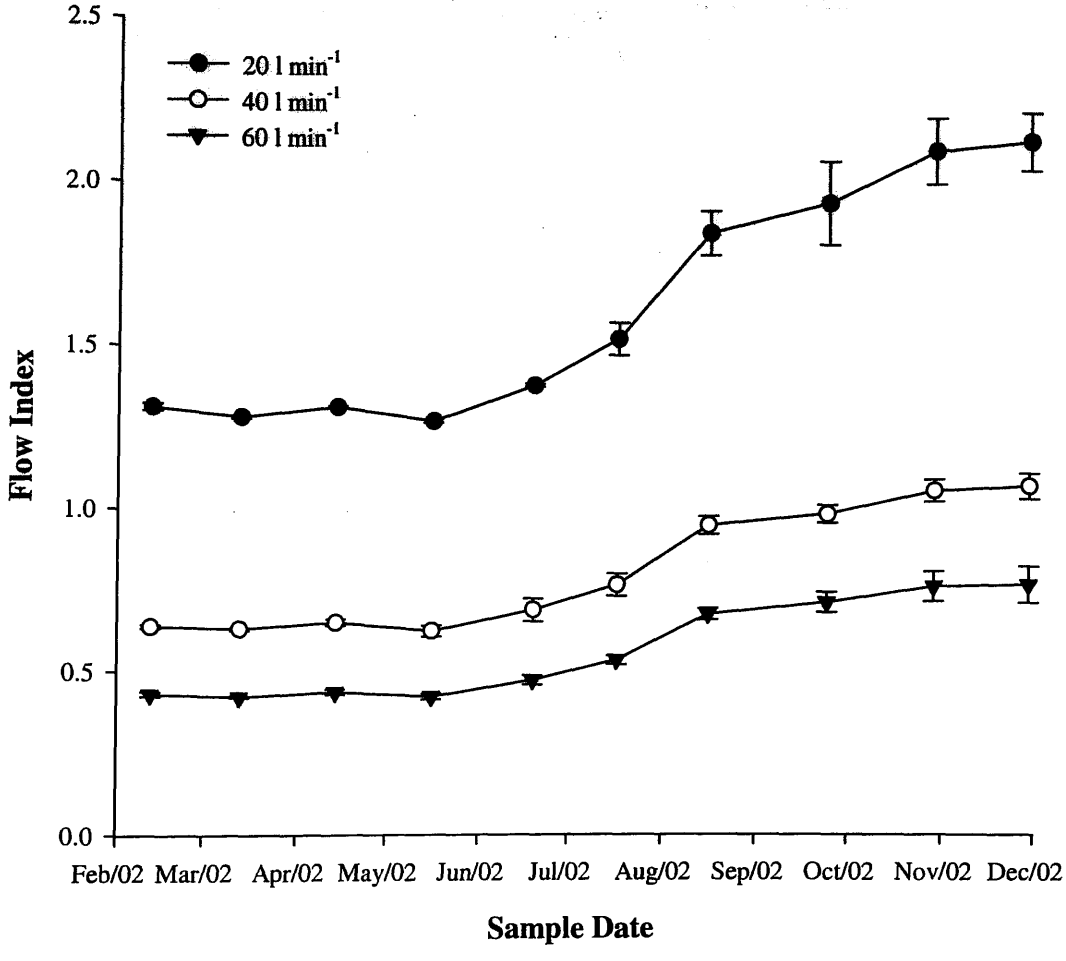


Figure 5.8. Flow Indices of tanks of rainbow trout with different inflow rates; Mean ± SEM of 3 replicates (only 2 replicates for 20 l min^{-1} treatment following the loss of one replicate).

Table 5.3. Mortality of rainbow trout maintained in tanks with different inflow rate.

Treatment	Replicate	Mortality	
		Replicate Mortality (%)	Total Treatment Mortality (%)
20 l min^{-1}	1	62.96	34.05
	2	6.19	
	3	0.00	
40 l min^{-1}	1	0.84	0.80
	2	0.84	
	3	0.84	
60 l min^{-1}	1	0.84	0.30
	2	0.00	
	3	0.00	

5.3.4 Growth

Over the course of the trial, the fish grew from a mean weight of around 140g, to final mean weights of 580, 595 and 652g in the 20, 40 and 60 l min^{-1} treatments respectively (Figure 5.9). When the individual weight of all PIT-tagged fish was used as a dependent variable in a GLM with time, inflow rate and replicate as a random factor, there was a significant effect of inflow rate, with growth significantly higher in the 60 l min^{-1} compared with the 20 and 40 l min^{-1} treatments (P>0.01). There was also a significant combined effect of time and inflow rate, and post-hoc comparison showed that the fish in the 60 l min^{-1} were significantly larger than those in the 20 and 40 l min^{-1} treatments from September through until the conclusion of the trial at the end of November (P>0.05; Tukey's).

5.3.4.1. Specific Growth Rate

Growth was poor during the first month of the trial and this was reflected by the low SGR for all treatments, with many fish actually losing weight. SGR began to increase in April as the water temperature increased and this trend continued until August when SGR peaked at levels of above 0.8 % in all treatments (Figure 5.10).

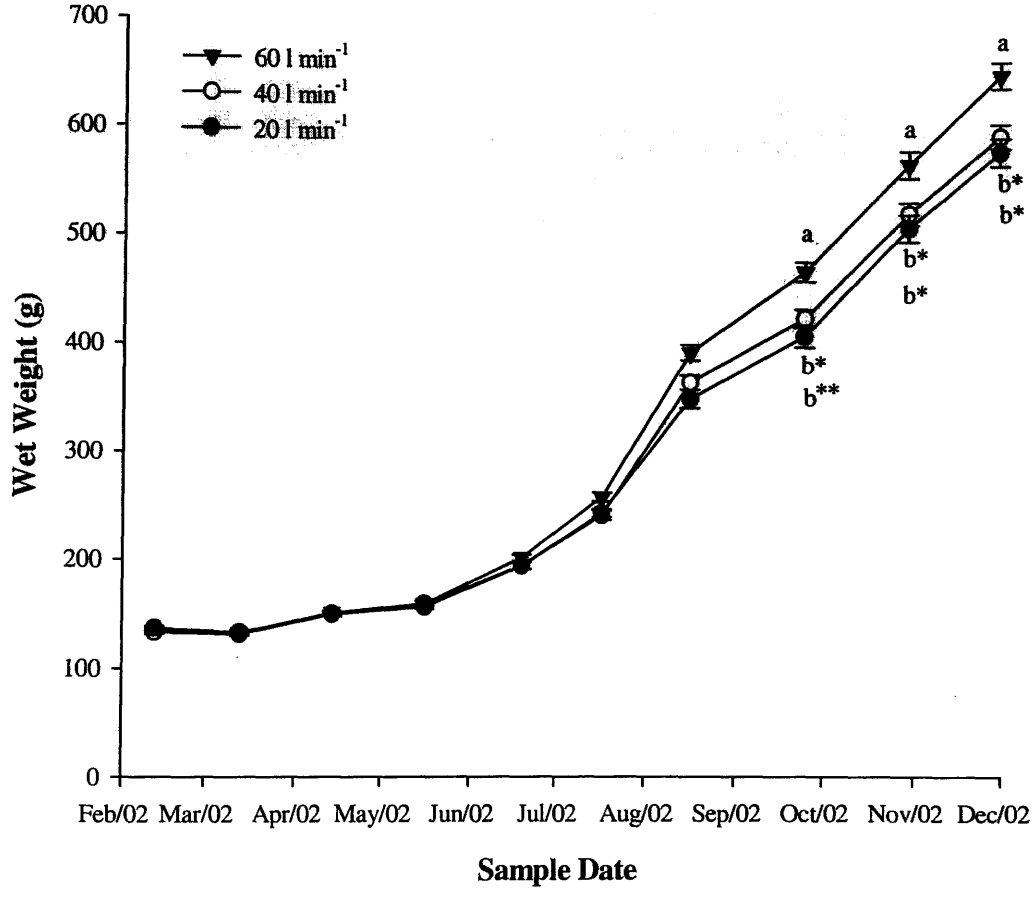


Figure 5.9. Weight gain of rainbow trout cultured in tanks with different inflow rates. Each point represents the mean (± SEM) individual weight for all PIT-tagged fish within each treatment; n = 120 for 40 and 60 l min^{-1} treatments, and 80 for 20 l min^{-1} treatment following the loss of 1 replicate. Treatments not sharing a common letter are significantly different at that time point (*P<0.05, **P<0.01; Tukey's multiple comparison post-hoc following GLM with weight as a dependent variable and time and inflow rate as categorical predictors, with replicate as a random factor).

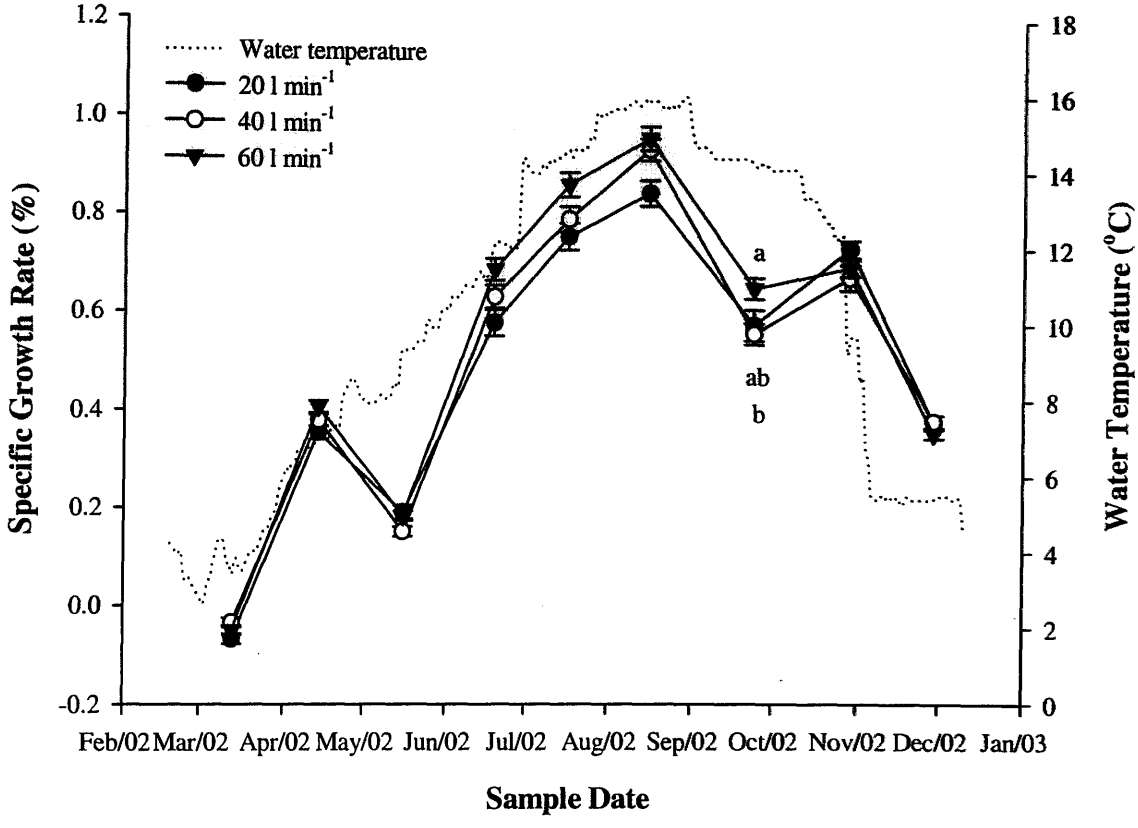


Figure 5.10. Specific growth rate of rainbow trout cultured in tanks with different inflow rates. Each point represents the mean (± SEM) individual SGR for all of the tagged fish within each treatment (n = 120 for 40 and 60 l min^{-1} treatments, and 80 for 20 l min^{-1} treatment following the loss of 1 replicate). Treatments not sharing a common letter are significantly different at that time point (P<0.05; Tukey's multiple comparison post-hoc following GLM with weight as a dependent variable and time and inflow rate as categorical predictors, with replicate as a random factor).

During the summer months (June – September), SGR appeared higher in the 60 l min^{-1} treatment compared with the 20 and 40 l min^{-1} treatments. There was a significant effect of inflow rate on SGR ($P<0.05$), with significantly higher SGR in the 60 l min^{-1} treatment compared with the 40 and 20 l min^{-1} treatments ($P<0.01$; Tukey's). There was also a significant interaction between time and inflow rate and a post-hoc difference was observed between August and September, when SGR was significantly higher in the 60 compared with the 40 l min^{-1} inflow treatment ($P<0.05$).

5.3.4.2. Feed Conversion Ratio

FCR was estimated for each PIT-tagged fish from the change in weight between sampling periods and the amount of feed 'presented' (total food fed per tank / fish numbers) over the same period. Throughout the course of the trial the mean FCR for the various treatments ranged between 1 and 2.5 (Figure 5.11). Many fish lost weight or grew very little during the first month of the trial (February – March), making FCR for these individuals impossible to compute. Due to the high number of negative values for the period between sample points, FCR data for this period were removed from statistical analysis.

The lowest (best) FCR occurred between the March and April sample points, which was surprising considering water temperature was still relatively low during this period. It may have been that following a prolonged period of low growth, the fish displayed compensatory growth as water temperature began to increase. Feed rates were lower in March and April compared with the summer months, so this may also have contributed to the low FCR through this period. It may also have been possible that the fish were underfed in March and April and had the ration been increased during this period, FCR may not have been so low.

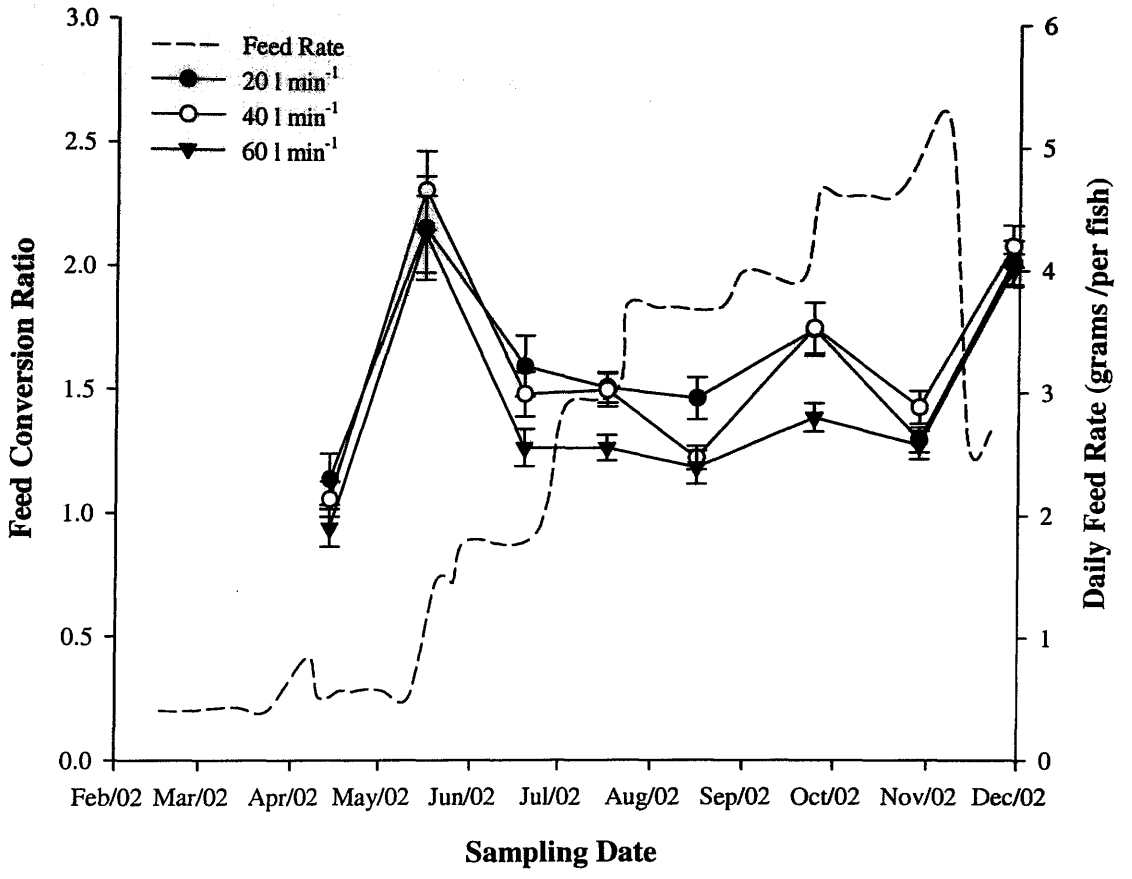


Figure 5.11. Estimated Feed Conversion Ratio of rainbow trout cultured in tanks with different inflow rates. Each point represents the mean (± SEM) individual FCR of all of the tagged fish within each treatment (n = 120 for 40 and 60 l min^{-1} treatments, and 80 for 20 l min^{-1} treatment following the loss of 1 replicate).

The estimated FCR data for each PIT-tagged fish were included as a dependent variable in a GLM with time, inflow rate and replicate (log transformed with negative values removed). There was a significant effect of time ($P<0.001$), but there was no significant effect of inflow rate ($P=0.312$). There was no interaction between time and inflow rate ($P=0.849$), but when corrected for replicate there was a significant overall effect of the model ($P<0.001$).

5.3.5. Somatic Indices

5.3.5.1. Condition Factor

Following an initial decrease during the first 3 months of the experiment, CF increased steadily in all treatments until the end of the experiment (Figure 5.12). There was a significant interaction between inflow rate and time ($P<0.01$) and post-hoc analysis showed CF to be significantly higher in the 60 l min^{-1} compared with the 20 l min^{-1} treatment in August ($P<0.05$; Tukey's), and 40 l min^{-1} treatment in September ($P<0.05$; Tukey's). Although CF appeared to be highest in the 60 l min^{-1} treatment until the conclusion of the experiment, the differences were no longer statistically significant in November or December.

5.3.5.2. Hepatosomatic Index

HSI remained at around 1% body weight in all inflow rate treatments for the first 6 months of the trial, but then showed a steady increase after the August sample point, eventually reaching around 1.5% in all treatments at the end of the experiment (Figure 5.13a). The HSI values (arcsine transformed) from the monthly samples were included as a dependent variable in a GLM with time, inflow rate and replicate (random factor).

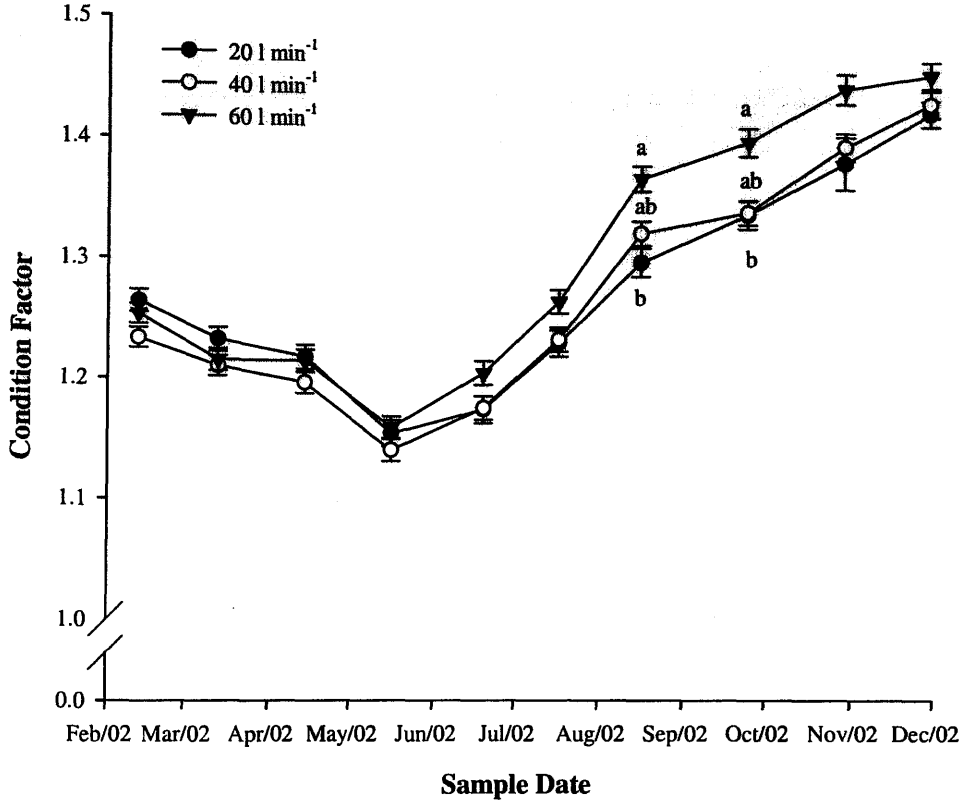
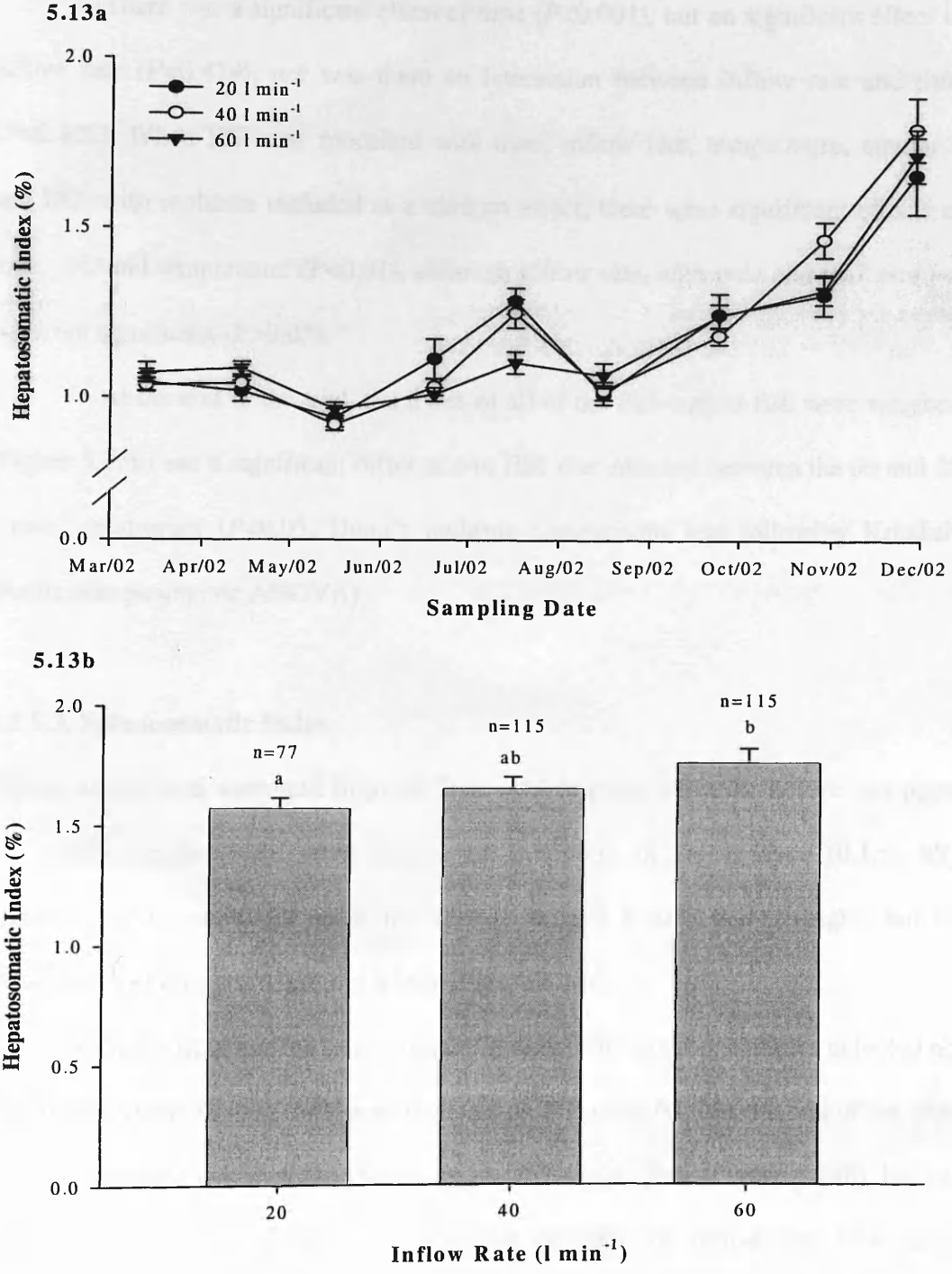


Figure 5.12. Condition Factor of rainbow trout cultured in tanks with different inflow rates. Each point represents the mean (± SEM) individual Condition Factor of all of the tagged fish within each treatment (n =120 for 40 and 60 l min^{-1} treatments, and 80 for 20 l min^{-1} treatment following the loss of 1 replicate). Treatments not sharing a common letter are significantly different at that time point (P<0.05; Tukey's multiple comparison post-hoc following GLM with weight as a dependent variable and time and inflow rate as categorical predictors, with replicate as a random factor).



Figures 5.13. Hepatosomatic Index of rainbow trout cultured in tanks with different inflow rates. 5.13a shows HSI from monthly samples (n=30 for 40 and 60 l min^{-1} treatments, and 20 in the 20 l min^{-1} treatment). 5.13b shows HSI of all tagged fish at the end of the trial (columns with different letters denote significant differences; $P<0.05$).

There was a significant effect of time ($P<0.001$), but no significant effect of inflow rate ($P=0.419$), nor was there an interaction between inflow rate and time ($P=0.102$). When HSI was modelled with time, inflow rate, temperature, ammonia and DO, with replicate included as a random effect, there were significant effects of time, DO and temperature ($P<0.01$), although inflow rate, ammonia and tank number were not significant ($P>0.05$).

At the end of the trial, the livers of all of the PIT-tagged fish were weighed (Figure 5.13b) and a significant difference in HSI was detected between the 60 and 20 l min^{-1} treatments ($P<0.05$; Dunn's multiple comparisons test following Kruskal-Wallis non-parametric ANOVA).

5.3.5.3. Splenosomatic Index

Spleen weight was measured from the June sample point onwards, before this point the spleen weights were often below the sensitivity of the balance (0.1g). SSI remained fairly stable for most the trial at around 0.20% body weight, but in November SSI dropped to around 0.15% (Figure 5.14a).

A GLM using arcsine transformed SSI data from monthly samples detected no significant effects of time, inflow or replicate on SSI ($P<0.05$). At the end of the trial the spleen weight was measured for all of the PIT-tagged fish (Figure 5.14b), but no significant differences in SSI were observed between the inflow rate treatments (Kruskal-Wallis non-parametric ANOVA on transformed SSI values).

5.3.6. Maturation and sex differentiation

It was only possible to confidently determine the sex of fish by gross examination from July onwards and before this point sex determination was not carried out. At the end of the experiment all remaining fish (tagged and untagged) were sexed. The ratio of male to female fish in the PIT-tagged populations were 1.14, 0.83 and 0.95:1 respectively in the 20, 40 and 60 l min^{-1} treatments (Table 5.4). The overall ratio of male to female fish for all fish within each treatment (tagged and untagged) was 1.07, 1.02 and 0.90:1 respectively in the 20, 40 and 60 l min^{-1} treatments.

The gonads of all PIT-tagged fish were excised and weighed at the end of the experiment to allowing the sex and GSI of each fish to be determined (Table 5.4 and Figure 5.15).

Table 5.4. Maturation assessment and sex allocation of all PIT-tagged rainbow trout within each treatment at the end of the experiment.

Treatment	GSI data of PIT-tagged fish (%)			Sex		
	Male	Female	All	Male	Female	M : F
20 l min^{-1}	4.1 ± 1.6	1.0 ± 1.3	2.7 ± 2.1	41	36	1.14
40 l min^{-1}	3.6 ± 1.8	1.2 ± 1.7	2.3 ± 2.1	52	63	0.83
60 l min^{-1}	4.4 ± 1.2	1.7 ± 1.7	2.8 ± 1.9	56	59	0.95

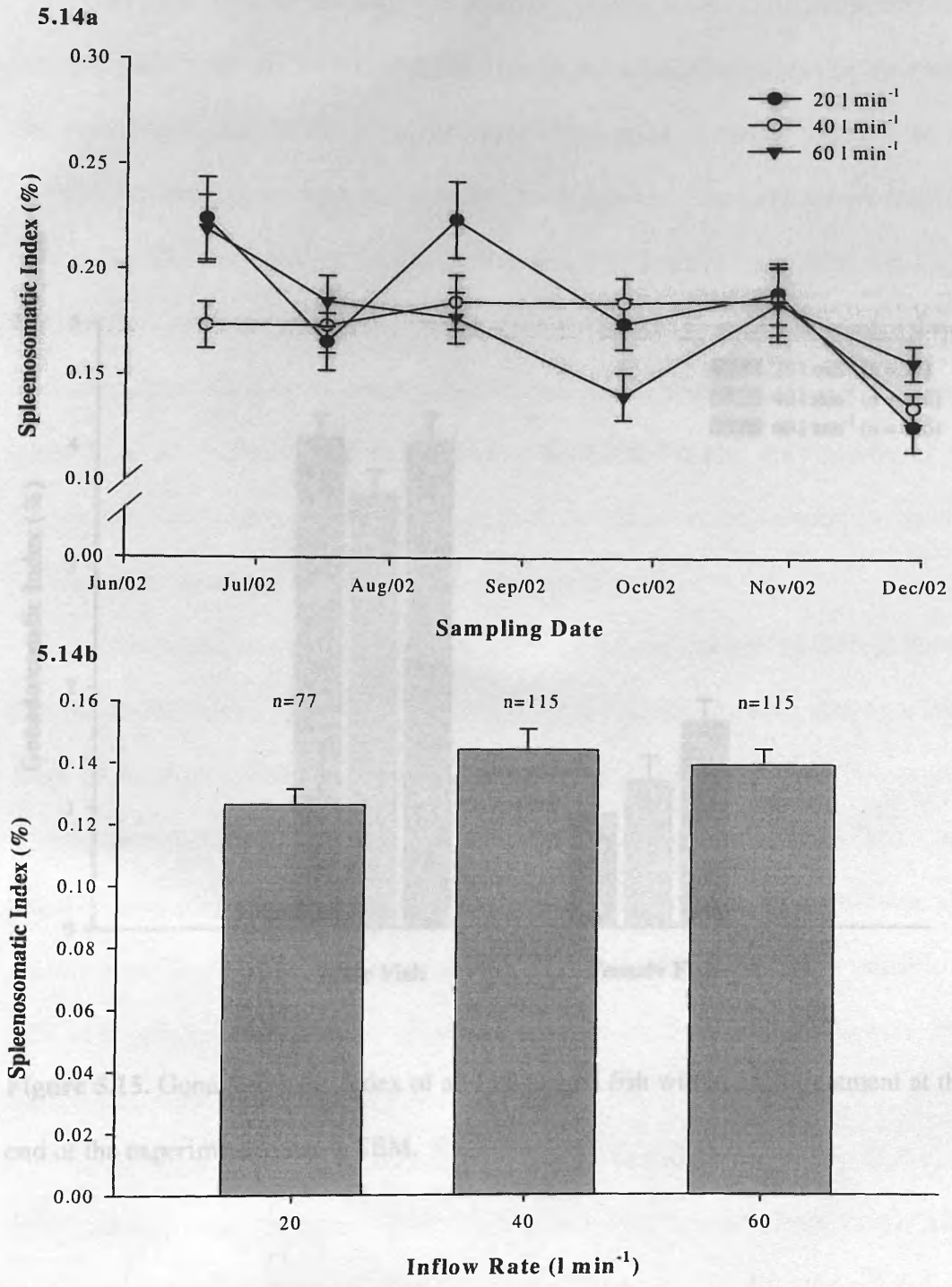


Figure 5.14. Splenosomatic Index of rainbow trout cultured in tanks with different inflow rates. 5.14a shows SSI from monthly samples (n=30 for 40 and 60 l min^{-1} treatments, and 20 in the 20 l min^{-1} treatment). 5.14b shows HSI of all tagged fish at the end of the experiment; mean ± SEM.

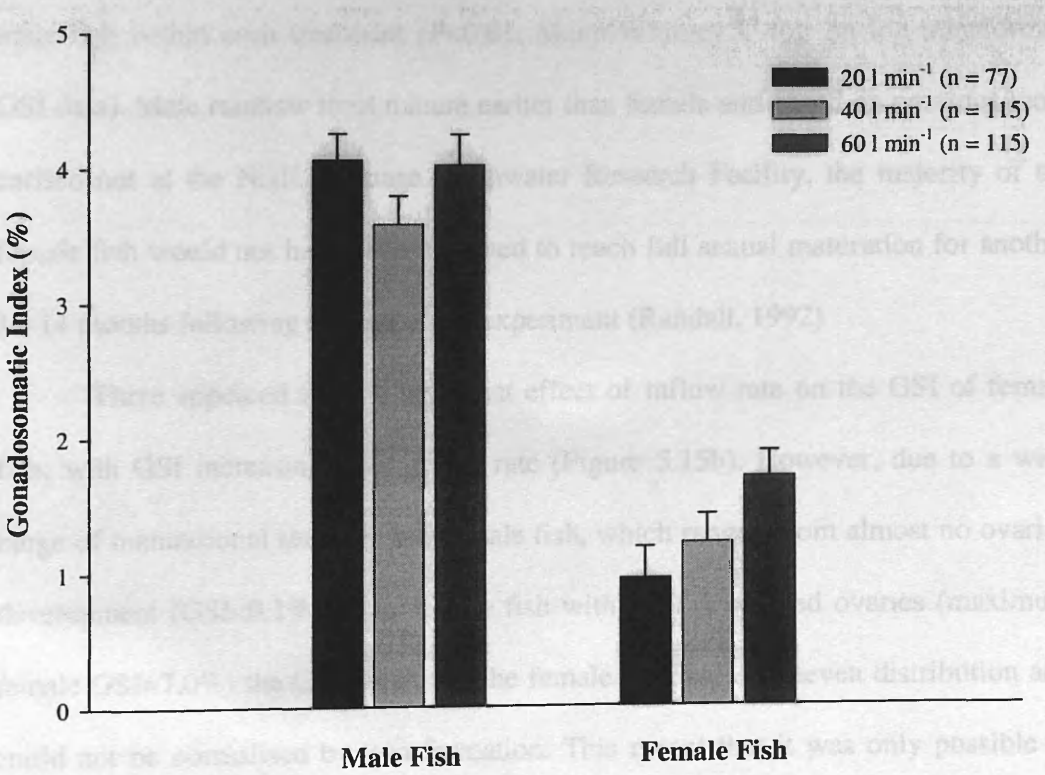


Figure 5.15. Gonadosomatic Index of all PIT-tagged fish within each treatment at the end of the experiment; mean ± SEM.

The GSI data for the male fish was very similar in all of the treatments (4.1, 3.6 and 4.0% in the 20, 40 and 60 l min^{-1} treatments respectively), and by the end of the experiment most of the male fish were either at an advanced stage of sexual maturation (displaying a kype and darkened body colour) or sexually mature (running with milt). The GSI data for the female fish was significantly lower than that of the male fish within each treatment ($P<0.01$; Mann-Whitney U-test on log transformed GSI data). Male rainbow trout mature earlier than female and based on previous work carried out at the Niall Bromage Freshwater Research Facility, the majority of the female fish would not have been expected to reach full sexual maturation for another 12-14 months following the end of the experiment (Randall, 1992).

There appeared to be a treatment effect of inflow rate on the GSI of female fish, with GSI increasing with inflow rate (Figure 5.15b). However, due to a wide range of maturational stages in the female fish, which ranged from almost no ovarian development (GSI<0.1%) up to female fish with well developed ovaries (maximum female GSI=7.0%) the GSI data from the female fish had an uneven distribution and could not be normalised by transformation. This meant that it was only possible to carry out non-parametric statistical analysis where no significant difference was observed between the treatments ($P=0.172$).

A Kruskal-Wallis non-parametric ANOVA was carried out on the GSI data, though no significant differences were detected following separate comparison of GSI from male and female fish within each treatment. When the GSI of male and female fish was analysed as a single variable in for each treatment, there was again no significant effect of inflow rate ($P=0.079$).

Comparison of total body weight of the PIT-tagged male and female fish within each treatment found the male fish to be significantly larger than the female

fish in the 40 l min^{-1} treatment (P=0.012), but no significant difference was observed in the 20 l min^{-1} (P=0.099) or 60 l min^{-1} treatments (P=0.923); the weights of the male and female fish in the 60 l min^{-1} treatment were very similar (Table 5.5).

Table 5.5. Comparison of the mean weights of male and female PIT-tagged rainbow trout that were cultured for 10 months under different flow regimes.

Inflow rate (l min^{-1})	Mean weight (± SEM) of male fish (g)	Mean weight (± SEM) of female fish (g)	Student's T-test P
20	600.6 ± 19.2	558.1 ± 16.2	0.099
40	626.1 ± 16.2	570.1 ± 14.9	0.012
60	650.6 ± 19.7	652.8 ± 15.3	0.923

5.3.7. Blood analysis

Two different GLMs were used to test to for significant effects on each of the welfare indicators from the blood. The effects of time and inflow rate were examined by modelling each indicator as a dependent variable in a GLM with time and inflow rate included as categorical predictors, and replicate as a random effect; this model will be referred to as GLM1 and the results are summarised in Table 5.6. GLM1 allowed for post-hoc analysis and also presented clear information regarding the bearing of any significant effects and/or changes over time.

Data for each welfare indicator were subsequently included as a dependent variable in a GLM with time, inflow rate, DO, ammonia and temperature as continuous independent variables, with replicate as a random factor; this model will be referred to as GLM2 and the results are displayed in Table 5.7. GLM2 allowed more parameters to be modelled, but did not permit post-hoc analysis, nor was it possible to determine derive details of the nature of any significant effects of independent variables on the welfare indicators.

Table 5.6. Summary of results from statistical analysis of blood parameters as a dependent variable in GLM1, with time and inflow rate as categorical predictors and replicate as a random categorical factor.

Dependent Variable	Effect		Degrees of Freedom	F	*P*
Haematocrit	Intercept	Fixed	1	46126.68	0.000
	Time	Fixed	8	20.30	0.000
	Inflow Rate	Fixed	2	1.13	0.389
	Replicate	Random	2	3.25	0.714
	Time*Inflow Rate	Fixed	16	0.71	0.759
	Time*Replicate	Random	16	0.83	0.649
	Inflow Rate*Replicate	Random	4	0.24	0.911
	Time *Inflow Rate*Replicate	Random	24	3.79	0.000
	Error		633		
Glucose	Intercept	Fixed	1	3894.76	0.000
	Time	Fixed	8	33.96	0.000
	Inflow Rate	Fixed	2	0.42	0.681
	Replicate	Random	2	1.44	0.363
	Time*Inflow Rate	Fixed	16	1.52	0.172
	Time*Replicate	Random	16	0.87	0.611
	Inflow Rate*Replicate	Random	4	1.34	0.283
	Time *Inflow Rate*Replicate	Random	24	1.93	0.005
	Error		632		
Lysozyme activity	Intercept	Fixed	1	3510.16	0.000
	Time	Fixed	8	44.59	0.000
	Inflow Rate	Fixed	2	8.35	0.010
	Replicate	Random	2	3.61	0.372
	Time*Inflow Rate	Fixed	16	2.33	0.030
	Time*Replicate	Random	16	1.21	0.327
	Inflow Rate*Replicate	Random	4	0.08	0.988
	Time *Inflow Rate*Replicate	Random	24	1.43	0.083
	Error		635		
Cortisol	Intercept	Fixed	2	443.26	0.001
	Time	Fixed	15	16.79	0.000
	Inflow Rate	Fixed	4	0.51	0.635
	Replicate	Random	4	0.56	0.611
	Time*Inflow Rate	Fixed	24	1.21	0.327
	Time*Replicate	Random	24	1.03	0.462
	Inflow Rate*Replicate	Random	24	1.33	0.286
	Time *Inflow Rate*Replicate	Random	631	2.43	0.000
	Error				

Table 5.7. Summary of results from statistical analysis of blood parameters as dependent variables in GLM2, with time, inflow rate, temperature, dissolved oxygen and ammonia as continuous predictors and replicate as a random factor.

Dependent Variable	Whole model effects			*P* values for univariate results for continuous predictors						Random Effects
	Adjusted R^2	F	*P*	Intercept	Time	Flow Rate	Temp	DO	Ammonia	Replicate
Hepatosomatic Index (arcsine transformed)	0.46	44.50	0.000	0.000	0.000	0.727	0.000	0.086	0.468	0.676
Haematocrit (arcsine transformed)	0.24	32.3	0.000	0.000	0.000	0.034	0.534	0.331	0.002	0.306
Glucose	0.09	9.51	0.000	0.029	0.802	0.905	0.000	0.000	0.163	0.197
Lysozyme activity	0.42	75.8	0.000	0.000	0.149	0.223	0.000	0.000	0.022	0.670
Cortisol	0.18	23.2	0.000	0.001	0.000	0.015	0.001	0.000	1.000	0.259

5.3.7.1. Haematocrit

Similarly to the experiment outlined in Chapter 4, there appeared to be an effect of water temperature on haematocrit levels, with haematocrit generally higher during periods of low water temperature (Figure 5.16). There was a significant effect of time on arcsine transformed haematocrit values ($P>0.001$), but there was no significant effect of inflow rate ($P=0.389$), with very similar haematocrit levels in all of the treatments throughout the course of the experiment (Table 5.6). There was a significant interaction between time, inflow rate and replicate on haematocrit levels ($P<0.001$), but this was likely to have largely been a result of the large temporal fluctuations in haematocrit, as there was no significant effect of inflow rate ($P=0.389$) or replicate ($P=0.714$). The results from GLM2 detected significant effects of time, ammonia ($P<0.01$) and inflow rate ($P<0.05$) on haematocrit (Table 5.7). The effect of time was expected, but the direction of the significant effect of inflow rate was unclear, given the apparent lack of differences between the treatments in GLM1.

5.3.7.2. Glucose

Plasma glucose concentrations remained similar in all of the treatments throughout the course of the trial (Figure 5.17). There was no significant effect of inflow rate on plasma glucose levels ($P=0.681$), nor was there a significant interaction between inflow rate and time ($P=0.172$). Similarly to haematocrit, there was a highly significant effect of time on plasma glucose concentration ($P<0.001$) and this was reflected in a significant interaction between time, inflow rate and replicate on GLM1 ($P=0.005$). Significant effects of temperature and DO ($P<0.001$) were observed in GLM2, suggesting possible reasons for the temporal fluctuations in glucose (Table 5.7).

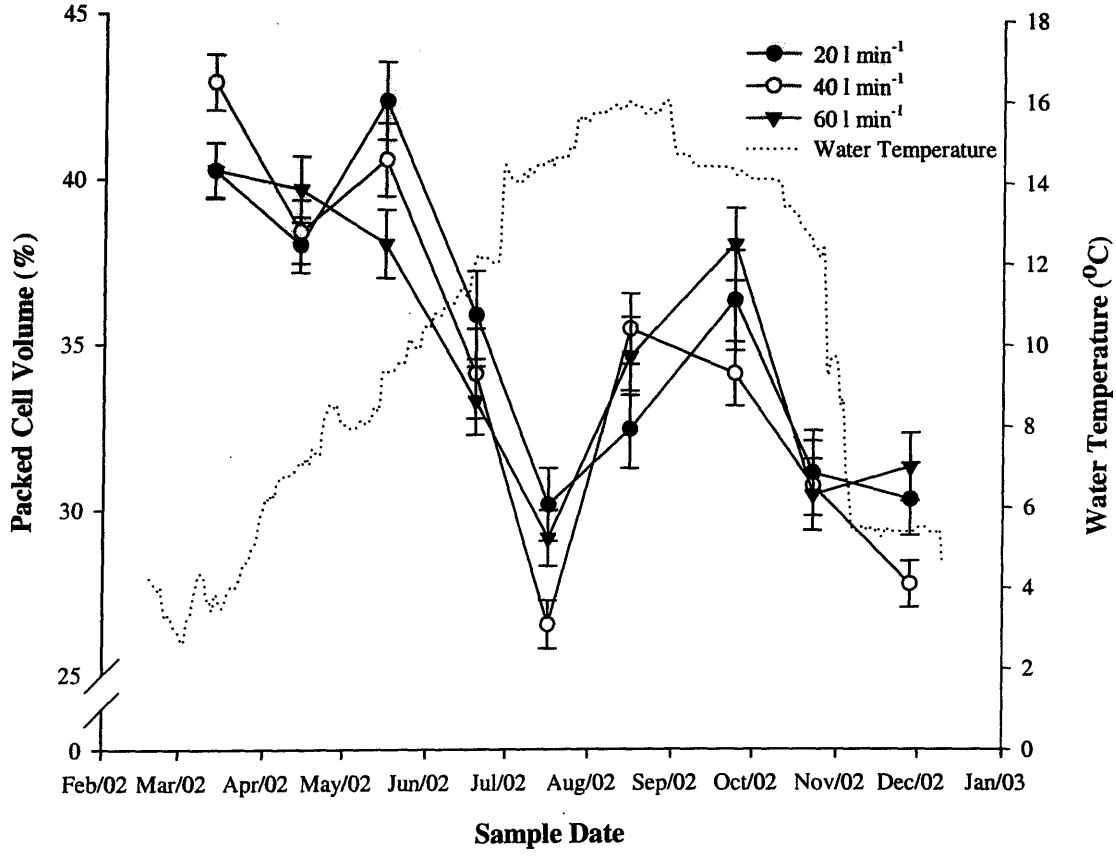


Figure 5.16. Haematocrit of rainbow trout cultured in tanks with different inflow rates (mean ± SEM; n=30 for 40 and 60 l min^{-1} treatments, and 20 in the 20 l min^{-1} treatment).

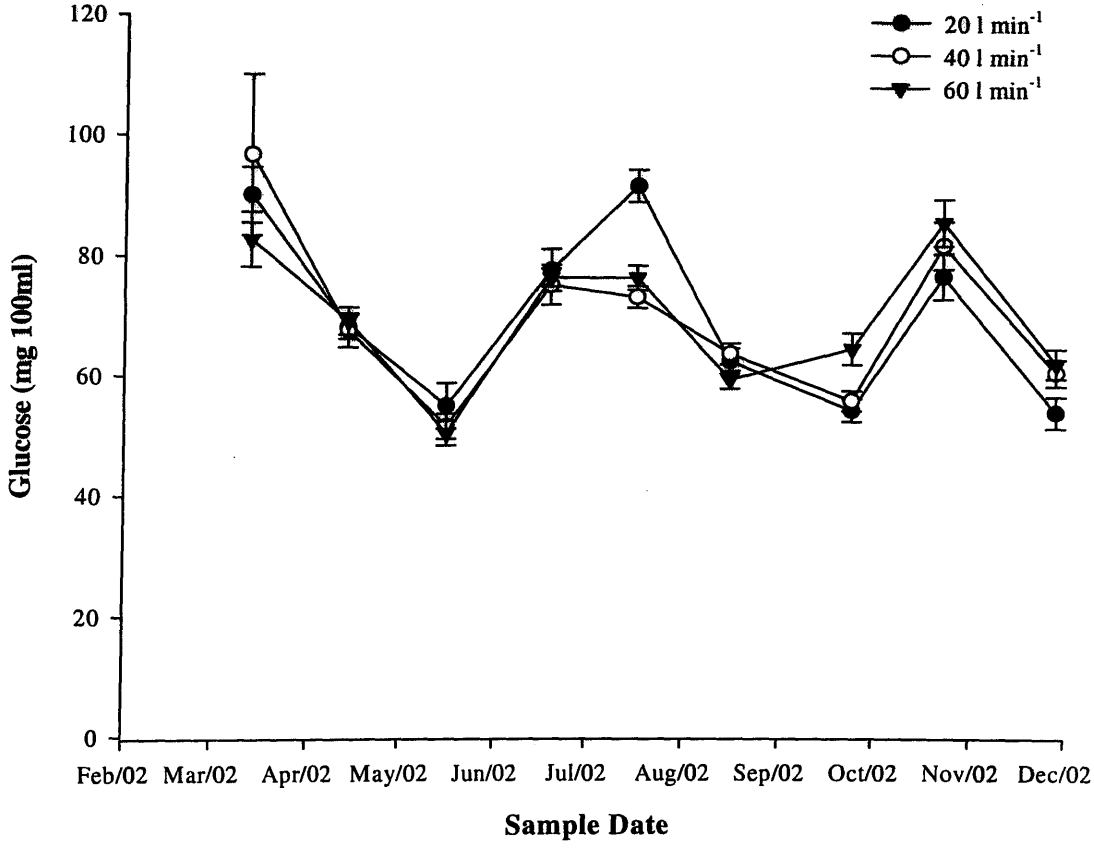


Figure 5.17. Plasma glucose of rainbow trout cultured in tanks with different inflow rates (mean ± SEM; n=30 for 40 and 60 l min^{-1} treatments, and 20 in the 20 l min^{-1} treatment).

5.3.7.3. Lysozyme activity

Lysozyme activity increased and decreased synchronously with water temperature, with lowest levels observed in March and highest levels occurring during the summer months (Figure 5.18). There was a significant effect of inflow rate on the lysozyme activity in GLM1 (P=0.01), and the effect was also a significant interaction between inflow rate and time (P=0.03). Levels of lysozyme activity appeared to be higher in the 20 l min^{-1} treatment in May and June, with a similar increase observed in the 40 l min^{-1} treatment during June and July. Post-hoc analysis confirmed lysozyme activity to be significantly higher in the 20 l min^{-1} treatment compared with the 60 l min^{-1} treatment in June (Tukey's P<0.001). There were significant effects of temperature, DO (P<0.001) and ammonia (P<0.05) on lysozyme activity in GLM2 (Table 5.7).

5.3.7.4. Cortisol

Levels of cortisol were low in all treatments for the duration of the experiment, and at no point did there appear to be any elevation from baseline levels (Figure 5.19). Cortisol levels appeared slightly higher in the in the 40 l min^{-1} treatment in March and April, but even at these times, the treatment mean remained low (> 6 ng ml^{-1}). When log-transformed cortisol data was included as a dependent variable in GLM1 there was a significant effect of time (P<0.001) and also an interaction between time, inflow rate and replicate (P<0.001; Table 5.6). GLM2 detected significant effects of time, DO (P<0.001), temperature (P=0.001) and inflow rate (P<0.05) on plasma cortisol levels (Table 5.7).

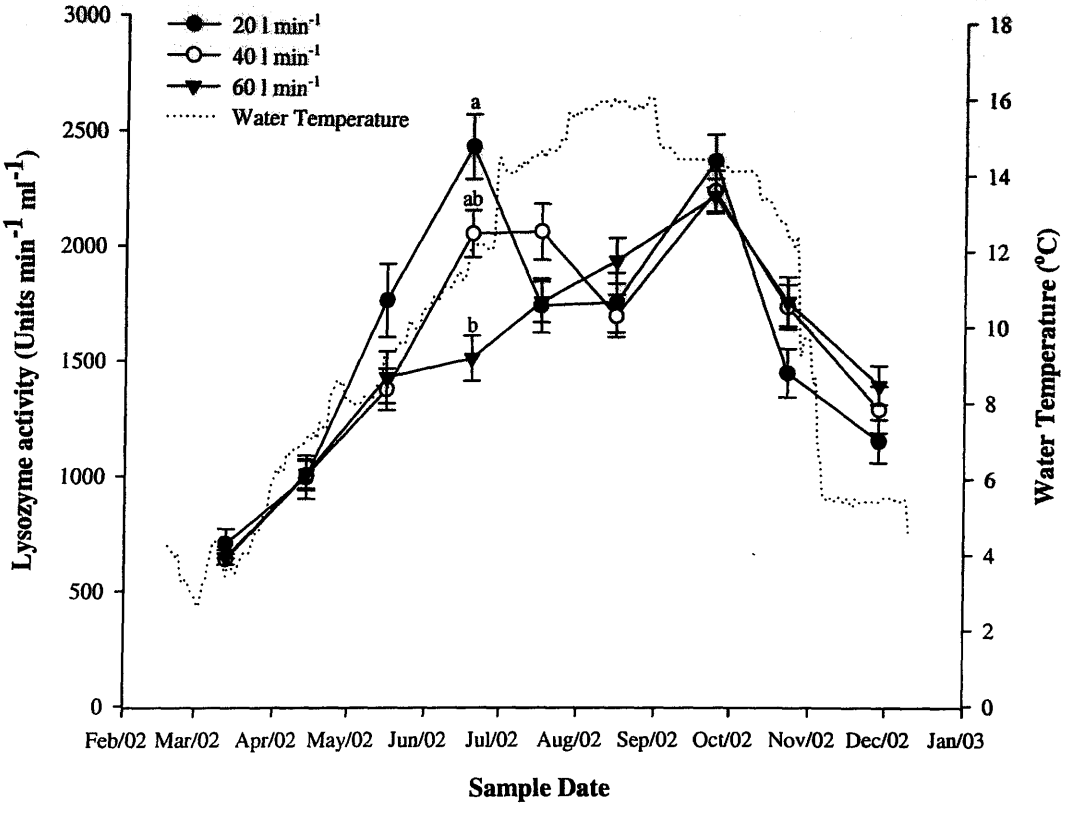


Figure 5.18. Lysozyme activity of rainbow trout cultured in tanks with different inflow rates (average ± SEM; n=30 for 40 and 60 l min^{-1} treatments, and 20 in the 20 l min^{-1} treatment). Different letters denote significant differences within that time point (P<0.05; Tukey's multiple comparison post-hoc following GLM with weight as a dependent variable and time and inflow rate as categorical predictors, with replicate as a random factor)

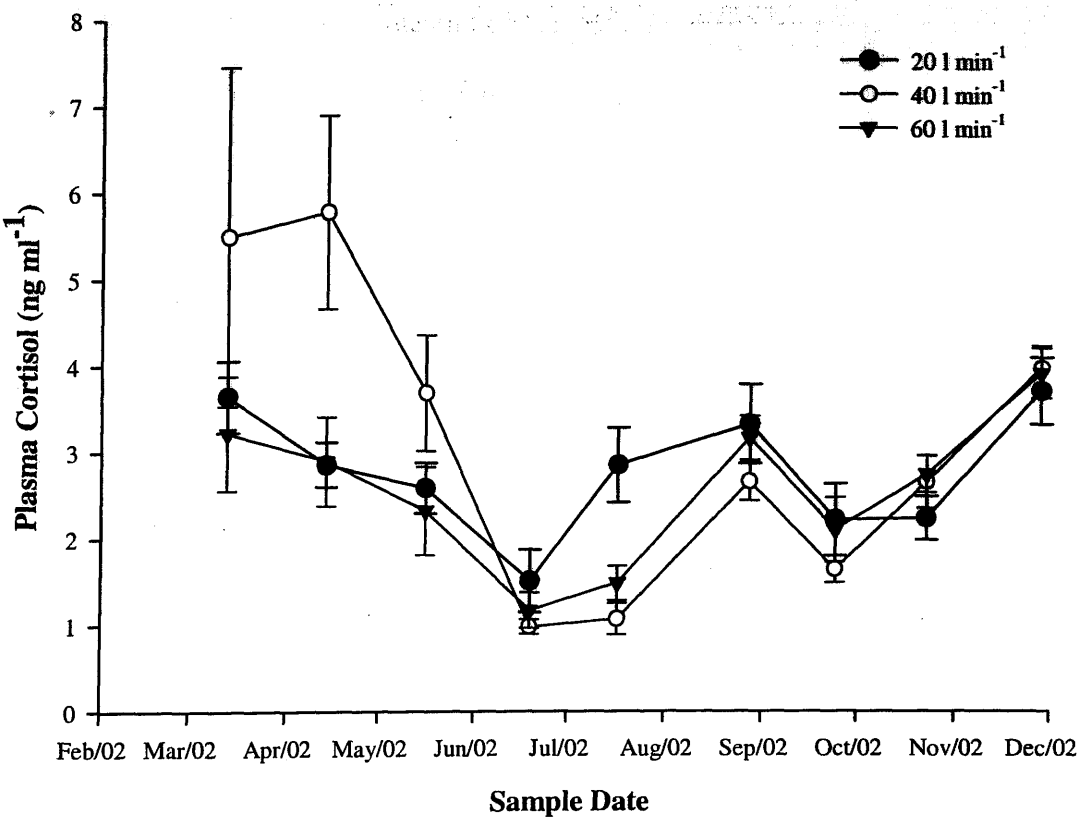


Figure 5.19. Plasma cortisol of rainbow trout cultured in tanks with different inflow rates (average ± SEM; n=30 for 40 and 60 l min^{-1} treatments, and 20 in the 20 l min^{-1} treatment).

5.3.8. Fin condition

The data for the RFL of the PIT-tagged fish measured at the start, middle and end of the experiment are presented in Figure 5.20. The result of repeated measures ANOVA was used to compare the RFL of each fin, of each of the PIT-tagged fish, as the trial progressed (Table 5.8). A one-way ANOVA was used to test for treatment effects on RFL of the PIT-tagged fish at the end of the experiment. Additionally, the RFL data for the 10 untagged fish that were sacrificed from each tank at the monthly samples were included as dependent variables in a GLM with time and inflow rate and as categorical predictors and replicate as a random factor (Table 5.9).

5.3.8.1. Dorsal fin

There was a significant increase in dorsal RFL between the start and middle of the experiment in the 20 l min^{-1} treatment ($P<0.01$), but no differences in dorsal RFL were observed between the start and end of the experiment in any of the treatments ($P>0.05$). There was no significant difference in dorsal RFL between the treatments at the end of the experiment ($P=0.226$). There was a significant effect of time on dorsal RFL collected from monthly sampling ($P<0.001$) (Table 5.9).

5.3.8.2. Caudal fin

There was a significant reduction in caudal RFL in all treatments at the middle and end of the experiment compared with the start ($P<0.001$), with mean caudal RFL above 10% at the start of the experiment and below 8% at trials conclusion (Figure 5.20). There were no significant differences in caudal RFL between the inflow rate treatments ($P=0.837$). The GLM detected a significant effect of time on caudal RFL $P=0.009$), and an interaction between time (inflow rate and replicate ($P=0.001$).

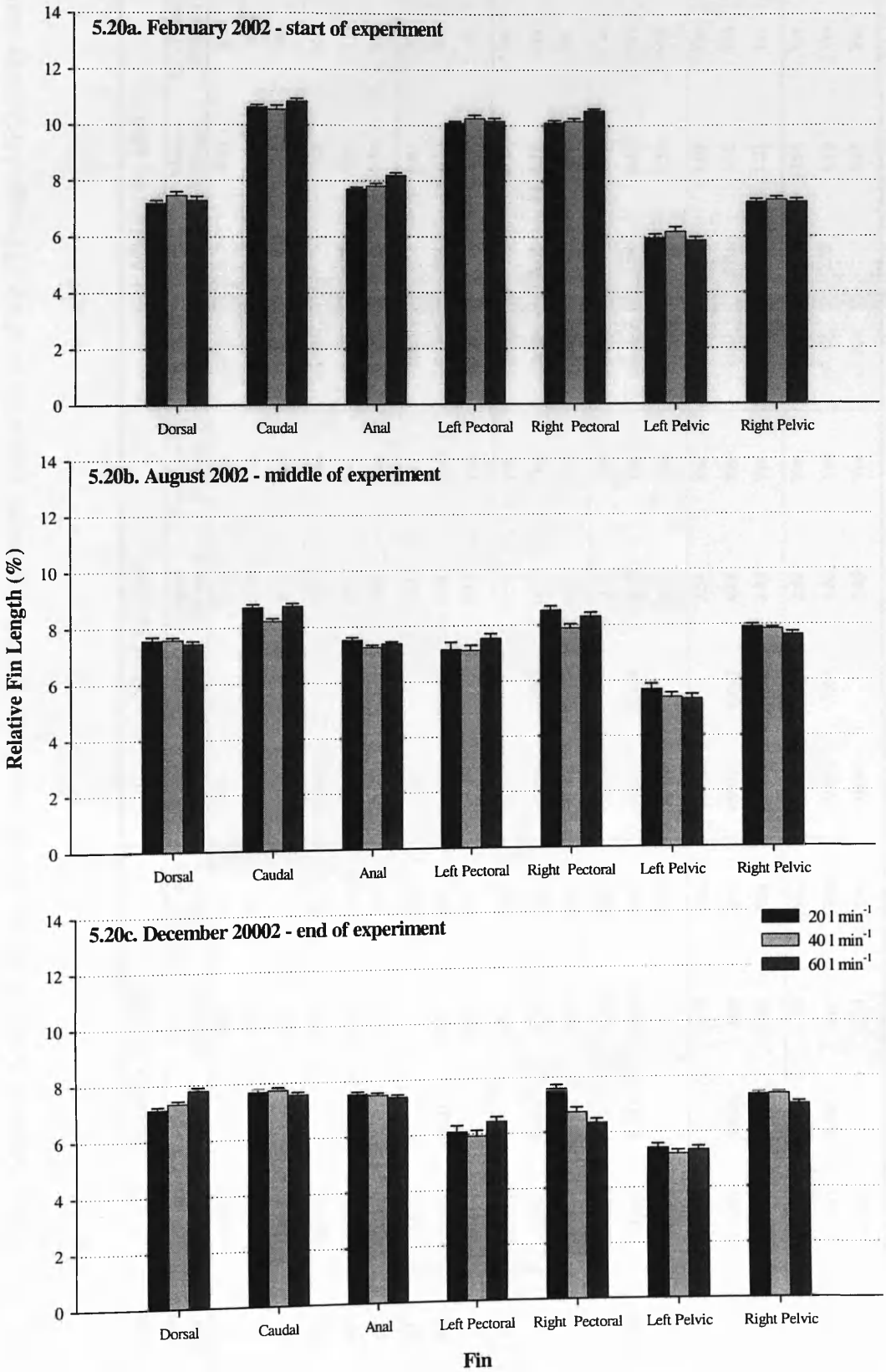


Figure 5.20. Relative fin index of PIT-tagged rainbow trout reared under different flow regimes at the start, middle, and end of the experiment (mean ± SEM).

Table 5.8. Summary a repeated measures ANOVA for the arcsine transformed relative fin length values for all of the PIT-tagged fish at the start, middle and end of the trial.

Fin	Flow Rate (l min^{-1})	Start vs. Middle				Start vs. End				Middle vs. End			
		Unadjusted P Value	Critical Level	Mean difference	Significantly different?	P Value	Critical Level	Mean difference	Significantly different?	P Value	Critical Level	Mean difference	Significantly different?
Dorsal	20	0.002		-0.41	Yes	0.936		0.01	No	<0.001		0.44	Yes
	40	NS	0.025	-0.27	No	NS	0.017	-0.02	No	NS	0.05	0.26	No
	60	NS		-0.14	No	NS		-0.11	No	NS		0.03	No
Caudal	20	<0.001		1.87	Yes	<0.001		2.91	Yes	<0.001		1.03	Yes
	40	<0.001	0.025	2.22	Yes	<0.001	0.017	2.81	Yes	<0.001	0.05	0.49	Yes
	60	<0.001		2.12	Yes	<0.001		3.09	Yes	<0.001		0.99	Yes
Anal	20	NS		0.15	No	NS		0.15	No	NS		0.01	No
	40	<0.001	0.025	0.47	Yes	0.003	0.017	0.24	Yes	0.051	0.05	-0.21	No
	60	<0.001		0.83	Yes	<0.001		0.82	Yes	0.952		0.01	No
Left Pelvic	20	<0.001		0.31	Yes	0.012		0.56	Yes	0.195		0.26	No
	40	<0.001	0.025	0.70	Yes	<0.001	0.017	0.90	Yes	0.342	0.05	0.20	No
	60	<0.001		0.56	Yes	<0.001		0.53	Yes	0.477		-0.02	No
Right Pelvic	20	<0.001		-0.80	Yes	<0.001		-0.21	Yes	0.034		0.59	Yes
	40	<0.001	0.025	-0.59	Yes	<0.001	0.017	-0.12	Yes	0.446	0.05	0.47	No
	60	<0.001		-0.40	Yes	0.204		0.12	No	0.001		0.59	Yes
Left Pectoral	20	<0.001		2.96	Yes	<0.001		2.54	Yes	<0.001		1.03	Yes
	40	<0.001	0.025	3.04	Yes	<0.001	0.017	4.24	Yes	<0.001	0.05	1.20	Yes
	60	<0.001		2.62	Yes	<0.001		3.89	Yes	<0.001		1.23	Yes
Right Pectoral	20	<0.001		1.58	Yes	<0.001		2.57	Yes	<0.001		0.94	Yes
	40	<0.001	0.025	2.21	Yes	<0.001	0.017	3.36	Yes	<0.001	0.05	1.16	Yes
	60	<0.001		2.23	Yes	<0.001		3.45	Yes	<0.001		1.25	Yes

Table 5.9. Summary of results from statistical analysis of arcsine transformed relative fin length data of untagged fish as a dependent variable in GLM with time and inflow rate as categorical predictors and replicate as a random factor.

Fin		Degrees of Freedom	F	*P*
Dorsal	Intercept	1	564354.7	0.000
	Time	8	4.5	0.006
	Inflow Rate	2	0.8	0.512
	Replicate	2	0.1	0.949
	Time*Inflow Rate	16	0.7	0.734
	Time*Replicate	16	0.9	0.552
	Inflow Rate*Replicate	4	1.5	0.233
	Time *Inflow Rate*Replicate	24	1.5	0.055
	Error	633		
Caudal	Intercept	1	3461.9	0.000
	Time	8	48.5	0.009
	Inflow Rate	2	0.5	0.634
	Replicate	2	0.4	0.693
	Time*Inflow Rate	16	0.7	0.746
	Time*Replicate	16	0.9	0.610
	Inflow Rate*Replicate	4	0.5	0.759
	Time *Inflow Rate*Replicate	24	2.1	0.001
	Error	633		
Anal	Intercept	1	21633.1	0.000
	Time	8	8.0	0.000
	Inflow Rate	2	1.1	0.392
	Replicate	2	16.8	0.706
	Time*Inflow Rate	16	0.5	0.895
	Time*Replicate	16	0.6	0.883
	Inflow Rate*Replicate	4	0.5	0.754
	Time *Inflow Rate*Replicate	24	3.8	0.000
	Error	633		
Left Pelvic	Intercept	1	5283.5	0.000
	Time	8	1.8	0.273
	Inflow Rate	2	0.2	0.839
	Replicate	2	0.1	0.894
	Time*Inflow Rate	16	0.5	0.930
	Time*Replicate	16	1.5	0.170
	Inflow Rate*Replicate	4	0.4	0.799
	Time *Inflow Rate*Replicate	24	2.1	0.002
Right Pelvic	Intercept	1	10044.5	0.000
	Time	8	14.2	0.042
	Inflow Rate	2	0.4	0.663
	Replicate	2	2.0	0.161
	Time*Inflow Rate	16	0.7	0.767
	Time*Replicate	16	0.9	0.594
	Inflow Rate*Replicate	4	0.8	0.540
	Time *Inflow Rate*Replicate	24	2.0	0.003
Left Pectoral	Intercept	1	817.0	0.000
	Time	8	64.2	0.001
	Inflow Rate	2	0.0	0.989
	Replicate	2	0.3	0.748
	Time*Inflow Rate	16	0.8	0.671
	Time*Replicate	16	1.0	0.502
	Inflow Rate*Replicate	4	0.3	0.857
	Time *Inflow Rate*Replicate	24	1.3	0.176
Right Pectoral	Intercept	1	2079.9	0.000
	Time	8	12.2	0.000
	Inflow Rate	2	1.8	0.198
	Replicate	2	4.4	0.027
	Time*Inflow Rate	16	3.6	0.002
	Time*Replicate	16	1.8	0.086
	Inflow Rate*Replicate	4	0.8	0.516
	Time *Inflow Rate*Replicate	24	1.0	0.530

5.3.8.3. Anal

There was a significant reduction in the anal RFL of the PIT-tagged fish in the 40 and 60 l min^{-1} treatments ($P<0.001$), but no significant difference was observed in the 20 l min^{-1} treatment ($P>0.05$). There was a significant effect of time ($P<0.001$) and also a significant interaction between, inflow rate and replicate on anal RFL ($P<0.001$; Table 5.9). However, there were no differences in anal RFL between the inflow rate treatments at the end of the experiment ($P=0.483$).

5.3.8.4. Pelvic fins

There was a significant reduction in left ventral RFL in all of the treatments between the start and end of the experiment ($P<0.001$). However, no differences were observed in the right pelvic RFL at the start compared with the end of the trial ($P>0.05$; Table 5.8), and there was a significant increase in right pelvic RFL in the 20 and 40 l min^{-1} treatments at the mid-point compared with the start (Table 5.8). There were no differences between left ventral RFL of the PIT-tagged fish in the different inflow treatments at the end of the experiment ($P=0.445$), but there was a significant treatment effect on right pelvic RFL ($P=0.02$). Post-hoc analysis detected a significant difference in right ventral RFL between the 40 and 60 l min^{-1} treatments (Tukey's; $P<0.05$). The GLM detected a significant effect of time on right pelvic RFL ($P=0.042$) and there was a significant interaction between time, inflow rate and replicate on both the left and right pectoral fins of the untagged fish ($P<0.01$; Table 5.9). Finally, paired *T*-test between the left and right pelvic fins of each PIT-tagged fish found the left pelvic RFL to be significantly lower than the right pelvic RFL in all of the treatments ($P<0.001$ in all cases).

5.3.8.5. Pectoral fins

Similarly to the pelvic fins, the RFL of the left pectoral fins appeared lower than the right pectoral fins (Figure 5.20). A paired *T*-test of the left and right pectoral RFL values confirmed this difference to be statistically significant in all of the treatments $P<0.001$). At the start of the experiment the RFL for the right and left pectoral fins was between 10 and 10.5%, but by the end of the experiment pectoral fin RFL was between 7.1 and 8.5% (Figure 5.20). A paired *T*-test comparing the pectoral RFL data for the PIT-tagged fish at the start compared with the middle and end of the experiment found significant decreases in the both right and left pectoral fins in all of the inflow rate treatments ($P<0.001$ in all cases; Table 5.8). The GLM for the untagged fin data detected a significant effect of time on both the right and left pectoral fins ($P<0.001$; Table 5.9).

Between treatment comparisons of the RFL of the pectoral fins at the end of the experiment found no significant differences for left pectoral fin ($P=0.259$), but there were significant differences between treatments for the right pectoral RFL ($P<0.001$). Post-hoc analysis found the right pectoral RFL to be significantly higher in the 20 compared with the 40 l min^{-1} treatment (Tukey's; $P<0.001$).

5.3.9. Principal Components Analysis (PCA)

Data collected from the 30 fish blood sampled monthly from each treatment was used in the PCA analysis. Case-wise deletion was used to remove any fish that had missing data for any of the parameters from the analysis. Any variables expressed as a percentage (HSI, RFL and haematocrit) were arcsine transformed before inclusion in the PCA.

5.3.9.1. PCA of blood parameters

PCA of welfare indicators measured from the blood produced two viable PCs with Eigenvalues >1, which together accounted for more than 60% of the observed variation of these indicators (Figure 5.21); these PCs for the blood parameters will be referred to as B-PC1 and B-PC2. An indication of the magnitude and direction of contribution for each of the blood parameters is shown in Table 5.10.

Table. 5.10. Contribution and factor coordinates and of the variables included in blood PCA.

Variable	B-PC1		B-PC2	
	Contribution	Co-ordinates	Contribution	Co-ordinates
Cortisol	0.313	0.664	0.019	-0.140
Lysozyme	0.348	-0.700	0.063	-0.254
Glucose	0.002	0.058	0.878	0.952
Haematocrit	0.336	0.688	0.040	-0.204

B-PC1 consisted of positive contributions from cortisol and haematocrit, a negative contribution from lysozyme and a negligible contribution from glucose. A fish with a high factor score for B-PC1 would be displaying signs characteristic of the acute stress response. The main variable contributing to B-PC2 was glucose (0.878). High positive factor co-ordinates for glucose (0.952) indicate that a fish with a high factor score for B-PC2 would have had a high plasma glucose concentration.

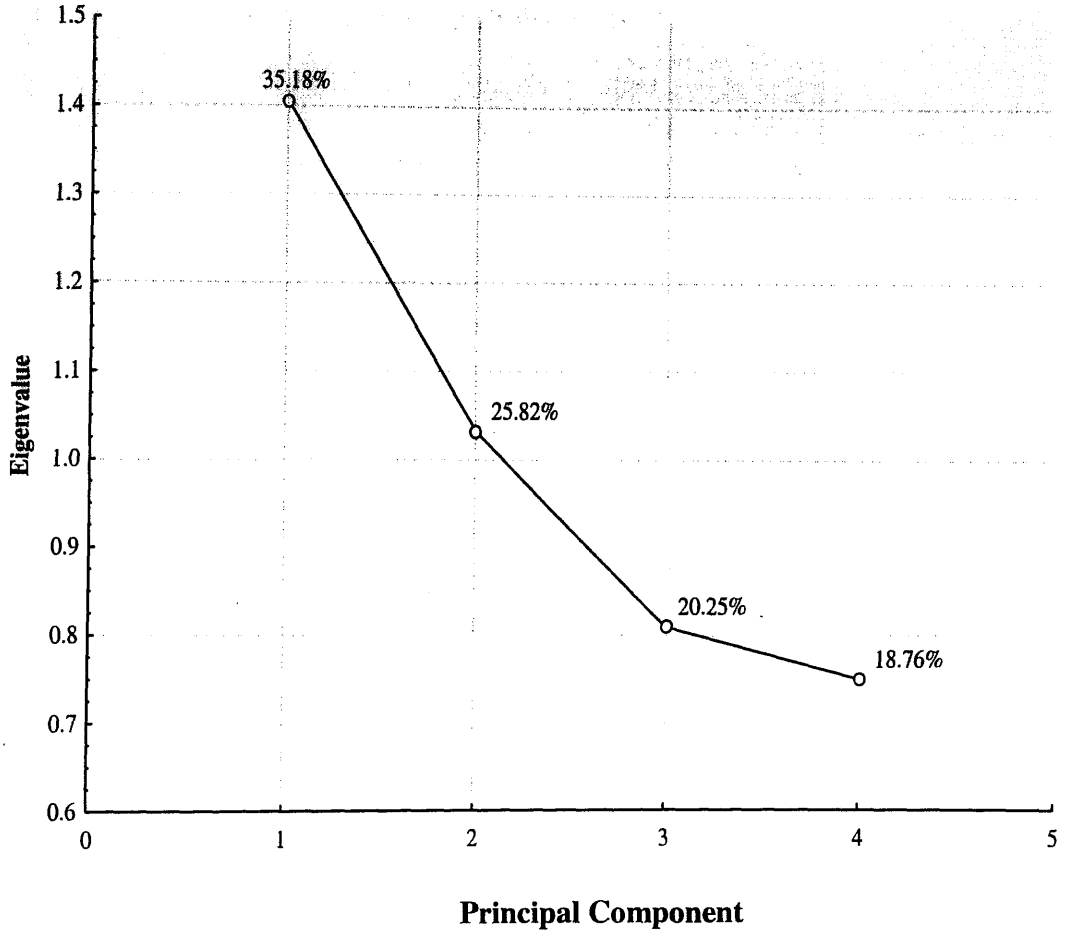


Figure 5.21. Scree-plot showing Eigenvalues for PCs of blood parameters; plasma cortisol, lysozyme activity and glucose, with arcsine transformed haematocrit values; percentage values indicate the proportion of explained variability for each PC.

The factor scores for each of the fish were included in the GLMs using time, inflow rate and water quality parameters as continuous predictors with tank number as random categorical factor. Both models were highly significant, although the R^2 of the models suggested that the model using B-PC1 was a much better fit (R^2= 0.38 and 0.06 for B-PC1 & B-PC2 respectively; Table 5.11).

Table 5.11. Whole model effects for GLM including factor scores for PCs derived from based from blood parameters.

Dependent Variable	Adjusted R^2	SS	Degrees of Freedom	F	*P*
BloodPC1	0.38	267.82	7	61.24	0.000
BloodPC2	0.06	56.82	7	4.33	0.000

Table 5.11 shows the results for univariate tests of significance for the variables in the GLMs. Interestingly, neither time or flow rate had a significant effect in either model. There was, however, a significant effect water temperature (P<0.05) and DO (P<0.001) and in both models. Ammonia was also shown to have a significant effect on B-PC2 (P=0.021).

Table 5.12. Univariate tests of significance for B-PCs 1 and 2.

Dependent Variable		Effect	SS	Degrees of freedom	F	*P*
B-PC1	Intercept	Fixed	3.40	1	5.43	0.020
	Time	Fixed	1.33	1	2.13	0.145
	Flow Rate	Fixed	1.44	1	2.31	0.129
	Temp	Fixed	25.20	1	40.34	0.000
	DO	Fixed	24.63	1	39.44	0.000
	Ammonia	Fixed	0.21	1	0.34	0.559
	Tank No.	Random	2.12	2	1.70	0.183
	Error		434.17	695		

Table 5.12. (continued) Univariate tests of significance for blood PCs

Dependent Variable		Effect	SS	Deg. of freedom	F	*P*
B-PC2	Intercept	Fixed	29.40	1	32.04	0.000
	Time	Fixed	0.00	1	0.00	0.948
	Flow Rate	Fixed	0.48	1	0.52	0.471
	Temp	Fixed	3.80	1	4.16	0.042
	DO	Fixed	32.06	1	35.06	0.000
	Ammonia	Fixed	4.90	1	5.36	0.021
	Tank No.	Random	2.88	2	1.57	0.208
	Error		635.52	695		

5.3.9.2. PCA of fin measurements

Similarly to the blood PCA, two viable PCs were derived from the RFI data for all of the fins (Figure 5.22). The relative contribution and factor co-ordinates of the variables for Fin-PCs 1 and 2 are shown in table 5.13.

Table 5.13. Factor co-ordinates of the variables included in PCA for arcsine transformed relative fin index data.

Variable	Fin-PC1		Fin-PC2	
	Contribution	Co-ordinates	Contribution	Co-ordinates
Dorsal	0.098	-0.487	0.013	-0.136
Caudal	0.186	-0.670	0.075	0.329
Anal	0.154	-0.609	0.041	-0.243
Left Pelvic	0.079	-0.436	0.273	-0.627
Right Pelvic	0.036	-0.294	0.434	-0.790
Left Pectoral	0.213	-0.717	0.102	0.383
Right Pectoral	0.233	-0.750	0.063	0.301

The factor co-ordinates of Fin-PC1 were all negative, with relatively strong contributions from the caudal, anal and pectoral fins. In contrast, there was a strong contribution for the ventral fins in Fin-PC2, with a much smaller contribution from the other fins. A fish with a high factor score for Fin-PC1 would have a low RFI for caudal, anal and pectoral fin, whilst a high factor score for Fin-PC2 would signify a low RFI for pelvic fins.

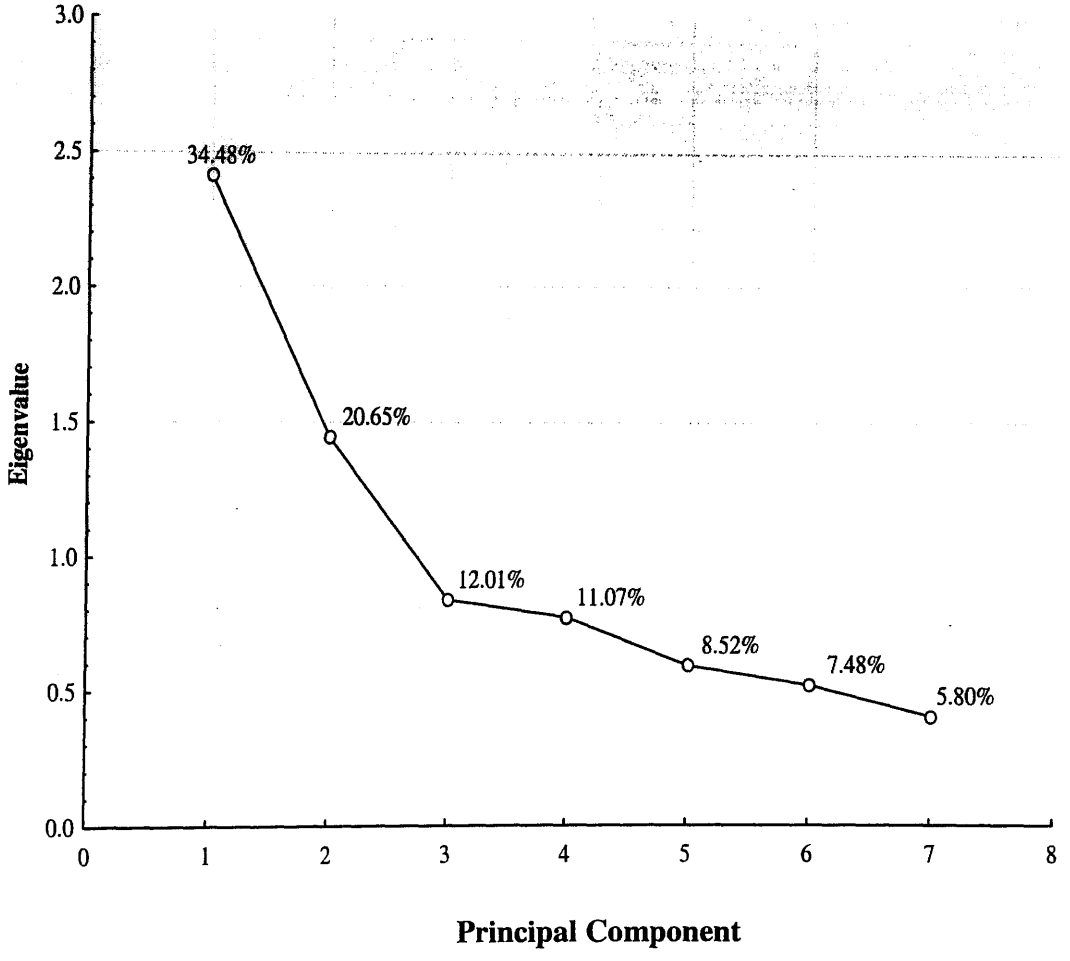


Figure 5.22. Scree plot showing Eigenvalues from PCA for arcsine transformed relative fin index values; percentage values indicate the proportion of explained variability for each PC.

The Fin PCs were included in the GLM and produced similar R^2 values of 0.345 and 0.301 respective (Table 5.14).

Table 5.14. Whole model effects for GLM including factor scores for PCs derived from relative fin length data.

Dependent Variable	Adjusted R^2	SS	Degrees of Freedom	F	*P*
Fin-PC1	0.345	247.72	7	54.02	0.000
Fin-PC2	0.301	217.53	7	22.85	0.000

The univariate results for the GLMs for Fin-PCs 1 and 2 are shown in (Table 5.15). Both fin PCs were influenced by time and temperature, and there was also a significant random effect of replicate on both models ($P<0.05$). The GLM for Fin-PC2 was also detected significant effects of inflow rate, DO and ammonia ($P<0.01$).

Table 5.15. Univariate tests of significance for GLMs using factor scores for Fin-PCs 1 and 2 as dependent variables.

Dependent Variable		Effect	SS	Degrees of Freedom	F	*P*
Fin-PC1	Intercept	Fixed	12.52	1	18.86	0.000
	Time	Fixed	41.84	1	63.87	0.000
	Flow Rate	Fixed	0.02	1	0.03	0.866
	Temp	Fixed	7.23	1	11.04	0.001
	DO	Fixed	0.75	1	1.15	0.284
	Ammonia	Fixed	0.07	1	0.11	0.739
	Replicate	Random	4.35	2	3.32	0.037
	Error		457.27	698		
Fin-PC2	Intercept	Fixed	17.91	1	25.19	0.000
	Time	Fixed	71.94	1	103.01	0.000
	Flow Rate	Fixed	6.93	1	9.92	0.002
	Temp	Fixed	7.70	1	11.03	0.001
	DO	Fixed	7.39	1	10.58	0.001
	Ammonia	Fixed	4.15	1	5.95	0.015
	Replicate	Random	5.61	2	4.02	0.018
	Error		487.47	698		

5.3.9.3. PCA combining all welfare indicators

The PCA analysis concluded by combining all of the welfare indicators in turn with Fin-PC1 and Fin-PC2. The two sets of analysis both produced just one viable PC each that explained approximately 30% of the observed variability within the indicators; these PCs will be referred to as C-PC1 and C-PC2 (Figure 5.23). The contributions and co-ordinates of the variables in C-PC1 and C-PC2 are shown in are shown in tables 5.16 and 5.17.

Table 5.16. Factor co-ordinates and contributions of variables included in C-PC1

Variable	Combined PC including Fin-PC1	
	Contribution	Co-ordinates
CF	0.267	0.765
HSI	0.239	0.723
Haematocrit	0.194	-0.653
Cortisol	0.001	-0.048
Lysozyme	0.092	0.450
Glucose	0.000	-0.029
Fin-PC1	0.206	0.671

Table 5.17. Factor co-ordinates and contributions of variables included in C-PC2

Variable	Combined PC including Fin-PC2	
	Contribution	Co-ordinates
CF	0.283	0.780
HSI	0.271	0.763
Haematocrit	0.177	-0.617
Cortisol	0.004	-0.088
Lysozyme	0.079	0.411
Glucose	0.000	-0.019
Fin-PC2	0.186	-0.632

The pattern of the contribution and co-ordinates of the variables was very similar for both C-PC1 and C-PC2, though the co-ordinates of the fin component suggested a positive contribution of the Fin-PC1, but a negative contribution of Fin-PC2.

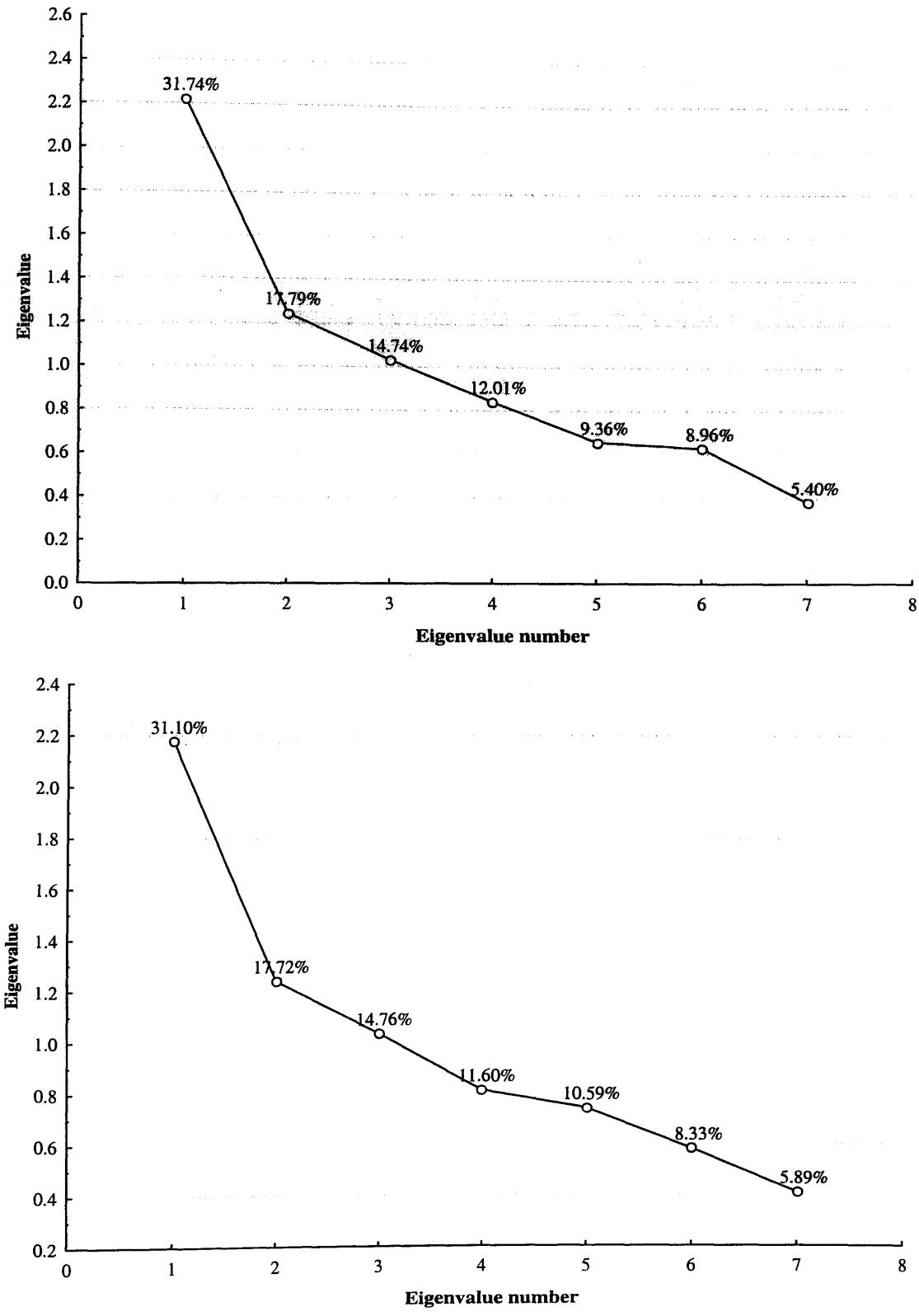


Figure 5.23. Scree-plots showing Eigenvalues for PCs that combined morphological and blood parameters with Fin-PCs 1 (5.23a) and 2 (5.23b); percentage values indicate the proportion of explained variability by each PC.

The nature of the contributions of the variables to the C-PC1 was complex. Positive co-ordinates for HSI, CF and lysozyme activity, with a negative contribution from haematocrit, suggested that a high factor score for C-PC1 would be indicative of a fish with good welfare. However, positive factor co-ordinates of Fin-PC1 suggested that low RFL values also contributed towards a high score for C-PC1.

The contribution of the variables in C-PC2 was almost identical to C-PC1, except for the bearing of Fin-PC2, which was negative. A high factor score for Fin-PC2 was typical of fish with low RFL for pelvic fins (Table 5.13). Therefore, a fish with a high factor score for C-PC2 would typically have high HSI, CF and lysozyme activity and ventral RFL, with low haematocrit, all of which would normally be associated with good welfare.

When the factor scores for the combined PCs were included as a dependent variable in the GLM, they produced very similar results (R^2 = 0.694 & 0.690 respectively; Table 5.18).

Table 5.18. Whole model effects for GLMs using factor scores for CPCs 1 and 2 as dependent variables.

Dependent Variable	Adjusted R^2	SS	Deg. of freedom	F	*P*
C-PC1	0.694	489.6	7	228.93	0.000
C-PC2	0.690	486.5	7	224.22	0.000

There was a significant effect of time on both of the models ($P<0.001$), but there was no significant effect ($P>0.05$) of flow rate, temperature or ammonia on either of the models (Table 5.19). There was, however, a significant effect of DO and random effect of replicate on C-PC2, which was not present on C-PC1.

Table 5.19. Univariate tests of significance for GLMs using factor scores for C-PCs 1 and 2 as dependent variables.

Dependent Variable		Effect	SS	Deg. of Freedom	MS	F	*P*
C-PC1	Intercept	Fixed	16.14	1	16.14	52.51	0.000
	Time	Fixed	102.68	1	102.68	336.04	0.000
	Flow Rate	Fixed	0.32	1	0.32	1.04	0.308
	Temp	Fixed	0.05	1	0.05	0.17	0.677
	DO	Fixed	0.47	1	0.47	1.55	0.213
	Ammonia	Fixed	0.83	1	0.83	2.71	0.100
	Replicate.	Random	1.23	2	0.61	2.01	0.135
	Error		212.35	695	0.31		
C-PC2	Intercept	Fixed	18.71	1	18.71	58.65	0.000
	Time	Fixed	120.29	1	120.29	388.04	0.000
	Flow Rate	Fixed	0.05	1	0.05	0.16	0.686
	Temp	Fixed	0.21	1	0.21	0.67	0.413
	DO	Fixed	1.84	1	1.84	5.95	0.015
	Ammonia	Fixed	0.19	1	0.19	0.61	0.433
	Replicate	Random	3.59	9	1.79	5.79	0.003
	Error		215.45	692	0.31		

5.4 Discussion

5.4.1. Water Quality

There were clear differences in water quality between the inflow rate treatments. It was necessary to supplement the oxygen in the 20 l min^{-1} treatment to maintain the levels above 5 mg l^{-1} from the middle of April, and the same was true of the 40 and 60 l min^{-1} treatments in May and August respectively. If oxygen had not been supplied to the tanks, mass mortality would have been very probable and feeding would have needed to be restricted or suspended. The oxygenation system used in the study did not facilitate total control of DO within the tanks and there were fluctuations in DO though the course of a day, with peaks and troughs corresponding to feeding and activity of the fish (Figure 5.4). The oxygenation system did permit DO to be maintained above 5 mg l^{-1}, and there were no significant statistical differences between the monthly average daily oxygen levels of the treatments. During periods when the tanks were not receiving additional oxygen, the DO in the tanks was proportional to inflow rate as would be expected. However, once oxygen was administered, DO was no longer related to inflow rate; this is highlighted in Figure 5.4 where average DO was higher the 40 l min^{-1} treatment throughout a 24 h period.

There was a significant effect of inflow rate on post-feed TAN, although similar to the SD experiment (Chapter 4), the prevailing pH (6-7) and water temperature (max 16°C) meant that only a very small percentage of the TAN (0.135% maximum) existed as the more toxic NH_3. Levels of NH_3 remained well below the generally accepted safe limit of 0.02 mg l^{-1} (Wedemeyer, 1996).

A 24 h sample period of water quality in each of the tanks showed ammonia levels peaked in the middle of the night (Figure 5.6). This midnight peak of ammonia was unexpected, as the design of the sampling regime had assumed that highest levels

of ammonia would occur several hours after first feeding. In a study that examined ammonia excretion in starved and fed rainbow trout over a 24 h period, Rychly and Marina (1977) observed a significant increase in ammonia 4 h after feeding in the fed compared with starved treatments. The same study also found significant differences between the highest and lowest daily ammonia levels within both treatments and suggested that peaks of ammonia (occurring between 2-4 pm and 6-8 am) may have been due to a circadian rhythm. Wagner *et al.* (1995) measured ammonia excretion by rainbow trout over a 24 h period and found peaks to be highly variable. Wagner *et al.* (1995) concluded that it was not possible to accurately predict peaks of ammonia based on feeding time alone, although they did note that peaks generally occurred during daylight h with lowest levels occurring close to dawn. Paulson (1978) modelled ammonia excretion in rainbow trout and observed a pulse of excretion 7-8 h after feeding. Ammonia excretion has been shown to increase with exercise in the rainbow trout (Holeton *et al.* 1984); suggesting that excretion would also be expected to be reduced at night when activity levels would be assumed to be lower. There is some evidence in the literature to support the midnight peak in ammonia observed in this trial provided by Rosenthal *et al.* (1984), where two 12 h ammonia profiles showed levels to increase steadily throughout the day and peak at 10 pm. Smart (1981) also found ammonia excretion of rainbow trout to peak around midnight.

Though unexpected, the midnight peak of ammonia observed in the present experiment highlighted the fact that point samples of water quality analysis provide only patchy data. It is also likely that the maximum level of ammonia to which the fish were exposed during this experiment was higher than the levels assumed by the post-feed water quality measurements.

5.4.2. Mortality

The mortality events that occurred in this study were both due to plumbing failures, and it may have been coincidental that both events occurred within the 20 l min^{-1} treatment. However, the fact that the 20 l min^{-1} treatment was running at the highest loading levels meant that there was an increased dependence on the supplementary oxygen for life support and that there was less time to react before levels of DO became critical in the event of a system failure. Kindschi *et al.* (1991a) reported a similar mass mortality event in a high density treatment following system failure and Miller *et al.* (1995) suggest that though feasible, high density culture with oxygenation systems require increased supervision and the requirement of appropriate back-up systems.

Apart from the system failures, mortality was very low in all treatments and it is likely that during this experiment water quality did not deteriorate sufficiently to result in levels of mortality observed in other studies that investigated the effects of water quality deterioration (Rosenthal *et al.*, 1984; Soderberg *et al.* 1983).

5.4.3. Growth

For the first 3 months of the experiment the growth of the fish in the different treatments was very similar and it was only when water temperature began to increase that differences in growth were apparent. The mean weight of the PIT-tagged fish in the 60 l min^{-1} treatment was significantly larger than those in the 20 and 40 l min^{-1} treatments from September through to the end of the experiment (Figure 5.9).

In terms of the rate of growth, the only period that a significant difference occurred was between the August and September sample points, when SGR in the 60 l min^{-1} treatment was significantly higher than in the 20 and 40 l min^{-1} treatments

(Figure 5.10). Although August/September was the only period in which the differences SGR were significant, SGR in the 60 l min^{-1} treatment appeared higher than the other treatments for the whole summer period from the May through to October. Growth rates at all other points were very similar, suggesting that it was only during the periods of high water temperature that inflow rate had an effect on growth.

Several studies have investigated the effects of water quality deterioration associated with increased SD on rainbow trout (Larmoyeux & Piper, 1973; Mayer & Kramer, 1973; Brauhn *et al.*, 1976; Rosenthal *et al.*, 1984; Baker & Ayles, 1990). Similarly to the present experiment, these studies simulated water quality deterioration caused by increased SD while removing the potential interference from behavioural related effects resulting from keeping different numbers of fish together. Keeping stocking densities equal in a number of tanks, these studies simulated water quality deterioration by either successively passing water through a series of tanks to simulate water reuse (Larmoyeux & Piper, 1973; Mayer & Kramer, 1973; Brauhn *et al.*, 1976), or by the manipulation of flow rate to simulate high loading rate (Brauhn *et al.*, 1976; Baker & Ayles, 1990). All of these experiments found adverse effects of increased loading rate or water reuse on growth and feed conversion of rainbow trout.

Further evidence to support the hypothesis that water quality deterioration is the root cause of reduced growth associated with increased SD is provided by Soderberg and Krise (1986) and Kebus *et al.* (1992). These studies effectively removed the effect of water quality deterioration by stocking fish at a range of densities within the same compartmentalised tank, and observed no effect on growth.

The only contradictory evidence for water quality being the root cause of reduced growth is provided by Procarione *et al.* (1999), who adopted the same approach as Soderberg and Krise (1986) and Kebus *et al.* (1992), but found an effect

of fish numbers on growth. Acknowledging the fact that their results were in not consistent with the findings and conclusions of several similar studies, Procarione *et al.* (1999) suggested that growth might not be impaired until a critical limit of temperature or loading is exceeded, after which the differences become apparent. A similar conclusion could be made of the present study, as inflow rate only appeared to effect growth rate during the periods of warmer water temperature.

Several studies have shown a relationship between water quality deterioration and reduced growth, but there is a lack of evidence or consensus on the mode of action by which the reduction in growth occurs. The lower growth in the 20 and 40 l min^{-1} treatments was either due to reduced food intake, or poorer conversion of the food that was consumed. As direct measurement of feed intake or uneaten pellets was not carried out in this study, it was not possible to ascertain whether reduced feed intake was the cause of reduced growth, though there is some evidence in the literature to support this theory. Several authors have observed reduced vacuolation of liver hepatocytes in rainbow trout exposed to chronically high levels of ammonia (Larmoyeux & Piper, 1973; Soderberg *et al.*, 1984; Soderberg, 1985). The authors suggested that this was due to reduced feed intake, as vacuolation of liver hepatocytes occurs mainly as a result of glycogen accumulation (Simon *et al.*, 1967: cited in Soderberg *et al.*, 1984). Similarly, Leatherland (1993) suggested fish reared at higher SD showed signs of reduced feed intake. Three other studies also reported food intake of rainbow trout to be lower at higher SD (Papoutsoglou *et al.*, 1979; Alanärä & Brännäs, 1996; Boujard *et al.* 2002). Boujard *et al.* (2002) suggested that the reduced feed intake at higher SD was due to restricted accessibility to food rather than water quality deterioration, as flow rates were set relative to the density of fish in each of their treatments.

There is enough evidence here to support a link between water quality deterioration and reduced appetite and this is certainly an area that warrants further research.

5.4.4. Sex allocation and maturation

The UK rainbow trout farming industry generally uses single sex populations of female fish (Anon., 1996a). Male fish are generally considered less desirable due to problems associated with early sexual maturation, which may occur in fish as small as 20g (precocious males), or more naturally when male fish are around 500g in weight (Randall, 1992). However, as a preventative measure for the introduction of diseases into the Niall Bromage Freshwater Research Facility a policy change in October 2000 meant that it was no longer possible to import fish from external sources, and instead a mixed sex population of fish that were hatched on site was used. Hand grading removed any precocious males from the study, although there was always a risk that the numbers of male and female fish would not be uniform in the different treatments. The proportion of male and female fish in the groups was fairly uniform with male:female ratios ranging from 1.1:1 to 0.9:1 (Figure 5.15).

Throughout the trial the weights of the PIT-tagged fish in each tank were used to estimate the mean weight and the male:female ratios of the tagged fish were 1.14, 0.83 & 0.95:1 for the 20, 40 and 60 l min^{-1} treatments respectively (Table 5.5). There were significant differences in weight between the male and female fish in the 40 l min^{-1} treatment ($P>0.05$; unpaired T-test), and although not statistically significant, there was also a 40g difference between the mean weight of the male and female fish in the 20 l min^{-1} treatment. Interestingly, the mean weight of the PIT-tagged male and female fish in the 60 l min^{-1} treatment was very similar (651 vs. 653g).

Though not statistically significant, there was some evidence to suggest that the female fish in the 60 l min^{-1} were at a more advanced state of maturation than the other treatments (Figure 5.15), possibly as a result of the growth achieved in this treatment. The reproductive success of salmonids is affected by a multitude of environmental and nutritional factors (see review by Bromage *et al.*, 2000). In this study the onset of maturation may have been delayed in the fish in the 20 and 40 l min^{-1} treatments, either through reduced feed intake and/or poorer FCR during the summer months as a result of poorer water quality in these treatments.

5.4.5. Welfare Indicators

5.4.5.1. Somatic Indices

Condition Factor and Feed Conversion Efficiency

A significant effect of inflow rate on CF was observed in this study, and during September and October the CF of fish in the 60 l min^{-1} treatment was significantly higher than the other treatments. Following a steady increase from May through to October, the CF of the fish in the 60 l min^{-1} treatment appeared to have reached a plateau in November, while CF of fish in the 20 and 40 l min^{-1} treatments was still increasing. In November and December the differences in CF between the treatments were no longer significant; this may have been due to compensatory fattening in the 20 and 40 l min^{-1} treatments, although another explanation could be increased CF with the onset of sexual maturation.

The most relevant studies with which to draw comparisons were conducted by Larmoyeux and Piper (1973) and Rosenthal *et al.* (1984). Despite observing a significant reduction in growth rates, Larmoyeux and Piper (1973) observed no significant differences in CF associated with decreasing water quality. Rosenthal *et al.*

(1984) found no significant differences in CF at lower compartments of their channel system, but concluded that there was a trend for reduced CF with increased water quality deterioration.

Other studies that have measured the effects of SD on CF found either no effect (Kilambi *et al.*, 1977; Winfree *et al.*, 1998; Makinen & Ruohonen 1990; Miller *et al.*, 1995) or a significant reduction in CF with increased SD (Refstie, 1977; Pickering & Pottinger, 1987a; Atay *et al.*, 1988; Rigolino *et al.*, 1988; Unlu & Baran, 1992; Wagner *et al.*, 1996a).

CF provides a crude indication of the energy reserves of a fish (Goede & Barton, 1990). A fish with high CF has either eaten more food, or converted the food that it has eaten more efficiently into fat and muscle than a fish with lower CF. Drawing conclusions from CF data from fish in this experiment was hindered by the on-set of sexual maturation and the resulting differences in CF between the male and female fish. However, the higher CF in the 60 l min^{-1} treatment in August and September and lower FCR in the 60 l min^{-1} treatment in June, July and September suggested that there was a genuine effect of inflow rate. It was not possible to measure uneaten feed in this experiment, so FCR was estimated based on the amount of food presented to each fish over a given period of time. It was therefore not possible to determine whether differences in FCR were genuine, or due to the fact that less food was consumed.

HSI

The HSI data of the 30 fish sampled monthly from each treatment showed no significant effect of inflow rate (Figure 5.13a). However, the HSI data collected from all of the tagged fish at the end of the experiment showed the relative size of the liver

in the 60 l min^{-1} treatment to be significantly larger than those in the 20 l min^{-1} treatment. This suggested that the differences in HSI were subtle and only became significant in the comparatively large sample size at the end of the experiment (approximately 120 fish per treatment). Two previous studies measured the effect of SD on HSI, both of which reported a negative effect of increased SD (Leatherland & Cho 1985; Leatherland, 1993). In both studies the authors suggested that the reduced HSI of the fish reared at high SD was consistent with other physiological signs characteristic of food deprivation (reduced growth, thyroid activity, and protein concentration), even though both studies fed fish to satiation. Furthermore, Leatherland and Cho (1985) suggested that the reduction in HSI that they observed at higher SD was not an effect of reduced water quality, as they used vigorous aeration and high rates of water exchange (3-4 times per h). However, details of the frequency and methods of water quality monitoring are vague in one study (Leatherland & Cho, 1985) and non-existent in the other (Leatherland, 1993). Both studies suggested that growth was reduced at high SD as a result of a reduced ability of fish to locate feed and that reduced thyroid activity and HSI are consistent with what the authors termed as a 'ration restricted state'. Water quality deterioration has also been suggested as a possible cause of reduced feed intake at higher SD and several authors have attributed a reduction in hepatocyte vacuolation to chronic exposure to high levels of ammonia, leading to reduced feed intake, independent of fish numbers (Larmoyeux & Piper, 1973; Soderberg *et al.*, 1984; Soderberg, 1985).

SSI

In the months that spleen weight was measured, there was no effect of inflow rate on SSI. The SSI data from the PIT-tagged individuals at the end of the experiment

suggested that SSI was slightly lower in the 20 l min^{-1} treatment, but there was no significant difference (ANOVA; P=0.30). The only other study that used spleen condition as an indicator of the effects of SD or loading rate on rainbow trout observed a trend for reduced spleen size (lower SSI) with increasing water quality deterioration (Rosenthal *et al.*, 1984). It may be that in the present study water quality did not deteriorate sufficiently to elicit an effect on SSI.

5.4.5.2. Blood parameters

Haematocrit

There was no effect of inflow rate on haematocrit levels, which at all points of the experiment remained within the range of 24 – 43% reported for clinically healthy rainbow trout (Wedemeyer, 1996). Similar to the experiment described in Chapter 4, haematocrit displayed an inverse correlation with water temperature; although in this experiment the effect was not as consistent, with relatively low haematocrit levels in October and November when water temperature had dropped to around 5°C. Most studies that have measured the effect of SD and loading rate on haematocrit have found no effect (Leatherland & Cho, 1985; Pickering & Pottinger, 1987a; Papousoglou *et al.*, 1987; Kindschi *et al.*, 1991a; Kebus *et al.*, 1992; Miller *et al.*, 1995).

There is some evidence in the literature of elevated haematocrit levels at higher SD (Wagner *et al.*, 1996a). Furthermore, Thurston *et al.* (1984) found differences in haematocrit levels of fish that were reared for 9 months in different levels of ammonia, with lower haematocrit observed in fish that were held in highest ammonia concentrations (>0.047 mg l^{-1}). Perhaps most relevant to the present study, Larmoyeux & Piper (1973) found levels of haematocrit to increase with decreasing

water quality. Swift (1981) also observed a short-lived acute stress response that included elevated levels of haematocrit following sub-lethal exposure of rainbow trout to a range of pollutants and low DO.

Glucose

There was no significant effect of inflow rate on glucose levels during this experiment, which is in agreement with several other studies that similarly found no effect of SD or loading rate on glucose concentrations (Larmoyeux & Piper, 1973; Wedemeyer, 1976; Leatherland & Cho, 1985; Laidley & Leatherland, 1988; Papousoglou *et al.*, 1987; Procarione *et al.*, 1999). Although glucose levels were very similar in all of the inflow treatments for most of the trial, levels appeared to be elevated in the 20 l min^{-1} in July (Figure 5.17), coinciding with the period in which water temperature and ammonia were near their peak (Figures 5.2 & 5.5).

Swift (1981) showed levels of glucose to rise dramatically in response to hypoxia and un-ionised ammonia and it may have been that a similar effect was observed in the 20 l min^{-1} in July in response to water quality deterioration. Swift (1981) also commented on the difficulties of interpreting changes in plasma glucose levels in relation to stress and discussed the complications that the nutritional state of the fish may have on plasma glucose levels. This may have implications for this study as fish were routinely starved for at least 24 h prior to sampling in an effort to reduce the chance of mortality during anaesthesia and prevent gill damage caused by regurgitation of gut contents.

Lysozyme activity

There was a significant difference in lysozyme activity in the 20 l min^{-1} compared with the 60 l min^{-1} treatment in June ($P<0.01$). Although not statistically significant, lysozyme activity also appeared elevated in the 40 l min^{-1} in June and July (Figure 5.18). It is unclear why lysozyme activity was elevated at these points in these treatments, because at all other points in the trial the levels of lysozyme activity were very similar in the treatments.

Lysozyme activity has previously been shown to display a close link with cortisol response (Fevolden *et al.*, 1999), but there has been limited use of lysozyme activity as a welfare indicator in fish. The widespread use of arbitrary units also makes cross-referencing between studies very difficult. Möck and Peters (1990) observed significantly decreased levels of lysozyme activity at 36 h following acute exposure to NH_3, although it is likely that the exposure would have elicited an acute stress response and the reduce lysozyme may have been a result of higher levels of cortisol (not measured) following stimulation of the HPI-axis.

In Chapter 3 of this thesis lysozyme was measured in conjunction with several blood parameters under situations of chronic and acute stress. Following an acute handling stressor lysozyme appeared to decrease from pre-stress levels (Figure 3.4). Similarly, in Chapter 4, there was a significant, negative correlation between lysozyme activity and plasma cortisol (Figure 4.18). However, the fact that cortisol remained low in all treatments throughout the experiment suggested that the change in lysozyme activity observed in this experiment was unlikely to have been stress mediated.

Cortisol

Levels of cortisol remained very low throughout the course of the experiment and at no point did they appear to be elevated above basal levels. The highest mean cortisol level in a treatment was around 6 ng ml^{-1}, which occurred in the 40 l min^{-1} treatment in April, but in comparison the 2000/1 SD experiment, plasma cortisol concentration were very low throughout. A number of factors may have contributed to the lower levels of cortisol in this study, such as the fact that tanks were covered with opaque fibreglass lids as opposed to the jump-nets used in the SD experiment, and also that hand feeding was replaced with automatic feeders. The strain of rainbow trout used in the 2000/1 SD experiment also appeared to display a greater cortisol response following a standardised handling stress. The mean peak level of cortisol for the strain of fish used in this study was around 40 ng ml^{-1} (Figure 3.5; Chapter 3), whilst the strain of fish used in Chapter 4 had peak cortisol levels of around 160 ng ml^{-1} (Figure 3.4; Chapter 3). Mean peak cortisol levels of 160 ng ml^{-1} and 40 ng ml^{-1} would be characteristic of high (HR) and low responding (LR) strains of rainbow trout (Pottinger *et al.*, 1991; Pottinger & Carrick, 1999). Direct comparison between the fish used in this experiment and the 2000/1 SD experiment are hindered by differences in fish size, season and water temperature, but a three-fold difference in peak cortisol level may suggest that the fish used in this experiment may have been a lower cortisol responding strain than those used in the Chapter 4.

The majority of studies that have attempted to measure the effect of increasing SD on plasma cortisol levels in rainbow trout have found no, or inconclusive effects (Leatherland & Cho, 1985; Laidley & Leatherland, 1988; Pickering *et al.*, 1991; Kebus *et al.*, 1992; Procarione *et al.*, 1999). However, there is evidence for cortisol levels to increase in response to acute changes in water quality in a number of

salmonid species in response to a wide range of pollutants (reviewed by Donaldson, 1981). Swift (1981) found increased plasma cortisol levels in rainbow trout following exposure to concentrations of 0.24 mg l^{-1} un-ionised ammonia or greater, but found no increase of cortisol in response of hypoxia.

Another consideration is the effect that combined deterioration of water quality parameters may have on the HPI-axis. Pickering and Pottinger (1987b) demonstrated that whilst elevated NH_3 alone had no effect on the acute stress response of brown trout, but when elevated NH_3 was combined with lowered pH, a significant increase in plasma cortisol response was observed.

There appeared to very little stimulation of the HPI-axis in this experiment and levels of cortisol were low in all treatments throughout. There is evidence to suggest that fish become acclimatised to water quality conditions (Larmoyeux & Piper, 1973). Cortisol levels have been shown to increase in response to acute changes in water quality, but plasma cortisol measurement appears to be of limited use as an indicator of chronic water quality deterioration.

5.4.6. Fin Condition

There was generally a significant decrease in RFL as the trial progressed and with the exception of the dorsal and right pectoral fins, RFL was lower in the PIT-tagged fish at the end compared with the start of the experiment. The fins were generally unaffected by the inflow rate treatments and comparison of the RFL values for the PIT-tagged fish at the end of the experiment found the right-sided pelvic and pectoral fins to be the only fins affected by inflow. The bearing of this significant effect was not very consistent, although the general pattern suggested that RFL of the right sided fins decreased with increasing inflow rate (Figure 5.20). Similarly to the 2000/1 SD

experiment (Chapter 4), significant differences were observed between the right and left pelvic and pectoral fins, with the RFL of the left sided fins significantly lower than the right-sided fins.

The experimental design endeavoured to reduce the effect that different rates of inflow had on water current by using down-pipes that directed the inflowing to the bottom of the tanks. However, the nature of the round tanks and the Coriolis effect meant that a clockwise water current was still established, and although not measured, water current would theoretically have been stronger in the higher inflow rate treatments. The fish in all treatments were observed to generally orientate themselves to swim against the flow, so that the right of the fish was predominantly facing the outside wall of the tank. The fact that the inside fin should be more prone to damage again remained unexplained, although it appeared to be a consistent effect of rearing fish in this system of round fibreglass tanks. An explanation for the apparent treatment effect on the right sided fins may also have been due to differences in water current. It may be that the fish situated themselves closer to the walls and floor of the tank to reduce the drag caused by the current. There is some evidence to support this from a study that assessed the effect of rearing-unit design on the behaviour of rainbow trout, where contact time with the bottom of the tank was shown to increase significantly at higher rates of water exchange (Ross *et al.*, 1995).

The PCA of the fin data confirmed some of the patterns observed in the univariate analysis of individual fins. Fin-PC1 comprised of negative contributions from all of the fins, while Fin-PC2 was predominantly made up of negative contributions from the pelvic fins. The GLMs including the factor scores for the Fin-PCs found there a significant effect of time and water temperature (Table 5.15). There was also a significant random effect of replicate on both of the Fin-PCs, with factor

scores for both PCs appearing higher (worse fins) in replicate 3, although it is unclear why this should have occurred. The effect of water temperature appeared to be positive on both of the Fin-PCs (i.e. worse fins at higher temperatures), although this may have been a confounding influence of time, as water temperature generally increased as the trial progressed. Significant effects of ammonia and DO were also observed on Fin-PC2, with scatter plots suggesting that Fin-PC2 decreased with increasing ammonia (i.e. better pectoral fins at higher levels of ammonia), and increased with increasing DO (i.e. worse pectoral fins at higher DO concentrations). A confounding influence of water current may have caused this effect, as water quality was higher in the tanks with high inflow rate. Fin damage would generally not be expected to increase at higher levels of water quality, although Larmoyeux & Piper, (1973) observed that the fish from the final two troughs in their cascade system had less dorsal fin erosion than those from higher in the cascade, which they attributed to reduced activity in the lower oxygen environment. Bosakowski and Wagner (1994b) associated increased ammonia with increased fin damage and suggested that other water quality problems associated with high levels of ammonia such as increased metabolic wastes, microbes, and suspended solids, may also have contributed to the effect.

5.4.7 Principal Components Analysis

The PCA of the blood parameters produced two viable PCs, one of which was based on associations between the cortisol, lysozyme activity and haematocrit (B-PC1). The second B-PC was comprised almost solely of glucose and when modelled produced a similar result to the earlier univariate analysis of glucose. The GLM including factor scores for B-PC1 found significant effects of temperature and DO. Scatter plots of

factor scores for B-PC1 against temperature and DO indicated a significant positive correlation with DO ($P<0.001$, correlation coefficient = 0.574, R^2 = 0.32) and a negative correlation with temperature ($P<0.001$, correlation coefficient = -0.579, R^2 = 0.33). Water temperature and DO were also highly correlated (R^2 = 0.58), so the result of the GLM for B-PC1 suggested that the fish were more stressed in the winter months when temperature was lower and DO higher.

When PCA was carried out on the blood parameters, somatic indices and the Fin-PCs to create a combined welfare index, the resulting PCs were harder to explain. There appeared to be a contradictory contribution of Fin-PC1 in C-PC1, which may have been explained by the general lack of differences that were observed on the RFL values between the treatments. The only significant effect on the GLM with factor scores of CPC1 was time, which was not surprising given the general absence of treatment effects on any of the welfare parameters.

The contribution of Fin-PC2 in C-PC2 was more logical and in addition to a significant effect of time, the GLM for C-PC2 factor scores detected a significant effect of DO. A linear regression between factor scores for C-PC2 and DO suggested a decrease in C-PC2 with increased DO ($P<0.001$, correlation coefficient = -0.580, R^2 = 0.337). The nature of this result was again counter intuitive as high dissolved oxygen would be expected to be beneficial for fish welfare.

Explanations for these results could be that water quality did not deteriorate sufficiently to elicit an effect on the welfare indicators, or that the welfare indicators used in this study were not sensitive to water quality deterioration. However, the fact that there was a significant treatment effect on growth and CF, suggested that at some point in the trial welfare was infringed in the 20 and 40 l min^{-1}.

5.4.8. Possible improvements and future work

If feed intake had been measured in this study it would have been possible to determine if reduced appetite was the cause of the reduced growth observed in the 20 and 40 l min^{-1} treatments. Any future study assessing the effects of water quality on fish welfare would also benefit from inclusion of some form of assessment of gill condition. Rosenthal *et al.* (1984) observed a reduction in the length of the primary gill lamellae and Soderberg *et al.*, (1984; 1985) related numerous pathologies of secondary gill filaments such as blood filled aneurysms, hyperplasia fusion and epithelial oedema to NH_3 levels. The sampling of the water quality parameters in this study was inadequate to confidently estimate the extent of the water quality deterioration. The 24 h profile of ammonia highlighted the general lack of information available regarding production and excretion of a fundamental metabolic waste product. The use of data-logging probes for ammonia and pH in addition to temperature and DO should be a requirement of any future study.

5.4.9. Summary

In summary, this experiment, and several other previously published studies have demonstrated the potential for water quality deterioration to result in reduced growth of rainbow trout. There is also further evidence to suggest that when the effects of water quality deterioration have been removed from studies (Soderberg & Krise, 1986; Kebus *et al.*, 1992), or steps have been taken to ensure that water quality remains at a high standard (Kindschi *et al.* 1991a; Miller *et al.* 1995) the commonly observed adverse effects of increasing SD is removed or greatly reduced.

The lower growth and condition factor observed in the 20 and 40 l min^{-1} treatment was not accompanied by changes in the other welfare indicators, suggesting

either that they were not sensitive indicators of water quality deterioration, or that the deterioration in water quality was not sufficient to elicit and effect.

The two, mass mortality events that occurred in the 20 l min^{-1} treatment are also worthy of comment. Both mortality events were the result of plumbing failures and it may have just been a chance occurrence that both events occurred in the treatment with the lowest flow (highest loading rate). Had the events occurred in the higher flow treatments, mortality may have still have occurred. There are, however, similar reports in the literature of mass mortality event in tanks with high loading rates (Kindschi *et al.*, 1991a) and the need for increased supervision and the requirement of appropriate back-up systems has been emphasised by other authors (Miller *et al.*, 1995).

Chapter 6: Questionnaire survey of stocking density practices on UK rainbow trout farms

6.1. Introduction

This chapter of the thesis discusses SD in the context of commercial practices in the UK rainbow trout farming industry. Following a brief overview of the UK industry, the results of a postal questionnaire requesting information on stocking density practices are discussed.

The earliest reports of rainbow trout culture in the UK date back to 1885 where stocks were established in Buckinghamshire and at the Howietoun fishery near Stirling, following shipments from North America to the National Fish Culture Association of London (MacCrimmon, 1971: cited in Gall, 1992). The 2000 survey of trout production in the UK reported 362 active farms, 269 in England and Wales, 63 in Scotland and a further 30 sites in Northern Ireland (Dunn, 2002). The UK was the seventh largest producer of rainbow trout in Europe (Figure 6.1). The total UK production rainbow trout was reported to be around 15,000 tones a year (Anon., 1996a). The most recent published figures reported total UK trout production in 2000 to stand at around 15,805 tonnes, 11,335 tonnes (71.7%) of which went to the table, and 4,470 tonnes (28.3%) for restocking (Table 6.1). The 2000 figure for trout production was down slightly on the industry peak in 1999 where 17,185 tonnes of trout was produced (Dunn, 2003).

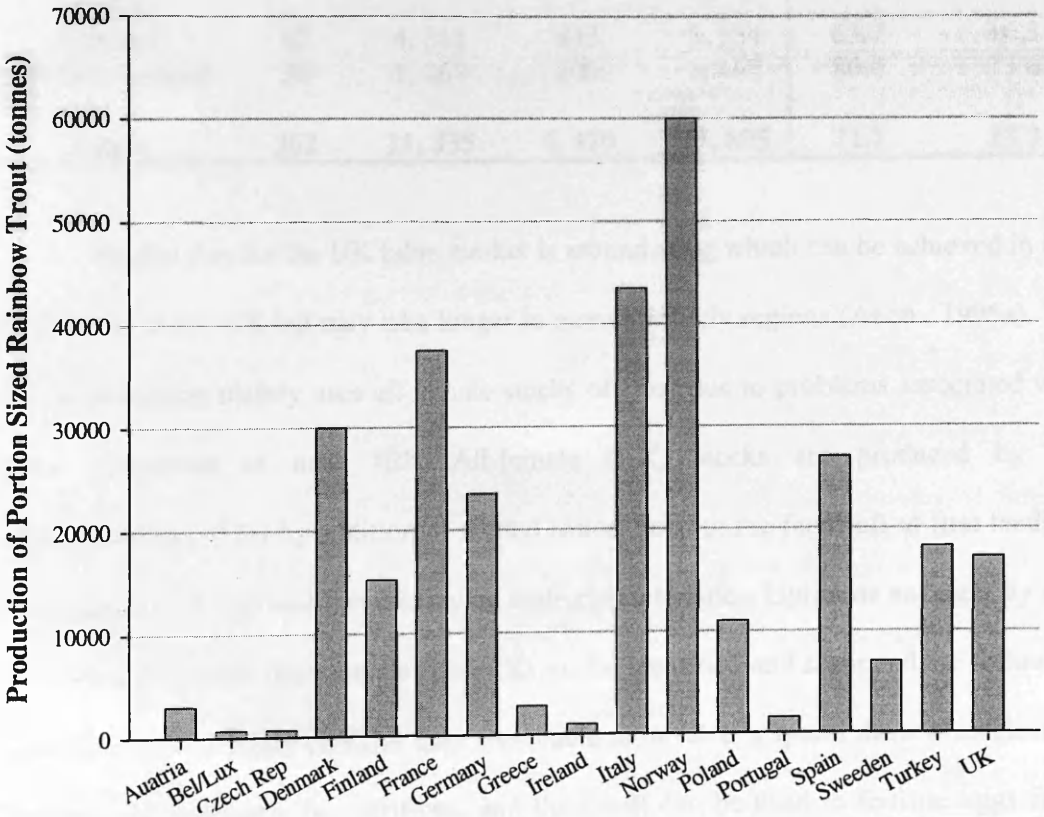


Figure 6.1. European production of portion sized rainbow trout; constructed from data on Federation of European Aquaculture Producers website (www.feap.com).

Table 6.1. Summary of UK trout production in 2000 (reproduced from Dunn, 2002).

	No of sites	Annual Production (tonnes)			Percentage of total (%)	
		Table	Restocking	Total	Table	Restocking
England and Wales	269	5, 757	3, 427	9, 184	62.7	37.3
Scotland	63	4, 311	843	5, 154	83.7	16.3
Northern Ireland	30	1, 267	200	1, 467	86.4	13.6
Totals	**362**	**11, 335**	**4, 470**	**15, 805**	**71.7**	**28.3**

Market size for the UK table market is around 400g which can be achieved in less than 1 year in the UK but may take longer in more northerly regions (Anon., 1996a). The UK table market mainly uses all-female stocks of trout due to problems associated with early maturation in male fish. All-female (XX) stocks are produced by the masculinisation of fry by addition of methyl testosterone to the feedstuff at first feeding; this results in all exposed fish displaying male characteristics. Hormone exposed fry that would naturally have been female fish (XX) can be identified and separated the following year from natural males (XY) as they are unable to develop a sperm duct. Masculinised females will eventually be sacrificed, and their milt can be used to fertilise eggs from normal females, effectively eliminating the male determining Y chromosome from the stock (see Olito & Brock, 1991).

In terms of the stocking densities applied on UK trout farms, Ellis *et al.* (2002) suggested that farmers use a combination of intuition and experience to decide upon the most appropriate SD, with codes of practice and hand books used guides. The guidance available to farmers is in the range of 2-80 kg m^{-3}, depending on type of holding systems and size of fish, although commercial farmers would normally be expected to operate somewhere in the range between 15 – 40 kg m^{-3} with 60 kg m^{-3} being seen as a maximum

(Ellis *et al.*, 2002). There are reports of fish being held at higher densities ≥80 kg m^{-3} in experimental studies with the aid of high rates of water exchange and/or additional aeration/oxygenation (Buss *et al.* 1970; Baker & Ayles, 1990 & Kebus *et al.*, 1992). However, reports of such high densities being applied commercially are limited (Anon, 1999).

The FAWC report (Anon., 1996a) made four recommendations expressly concerning SD of rainbow trout; presented earlier in Table 1.1 (page 2). Until the time of the present study there had been no collection of data regarding SD practices of UK trout farms, although both Anon. (1996a) and Lymbery (2002) suggested that SD were too high and that densities of 30-40 kg m^{-3} were potentially detrimental to trout welfare. There is currently no legislation regulating the density at which trout can be farmed. However, farms that are members of the BTA are encouraged to comply with the code of practice, the latest revision of which (Anon., 2002) makes the following references to SD:

- Stocking fish at too high a density should be avoided as this is likely to lower water quality and may inflict physical damage and possibly induce stress and disease or changes in behaviour and thereby compromise fish welfare. As a general rule each litre of water inflow per minute will support 1-4 kg of fish depending on their size, although experience may allow some farms to stock at higher levels.
- SD should be kept at an appropriate level to avoid detrimental effects on fish health and welfare. Fish should have enough space for swimming but not so large as to encourage aggressive territorial behaviour. It should also be borne in mind that the optimal SD will vary depending on water flow, current, oxygen concentration and temperature and other water quality characteristics and the size, age, sex, health status

and feeding regime of the fish under culture. If water quality parameters are maintained at recommended levels then the health and welfare of the fish should be optimal.

The BTA code of practice (Anon., 2002) also makes references to the importance of maintaining high levels of water quality:

- Trout farms require large quantities of good quality water. Trout generally require supplies of water, which provide a minimum of 6 mg l^{-1} (ppm) of oxygen in the farm outflow, although performance may be optimised by using higher levels. Temperature also affects carrying capacity with lower temperatures enabling more oxygen to be carried in the water and hence more fish to be safely supported. Aside from welfare issues, the amount of oxygen carried in the water is generally the primary limiting factor on stocking rates and therefore production. As an approximate guide to carrying capacity it is suggested that a maximum of 3-4 tonnes of annual production may be raised with every one million litres of water per day inflow depending on the quality of the water. Aeration/oxygenation or water re-use may increase this.
- Appropriate water flow rates should be maintained and the water should be well oxygenated.
- Water quality parameters should be maintained at optimal levels to achieve good health and growth of the stock. In particular low levels of ammonia, suspended solids and BOD should be maintained with oxygen values as near saturation as possible.

The revised 2002 version of the BTA code of practice included a section specifically relating to fish welfare, the first section of which stated that "trout farmers should recognise that by engaging in the act of farming they have a duty to care for the welfare of their stock from egg to harvest". In a recent meeting organised by Defra on the subject of fish welfare, the concept of stewardship, defined as ownership with responsibility, was used to encapsulate this aspect of the code of practice (Davies, 2002).

Quality tout UK (QTUK) is a scheme that operates in the UK. Farmers wishing to join QTUK must adhere to the operational procedures and standards laid out in the Certification Scheme, which makes the following recommendation regarding SD (QTUK, 2004):

- Fish must be stocked at densities appropriate to their size, water temperature and flow, available oxygen, stage in production cycle and type of fish holding unit in order to reduce the risk of poor water quality, physical damage, stress and disease. Fish must be allowed to exhibit normal swimming behaviour. Appropriate stocking densities will be decided on a farm-to-farm basis and must be such that there is no adverse effect on the condition and welfare of the fish. Dissolved oxygen after feeding should not fall below 6 mg l^{-1}.

In addition to the quality schemes and codes of conduct in the UK, there are also pan-European organisations and regulatory bodies that make specific recommendations regarding SD. The FEAP code of conduct suggests that SD should be adjusted to the specific requirements of the species, and include respect for:

- The average live weight of the fish.
- The population's health and behavioural needs.
- The population's demand on the growing environment, in particular they're behavioural needs, the availability of adequate oxygen supply and the removal of wastes to avoid excessive accumulation of substances that may cause stress or toxic effects (*e.g.* CO_2 and ammonia).

At the time of writing the Council of Europe (CoE) was in the process of drafting a Resolution regarding the welfare of farmed fish in which references to SD are likely to feature. The Standing Committee of the European Convention for the Protection of Animals kept for Farming Purposes first started drafting fish welfare conditions in 1998 and at the time of writing the draft was in its 13th Revision (FEAP, 2004). Once complete, the introduction of the resolution will provide the framework for subsequent legislation that will become law in participating member states of the European Economic Community.

Whilst there are no current legislative powers that exist to limit stocking densities, farmers are under mounting pressure from retailers to address stocking densities. Supermarket chains are becoming increasingly stringent in the demands they make of their suppliers, and fish farms are no exception. Supermarkets are ultimately driven by consumer demands, and animal welfare is becoming an increasingly important issue in the decisions made regarding what people eat and where they purchase food stuffs (Cooke, 2001). Supermarkets are already making demands on fish farmers in terms of

standards of fish welfare, maximum stocking densities and methods of slaughter, and enforce these demands with site audits.

6.2 Aims of the Questionnaire

The questionnaire aimed to collect data regarding the SD practices on rainbow trout farms in the UK. The questionnaire also aimed to gain an idea of the way that SD was perceived by farmers *i.e.* what they considered to be a high SD and the welfare implications of high SD.

6.3. Materials and Methods

The format and content of the questionnaire was initially decided in consultation with the late Professor Niall Bromage who was the Scientific Advisor to the BTA. The pilot questionnaire was sent to Defra and Council Members of the BTA for comments and suggestions, which were incorporated into the final format. In order to reduce the burden on respondents, the questionnaire was kept fairly simple and where possible tick-boxes were provided, which also aided with analysis. A copy of the questionnaire can be seen in Appendix I.

A total of 295 questionnaires were distributed through the BTA, of which 99 were BTA members and 196 non-members (obtained from the BTA). The resulting data were then analysed and sorted to allow the stocking practices of the main types of farming system to be analysed separately. The main focus of the analysis was on-growing farms, as it was concern over stocking practices on such farms expressed in the FAWC report (Anon., 1996a) that initiated the present study.

The questionnaire was designed to incorporate all forms, stages and methods of trout culture that existed in the UK. This was achieved primarily by breaking down the types of farms into four categories; table farms (*i.e.* producing portions sized trout for the table market), fisheries, restocking farms and hatcheries. Questions relating to site, husbandry practices and perceptions of SD were also asked. In order to encompass the full range of culture facilities in use in on UK trout farms, a density matrix was constructed requesting information for fish of different size ranges *e.g.* minimum and maximum (start/finish) SD (kg m^{-3}), and information regarding the type, volume and flow rates of holding systems.

6.4. Results

6.4.1. Response Rate

Of the 295 questionnaires distributed, a total of 88 copies of the questionnaire were returned, representing a 29.8% return rate. The return rates were 64.6% for BTA member farms (99 BTA members in total), and 11.7% for non-member farms. The BTA member farms accounted for more than 80% of all UK trout production (Niall Bromage, *perrs com.*), suggesting that the data collected from this questionnaire represented a significant proportion of UK trout production.

6.4.2. Species of fish farmed

Question 1 asked what species of fish were farmed and the cover letter that accompanied the questionnaire asked that separate questionnaires were filled out for different species.

The data that is discussed in this thesis relates specifically to the responses that selected rainbow trout production for this question.

6.4.3. Types of trout production

Farms were categorised depending on their response to question 2, which asked for the types of production carried out on their site i.e. hatchery, fishery, restocking, or table farm. Many responses selected more than one type of production, indicating that a large number farms carried out more than one type of production. Throughout this chapter the results will firstly be discussed for farms that selected more than one type of production i.e. farms that may have selected both restocking and table trout production. These farms were referred to as multi-output farms.

In order to provide more accurate information regarding stocking practices for specific types of trout production, farms that produced trout exclusively for the table market were separated from those that produced trout for fisheries and restocking markets. Fisheries and restocking operations were grouped because restocking production is ultimately aimed at supplying sport fisheries, and also because all of the questionnaire responses that selected fisheries in question 2, also selected restocking production. These farms were referred to as single-output farms.

A large number of restocking and table farms also had hatcheries. Responses that were categorised into either table or restocking/fisheries production, but also had hatcheries, were not analysed as a separate group as this would have greatly reduced the numbers of farms in the single-output categories. There was a third group of interest,

namely farms that solely carried out hatchery production, but this group accounted for a much smaller number of farms and therefore there was less data available for hatcheries.

The predominant forms of trout production among farms were for table and restocking purposes, with respective percentages of 62 and 59% of all questionnaire responses (Figure 6.2). Hatchery production was carried out on 42% of responding farms, indicating that farms with hatchery operations may be supplying more than one table farm or restocking farm with fry. Fisheries made up the smallest cohort of production with 16% of responding farms carrying out this form of trout farming. With 59% of farms carrying out restocking production but just 16% carrying out fishery operations, we can infer that fisheries are likely to source fish from numerous different suppliers.

Single-output farms producing trout specifically for the table or fishery/restocking markets

There were 25 farms that carried out production specifically for the table market and 24 that carried out production specifically for the restocking or fisheries markets. Additionally there were 4 farms that operated solely as hatcheries.

6.4.4. Annual production of table farms

Analysis of annual production was carried out for all questionnaire responses that produced trout for the table (from question 2). Responses were categorised by size based on total annual production and the results showed the biggest proportion of table farms that responded to the questionnaire were producing >200 tonnes per year (Figure 6.2).

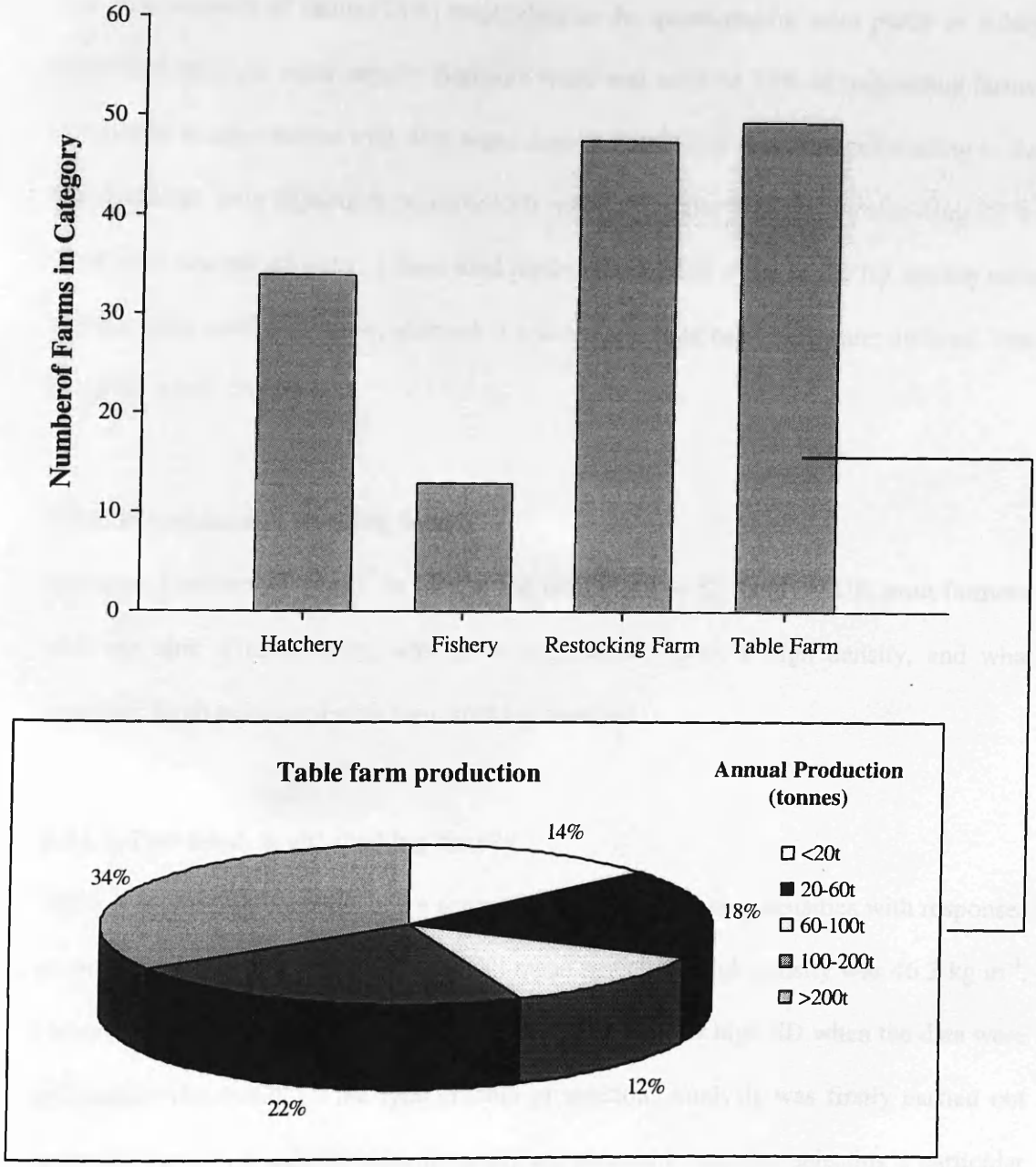


Figure 6.2. Analysis of the main types of rainbow trout production from all farms responding to the questionnaire (above) and the composition of the table farms based on total annual production for table farm production (below).

6.4.5. Water Supply

The vast majority of farms (78%) responding to the questionnaire were partly or solely dependent on river water supply. Borehole water was used on 32% of responding farms, but mostly in conjunction with river water supply. Just 7% of the farms responding to the questionnaire were dependent on lakes/loch water. A further 2 farms, representing 2.5%, used other sources of water; 1 farm used partly-recirculated water in the fry rearing units and the other used well water, although it was not specified how well water differed from borehole water (Figure 6.3).

6.4.6. Perceptions of stocking density

Question 5 attempted to gain an idea of the perception of SD held by UK trout farmers, with the aim of establishing what farmers perceived to be a high density, and what problems were associated with high stocking densities.

6.4.6.1. Perceived 'high' stocking density

There were marked contrasts in the perception of 'high' stocking densities with responses ranging from 7 to 200 kg m^{-3}. The overall mean perceived high density was 46.3 kg m^{-3}. However, there were marked differences in the perception of high SD when the data were categorised depending on the type of trout production. Analysis was firstly carried out using the perceived high densities from any questionnaire response selecting a particular type of production, even if more than one type of production was selected for question 2 (Figure 6.4). Subsequent analysis was carried out for the single-output farms producing trout for specific markets.

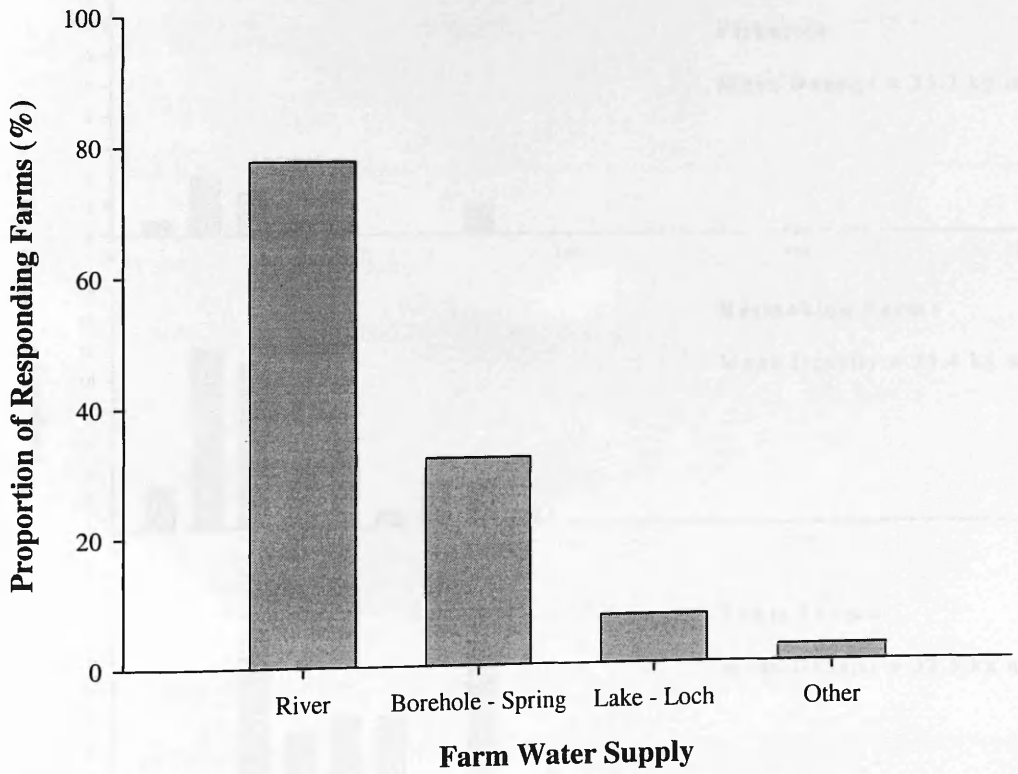


Figure 6.3. Water supply of UK rainbow trout farms responding to the questionnaire.

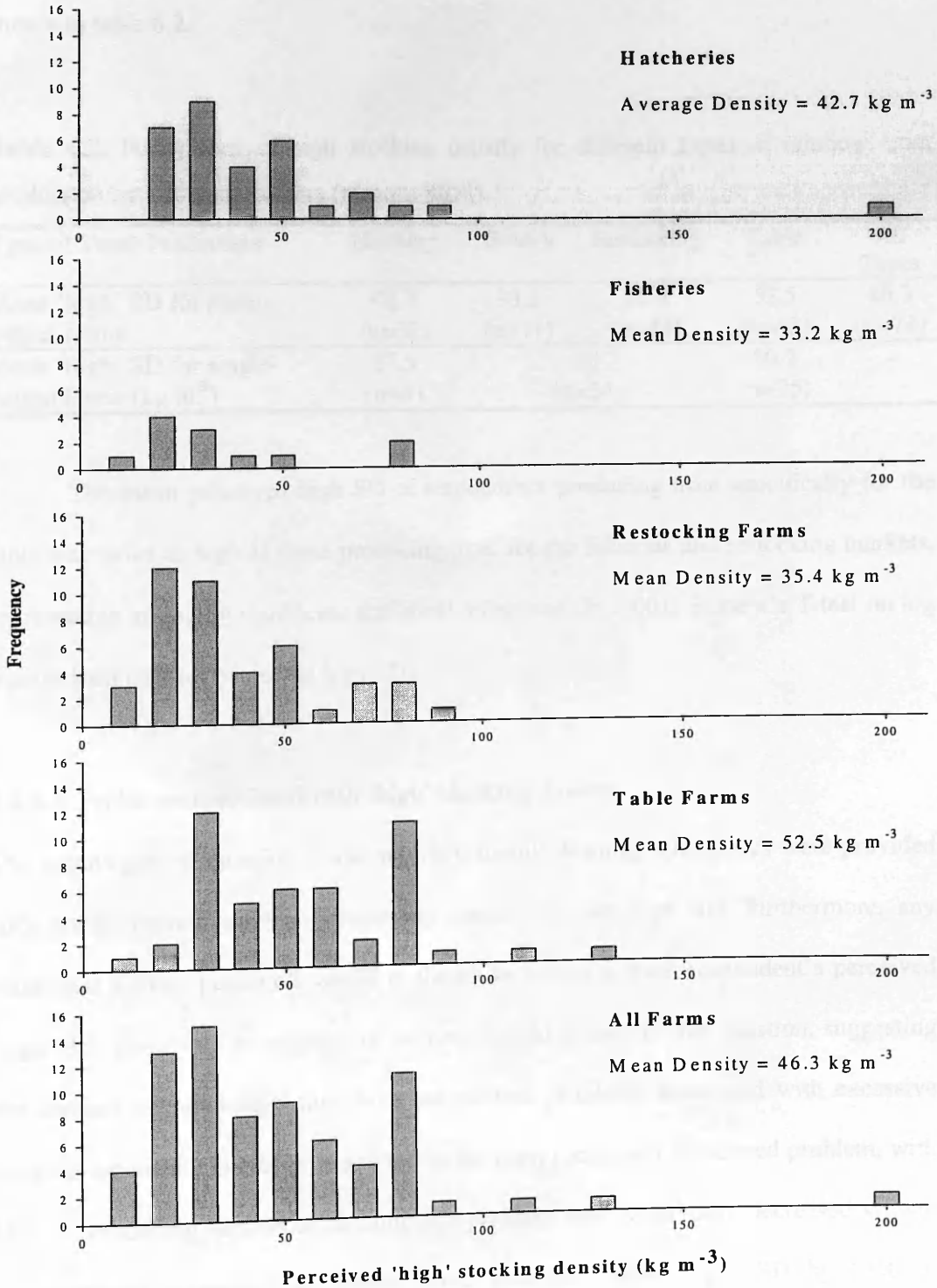


Fig. 6.4. Perception of a 'high' stocking density by British trout farmers.

The mean perceived high SD for multi-output and single-output trout farms are shown in table 6.2.

Table 6.2. Perceptions of high stocking density for different types of rainbow trout production by UK trout farmers (mean ± SEM).

Type of Trout Production	Hatchery	Fishery	Restocking	Table	All Types
Mean 'high' SD for multi-output farms	42.7 (n=32)	33.2 (n=11)	35.4 (n=44)	52.5 (n=48)	46.3 (n=74)
Mean 'high' SD for single-output farms (kg m^{-3})	87.5 (n=4)	28 (n=24)		60.2 (n=25)	-

The mean perceived high SD of respondents producing trout specifically for the table was twice as high as those producing trout for the fisheries and restocking markets, representing an highly significant statistical difference ($P<0.001$; Student's T-test on log transformed data for perceived high SD).

6.4.6.2. Problems associated with 'high' stocking density

The second part of question 5 was arguably slightly leading, with boxes were provided with pre-conceived problems commonly associated with high SD. Furthermore, any associated welfare problem/s would in theory be linked to each respondent's perceived 'high' SD. However, the majority of farmers elected to answer this question, suggesting that farmers acknowledged that there are welfare problems associated with excessive stocking densities. Fin erosion was cited as the most commonly associated problem, with 84% of responding farmers associating this problem with 'high' SD. Decreased growth and increased prevalence of disease were associated with 'high' SD by 63% of responding farms and increased mortality by 58% (Figure 6.5).

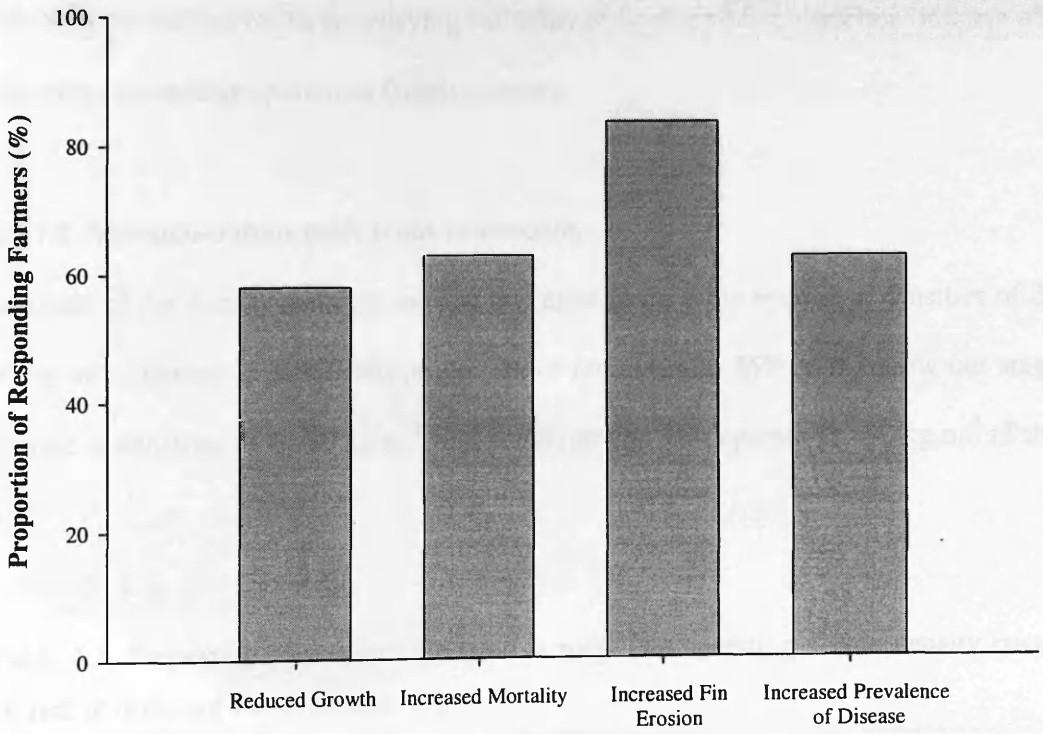


Figure 6.5. Problems associated with 'high' stocking density by UK trout farmers responding to the questionnaire.

6.4.7. Stocking density practices

Analysis was firstly carried out on the density matrices of all farms that selected table production in question 2 (multiple and single-output) allowing a good indication of SD practices to be gained for the bulk of the annual tonnage of trout farmed in UK. Analysis was then carried out for farms carrying out either exclusive table production or those with fisheries / restocking operations (single-output).

6.4.7.1. Multiple-output table trout production

Analysis of the density matrices showed that most farms table operate at densities of 20-40 kg m^{-3}, although a significant proportion of farms (up to 45% at the grow-out stage) operate at densities of 40-80 kg m^{-3}, and some (around 4%) operate at >80 kg m^{-3} (Table 6.3).

Table 6.3. Proportion of non-specific table farmers (%) operating within density ranges for fish of different size brackets.

Stocking Density (kg m^{-3})	Size Range											
	< 5g		5 – 50g		50 – 100g		150 – 250g		250 – 500g		> 500g	
	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End
0 – 20	86	46	83	22	69	17	63	17	64	16	72	26
40 – 60	11	42	15	51	29	49	33	43	26	39	22	54
60 – 80	4	4	2	20	2	19	4	21	8	20	3	14
> 80	0	4	0	2	0	2	0	2	0	4	0	0
No. of farms in weight category	28	26	46	45	48	47	48	47	50	49	36	35
Percentage of farms exceeding 40 kg m^{-3}	4%	12%	2%	27%	2%	34%	4%	40%	10%	45%	6%	20%

6.4.7.2. Density analysis for single-output farms producing trout specifically for the table or restocking and fisheries markets

Analysis of the density matrices showed there to be differences in the ranges of SD for specific types of trout production, with a higher percentage of table farms operating above 40 kg m^{-3} for all size ranges of fish (Table 6.4). It was possible to estimate the mean maximum SD for fish of different sizes by selecting the mid-point of the density brackets. The mean maximum SD for single-output table farm production increased steadily from 28 kg m^{-3} for fry (>5g) to a maximum of around 50 kg m^{-3} for fish of 250-500g. In contrast, the average maximum SD for fisheries/restocking farms remained relatively constant at 20 kg m^{-3} for fish of all sizes (Figure 6.6).

At least 95% of all respondents carrying out restocking/fishery production used initial SD in the 0-20 kg m^{-3} range, whilst for table production the initial starting densities were often in the 20-40 kg m^{-3} range, with the exception being for fry (>5g) where 82% of table farms started in the lowest SD bracket (Figure 6.7). The fact that the table farmers responding to the questionnaire used lower start and finishing ranges of SD for fry suggested that farmers perceived that small fish may be less tolerant to higher stocking densities. The ranges of SD applied on restocking farms and fisheries remained fairly uniform for all size ranges of fish, whereas the SD applied on table farms increased with fish size, peaking with fish of 250-500g at the end of production. This meant that by the end of production, 63% of table farms exceeded 40 kg m^{-3} compared with just 5% of fisheries and restocking operations. For ease of comparison, an alternative graphical presentation of the stocking policies for table and restocking/fishery operations is shown in Figure 6.8.

Table 6.4. Proportion of responding farmers (%) operating within density ranges for fish of different size brackets.

Stocking Density Range (kg m^{-3})	Type of Farm	Fish Size Range											
		< 5g		5 – 50g		50 – 100g		150 – 250g		250 – 500g		> 500g	
		Start	End	Start	End	Start	End	Start	End	Start	End	Start	End
0 – 20	Table	82	40	73	18	67	8	50	4	46	4	42	0
	Restocking / Fishery	*100*	*81*	*94*	*61*	*95*	*48*	*95*	*52*	*95*	*55*	*95*	*48*
20 – 40	Table	18	40	27	32	33	50	42	42	38	33	42	50
	Restocking / Fishery	*0*	*13*	*6*	*33*	*0*	*48*	*5*	*43*	*5*	*40*	*5*	*48*
40 – 60	Table	0	10	0	41	0	25	8	29	13	33	8	17
	Restocking / Fishery	*0*	*0*	*6*	*33*	*5*	*5*	*0*	*5*	*0*	*5*	*0*	*5*
60 – 80	Table	0	10	0	5	0	13	0	21	4	21	8	33
	Restocking / Fishery	*0*	*6*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
> 80	Table	0	0	0	5	0	4	0	4	0	8	0	0
	Restocking / Fishery	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
No. of farms in weight category	Table	11	10	22	22	24	24	24	24	24	24	12	12
	Restocking / Fishery	*16*	*16*	*18*	*18*	*21*	*21*	*21*	*21*	*20*	*20*	*21*	*21*
No. of farms > 40 kg m^{-3}	Table Restocking / Fishery	0	20	0	50	0	42	8	54	17	63	17	50
		0	*6*	*0*	*6*	*5*	*5*	*0*	*5*	*0*	*5*	*0*	*5*

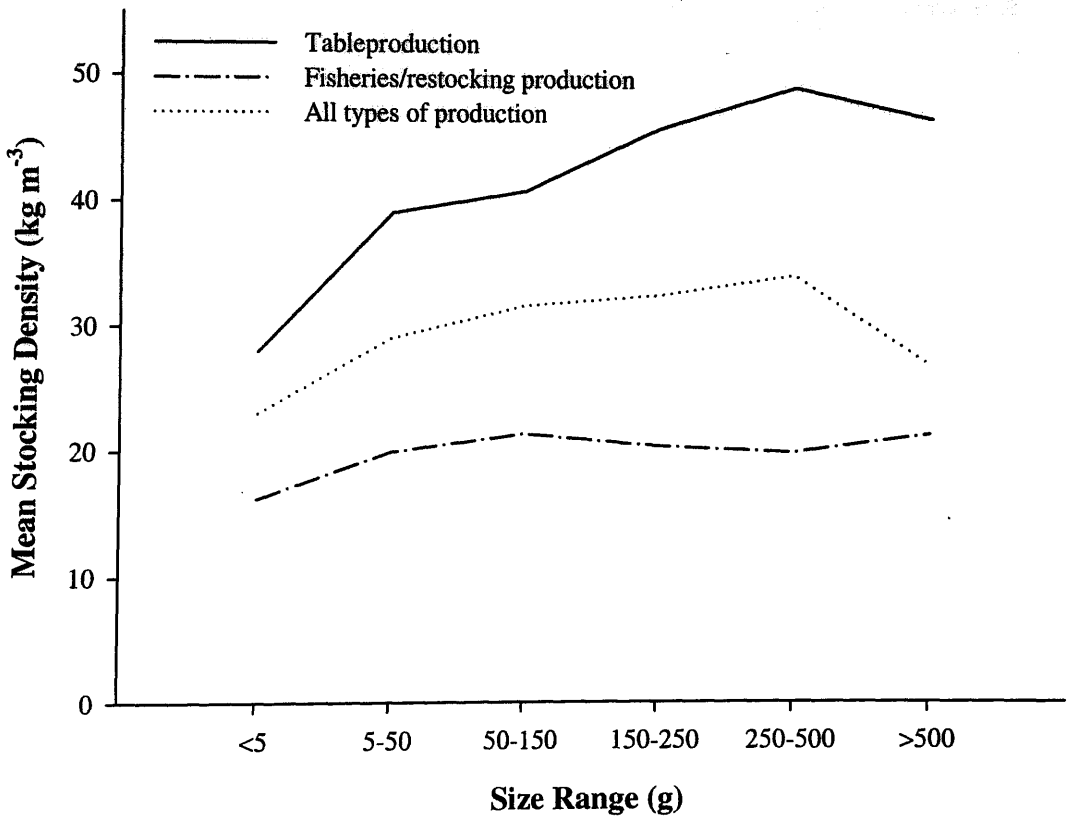


Figure 6.6. Estimated mean stocking density for fish of different sizes ranges on UK trout farms based upon the mid-point of the stocking density ranges.

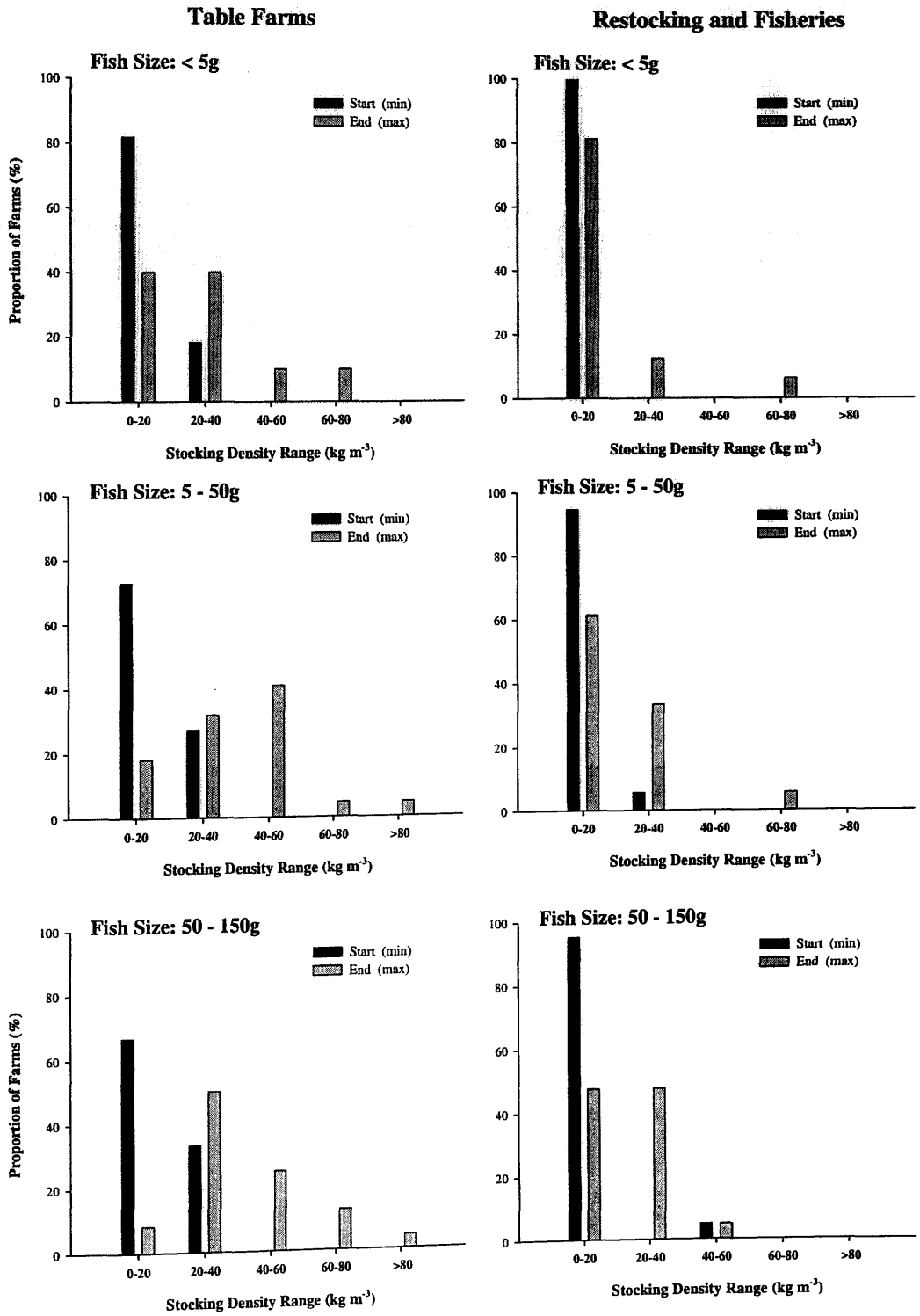


Fig. 6.7. Stocking density practices for fish of different sizes on UK rainbow trout farms producing trout for the table (left) and restocking/fisheries markets (right).

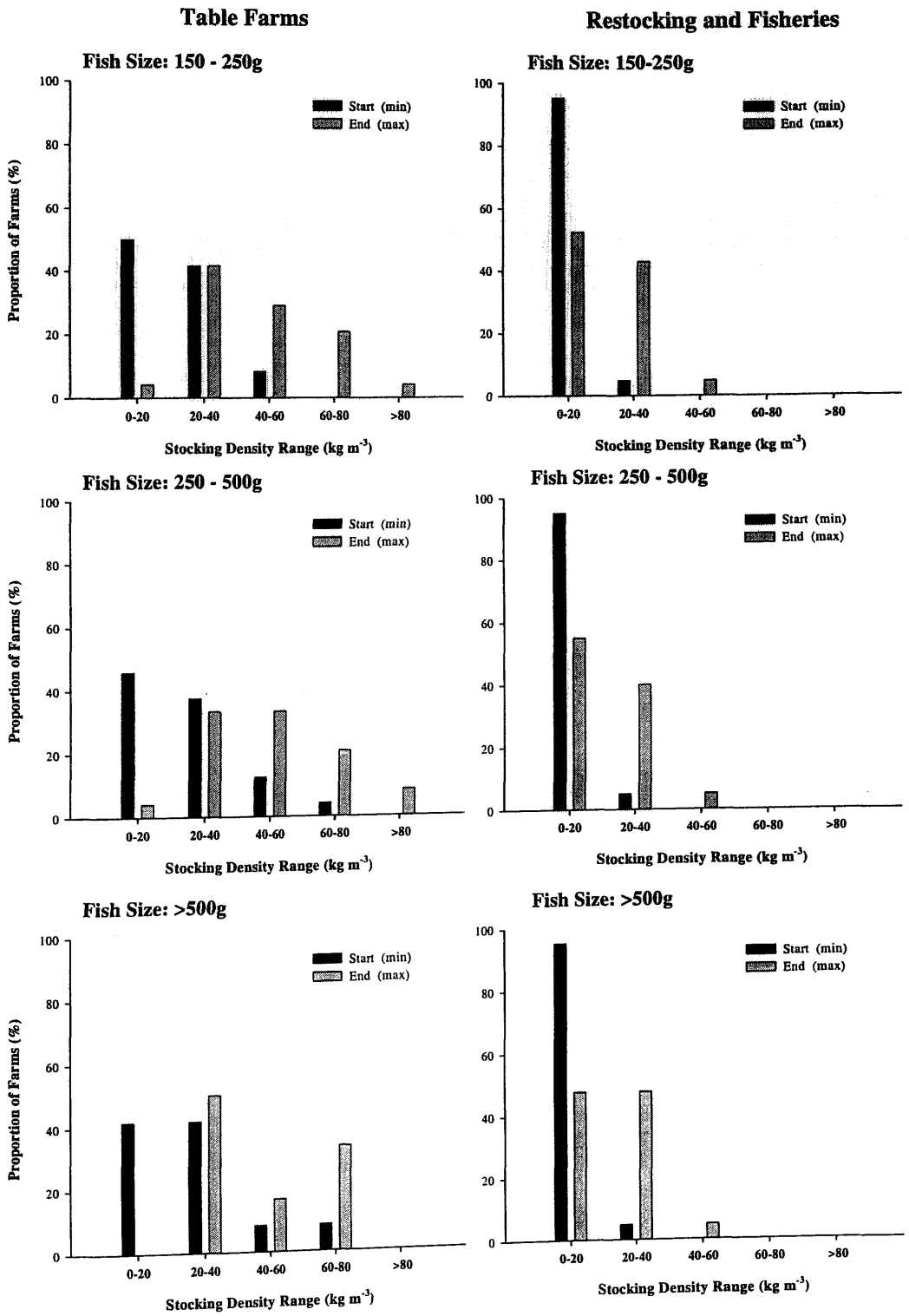
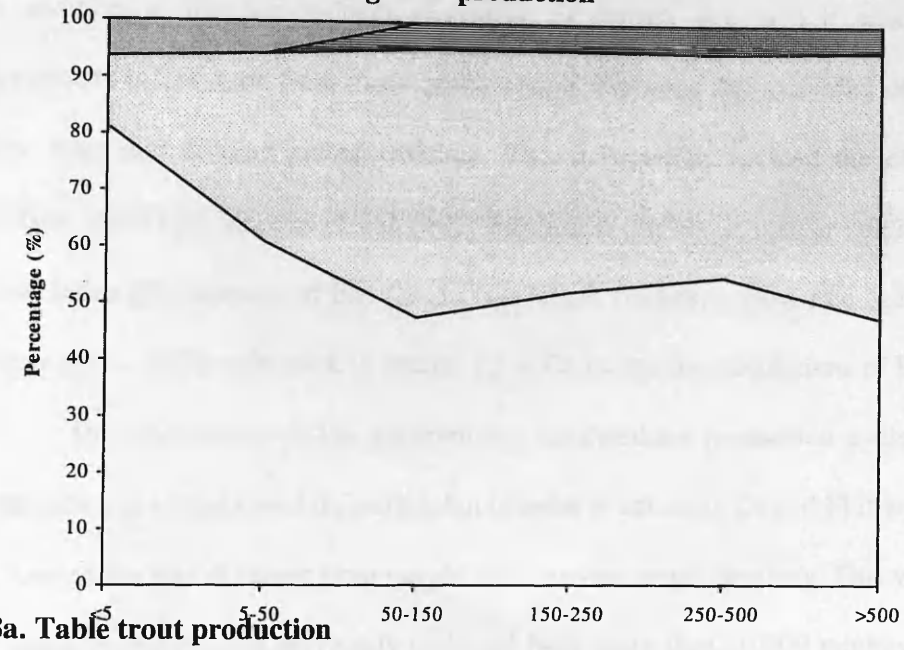


Fig. 6.7. (continued). Stocking density practices for fish of different sizes on UK rainbow trout farms producing trout for the table (left) and restocking/fisheries markets (right).

6.8a. Fisheries and restocking trout production



6.8a. Table trout production

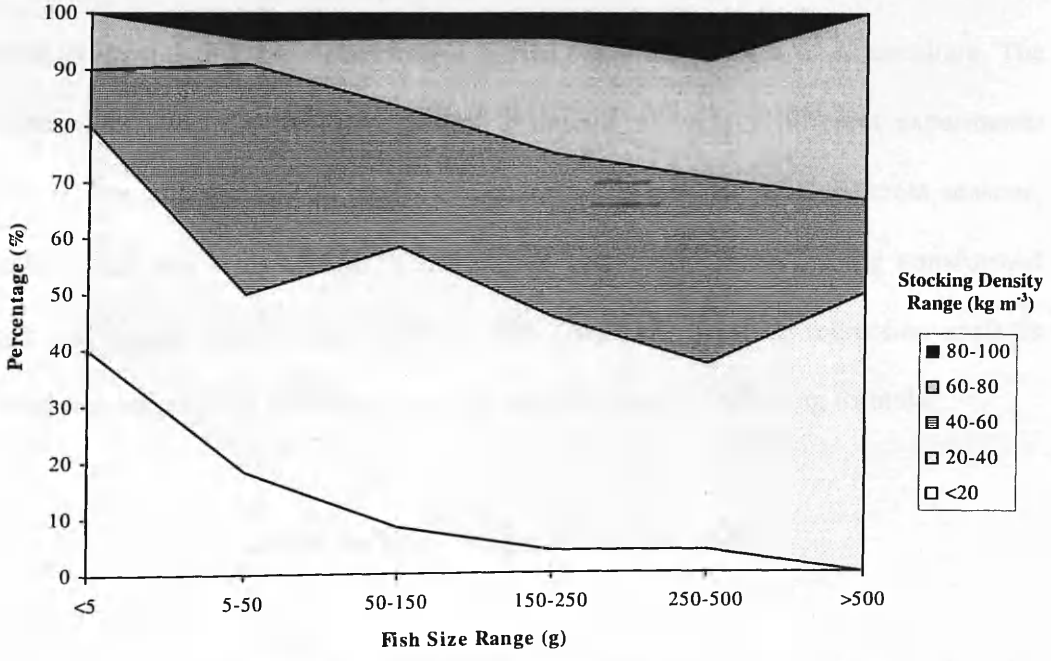


Figure 6.8. Stocking density practices for rainbow trout of different sizes on UK farms producing trout for the restocking/fisheries (6.8a) and table markets (6.8b). The X-axis in represents the different size ranges of fish and the Y-axis shows the percentage of responding farms operating within each of the pre-stated stocking density ranges.

6.4.8. Alternative expressions of stocking density

In addition to the conventional expression of density (kg m^{-3}) it was possible to extrapolate information from those questionnaire responses that provided data regarding flow rates and holding system volumes. This information enabled the calculation of Density Index [DI: biomass of fish (lbs) / fish length (inches) x system volume (ft^{-3})] and Flow Index [FI: biomass of fish (lbs.) / fish length (inches) x flow rate (gallons min^{-1})] (Piper *et al.*, 1982); refer back to section 1.3 in Chapter 1 for calculations of DI and FI.

The information on the questionnaire separated the production cycle of rainbow trout into size ranges based on weight, but in order to calculate DI and FI it was necessary to convert the size divisions from weight brackets into length brackets. This was achieved by using biometric data previously collected from more than 20,000 rainbow trout from control treatments of growth experiments carried out at the Institute of Aquaculture. The biometric data was derived from control treatments of various different experiments (2000-02) encompassing wide ranges of conditions representative of different seasons, genetic strains and sizes of fish. A scatter plot was created from the log transformed length and weight data for the individual fish (Appendix II). The regression analysis allowed fish length to be estimated from fish weight using the following formula:

$$\textbf{Length} = e^{x}[(\textbf{LN Weight} \times 0.332) + 3.749]$$

This calculation was found to be in agreement with other published data for length-weight relationships in rainbow trout (Piper *et al.*, 1982). The converted values for weight brackets are shown in Table 6.5.

Table 6.5. Weight to length conversion values for calculation of DI and FI.

Weight (g)	Estimated Length (cm)
>5	>7.25
5 - 50	7.25 – 15.5
50 -150	22.4 – 26.5
250 - 500	26.5 – 33.4
> 500	>33.4

In addition to the weight:length conversion, it was also necessary to make an estimation of the biomass of fish being held in the holding systems of respondents. The biomass estimation was made using the upper-limits of the ranges of SD together with system volumes for the start and end of production. *e.g.*

System volume = 250 m^3

Stocking Density Ranges = Start (min) 0-20 kg m^{-3}; End (max) 60-80 kg m^{-3}

Estimated Biomass = Minimum = 250 x 20 = 5000 kg

Maximum = 250 x 80 = 20000 kg

Piper *et al.* (1982) recommended a maximum DI of 0.5 for trout *i.e.* SD should not exceed half the fish length in inches, which can be converted to a metric recommendation of 3.2 (x 6.314). Using Piper's recommendation for DI, the maximum SD for fish of 25cm would be 80 kg m^{-3}. Piper's recommended maximum FI ranged from a minimum of 0.83 (18 °C 9,000 ft. above sea level) to a maximum of 2.7 (4.5°C and sea level). Imperial units of measurement of FI can be converted into metric equivalents by multiplying by 0.039.

DI and FI were calculated for all respondents that provided information regarding rearing unit volumes and the flow rates, firstly for all farms and then separately for specific types of trout production (Table 6.6).

Table 6.6. Ranges of Density and Flow Indices on UK rainbow trout farms; (Mean ± SEM) for different types of farming production and different sizes of fish.

Size Range (g)	Type of Farm	Density Index					Flow Index				
		Start (min)		End (max)		*n*	Start (min)		End (max)		*n*
		Mean	*SEM*	Mean	*SEM*		Mean	*SEM*	Mean	*SEM*	
< 5	All farms	0.44	*0.03*	0.61	*0.07*	21	4.30	*1.03*	5.09	*1.04*	18
	Table Farms	0.44	*0.00*	0.73	*0.15*	3	4.34	*1.39*	6.34	*0.83*	3
	Fisheries /Restocking	0.38	*0.05*	0.46	*0.10*	6	2.66	*1.28*	2.67	*1.27*	5
5 - 50	All farms	0.25	*0.02*	0.44	*0.05*	31	2.49	*0.45*	3.85	*0.62*	29
	Table Farms	0.32	*0.06*	0.64	*0.12*	11	2.13	*0.52*	3.85	*0.99*	11
	Fisheries /Restocking	0.19	*0.02*	0.29	*0.05*	8	2.57	*1.18*	3.45	*1.16*	6
50 - 150	All farms	0.17	*0.01*	0.30	*0.03*	32	2.84	*0.78*	3.75	*0.79*	29
	Table Farms	0.21	*0.03*	0.37	*0.05*	12	2.47	*1.11*	2.73	*0.66*	12
	Fisheries /Restocking	0.13	*0.01*	0.20	*0.03*	9	2.48	*1.10*	3.96	*1.97*	7
150 - 250	All farms	0.15	*0.01*	0.25	*0.02*	31	2.57	*0.68*	3.43	*0.69*	28
	Table Farms	0.18	*0.02*	0.32	*0.03*	12	2.18	*0.92*	2.80	*0.58*	12
	Fisheries /Restocking	0.11	*0.01*	0.17	*0.03*	9	2.76	*0.85*	4.09	*1.54*	7
250 – 500	All farms	0.12	*0.01*	0.20	*0.02*	31	1.84	*0.48*	2.45	*0.49*	28
	Table Farms	0.15	*0.02*	0.26	*0.03*	12	1.06	*0.20*	1.89	*0.42*	12
	Fisheries /Restocking	0.09	*0.00*	0.12	*0.02*	9	2.07	*0.72*	2.35	*2.35*	7

2.8.4.1. Density Index

The mean values for DI fell on, or below the recommended level of 0.5 for all but the smallest size bracket (<5g) when all farms were grouped together, and all but the two smallest size brackets of fish (<5g & 5–50g) on single-output table farms (Figure 6.9). DI was consistently lower for fisheries and restocking, compared with table production. There was also a trend for DI to decrease with increasing fish size (Figure 6.9). Analysis of SD practices showed that SD generally increased with increasing fish size (Figure 6.6), but the fact that DI showed such a marked decrease with increasing fish size suggested that fish length may have a strong influence on the DI results.

2.8.4.2. Flow Index

The highest FI values occurred at the fry (<5g) stage of production, with the mean FI for multi-output table farms (FI = 5.09), and also for single-output farms producing trout solely for the table market (FI = 6.34), well above Piper's maximum recommended FI value (2.7). At the start of the production cycle FI was generally at, or below, the recommended maximum value of 2.7, but by the end of the cycle, FI was above this value for all sizes of fish except the 250-500g fish (Figure 6.10).

The FI values for the fisheries and restocking farms were higher than the values for the table farms in the 50-150, 150-250 and 250-500g size divisions, which was surprising, considering how much higher the SD used by the table farmers was. An explanation for the high FI values on the fisheries and restocking farms could be the comparatively low inflow rates in large still-water ponds, compared with the more compact raceway systems used in table trout production, where rates of water exchange and inflow are much higher.

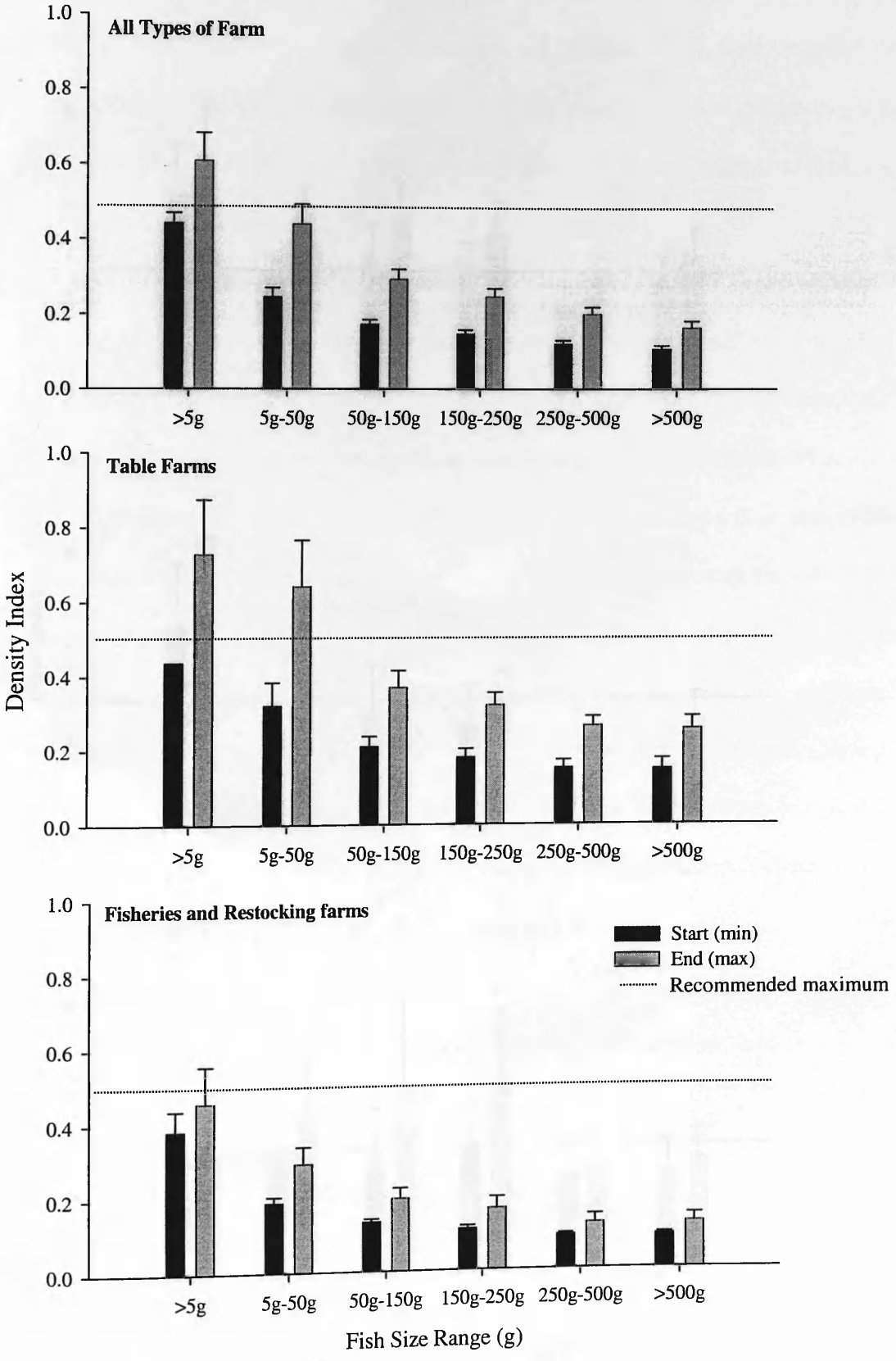


Figure 6.9. Estimated ranges of Density Index for different types of UK trout farms.

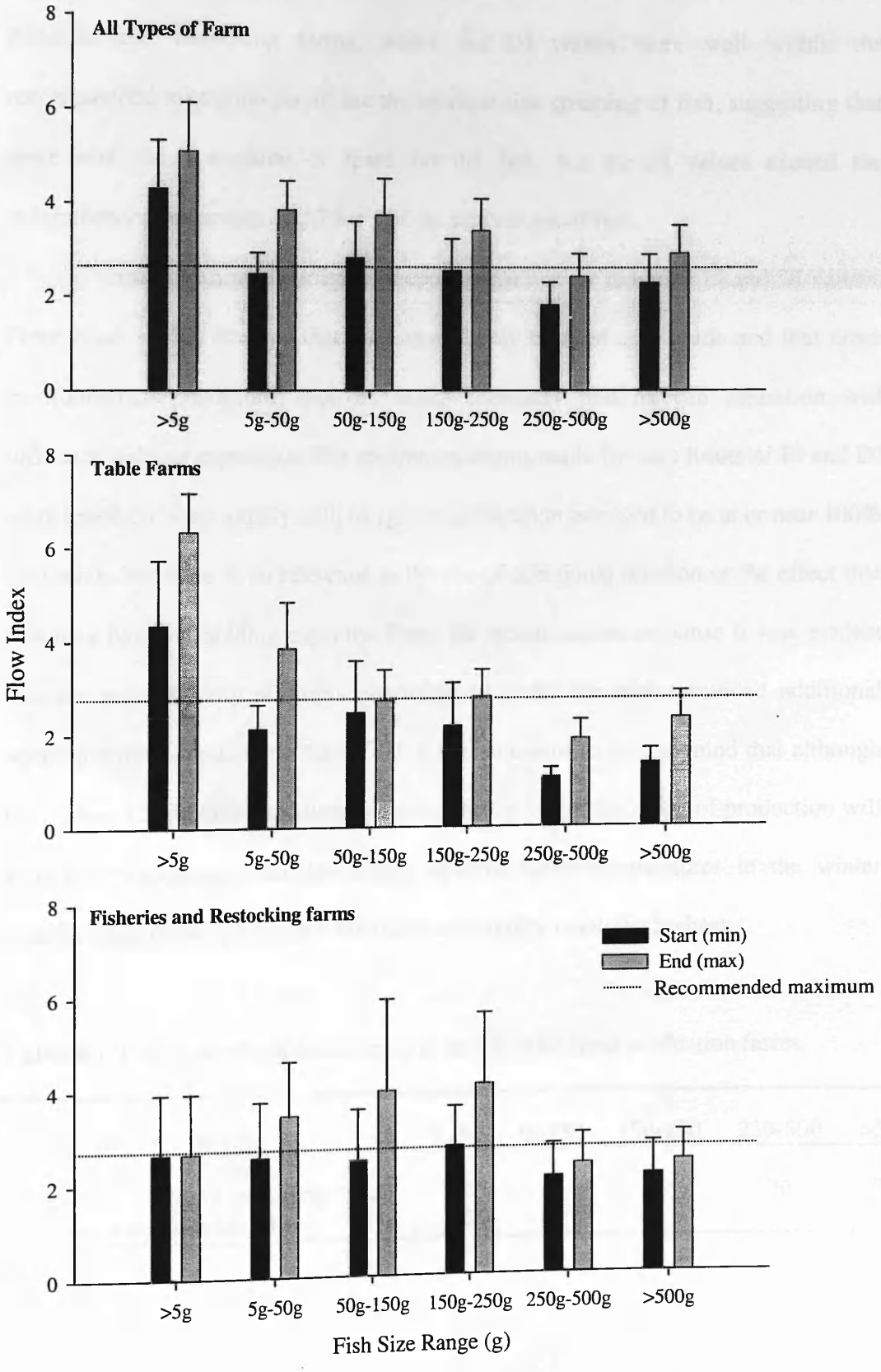


Figure 6.10. Estimated ranges of Flow Index for different types of UK trout farms.

An interesting comparison can be made between the DI and FI values for the fisheries and restocking farms, where the DI values were well within the recommended maximum for all but the smallest size grouping of fish, suggesting that there was good provision of space for the fish, but the FI values exceed the recommended maximum of 2.7 for 3 of the size ranges of fish.

When proposing the original recommendations for maximal FI and DI values, Piper *et al.* (1982) stressed that they should only be used as a guide and that other environmental conditions such as water chemistry and oxygen saturation will influence holding capacities. The recommendations made for safe limits of FI and DI were based on water supply with oxygen concentration assumed to be at or near 100% saturation, but there is no reference to the use of additional aeration or the effect that this may have on holding capacity. From the questionnaire response it was evident that the vast majority of farms producing trout for the table provided additional aeration during production (Table 6.7). It is also useful to bear in mind that although the highest FI and DI values were recorded for fry (<5g), this stage of production will generally correspond with the lowest seasonal water temperatures in the winter months when dissolved oxygen and water availability would be highest.

Table 6.7. Provision of additional aeration on UK table trout production farms.

Size Range (g)	<5	5 -50	50-150	150-250	250-500	>500
Percentage of farms providing additional aeration (%)	49	57	74	79	76	79

6.4.9. Additional Comments

This section summarises some of the additional comments that were added to questionnaires.

6.4.9.1. Aeration

The vast majority of on-growing farms use additional aeration (Table 6.7) and from the comments added to the questionnaires, aeration would appear to be perhaps the most important factor when considering SD. Several respondents stated that DO is the means by which decisions are made in regard to how heavily fish are stocked. Two respondents stated that SD was determined on the premise that DO in outlet water was maintained above a specific concentration (>6.5 – 7.0 mg l^{-1} in one case and >70% saturation for the other). Other respondents attributed the associated welfare problems in question 5 to insufficient provision of oxygen rather than SD *per se*.

6.4.9.2. Feeding

Feeding is linked to aeration, particularly in summer months when feeding may be restricted or stopped all together if dissolved oxygen is low. Most manufacturers' feed tables' state that at water temperatures in excess of 16°C, feeding should be ceased or fish should be fed according to DO levels. From the comments on some questionnaires it was clear that sub-optimal feeding routinely occurs due to low DO in summer months and this in turn could result in, or contribute to, some of the welfare problems associated with high stocking densities.

6.4.9.3. Fish Size

From the questionnaire response data and additional comments added to some of the replies, it would appear that there is a general consensus that tolerance to SD varies with fish size. However, there are contradictions between respondents in how this relationship actually works. The collated SD information in section 1 indicated that higher densities (kg m^{-3}) occur during the on-growing stage (150g – 500g), but the opposite is true for FI and DI data, which both decreased with increasing fish size.

One farmer producing trout for the table market specified differences in the perceived high SD based on fish size, suggesting 30 kg m^{-3} for 500g fish and 60 kg m^{-3} for 5g fish. In contrast, another response from a restocking farm stated the opposite, highlighting the period between fingerlings up to 200g as being the crucial time at which to keep SD low (specifying >14 kg m^{-3}). They also added that fin damage and reduced growth occurred at early stages will worsen, almost regardless of future stocking densities. These differences in opinion may reflect how different types of trout production have different priorities *i.e.* the priority for a restocking farms may be to produce fish with good quality fins, whereas the priority for table production will be maximal growth.

6.4.9.4. Season

Several respondents were eager to stress the importance of considering season when addressing SD, as during the colder months when water temperature is lower there will not only be higher DO but also more water available (78% responding farms are supplied by river water). Seasonal effects should also be taken into account when considering information already discussed for stocking practices; this is especially true for the smaller sizes of fish, as this stage of production coincides with the colder

months (December – April), when water temperature and consequently feeding will be at their lowest.

6.4.9.5. Sceptics and Advocates

There was some objection to the use of SD as a means of safeguarding fish welfare. One response commented that "the use of SD as a basis for a welfare measurement is purely theoretical and could not be applied to a practical situation because fish naturally shoal and therefore the SD constantly changes". It was also suggested that SD practices are totally site specific. On the opposite scale of the spectrum, other farmers were keen to see stocking densities reduced and suggested that quality schemes and marketing be introduced akin to 'free-range' and 'barn-reared' chickens. Other respondents suggested that "lower SD would result in better quality of life and that welfare and quality go hand in hand".

6.5. Discussion

The good response rate to the questionnaire and the willingness to supply information, opinions and offers of farm visits, suggested that the trout farming industry was aware of welfare issues and was prepared to disclose information regarding stocking practices. BTA member farms accounted for around 80% of UK trout production and with 65% of BTA member farms responding to the questionnaire it would also appear that the questionnaire was successful in collecting information from a good proportion of the major trout producers in the industry.

There were major differences in the perceptions of a 'high' SD ranging from 7 – 200 kg m^{-3}, but when separated into specific types of production the ranges became

much tighter with the perceived high density for single-output table farm production more than double that of fisheries and restocking operations (60 vs. 33.2 kg m^{-3}).

The majority of farms producing trout exclusively for the table were shown to operate at average stocking densities of around 40 -50 kg m^{-3}, with highest densities occurring in the final grow-out stage of production (250-500g). The SD used for fisheries and restocking operations was around half the figure for table farms, with a mean maximum SD of around 20 kg m^{-3} for fish of all sizes. It is probable that these differences in SD practices reflected different production priorities, with the table farmers focusing on maximising output, while the fisheries and restocking farms concentrated on producing fish with intact fins and no visible blemishes. Parallels may be drawn with other forms of farming, where the way in which broiler chickens or bullocks are farmed for meat is very different to the way in which laying hens or dairy cattle are farmed.

For the most part, the estimated DI values were within the recommended maximum of 0.5 and only exceeded this level for the smallest fish sizes (Piper *et al.*,1982). Fisheries and restocking operations were well within the 0.5 limit for fish of all sizes. The results for FI suggested that the recommended maximal level to be exceeded for the smallest groups of fish but there after there was a general trend for the FI to be well within the limit at the start of stocking, but by the end of a growing phase the FI would be on or slightly in excess of the recommended maximal level.

A major problem experienced with the questionnaire responses was the lack of accurate information regarding flow rates, with very little accurate data available. Respondents predicted flow rates by either timing how long it took for a particular system to fill up from empty with knowledge of the system's volume, or estimated flow rates from knowledge of the total throughput of a farm (usually in millions of

gallons per day), which could then be divided depending on the layout of the farm *e.g.* if all first-use water entering a farm passed though six identical raceways an estimation of flow rates could be made. The lack of accurate flow rate data highlighted the difficulties of trying to apply alternative methods of quantifying SD rather than the conventional unit of kg m^{-3}.

The vast majority (78%) of respondents were either totally or partially dependent on river water supply. River water supply is subject to seasonal and annual variations, thus adding to the difficulty of estimating flow rates. Flow rate and system volume data was generally much better for raceways and smaller tanks, but data provided for larger ponds was very limited and obviously no flow data was available for cage farms.

In addition to collecting information regarding the SD practices from a good proportion of the UK trout industry, this questionnaire also provided an insight into the way in which farmers regard SD and trout welfare. Although farmers acknowledged the potential of SD to result in poor welfare, there were marked differences of opinion regarding exactly what comprised of a 'high' SD. It is also likely that the priorities of farmers producing trout for the table and restocking/fishery markets is very different and that there may be lessons to be learnt from the way in which these industries have evolved regarding safeguarding different aspects of fish welfare.

Chapter 7: On-farm welfare assessment

7.1 Introduction

There is a growing demand for systems for on-farm welfare assessment in all areas of livestock production. Such systems have a wide range of benefits to farm animal welfare and can be used to aid policy makers, provide feedback to farm managers on areas in which they can improve the welfare of their stock, and can be developed as a marketing tool (Spoolder *et al.* 2003). An example of a commercially successful application of stringent welfare policy as a marketing tool is provided by the Dutch veal farming company, 'Peter's Farm'. By increasing the amount of space for the calves, using communal housing with environmental enrichment and only selecting suppliers that can provide high quality animal housing, many of the welfare concerns over traditional veal production have been addressed. These practical steps have been combined with modern technology to provide total traceability, whereby consumers with internet access can trace meat back to the farm where it was grown via a batch code, allowing images of the calves at different ages to be viewed along with information on the farmers who grew that batch of animals (www.petersfarm.com). Although such an approach could be viewed cynically as a mere marketing ploy, this approach has been successful in restoring consumer confidence in the ethics of veal production and in terms of animal welfare, is a vast improvement upon the traditional narrow crate intensive veal production (a practice which has been banned in the UK since 1990).

In the absence of a 'Gold Standard', the objective determination of welfare relies on the selection, collection and interpretation of different parameters. The choice and

relative weightings of these parameters will depend largely upon the opinion and experience of the 'experts' involved in the decision who may be laypeople (no involvement with animal husbandry), specialists (*e.g.* veterinarians), farmers and welfare scientists (Spoolder *et al.*, 2003).

The concept of welfare assessment of fish farms is still in its infancy though developments in other areas of livestock production offer a wide variety of different approaches. Spoolder *et al.* (2003) suggested five different approaches in which parameters can be integrated for on-farm welfare assessment (Table 7.1).

On-farm welfare assessment of UK trout farms is presently almost non-existent, with the only regulatory inspection coming from the Fish Health Inspectorate, which has a very limited brief with regard to safeguarding fish welfare. One of the main driving forces in promotion of fish welfare policy are the increasing demands made by the supermarkets chains. It is now common place for supermarkets to carry out their own audits of fish farms and there is an increasing demand for farmers to demonstrate that fish welfare is being safeguarded. In contrast to the limited power that regulatory bodies currently have to enforce welfare legislation, supermarket chains have considerable power over farmers, whereby, a contract can be terminated if a farm is not complying with their requirements. The welfare policy of supermarkets ultimately reflects a consumer concern over the ethics of livestock production (Cooke, 2001), but it is important that welfare criteria are determined on a scientific basis rather than an emotive response to the demands of pressure organisations.

Table 7.1. Suggested approaches for on-farm welfare assessment (after Spoolder *et al.* 2003).

Approach	Key steps	Advantages	Disadvantages
1. Scoring Systems	1. Parameters selected based on expert opinion and literature evidence 2. Parameters weighted by experts on the basis of literature and experience. 3. Usually involves setting of threshold limits 'pass' and 'fail' 4. On-farm testing. Steps 1-3 may be adjusted if perceived and observed welfare status is mismatched.	Simple. Logical. Transparent. Based on recognised 'accepted' measures.	Scoring can be subjective. Over- or under compensation' if threshold limits are inaccurate.
2. A Decision Support System	1. Defines welfare and breaks down into functional welfare needs, thus defining the model's domain. 2. Quantifies available statements in literature and links to welfare needs. 3. Assigns relative weights to needs. 4. Validates the model by comparing welfare scores from the model to those of internationally recognized welfare experts.	Integrates complex information into one model High level of objectivity.	Method is a 'black box' with only the developer knowing the true strengths and weaknesses of the model.
3. Multivariate statistics to determine relative weights	1. Experts give an overall welfare score based on own experience to a range of husbandry situations. 2. As many animal- and housing related parameters as possible are measured in each husbandry situation. 3. Scores of experts are linked to parameters measured through multivariate statistical techniques to identify relevant (and irrelevant) parameters and allocate a weighting to each. 4. Model is validated by testing outcome using different husbandry situations and different experts.	Using other Expert's opinions makes the selection and weighting of parameters objective.	Design and quality of model relies heavily on the experts who provide initial scores. Large dataset required to build model with all of its parameters.
4. 'Classic' post-hoc interpretation of results	1. Parameters selected to be measured on-farm 2. Data collected for each parameter. 3. Conclusions drawn from the outcome of each parameter and conclusions drawn on level of welfare from author's opinion of the relative weighting of each parameter.	Transparent with records of individual parameters. Easy to apply.	Final conclusions can not be standardised - depends on initial choice of parameters and researcher's interpretation.
5. Qualitative assessment – integrating parameters through 'whole animal' observations	1. 'Whole animal' qualitative scores are assigned to groups of animals during farm visits. 2. Scores are used to interpret measured quantitative parameters through multivariate statistical mapping techniques.	Potentially, cheap, flexible and includes all aspects of welfare.	Relies heavily on expert's interpretation of behaviour. Poor traceability

There is much debate over the most appropriate means of on-farm welfare assessment to use. The current approach with terrestrial livestock is moving away from total reliance on objective welfare indicators and back to systems that focus on more subjective assessments (Bracke *et al.* 1999; Bartussek, 1999; Wehmelsfelder *et al.* 2001; Dawkins, 2004).

Documented evidence regarding stocking density practices of UK trout farms is very limited and the results of the questionnaire presented in Chapter 6 offer perhaps the first insight into the ranges of SD applied on different types of trout farming systems in the UK. There is also no proposed system of on-farm welfare assessment of fish farms, although systems such as the health and condition profile (Goede & Barton, 1990) provide possible directions for ways to assess individual fish.

This Chapter focuses on the pilot-scale application of on-farm welfare assessment in relation to stocking density practices from a selected range of UK trout farms. The work outlined in this Chapter aimed to:

1. Provide detailed information on stocking density practices from a range of representative trout farms.
2. Field-test the system of welfare assessment that was used in the experimental studies.
3. Assess the welfare of batches of fish from representative trout farms through the grow-out stage of the production cycle (100g to harvest).
4. Identify potential areas of improvement for future development of systems of on-farm welfare assessment in trout farms.

7.2. Materials and Methods

For this pilot exercise a purposive sampling strategy was adopted because of a lack of resources and anticipation that compliance may have been problematic. This meant that the preferred sampling approach of a randomised epidemiological survey was not feasible. Instead, farms that were representative of high, medium and low intensities of production were selected for inclusion in the study based on the following criteria:

- Agreement to co-operate with the study.
- Provision of reliable information from the postal questionnaire.
- The agreement to keep batches of fish as distinct groups without mixing or grading.

7.2.1. Sampling strategy

A longitudinal sampling strategy was adopted, which comprised of repeated of visits between July 2002 and November 2003 to selected farms that were representative of the main types of trout farming systems used in the UK. The original sampling design proposed to follow batches of fish at monthly intervals on four sites, two of which would represent low-density production (*e.g.* less than 30 kg m^{-3}) and another two representing high-density production (>60 kg m^{-3}). However, it was not always possible to follow distinct batches of fish, and from the original four sites it was only possible to follow three batches for more than one visit. The sampling was later extended to include a wider range of systems, fish sizes, and to cover a larger geographic area with data collected from a total of seven different farms. A brief description of each of the farms included in the study is shown in Table 7.2.

Table 7.2. Description of the trout farms included in the longitudinal sampling.

Farm	Approx. production (tonnes per annum)	Type of culture systems	Max. SD (kg m^{-3})		Water Quality characteristics		Approximate location
			Fry	On-growing	pH	Alkalinity (mg l^{-1})	
1	200	Concrete raceways, paddle wheel aeration	40	30-40	6.20-6.86	28.2	SW Scotland
2	120	Earth raceways with paddle wheel aeration	60	30-40	6.43-7.47	84.6	SE Scotland
3	300	Concrete raceways, liquid oxygen injection	60	140	6.76	42.3	NE England
4	250	Concrete raceways, liquid oxygen injection	60	120	7.72-8.14	305	SE England
5	400	Concrete raceways, round tanks and large concrete outlet channels with oxygen injection	60	40	7.61-8.02	315	SE England
6	60	Concrete raceways and earth ponds (no additional aeration)	20	10	6.75	not measured	SW England
7	40	Concrete raceways and earth ponds (no additional aeration)	40	10	7.95-8.5	330	SE England

7.2.2. Sampling Protocol

On each farm visit 24 fish were sampled and killed (Home Office Schedule 1); the time taken to do this was always less than 5 minutes. Cadavers were:

- Weighed (±0.1g) and measured for fork and total length (±1mm)
- All rayed fins were measured using calipers (±0.1mm)
- Blood sampled for analysis of:
  - Haematocrit
  - Glucose
  - Cortisol
  - Lysozyme activity

Sampling generally commenced at around 10 am on each visit approximately 2 h following the first daily feed

Water quality analysis was also carried out for the following parameters from the inflow and outflow of each system as described in Chapter 2:

- Dissolved oxygen
- Ammonia (TAN)
- pH
- Temperature
- Alkalinity

For improved consistency, water samples were always collected between 2 and 3 pm at each visit.

Farmers were also asked to keep a log of mortality, feeding, water temperature and any stock movements during the period of sampling.

7.3. Results

7.3.1. Stocking density practices on a selection of UK trout farms

Changes in SD were monitored through the course of the production cycle on farms 1, 2, 4 and 5. Fish would be stocked into different systems on these farms at initial SD ranging from 12.3 to 32.0 kg m^{-3}. When fish were initially stocked into systems the farmers had a reasonable estimate of the mean fish weight and total number of fish. Fish numbers were determined by either automated fish counters built onto grading machines, or estimated from knowledge of the total biomass and mean individual fish weights. Mean weights were estimated from batch weights, the frequency and method of which, varied between sites. For smaller fish mean weight was normally calculated by counting a known weight of fish in or out of a bucket and for larger fish, between 50 to 100 fish were weighed into a pre-weighed bin part-filled with water.

With knowledge of the number and weight of fish initially stocked into a system, farmers would then feed either a set ration, or use demand feeders to allow fish to feed to appetite. Of the 7 farms visited, 6 used the software packages 'D-Journal' or 'Farm Navigator' to plot and predict the performance of batches of fish. The software packages estimated the growth of fish based upon an assumed FCR, which could be calculated by recording the amount of food fed each day. Drawing comparison between the estimated and actual weights of fish, this system appeared very accurate (see Figure 7.1). However, the accuracy of the software for predicting growth was ultimately dependent on the accuracy with which fish numbers and the weight of feed supplied to the fish was recorded.

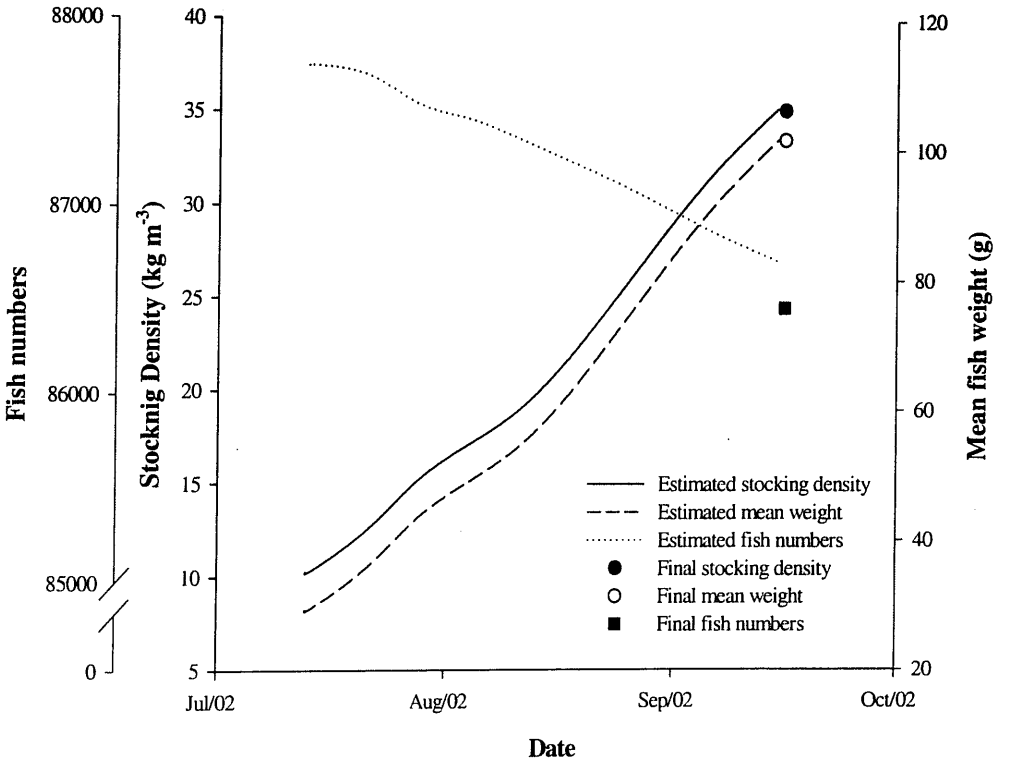


Figure 7.1. Relationship between changes in stocking density, fish growth and mortality in a raceway of fingerling rainbow trout from Farm 1.

Due to the large volumes of food fed to fish each day, the amount was often recorded based on the number of bags of food used (25kg) *e.g.* 80,000 fish at 100g fed on a 1.5% body ration would require 120 kg or approximately 5 bags of food per day. With feed costs accounting for up to 50% of total production costs (Westers, 2001) farmers were very conscious of how much feed was given to the fish.

As fish grew, SD increased and the effect of growth overrode any effect that mortality had on reducing SD (Figure 7.1). Farmers are required by law to keep a record of daily mortalities and this information was available on all of the farms visited. It was usually the case that when fish were graded out of a system, the actual number of fish would differ slightly from the estimated number. An example from Farm 2 shows that the actual number of fish at grading (86,466) was approximately 250 lower than the estimated number 86,719 (Figure 7.1). However, this difference of 250 fish represented less than a 0.5% margin of error and could have been attributed to a number of factors such as predation (*e.g.* herons, mink and otters), escapees, or human error.

The way in which SD was regulated was largely site specific, depending on factors such as the size, number and availability of culture systems on a particular farm, water temperature, water availability, dissolved oxygen and market demand for fish. Figure 7.2 follows a batch of around 112,000 fish from their arrival on Farm 5 from a size of around 15 g through until harvest. Farm 5 was a particularly large site with a wide variety and numerous rearing units, offering a degree of flexibility throughout the production cycle. Other farms that were visited were more restricted with fewer systems and fewer movements of fish, though in all cases fish would generally be moved or graded at least twice during production cycle.

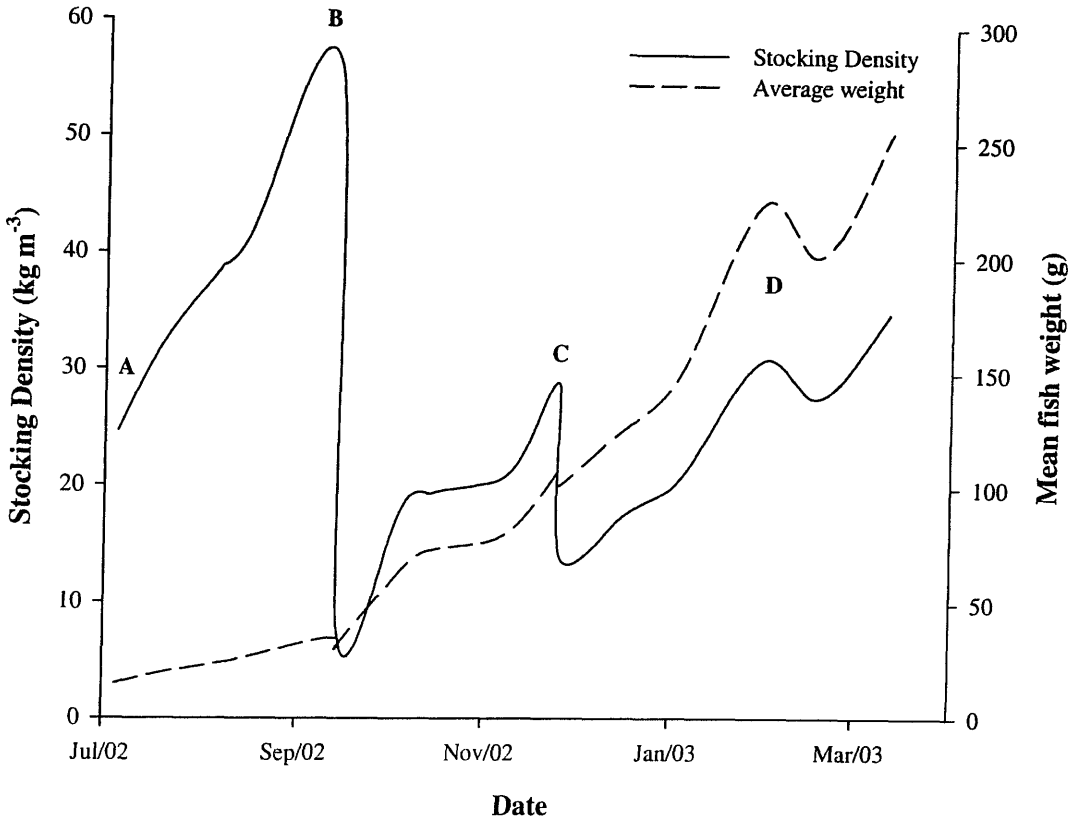


Figure 7.2. Growth and stocking density regulation of a batch of fish from arrival until harvest on Farm 5.

Key:

A. Batch of approximately 112000 fish (mean weight 15g) arrived on-site and were split into 5 raceways (13.5 m^3; 1st use water)

B. Each raceway of fish is transferred into a larger round tank (80 m^3; 1st use water)

C. Fish are transferred from round tanks and mixed with other fish from the same original batch into an outlet channel (450 m^3; 3rd use water)

D. Fish are graded and the top grade of fish at harvest size is removed

Farms 3 and 4 were examples of modern, intensive trout farming systems, where parallel banks of concrete raceways allowed for a compact site with the capacity to produce a large biomass of fish (Farm 4 produced around 250 tonnes of fish per annum from a site covering around 400 m^2). Farms 3 and 4 both operated at maximum SD of above 100 kg m^{-3} and were dependent on high rates of water exchange and liquid oxygen injection to maintain DO above critical levels. Space limitations on Farms 3 and 4 meant that it was difficult for farmers to maintain fish as distinct batches for prolonged periods of time, although a batch of fish on Farm 4 was followed for a 6 month period, from a size of around 40 g until harvest (Figure 7.3). The SD of the batch of fish from Farm 4 was reduced after around two months by the removal of a screen that had been separating this batch from another batch of fish positioned downstream in the raceway. When the fish reached an average weight of around 250 g and a SD of more than 120 kg m^{-3} they were graded, with the top grade (>400g) being harvested and the remainder of the fish returned to the raceway before subsequent harvest at approximate monthly intervals (Figure 7.3).

7.3.2. Water quality

On most of the sites visited, the main factor influencing farmers' decisions with regard to the maximum SD was oxygen availability. Farms 3, 4 and 5 all used liquid oxygen injection, allowing oxygen to be diffused into the water in the inlet channel to the farm. Smaller, dome-shaped diffusers positioned strategically around the farm could provide top-up injections, allowing the water to be re-used through different rearing systems without DO becoming limiting.

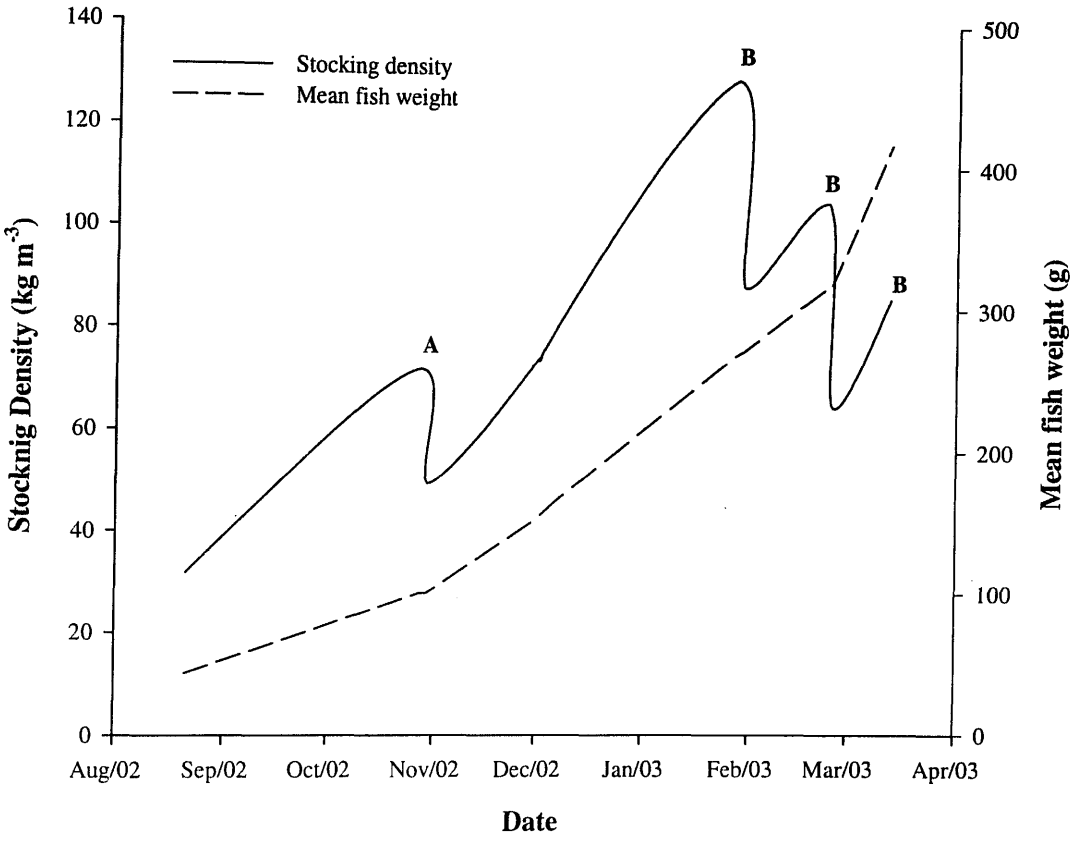


Figure 7.3. Growth and stocking density regulation on Farm 4, an example of a modern, high density trout farm.

Key:

A. Removal of a dividing screen in the raceway provided fish with more space

B. Fish were partially harvested, where 'top-grade' of largest fish was removed

The oxygen injection systems used on Farms 3, 4 and 5 were all fully automated and worked on a feed-back loop, whereby DO measurements from probes based at strategic points around the farm were relayed back into a control system. The oxygenation systems on Farms 4 and 5 were also capable of logging the DO measured from the probes into a PC at pre-set time intervals.

The data-logging capacity afforded by monitoring systems such as those used on Farms 4 and 5 is illustrated in Figures 7.4 and 7.5. Measurements of water temperature and DO taken at 20 min intervals over a 24 h period show how the injection system on Farm 4 maintained inflow DO at a steady 12 mg l^{-1}, even though the DO in the river supplied the farm ranged between 9 and 11 mg l^{-1}. The night-time drop in river water DO is typical of the region in which Farm 4 was located (SE England), occurring when the large plant biomass that grows naturally in the rivers stopped photosynthesising during hours of darkness (Figure 7.4). Data is also presented for the entire 6 month sampling period during which a batch of fish was monitored on Farm 4 (Figure 7.5).

Farm 5 also used oxygen injection, but the SD policy differed substantially from that of Farms 3 and 4. The highest SD on Farm 5 (approx. 60 kg m^{-3}) occurred when the fish were relatively small (>50g) and were housed in 13.5 m^3 concrete raceways, but when moved into larger systems, the maximum SD was around 30 kg m^{-3} (Figure 7.2). Instead of using the oxygen injection system to achieve very high SD, Farm 5 used the system to enable the water to be re-used through numerous different systems in a multi-pass or 'maze' system.

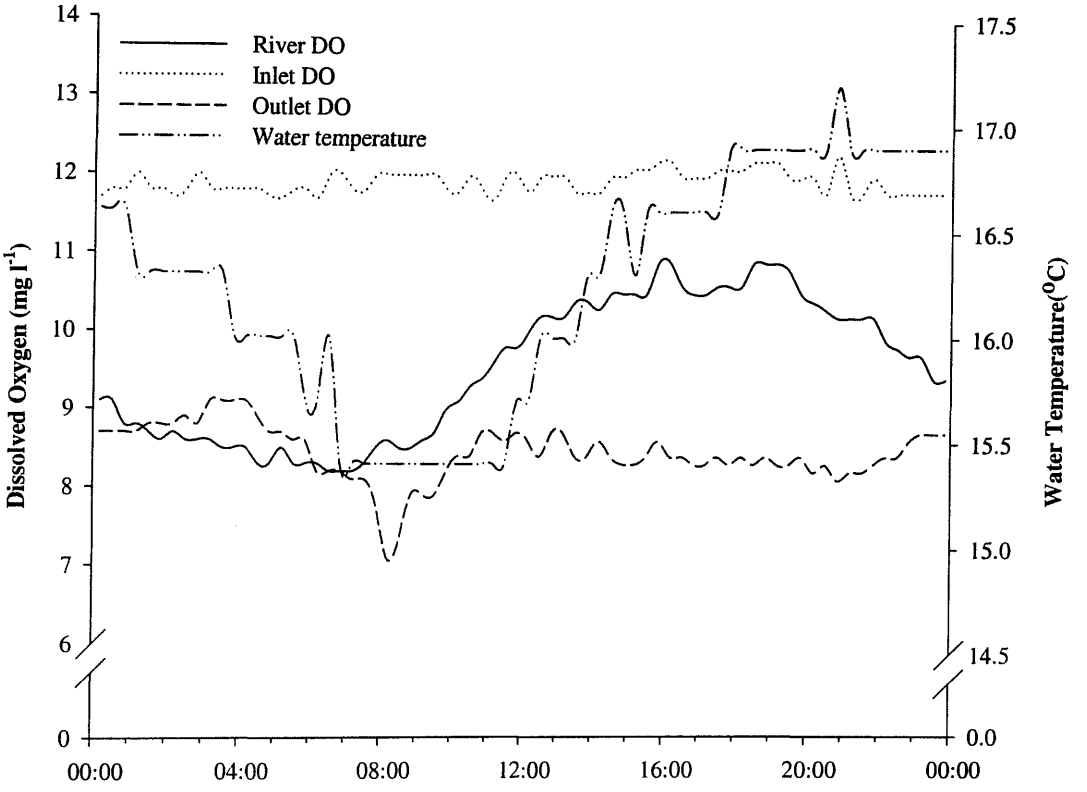


Figure 7.4. Fluctuations in dissolved oxygen and water temperature over a 24 h period on Farm 4; automated recordings taken at 20 min intervals by an Oxyguard™ monitoring system.

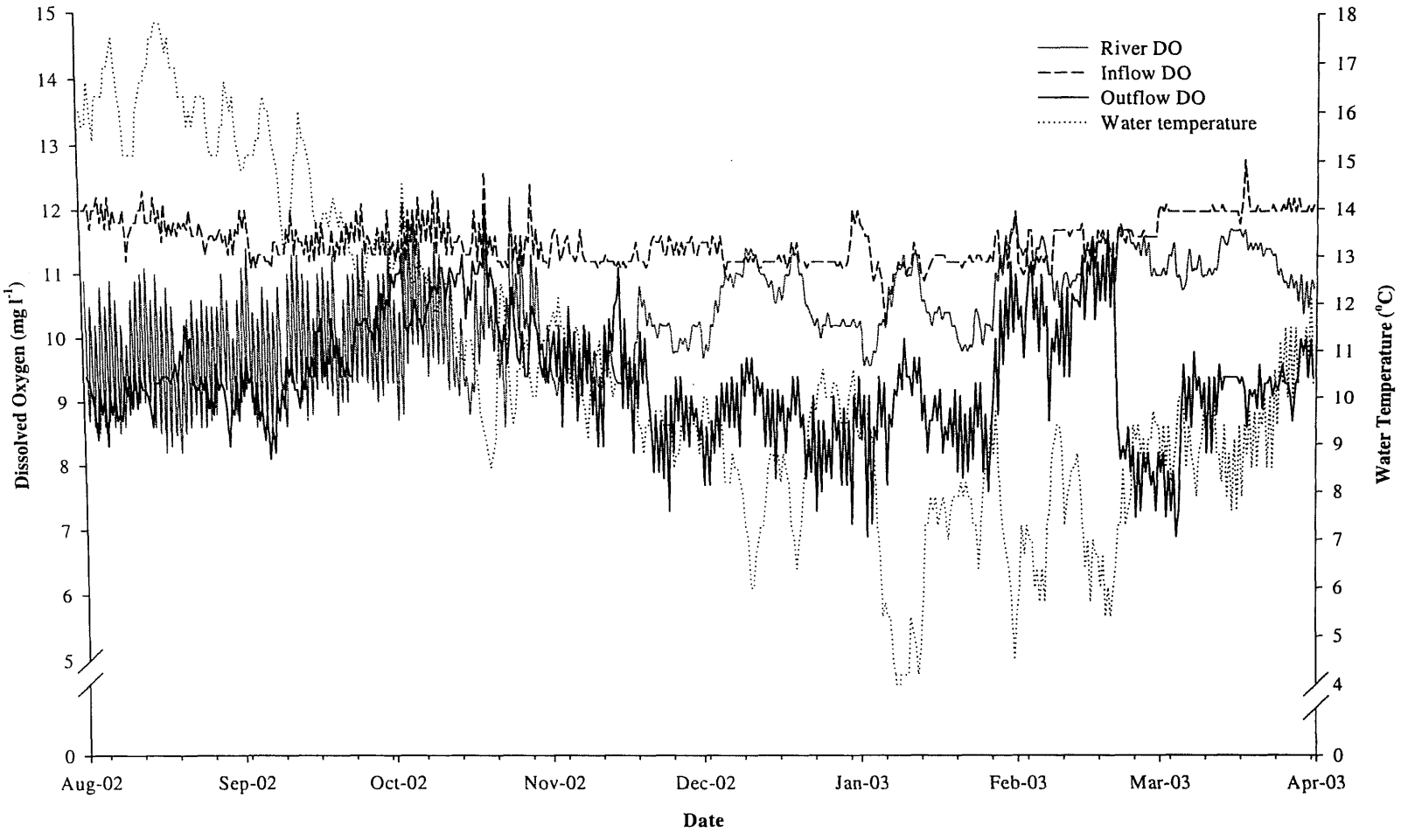


Figure 7.5. Dissolved oxygen and water temperature profiles for the 6-month sampling period for fish sampled from Farm 4.

Farms 1 and 2 represented the more traditional style of table trout farm, where aeration was provided by paddle wheels and DO was ultimately the limiting factor for maximum SD. Farms 1 and 2 both operated at a higher maximum SD during the winter months when DO was higher and where maximum SD of around 40 kg m^{-3} would be used compared with the summer maximum of around 30 kg m^{-3}.

Farms 6 and 7 both produced trout mostly for restocking fisheries. The fry stages of production on Farms 6 and 7 were similar to the other sites, where fry were grown in compact concrete raceways and Farm 7 operated up to a maximum SD of around 40 kg m^{-3}. However, the maximum SD applied during the on-growing stages (>100g) was less than 10 kg m^{-3} on both sites, with fish grown in large earth ponds. The main driver limiting SD on Farms 6 and 7 was the farmers desire to prevent fin and body damage.

7.3.3. On-farm welfare assessment

Over the course of 39 separate visits a total of 914 fish were sampled from the 7 different farms. Following analysis, the data was entered into a spreadsheet for Principal components analysis (PCA).

7.3.3.1. Principal Components Analysis of farm data

Haematocrit data was arcsine transformed and case-wise deletion was used, removing any individual fish with missing data for any of the parameters leaving a total of 804 fish being included in the final analysis.

Fin Principal Component (Fin-PC)

The first step of the analysis involved creating the PC for the RFL scores for all of the fins from measured from each fish. This resulted in a single PC (Fin-PC), which accounted for over 50% of the total variance observed within the fin data (Figure 7.5). In addition to accounting for more than half of all the variability within the fin data, the factor co-ordinates for the fin measurements that made up the Fin-PC indicated that there was a unidirectional contribution from each fin i.e. a low RFL value for each fins contributed towards a high factor score for Fin-PC (Table 7.3).

Table 7.3. Contribution and co-ordinates of the variables included in Fin-PC for arcsine transformed relative fin index data.

Variable	Fin-PC1	
	Contribution	Factor co-ordinates
Dorsal	0.065	-0.479
Caudal	0.082	-0.538
Anal	0.151	-0.728
Left Pelvic	0.182	-0.800
Right Pelvic	0.190	-0.817
Left Pectoral	0.187	-0.809
Right Pectoral	0.142	-0.707

To make the Fin-PC a more intuitive reflection of welfare status, the individual factor scores were multiplied by –1, so that a high Fin-PC factor score indicated a fish with good fins (high RFL values). It was also necessary to ensure that all values were greater than zero for subsequent statistical modeling, and therefore 4 was added to each Fin-PC factor score. The converted Fin-PC factor scores [*-1(+4)] for each case were used as an independent variable in a GLM that included farm as a categorical predictor with the addition of environmental and water quality parameters as continuous predictors.

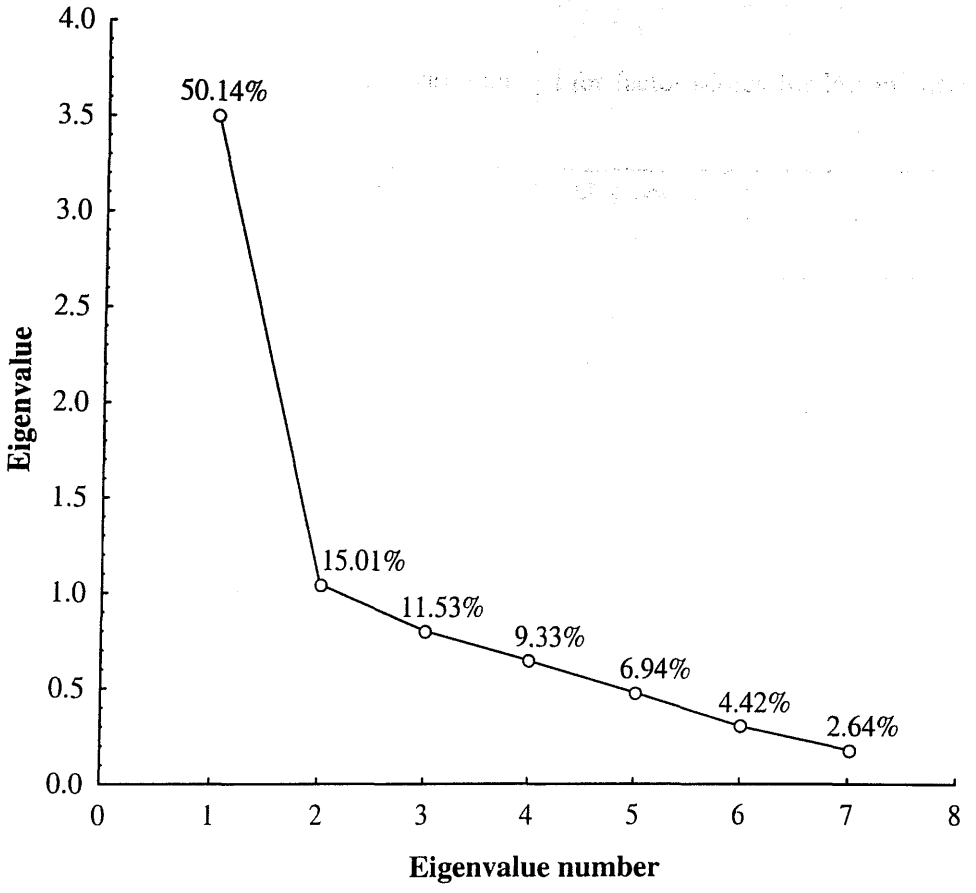


Figure 7.6. Scree plot of Eigenvalues for principal components derived from arcsine transformed relative fin index values for rainbow trout collected from 7 UK farms; percentages indicate the amount of variability taken into account by each PC (Eigenvalue number).

The whole model results are shown in Table 7.4 and the high R^2 (0.72) indicated that the model was taking account of a large proportion of the variability observed in the factor scores for Fin-PC.

Table 7.4. Whole model effects based on test for factor scores for Fin-PC derived from relative fin index.

Independent Variable	Adjusted R^2	SS	Degrees of Freedom	F	*P*
Fin-PC	0.72	579	10	206	0.000

Univariate analysis indicated that the predictors that were having a significant effect on Fin-PC were SD, temperature, pH, NH_3 and farm (Table 7.5).

Table 7.5. Univariate tests of significance for variables included in the GLM using factor scores for Fin-PC [*-1(+4)] as an independent variable.

Independent Variable		SS	Degrees of freedom	MS	F	*P*
Fin-PC	Intercept	31.62	1	31.623	112.652	0.000
	Density	5.29	1	5.290	18.844	0.000
	Loading rate	0.04	1	0.039	0.138	0.710
	Temp	5.61	1	5.608	19.977	0.000
	pH	10.43	1	10.428	37.147	0.000
	DO	0.11	1	0.113	0.401	0.527
	Ammonia	0.26	1	0.258	0.920	0.338
	NH_3	1.18	1	1.176	4.188	0.041
	Farm	144.92	6	24.154	86.045	0.000
	Error	220.92	787	0.281		

The residuals for the GLM were not normally distributed (Kolmogorov and Smirnov d = 0.056; P= 0.0136), but when the same model was analysed using a GLZ model with a log-link function, the same parameters were found to be significant (Table 7.6).

Table 7.6. Univariate tests of significance for variables included in the GLZ using factor scores for Fin-PC [*-1(+4)] as an independent variable.

Independent Variable		Degrees of freedom	Wald statistic	P
Fin-PC	Intercept	1	139.0	0.000
	Density	1	19.1	0.000
	Loading rate	1	0.2	0.670
	Temp	1	23.6	0.000
	pH	1	32.6	0.000
	DO	1	1.2	0.280
	Ammonia	1	0.6	0.427
	NH3	1	4.0	0.046
	farm	6	486.6	0.000

Combined Principal Component (C-PC)

The next stage in the analysis was to include the converted factor scores for the Fin-PC along with the values for the other welfare indictors to create a combined principal component (C-PC). The PCA resulted in the selection of two C-PCs, which together accounted for around 45% of the variability observed within the welfare indicators (Figure 7.7). The relative contribution and factor-co-ordinates of variables included in the C-PCs are shown in table 7.7.

Table. 7.7. Contribution and factor coordinates of the variables included in the PCA analysis for the creation of a combined PC welfare index.

Variable	C-PC1		C-PC2	
	Contribution	Co-ordinates	Contribution	Co-ordinates
Condition factor	0.170	0.520	0.048	-0.236
Cortisol	0.019	0.173	0.023	0.164
Glucose	0.276	-0.661	0.052	-0.245
Lysozyme activity	0.001	-0.040	0.662	-0.875
Haematocrit	0.228	-0.602	0.075	-0.295
Fin PC [(*-1)+4]	0.306	0.697	0.140	-0.402

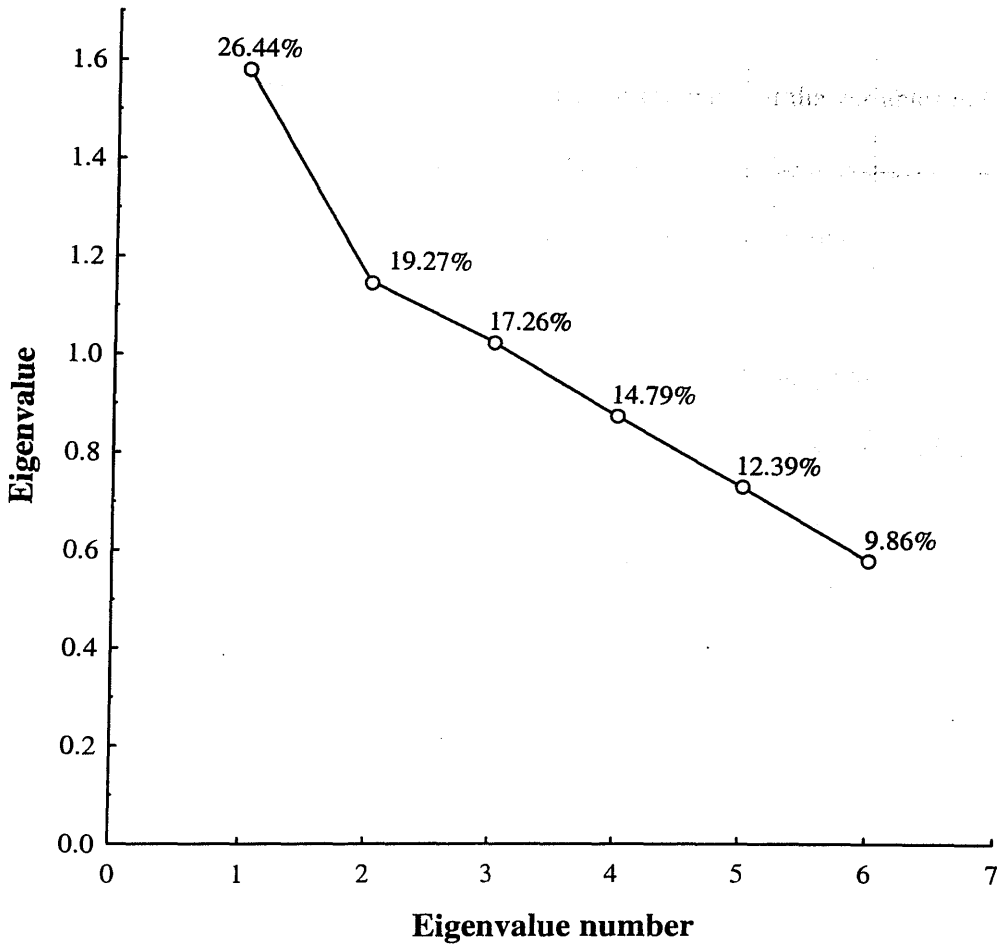


Figure 7.7. Scree plot of Eigenvalues for principal components derived from welfare indicators measured from rainbow trout collected from 7 UK farms; percentages indicate the amount of variability taken into account by each PC (Eigenvalue number).

The co-ordinates of the variables included in C-PCs 1 and 2 fitted intuitively with what would be expected for a fish with good or bad welfare status. A high score for C-PC1 would be indicative of good welfare *e.g.* a fish with high condition factor, good fins (high RFL), low glucose and low haematocrit. The co-ordinates of the variables in C-PC2 were also very coherent, with the main contributions coming from lysozyme activity, condition factor and Fin-PC, although the negative co-ordinates for these variables meant that a low score for C-PC2 indicated good welfare.

The factor co-ordinates of glucose and haematocrit were negative in C-PC2, but this counter intuitive result had little bearing on the factor scores for C-PC2 as the contributions of these variables was very low (0.052 and 0.075 respectively; Table 7.6). The strong contribution of lysozyme activity in C-PC2 when it had almost no contribution in C-PC1 was also encouraging, suggesting that C-PCs 1 and 2 reflect different aspects of fish welfare. Cortisol contributed very little to either of the C-PCs (0.019 and 0.023 in C-PCs 1 and 2 respectively).

The relationship between C-PCs 1 and 2 is illustrated in Figure 7.8; the arrows indicate the change in welfare status at different positions on the factor plane *e.g.* a fish with a low score for C-PC1 and a high score for C-PC2 was assumed to have poor welfare status. The position of each of the variables contributing to the C-PCs on the factor plane, illustrates the bearing of their contribution.

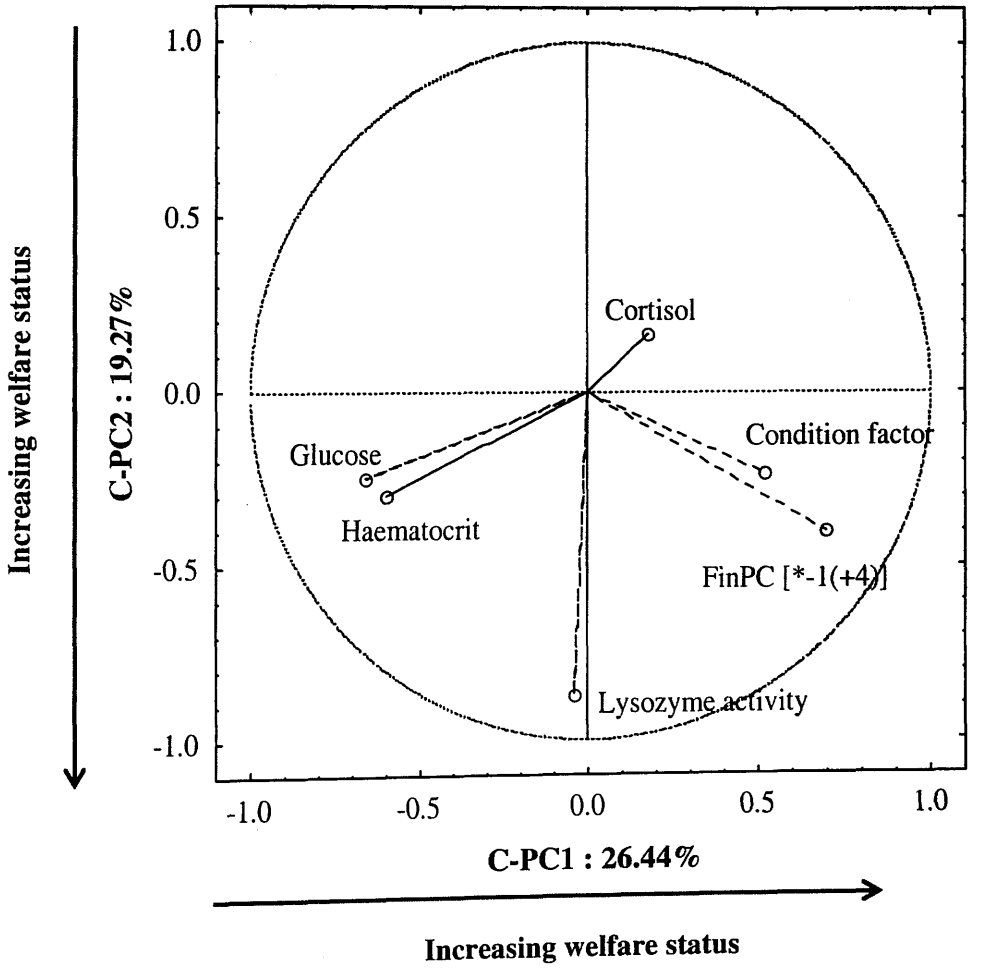


Figure 7.8. Projection of the variables for C-PCs 1 and 2 on the factor-plane.

The factor scores for C-PCs 1 and 2 were included in a GLM with the same independent variables that were used for the Fin-PC. The R^2 values for the whole model effects, suggested that the GLMs accounted for around 65% of observed variability for C-PC1 and 28% from C-PC2 (Table 7.8). The residuals for both models were within accepted limits for normality tests (Kolmogorov and Smirnov '*d*' statistic was 0.024 and 0.037 for C-PC1 and 2 respectively; $P>0.20$ in both cases).

Table 7.8. Summary of whole model effects for GLMs using C-PCs 1 and 2 as independent variables.

Independent Variable	Adjusted R^2	SS	Degrees of Freedom	F	P
C-PC1	0.646	521.5	13	113.4	0
C-PC2	0.278	232.5	13	24.8	0

The main influence on both of the models was the effect of farm. However, there were differences in effects of other variables, with DO, TAN and UIA all having significant effects on C-PC1, while the only variables that had a significant effect on C-PC2 were SD and pH (Table 7.9).

The factor scores for the fin and combined PCs were plotted against the different variables included in the models to illustrate the magnitude and bearing of any effects that the variables had on the various components of fish welfare. For improved clarity, CPC-2 was multiplied by -1 (as with Fin-PC), so that for all of the PCs, a high score represented good welfare (Figures 7.7 – 7.15).

Table 7.9. Univariate tests of significance for effects of variables included in GLMs using C-PCs 1 and 2 as independent variables.

Independent Variable		SS	Degrees of freedom	MS	F	*P*
C-PC1	Intercept	7.8	1	7.8	22.2	0.000
	Density	0.1	1	0.1	0.1	0.703
	Loading rate	0.3	1	0.3	0.9	0.342
	Temperature	0.0	1	0.0	0.1	0.753
	pH	0.1	1	0.1	0.2	0.688
	DO	2.1	1	2.1	5.9	0.016
	Ammonia	1.7	1	1.7	4.8	0.029
	NH_3	3.2	1	3.2	9.0	0.003
	Farm	154.1	6	25.7	72.6	0.000
	Error	278.5	787	0.4		
C-PC2	Intercept	0.9	1	0.9	1.3	0.253
	Density	19.1	1	19.1	26.5	0.000
	Loading rate	0.3	1	0.3	0.5	0.487
	Temperature	0.0	1	0.0	0.0	0.904
	pH	9.5	1	9.5	13.2	0.000
	DO	0.4	1	0.4	0.5	0.463
	Ammonia	2.1	1	2.1	2.9	0.089
	NH_3	1.4	1	1.4	1.9	0.169
	Farm	51.4	6	8.6	11.9	0.000
	Error	567.5	787	0.7		

7.3.3.2. Summary of effects of variables on welfare PCs

Stocking Density

During the farm sampling fish were collected from systems operating at a wide range of SD (2.0 – 117.3 kg m^{-3}). The mean SD for the systems included in the study was 43.2 kg m^{-3}. There was a significant effect of SD on Fin-PC ($P<0.001$), with a trend for mean factor score to decrease with increasing SD (Figure 7.9). A high factor score ($\approx$1.25) indicated a high RFL (good fins) within the lowest density category (<10 kg m^{-3}), which was followed by a sharp drop to a mean factor score of around 0 in the 11-20 kg m^{-3} SD category. There was then a steady decrease in the mean Fin-PC factor score as SD increased, to reach it's lowest in the 100-110 kg m^{-3} SD category.

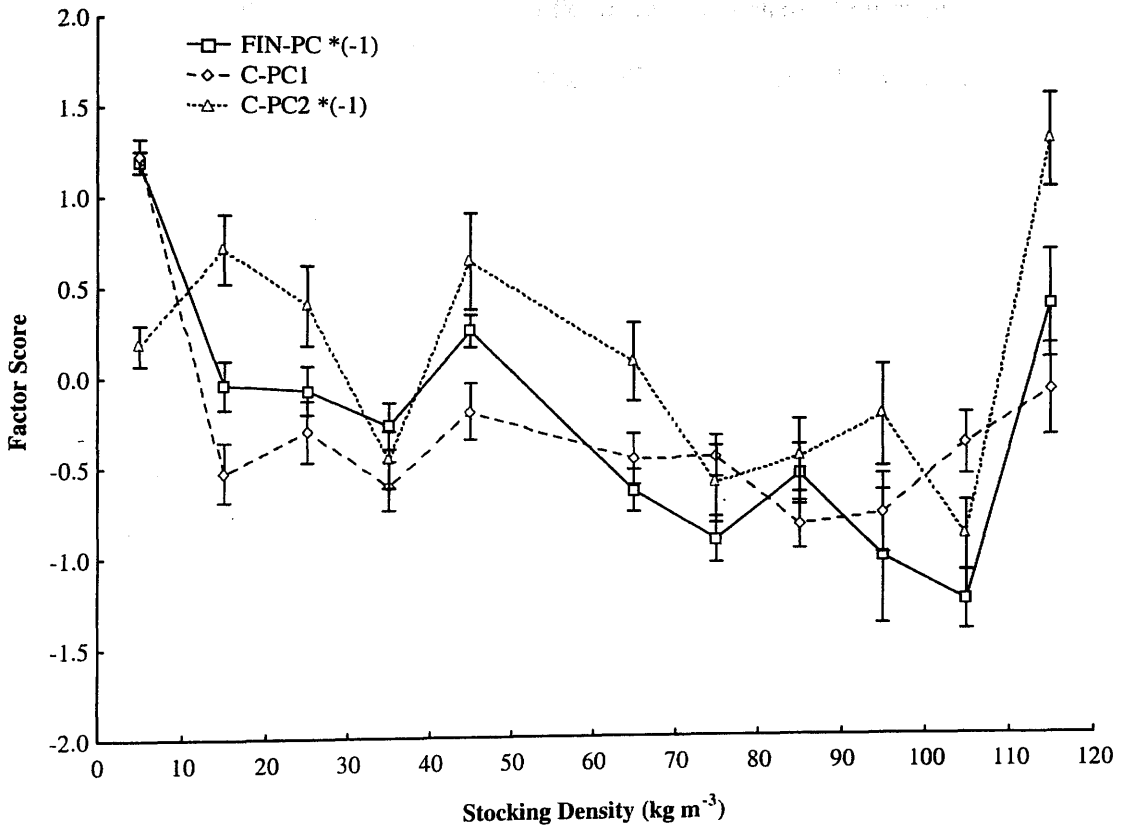


Figure 7.9. The effect of stocking density on principal components for welfare indicators measured from UK rainbow trout farms; box shows mean ± 95% confidence interval.

The sharp increase in Fin-PC from around -1.25 up to 0.4 (the second highest mean over all SD ranges) at a SD of 110-120 kg m^{-3}, which was due entirely to a single batch of fish sampled from Farm 3 that had particularly good fins and were the only fish that fell into the 110-120 kg m^{-3} SD category. There was no significant effect of SD on C-PC1 (P = 0.703), although similar to the Fin-PC there was a sharp drop in mean factor score moving from the <10 kg $m^{-3}$to the 10-20 kg m^{-3} SD categories. However, after this initial drop, the mean factor score for C-PC1 remained fairly steady at around -0.5 over the remaining SD categories. The effect of SD on C-PC2 was found to be significant and (P<0.001) and Figure 7.9 suggested that increasing SD generally had a negative overall effect, with negative mean factor scores observed over the SD range of 70 to 110 kg m^{-3}, although up until 60-70 kg m^{-3}, C-PC2 seemed relatively unaffected by SD. Similar to the Fin-PC, there was also a sharp increase in mean C-PC2 at the highest SD categories where mean factor score increased from its lowest value of around -0.75 at the 100-110 kg m^{-3} category up to 1.25 in the 110-120 kg m^{-3}, which was incidentally the highest mean factor score for C-PC2.

The stocking density categories were separated into 20 kg m^{-3} divisions and a scatter plot of CPCs 1 and 2 is shown in Figure 7.10; ellipses indicate 70% confidence intervals of the data points for each density range (refer back to Figure 7.6 for explanation in the direction of change of welfare status).

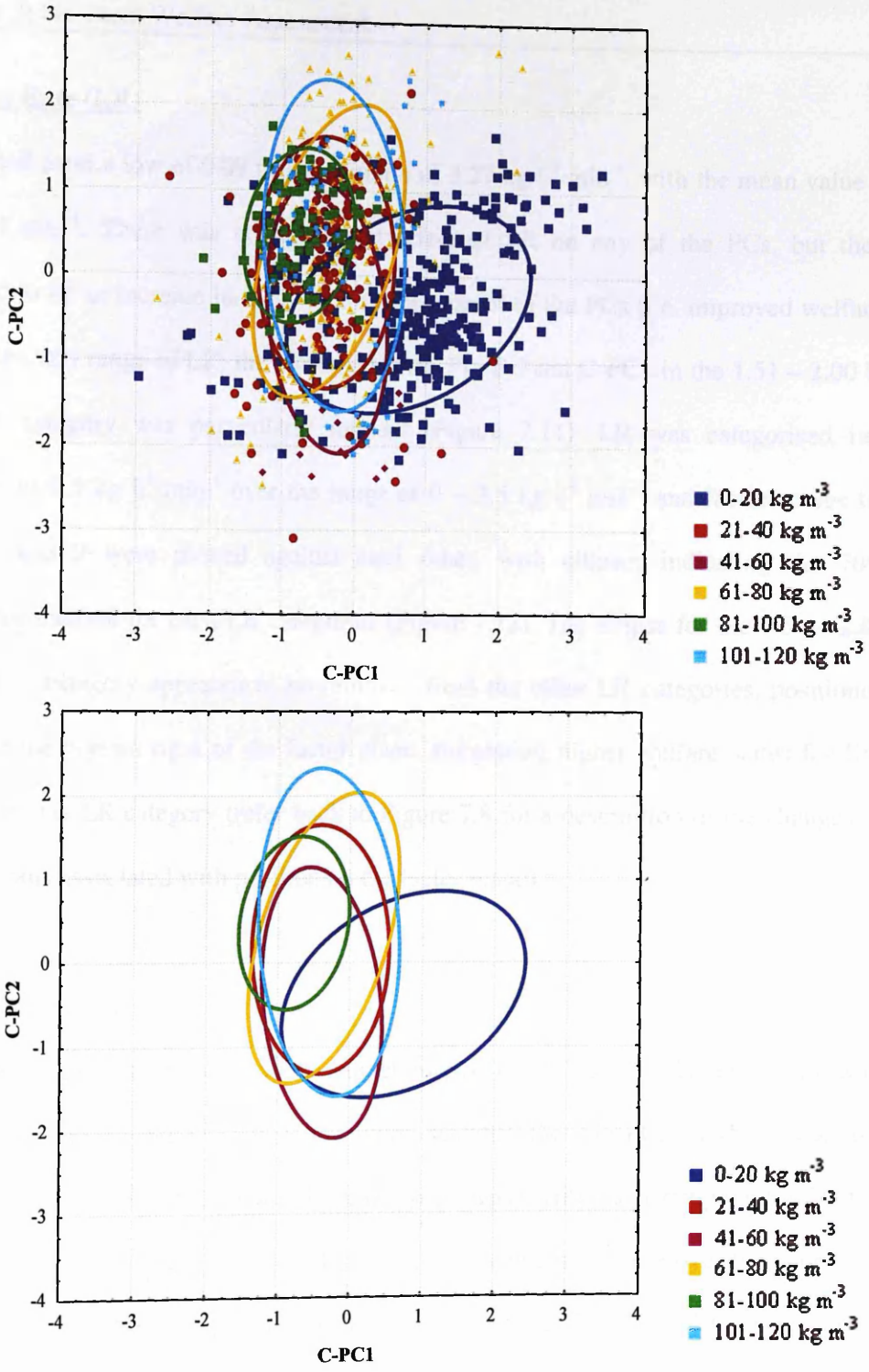


Figure 7.10. The effect of stocking density on the projection of factor scores for PCs created from welfare indicators measured on UK trout farms; ellipses indicate 70% confidence intervals, data points removed from lower plot for improved clarity.

Loading Rate (LR)

LR ranged from a low of 0.09 to a maximum of 3.27 kg l^{-1} min^{-1}, with the mean value of 1.4 kg l min^{-1}. There was no significant effect of LR on any of the PCs, but there appeared to be an increase in mean factor score for all of the PCs (i.e. improved welfare) around the mid range of LR; the increase for the Fin-PC and C-PC1 in the 1.51 – 2.00 kg l^{-1} min^{-1} category was particularly marked (Figure 7.11). LR was categorised into divisions of 0.5 kg l^{-1} min^{-1} over the range of 0 – 3.5 kg l^{-1} min^{-1} and factor scores for CPCs 1 and 2 were plotted against each other, with ellipses indicating the 70% confidence interval for each LR categories (Figure 7.12). The ellipse for the 1.51 – 2.00 kg l^{-1} min^{-1} category appeared to be removed from the other LR categories, positioned further to the bottom right of the factor plane, suggesting higher welfare status for fish sampled in this LR category (refer back to Figure 7.8 for a description of the changes in welfare status associated with position on the factor plane).

Water temperature

There was a significant effect of water temperature on Fin-PC ($P<0.001$), although it was difficult to interpret how water temperature affected the Fin-PC, as there was no consistent trend for Fin-PC to either increase or decrease with water temperature. Water temperature ranged between 3.5 and 18.6°C and the mean temperature over the course of the sampling was 12.0°C. The mean factor scores for both Fin-PC and C-PC1 peaked between 12 and 14°C. The observed mean values for Fin-PC and C-PC1 were very similar over the full range of water temperatures. There was no significant effect of water temperature on either C-PC1 ($P=0.703$) or C-PC2 ($P=0.904$).

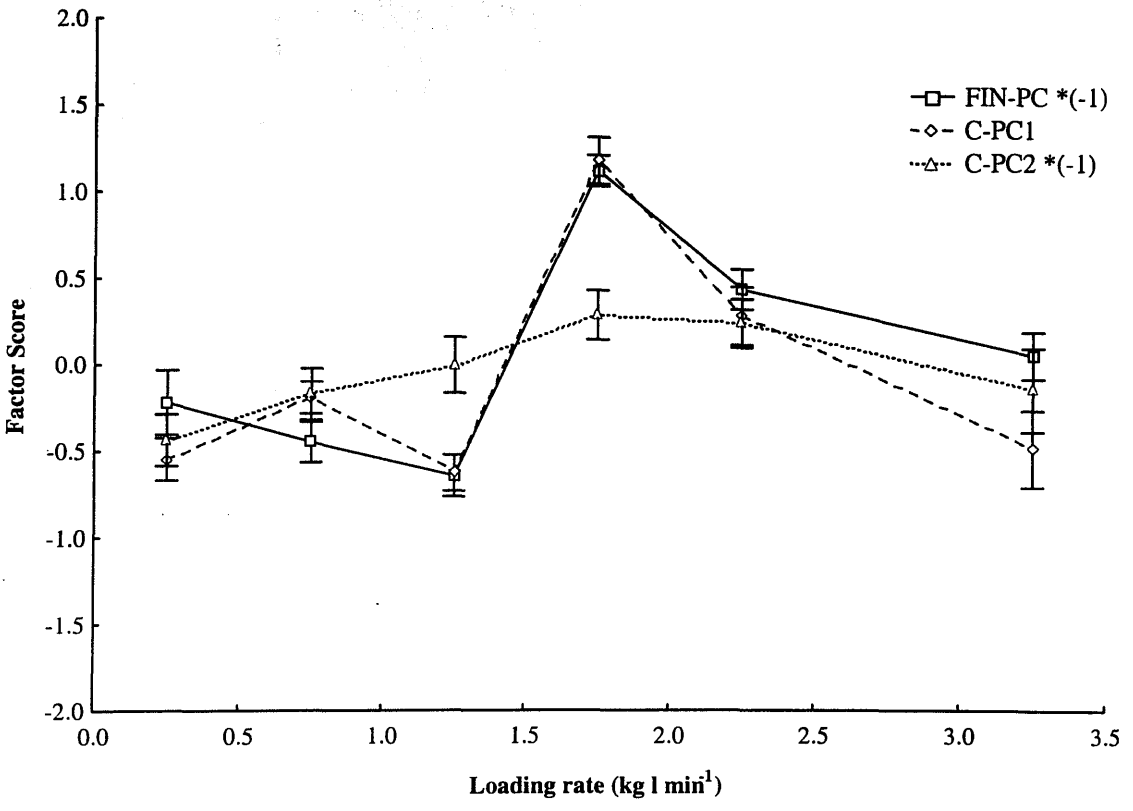


Figure 7.11. The effect of loading rate on principal components for welfare indicators measured from UK rainbow trout farms; box shows mean ± 95% confidence interval.

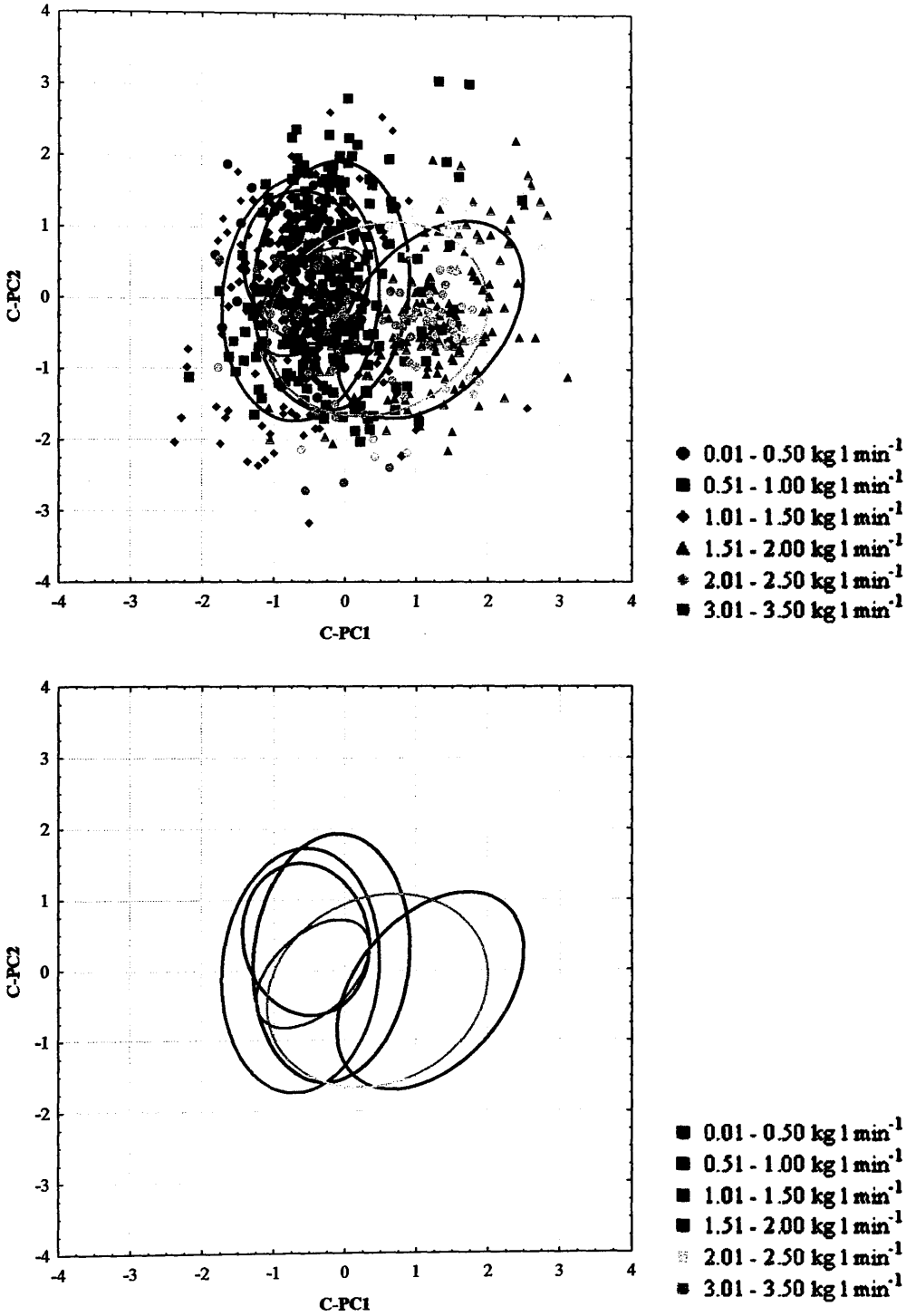


Figure 7.12. The effect of loading rate on the projection of factor scores for C-PCs derived from welfare indicators collected from UK trout farms; ellipses indicate 70% confidence intervals.

PH

The average pH measured from the outflow of the systems was 7.9, with the lowest pH observed on Farm 1 (pH 6.2) and the highest on farm 7 (pH 8.5). The water on the farms located in South East England (Farms 4, 5 & 7) was alkaline (pH 7.61-8.50), while water in the Northern and Scottish farms (Farms 1, 2 & 3) was neutral or slightly acidic (pH 6.20-7.47). There was a significant effect of pH ($P<0.001$) on Fin-PC1 and C-PC2, but there was no effect of pH on C-PC1 ($P=0.688$).

Dissolved oxygen (DO)

The mean DO measured from the outflow of the systems included in the sampling was 8.7 mg l^{-1}, and at all points the outflow DO remained above 6.5 mg l^{-1}. There was a significant effect of DO on C-PC1 ($P= 0.016$), with an increase in mean factor score with increasing DO. The mean factor scores for the different PCs were generally consistent over the range of DO.

Total ammonia nitrogen (TAN)

There was a significant effect of TAN on both of the combined PCs ($P=0.029$ for C-PC1 and $P=0.089$ for C-PC2), but there was no effect of ammonia on the Fin-PC ($P=0.427$). The highest concentration of TAN was 0.48 mg l^{-1}, measured from Farm 1 and the mean concentration was 0.22 mg l^{-1}; the concentration of TAN measured from the outflow was greater than the inflow concentration in all cases. There appeared to be an effect of decreasing factor score for C-PC1 with increasing ammonia concentration, suggesting poorer welfare at increased ammonia concentration.

Unionised ammonia (UIA)

There was a significant effect of UIA on C-PC1 (P=0.003) and the Fin-PC (P=0.041) with factor scores decreasing with increased UIA concentration. The levels of UIA were generally much higher on Farms 3 and 4 due to a combination of high SD and alkaline water. The maximum observed concentration of UIA was 0.038 mg l^{-1} (Farm 4), almost double the recommended safe level of 0.02 mg l^{-1} (Wedemeyer, 1996).

Farm

Farm had a significant effect on all of the PCs. Farm 7 stood out as having particularly high factor scores for CPC1 and Fin-PC, while Farm 3 had high factor scores for C-PC2 (Figure 7.13). Farms 4 and 5 had low factor scores for all of the PCs. The factor scores generally displayed a similar pattern of change between farms suggesting that there was a good degree of coherence between the PCs. A scatter plot of C-PCs 1 and 2 with ellipses indicating 70% confidence intervals of the data points for each farm is shown in Figure 7.14 (refer back to Figure 7.8 for explanation of the direction of change of welfare status). Although there was a large degree of overlap between the different sites, Farm 7 stood out clearly with particularly high scores for C-PC1.

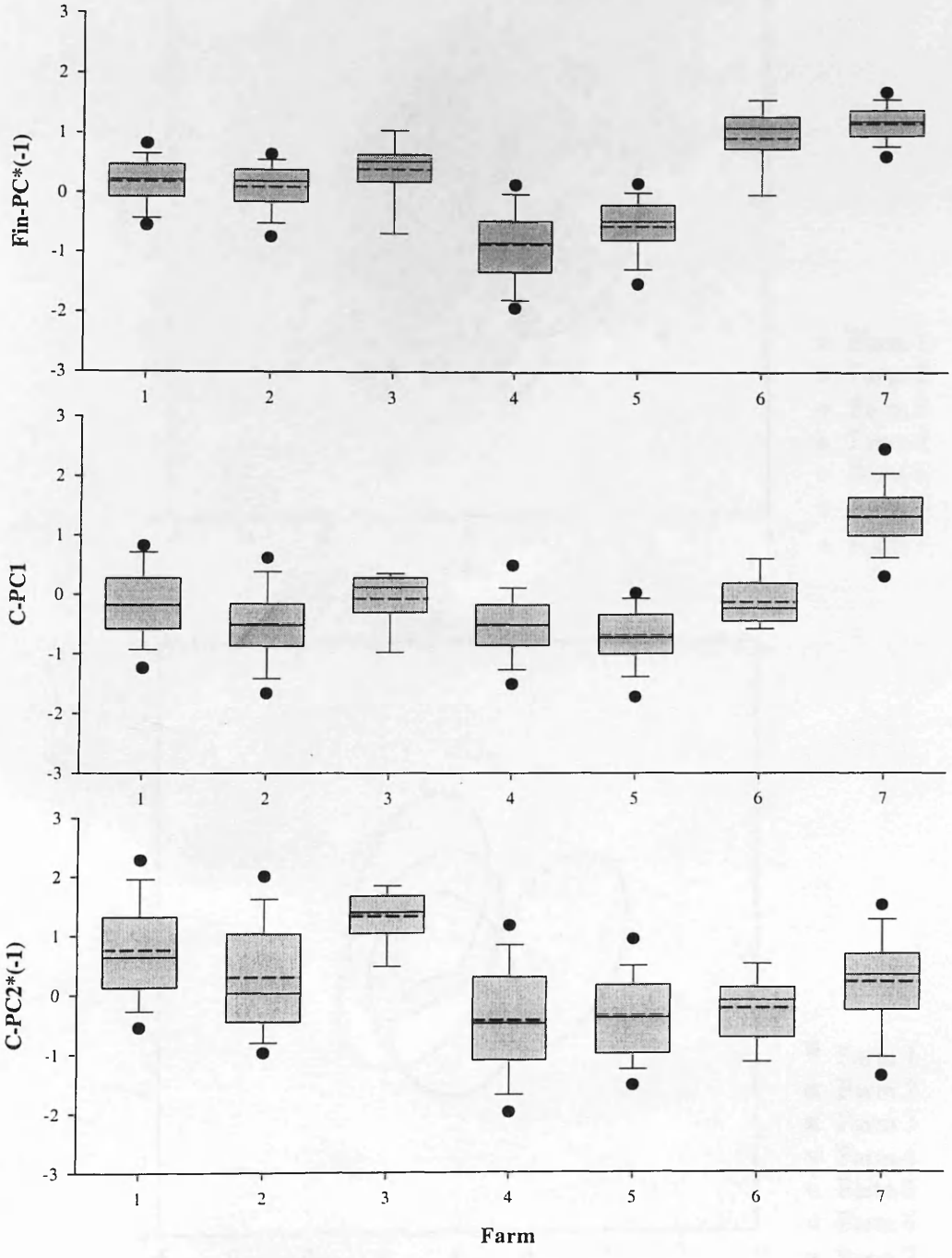


Figure 7.13. Effect of farm on principal components for welfare indicators measured from UK rainbow trout farms. Boxes represent 25th to 75th percentiles, with the mean and median factor scores for each farm represented respectively by dashed and solid lines; error bars denote 10th and 90th percentiles, with dots representing outliers (>95% confidence interval).

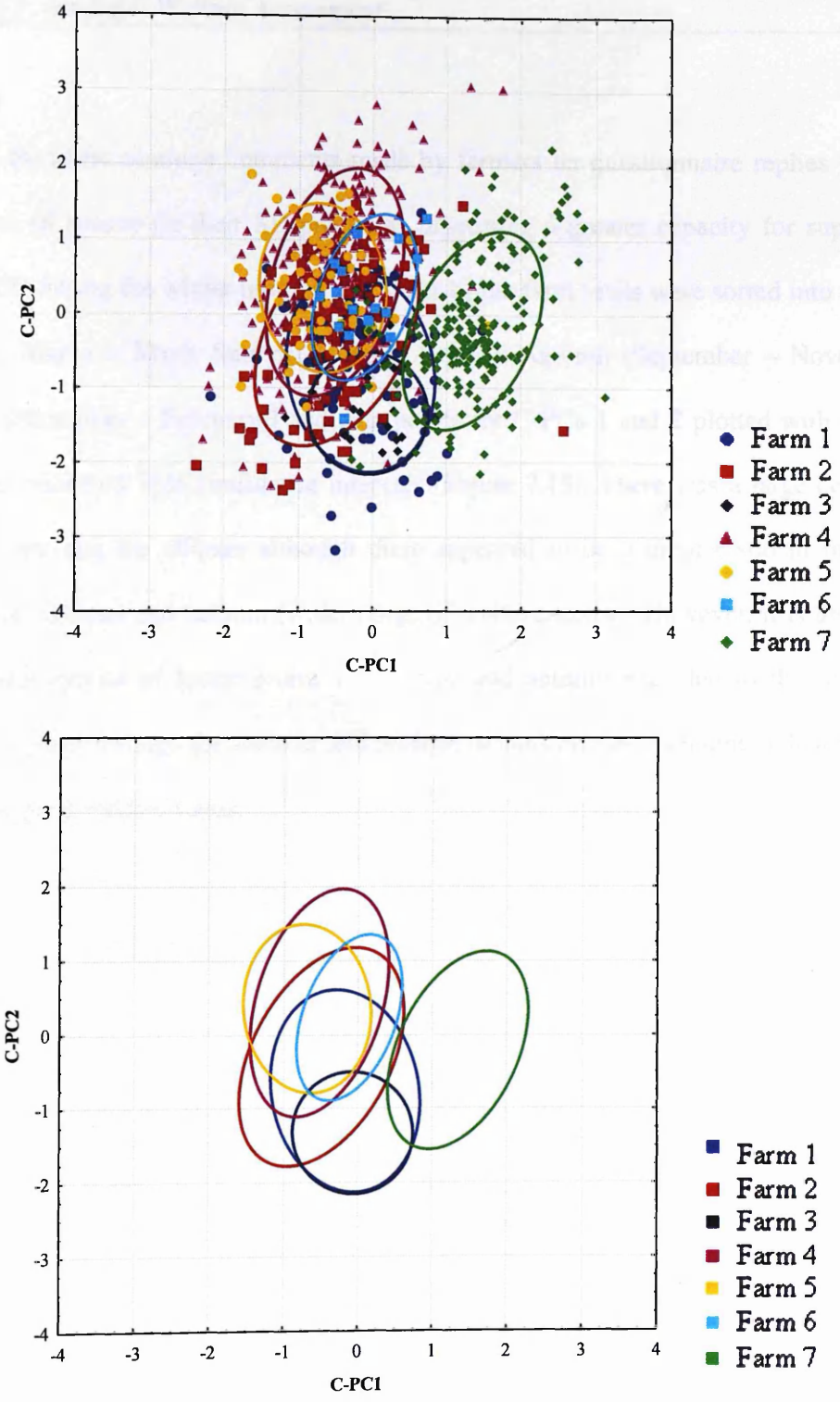


Figure 7.14. The effect of farm on the projection of factor scores for PCs created from welfare indicators measured on UK trout farms; ellipses indicate 70% confidence intervals, data points removed from lower plot for improved clarity.

Season

One of the most common comments made by farmers on questionnaire replies was the influence of season on their SD practices, suggesting a greater capacity for supporting higher SD during the winter months. The dates of the farm visits were sorted into seasons [Spring (March – May); Summer (June – August); Autumn (September – November); Winter (December – February)] and data points for C-PCs 1 and 2 plotted with ellipses added to indicated 70% confidence intervals (Figure 7.15). There was a large degree of overlap between the ellipses although there appeared to be a greater spread of factor scores for summer and autumn (wider range of welfare status). However, it is unclear if the greater spread of factor scores in summer and autumn was due to the increased sampling effort through the summer and autumn months or was a genuine indication of a wider range of welfare status.

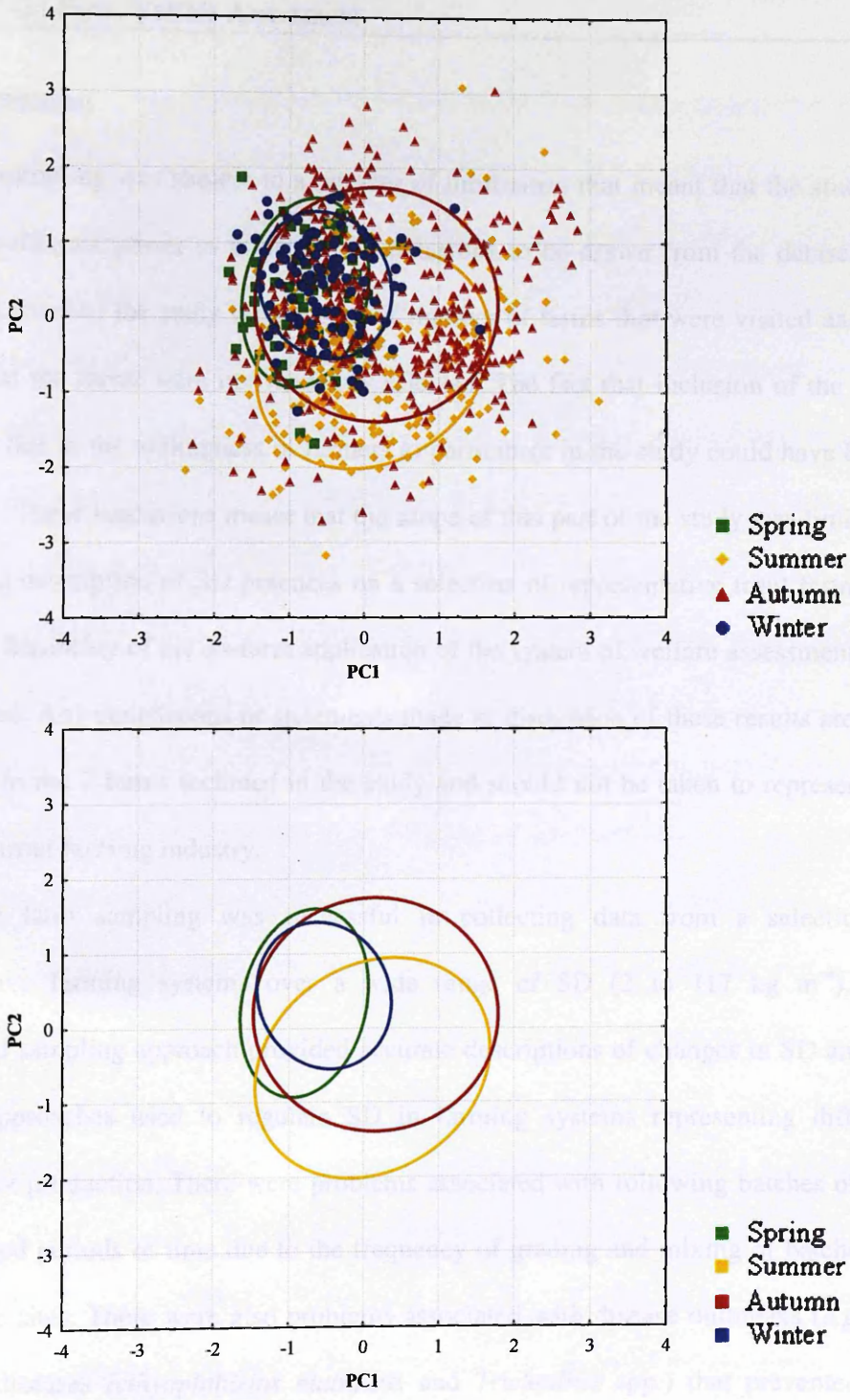


Figure 7.15. The effect of season on the projection of factor scores for PCs created from welfare indicators measured on UK trout farms; ellipses indicate 70% confidence intervals, data points removed from lower plot for improved clarity.

7.4. Discussion

The farm sampling was subject to a number of limitations that meant that the study did not have sufficient power to allow firm conclusions to be drawn from the dataset. The main limitations of the study were the small number of farms that were visited and also the fact that the farms were not randomly selected. The fact that inclusion of the farms was partly due to the willingness of farmers to participate in the study could have biased the dataset. These limitations meant that the scope of this part of the study was limited to providing a description of SD practices on a selection of representative trout farms and testing the feasibility of the on-farm application of the system of welfare assessment used in this thesis. Any conclusions or statements made in discussion of these results are only applicable to the 7 farms included in the study and should not be taken to represent the whole UK trout farming industry.

The farm sampling was successful in collecting data from a selection of representative farming systems over a wide range of SD (2 to 117 kg m^{-3}). The longitudinal sampling approach provided accurate descriptions of changes in SD and the different approaches used to regulate SD in farming systems representing different intensities of production. There were problems associated with following batches of fish for prolonged periods of time due to the frequency of grading and mixing of batches on some of the sites. There were also problems associated with disease outbreaks (*e.g.* the protozoan diseases *Icthyophthirius multifiliis* and *Trichodina* spp.) that prevented the continuation of sampling of some batches of fish.

The PCA appeared to be an effective method for determining welfare status based on correlations within the different welfare indicators that were measured on farm. The

PCs that resulted from the analysis appeared to be both coherent and biologically meaningful indications of different components of welfare. It is generally accepted that animal welfare can not accurately assessed by measuring any single variable in isolation (FSBI 2002; Spoolder *et al.*, 2003) and requires a selection of variables to be measured that are indicative of the different aspects of welfare status. However, one of the draw backs of such an approach is that there is ultimately a degree of subjectivity associated with the weighting of the different components of welfare (Spoolder *et al.*, 2003). The use of PCA effectively removed any subjectivity associated with weighting of the different variables by extracting statistical significance from the data, resulting in the generation of objective welfare indices.

When the results of the farm sampling are used in conjunction with the results of the tank based studies there appeared to be a degree of agreement between the data sets. Similar to the tank studies there was an effect of increasing SD on fin damage (lower RFL) with increasing SD. The sharp drop in factor score for the Fin-PC moving from 0-10 kg m^{-3} up to the 10.1-20.0 kg m^{-3} density category (Figure 7.9) may suggest that very low SD may be required to preserve good fin quality. However, the relatively small sample size meant that only the data from the two restocking farms (Farm 6 & 7) fell into the <20 kg m^{-3} SD category. Furthermore, the pronounced effect that a single batch of fish with good fins that were being farmed at a high SD (100-120 kg m^{-3}) demonstrated how one anomalous result can have a large influence on observed trends within such a small data set (Figure 7.9). This anomalous result of a batch of fish at high SD with good fins did however suggest that it may be possible to preserve fin quality even at very high SD, as these fish were being farmed at 117 kg m^{-3}.

One of the points of contradiction in the SD questionnaire responses was the critical time at which SD had to be kept low to maintain good fins. Some farmers suggested that it was at the fry stage, while others suggested the on-growing stages. The policy of Farm 7, which had the highest factor scores for Fin-PC was to stock fry at SD of up to 40 kg m^{-3} but to operate a maximum SD of around 10 kg m^{-3} for larger fish.

There was a wide range in the degree of fin damage observed on the farms, from fins with no visible damage down to fins that were completely absent. Fish sampled from Farms 4 and 5 generally had poor fins, with the pectoral and pelvic fins appearing particularly vulnerable. It also appeared apparent that if a fin was severely damaged it was unable to regenerate properly; with heavy scar tissue often evident on twisted and deformed fins suggesting a history of damage.

Other factors that had a significant effect on the Fin-PC were temperature, pH and UIA. Factor scores for Fin-PC appeared to decrease with increasing pH and UIA concentration, but the relationship between temperature and Fin-PC was not clear. There are limited reports of fin measurements being recorded for rainbow trout from UK trout farms, and as a non-native fish species, there is no reference wild population of rainbow trout with which to make to make comparisons. The most relevant piece of literature comes from a North American study into the prevalence of fin erosion in hatchery and wild reared rainbow trout in the state of Utah (Bosakowski & Wagner, 1994b). This study found lower alkalinities, unnatural bottom substrates (steel or concrete), higher UIA concentration and higher SD to be significantly correlated with fin erosion. The results of the present study are in general agreement with those of Bosakowski and Wagner with significant effect observed for UIA and SD. The comparatively small sample size in the

present study meant that it was not possible to model factors such as substrate or system type. As alkalinity was not measured on all visits it was also excluded from the models.

Two PCs were selected from the combined PCA (C-PC1 & C-PC2). A fish with a high factor score or C-PC1 had high CF, good fins, low plasma glucose and low haematocrit, suggesting good welfare status. In contrast, C-PC2 was predominantly a reflection of lysozyme activity, with very low contributions from the other welfare indicators (Table 7.7).

The factors that had a significant effect on C-PC1 were DO, TAN, UIA and farm, suggesting that water quality deterioration had a greater effect on welfare than SD. The significant effect of DO on C-PC1 was interesting as DO was above 6.5 mg l^{-1} in all of the systems sampled (6.5 mg l^{-1} is a conservative estimate of minimum requirement for trout culture quoted in the BTA Code of Practice; Anon., 2002). A similar significant effect of DO was observed in the tank study in Chapter 5 when DO was maintained above 5 mg l^{-1} at all times. This might suggest that there may be beneficial effects of maintaining DO at higher concentrations than are currently recommended for rainbow trout.

The change in factor scores for Fin-PC and C-PC1 were generally very similar over the ranges of the different variables. The Fin-PC was variable with the strongest contribution towards C-PC1 (Table 7.7), so it was not surprising that factor scores for these PCs displayed a strong correlation (adjusted R^2 = 0.48). However, the GLMs showed that Fin-PC and C-PC1 were affected by different factors, suggesting that there was a greater degree of complexity in the models than was suggested from the responses to individual parameters.

The effects of the various parameters on C-PC2 were less clear and the comparatively low R^2 value for the best-fit model for C-PC2 ($R^2 = 0.278$) compared with C-PC1 and the Fin-PC suggested that the model accounted for less of the variability observed within the C-PC2. The main variable contributing to C-PC2 was lysozyme activity so this PC would have been providing a crude indication of the non-specific immune status of the fish at the time of sampling.

Farm was a major influence on all of the models and was likely to be a reflection of the small number of farms that were visited and the fact that the farms included in the study were deliberately selected to cover as wide a range of farming systems as possible. The factor scores of the PCs suggested that from the farms sampled, welfare was highest on Farm 7 (Figures 7.13 & 7.14).

The fact that cortisol had very little influence on either of the C-PCs was surprising considering how prominent it was in the tank based study in Chapter 4. Some of the factors influencing cortisol response were discussed in depth in Chapter 4, but it may be that the production environment of the farms included this study was less stressful than the experimental tank system. Alternatively, it may have been that the interrenal cortisol response of the fish was acclimated or exhausted.

Considering the significant effects of water quality parameters on fish welfare, any future application of fish welfare assessment might benefit from inclusion of some form of gill assessment. Various methods of gill assessment have been proposed in the literature such as measurement of the primary gill lamellae (Rosenthal *et al.*, 1984), histological examination (Soderberg *et al.*, 1984), and a light-microscopic morphometric examination of gill tissue (Hughes & Perry, 1976). Another factor that should be included

in any future method of welfare assessment is the assessment of feed intake. Reduced growth is commonly reported as a negative effect of increasing SD and water quality deterioration, but evidence of a mode of action is rarely provided (Ellis *et al.*, 2002).

There was generally good co-operation from farmers participating in this study although the degree of enthusiasm was lower in the two restocking farms. The restocking farms included in the study both saw low SD as a prerequisite for producing fish with good quality fins. There also appeared to be a difference in the way the farmers viewed the fish, with the restocking farmers viewing each fish as a potential 10 lb trout to stock into a lake, whilst the table farms were more inclined to see each fish a 450g food portion. There was likely to have been an element of financial value associated with the way in which the fish were viewed by farmers, with a portion sized fish being worth less than £1, while restocking fish will be worth much more than their relative value as a food product, with anglers prepared to pay in excess of £30 a day to attempt to catch and take home a maximum of one or two fish.

The accessibility and ability to provide data from the larger table farms was generally much better than smaller farms. Most of the staff of the larger farming companies with more than one site had been educated to degree or higher national diploma level in some form of fish husbandry course and were confident with the use of computers. The larger farms, especially those with liquid oxygen injection were very much reliant on computers and software such as D-Journal or Farm Navigator to keep track of batches of fish. Larger farms were generally able to provide detailed information regarding fish numbers, mean weights and SD with very little effort. Two of the farms

with liquid oxygen injection were also able to provide accurate logs of DO and water temperature dating back over previous months.

The highest SD were found on table farms using liquid oxygen injection and SD in excess of 100 kg m^{-3} were routinely used on two such sites included in this study (Farms 3 & 4). A third site using oxygen injection operated at a maximum SD around the industry norm and used the injection system to facilitate the sequential re-use of water through different systems, so that the fish in the final outlet channel of this site were receiving what was in effect 7^{th} use water. Although the oxygen injection systems allowed DO to be maintained above critical levels, such heavy re-use of the water would greatly increase the build up of nitrogenous wastes and CO_2.

The larger table farms are now almost totally reliant on the business of supermarkets chains but are facing increased pressure from these retailers to be seen to address welfare concerns. One of the main concerns for retailers is SD, but the results from this preliminary attempt to assess fish welfare in relation to stocking density suggest that although there was a significant effect of increased SD on fins erosion, high SD did not necessarily result in poor welfare. It is necessary to further improve our understanding the implications of fin erosion to fish welfare and also to identify risk factors other than SD associated with fin erosion *i.e.* how did one farm operating at SD in excess of 100 kg m^{-3} manage to maintain better fins than other farms operating at less than 40 kg m^{-3}? There is also the need to build upon the base of welfare indicators used in these studies with other indicators that provide a reflection of different aspects of fish welfare.

Chapter 8: General Discussion and Future Work

The aim of this study was to investigate the effects of stocking density on the welfare of farmed rainbow trout. The first step in the investigative process involved the distinction of the two main routes by which stocking density can potentially infringe fish welfare, namely, through behavioural interactions associated with fish numbers, and through deterioration of water quality bought about by the demand for oxygen and toxic metabolite production. Identifying these two root causes of welfare infringement was largely achieved by reference to the literature and the focus of the main body of experimental work (Chapters 4 and 5) was a direct result of this process.

In order to assess the effects of stocking density on trout welfare it was first necessary to establish a system of welfare assessment. This again relied heavily on parameters measured in previous studies, but the process was taken a step further by incorporating a wider range of indicators, relevant to different aspects of welfare infringement. Principal components analysis was used to identify coherence between the different welfare indicators and to condense numerous different measurements into fewer numbers, which represented a fish's welfare status.

The final stage of the process was to examine SD from a commercial perspective and to confirm the findings from controlled experiments in the farm environment. A questionnaire identified the main types of farming system and also established an understanding of the practices, perceptions and key issues relating to SD on UK trout farms. Finally, welfare was assessed on-farm from a selection of representative farming

systems. This final chapter will summarise the main findings of these diverse approaches to examine the contribution of SD to fish welfare.

8.1. Welfare assessment (Chapter 3)

The approach used to assess welfare in this thesis incorporated aspects of the stress response, immune function, nutritional status and body condition. The experiments described in Chapter 3 confirmed the widely reported acute cortisol stress response observed following a standardised handling stressor, where basal cortisol levels of around 5 ng ml^{-1} rose to peak levels between 30 min and 1 h post-stress (Barton *et al.*, 1980; Donaldson, 1981). Although the pattern of cortisol response was similar, there were large differences in the peak response from the two exposure experiments, but it was not possible to establish if these differences were due to a strain effect, or resulted from other factors such as differences in fish size, water temperature and season. It would have been preferable to have carried out the stress exposures at a similar water temperature on fish of the same size. In addition to measuring the cortisol response, lysozyme activity and haematocrit were also measured. The changes in lysozyme activity following the standardised handling stressor were not consistent, although in one of the first exposure trials lysozyme activity generally displayed a negative correlation with high levels of plasma cortisol, which was in general agreement with other published work (Möck & Peters, 1990; Fevloden *et al.*, 1999). The data collected in Chapter 3 was important in the validation and development of the cortisol radioimmunoassay and it also highlighted the profound effect of water temperature and other environmental factors on the welfare indicators.

8.2. The effects of stocking density on trout welfare (Chapter 4)

The experiment outlined in Chapter 4 maintained rainbow trout at 10, 40 and 80 kg m^{-3} for 10 months and found no effect of SD on growth. This result was unexpected as a literature review found a negative effect of increasing SD on growth in 31 of the 42 studies in which it was assessed (Ellis *et al.*, 2002). Possible reasons for the lack of effect of increased SD on growth in this study include the relatively high rates of water exchange that were used (2 tank volumes per hour) preventing metabolites reaching critical levels and the provision of aeration to maintain DO above 5 mg l^{-1}. The fact that the fish were fed a set ration instead of fed to appetite may also have contributed to the lack of density effect.

There was, however, a significant, cumulative effect of SD on increased fin erosion. It was not possible to elucidate the exact cause of the increased incidence of fin erosion, although increased aggressive behavioural interactions, abrasion against tank surfaces, accidental damage due to feeding/collisions, and water quality deterioration with increased SD could all have been contributing factors.

Perhaps the most unexpected result of this study was the significant elevation of cortisol observed in the 10 kg m^{-3} treatment. The potential confounding effect(s) of the tank position and sampling sequence failed to provide an explanation, suggesting that the high levels of cortisol were a genuine affect of the low SD rather than sampling error. There was limited evidence in the literature to support either negative or beneficial effects of stocking density on plasma cortisol in rainbow trout, although Vijayan and Leatherland (1988) found a similar inversely proportional relationship between cortisol

and SD in brook charr over a similar range of SD to those used in this study (30, 60 & 120 kg m^{-3}).

The size distribution within the SD treatments indicated that there was greater size variation in the 10 kg m^{-3} treatment, suggesting the possible presence of a dominance hierarchy. There is some evidence in the literature to support this theory, as increased levels of cortisol have been observed in subordinate rainbow trout (Laidley & Leatherland, 1988; Pottinger & Pickering, 1992). However, the association between lower SD and the increased prevalence of dominance hierarchies is tentative and could only have been confirmed through investigation of behavioural interactions. Despite rainbow trout being one of the most well studied fish species, there is a relatively poor understanding of its behaviour, especially under aquaculture conditions and this certainly warrants further research.

The elevated haematocrit and lower lysozyme activity observed in the 10 kg m^{-3} treatment fitted with the elevated levels of cortisol in this treatment, further suggesting an acute stress response. There was a very pronounced effect of water temperature on both haematocrit (increased at lower temperatures) and lysozyme activity (increased at higher temperatures), suggesting that if incorporating of these parameters in a welfare index, it is necessary to account for environmental variation. A temperature controlled experiment would have removed the seasonal fluctuation in some of the variables, but the applied nature of this study meant that controlling temperature would have removed the focus from commercial farm practices in the UK where temperature control is not common.

A final point to make in regard to the experiment described in Chapter 4 was the fact that this study highlighted the important issue of the potential for welfare infringement at low as well as high SD.

8.3. Effects of water quality deterioration on trout welfare (Chapter 5)

The experiment described in Chapter 5 tested the hypothesis generated in Chapter 4 and also sought to improve upon the experimental design through a combination of upgrading the experimental system and introducing the use of PIT-tags to enable individual fish to be followed throughout the course of the experiment.

There were distinct differences in water quality depending on the inflow rate, although measured ammonia and DO remained within published 'safe' limits at all times (Wedemeyer, 1996). This highlighted two further points of import, firstly that point samples for water quality parameters are of limited value in reflecting maximum and mean levels of a particular parameter, and secondly, that reduced growth may occur within the recommended 'safe' levels. DO was kept above 5 mg l^{-1} at all times during the study but still featured as a significant factor in the GLM. A 24 h water quality profile showed that TAN peaked around midnight, a previously unreported observation. The poor understanding regarding the production and release of the main nitrogenous waste product in a well studied and commercially valuable fish species certainly justifies further work.

The main finding of Chapter 5 was that growth and body condition were reduced in the 20 and 40 l min^{-1} compared with the 60 l min^{-1} flow treatments. The treatment related differences in growth and condition factor coincided with the periods of highest

water temperature. All of the inflow rate treatments were fed exactly the same amount of food through the course of the experiment, so it was not possible to determine if the reduced growth was due to reduced feed intake, or poorer conversion of feed into somatic growth. This inability to identify a causative mechanism is common to many studies reporting reduced growth. Any future study should attempt to ascertain the causative mechanism by measuring feed intake. Feed intake could be assessed by feeding to appetite, measuring uneaten feed, or estimated by measurement of gut contents or x-ray photography.

There was no observed effect of inflow rate on fin length suggesting that the increased erosion at higher SD observed in Chapter 4 was due to behavioural interactions or space limitation rather than water quality deterioration. There was no effect of flow rate on any of the other welfare indicators and cortisol levels remained very low for the duration of the experiment.

There were two mass mortality events in the 20 l min^{-1} treatment suggesting that systems running at high loading rates may run an increased risk of mass fish kills in the event of episodic system failures. Similar reports in the literature of mass mortality following system failures at high loading rates support this intuitively obvious risk (Kindschi *et al.*, 1991a). It is appropriate to echo the recommendations made by other researchers for the need for increased supervision and provision of appropriate back-up for systems operating at high SD or loading rates (Kindschi *et al.*, 1991a; Miller ***et al.***, 1995; Ellis *et al.*, 2002).

8.4. On-farm welfare assessment and commercial stocking practices (Chapters 6 & 7)

There were marked differences in both the SD practices and the perception of what passes for a high SD between farmers producing trout for restocking and farmers producing trout for the table. Farmers acknowledged the potential for high SD to infringe aspects of trout welfare and with 84% of respondents associating increased fin erosion with high. There were marked differences in SD practices between the different types of trout production, with farms producing fish for restocking/fisheries markets operating at much lower SD than those producing fish for the table. If concerns regarding welfare implications of high intensity fish farming continue (e.g. fin erosion, water quality deterioration), it may be necessary to focus only on farms producing trout for the table rather than re-stocking farms. However, the findings of Chapter 4 suggested that aspects of fish welfare can be infringed at low as well as high SD. This is another subject that justifies further research to improve understanding of the factors affecting the establishment of dominance hierarchies and the implications of such structures on fish welfare.

The additional comments added to questionnaires and discussions during farm visits provided valuable insights into the priorities of farmers. Several farmers suggested that their SD policy was determined by DO levels rather than a specific SD limit. The inability of many farmers to provide accurate data regarding flow rates was another important point highlighted by the questionnaire, suggesting that applying alternative measures other than kg m^{-3} is unlikely to be possible on commercial farms.

The preliminary application of on-farm welfare assessment was successful in confirming many of the observations that were highlighted in the tank-based studies described in Chapters 4 and 5. The ability to generalise from the findings of the commercial application of the welfare index was limited by the small number of farms included in the study, differences in the number of visits to the different farms, and also by the non-random (purposive) farm selection. However, similar to the tank studies, it was shown that there was a significant effect of SD on the fin length (Fin-PC). There was also a significant effect of temperature and UIA on the fins, which was in general agreement with a previously published survey of fin erosion in rainbow trout (Bosakowski & Wagner, 1994b). When the Fin-PC was combined with the other welfare indicators, the resulting PCs (C-PC1 and C-PC2) appeared to be biologically meaningful, with the different variables contributing in a logical and biologically intuitive way. In addition to the effect of farm, which featured as the most significant factor in all of the models, there was a significant effect of DO, TAN and UIA on C-PC1; a similar finding was observed in the tank-based studies in Chapters 4 and 5, which showed no significant effect of increased SD on the welfare indices derived from the PCA.

In addition to the preliminary application of a system for on-farm welfare assessment, the commercial sampling provided detailed examples of the regulation of SD in different types of farming system. This further highlighted the impracticalities of establishing a maximum SD limit as the SD changed continuously throughout the production cycle. The original scope of the farm sampling was very broad and the work was later extended to incorporate a cross-sectional study to investigate the prevalence of fin erosion and a more rigorous longitudinal study to identify the variance that existed

within the welfare indicators before and after feeding, on a daily, weekly and monthly basis, with the aim of establishing the optimum sample size for future farm-based work. The extended farm sampling work is not presented in this thesis, although it was part of the same study and will form part of the final report that will be presented to the main sponsor, Defra.

8.5. Future work

The tank based experiments measured the effects of different numbers of fish in tanks with the same inflow rate (Chapter 4) and of different inflow rates in tanks containing the same numbers of fish (Chapter 5). A further experiment that could answer some remaining questions would be to maintain different numbers of fish at the same SD (kg m^{-3}) and loading rate (kg l min^{-1}) by adjusting tank volumes together with inflow rates between treatments *e.g.* one treatment with 200 x 100 g fish in 1 m^3 of water with 30 l min^{-1} inflow and another treatment with 100 x 100 g fish in 0.5 m^{-3} with 15 l min^{-1} inflow. Such an experimental design would allow any effects of fish numbers to be separated from those of spatial allocation and water quality deterioration; this would be particularly useful in addressing the causes of fin erosion and the formation of dominance hierarchies.

Fin erosion was the most reliable indicator of high SD in the tank based studies and this was further confirmed during the sampling of commercial farms. However, the exact cause of the fin erosion remains unclear and further work is necessary to establish how and why fin erosion occurs. There was also the suggestion from samples collected from a group of fish being maintained at 117 kg m^{-3} on Farm 3, that it is possible to

produce fish with relatively intact fins even when farming at high SD. An epidemiological survey to investigate the prevalence of fin erosion on UK trout farms could identify risk factors associated with fin erosion, or critical stage in the production cycle at which SD should to be limited to maintain fin quality. A research proposal adopting such an approach was recently approved by Defra and work is due to commence in the coming months.

It could be argued that before identifying the causes of fin erosion it would be better to first invest research effort to establish the implications of fin erosion in terms of fish welfare. The results of the experiment described in Chapter 4 suggested that the increased fin erosion observed in the 40 and 80 kg m^{-3} treatments did not affect growth, but good welfare entails more than good growth and further work is warranted to investigate the implications of fin erosion on fish welfare.

The work conducted in this thesis used larger fish (>100g) and further work focusing on the fry stages would be also beneficial. Such work would be particularly relevant in establishing key stages at which fin erosion might occur and there were certainly some contradictory comments attached to the questionnaire replies regarding the size at which fish are most vulnerable to fin erosion, with some farmers suggesting small fish were more vulnerable and another suggesting larger fish. There is some evidence to suggest that larger fish have a greater potential to tolerate poorer water quality (Piper *et al.*, 1980; Wedemeyer, 1996) and the questionnaire found that highest SD occurred during the latter stages of production. On all of the farms that were visited during this study, the fry rearing units received first-use water (cleanest), whereas larger fish often received water that had passed through other systems. This is likely to be carried out with

the objective of minimising the chance of cross-infection from older fish with the fry, but it could also suggest that smaller fish are perceived to be more sensitive to water quality deterioration.

The confounding influence of water quality deterioration at higher SD became apparent early on in this project. It also became apparent that there are contradictions in the acceptable limits of key water quality parameters for salmonid culture, especially when considered from the viewpoint of fish welfare rather than optimised production. There remains a need to establish 'safe' limits for key parameters (DO, TAN, UIA & CO_2) and also to identify sensitive fish-based indicators of water quality deterioration. These concerns were highlighted in the literature review conducted as part of this study (Ellis *et al.*, 2002) and Defra is presently funding a further research project to investigate the links between water quality and fish welfare.

Lysozyme activity appeared to be a useful indicator of fish welfare, especially when used in conjunction with other indicators, but there is no standardised protocol making comparison of results between studies very difficult. The development and publication of a reproducible and universally recognised protocol for determining lysozyme activity would therefore be valuable.

The system of welfare assessment applied in this study combined indicators representing different aspects of fish welfare through multivariate analysis to result in the generation of welfare indices. With the exception of lysozyme activity, the assay procedures used in this study were well established and have been applied in numerous other studies, but the PCA took the process further by extending analysis beyond classical post-hoc analysis of each individual variable. The welfare indices generated by the PCA

ultimately reflected the coherence that existed in the particular datasets analysed. The PCA analysis in Chapters 4, 5 and 7 produced different PCs, but the contribution of the variables in each of the PCs appeared to be biologically feasible, supporting the validity of this method of analysis. Different situations will inevitably affect different aspects of fish welfare, so the PCA would not be expected to always identify the same PCs. The PCA approach also removes problems associated with the subjective weighting of the variables in terms of fish welfare.

The development of the welfare indices is perhaps the most important requirement for further research and it is recommended that any future indices incorporate measurements of feed intake and some form of gill assessment. There is also a need for a simplified system of welfare assessment that is not reliant on laboratory equipment; such a system would be hugely beneficial for farmers and legislators alike.

There is a very poor understanding of fish behaviour, and more so in the farming environment. The study of welfare in terrestrial animals appears to be increasingly focused on behavioural measures and there is certainly an argument that although behaviour is more difficult to quantify, it can sometimes produce more meaningful results than objective indicators (Dawkins, 2004). This study opted for the application of objective indices rather than behavioural measurements, but many of the unanswered questions such as the causes and implications of fin erosion and dominance hierarchies on fish welfare could perhaps be better explained by behavioural studies. However, observation of behaviour in commercially farmed populations of fish can be problematic (James Turnbull *perrs com.*)

Although not discussed in much detail in this study, future work that would be of practical benefit to fish farmers would include the identification of risk factors, or critical points for each farming system at which welfare might be threatened. One of the arguments for not enforcing a maximum SD is the fact that each farming system is unique and that farmers have therefore developed systems to best meet the particular characteristics of each site. Parallels can be made with other industries, particularly the food factory environment where systems such as Hazard Analysis and Critical Control Points (HACCP) and Hazard Analysis and Risk Assessments (HARA) have been instrumental in establishing universally recognised industry standards across the range of factory environments *e.g.* British Retail Consortium; ISO9000. The farm visits highlighted the following points that should be considered if a HACCP or HARA approach is applied to fish farms:

Feeding

Work is required to investigate the welfare implications of feeding practices such as methods of feed delivery (hand, demand, automated) and the frequency of feeding. There is an argument that fish could benefit from demand feeders allowing fish to feed when they want, but there are also behavioural implications of demand feeders, whereby dominant fish may monopolise the food source.

Periods of Starvation

During the production cycle it is relatively common for feeding to be restricted, or for fish to be starved for prolonged periods of time. Sometimes this occurs with fish welfare

in mind *e.g.* it is considered to be less stressful for fish to be starved before being graded. It is also common practice to starve fish if water temperatures reach 16°C and farmers will certainly restrict feeding if DO becomes limiting. However, it is also common for farmers to 'hold back' batches of fish to meet production goals, especially now that there are very strict demands made by processors for fish to be of a certain shape and size. During the farm visits it was observed that if fish are to be smoked, there can be a period of up to two weeks prior to slaughter during which fish are starved to allow the flesh to firm, which results in a better final product. Fish differ from mammals in that they are not reliant on a constant feed intake to maintain body temperature and will naturally go for long periods in the wild without feeding, but establishing the welfare implications of restricted feeding is again an area that justifies further work.

Market Demand

It was apparent from the farm visits and from attendance of several annual conferences held by the British trout farming community (Sparsholt College, Whinchetser, 2000-2003) that supermarket chains account for an ever increasing proportion of fish sales. Supermarkets demand continuity of supply, increased flexibility to meet market demands and also conduct regular audits of farms which increasingly address welfare related aspects of production. Although there are obvious potential benefits for fish welfare through the auditing system, the fact that supermarkets demand such flexibility from farmers can generate welfare problems of its own. The issue of 'pushing' and 'holding back' batches of fish has already been touched upon, but there is also the problem of fish reaching market size with no buyer. This may be due to an oversight by the farmer, or

due to cancellation of an order by a supermarket, but the result is that a batch of fish at market size may have to spend a prolonged period of time on a restricted ration until a buyer is found. If the fish are being held at a high SD and/or the delay occurs during the summer months, this can result in high levels of mortality; this was a point that also arose in the FAWC report (Anon. 1996a). This may also cause a production bottleneck that passes down through the farm, especially if there are batches of smaller fish that need to be moved into the larger systems that are occupied by the harvest sized fish. Although this is ultimately an issue that should be addressed by industry rather than academia, it certainly appears to be an increasingly common problem and with the continued growth of supermarkets it is likely to worsen in the future.

Weather

During the course of this study there was a period of heavy flooding (Spring 2000) and also the hottest year on record (2003), both of which posed serious problems to farmers. There are particular welfare problems associated with the summer that have been discussed in some detail in this thesis. Although the weather will always remain an unpredictable and uncontrollable entity, there could certainly be benefits of planning for the worst-, rather than the best-case scenario as highly loaded systems have less capacity to cope. This is particularly true for predicting water availability since decisions regarding the number of fish that will be present on the farm during the summer months are made five or six months in advance when eggs/fry are purchased.

8.6. Placing this work in a fish welfare legislation context

The FAWC report (Anon., 1996a) has been the subject of much discussion and has received a considerable amount of criticism. Some of the recommendations such as the need for a universally applied maximum SD reflected a lack of understanding of fish biology and farming practice. However, the FAWC report has been instrumental in pushing forward the development of fish welfare research and the implications of the report will ultimately be of benefit to the aquaculture industry.

8.7. Summary

- It is apparent that a universally applied SD limit is not the answer to safeguarding fish welfare.
- Increased SD results in increased fin erosion and more work is required to identify the causes and welfare implications of fin erosion.
- Systems applying high SD or loading rates face an increased risk of mass mortality in the event of system failure, necessitating the need for increased supervision and appropriate back-up systems.
- Defining limits of key water quality parameters may prove to be a more effective measure than limiting SD, although further work is required to establish these thresholds of these parameters to safeguard fish welfare.
- There may also be welfare implications at low as well as high SD.

This thesis has addressed just one of the many welfare issues highlighted in the FAWC report and in doing so has perhaps generated more questions than answers. The Council of Europe is presently drafting its Resolution regarding fish welfare, which will ultimately become law in member states of the European Union. Work is also underway by Defra to produce a 'Code of Recommendation for the Welfare of Livestock' for Fish, similar to codes that are already in place for terrestrial livestock. There is also a growing emphasis on fish welfare driven by industry codes of practice (Anon., 2002; QTUK, 2004) and farm audits carried out by supermarkets. The profile of fish welfare as an area of research is likely to continue to rise in coming years and the scope of the subjects addressed in this thesis stretches far beyond the remit of stocking density alone.

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Appendix 1. Summary of studies examining the effects of stocking density on indicators of trout welfare; authors' conclusions concerning the effect of density indicated by ' +, -, 0' signifying beneficial, adverse, and no/inconclusive effect. After Ellis *et al.* (2001).

	Production and nutritional status														Health and condition profile											Stress indicators					
Study	Mortality	Food intake	Food conversion efficiency	Body condition index	Hepatosomatic index	Hepatic glycogen	Hepatic lipid	Plasma protein	Plasma Lipid	Carcass protein	Carcass lipid	Carcass water	Carcass ash	Growth	Size variation	Haematocrit/erythrocyte count	Leucocrit	Total lymphocyte count	Specific lymphocyte count	Fin condition	Gill condition	Spleen condition	Thymus index	Interrenal cell nuclear diameter	Plasma cortisol	Responsiveness of HPI axis	Plasma glucose	Plasma chloride	Oxygen consumption	Gastric mucosa
Alanärä & Brännäs, 1996		-												-																
Atay *et al.*, 1986	0		-	-										-																
Bagley *et al.*, 1994	+													0	0															
Baker & Ayles, 1990														-																
Bebak *et al.*, 2002	-																													
Bircan, 1997	0		0	0										0																
Boydstun & Hopelain, 1977	0		-											-						-										
Boujard *et al.* 2002	0	-	0	0										-																
Brauhn *et al.*, 1976			-											-																
Collins, 1972	0		0											0																
Donald & Anderson, 1982														-																
Holm *et al.*, 1990	-													-																
Iwamoto *et al.*, 1990														-																
Kebus *et al.*, 1992				0										0		0								0	0					0
Kilambi *et al.*, 1977	0		-	0										-	0															
Kincaid *et al.*, 1976	-		-											-																

Study	Production and nutritional status													Health and condition profile										Stress indicators					
	Mortality	Food intake	Food conversion efficiency	Body condition index	Hepatosomatic index	Hepatic glycogen	Hepatic lipid	Plasma protein	Plasma Lipid	Carcass protein	Carcass lipid	Carcass water	Carcass ash	Growth	Size variation	Haematocrit/erythrocyte count	Leucocrit	Total lymphocyte count	Specific lymphocyte count	Fin condition	Gill condition	Spleen condition	Thymus index	Interrenal cell nuclear diameter	Plasma cortisol	Responsiveness of HPI axis	Plasma glucose	Plasma chloride	Oxygen consumption	Gastric mucosa
Kindschi *et al.*, 1991a	-		-					0						+, -	-	0	0	0	0	0										
Kindschi *et al.*, 1999b																													0	
Laidley & Leatherland, 1988								0																	0		0			
Leatherland, 1993		-			-	0	0							-											+,0	0				
Leatherland & Cho, 1985					-	0	0	-	0	0	0	0	0	-		0								0	+		0			
Lefrançois *et al.*, 2001																													0	
Li & Brocksen, 1977			-											-																
Mäkinen & Ruohonen, 1990			0	-										-						-										
Miller *et al.*, 1995	0		0	0				-			0			0, -		0	0			-			0						0	
Murai & Andrews, 1972	-		-											-																
Papoutsoglou *et al.*, 1979	-	-	-											-																
Papoutsoglou *et al.*, 1980														-																
Papoutsoglou *et al.*, 1987	+		-				-		-		-		-	-		0											0			
Pickering & Pottinger, 1987a				-										0		0		-	-						-					
Pickering *et al.*, 1991	-			-										-											-, 0					
Pickering & Pottinger, 1989																									-					
Piper, 1970			0											0																
Procarione *et al.*, 1999			-											-											+	-	0	0		
Purser & Hart, 1991	-													-	0					-										
Refstie, 1977	+, -			-										-																
Rigolino *et al.*, 1989	0		0	-										0																

Study	Production and nutritional status															Health and condition profile										Stress indicators					
	Mortality	Food intake	Food conversion efficiency	Body condition index	Hepatosomatic index	Hepatic glycogen	Hepatic lipid	Plasma protein	Plasma Lipid	Carcass protein	Carcass lipid	Carcass water	Carcass ash	Growth	Size variation	Haematocrit/erythrocyte count	Leucocrit	Total lymphocyte count	Specific lymphocyte count	Fin condition	Gill condition	Spleen condition	Thymus index	Interrenal cell nuclear diameter	Plasma cortisol	Responsiveness of HPI axis	Plasma glucose	Plasma chloride	Oxygen consumption	Gastric mucosa
Roell *et al.*, 1986	0		0											0																
Rosenthal *et al.*, 1984				-										-							-	-								
Sahin *et al.*, 1999	0													0, -																
Soderberg *et al.*, 1983	0													0																
Teskeredžić *et al.*, 1986	-													-																
Trzebiatowski *et al.*, 1981	+		-											-																
Tsintsadze, 1981				-										-																
Unlu & Baran, 1992				-				0			0			-																
Wagner *et al.*, 1996b			0	-										0		-	0			0			-							
Wedemeyer, 1976																											0			
Winfree *et al.*, 1998	-		+	0						0	0	0	0	0						-										
Wonjo, 1976	0		+											-																
No. study	26	4	24	15	2	2	3	5	2	2	5	2	3	43	4	7	3	2	2	7	1	1	2	2	7	2	5	1	3	1
No. adverse	11	4	13	9	2	0	1	2	1	0	1	0	1	32	1	1	0	1	1	5	1	1	1	0	2	1	0	0	0	0
No. none/inconclusive	12	0	9	6	0	2	2	3	1	2	4	2	2	13	3	6	3	1	1	2	0	0	1	2	4	1	5	1	3	1
No. beneficial	4	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0

Appendix 2. Questionnaire of stocking density practices sent to UK trout farms.

Name of Farm ..... **Contact Person** .....

Telephone ..... **Email** .....

1. Which species of fish do you farm?

☐ Rainbow trout ☐ Brown Trout ☐ Other (please specify) _____

2. Which of the following categories best classifies your facility? (you may tick more than one)

☐ Hatchery ☐ Fishery ☐ Restocking farm ☐ Table farm

3. How is your farm supplied with water?

☐ River ☐ Borehole/Spring ☐ Lake/Loch ☐ Other

4. Please give us an idea of the annual production of your facility (tonnes).

☐ <20 t. ☐ 20 – 60 t. ☐ 60 – 100 t. ☐ 100 – 200t. ☐ >200 tonnes

5. a. What do you consider to be a high stocking density? .....kg m^{-3}

b. Do you associate the high stocking density with any of the following:

☐ increased mortality ☐ decreased growth ☐ fin erosion ☐ disease outbreaks

6. Do you use additional lighting in your production cycle other than in the hatchery or during working hours?

☐ Yes ☐ No

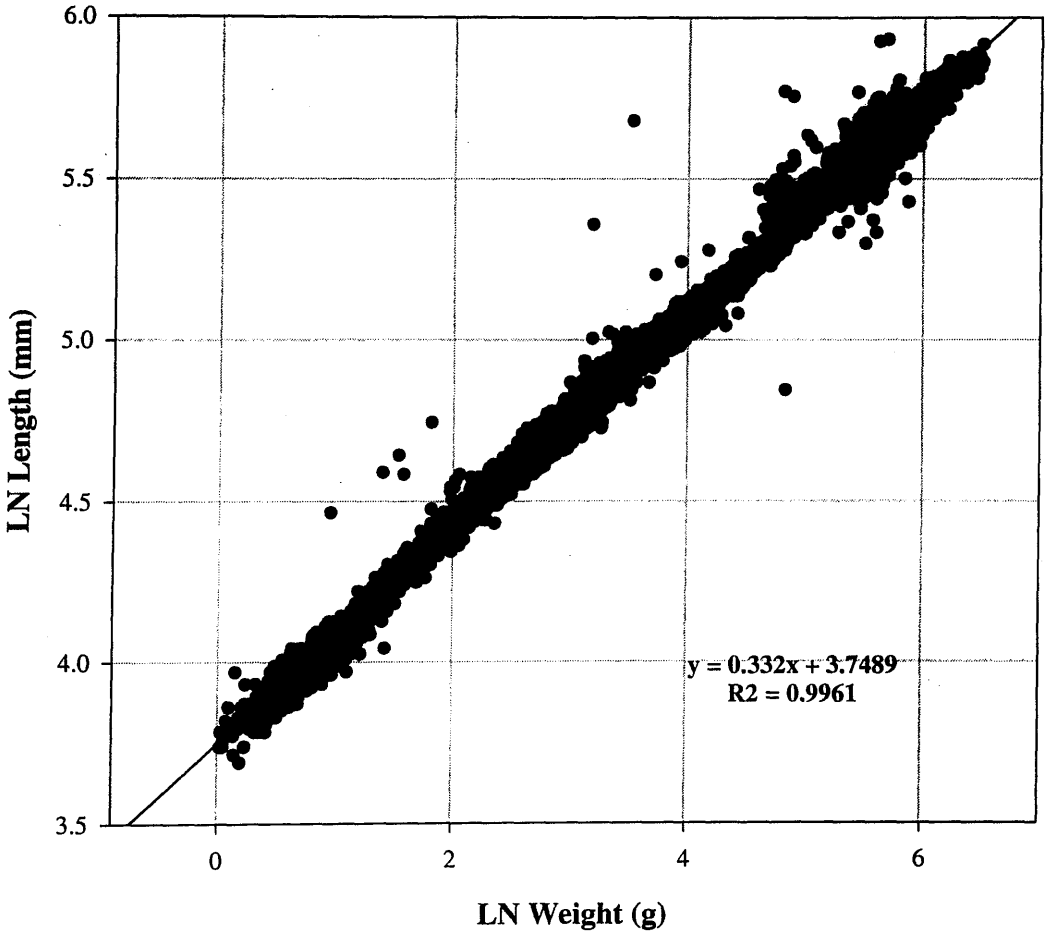
If yes, please state when .....

7. How do you feed your fish?

☐ Hand ☐ Demand Feeders ☐ Timed Hopper ☐ Belt Feeders ☐ Bulk Feeders

In order to encompass the wide range of trout farming facilities and the various stages of the production cycle, the following table has been constructed. You are asked to fill in sections that are relevant to you as accurately as possible to provide us with an idea of the range of stocking densities that you apply and some of the basic husbandry conditions for fish of different sizes. ***Please tick the relevant boxes where possible.***

Size of Fish	Minimum Stocking Density	Maximum Stocking Density	Typical Holding Unit	Typical Flow Rate (please state units)	Additional Aeration
Less than 5g	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ Pond ☐ Raceway ☐ Cage ☐ Tank Volume:		☐ Pump & stone ☐ Oxygen ☐ Paddle Wheel ☐ Other
5g - 50g	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ Pond ☐ Raceway ☐ Cage ☐ Tank Volume:		☐ Pump & stone ☐ Oxygen ☐ Paddle Wheel ☐ Other
50g - 150g	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ Pond ☐ Raceway ☐ Cage ☐ Tank Volume:		☐ Pump & stone ☐ Oxygen ☐ Paddle Wheel ☐ Other
150g - 250g	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ Pond ☐ Raceway ☐ Cage ☐ Tank Volume:		☐ Pump & stone ☐ Oxygen ☐ Paddle Wheel ☐ Other
250g - 500g	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ Pond ☐ Raceway ☐ Cage ☐ Tank Volume:		☐ Pump & stone ☐ Oxygen ☐ Paddle Wheel ☐ Other
500g +	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ 0 - 20 kg/m^3 ☐ 20 - 40 kg/m^3 ☐ 40 - 60 kg/m^3 ☐ 60 - 80 kg/m^3 ☐ 80 kg/m^3 +	☐ Pond ☐ Raceway ☐ Cage ☐ Tank Volume:		☐ Pump & stone ☐ Oxygen ☐ Paddle Wheel ☐ Other



Appendix 3. Correlation of body weight and length in farmed rainbow trout