# Environmental and Behavioural Stressors: Effects on Physiological Function in Salmonid Fish

by

Katherine Anne Sloman B.Sc. (Swansea)

Institute of Biomedical and Life Sciences
Division of Environmental and Evolutionary Biology
University of Glasgow

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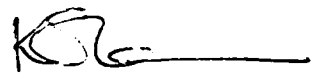
For my parents, Penelope and David Sloman

Declaration

I declare that this thesis is the results of my own work, unless otherwise stated, and has been composed by myself.



Katherine A Sloman

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## Summary

Social interactions and aggression among salmonid fish are known to induce stress responses, particularly in subordinate fish, and the physiological effects of these stress responses have implications for fish in both aquaculture and natural environments. The physiological responses exhibited by subordinate fish (*i.e.* those fish that are low ranking in a social hierarchy) vary depending on the nature and extent of the social interaction and can also be influenced by environmental factors. Artificial environments - typically aquaria - generally elicit a larger stress response in the subordinate fish due to the fish being held in close confinement. A time course study investigating the effects of social stress in rainbow trout, *Oncorhynchus mykiss*, and brown trout, *Salmo trutta*, confined in pairs demonstrated that after 48 h confinement subordinate fish exhibited elevation of the stress hormone cortisol. In the more aggressive rainbow trout, cortisol elevation was apparent after 4 h of confinement, demonstrating that the degree of social interaction and aggression has an effect on the cortisol response and that it can vary between species. Those fish that became subordinate were found to display higher plasma cortisol concentrations even before being subjected to social stress than those fish that became dominant. It is therefore suggested that, in these species, elevated cortisol concentrations (and/ or conditions leading to elevated plasma cortisol) may be indicative of subordinance.

An increase in stress may also cause an elevation of basal metabolic rates. When brown trout were confined in pairs, the subordinate fish showed a significant increase in standard metabolic rate. Confinement of rainbow trout in pairs may affect the ability of subordinate

fish to respond to other stressors, in that subordinate fish were found to show a reduced secretion of cortisol upon stimulation of the interrenal cells in an *in situ* perfused head kidney preparation with ACTH. However, using the same preparation, the ability of the subordinate to secrete catecholamines was not impaired and thus the chronic elevation of plasma cortisol concentrations seen in the subordinate fish appears only to affect further secretion of cortisol through negative feedback mechanisms.

The potential for the increases in blood plasma cortisol concentrations associated with subordinance to elicit chloride cell proliferation in the gill epithelia was also investigated, since artificial elevation of cortisol concentrations (*i.e.* cortisol injection at supraphysiological levels) is known to induce proliferation of chloride cells under certain conditions. However, when trout were confined in pairs no chloride cell proliferation was observed, even though cortisol elevation occurred in subordinates.

Cortisol elevation was also documented in rainbow trout subjected to brief periods (30 s) of air emersion stress, but again, no evidence of chloride cell proliferation was found, suggesting that an ionoregulatory challenge is essential for the natural stimulation of chloride cell proliferation. The role of cortisol in chloride cell proliferation was investigated further by blocking corticoid receptors before acclimation of rainbow trout to ion deficient water. A glucocorticoid receptor blocker, RU486, and a mammalian mineralocorticoid receptor blocker, spironolactone, were used. Chloride cell proliferation in response to soft water acclimation was observed in the fish treated with RU486 but not in those treated with spironolactone, suggesting that cortisol elevation is involved in

chloride cell proliferation, in a mineralocorticoid role. The lack of chloride cell proliferation associated with chronic (social) or acute (air emersion) elevation of cortisol may reflect a lack of mineralocorticoid receptors in the absence of an ionoregulatory challenge.

A major finding of the present research was that dominance hierarchies do not always lead to stress responses in subordinates. In a study carried out under semi-natural conditions (in stream tanks), the formation of dominance hierarchies amongst groups of four brown trout did not elicit plasma cortisol elevation in subordinate fish. However, dominant fish still appeared to have a physiological advantage over subordinate fish in terms of growth; the growth rates of dominants were significantly higher than those of the other three ranks of fish. In this particular study, sub-dominant (second-ranking) fish had the lowest growth rate perhaps because these fish adopted a high cost/ high return strategy of competing with the dominant fish and therefore expended more energy than the other two ranks of subordinate fish, which adopted a low cost/ low return strategy. Interestingly, sub-dominant fish demonstrated significantly higher numbers of chloride cells than the other ranks of fish, but whether the proliferation of chloride cells is related to changes in plasma cortisol concentrations was not clear.

Further studies using semi-natural conditions investigated the effects of environmental perturbations on previously established dominance hierarchies. The environmental perturbations of lowered water levels (simulating drought) and increased flow rates (simulating spates) were found to disrupt dominance hierarchies and the social structure of

groups of four brown trout. The normal growth advantages gained by dominant fish under constant semi-natural conditions were lost; all fish exhibited similar growth rates. No effects of environmental perturbations on plasma cortisol concentrations were detected, suggesting that while environmental perturbations may disrupt social hierarchies, these perturbations do not represent a chronic stress. Therefore, the lower growth rates in groups subjected to environmental perturbations was presumably a reflection solely of the changes in social structure.

In conclusion, the present study has clearly demonstrated that the physiological responses to social interaction and the formation of dominance hierarchies in rainbow trout and brown trout are affected by the environment of the fish e.g. whether the environment is artificial or natural, and stable or subject to fluctuations. In an artificial environment, the present study has confirmed that the effects of the social stress encountered by subordinate fish include decreases in growth rate and condition and increases in plasma cortisol but has also demonstrated decreases in the ability to secrete further cortisol and increases in the standard metabolic rate of subordinates. In a semi-natural environment these physiological consequences of subordinance were seen to be reduced; indeed with the presence of environmental perturbations no physiological differences were noted between dominant and subordinate fish. Finally the present study also investigated the role of cortisol in chloride cell proliferation and the determination of social status and concluded that cortisol appears to play a mineralocorticoid role in the proliferation of chloride cells and may also influence the outcome of social interactions.

## Contents

# CHAPTER 1

# Chapter 1: Environmental and Behavioural Stressors: Effects on Physiological Function in Salmonid Fish

## 1.1 Stress in vertebrates

That stress occurs in animals is a widely accepted concept, but methods of defining and measuring stress present a greater problem and 'there are few concepts which have evoked as much discussion and disagreement as that of stress when applied to biological systems' (Pickering 1981). The stress response was first recognised by Hippocrates (460-377 BC) as the ability of organisms to restore the body to health after exposure to pathogens. The physiologist Walter Bradford Cannon first used the term 'homeostasis' to describe the ability to maintain a set of conditions, and demonstrated the autonomic response of organisms, inducing metabolic and cardiovascular changes preparing the body for 'flight or fight' (Cannon 1939). The phases of stress through which a vertebrate passes are most famously described as the General Adaptation Syndrome, G.A.S. (Seyle 1971). Phase one is an alarm reaction during which there is an increase in sympathetic stimulation and in the synthesis and secretion of stress hormones. During the second, resistance, phase the organism is adapting to the stressful stimuli and the phase is characterised by prolonged elevation of glucocorticosteroids. The third phase, exhaustion, is where, under continuous stressful conditions, the ability of the organism to function normally is impaired. The study and understanding of the stress response was originally based on the human body with the emphasis being on its importance for medicine. However, an understanding of the stress response in other vertebrates has now become exceedingly important for both

conservation and economic reasons. Within vertebrates, the anatomical organisation of the endocrine tissues varies greatly, the only similarity being the tendency for both adrenocortical homologues and chromaffin cells to be located in one organ (Norris 1980). Therefore, although the stress responses themselves are very similar throughout all vertebrates, to avoid confusion the following discussion will consider only the stress responses of teleost fish.

## 1.2 Primary responses to stress in teleost fish

The primary response to stress is elevation of circulating concentrations of the "stress hormones" adrenaline, noradrenaline and cortisol (Sumpter 1997) which induce physiological changes allowing the fish to deal with stress. The release of adrenaline and noradrenaline, the catecholamines, is known as the adrenergic response (Mazeaud and Mazeaud 1981). The adrenergic response is an immediate response to stress, occurring within seconds. In teleosts the chromaffin cells, from which catecholamines are released, are located in the head kidney. Catecholamine release is stimulated primarily by the release of acetylcholine from pre-ganglionic fibres of the sympathetic nervous system (Perry *et al.* 1991). Release of catecholamines does not result in a decrease of catecholamine concentrations in chromaffin cells, suggesting that stress also stimulates catecholamine synthesis (Reid *et al.* 1994). The effects of catecholamines can be direct or indirect; they elicit increases in or maintenance of energy turnover and oxygen supply under adverse conditions (Randall and Perry 1994). Practical difficulties in the timing of sampling for catecholamines mean that measurement of catecholamines is not often used in the

investigation of stress except under highly controlled laboratory conditions (Sumpter 1997).

The release of glucocorticosteroid hormones during stress is known as the hypothalamo-pituitary-interrenal (HPI) response and has a much slower response time than the adrenergic response (Donaldson 1981) (occurring over a period of hours rather than seconds), as cortisol is synthesised at the time of release (Sumpter 1997). Cortisol, the most recognised of these glucocorticosteroids, has two different chemical names, 11β,17α21-trihydroxypregn-4-ene-3,20-dione and 11β,17α21-trihydroxy-Δ4-pregnene-3,20-dione and three common names; cortisol, 17-hydroxycorticosterone, and hydrocortisone (Norris 1980). Its chemical structure is illustrated in Fig. 1.1. The HPI axis itself is a cascade of hormones resulting in the eventual release of cortisol (Fig. 1.2).

The first hormone involved in the HPI response is corticotropin-releasing hormone, CRH (or corticotropin-releasing factor, CRF). The main role of CRH is to stimulate the anterior pituitary to release adrenocorticotropin, ACTH (Sumpter 1997), which occurs rapidly in response to stress. In coho salmon, *Oncorhynchus kisutch*, and rainbow trout, *O. mykiss*, ACTH concentrations increased within two minutes of handling and confinement and reached concentrations five to eight times higher than basal concentrations after 30 minutes (Sumpter *et al.* 1986). ACTH then acts on the interrenal tissue, resulting in the secretion of corticosteroids. ACTH is generally considered as the most important factor in mediating pituitary control of cortisol secretion during stress (Wendelaar Bonga *et al.* 1995). Confirmation of the presence of the HPI axis in fish has been demonstrated by the

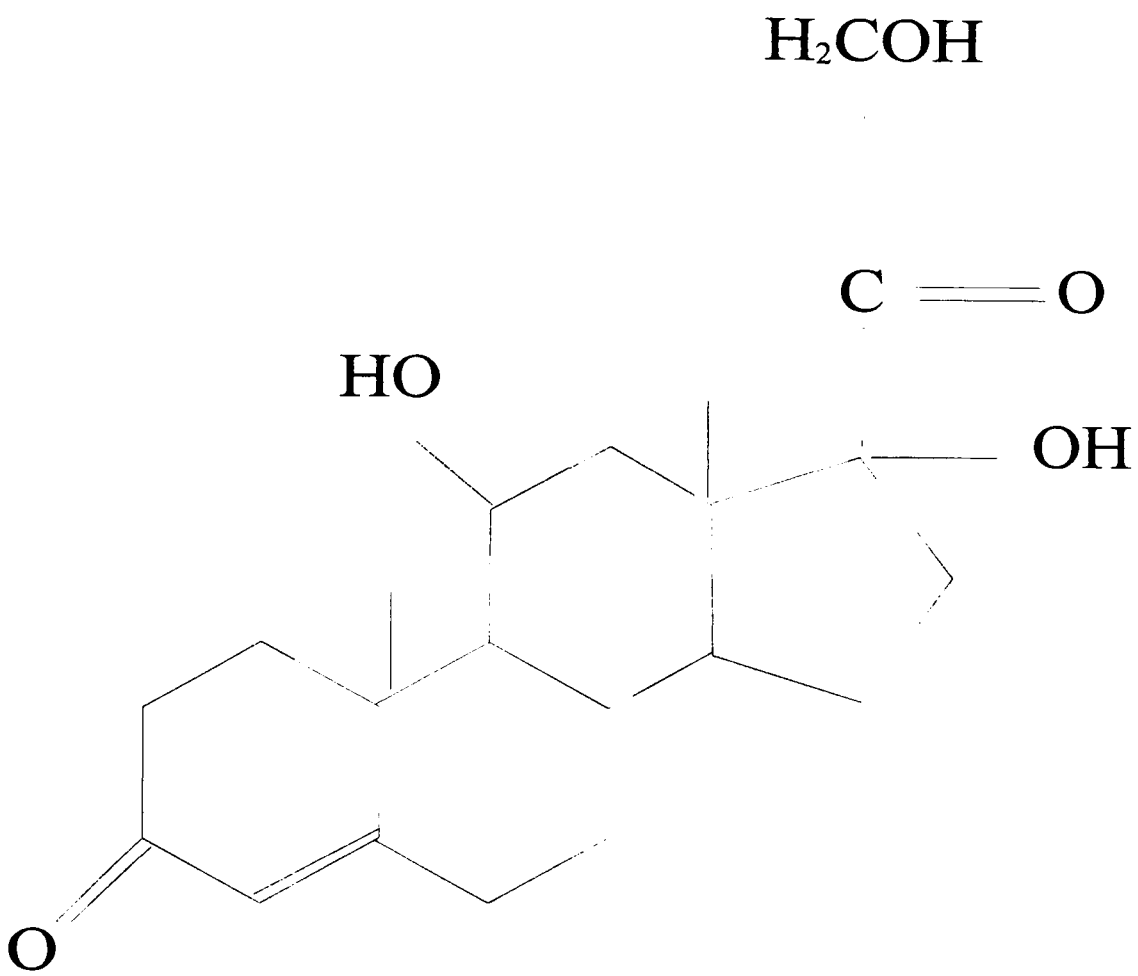



Figure 1.1: Chemical structure of the glucocorticosteroid stress hormone, cortisol (Norris 1980).

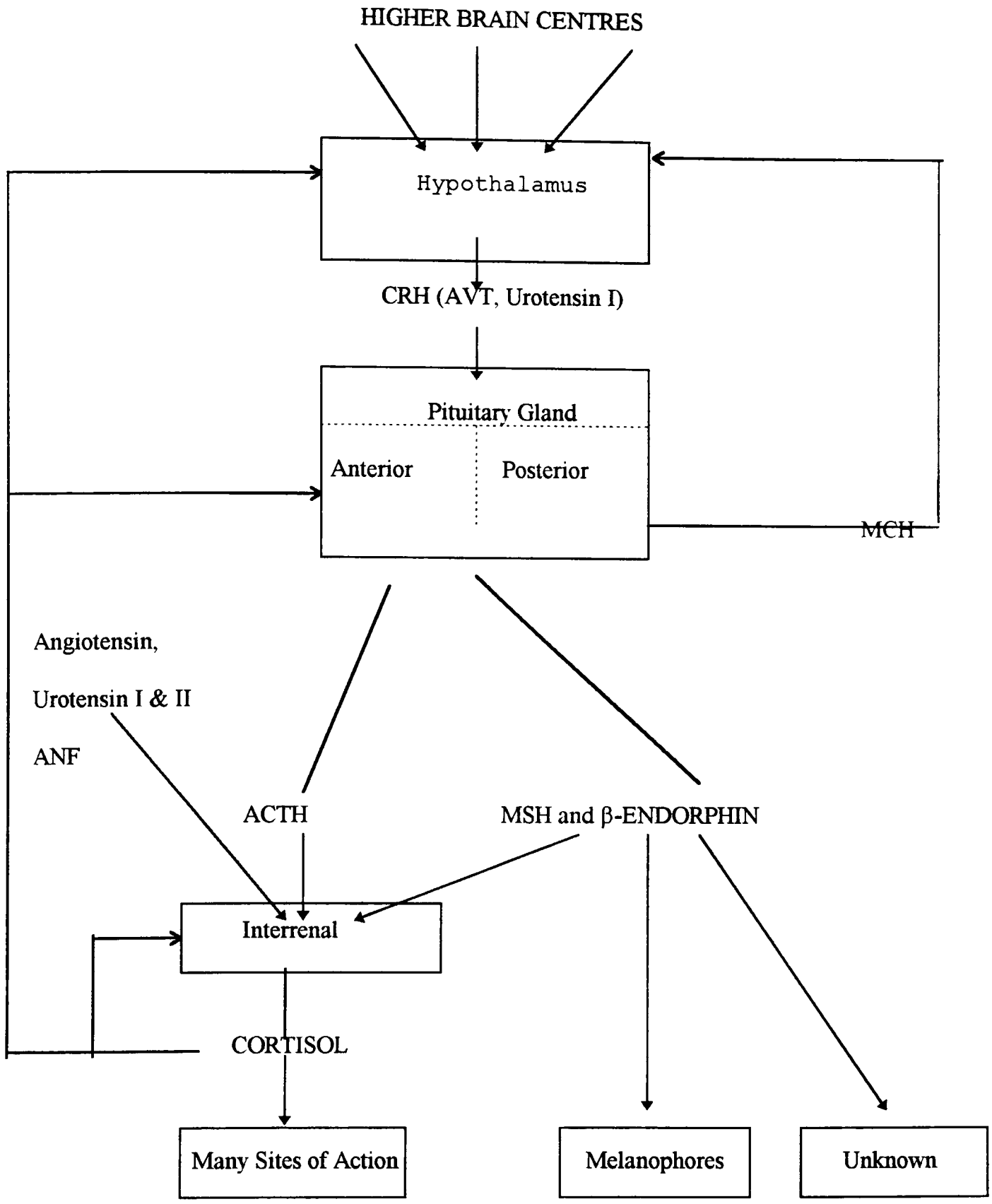



Figure 1.2: The hypothalamo-pituitary-interrenal axis (HPI) (Sumpter (1997)) [CRH = corticotropin-releasing hormone]

effects of hypophysectomy (removal of the pituitary gland). Donaldson and McBride (1967) found that the interrenal tissue of rainbow trout had undergone considerable atrophy three months after hypophysectomy. Plasma cortisol concentrations in the hypophysectomised fish decreased over the three month period, and the fish showed no increase in cortisol concentrations when subsequently subjected to the acute stress of lowered water levels. Although cortisol is the major corticosteroid released through the activation of the HPI axis, other corticosteroids may also be released, e.g. cortisone (Pottinger and Moran 1993), although these may also arise from peripheral conversion of cortisol (Patiño *et al.* 1987). However, due to the ease of measurement of plasma cortisol concentrations and the ubiquitous elevation of cortisol in response to stress, cortisol concentrations are now widely used for stress quantification in teleost fish (Pickering 1993a).

The adrenergic and HPI responses to stress are very different, and are often described separately, but it should be noted that the two do not function in isolation. Little research has been carried out on the interactions between these responses to stress but it is believed that the adrenergic response can enhance the activation of the HPI axis and the HPI axis in turn can enhance activation of the adrenergic response (Sumpter 1997). For example, chronic elevation of plasma cortisol concentrations has been shown to increase the responsiveness of red blood cells to catecholamines. Thus, cortisol plays an adaptive role in increasing the sensitivity of the β-adrenergic signal transduction system of red blood cells (and also hepatocytes) involved in the relieving of acute stress (Perry and Reid 1993).

## 1.3 Secondary responses to stress in teleost fish

The primary hormonal response to stress gives rise to secondary responses, which are far more wide-ranging in their effects and can involve both short and long term physiological changes; some examples of parameters (secondary responses) used as indicators of stress in fish are given in Table 1a. Chan and Woo (1978) injected control and hypophysectomised eels, *Anguilla japonica*, with cortisol and found that the effects included an increase in oxygen consumption rate and a depression of the respiratory quotient (where respiratory quotient is the quantity of $CO_2$ produced per quantity of $O_2$ consumed (Cech 1990)). Other metabolic effects included a rapid increase of blood plasma glucose from $33.9 \pm 6.1$ mg $dl^{-1}$ to $88.9 \pm 9.2$ mg $dl^{-1}$ at 2 h after the hormone treatment. These values continued to rise until 9 h after injection. It was concluded that the carbon skeleton used for synthesis of blood glucose and liver glycogen comes from breakdown of peripheral tissues.

Stress can also cause osmoregulatory disturbances, including an effect on sodium balance. Randall *et al.* (1972) found that electric shocks or prodding, inducing violent escape reactions in rainbow trout, caused a loss of sodium across the gill. Capture and handling have been shown to induce an increase in $Mg^{2+}$ and $Na^{+}$ concentrations in the blood plasma of rainbow trout (Laidley and Leatherland 1988), although plasma $Ca^{2+}$ concentrations remained unchanged. Stress is also known to induce cellular changes in the

Table 1a: Different types of secondary indicators of stress. Modified from Barton and Iwama (1991).

| | |
|---|---|
| Metabolic: | Increase in plasma glucose (Chan and Woo 1978)<br>Increase in plasma lactic acid (Pickering and Pottinger 1995)<br>Decrease in liver glycogen (Andersen *et al.* 1991)<br>Decrease in liver and muscle adenylate energy charge (Pickering and Pottinger 1995) |
| Haematological: | Increase in haematocrit (Barton *et al.* 1987)<br>Decrease in lymphocyte numbers (Barton *et al.* 1987) |
| Hydromineral: | Increase in plasma chloride (Eddy 1981)<br>Increase in plasma sodium (Laidley and Leatherland 1988)<br>Decrease then increase in plasma potassium (Laidley and Leatherland 1988)<br>Increase in plasma amino acids (Chan and Woo 1978) |
| Structural: | Increase in interrenal nuclear diameter (Noakes and Leatherland 1977)<br>Decrease in hepatosomatic index (Goede and Barton 1990)<br>Decrease in condition factor (Barton *et al.* 1987) |

haemopoietic tissue of the spleen and head kidney (Peters and Schwarzer 1985). Peters and Schwarzer (1985) demonstrated that there is an increase in the abundance of macrophage-like cells, a decrease in the number of haemoblasts and lymphocytes and an increase in red blood cell degeneration in rainbow trout stressed by handling and social conflict. Similarly, Noakes and Leatherland (1977) found that social stress in rainbow trout caused a significant increase in interrenal cell activity.

### 1.4 Tertiary responses to stress

As well as secondary responses to stress, tertiary factors are also often considered when investigating stress. Tertiary factors result from a combination of secondary responses rather than directly from the primary responses to stress. The two most commonly measured tertiary responses to stress are growth and condition factor, an indicator of fitness. Another tertiary factor that can be affected by stress is reproduction. Data on the lengths and weights of fish are easily obtained and are indicative of growth (Wedemeyer *et al.* 1990). Depression of growth under stressful conditions is probably linked to the secondary effects of stress on metabolism. Another secondary effect of stress affecting growth involves growth hormone. Pickering *et al.* (1991) demonstrated that rainbow trout subjected to the acute stress of handling followed by confinement for a period of 1 or 24 h exhibited depressed plasma growth hormone (GH) concentrations. Pituitary growth hormone (GH) is known to play a major role in the growth of teleost fish (see review by Weatherley and Gill 1987). However, when oxygen concentrations in both crowded and uncrowded tanks were reduced as a chronic stressor, the fish showed elevated plasma GH

concentrations. It was suggested that the effects of chronic stress seen in the experiment could be caused by starvation, since stress is known to affect appetite and starvation in rainbow trout can induce elevated GH concentrations (Sumpter *et al.* 1991). Although there does appear to be a relationship between stress and GH concentrations, and possibly between GH and plasma cortisol concentrations, further research into these specific interactions is needed (Sumpter 1997).

Abbott and Dill (1989) demonstrated that when dominant and subordinate rainbow trout were fed equal rations dominant fish grew faster than subordinates, suggesting a possible metabolic disadvantage of being subordinate. Intra-arterial injection of eels, *Anguilla anguilla*, with cortisol resulted in suppression of the thyroid hormone, $T_3$ and $T_4$, which could also have a growth-inhibiting effect (Redding *et al.* 1986). The specific pathways through which stress is linked to tertiary effects may not be completely understood, but the fact that growth suppression can be linked to stress allows specific growth rate (% change in weight of fish per day) to be used as an indicator of stress. Condition factor is also a useful tool in monitoring stress and is calculated as (weight/ fork length$^c$) x 100, where c is the slope of the regression of log(weight) on log(fork length) (Bolger and Connolly 1989). Although the stress response in fish is far from being understood, these characteristic primary, secondary and tertiary physiological changes provide ample tools for measuring responses to stress.

Natural populations of fish are affected by different stressors to those experienced by fish in aquaculture, but research into stress induced by procedures used in aquaculture can help

to identify the physiological effects of stress occurring in natural populations. Stressors can be defined as acute, where the stress applied is severe but short-lived, or chronic, where the stress is less severe but applied for a longer time period. The majority of previous research has examined either social or environmental stressors, and the two have rarely been considered together.

## 1.5 Behavioural Stressors

Behavioural stressors result when fish encounter other fish and social interactions occur. One of the ways by which a fish may become stressed due to social factors is through aggression from conspecifics. Inter-individual aggression among salmonids can lead to the less successful individuals within a population being deprived of resources, including food and shelter (Newman 1956). Fausch (1984) showed that intraspecific hierarchies formed within groups of coho salmon, brook trout, *Salvelinus fontinalis*, and brown trout, *Salmo trutta*, released into stream aquaria, with dominant fish holding positions with the maximum potential for profit (and so achieving the fastest growth rate). Within natural populations of brown trout, Bachman (1984) found that the social structure consisted of linear dominance hierarchies, dependent on the size of the fish.

A high ranking position in a dominance hierarchy can result in a higher specific growth rate. Indeed, Abbott and Dill (1989) found that, even when fed equal rations, dominant fish grew faster than subordinate fish. Dominance status can also affect the timing of migration to sea in anadromous salmonids. Metcalfe *et al.* (1989) demonstrated that the

more dominant Atlantic salmon, *S. salar*, were more likely to migrate to sea after spending only one year in freshwater. However, the factors determining which fish becomes dominant over its conspecifics are less well defined. Although dominant fish are in general larger than subordinates it is now believed that size is an effect rather than a cause of dominance (Huntingford *et al.* 1990; Yamamoto *et al.* 1998). Metcalfe *et al.* (1995) found a strong relationship between social status and standard metabolic rate (SMR) in juvenile Atlantic salmon, with more dominant fish having a higher SMR. Cutts *et al.* (1999) also demonstrated that SMR was a better predictor of dominance than body size in Atlantic salmon, in which again a high SMR was indicative of dominance. Yamamoto *et al.* (1998) also found that resting metabolic rate of masu salmon, *Oncorhynchus masou*, was significantly correlated with dominance status and concluded that a high metabolic rate can influence dominance status. Winberg and Nilsson (1992) demonstrated that administration of the immediate precursor of the neurotransmitter dopamine, associated with aggressive behaviour in fish, could influence the determination of social status and make a juvenile Arctic charr, *Salvelinus alpinus*, dominant. This result would suggest that circulating hormone concentrations may influence social status.

Under simulated natural conditions, the existence of dominance hierarchies has been shown to result in stress in subordinate fish. Noakes and Leatherland (1977) placed groups of six rainbow trout in stream tanks and illustrated that social position impacted on interrenal cell activity. Subordinate fish showed a higher interrenal cell activity, indicative of activation of the HPI axis (McLeay 1975), than more dominant fish. It was concluded that as subordination increased, so did social stress. Li and Brocksen

(1977) also utilised stream tanks to investigate competition amongst rainbow trout. It was found that within groups of trout, subordinate fish tended to grow more slowly than dominant fish and had a lower fat content than dominant fish. Another example of the effect of social stress on subordinate fish was demonstrated by Ejike and Schreck (1980). Groups of six coho salmon parr were placed into aquarium tanks and monitored for social interactions and dominance hierarchies for a two week period. It was found that plasma cortisol concentrations and interrenal cell nuclear diameters were highest in subordinate fish and that hepatic glycogen content also varied directly with social position.

Within groups of three to eight rainbow trout confined together for 17 days, it was found that social rank had an effect on type IV cells of the adenohypophysis (pituitary gland), with submissive animals having type IV cells with a higher synthetic activity than more dominant fish (Boddingius 1976). Type IV cells are defined as proximal pars distalis cell types which are slightly basophil. It was concluded that submissive trout were exhibiting high adrenal activity, and that the high synthetic activity of the type IV cells was due to the release of ACTH from these cells. The stressful effect of the presence of a conspecific has also been demonstrated to occur without the need for physical contact. Adult blennies, *Blennius pholis*, in visual contact with a conspecific exhibited lower growth rates, food conversion efficiency, liver weight, percentage of glycogen in the liver tissue and a higher water content of muscle tissue than control fish (Wirtz 1975). In a similar study on blennies, oxygen consumption was seen to increase due to an increase in metabolic rate at the sight of a conspecific (Wirtz and Davenport 1976). Koebele (1985) also found in

cichlids, *Tilapia zillii*, that the sight of a more dominant conspecific can suppress the appetite of subordinate fish, even when food is readily available.

A more extreme social relationship exists when two fish are placed together in a confined area. Under these circumstances, the difference in physiological condition between the dominant and subordinate fish can be great. Pottinger and Pickering (1992) placed different numbers of rainbow trout in confined areas for a period of several weeks and demonstrated that those fish held in pairs showed the greatest variation in physiological condition. One fish of the pair, the dominant, gained weight or remained the same weight, whilst the other, the subordinate fish, lost weight and in many cases died through disease. Subordinate fish also exhibited elevated plasma cortisol concentrations. Peters *et al.* (1980) noted that under captive conditions, aggressive interactions among eels, *Anguilla anguilla*, increased when stocking density was low. Subordinate members of pairs of eels held together for 5-10 days had increased plasma cortisol, glucose and lactate concentrations. Subordinate fish also exhibited a significantly lower hepatic glycogen content and lower numbers of leucocytes. The 'removable blood quantity' (defined as the amount of blood available for extraction through the bulbus arteriosus) was significantly less in subordinate eels than in dominants. In a similar study, Laidley and Leatherland (1988) also found that in rainbow trout confined in pairs, the subordinates showed increased plasma cortisol concentrations and mortality rates. Winberg *et al.* (1992) demonstrated that subordinate Arctic charr displayed higher activities of brain serotonin in comparison with dominant fish. Confinement of juvenile rainbow trout in pairs for 11 h combined with exposure to the opportunistic ubiquitous bacterium, *Aeromonas*

*hydrophila*, demonstrated that the pathogens spread to more organs and were found in greater numbers in subordinate than in dominant fish (Peters *et al.* 1988). Infected dominant fish illustrated a higher level of aggression towards subordinate fish than non-infected dominant fish and infected subordinate fish were more easily exhausted than non-infected subordinates.

### 1.6 Physical Stressors

Physical stressors can be divided into two categories; those that are normally encountered by fish in their natural habitat and those that occur with conditions found in aquaculture. Stressors associated with aquaculture are easily manipulated and have been investigated in some detail as they provide a valuable tool for researching the stress response in fish. The deleterious effects of stress can have important economical implications within aquaculture; thus, it is important to understand the effects of stress to optimise fish health. The two most common physical stressors associated with aquaculture are handling and confinement. A handling stress (as occurs, for instance, when fish are being routinely weighed or moved) would generally involve a combination of catching the fish, a short period of confinement in a net and most likely a period of air exposure. The stress is usually short-lived and hence termed an acute stressor. Confinement occurs when fish are being collected prior to being moved, size-graded or given a disease treatment such as vaccination. Confinement may be solitary in a small area, or with a large number of other fish resulting in a high stocking density. The consequence for the fish in either case is a decrease in available space, with the latter also introducing the aspect of social stressors.

Generally, confinement is for a longer period of time than handling and is therefore a chronic stressor. Confinement of fish in high densities can also be stressful in terms of the effects on water quality. Increases in stocking density result in deterioration of water quality, in particular ammonia concentrations are seen to increase, which can also elicit elevation of plasma cortisol concentrations (see review by Pickering 1992).

A very characteristic response to handling is a large increase in plasma cortisol concentrations; cortisol returns to basal levels several hours after the stressor has been removed. Barton *et al.* (1980) found that netting fingerling rainbow trout from a tank did not cause an elevation of plasma cortisol concentrations but further handling for 9 s elicited a sharp increase in plasma cortisol 15 minutes after handling, returning to basal concentrations after two hours. Mazur and Iwama (1993) found an increase in plasma cortisol 30 minutes after handling in wild and hatchery-reared chinook salmon, *O. tshawytscha* and a similar result was shown by Sumpter *et al.* (1986) in coho salmon and rainbow trout. Other hormones affected by the acute stress of handling include ACTH. Concentrations of ACTH were demonstrated to increase in coho salmon and rainbow trout after handling stress, peaking earlier, but at a lesser magnitude, than plasma cortisol concentrations (Sumpter *et al.* 1986).

The increase in plasma cortisol concentrations illustrated by Davis and Schreck (1997) in juvenile coho salmon, which were induced by moderate handling stress, were correlated with an increase in oxygen consumption. Severe handling stress produced a rate of oxygen consumption 39-98% higher in experimental than control animals. Similarly, Mohamed

(1982) also found that handling caused a 260 % rise in routine metabolic rate and a 291 % increase in standard metabolic rate in the freshwater mullet, *Rhinomugil corsula*. Handling of rainbow trout caused an increase in both plasma adrenaline and noradrenaline and also liver and heart adrenaline concentrations (Nakano and Tomlinson 1967). Plasma glucose concentrations also increased, while heart and skeletal muscle concentrations of glycogen decreased. A depression of androgen concentrations in mature male brown trout, *Salmo trutta*, has also been associated with handling stress (Pickering *et al.* 1987a) and was accompanied by an increase in gonadotrophin hormone (GTH) concentrations.

The chronic stress of confinement is known to generate a different cortisol response than that of acute stressors; cortisol concentrations are generally elevated to a lesser extent but for a longer period of time. Plasma cortisol concentrations in brown trout subjected to prolonged crowding have been shown to increase to approximately 10 ng $ml^{-1}$ and remain elevated for the first four weeks of the confinement period (Pickering and Pottinger 1989). Continuous moderate confinement of juvenile chinook salmon also elicited an increase in plasma cortisol, with concentrations returning to basal levels within six to eight days as the fish acclimated to the stress (Strange *et al.* 1978). During this experiment, a depression in gill $Na^+K^+$ATPase activity was also noted after three weeks confinement (Strange *et al.* 1978). Suppressed concentrations of plasma testosterone and 11-ketotestosterone have been demonstrated in mature male brown trout subjected to chronic confinement for one month, accompanying an increase in plasma cortisol concentrations (Pickering *et al.* 1987a).

Stressors encountered by fish in their natural environments are often harder to manipulate than stressors occurring in aquaculture and have therefore been less researched. Where these stressors have been investigated, in terms of their physiological effects, they have been carried out under laboratory conditions. A change in water level, and therefore territory size, is probably one of the most common environmental stressors encountered by a stream-dwelling fish. Decreasing water levels under semi-natural conditions have been shown to have an effect on behaviour (Huntingford *et al.* 1998b) but the potential impacts on physiology are less well understood. Einarsdóttir and Nilssen (1996) found that in Atlantic salmon, water level reduction within rearing tanks caused an increase in plasma cortisol concentrations. Hypoxia is another environmental stressor that a fish may encounter in its natural environment and is known to affect catecholamine concentrations (see review by Perry and Wood 1989).

Other environmental stressors to which a fish may be exposed include suspended solids, pollutants, hypercapnia (increased partial pressures of $CO_2$), changes in overhead cover, and environmental acidification. Redding *et al.* (1987) found that coho salmon demonstrated elevated cortsiol concentrations when exposed to 2-3g $L^{-1}$ of suspended top soil. A similar effect was found when steelhead trout were exposed for two days to concentrations of topsoil, kaolin clay or volcanic ash. High concentrations of these sediments also caused an increase in blood haematocrit. Brown *et al.* (1984) showed that exposure of rainbow trout to a pH value below 5.2 caused an increase in circulating plasma cortisol concentrations and eight days of acid exposure (pH 4.7) were required to

elevate plasma cortisol concentrations significantly. Exposure of salmonids to hypercapnia has been shown to affect branchial ionic fluxes and acid-base regulation (Perry *et al.* 1987; Goss *et al.* 1992). Rainbow trout exposed to sub-lethal concentrations of the pesticide endrin are also known to exhibit elevated plasma cortisol concentrations (Bennett and Wolke 1987). These are just a few examples of environmental stressors that have been studied previously.

An environmental stressor which has perhaps received less attention in the past is exposure to ion deficient water. The acute effects of acclimation to ion-poor water in freshwater salmonids include a decrease in active NaCl uptake (McDonald and Rogano 1986) and an increase in passive loss of NaCl resulting in an overall net loss of $Na^+$ and $Cl^-$ ions to the environment (Perry and Laurent 1989). Overall net ion loss leads to a decrease in the levels of plasma electrolytes. For example, McDonald and Rogano (1986) found that rainbow trout placed in extremely soft water ($[Ca^{2+}]$ = 0.025 mM, [NaCl] = 0.06 mM) lost $Na^+$ and $Cl^-$ at about 200 $\mu eq\ kg^{-1}\ h^{-1}$ over the first 12 hours and after nine days of exposure had lost 8 % and 13 % of total body $Na^+$ and $Cl^-$ ion content, respectively. However, they found that the trout showed a persistent uptake of calcium. The overall net loss of ions to the environment is due to an increase in passive (diffusional) efflux of ions across the gill. As freshwater fish become acclimated to ion-poor water adaptations occur both to increase active ion uptake across the gills and to decrease passive ion loss (see review by Marshall (1995)). Increased transport capacity is believed to be linked to the proliferation of chloride cells as an adaptation to the lack of ions in the surrounding media (Laurent *et al.* 1985; Laurent and Hebibi 1989; Perry and Laurent 1989).

Proliferation of the chloride cells in freshwater fish acclimated to ion-poor water is particularly apparent in the lamellar epithelium (Laurent 1984; Greco *et al.* 1996) and thus increases the blood-water barrier thickness. Perry and Laurent (1989) found a 1.5 - 2.5 fold increase in total filament surface area occupied by chloride cells within 24 h when rainbow trout were placed in ion-poor water. Greco *et al.* (1996) found that rainbow trout placed in ion-poor water ($[Na^+]$ = 0.055 mmol $l^{-1}$, $[Cl^-]$ = 0.029 mmol $l^{-1}$, $[Ca^{2+}]$ = 0.059 mmol $l^{-1}$ and $[K^+]$ = 0.007 mmol $l^{-1}$) showed a doubling of the gill epithelial surface area occupied by chloride cells (chloride cell fractional area). The increase in chloride cell fractional area was due to increases in both the average chloride cell area and chloride cell density. Perry *et al.* (1996) found that soft water-acclimated rainbow trout displayed an increase in chloride cell numbers from 2,390 ± 625 to 6,583 ± 283 per square millimetre of lamellar surface area.

Proliferation of chloride cells in the gill epithelia of salmonid fish is particularly relevant to the present study due to observed and presumed links between increases in blood plasma cortisol concentrations and increases in chloride cell densities, both during transfer of salmonids from freshwater to seawater and in freshwater salmonids placed in ion-poor water (Madsen 1990a; Laurent and Perry 1990; Perry *et al.* 1992; Bindon *et al.* 1994a, 1994b; Laurent *et al.* 1994). Experimental administration of cortisol has also been shown to induce chloride cell proliferation. Intraperitoneal injections of cortisol (at pharmacological doses) in rainbow trout caused significant proliferation of chloride cells (Laurent *et al.* 1994). The effect occurred 12 - 24 hours after injection. Daily

intramuscular injections of cortisol in rainbow trout produced significant increases in chloride cell fractional area in 10 days (Laurent and Perry 1990). Chloride cells covered 10 % of the trailing edge of the gill filaments in fish treated with cortisol, four times that of controls. However, the evidence for a link between naturally elevated cortisol concentrations and chloride cell proliferation is strictly correlative; a causal relationship has not yet been demonstrated. Endogenous concentrations of cortisol increase when rainbow trout are transferred to ion-poor water (Perry and Wood 1985; Flik and Perry 1989; Perry and Laurent 1989). Young immature chloride cells appear to be the target for cortisol which appears to influence their differentiation (Laurent *et al.* 1984). Laurent and Perry (1989) found that rainbow trout transferred to ion deficient water showed an increase in plasma cortisol concentrations of 3 - 4 fold between 12 - 48 h after transfer. The concentrations returned to basal levels after one week. The mechanism of action on the gills has not yet been determined but there is evidence for the presence of glucocorticoid receptors in the branchial tissue (Sandor *et al.* 1984).

The proliferation of chloride cells on the lamellae increases the ion uptake capability of the gills in ion-poor media, but results in an increased diffusion distance for respiratory gases. Experiments on rainbow trout acclimated to ion-poor water (Greco *et al.* 1996) showed that chloride cell proliferation resulted in an increase in the blood-water diffusion distance from $3.26 \pm 0.08$ μm to $6.58 \pm 0.43$ μm. The increase in blood-water diffusion distance also caused a narrowing of the interlamellar water channels. Similarly, work by Bindon *et al.* (1994b) showed a positive correlation between artificially-induced chloride cell proliferation in rainbow trout caused by the administration of cortisol and an increase in

lamellar epithelial thickness. Due to the trade-off between increased ability for osmoregulation and decreased efficiency of oxygen transfer between blood and water, a number of studies have been carried out on the effect of chloride cell proliferation itself and/or acclimation to ion-poor water on the ability of fish to cope with hypoxia (Thomas *et al.* 1988; Bindon *et al.* 1994a; Greco *et al.* 1996; Perry *et al.* 1996). In all cases, it was found that freshwater fish exhibiting chloride cell proliferation and/or acclimated to ion-deficient water were less tolerant of hypoxic conditions.

## 1.7 Manipulation of exogenous and endogenous cortisol concentrations

As well as investigation into the effects of behavioural and physical stresses, research has been carried out into the stress response of fish by manipulating stress hormones. In this way the primary response to stress in fish is artificially controlled and the associated secondary effects can be studied. In particular, due to the ease of measurement and administration, plasma cortisol concentrations have often been manipulated. There are several different methods of artificially elevating plasma cortisol concentrations, including incorporation into the diet, intra-peritoneal or intra-muscular injection, intra-arterial injection, silastic implants and mini-osmotic pumps (Gamperl *et al.* 1994). Using these techniques it is possible to investigate the physiological effects of elevated plasma cortisol concentrations in a relatively controlled manner. Intra-peritoneal implants given to cutthroat trout parr, *O. clarki clarki*, increased oxygen consumption and plasma glucose concentrations (Morgan and Iwama 1996). These physiological changes also caused a

slight adaptation of the fish for seawater transfer indicating that cortisol plays a role in seawater adaptation. Cortisol administration via the diet to yearling channel catfish, *Ictalurus punctatus*, caused decreases in body weight, liposomatic index, condition factor and hepatosomatic index (Davis *et al.* 1985). Intra-muscular injection of eels, *A. japonica*, with cortisol caused an increase in oxygen consumption associated with a depression in respiratory quotient, changes in liver glycogen content and increases in blood plasma glucose (Chan and Woo 1978). On the basis of these studies, cortisol is an important metabolic hormone that elicits the synthesis of blood glucose and liver glycogen from the breakdown of peripheral tissue.

The use of exogenous sources of cortisol has made it possible to manipulate cortisol concentrations *in vivo* but in general the concentrations of cortisol produced are pharmacological (*i.e.* high) rather than physiological. Some surgical procedures that can be used to manipulate endogenous production of cortisol include hypophysectomy (Donaldson and McBride 1967) and adrenalectomy (Butler *et al.* 1969). However, both these procedures involve the stress associated with major surgery and the former results in the removal of all pituitary hormones, the latter in possible electrolyte imbalance (Vijayan *et al.* 1994). Alternatives include the administration of pharmacological drugs to inhibit cortisol secretion which include synthetic corticosteroids (Leatherland 1985; Pickering *et al.* 1987b) and adrenal enzyme blocking drugs (Fagerlund *et al.* 1968). Metopirone is an example of an adrenal enzyme blocking drug and blocks the final step in the pathway of cortisol synthesis (Williamson and O'Donnell 1969) by binding to cytochrome P-450 in mitochondria. Unfortunately, metopirone may cause increases in products such as 11-

deoxycortisol and deoxycorticosterone which may interfere with experiments (Eros and Milligan 1996). The use of synthetic corticosteroids such as dexamethasone to explore the physiological roles of cortisol can be limited by the cortisol-like effects of these compounds on other steroid-sensitive tissue (Pickering *et al.* 1987b).

An alternative method of controlling the effects of endogenous concentrations of plasma cortisol is the use of cortisol receptor blockers. The steroid analogue RU486 (11β-[4-dimethylaminophenyl]-17β-hydroxy-17α-[prop-1-ynyl]-estra-4,9-dien-3-one) was developed by the French pharmaceutical company Roussel-Uclaf (Vijayan and Leatherland 1992) and is known to be a strong antiglucocorticoid and antiprogesterone steroid. In mammals it binds to cytosolic glucocorticoid receptors without any agonistic effects (Moguilewsky and Philibert 1984). *In vitro*, RU486 is known to block the cortisol-mediated inhibition of $^{3}$H-thymidine incorporation into DNA in trout fibroblasts (Oostrom and Bols 1991). It is also known to bind to cytosolic cortisol receptors in rainbow trout liver preparations (Pottinger 1990).

In an experiment by Vijayan and Leatherland (1992), brook charr were given RU486 using a regular intraperitoneal injection protocol to investigate the *in vivo* effects of RU486. Due to the handling stresses involved with the protocol, the effects of RU486 on plasma cortisol concentrations could not be established satisfactorily. However, RU486 administration suppressed hepatic 5`-MD activity and hepatic $T_3$ content which would suggest that blocking of cortisol receptors reduced the ability of the liver to convert $T_3$ to $T_4$, and therefore this process is likely to be cortisol mediated. RU486 also inhibited the

stressor-related elevation of plasma glucose concentrations and provided evidence that RU486 is a useful tool in examining the effects of cortisol on fish physiology.

The approach of intraperitoneal slow-release implants instead of repeated injections was utilised by Vijayan *et al.* (1994) to investigate further the effect of RU486 on cortisol concentrations. The use of implants as opposed to injections meant that the stresses involved with regular handling could be avoided. However, it was found that there was no effect of RU486 on cortisol concentrations and it was suggested that there may be a complex interaction between RU486 and the HPI axis in fish, unlike in mammals where RU486 is known to cause an increase in cortisol concentrations due to altered negative feedback control of hormone release from the adrenal cortex (Gaillard *et al.* 1985). It was also demonstrated that RU486 significantly increased *in vitro* hepatocyte glycogen breakdown and blocked the cortisol-elicited increases in alanine-glyconeogenesis and glycogen utilisation for endogenous use.

Another experiment utilising implants of RU486 was carried out by Reddy *et al.* (1995). Here four treatments were used in both fed and fasted rainbow trout, namely oil only implants, cortisol implants, RU486 implants and cortisol + RU486 implants. Firstly, changes in cortisol over time were investigated in the four treatments. In the cortisol and cortisol + RU486 treatments, cortisol concentrations were elevated 3 days after administration of the implants. In the RU486 treatment cortisol concentrations were depressed after 3 days. By day 7, cortisol concentrations in the RU486 treatment were still lower than those of the cortisol + RU486 group but by day 14 there were no significant

differences in cortisol concentrations between the treatments. In the second part of the experiment the effect of RU486 on the cortisol response of fish to an acute handling stress was investigated. The same four treatment groups were used and the fish were stressed two weeks after implant administration. However, RU486 did not prevent the stressor-induced cortisol changes in either fed or fasted fish.

Other experiments that have used RU486 to investigate the effects of cortisol on fish physiology have found that the effect of cortisol on metabolic recovery from exhaustive exercise is not linked to the RU486-sensitive cortisol receptor (Eros and Milligan 1996) and that both cortisol-induced apoptosis of B cells and the inhibition of neutrophil apoptosis by cortisol in the immune system of the common carp, *Cyprinus carpio* are receptor mediated (Weyts *et al.* 1998a, b). Thus, although RU486 is a relatively new technique in determining the physiological effects of cortisol it has already been used successfully in many experiments.

The literature available on the stress response in fish is vast, and yet much remains to be understood about this complex concept. The majority of work has concentrated on either physical or behavioural stressors and these experiments have mainly been carried out under laboratory conditions with little, if any, reference to the conditions normally experienced by fish in their natural environment. Little is known about the possibility of chloride cell proliferation in the absence of an ionoregulatory disturbance and the possibility of epithelial thickening occurring during other stressors involving elevation of plasma cortisol concentrations. Also, the effect of behavioural interactions occurring under

natural conditions on the physiology of fish is not well understood. This thesis therefore examines some of these unresolved questions regarding stress physiology in fish.

Approximately two thirds of scientific research on freshwater teleosts has been carried out on fish from the order Salmonidae (Barton and Iwama 1991). Salmonid fish therefore make an appropriate choice for the present study as a great deal of background information on their behaviour and physiology is available. The two main salmonids used in the present study were the rainbow trout, *O. mykiss* (Walbaum), and the brown trout, *S. trutta* (L.). Although these species are not exclusively fresh water fish, the strains used in the present study experienced only fresh water environments. Migration from fresh water to sea water occurs in the life history of many salmonid species (Elliott 1994) but studies on migratory fish must take into account physiological changes that occur during this transition. As there is a possibility that some hormones involved in the stress response are also involved in seawater adaptation in anadromous salmonids (Patiño *et al.* 1987; Madsen 1990a), the use of migratory fish was avoided.

## 1.8 The rainbow trout and the brown trout

The rainbow trout is a species of fish originally native to the eastern Pacific Ocean and fresh waters from north west Mexico to the Kuskokwin River, Alaska (Scott and Crossman 1973), but has now been artificially introduced throughout the world. Although rainbow trout are found in similar habitats to brown trout, they are generally able to withstand higher temperatures and grow faster (Wheeler 1969). Rainbow trout can be

exclusively fresh-water dwelling or may be anadromous, migrating from fresh water to sea water, and the common names for these fish vary, with those remaining in fresh water known as rainbow trout and anadromous examples known as steelhead trout (Scott and Crossman 1973).

There are about 50 named forms of brown trout with 10 located in the British Isles. All were grouped together by Regan (1911) as one polytypic species but, in some cases, they are divided into those that migrate to sea, *Salmo trutta trutta* (sea trout), and those that do not, *S. trutta fario*. Essentially a European species, the brown trout is found as far north as Iceland, Scandinavia and Russia and as far south as the northern coastline of the Mediterranean sea and the islands of Corsica and Sardinia (Elliott 1994). Anadromous brown trout populations occur in western Europe and can be found in many areas including the Firth of Clyde (Mackay and Doughty 1986). At least 24 other countries outside of Europe now have introduced populations of brown trout (Elliott 1994) and so successful has been the introduction of brown trout world-wide that most areas capable of supporting populations have received them (MacCrimmon and Marshall 1968).

Using the rainbow and brown trout as the study species, the aim of this project was to examine the complex interrelationships between behaviour and physiology, using responses to stress as a probe. Specifically, the objectives were:

- To investigate further the effects of social stress by manipulating pairs of fish under laboratory conditions; in particular to look at the effects on respirometry, the stress response and also plasma cortisol concentrations over time.
- To assess the physiological effects of social stress within hierarchies of salmonids held under semi-natural conditions.
- To examine the effect of environmental perturbations on social hierarchies formed under semi-natural conditions and the potential consequences for physiological function.
- To investigate the potential for chloride cell proliferation in response to stressors other than ion-deficient water.

# CHAPTER 2

## Chapter 2: Plasma Cortisol Concentrations Before and After Social Stress in Rainbow Trout and Brown Trout.

A version of this chapter has been submitted to Physiological and Biochemical Zoology with co-authors: Neil B. Metcalfe, Alan C. Taylor and Kathleen M. Gilmour.

### 2.1 Abstract

The present study examines the relationship between plasma cortisol concentration and development of social hierarchies in fish, by means of two related experiments. In the first, rainbow trout, *Oncorhynchus mykiss*, and brown trout, *Salmo trutta*, were observed for dominance interactions when confined within single-species pairs for 4, 48 or 168 hours. Subordinate members of a pair exhibited significantly higher cortisol concentrations than dominant and single fish, but the pattern of cortisol elevation differed between the two species, being quicker to rise, and increasing to a higher level, in rainbow trout. Cortisol concentrations were correlated with behavioural measurements; the more subordinate the behaviour exhibited by a fish, the higher its cortisol concentration. Social stress was a chronic stressor and no acclimation to social status occurred during the week. In the second experiment, measurements of plasma cortisol were made prior to pairing of rainbow trout and then after 48 h of confinement in pairs. Subordinate fish demonstrated significantly higher concentrations of plasma cortisol both before and after social stress and it therefore appears that, in addition to cortisol being elevated during periods of social

stress, there may be an association between initial and cortisol levels and the likelihood of a fish becoming subordinate.

## 2.2 Introduction

The primary response of fish to stress includes the activation of the hypothalamo-pituitary-interrenal (HPI) axis, resulting in the release of stress hormones. The glucocorticosteroid cortisol is one of the most commonly measured hormones used as an indicator of stress (Pickering 1993b). Blood plasma cortisol concentrations in unstressed fish are typically 0-5 ng $ml^{-1}$ (Pickering and Pottinger 1989), but may be elevated 20-fold or more during periods of stress. Many studies have investigated the changes in plasma cortisol concentrations over time in response to various physical stressors (e.g. Barton *et al.* 1980; Pickering *et al.* 1982), and the extent and duration of cortisol elevation has been shown to influence the type of secondary responses to stress exhibited.

An immediate and dramatic increase in plasma cortisol concentration, as demonstrated by Strange *et al.* (1978) in juvenile chinook salmon (*Oncorhynchus tshawytscha*), is generally considered to be caused by an acute stressor, *i.e.* a severe but short-lived period of stress. For example, Pickering and Pottinger (1989) demonstrated that the extent of cortisol elevation in response to an acute stressor varied between brown trout and rainbow trout but in both cases, an acute stressor of handling caused a temporary elevation of plasma cortisol to concentrations ranging from 40-200 ng $ml^{-1}$ compared with resting concentrations of 1-2 ng $ml^{-1}$. In comparison, chronic stressors, *i.e.* those involving a less

severe but extended period of stress, characteristically involve a more prolonged but less extreme cortisol elevation. For example, prolonged confinement or crowding can increase cortisol concentrations to 10ng $ml^{-1}$ in rainbow trout and cortisol may remain elevated for up to 4 weeks before acclimation to confinement occurs (Pickering and Pottinger 1989). These studies illustrate that the duration and severity of the stress are of great importance in determining the nature of the cortisol response itself, as well as its physiological consequences (Pickering 1992).

Because the pattern of cortisol elevation (the primary stress response) affects the physiological manifestation of the stressor (*i.e.* secondary and tertiary stress responses), cortisol responses to a variety of different environmental stressors have been investigated. However, less is known about the pattern of cortisol responses to behavioural stressors. It has been demonstrated previously that confinement of fish in pairs generally results in one fish becoming dominant over the other (subordinate) fish (e.g. Peters *et al.* 1980, 1988; Pottinger and Pickering 1992; Sloman *et al.* 2000a (Chapter 3)) and that, characteristically, the subordinate fish exhibits an increase in circulating cortisol concentrations. The subordinate fish may also exhibit other physiological changes including weight loss (Pottinger and Pickering 1992), reduced disease resistance, and an increase in plasma glucose concentrations (Peters *et al.* 1988). Previous studies have examined cortisol elevation for social stress lasting a few hours or several weeks, but data on the elevation of cortisol during the first few days of social stress remain sparse.

Previous studies have investigated the characteristic elevation of cortisol in subordinate fish as a result of social stress but whether cortisol concentrations also play a role in the determination of dominance remains unstudied. Huntingford *et al.* (1990) suggested that social status in salmonids may depend on behavioural properties rather than size. Resource holding power (RHP, Parker 1974) is a measure of the absolute fighting ability of an individual. Whereas body size may appear to be the best indicator of RHP, during social interactions it may not always be a reliable indicator if physiological states are taken into account (Beaugrand *et al.* 1996). Particularly within size-matched pairs the factors for determining social status remain unknown. There is some evidence that standard metabolic rate (SMR) may be a possible indicator of dominance in juvenile Atlantic salmon, *Salmo salar* (Metcalfe *et al.* 1995). Winberg *et al.* (1991) suggested that high serotonin activity in the brain of Arctic charr, *Salvelinus alpinus*, may be a precursor for subordinance, but in a subsequent study concluded that high serotonin concentrations were in fact an effect of subordination rather than a cause (Winberg *et al.* 1992). In size-matched pairs of salmonid fish, it is possible that the prior physiological condition of each fish predetermines its social status. Elevated cortisol concentrations may be symptomatic of poor physiological condition and/or may result themselves in physiological disadvantage. It is also possible that increased cortisol concentrations may be due to prior experience of being subordinate. Thus, the hypothesis that the fish exhibiting the higher plasma cortisol concentration would become subordinate when paired with a size-matched conspecific was tested in the present study. Therefore, the aims of the present study were to monitor the pattern of cortisol elevation elicited by social stress and also to investigate whether an

association exists between cortisol concentrations prior to pairing and subsequent social status.

## 2.3 Methods

### *2.3.1 Experiment 1 - Time course of circulating cortisol concentrations*

Rainbow trout (weight 71.61 ± 2.2 g, length 18.9 ± 0.18 cm, mean ± S.E.M., n = 79) were obtained from College Mill Trout Farm, Perth (Scotland) while brown trout (weight 49.48 ± 1.44 g, length 16.7 ± 0.14 cm, mean ± S.E.M., n = 86) were obtained from Howietoun fish farm, Stirling (Scotland). The fish were held in 70 L stock tanks in aerated, flowing, dechlorinated tap-water at 12 L: 12 D light and 12.3 ± 0.09 °C temperature conditions. After four weeks acclimation to the laboratory conditions, fish were anaesthetised in a solution of benzocaine (0.05 mg benzocaine $ml^{-1}$ water) and given unique combinations of alcian blue dye marks (Kelly 1967) injected into their fins. One week later, fish were allocated to one of two treatments; either single fish (n = 30 for each species), or size-matched conspecific pairs (size difference, rainbow 0.24 ± 0.04 cm, n = 30; brown trout 0.26 ± 0.04 cm, n = 30 (mean ± S.E.M.)).

Those fish held singly were placed into 33.5 L glass aquaria, while paired fish were placed in 67 L glass aquaria so that the volume of water per fish was constant. Water quality was closely monitored and the water constantly aerated by box filters. Paired fish were initially kept separated from each other by an opaque Foamex® partition which divided the tank in half. After 48 h, the Foamex® partitions were removed from the tanks containing paired

fish and the fish were then observed for the subsequent four hours and scored for behavioural interactions (see below for details). A similar piece of Foamex® was also removed from the tanks containing single fish so that all fish were exposed to a similar level of disturbance. Tanks within each experimental treatment were allocated randomly to one of three sampling times - 4 h, 48 h and 168 h after the removal of the partitions. Once the behavioural observations were completed, paired and single fish allocated to the 4 h treatment were terminally sampled while PVC tube refuges were added to aquaria of fish allocated to the 48 h and 168 h treatments to allow subordinates to evade the dominants. Fish in these aquaria were terminally sampled 44 or 164 h later as appropriate. During the experiment, fish were fed to satiation on bloodworm twice daily. Although diet type and/or fasting are not believed to have a significant effect on the response of cortisol concentrations to stress (Barton *et al.* 1988), a gap of at least 4 h was always left between feeding and sampling.

*2.3.2 Experiment 2 - Cortisol concentration as a predictor of social status*

Rainbow trout (weight 217.64 ± 10.63 g; length 27.5 ± 0.26 cm (mean ± S.E.M.) n = 36) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). The fish were held in a two large stock tanks (1250 L) supplied with aerated, flowing, dechlorinated City of Ottawa tap-water at a temperature of 13 °C. A 12L:12D photoperiod was utilised. After at least four weeks acclimation to the laboratory conditions, fish were anaesthetised in a solution of benzocaine and given unique combinations of alcian blue dye marks injected into their fins. A blood sample of 0.5 ml was withdrawn by caudal venipuncture.

Following a 48 h recovery period in a holding tank, fish were allocated to size-matched pairs (size difference, 0.47 ± 0.13 cm (mean ± S.E.M.), n = 18 pairs).

Pairs of fish were held in aerated, flowing, dechlorinated City of Ottawa tap-water (33 L). Behavioural observations were carried out on the paired fish for 48 h and the fish were then terminally sampled, with the exception of two pairs of fish which were sampled after 24 h due to the obvious increasing distress of the subordinate. During the experiment fish were fed to satiation on pellets twice daily, following behavioural observations.

*2.3.3 Behavioural Measurements*

Behavioural observations were carried out continuously during the first 4 h of pairing in experiment one and twice daily in experiment two. Dominance was measured by assigning points, a method that has been used previously for determining social status among salmonids (e.g. Metcalfe *et al.* 1989; Johnsson *et al.* 1996; Sloman *et al.* 2000a, b (Chapters 3 and 8)). In experiment one (time course study), fish that chased and attacked another fish scored one point for each attack. However, since fights to establish dominance were always concluded within the initial 4 h period of confinement in experiment 1 and overt aggression was seen to be rare, social interactions were not scored in experiment two (cortisol as a predictor of social status). Fish were also scored according to their position in the tank for both experiments. Fish that maintained their position in the water column scored 10 points, fish that rested on the bottom of the tank scored 5 points, and those fish that either attempted to hide or swam at the water surface (indicative of subordinance, Sloman *et al.* 2000a (Chapter 3)) scored zero points. In

experiment two, the additional behavioural observation of food acquisition was included; the first fish to take a food item introduced to the tank scored one point and the other fish scored zero points. Although different behavioural parameters were measured in the two experiments due to differences in the experimental protocol, in both cases these behavioural scores allowed clear determination of which fish became dominant and which subordinate.

*2.3.4 Physiological Measurements*

When the fish were terminally sampled, they were killed rapidly by immersion in a lethal dose of anaesthetic (benzocaine; 0.5 mg $ml^{-1}$). Paired fish were killed within one minute of each other, and the sampling order of dominant and subordinate fish was alternated among pairs to control for any sequential sampling effects. All blood samples were removed from all fish (0.5 ml) by caudal venipunture. Blood samples were centrifuged (13,000 g), and the plasma was removed and stored at -70 °C for later analysis of plasma cortisol concentrations using a commercial radioimmunoassay (ICN Pharmaceuticals Ltd).

*2.3.4 Statistical Methods*

The social interactions and position scores for paired fish were processed using a principal components analysis (PCA), which combined the different measurements (weighting them according to the extent to which they correlated with the derived principal axis) and so generated an overall behaviour score for each fish (Sloman *et al.* 2000a, b (Chapters 3 and 8)). The PCA results allowed each fish to be classified as either dominant or subordinate, the fish with the highest score within each pair being the dominant and the fish with the

lower score being the subordinate fish. In the first experiment (time course study), cortisol concentrations among the status categories of fish (dominant, subordinate or single), time periods (4, 48 or 168 h) and species (rainbow trout vs. brown trout) were compared using analysis of variance (ANOVA) models. In experiment two (cortisol as a predictor of social status), cortisol concentrations in dominants and subordinates before and after pairing were compared using both ANOVA and paired t-test analyses. Cortisol concentrations were normalised by means of a logarithmic transformation due to the wide variety in cortisol values between pairs of fish. Data are presented as means ± S.E.M. The fiducial limit of significance in all analyses was 5 %.

## 2.4 Results

### *2.4.1 Experiment 1- Time course of circulating cortisol concentrations*

Rainbow trout exhibited significantly higher levels of aggression than brown trout during the four hours after being first placed in pairs (ANOVA: $F_{1,106} = 20.46$, $P < 0.001$; mean number of aggressive interactions per pair per 4 h: rainbow trout = 6.92 ± 0.82; brown trout = 0.06 ± 0.02). Fighting between paired rainbow trout was very characteristic (occurring in 67 % of pairs), and generally varied only with respect to when it started. In some pairs, fighting started as soon as the partition was removed whilst in others a short delay was observed before fighting commenced. Fights started with both fish circling the tank, followed by interactions. In most cases, aggressive interactions were only initiated by one member of the pair and dominance was quickly established. The subordinate fish would then retreat to either the bottom of the tank or to the water surface. In 4% of pairs

of rainbow trout, both fish carried out aggressive acts. By contrast, interaction was minimal between pairs of brown trout, with attacks only being observed in 7 % of pairs. In general, after a period of movement by both brown trout around the tank, dominance was established even in the absence of aggression, and one fish (the subordinate) would take refuge.

In both species, there was a significant effect of social status on plasma cortisol concentrations, with subordinate fish having higher concentrations of plasma cortisol than dominant or single fish (three-way ANOVA: $F_{2,17} = 35.58$, $P < 0.001$). Sampling time, on the other hand, had no significant effect on plasma cortisol concentrations in either species ($F_{2,17} = 1.167$, $P > 0.1$). A significant 2-way interaction between species and status (subordinate, dominant or single) ($F_{2,17} = 3.371$, $P < 0.05$) showed that the effect of status on the elevation of cortisol concentrations was different in rainbow and brown trout.

Figure 2.1a demonstrates that the cortisol concentrations of subordinate rainbow trout were already greatly elevated after 4 h and remained much higher than those of dominant and single fish for the whole experiment. In contrast, the differences in cortisol concentrations among categories of brown trout were less pronounced and were slower to become established, being greatest after 48 h (Fig. 2.1b).

For paired fish of both species, there was a correlation between the behaviour score on the principal components analysis and plasma cortisol concentrations: the lower the score (and hence the more subordinate the behaviour of the fish), the higher the concentration of cortisol (Fig. 2.2); this relationship was significant (covariance analyses; effect of covariate

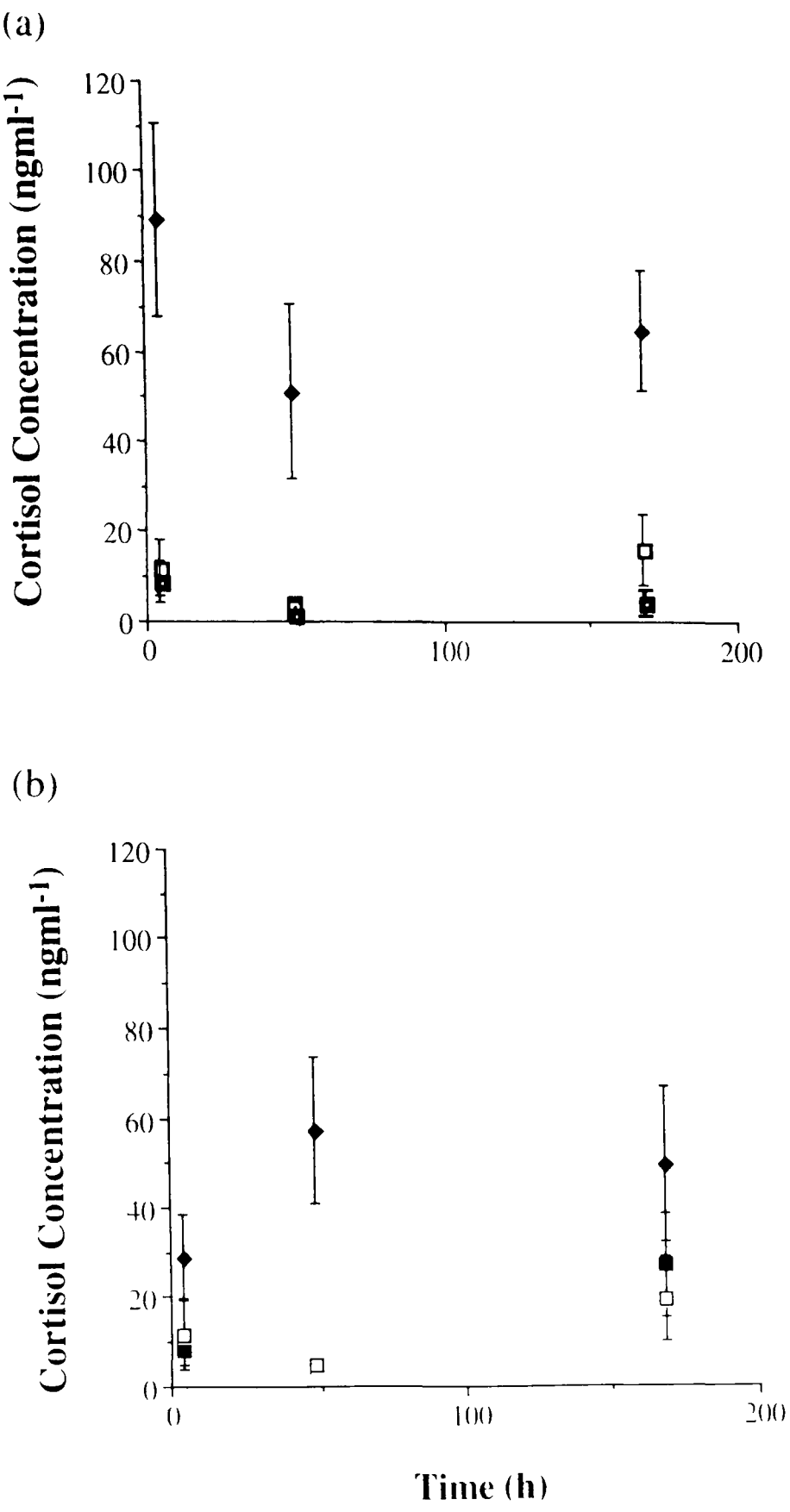



Figure 2.1: Plasma cortisol concentrations as a function of time of sampling in □ dominant, ◆ subordinate and ■ single rainbow trout (a) and brown trout (b). Data are presented as means ± S.E.M. (rainbow trout: 4 h, N = 8-9; 48 h, N = 9-10; 168 h, N = 8; brown trout: 4 h, N = 9-10; 48 h, N = 7-10; 168 h, N = 10).

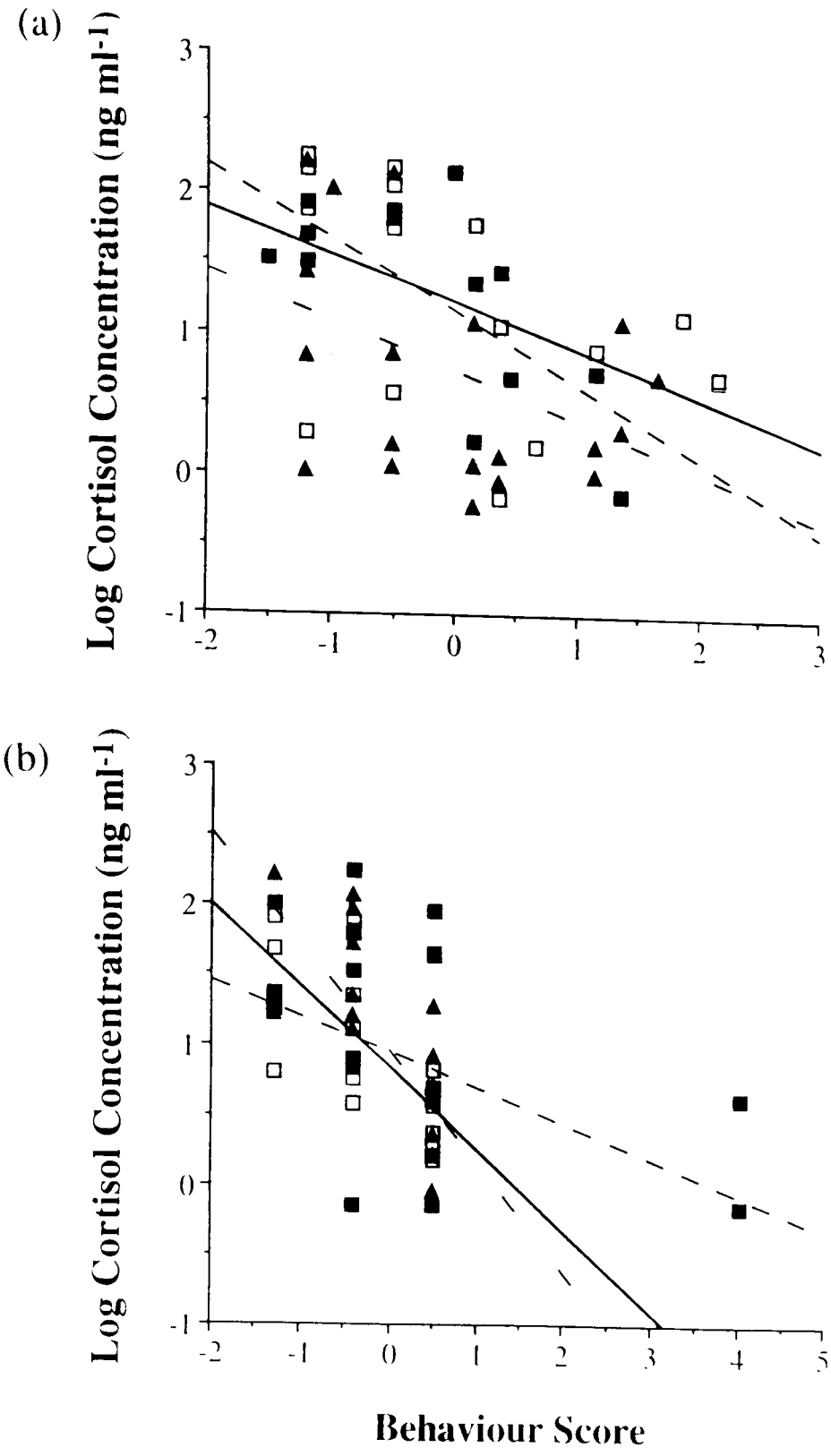



Figure 2.2: Relationship between behaviour score and plasma cortisol concentration in (a) rainbow trout and (b) brown trout after 4 h (□), 48 h (▲), 168 h (■) since the formation of pairs. Higher positive behaviour scores indicate more dominant fish. Behaviour score is inversely correlated with cortisol concentration; although separate regression lines are shown for each sampling time there was no effect of sampling time on this relationship (see text for analysis). Solid line = 4 h; wide spaced dashed = 48 h; narrow spaced dash = 168 h.

$F_{1,46} = 16.57$, $P < 0.001$; $F_{1,54} = 10.48$, $P < 0.05$ for rainbow and brown trout respectively). There was no significant effect of sampling time on the relationship between behaviour score and cortisol concentration in either species (rainbow trout: $F_{2,46} = 1.63$, $P > 0.1$, brown trout: $F_{2,54} = 1.54$, $P > 0.1$).

*2.4.2 Experiment 2 - Cortisol concentration as a predictor of social status*

Analysis of plasma cortisol concentrations in rainbow trout before and 48 h following confinement in size-matched pairs indicated that there was a significant effect of rank on cortisol elevation (Repeated measures ANOVA: $F_{1,34} = 12.24$, $P < 0.005$). However there was no interaction with time (repeated measures ANOVA: rank*time interaction: $F_{1,34} = 0.004$, $P > 0.1$) suggesting that there was a significant effect of rank on cortisol concentrations both before and after pairing. Subordinate fish had significantly higher plasma cortisol concentrations both prior to (paired Student's t-test: $t = -2.302$, $P < 0.05$, $n = 17$), and following confinement in pairs (paired Student's t-test: $t = -2.676$, $P < 0.05$, $n = 17$) (Fig. 2.3). For the blood plasma cortisol samples taken before pairing, there was no significant sampling effect even though all fish could not be sampled simultaneously (linear regression: $F_{1,36} = 0.313$, $P > 0.5$).

## 2.5 Discussion

The present study examined the effect of social status on the elevation of blood plasma cortisol concentrations in both rainbow and brown trout. Previous studies have investigated the patterns of cortisol elevation induced by physical stressors (Barton *et al.*

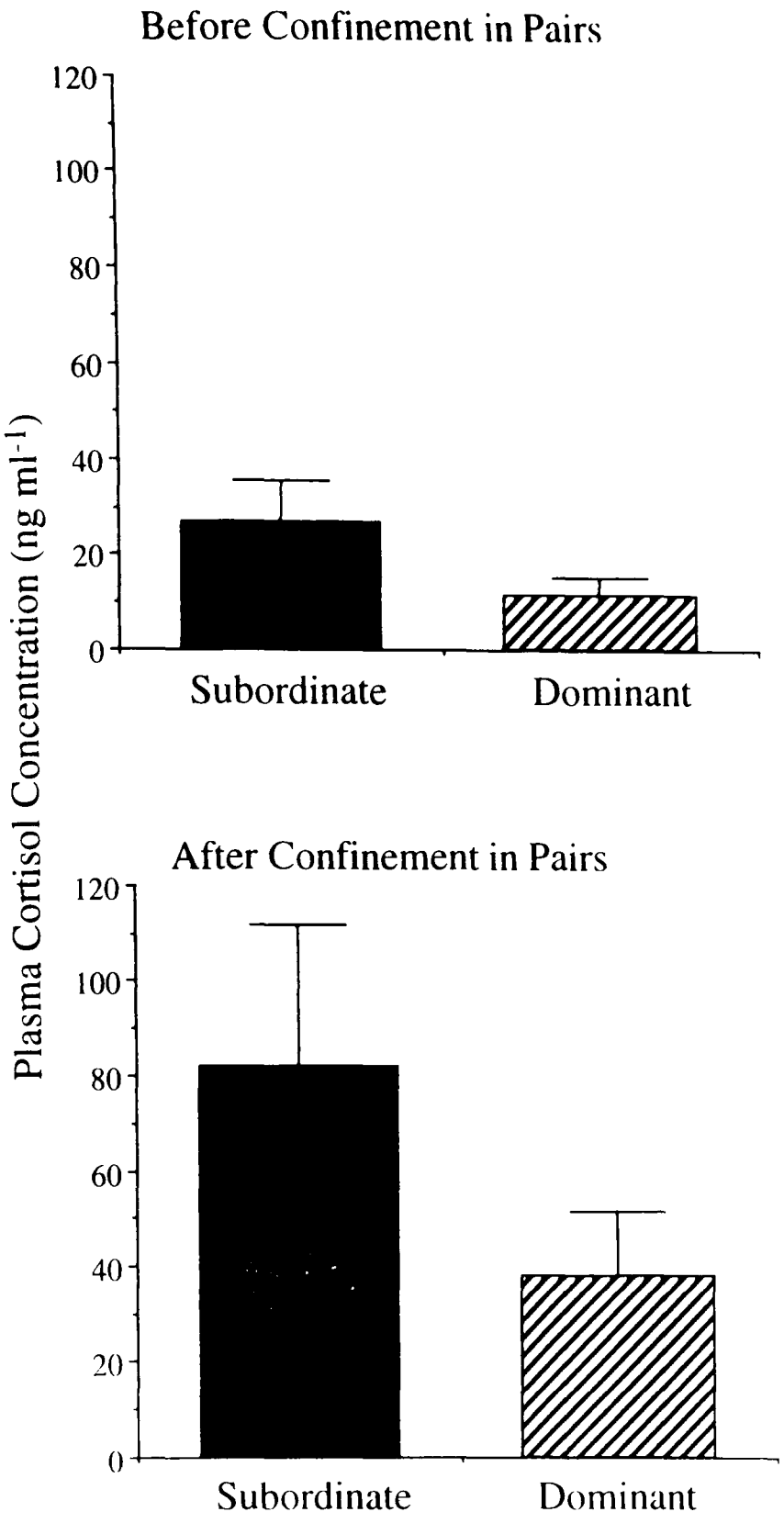



Figure 2.3: Plasma cortisol concentrations for before and after confinement in pairs. ■ = subordinate, □ = dominant. Data are presented as means ± S.E.M. (n = 18 fish per social status).

1980; Pickering *et al.* 1982; Einarsdóttir and Nilssen 1996), but to our knowledge the present study is the first to look at both the concentrations of cortisol before pairing and the time trajectory of cortisol concentrations for the duration of one week after the imposition of social stress. In the first experiment (time-course study), the plasma cortisol concentrations of subordinate fish were significantly elevated over those of dominant and solitary fish. Previous studies have also demonstrated that subordinates characteristically have higher plasma cortisol concentrations than dominant fish (Peters *et al.* 1980; Laidley and Leatherland 1988; Sloman *et al.* 2000a (Chapter 3)). It is noteworthy, however, that in the present study, the time of sampling had no significant effect on cortisol concentrations. This result implies that subordinate fish remained stressed for the entire experimental period and that no acclimation to social status occurred.

The pattern of cortisol elevation exhibited by both subordinate rainbow and brown trout in the present study appears to be characteristic of the response to a chronic stressor, in that a relatively low but continuous elevation of plasma cortisol was observed. Øverli *et al.* (1999) carried out a similar but shorter time course study in paired rainbow trout. They found that an increase in cortisol concentration to around 35 ng $ml^{-1}$ occurred in both dominant and subordinate fish within minutes of the end of aggressive interactions. After 3 h, however, cortisol concentrations in subordinate fish remained elevated, whilst those in dominant fish had returned to basal levels.

Although subordinate fish of both species had higher cortisol concentrations than dominants, there was a significant interaction between species and status indicating that

the pattern of cortisol elevation was not identical in subordinate rainbow and brown trout. The difference in the pattern of cortisol elevation appears to reflect the differences in behaviour between the two species. Rainbow trout were significantly more aggressive than brown trout; dominant rainbow trout carried out more aggressive acts on the subordinate with which they were paired than was the case for dominant brown trout. Figure 2.1 demonstrates that the elevation of plasma cortisol concentrations in subordinate rainbow trout was correspondingly greater and more rapid than in the brown trout.

The direct link between status and cortisol concentration is further indicated by the inverse correlation between behaviour scores and blood plasma cortisol concentrations. Thus, cortisol concentrations were not simply a reflection of categories of status, but were related to the extent to which each dominant fish suppressed its subordinate: the more suppressed the behaviour of the subordinate, the higher its plasma cortisol concentration. This relationship between behaviour and cortisol was not affected by the time of sampling, again suggesting that the pattern of cortisol elevation was characteristic of the response to a chronic stressor. Previous studies have also demonstrated a relationship between behaviour and plasma cortisol concentration (Sloman *et al.* 2000a (Chapter 3)).

Physiological and behavioural effects of social hierarchies in brown trout held in groups of four under semi-natural conditions have also been investigated previously and it has been found that, after two weeks, plasma cortisol concentrations of dominant fish were not significantly different from those of subordinates (Sloman *et al.* 2000b). It is possible that the pattern of cortisol elevation resulting from fish confined in pairs is different to that of

fish held in groups. The subordinate of a pair of fish is subjected to constant, chronic stress due to the continual presence of a dominant fish. Within a group of four fish there is likely to be a brief period of stress, *i.e.* an acute stress, during the establishment of the hierarchy only, after which subordinate fish have a greater opportunity to avoid the dominant fish. However, further work is needed to establish the pattern of cortisol elevation among groups of more than two fish.

The present study is among the first to examine the association between cortisol concentrations before pairing and resultant social status. In the present study, subordinate fish demonstrated higher plasma cortisol concentrations both before and after pairing suggesting that the fish with the higher cortisol concentration became subordinate. The metabolic consequences associated with elevated cortisol have long been known and therefore it is perhaps not surprising that elevated cortisol levels seem to be linked with subordinance. Gregory and Wood (1999) demonstrated that administration of cortisol had significant effects on individual appetite, growth rate, condition factor and food conversion efficiency and that the ability of a fish to compete with its conspecifics may be influenced by the initial condition of the fish. Also, Barton *et al.* (1987) found that the chronic administration of cortisol in rainbow trout elicited deleterious effects on growth, condition and circulating lymphocytes.

Clearly, prolonged elevation of circulating cortisol concentrations has a deleterious impact on physiological condition. Equally, poor physiological condition may be reflected in elevated circulating cortisol concentrations. In either case, poor physiological condition

seems likely to predispose a fish for lower social status during interactions with size-matched conspecifics. Therefore, any differences in circulating cortisol concentrations of the fish before social interaction may affect the outcome of such interactions and hence social status, implicating elevated cortisol concentrations as a predictor of subordinance. All of the fish in the present study were taken from the same holding environment and were subjected to the same conditions throughout the experiment, and no affect of sequential sampling was seen. Therefore, it is suggested that variations in physiological conditions and/or cortisol concentrations among both natural and aquacultural populations of salmonid fish significantly affect social interactions and the formation of dominance hierarchies. Whether elevated cortisol itself or poor condition resulting in elevated cortisol levels is the critical factor remains to be ascertained.

In summary, after the occurrence of social interaction, the stress imposed on a subordinate fish by the presence of a dominant fish is characteristic of a chronic stressor, and cortisol elevation occurs within the first four hours of confinement and is maintained for the remainder of a one week period. Socially-induced elevation of plasma cortisol concentrations was also demonstrated to vary between salmonid species. There exists an association between high cortisol concentrations pre-pairing and subordination suggesting that cortisol may indeed be a predictor of subordinance.

# CHAPTER 3

# Chapter 3: Does Socially-Induced Stress in Rainbow Trout Cause Chloride Cell Proliferation?

A version of this chapter is published in the Journal of Fish Biology, 56: 725-738 with co-authors: Kathleen M. Gilmour, Neil B. Metcalfe and Alan C. Taylor.

## 3.1 Abstract

Rainbow trout, *Oncorhynchus mykiss* (Walbaum), were confined in pairs for a period of two weeks to allow a dominant/ subordinate relationship to develop. At the end of the two week confinement period, the subordinate trout had significantly higher circulating concentrations of plasma cortisol than did the dominant fish with which they were paired. Physiological effects linked to elevated plasma cortisol concentrations in subordinate fish included loss of weight and a lowering of condition factor. However, there were no significant differences in gill epithelium chloride cell numbers or blood plasma ion concentrations between dominant and subordinate fish. It is concluded that elevated plasma cortisol concentrations elicited by the social stressors of the present study did not cause proliferation of chloride cells.

## 3.2 Introduction

Cortisol is a glucocorticosteroid hormone synthesised and released from fish interrenal cells in the head kidney as a ubiquitous response to stress. Elevation of the stress

hormone, cortisol, in the blood plasma of fish has been demonstrated to have a variety of physiological effects, including decreases in body weight and condition factor (Davis *et al.* 1985), hepatic glycogen content (Peters *et al.* 1980), and circulating lymphocytes and increases in blood glucose and haematocrit (Barton *et al.* 1987). Such changes are thought to enable the fish to access energy reserves during acute stresses but may be maladaptive during chronic stress owing to the suppression of immune function and continued mobilisation of energy reserves (Pickering 1993a).

Elevated plasma cortisol concentrations have also been implicated in the proliferation of chloride cells in the gill epithelium that occurs in response to ionoregulatory and/or acid-base disturbances in freshwater teleosts (reviewed by Perry 1997, 1998). Chloride cell proliferation is usually associated with an ion deficiency in the fish's environment (e.g. Greco *et al.* 1995, 1996), with acid-base disturbances (e.g. Goss *et al.* 1992), or with exposure to toxicants (Leino *et al.* 1987). It has been suggested that such increases in chloride cell density may be cortisol-mediated since injections of cortisol (at supraphysiological concentrations) cause chloride cell proliferation (Bindon *et al.* 1994a, b). Indeed, cortisol injections are routinely used as an experimental tool to induce chloride cell proliferation (e.g. Doyle and Epstein 1972; Laurent and Perry 1990; Madsen 1990b; Laurent *et al.* 1994). Additionally, transient increases in plasma cortisol concentrations have been documented during soft-water exposure in rainbow trout (Perry and Wood 1985; Perry and Laurent 1989).

Chloride cell proliferation is an adaptive response with respect to ionoregulation. The increase in chloride cell density translates into an increase in the ion transporting capacity of the gills (Bindon *et al.* 1994a) that is beneficial during acclimation to ion-deficient water. However, chloride cell proliferation also impairs respiration due to consequent thickening of the blood-to-water diffusion barrier (Bindon *et al.* 1994b; Greco *et al.* 1995, 1996). The fish can compensate for the impairment of gas transfer by hyperventilation (Perry *et al.* 1996), but consequently there is an increase in the energetic cost of respiration and the normal hyperventilatory responses to exercise and environmental disturbances, such as hypoxia, are reduced or prevented from occurring (Perry 1997).

Given that chloride cell proliferation during soft-water acclimation and other ionoregulatory disturbances is cortisol-mediated, then it is possible that other stressors causing elevation of plasma cortisol concentrations, such as those induced by social interactions and stocking density, might also cause chloride cell proliferation. Such increases in chloride cell density, in the absence of an ionic disturbance, could be detrimental to respiratory function and reduce the ability of the fish to cope with additional environmental stressors. Thus the present study was designed to test the hypothesis that social-stress-induced elevation of plasma cortisol concentrations can cause chloride cell proliferation in the gill epithelium of freshwater rainbow trout in the absence of ionoregulatory challenge.

Fish of many species establish dominance hierarchies when living in small groups (Noakes and Leatherland 1977), and when confined in pairs one fish will become dominant over the

fish with which it is paired, the subordinate (Pottinger and Pickering 1992). The establishment of a dominance hierarchy constitutes a 'social' stress that can elicit a marked elevation of plasma cortisol in the subordinate fish (Ejike and Schreck 1980; Pottinger and Pickering 1992). Although elevation of plasma cortisol concentrations in subordinate fish may not be as high as elicited by a more acute stressor, e.g. air exposure, current research (Sloman *et al.* submitted (Chapter 2)) suggests that cortisol concentrations are significantly elevated during initial confinement in pairs. It has also been found that confinement in pairs can lead to the death of a subordinate fish (Peters *et al.* 1980) suggesting that social stress can be very severe. If chloride cell proliferation occurs as a result of socially-induced elevation of plasma cortisol concentrations then there is the potential for the respiratory function of subordinate fish to be seriously impaired, an additional maladaptive consequence of elevated plasma cortisol levels. In the present study, confinement in pairs was used to investigate the hypothesis that social-stress induced elevation of plasma cortisol concentrations can induce chloride cell proliferation in rainbow trout.

## 3.3 Materials and Methods

Rainbow trout (weight 50.5 ± 2.2 g, length 16.8 ± 0.2 cm, mean ± S.E.M., n = 36) were obtained from College Mill Trout Farm, Perth (Scotland). The fish were held in a 70 L circular stock tank under ambient light (15L:9D) and temperature regimes (8.4 ± 0.2 °C) in aerated, flowing, dechlorinated tap-water. After two weeks to acclimate to the laboratory conditions, the fish were anaesthetised in a solution of benzocaine (0.05 mg

benzocaine $ml^{-1}$ water) and given unique combinations of alcian blue dye marks (Kelly 1967) injected into their fins. Two days later the fish were allocated to one of three treatments: paired fish, isolated fish, or grouped fish.

Pairs of size-matched fish were placed in each of six 45 L glass aquaria, while single fish were placed into six 22.5 L aquaria. Vigorous aeration served both to maintain water quality with respect to $O_2$ and $CO_2$ levels, and to circulate the water in the tank. The experimental set-up created a confined environment for the fish, designed to maximise social interaction. Each tank was provided with several rocks and two PVC tubes as refuges for although the aim of the present experiment was to induce stress in the subordinate fish it was intended that the stress level should not result in fatalities. The remaining 18 fish were kept in the 70 L holding tank. The stocking density was therefore 2.55 g $L^{-1}$ for paired and single fish and 14.73 g $L^{-1}$ for fish held in the group. Partial water changeover was carried out every three days using a siphon to slowly remove water and a steady inflow source to replace water at a similar rate. Even though great care was taken to minimise disturbance to the fish, the procedure may have resulted in some elevation of cortisol concentrations but any stress caused would have been at the same level for all fish.

Water ammonia concentrations (Red Sea Fish pHarm ammonia test kit) were closely monitored in the static tanks and were kept at levels similar to those of the stock tank. The concentrations of $Na^+$, $Ca^{2+}$ and $K^+$ ions in the water were also monitored. $[Na^+]$ and $[K^+]$ were measured by flame photometry and $[Ca^{2+}]$ by atomic absorption spectrophotometry. For atomic absorption spectrophotometry lanthanum chloride was added to the water

samples (1 part $LaCl_3$: 50 parts sample) to prevent interference of other ions present. The water chemistry did not vary greatly over the experimental period and was recorded as $[Na^+] = 0.189 \pm 0.007$ mM; $[Ca^{2+}] = 0.155 \pm 0.002$ mM and $[K^+] = 0.038 \pm 0.002$ mM. Twice daily behavioural observations were carried out on the paired fish and then fish in all the experimental treatments were fed to excess (*i.e.* until they had ceased feeding) on bloodworm (chironomid larvae).

*3.3.1 Behavioural measurements*

To determine the dominance hierarchy within the paired fish, several behavioural observations were conducted twice daily for two weeks, and dominance was measured by assigning points. First, at both the morning (10.00-11.00 h) and afternoon (2.00-3.00 h) feeding times, a single bloodworm was initially presented, and the identity of the fish that consumed it was noted. The fish that took the first blood worm was given one point, so the maximum feeding score a fish could obtain was two (morning and afternoon feeding). Secondly, fish were scored on a scale of 0 to 4, in both the morning and afternoon, according to their position in the tank, (Table 3a).

Table 3a. Method used to score pairs of fish according to their positions in the tank. Higher scores are indicative of more dominant behaviour. Fish resting at the bottom of the tank at the front scored higher than those at the back of the tank since they were closer to the point of food entry.

| Behaviour | Score |
|---|---|
| Hiding in refuges. | 0 |
| Swimming at the water-surface. | 1 |
| Resting on the bottom at the back of the tank. | 2 |
| Resting on the bottom at the front of the tank. | 3 |
| Patrolling in the water column. | 4 |

The weighted scoring method was chosen after preliminary behavioural observations, and was based on previous studies of dominance in salmonids (e.g. Metcalfe *et al.* 1989; Johnsson *et al.* 1996). Overall feeding and position scores for each fish in a pair were then calculated as the means of their respective daily scores, and the two types of score were combined into an overall dominance rank for each fish using principal components analysis (PCA).

*3.3.2. Physiological Measurements*

After two weeks in their respective social environments, paired and isolated fish were killed instantly by a blow to the head, a blood sample (0.5-1.0 ml) was withdrawn by caudal venipuncture and gill tissue was removed. One pair of fish was sampled after only 12 days due to the obvious and increasing distress of the subordinate. However, it was possible to identify the subordinate and dominant within this pair at this time. Paired fish were killed within a maximum of one minute of each other (and usually considerably less). To control for any effect of sampling stress, fish sampled first from each alternated

between the subordinate and the dominant. In addition, six fish randomly selected from the grouped fish were also sampled. Sampling all six fish took a total of three minutes. To ensure that this time delay did not affect the blood cortisol concentration, a correlation analysis between cortisol concentration of each fish and the order of sampling was carried out. No significant correlation was obtained ($P > 0.1$) between these two parameters indicating that removal of fish from the tank had no significant effect on the level of stress experienced by the remaining fish. Final fork lengths and weights were recorded to allow calculation of specific growth rates (% weight change per day; Ricker 1979) during the experimental period. Condition factor was calculated as (weight(g)/fork length(cm)$^{3.013}$)x100, where the constant 3.013 was the slope of the regression of log(weight) on log(fork length) ($r^2 = 0.919$; $n = 36$; $P < 0.01$) (Bolger and Connolly 1989).

Blood samples were centrifuged (13,000 g), and the plasma was removed, immediately frozen in liquid nitrogen and stored at -70° C for later analysis of plasma cortisol, $Na^-$ and $Ca^{2+}$ concentrations. A radioimmunoassay (ICN pharmaceuticals Ltd) was used for measurements of plasma cortisol concentrations (Gamperl *et al.* 1994). Plasma [$Na^-$] and [$Ca^{2+}$] were measured using the same methods that were used for analysis of water samples.

To determine chloride cell densities, the second gill arch on the left side of each fish was removed at the time of sampling and washed in 0.9 % saline. Pairs of filaments were then fixed in buffered glutaraldehyde (5 % glutaraldehyde in phosphate buffer; 1 h; 4 °C). The tissue was stained with an osmium-zinc iodide preparation according to the method of

Garcia-Romeu and Masoni (1970) (1 part 2 % $OsO_4$ to 4 parts 3 % $ZnI_2$; 18 h; 20 °C). This stain causes a reduction of osmic acid to osmium, which blackens the phospholipids. Chloride cells have an intricate plasma membrane with many invaginations and thus stain strongly. Following staining the tissues were dehydrated in an ethanol series (30 %, 50 %, 75 %, 95 %, 100 % x 2, 20 minutes in each; 20 °C), rinsed in Histoclear® (2 x 20 minute rinses; 20 °C) and paraffin wax (2 x 20 minute rinses; 55 °C), embedded in paraffin wax (BDH) and sectioned at 7 µm using a microtome (Leitz 1512).

Sections were viewed using a light microscope (40 x objective; Leitz Dialux® microscope) and photographs were taken with an attached camera (Wild Leitz MPS51 camera; Wild MPS45 photoautomat). Twenty slides, with eight sections per slide, were prepared for each fish for a total of 160 sections per fish. In general, 11 photographs were taken per fish from randomly selected sections. For each photograph, the slide was positioned so that the field of view contained approximately seven lamellae and a small portion of the filament at the bases of the lamellae. Photographs were taken from all areas of the gill tissue and also without prior knowledge of the status of the fish so that any data bias was avoided. Chloride cell numbers were quantified by viewing these 11 photographs per fish. For quantification, the gill tissue area in a photograph was digitised using a digitising tablet (BBC Cherry A3 Graphics Tablet). The number of chloride cells per unit tissue area was calculated for each photograph by visually counting the intensely stained cells, and the value for each fish was taken as the mean of the values for the 11 photographs.

### *3.3.3. Statistical Methods*

Data are presented as means ± 1 standard error of the mean (S.E.M.) or as values for individual fish. A principal components analysis (PCA) was carried out on the behavioural scores to provide an overall indication of dominance. Comparisons of physiological parameters among each of the four treatments were accomplished using a one way analysis of variance (ANOVA) followed by a Scheffé test for multiple comparisons. Comparisons of physiological parameters between pairs of fish were accomplished using a Wilcoxon signed ranks test (one-tailed). Linear regression analyses were carried out between behavioural and physiological data.

## 3.4 Results

### *3.4.1 Behaviour*

The principal components analysis carried out on the behavioural data for paired fish indicated that there was a close agreement between the feeding and position scores. Thus, subordinates could be readily distinguished from dominants among the paired fish, for in all cases the fish that had the highest feeding score also had the highest position score (Table 3b).

Table 3b. Behavioural scores for rainbow trout held in pairs. Separate scores were given for feeding behaviour and for position in the tank (see text). A principal components analysis (PCA) combined these scores to give an overall dominance score for each individual fish. Within each pair the dominant was defined as the fish with the highest PCA score.

| Pair Number | Food Score | Position Score | PCA Score | Fish Status |
|---|---|---|---|---|
| 1 | 2.00 | 3.89 | 1.0206 | Dominant |
| | 0.00 | 0.00 | -1.40303 | Subordinate |
| 2 | 1.67 | 3.83 | 0.81035 | Dominant |
| | 0.33 | 1.33 | -0.77884 | Subordinate |
| 3 | 1.50 | 3.58 | 0.63032 | Dominant |
| | 0.50 | 2.08 | -0.43615 | Subordinate |
| 4 | 2.00 | 4.00 | 1.05646 | Dominant |
| | 0.00 | 0.00 | -1.40303 | Subordinate |
| 5 | 2.00 | 3.92 | 1.03038 | Dominant |
| | 0.00 | 1.33 | -0.96954 | Subordinate |
| 6 | 1.75 | 4.00 | 0.91199 | Dominant |
| | 0.25 | 2.42 | -0.46987 | Subordinate |

[PCA result for food variable: EigenValue = 1.93243, % variance = 96.6; Position variable: EigenValue = 0.06757, % variance = 3.4]

Dominant fish generally took food items first and moved around the front of the tank, while subordinates had a tendency to hide in shelters or remain at the back of the tank, although towards the end of the two week period some subordinates spent prolonged periods swimming at the water surface. Swimming at the water surface was found to be characteristic of subordinate fish seeking shelter. Fish swimming at the top of the water column were generally left alone by dominant fish and it therefore appeared to be an effective method of avoiding aggression.

*3.4.2 Physiology*

Physiological measurements were carried out on four distinct categories of fish; dominants and subordinates from paired fish, isolated fish (held singly) and grouped fish (held as a group of 18). A significant difference was detected among the specific growth rates for the four categories of fish, with subordinates having a significantly lower specific growth rate than the other three groups (Fig. 3.1). The four categories of fish showed no significant difference in change in condition factor during the experimental period. However a more sensitive, paired, analysis comparing only subordinates and dominants revealed that within individual pairs each subordinate had a significantly greater reduction in condition factor over the course of the experiment than did the dominant fish with which it was paired, with one exception (Fig. 3.2). It was noted that growth rates for all fish in the present study were relatively low, reasons for which are considered in the discussion.

No significant differences in plasma cortisol concentrations were detected among the four groups but again paired comparisons between dominants and subordinates only, indicated that within individual pairs each subordinate had significantly higher circulating plasma cortisol concentrations than its respective dominant (Fig. 3.3). However, despite the difference in plasma cortisol concentrations between dominant and subordinate fish, no significant differences in gill epithelial chloride cell densities were found among the four groups of fish (Table 3c) nor between dominants and subordinates within a pair.

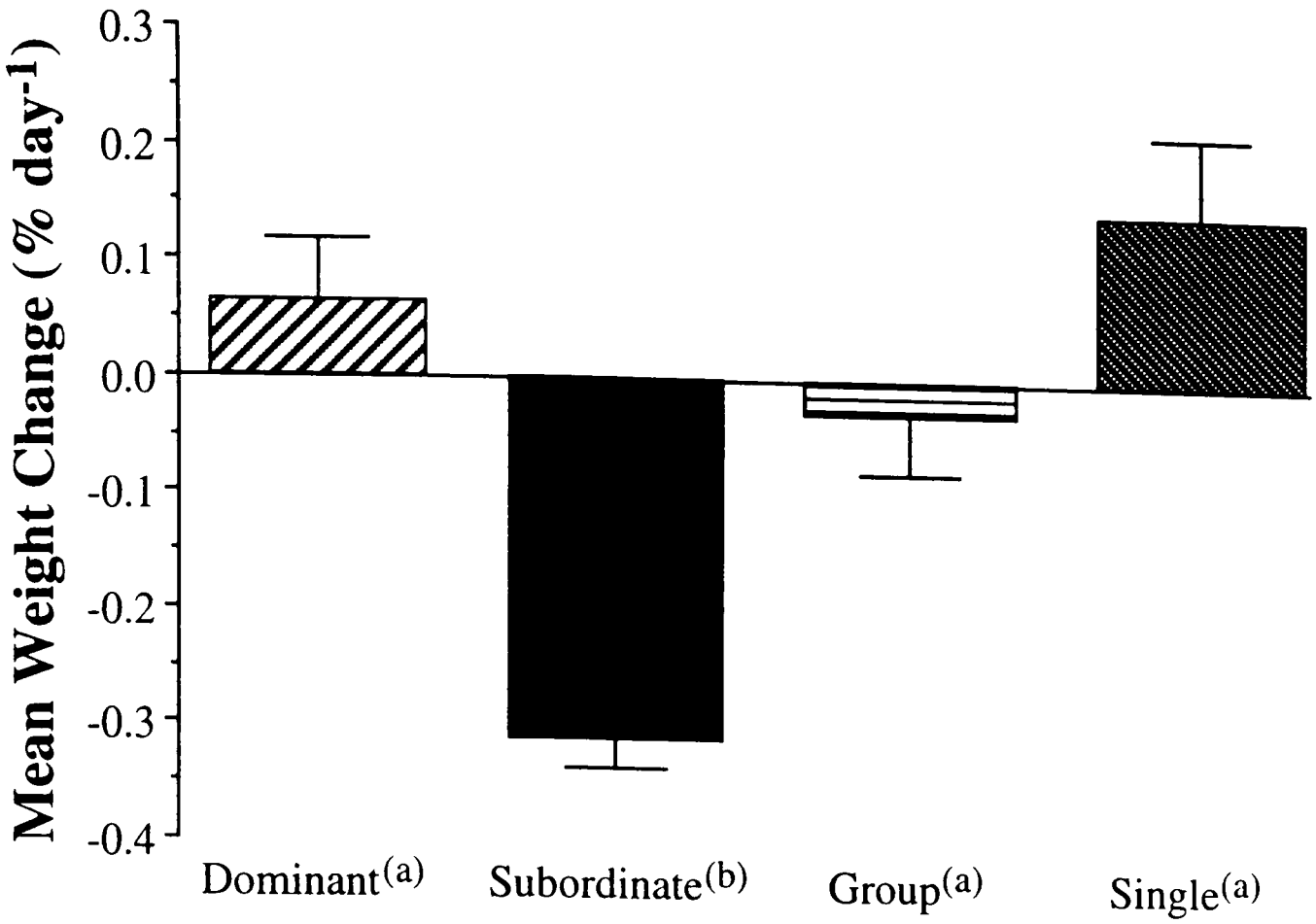



Figure 3.1: Specific growth rates (% change in weight per day) are presented for dominant and subordinate members of a pair, for solitary rainbow trout and trout held in a group of 18. Data given as means ± S.E.M. (n = 6). Statistical differences (one way ANOVA followed by Scheffé multiple comparisons test, $P < 0.05$) are indicated by the letters; groups sharing the same letter are not significantly different from one another.

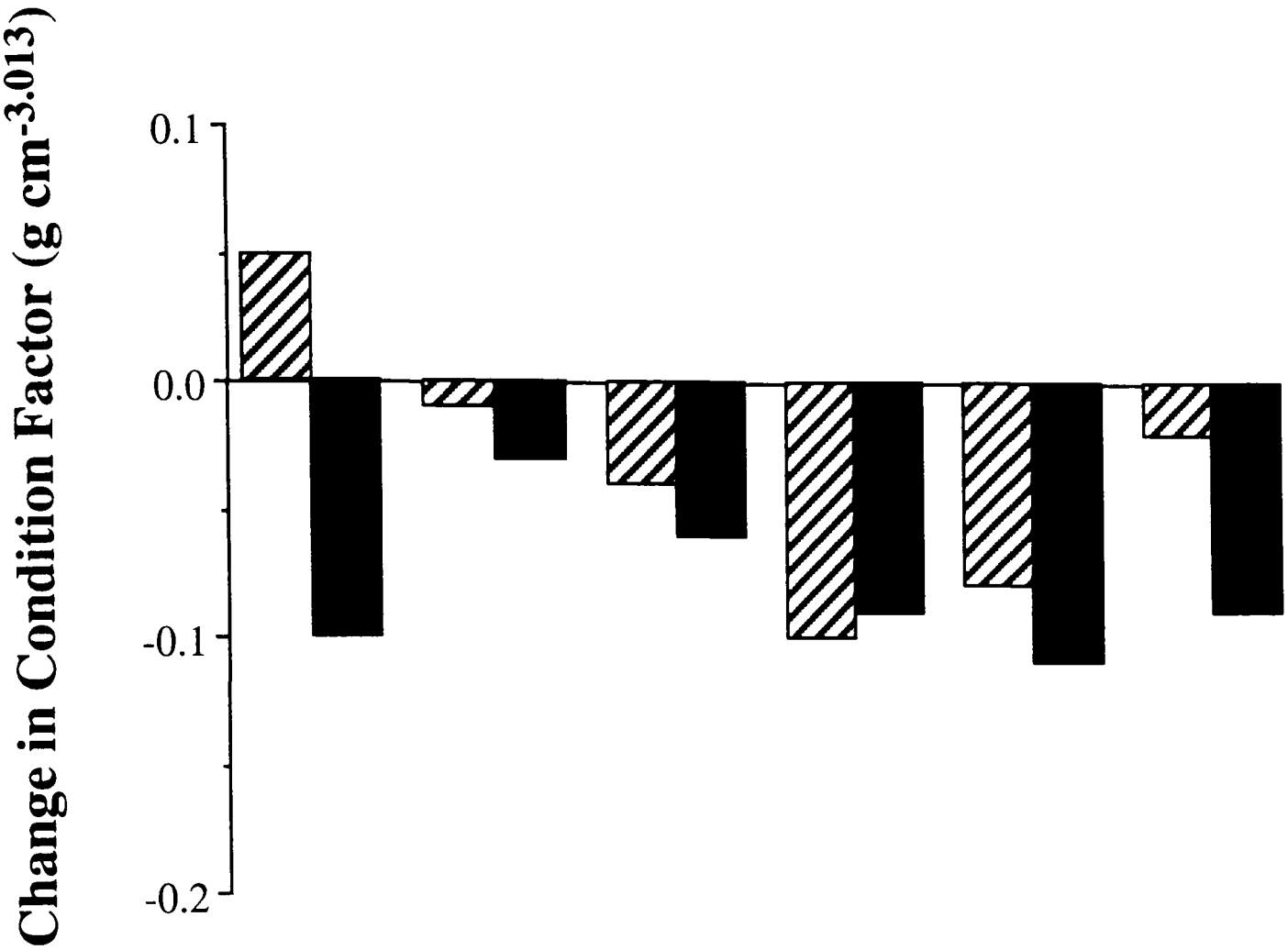



Figure 3.2: Change in condition factor for each fish in six dominant and subordinate pairs of rainbow trout. ▨ , dominant; ■ , subordinate. No significant differences were found between the four groups for change in condition factor (dominant = -0.03 ± 0.02; subordinate = -0.08 ± 0.01; group = -0.06 ± 0.03; single = -0.07 ± 0.03 (means ± S.E.M.)) $P > 0.05$.

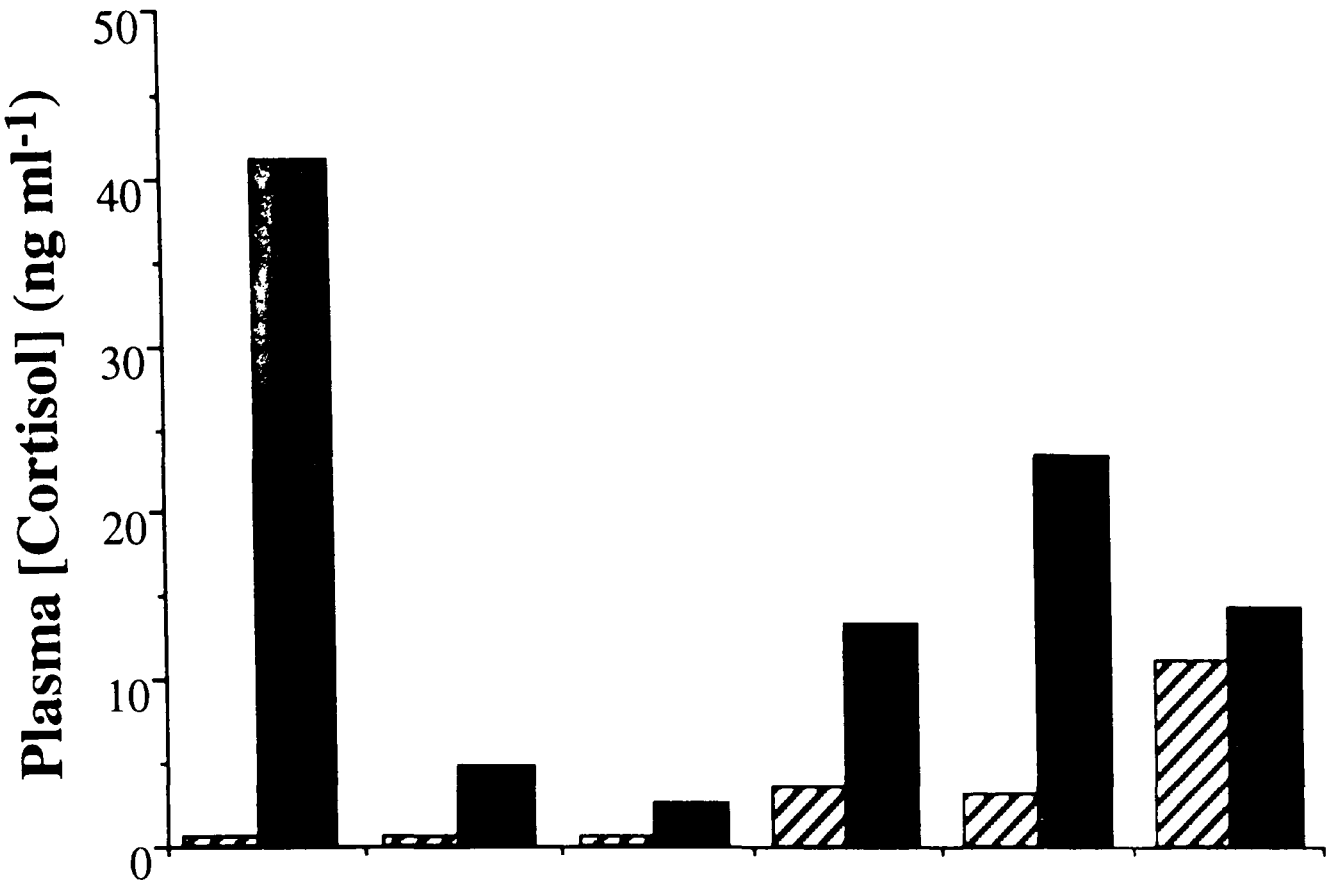



Figure 3.3: Plasma cortisol concentrations for each fish in six dominant and subordinate pairs of rainbow trout. ▨ , dominant; ■ , subordinate. No significant differences were found between the four groups for cortisol concentrations (dominant = 3.37 ± 1.66; subordinate = 17.16 ± 7.04; group = 16.02 ± 8.64; single = 2.75 ± 0.96 (means ± S.E.M.)) $P > 0.05$.

Table 3c. Chloride cell densities for dominant and subordinate members of a pair, solitary rainbow trout and trout held in a group of 18. Data given as means ± S.E.M. (n = 6).

| Fish Status | Chloride Cell Density (cells $mm^{-2}$ tissue) |
|---|---|
| Dominant | 1768 ± 102 |
| Subordinate | 1752 ± 87 |
| Group | 2066 ± 247 |
| Single | 1920 ± 184 |

Nor were there any effects of the social environment on blood plasma ion concentrations, either across all four categories of fish or within dominant/subordinate pairs, (Table 3d).

Table 3d. Blood plasma ion concentrations for dominant and subordinate members of a pair, and for grouped and single rainbow trout. Data given as means ± S.E.M. (n = 6).

| | Fish Status | | | |
|---|---|---|---|---|
| Blood Plasma Ions (mM) | Dominant | Subordinate | Group | Single |
| $Na^+$ | 149 ± 4 | 150 ± 6 | 148 ± 3 | 145 ± 2 |
| $Ca^{2+}$ | 2.09 ± 0.15 | 1.80 ± 0.18 | 2.00 ± 0.19 | 2.27 ± 0.28 |

3.4.3 *Interactions Between Plasma Cortisol Concentrations and Other Variables*

To determine whether differences in behaviour and physiological condition due to the social environment could be correlated with differences in plasma cortisol concentrations, regression analyses were carried out against log cortisol concentrations. A significant relationship existed between the difference in dominance score between the members of each pair and the difference in their log plasma cortisol concentrations, such that pairs with more pronounced dominance relationships also had greater signs of a stress response in the subordinate (Fig. 3.4). Within dominant/subordinate pairs, both specific growth rate and the change in condition factor were significantly correlated with log plasma cortisol

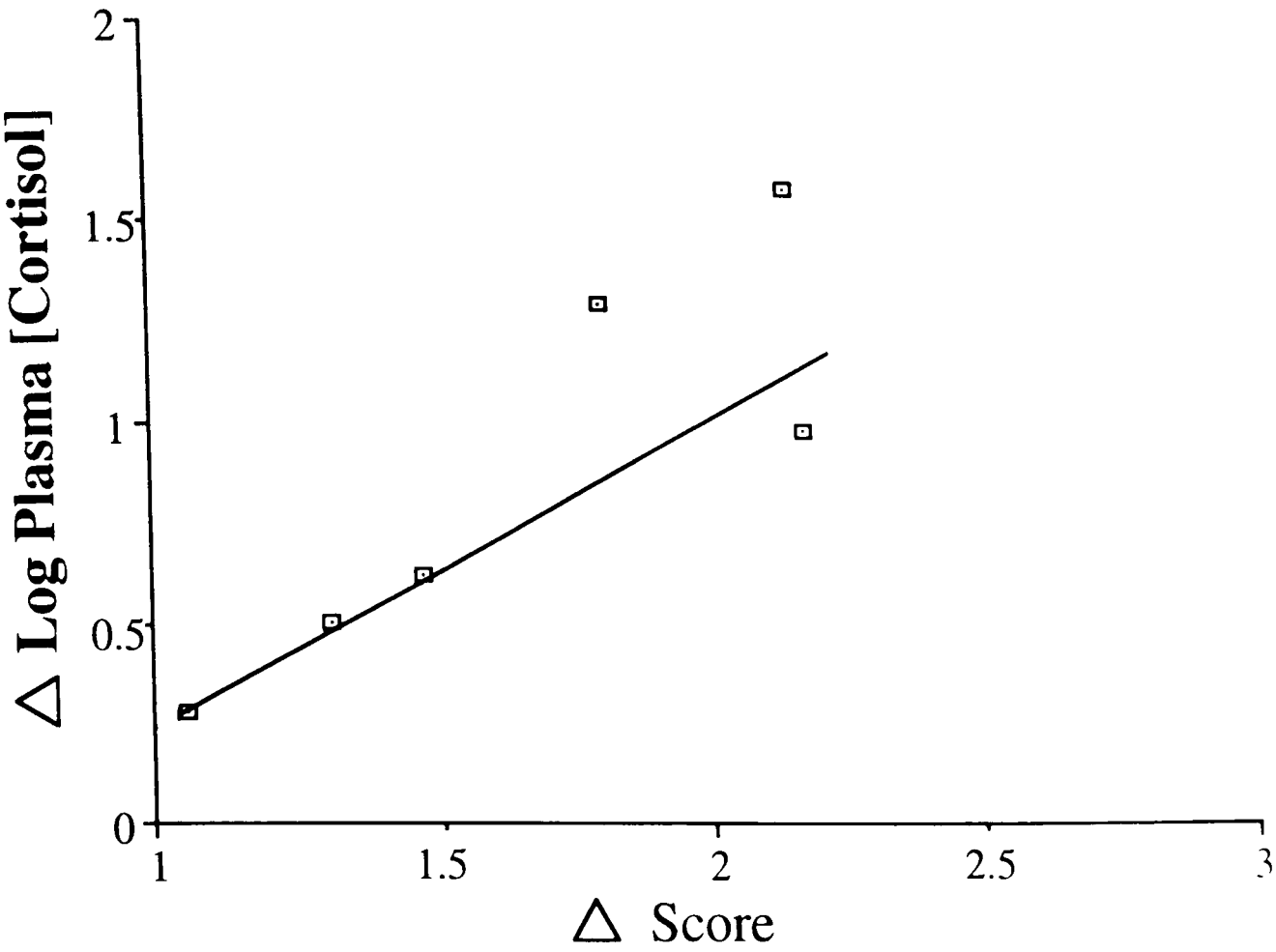



Figure 3.4: Relationship between the difference in behavioural score (Δscore) and the difference in log plasma cortisol concentrations (Δlog plasma [cortisol]) for dominant and subordinate members of each pair. The regression equation for these data is also plotted; the regression equation was Δlog plasma [cortisol] = 0.782 Δscore - 0.538, $r^2$ = 0.766, $P<0.05$.

concentrations (Fig. 3.5). Further analysis of these results indicated that the negative relationship between log plasma cortisol concentrations and growth rate was strongest in the subordinate fish. However, there was no significant relationship between the difference in dominance score and the difference in growth rate of the pairs of fish.

## 3.5 Discussion

Confinement of rainbow trout in pairs resulted in a significantly higher circulating concentration of the stress hormone, cortisol, in the blood of subordinate fish compared to dominant fish, indicating that the former were stressed by the social environment (Fig. 3.3). Pottinger and Pickering (1992) also found that rainbow trout confined in pairs developed large differences in plasma cortisol concentrations, with mean values of 19.7 ng $ml^{-1}$ *versus* 6.1 ng $ml^{-1}$ after 6 weeks confinement in pairs. Comparable plasma cortisol concentrations were measured in the present study after two weeks of confinement in pairs. Similar results have also been obtained by Ejike and Schreck (1980) for coho salmon (*Oncorhynchus kisutch* Walbaum) parr, and by Laidley and Leatherland (1988) for rainbow trout.

The moderate elevations of plasma cortisol concentrations observed in subordinate fish in the present study are indicative of chronic stress. Pickering and Pottinger (1989) found that acute stressors, such as handling, extreme but short-term confinement, or air exposure elicit rapid (over 2-4h) elevations of plasma cortisol in rainbow trout to much higher

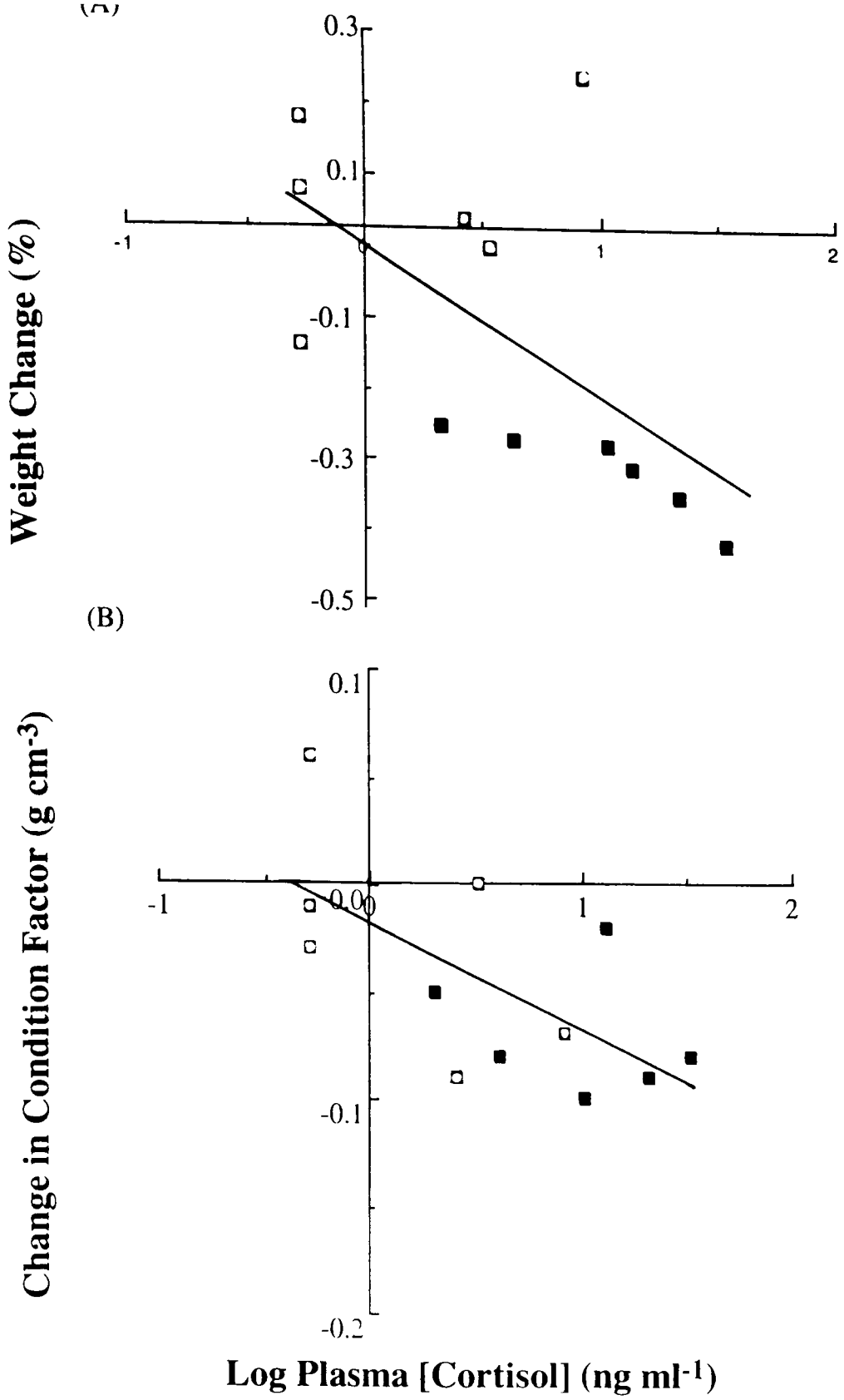



Figure 3.5: The relationship between log plasma cortisol concentrations (log plasma [cortisol] and (A) specific growth rate or (B) change in condition factor for dominant (□) and subordinate (■) rainbow trout. The calculated regression lines for these data are also plotted. The equations for these regression lines are (A) SGR = -0.210 log plasma [cortisol] + 0.0139, $r^2 = 0.354$, $P<0.05$ and (B) condition factor = -0.0459 log plasma [cortisol] - 0.0148, $r^2 = 0.606$, $P<0.05$.

concentrations of 40-200 ng $ml^{-1}$, with the levels returning to basal concentrations within 24-48 h. In contrast, continuous chronic stressors, such as high stocking densities or prolonged confinement, elevated plasma cortisol to concentrations of approximately 10 ng $ml^{-1}$, a level at which they remained for up to four weeks. The moderate elevation of plasma cortisol concentrations recorded in the present study suggests that subordinate fish were reacting to the chronic stress imposed by confinement with a dominant. Cortisol concentrations in the fish held singly were similar to those of dominant fish, and were characteristic of unstressed fish (i.e. < 5 ng $ml^{-1}$) (Pickering and Pottinger 1989). Similarly, Peters *et al.* (1980) found that isolated eels (*Anguilla anguilla* L.) also had cortisol values similar to those of the dominant members of fish kept in pairs.

In the present study, social stress due to confinement in pairs was found to cause weight loss (Fig. 3.1) and loss of condition (Fig. 3.2) in subordinate fish, effects that were correlated with plasma cortisol concentrations (Fig. 3.5). The inter-relationships among social stress, plasma cortisol concentrations, specific growth rate and condition factor have been characterised in a number of previous studies (e.g. Chan and Woo 1978; Barton *et al.* 1987; Laidley and Leatherland 1988; Pottinger and Pickering 1992), and the results of the present study are in agreement with the reported trends. Social stress (Pottinger and Pickering 1992) or elevation of plasma cortisol concentrations through cortisol injection (Chan and Woo 1978) or ingestion (Barton *et al.* 1987) are generally found to result in a decrease in condition factor and/or weight loss. These effects are usually attributed to the influence of cortisol on intermediary metabolism, that is, a cortisol-mediated switch of

metabolic processes from anabolic to catabolic that is accompanied by a mobilisation of energy stores (Pickering and Pottinger 1995).

Although there was a clear difference between growth rates of dominant fish and the subordinates with which they were paired, it does appear that growth rate of all fish in the experiment was minimal. No behavioural changes, such as lack of appetite, were noted during the experimental period, suggesting that the fish were healthy and were continuing to feed. Furthermore, the fish were always fed until they stopped feeding. The fact that growth, although minimal, did occur suggests that there were no compounding effects causing loss of weight. Loss of condition can also be attributed to low nutrient availability (Goede and Barton 1990), and therefore it seems likely that the lack of growth in the present study was attributable to the high water content and consequently low nutritional value of the bloodworm on which the fish were fed. Fish were thus provided with only slightly more than a maintenance ration despite food being provided in excess.

Chloride cell proliferation has been observed in response to a change in the ionic composition of the environment of the fish (e.g. Perry and Wood 1985; Laurent and Hebibi 1989; Perry and Laurent 1989; Greco *et al.* 1995, 1996), environmental factors affecting internal acid-base balance (hypoxia; hypercapnia) (Goss *et al.* 1992), environmental toxicants (Leino *et al.* 1987) or injection with cortisol (Madsen 1990b; Bindon *et al.* 1994a; Laurent *et al.* 1994). Proliferation is accompanied by an increase in $Na^+$ and $Cl^-$ influx due to increased transport capacity of the gill epithelia (Perry and Laurent 1989), enhancing the ability of the fish to deal with ionoregulatory disturbances.

While supraphysiological concentrations of cortisol are often used as a tool to induce chloride cell proliferation (e.g. Bindon *et al.* 1994 a, b), experimental evidence suggests that elevated plasma cortisol concentrations play a role in stimulating chloride cell proliferation. For example, rainbow trout placed in ion-poor water showed a large transitory increase in plasma cortisol, with concentrations after 24 h being more than three times higher in fish acclimated to soft water than those of control fish (104.2 ng $ml^{-1}$ compared with 30 ng $ml^{-1}$). These concentrations then decreased to concentrations similar to those of controls within a week, and were not significantly different after one month (Perry and Laurent 1989). Similarly, Perry and Wood (1985) measured large (120 ng $ml^{-1}$), transient (1 day) elevations of plasma cortisol in rainbow trout exposed to low environmental calcium concentrations.

The likely involvement of elevated plasma cortisol concentrations in eliciting chloride cell proliferation suggests that other stressors that also elevate plasma cortisol concentrations but which do not involve ionoregulatory challenge may have the potential to alter the density of gill chloride cells. The present study is the first to test this hypothesis. The social stress of confinement in pairs resulted in significant elevation of plasma cortisol concentrations in subordinate fish (Fig. 3.3) in the absence of ionoregulatory disturbance. However, no significant differences in gill chloride cell densities were found in the present study of rainbow trout subjected to this social stress. The ionic composition of the water used in the present study ($[Na^+] = 0.189 \pm 0.007$ mM, $[Ca^{2+}] = 0.155 \pm 0.002$ mM, $[K^+] = 0.038 \pm 0.002$mM) was similar to that of water used as a control condition in experiments in which chloride cell numbers were manipulated by exposure to ion deficient water

(Greco *et al.* 1995, 1996). As increases in chloride cell fractional area of 100 % were measured by Greco *et al.* (1996) it is unlikely that the lack of chloride cell proliferation in the present study was due to elevated chloride cell numbers.

The lack of chloride cell proliferation associated with elevated plasma cortisol concentrations in the present study may have several possible explanations. The elevation of plasma cortisol concentrations in subordinate fish (~ 17 ng $ml^{-1}$) may not have been sufficient to elicit chloride cell proliferation. Perry and Laurent (1989) for example, observed plasma cortisol concentrations of 100 ng $ml^{-1}$ in fish exposed to ion-deficient water, while the doses administered to induce chloride cell proliferation are likely to produce supraphysiological plasma cortisol levels (Bindon *et al.* 1994a). Alternatively, it is possible that physiologically-relevant elevations of plasma cortisol concentrations will only induce chloride cell proliferation when in conjunction with an ionoregulatory challenge, or that other hormones are involved in eliciting the proliferation of chloride cells. Many experiments using cortisol as a tool for causing chloride cell proliferation have also used combinations of hormones, in particular growth hormone, which give a greater response (Bindon *et al.* 1994a, b; Laurent *et al.* 1994). Growth hormone is known to be affected by environmental stressors (Pickering *et al.* 1991; Sumpter *et al.* 1991) and so there is a possibility that concentrations of growth hormone in the present study influenced the lack of chloride cell proliferation.

Finally, it is possible that the induction of chloride cell proliferation requires a different pattern of cortisol elevation than that of the present study. Acclimation to ion-poor water may cause plasma cortisol elevation typical of that in response to an acute stressor (Perry and Wood 1985; Perry and Laurent 1989), whereas confinement in pairs probably elicits a chronic cortisol response (see above). Initial exposure to ion-poor water causes a pronounced reduction in ion uptake rates resulting in net ion losses. Over the course of several days, a decrease in ion efflux rates in conjunction with increased ion uptake rates serves to re-establish ionic homeostasis (Perry and Laurent 1989). Large, transient elevations of plasma cortisol concentrations are observed during the initial stages of acclimation to ion-deficient conditions (Perry and Wood 1985; Perry and Laurent 1989) and probably play a role in stimulating chloride cell proliferation, an important contributor to the increase in ion uptake rates. The lower, prolonged elevation of plasma cortisol concentrations observed in response to social stress may not provide the appropriate stimulus to evoke chloride cell proliferation.

The social stress of subordinance in paired fish in the present study was sufficient to cause typical elevations in the stress hormone cortisol, and affected both the weight gain and condition factor of the fish. However, no effect on chloride cell densities was observed, suggesting that the concentrations and/or pattern of cortisol elevation produced during periods of chronic stress do not cause chloride cell proliferation. It therefore seems unlikely that social interactions, and probably other chronic stressors such as high stocking densities, will result in chloride cell proliferation or the respiratory problems associated with chloride

cell proliferation. The possibility that cortisol is involved in the proliferation of chloride cells remains to be demonstrated.

# CHAPTER 4

## Chapter 4: The Effect of Social Stress on the Standard Metabolic Rate (SMR) of Brown Trout, *Salmo trutta.*

A version of this chapter is published in Fish Physiology and Biochemistry with co-authors: Gordon Motherwell, Kirstine I. O'Connor and Alan C. Taylor.

### 4.1 Abstract

The effect of social stress, induced by confinement in pairs, on the SMR of the brown trout, *Salmo trutta* (L.) is investigated. Fish were confined in pairs under laboratory conditions and allowed to establish social hierarchies, with one fish becoming dominant and the other subordinate. The change in SMR of the subordinate fish was significantly greater than that of their respective dominant. Also, the more aggressive the dominant towards the subordinate with which it was paired, the greater the increase in the SMR of the subordinate fish. It is concluded that social stress causes an increase in SMR in subordinate fish and therefore imposes a metabolic disadvantage.

### 4.2 Introduction

A frequently used model to investigate the potential consequences of dominance hierarchies and social interaction is the confinement of pairs of fish in tanks with little or no refuge. Typically, one fish will become dominant over the other, the subordinate fish.

An elevation of plasma cortisol concentration is characteristic of subordinate fish and has been illustrated in paired rainbow trout, *Oncorhynchus mykiss* by Laidley and Leatherland (1988) and by Pottinger and Pickering (1992). Other physiological changes exhibited by subordinate fish, and which are probably associated with an increase in plasma cortisol concentrations, include decreases in weight (Pottinger and Pickering 1992), hepatic glycogen content (Ejike and Schreck 1980), disease resistance (Peters *et al.* 1988), and increases in interrenal cell activity (Noakes and Leatherland 1977) and plasma glucose concentrations (Peters *et al.* 1988).

Less is known about the effect of social status on metabolism. Cortisol injections or implants have been shown to lead to increased oxygen consumption rates in the eel, *Anguilla japonica* and cutthroat trout *Oncorhynchus clarki clarki* (Chan and Woo 1978; Morgan and Iwama 1996) but the relevance of these physiological manipulations of cortisol to the situation of naturally occurring responses to social interactions is not clear. Wirtz and Davenport (1976) showed that oxygen consumption rates increased in the blenny, *Blennius pholis*, simply in response to a conspecific, while measurements of growth efficiency led Abbott and Dill (1989) to suggest that there was a metabolic disadvantage associated with subordinance.

Therefore, the present study was designed to investigate the effect of a period of confinement with a more dominant or subordinate conspecific on the metabolism of brown trout, to test whether the experience of being subordinate produces an elevation of standard metabolic rate. SMR is important because it is the minimum metabolic rate

required to sustain life (Cech 1990) and therefore gives an indication of the overall physiological condition of the fish. Although SMR is difficult to measure directly, measurements of oxygen consumption of resting fish are thought to provide a close approximation.

## 4.3 Materials and Methods

### *4.3.1 Experimental protocol*

Brown trout (weight 58.67 ± 6.55 g; length 16.81 ± 0.59 cm (mean ± S.E.M.); n = 16 chosen to make eight size-matched pairs) were obtained from the University of Glasgow Field Station, Rowardennan (Scotland). They were the offspring of wild adults from Loch Awe, western Scotland, but had been reared under hatchery conditions. The fish were held in a 70 L stock tank under an ambient light (16 L: 8 D) and temperature regime (12.7 ± 0.1 °C) in aerated, recirculating, dechlorinated tap-water. After two weeks, the fish were anaesthetised using a solution of benzocaine (0.05 mg $ml^{-1}$) and unique combinations of alcian blue dye marks (Kelly 1967) were injected into their fins. The fish were then returned to the stock tank to recover. One week later the fish were placed in individual chambers of a flow-through respirometer and initial oxygen consumption measurements were carried out (see below). The fish were then allocated to size-matched pairs and placed in small experimental tanks so that behavioural observations could be made to determine dominance relationships. Finally, after 24 h confinement in pairs, the fish were returned to the respirometer and final oxygen consumption readings taken.

*4.3.2 Measurements of SMR*

Measurement of oxygen consumption was similar to that detailed in Metcalfe *et al.* (1995) in which the oxygen consumption of the fish was calculated by measuring the reduction in oxygen concentration of fully aerated water flowing past a stationary fish (Steffensen 1989). Single fish were placed in Perspex respirometer chambers (45 mm diameter by 275 mm in length) which were continually fed with aerated water from a header tank, where the flow was as low as possible to achieve continual replacement of water without disturbing the fish. The flow rate was kept constant and measured by weighing the amount of water dripping from the outflow from each tube during a measured time interval. All measurements were made at the temperature to which the fish had been acclimated and 'blank' runs were carried out with no fish in the tube to ensure that the reduction in oxygen concentration of the water caused by bacterial respiration was negligible. Water entering the header tank was passed through a UV steriliser to reduce bacterial growth. Fish were placed into their tubes and left for 15-20 h before measurements were made, to allow the fish to empty their guts (to ensure a post-absorptive state (Beamish 1978)) and to become acclimatised to the tube environment. To minimise disturbance to the fish the tubes were covered with black plastic sheet.

In order to determine the reduction in oxygen content of the water caused by the respiring fish, samples (0.5 ml) of the inflow and outflow water were collected using a hypodermic syringe and injected into a thermostatted cell (Strathkelvin Instruments, Glasgow)

containing an oxygen electrode connected to an oxygen meter. Calibration of the oxygen electrode used air-saturated water from the header tank as 100 % saturation and a solution of sodium sulphite in borax (0.01 M sodium tetraborate) as 0 %.

The oxygen consumption measured here is assumed to be the standard metabolic rate *i.e.* the metabolism of an inactive fish that is not digesting food (Brett and Groves 1979). Three measurements of oxygen consumption were made for each fish, with a delay of 20 minutes between successive readings to allow for flushing of the water in the respirometer (Steffensen 1989), and a mean value calculated. Flow rates were measured each time oxygen consumption was recorded. All data were collected between 0900 and 1100 h to avoid diurnal variation in metabolic rate. Rates of oxygen consumption ($VO_2$) were calculated as:

$$VO_2 \text{ (ml/min)} = V_w.\Delta C_wO_2$$

where $V_w$ is the flow rate of water through the respirometer (ml minute$^{-1}$) and $\Delta C_wO_2$ is the difference in oxygen concentration between the inflow and outflow water (ml L$^{-1}$). One hundred percent saturation of water at 12.7 °C was equivalent to 7.369 $O_2$ ml L$^{-1}$ (Weiss, 1970). Weight specific rates (ml $O_2$ kg$^{-1}$ h$^{-1}$) were then calculated. The change in SMR as a result of social interactions (ΔSMR) was calculated for each individual fish as the difference between the initial and final SMR (i.e. ΔSMR = final SMR - initial SMR).

### *4.3.3 Behavioural Measurements*

Once initial oxygen consumption measurements had been carried out on the fish they were allocated in size-matched pairs to one of eight 22.5 L experimental tanks (mean size

difference = 0.28 ± 0.08 cm). Each tank was bare except for a single Perspex tube that could be used as a shelter. Behavioural observations were then made at 30 minute intervals for the first six hours of confinement and dominance measured by assigning points (see below). The pairs of fish were presented with a single bloodworm every 30 minutes and the fish that consumed it was noted and was awarded one point. Fish that did not get the bloodworm scored zero. At the same time fish were also scored on a scale of 1 to 5 according to their position in the tank (Table 4a). The method of scoring was based on previous studies of dominance in salmonids (Metcalfe *et al.* 1989; Johnsson *et al.* 1996). A principal components analysis (PCA) was carried out using the feeding and position scores for each fish. The PCA was used to weight the data according to relative importance and produce an overall dominance score for each fish.

Table 4a. Method used to score pairs of fish according to their positions in the tank. Higher scores are indicative of more dominant behaviour. Fish resting at the bottom of the tank at the front scored higher than those at the back of the tank since they were closer to the point of food entry.

| Behaviour | | Score at each observation |
|---|---|---|
| Feeding: | Fish that took introduced food item | 1 |
| | Fish that did not take food item | 0 |
| Position: | Permanently hiding in refuges | 0 |
| | Frequently hiding in refuges | 1 |
| | Swimming at the water-surface | 2 |
| | Resting on the bottom at the back of the tank | 3 |
| | Resting at the bottom at the front of the tank | 4 |
| | Cruising in the water column | 5 |

*4.3.4 Statistical methods*

Comparisons of behavioural and physiological data of dominant and subordinate fish were accomplished using a one way analysis of variance (ANOVA) and changes between pre-confinement and post-confinement SMR for both dominant and subordinate fish were analysed using a paired t-test. The limit of significance in all analyses was 5 %. Although change in SMR is expressed here as % change no transformation of the data was needed due to the range of values being greater than 100.

## 4.4 Results

Behavioural observations allowed dominant fish (PCA score: $0.926 \pm 0.136$; $n = 8$ (mean $\pm$ S.E.M.)) to be distinguished from subordinates (PCA score: $-0.926 \pm 0.136$; $n = 8$ (mean $\pm$ S.E.M.)). Dominant fish took the initial bloodworm more frequently than subordinates ($P < 0.01$) and repeatedly scored higher than subordinates for their position in the tank ($P < 0.01$) resulting in higher mean behavioural scores. Initial SMR values were not significantly different between dominants and subordinates ($P > 0.05$). However, there was a significant difference between the dominants and their respective subordinates in the change in SMR during the experiment (Fig. 4.1; $P < 0.05$). There was no significant change in the SMR of dominant fish ($P > 0.1$) between pre-confinement and post-confinement but there was a significant increase in the SMR of subordinates ($P < 0.05$). These results are summarised in Table 4b.

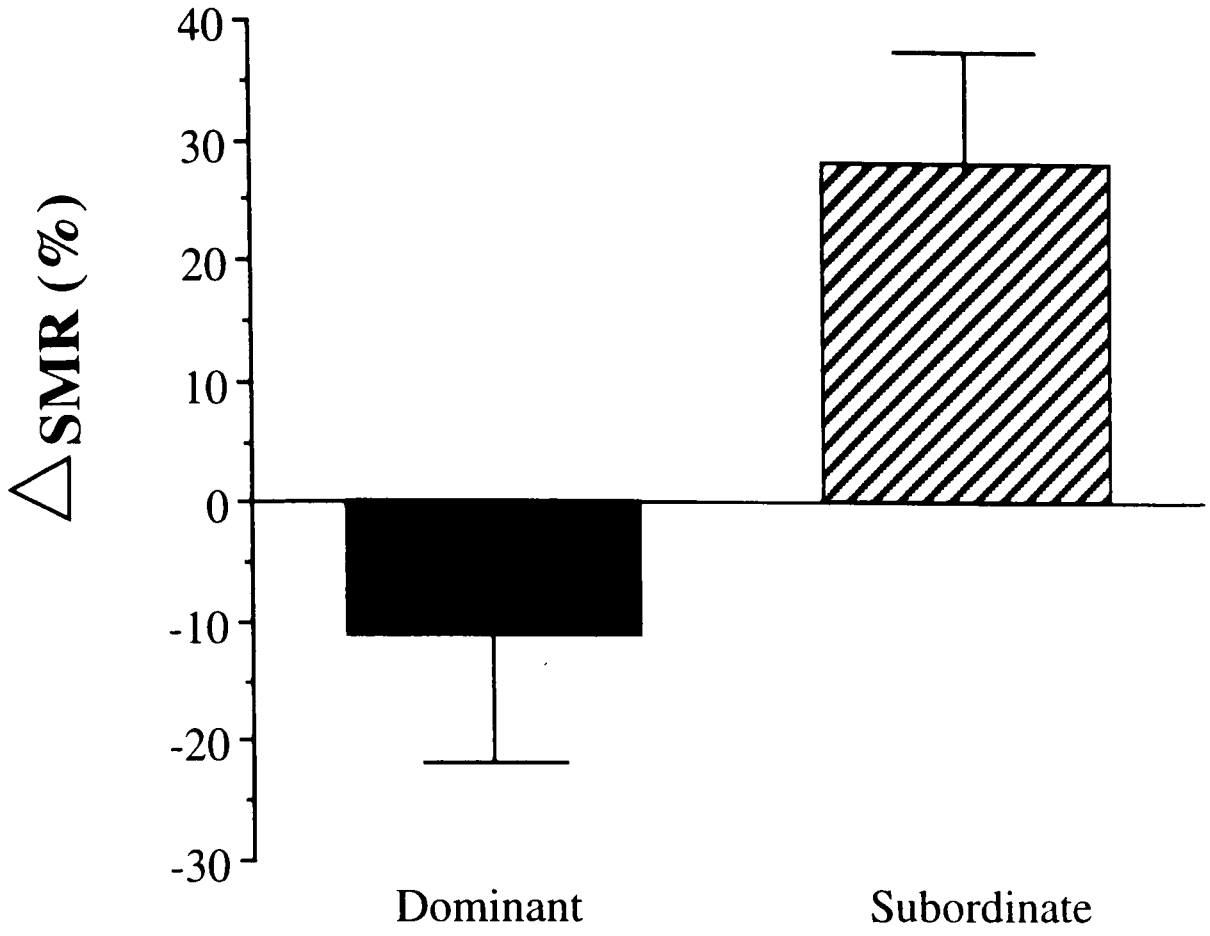



Figure 4.1: Percentage change in SMR between initial and final measurements for pairs of subordinate ▨ and dominant ■ fish. Data given as means ± S.E.M. (n = 8). The change in SMR between dominants and subordinates was significantly different (ANOVA: $P<0.05$).

Table 4b: Weight, length and SMR values for dominant and subordinate fish (mean ± S.E.M.)

| | Dominant | Subordinate |
|---|---|---|
| Length (cm) | 16.7 ± 0.9 | 16.9 ± 0.88 |
| Weight (g) | 56.75 ± 9.11 | 60.59 ± 10.0 |
| SMR before confinement (ml $O_2$ $kg^{-1}$ $h^{-1}$) | 68.25 ± 7.46 | 50.4 ± 4.5 |
| SMR after confinement (ml $O_2$ $kg^{-1}$ $h^{-1}$) | 53.25 ± 2.93 | 61.97 ± 4.23 |
| Change in SMR (%) | -11.21 ± 10.55 | 28.22 ± 9.10 |

There was a significant positive correlation between the difference in ΔSMR between dominant and subordinate fish, and the difference in the behavioural score of the dominant and subordinate of each pair (i.e. an indication of the polarity of the pair) (Fig. 4.2; $P < 0.05$). In pairs in which the dominant was very aggressive towards the subordinate, and so scored much higher than the subordinate, leading to a large difference in behaviour score, the difference between the ΔSMRs for the pair was also higher than in a pair in which the dominant was less aggressive.

## 4.5 Discussion

The effect of a short period of confinement in pairs on the standard metabolic rate of brown trout depended on social status. The period of confinement resulted in subordinates exhibiting an increase in SMR, whilst the SMR of dominants remained the same. Also, a significant relationship was found between the difference in behavioural scores of the pairs and the difference in ΔSMR values between the pairs. Wirtz and Davenport (1976)

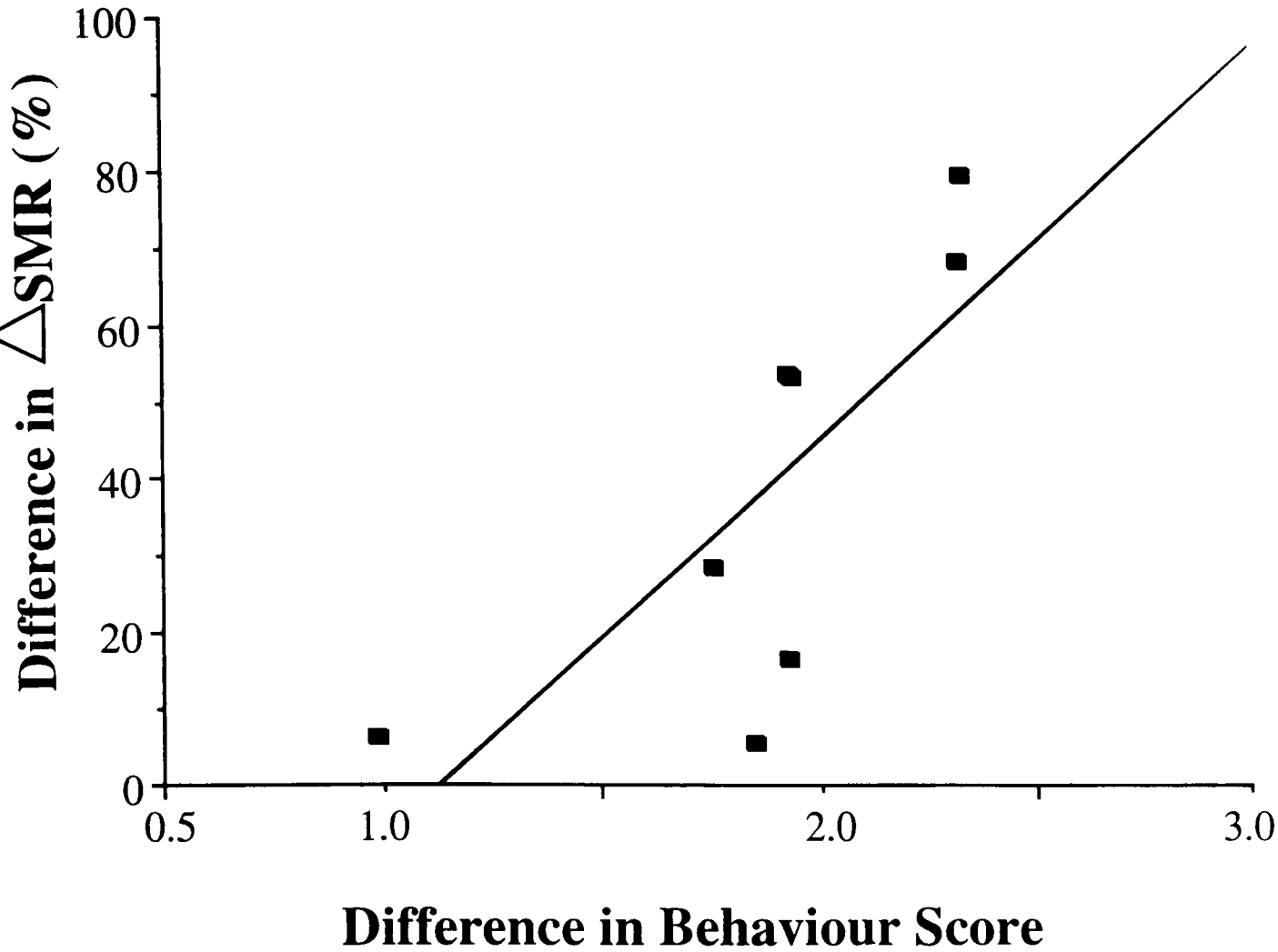



Figure 4.2: Relationship between the difference in behavioural score and the difference in percentage change in SMR between initial and final measurements for dominant and subordinate members of each pair. The regression equation for these data is also plotted; the regression equation was: change in SMR = 52.73 change in score - 62.11, $r^2 = 0.53$, $P < 0.05$.

demonstrated that the sight of itself in a mirror caused a 14 % increase in $O_2$ consumption of *Blennius pholis*. It was concluded that the increase in $O_2$ consumption resulted from the perception of an intruder; *B. pholis* is highly territorial (Gibson 1967), which resulted in increased metabolism and also increased activity. While the protocol used by Wirtz and Davenport (1976) did not enable distinctions to be made between dominant and subordinate fish (because the fish were shown their own mirror image), the increase in $O_2$ consumption did suggest that metabolism may be affected by social interactions. However, they noted an increase in activity with the presence of a mirror image and, therefore, the effect of this social stressor on SMR could not be distinguished.

There was no significant difference between the initial SMR values of dominants and subordinates but it can be seen (Table 4b) that the mean value for initial SMR in dominant fish was actually higher than in subordinates. A higher initial SMR in dominant fish is in agreement with work carried out by Metcalfe *et al.* (1995) who found that SMR could be used as an indicator of dominance; fish with a higher SMR subsequently become dominant. The absence of a significant difference between initial SMR values of dominant and subordinate fish in the present study is probably due to a small sample size.

Although blood plasma cortisol concentrations were not measured in the present study, evidence from other studies suggests that subordinate fish experience elevated cortisol concentrations (Peters *et al.* 1980; Pottinger and Pickering 1992). Measurement of SMR in the present study means that changes in activity cannot account for the elevation of metabolic rate measured in subordinate fish and so it is possible that the changes in SMR

(i.e. metabolic rate in the absence of activity) are due, at least in part, to elevated plasma cortisol concentrations in the subordinate fish. Davis and Schreck (1997) suggested that cortisol may influence oxygen consumption during moderate stress but that other factors such as the metabolism associated with exercise or hypoxia become involved when the stress becomes severe.

In conclusion, the present study indicates that the social stress experienced by subordinate fish when confined with a dominant increases the SMR of the subordinate fish, and therefore imposes a metabolic disadvantage. The lower growth rates and decreased condition of subordinate fish documented by numerous workers (Peters *et al.* 1980; Abbott and Dill 1989; Pottinger and Pickering 1992) may therefore result from an increase in the minimum metabolic rate required by fish for survival.

# CHAPTER 5

# Chapter 5: The Effect of Air Emersion on Chloride Cell Numbers in the Gills of Rainbow Trout (*Oncorhynchus mykiss* Walbaum)

A version of this chapter has been submitted to the Journal of Fish Biology as a short communication with co-authors: Alan C. Taylor, Neil B. Metcalfe and Kathleen M. Gilmour.

## 5.1 Abstract

The effect of air emersion on chloride cell proliferation in the rainbow trout was investigated. Emersion stress caused a characteristic elevation in blood plasma cortisol but this peak in cortisol did not elicit chloride cell proliferation.

## 5.2 Introduction

The glucocorticoid hormone, cortisol, is secreted as a primary response to stress in vertebrates (Sumpter 1997), and has become a widely recognised indicator of stress in fish (Pickering 1993a). Many studies have demonstrated that blood plasma cortisol concentrations in fish are elevated by various stressors, including handling (e.g. Nakano and Tomlinson 1967; Barton *et al.* 1980; Sumpter *et al.* 1986), pollutants (e.g. Bennett and Wolke 1987), confinement (e.g. Strange *et al.* 1978), and social interaction (e.g. Laidley and Leatherland 1988; Pottinger & Pickering 1992). The pattern and magnitude of cortisol elevation are dependent upon the nature and duration of the stress. Acute stress

(*i.e.* severe but short term such as caused by handling and brief exposure to air) elicits cortisol concentrations many times higher than basal (e.g 20-400 *vs* 0-5 ng $ml^{-1}$; Pickering and Pottinger 1989) but plasma cortisol quickly returns to normal (Pickering *et al.* 1982; Einarsdóttir and Nilssen 1996). In contrast, less severe but extended or chronic stress, e.g. due to social stress and crowding, does not elevate cortisol to such high concentrations, but the elevation can persist for several weeks (Pickering and Pottinger 1989).

The physiological effects of elevated cortisol are generally referred to as secondary stress responses and include increased oxygen consumption (Chan and Woo 1978), haemopoietic changes in the spleen and head kidney (Peters and Schwarzer 1985), increased plasma glucose concentrations and decreased disease resistance (Peters *et al.* 1988). Elevation of plasma cortisol concentrations has also been implicated in the proliferation of chloride cells in the gill epithelium. Chloride cells are involved in active ion uptake at the gills (see review by Perry 1997) and the proliferation of these cells has largely been associated with the acclimation of fish to ion deficient water (e.g. Laurent 1984; Perry and Laurent 1989; Greco *et al.* 1996). Perry and Laurent (1989) showed that the proliferation of chloride cells in ion deficient water was associated with an elevation of blood plasma cortisol. Also, injection of rainbow trout with supra-physiological concentrations of cortisol has been shown to cause chloride cell proliferation (Laurent and Perry 1990; Bindon *et al.* 1994a; Laurent *et al.* 1994).

The potential for endogenous elevation of plasma cortisol concentrations to elicit chloride cell proliferation in the absence of ionoregulatory challenge remains uncertain. Sloman *et*

*al.* (2000a (Chapter 3)) found no evidence of chloride cell proliferation in rainbow trout whose plasma cortisol concentrations had been elevated through chronic social stress. However, the extent and duration of this cortisol elevation differed substantially from that reported to occur during acclimation to ion-poor water (Perry and Laurent 1989). Thus, the aim of the present study is to investigate the effect of an acute stressor on chloride cell proliferation, as the elevation of plasma cortisol elicited by an acute stressor may in fact be more similar to that caused by cortisol injection or transfer to ion-poor water.

## 5.3 Methods

Rainbow trout (weight: 198.59 ± 6.10 g; length: 26.02 ± 0.19 cm (mean ± S.E.M.) n = 80) were obtained from College Mill Trout Farm, Perth (Scotland). Fish (n = 20) were placed in each of four 500 L stock tanks. The tanks were supplied with aerated, re-circulating, dechlorinated water and kept under ambient conditions (12.3 ± 0.09 °C (mean ± S.E.M.) 16L:8D). Fish were allowed to acclimatise to the holding conditions for one month and were fed to excess on pellets (BOCM Pauls Ltd Keystart; oil 16.0%; protein 55.0%).

The four stock tanks were randomly allocated as either control (n = 2) or experimental (n = 2) treatments. In the two experimental tanks, the water was drained quickly from the tank and the fish were exposed to air for a period of 30 s before the tanks were refilled; control tanks were not manipulated. Thirty minutes after the air emersion, one experimental and one control tank were sampled. The remaining control and experimental tank were kept under the same conditions and food ration for a further two weeks and

then sampled in the same way as the other two tanks. Fish were killed rapidly by a lethal dose of benzocaine (0.5 mg $ml^{-1}$), added to the tank to avoid sampling stress. A blood sample (1.0 ml) was withdrawn by caudal veni-puncture. Blood samples were centrifuged (13,000 g) and the plasma was removed, frozen in liquid nitrogen and stored at -70 °C for later analysis of cortisol concentrations using a radioimmunoassay (ICN Pharmaceuticals Ltd). The second left gill arch was removed and washed in 0.9 % saline. Small samples of filaments were removed and fixed in buffered glutaraldehyde (5 % glutaraldehyde in phosphate buffer; 1 h; 4 °C).

The gill tissue was stained with an osmium-zinc iodide preparation (Garcia-Romeu and Masoni 1970) (1 part 2 % $OsO_4$ to 4 parts 3 % $ZnI_2$; 18 h; 20 °C) which causes a reduction of osmic acid to osmium, blackening the phospholipids. Chloride cells stain strongly due to their intricate plasma membranes with many invaginations. Following staining, the tissues were dehydrated in an ethanol series (30 %, 50 %, 75 %, 95 %, 100 % x 2, 20 minutes in each; 20 °C), rinsed in Histoclear® (2 x 20 minute rinses; 20 °C) and paraffin wax (2 x 20 minute rinses; 55 °C), embedded in paraffin wax (BDH) and sectioned at 7 µm using a microtome (Leitz 1512).

Eight slides, with eight sections per slide, were prepared for each fish for a total of 64 sections per fish. Slides were viewed using a light microscope (40 x objective; Leitz Dialux® microscope) and digital pictures were taken (JVC TK1381 1/2"CCD digital video colour camera, 470 lines resolution PCI Image Capture card; Viglen P460 PC). Ten pictures were produced per fish from randomly selected sections with the slide positioned

so that the field of view contained approximately seven lamellae and a small portion of the filament at the bases of the lamellae. For quantification, the total gill tissue area in a picture was recorded using Scion Image software and the number of chloride cells per unit tissue area was calculated by visually counting the intensely stained cells. The value for each fish was determined as the mean of the values for the 10 images.

## 5.4 Results

Plasma cortisol concentrations were significantly higher in the air-emersed fish sampled immediately after the emersion stress was imposed, than in either the air-emersed fish sampled two weeks after the emersion stress, or both control groups of fish (ANOVA: $F_{3,74} = 54.722$, $P < 0.001$)(Fig. 5.1). In both control groups and in the air-emersed fish sampled two weeks after emersion stress, cortisol concentrations were low (Fig. 5.1) and characteristic of unstressed fish (Pickering and Pottinger 1989). Despite the elevation of cortisol due to emersion stress, there were no significant differences in chloride cell densities among any groups (Fig. 5.2).

## 5.5 Discussion

The pattern of cortisol elevation observed in the experimental fish of the present study is characteristic of an acute stressor. Cortisol was significantly elevated in the experimental fish immediately after the emersion stress was applied, but had returned to basal concentrations two weeks later. Similar results have been reported in response to the

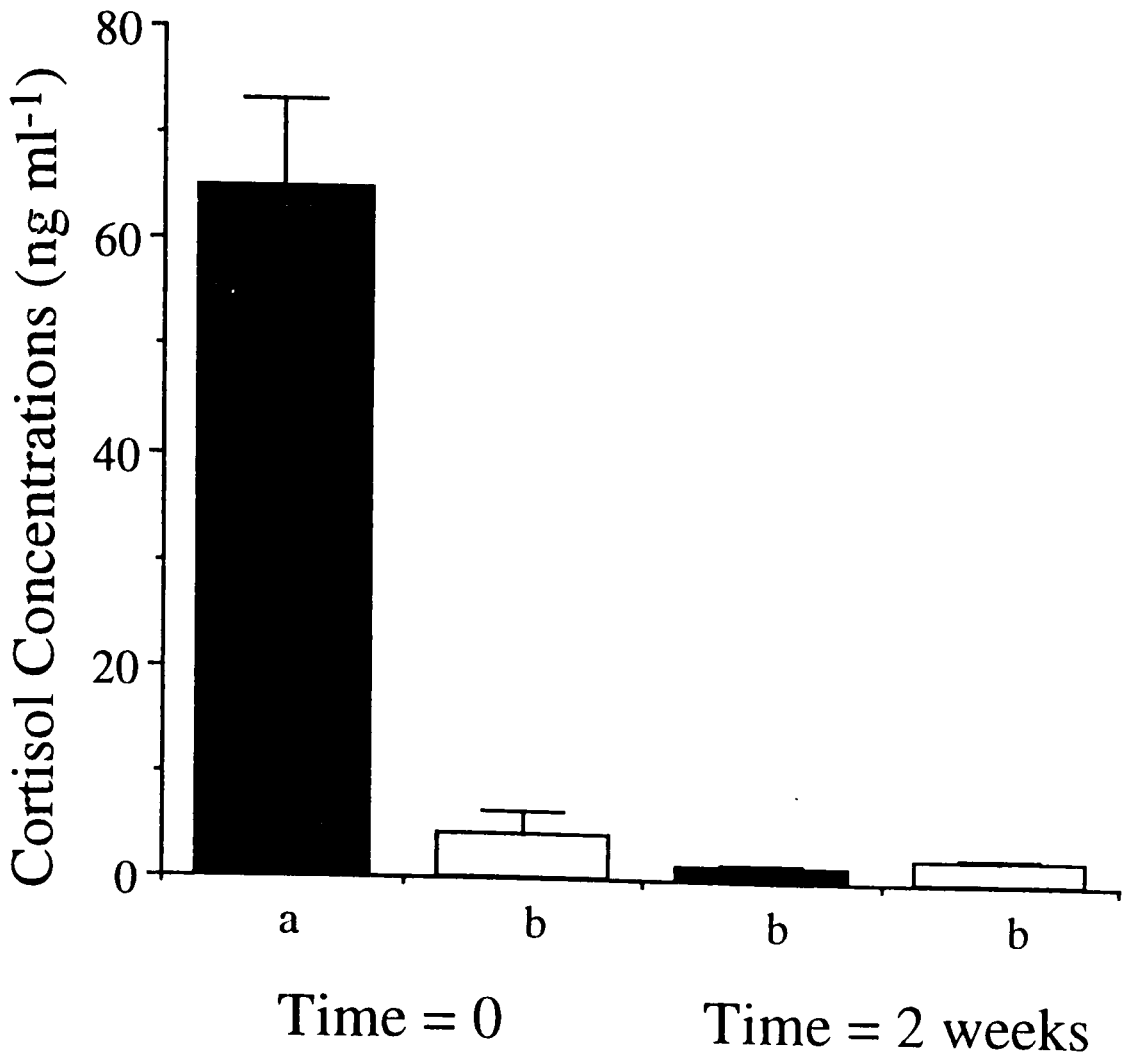



Figure 5.1: Plasma cortisol concentrations for ■ experimental and □ control fish sampled either immediately or two weeks after application of an acute stressor (n = 20 fish per treatment). Statistical differences (one way ANOVA followed by Scheffé multiple comparisons test, $P < 0.05$) are indicated by the letters; groups sharing the same letter are not significantly different from one another.

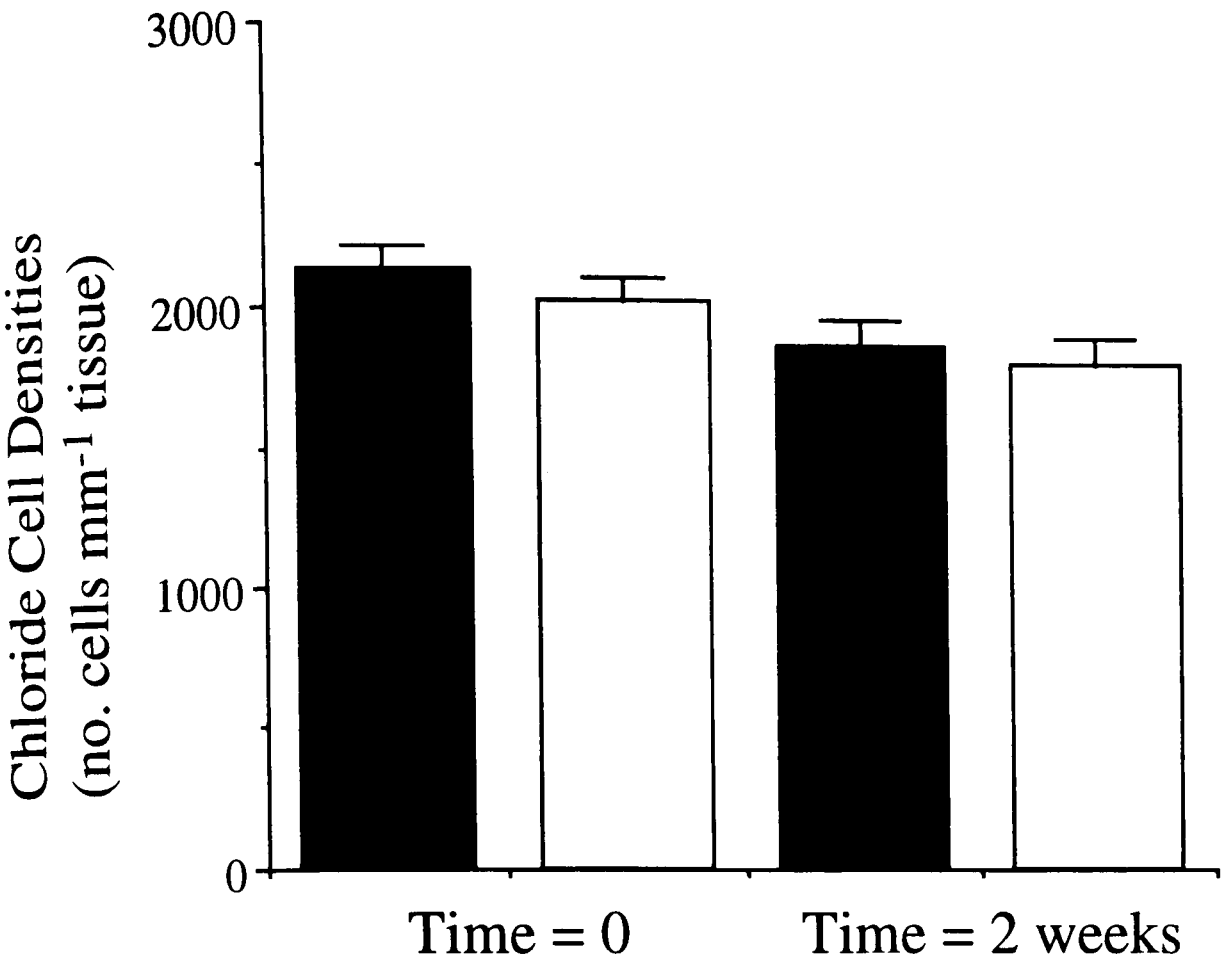



Figure 5.2: Chloride cell densities for ■ experimental and □ control fish sampled either immediately or two weeks after application of an acute stressor (n = 20 fish per treatment). No significant differences were found between the four groups for chloride cell densities ($P > 0.05$).

imposition of other acute stressors (e.g. Pickering *et al.* 1982; Einarsdóttir and Nilssen 1996), including air emersion (Pickering and Pottinger 1989). Therefore, the cortisol response was as predicted.

However, the elevation of cortisol experienced by the air-emersed fish does not appear to have induced chloride cell proliferation, suggesting that chloride cell proliferation requires an ionoregulatory challenge. The elevation of plasma cortisol elicited by acclimation to ion-poor water may play a role in eliciting chloride cell proliferation, but clearly the ionoregulatory challenge is a critical component of the chloride cell response. The proliferation of chloride cells induced by administration of cortisol (Laurent and Perry 1990; Bindon *et al.* 1994a; Laurent *et al.* 1994) is a likely artifact of the supraphysiological concentrations of cortisol that would be experienced by the fish (Bindon *et al.* 1994a).

Chloride cell proliferation causes an increase in the thickness of the gill epithelia, resulting in decreased respiratory efficiency, particularly under hypoxic conditions (see review by Perry 1998). Therefore, chloride cell proliferation appears to be a trade-off between increased ion uptake capacity and decreased respiratory efficiency. Chloride cell proliferation due to elevated cortisol concentrations in the absence of an ionoregulatory stress would presumably produce decreased respiratory efficiency without the apparent need for increased ion transportation, and therefore would appear only detrimental to the health of the fish. The apparent requirement for chloride cell proliferation during ionoregulatory challenge is therefore of functional significance.

The elevation of cortisol in air-emersed fish during the present study was not particularly high (64.85 ± 8.25 ng $ml^{-1}$) in comparison with other studies that have investigated acute stressors (*c*130 ng $ml^{-1}$ Pickering *et al.* 1982; *c*80 ng $ml^{-1}$ Pickering and Pottinger 1989). Thus, the possibility that very severe acute stress could trigger chloride cell proliferation still exists. However under levels of stress normally encountered by fish in the natural environment or in aquaculture, the potential for chloride cell proliferation to occur in the absence of an ionoregulatory disturbance is unlikely.

# CHAPTER 6

# Chapter 6: The Role of Cortisol in Chloride Cell Proliferation in the Rainbow Trout (*Oncorhynchus mykiss*)

A version of this chapter will be submitted for publication with co-authors: Patrick R. Desforges and Kathleen M. Gilmour,

## 6.1 Abstract

The role of cortisol in chloride cell proliferation was investigated by blocking cortisol at the receptor level before acclimation of rainbow trout to ion-deficient water. Fish were given implants of coconut oil containing either the glucocorticoid receptor blocker, RU486, or the mammalian mineralocorticoid receptor blocker, spironolactone, and were then either exposed to ion-deficient water or held in normal water. Sham implants (coconut oil alone) and untreated fish were used as controls. Whereas RU486 treatment was found to have no effect on chloride cell proliferation, treatment with spironolactone inhibited the proliferation of chloride cells normally associated with acclimation to soft water. No effects of any of the treatments were seen on plasma cortisol or plasma ion ($Na^+$, $K^+$, $Ca^{2+}$, $Cl^-$) concentrations seven days after implantation. The results of the present study support the hypothesis that cortisol plays an important mineralocorticoid role in freshwater fish, and that contrary to previous dogma, there may be more than one type of corticoid receptor in teleost fish.

## 6.2 Introduction

The primary response to stress in teleost fish and other vertebrates is the release of stress hormones in the form of the catecholamines (adrenaline and noradrenaline) and the corticosteroids (including cortisol). Catecholamines are released from chromaffin cells in the head kidney (fish) or from the adrenal medulla (mammals) in the adrenergic response, and corticosteroids are released from the interrenal cells in the head kidney (fish) or from the adrenal cortex (mammals) in the hypothalamo-pituitary-interrenal (HPI) response (Sumpter 1997). In eutherian mammals, the corticosteroid group of hormones, to which cortisol belongs, has been further divided into those hormones which influence aspects of carbohydrate metabolism, the glucocorticosteroids, and those which control electrolyte movement, the mineralocorticosteroids (Chester Jones and Phillips 1959). In mammals, cortisol, corticosterone and cortisone all act as glucocorticoids while aldosterone is the major mineralocorticoid hormone; the glucocorticoids and mineralocorticoids act through separate, well-characterised receptors. In fish, on the other hand, the dual roles of cortisol as both a glucocorticoid and a mineralocortoid hormone are well established (see review by Pickering and Pottinger 1995) and both effects have, until recently, been thought to be mediated through a single glucocorticoid receptor type. Fish are thought to lack aldosterone, although there is some evidence for the existence of aldosterone in *Fundulus* spp. (Matty 1985).

The division between glucocorticoids and mineralocorticoids in mammals is far from rigid. For example, cortisol can affect electrolyte excretion and aldosterone can affect

carbohydrate metabolism (Chester Jones and Phillips 1959). In fish, the actions of cortisol as a mineralocorticoid have been studied extensively, particularly in euryhaline and anadromous teleosts (Abo Hegab and Hanke 1984; Madsen 1990a; Shrimpton 1996). During acclimation of fish from freshwater to sea water, increases in plasma cortisol concentrations are seen which may last for several days (Abo Hegab and Hanke 1984) and it is widely accepted that this elevation of plasma cortisol plays a vital role in sea water acclimation by stimulating $Na^+/K^+$-ATPase enzyme activity in the ion-transporting epithelia of the gill and gut (Madsen 1990a). For example, treatment of sea trout parr (*Salmo trutta trutta*) with cortisol was found to increase the ability of fish to regulate plasma ions and muscle water during sea water transfer (Madsen 1990a). Similarly, Bisbal and Specker (1991) also demonstrated an improvement in the hypo-osmoregulatory ability of juvenile Atlantic salmon, *Salmo salar*, associated with cortisol treatment; where fish treated with cortisol and then transferred to sea water survived better than untreated fish.

There also appears to be a mineralocorticoid role of cortisol in the ionoregulation of freshwater fish. The acclimation of trout to ion-deficient or soft water has been shown previously to be associated with a transient elevation of plasma cortisol concentrations (Perry and Wood 1985; Flik and Perry 1989; Perry and Laurent 1989). In addition, injections of cortisol have been demonstrated to cause proliferation of the ion transporting cells in the gill epithelia (the chloride cells), and also to increase ion uptake and/ or fluxes across the gills (Foskett *et al.* 1983; Flik and Perry 1989; Laurent and Perry 1990; Bindon *et al.* 1994a; Laurent *et al.* 1994).

The peak in cortisol concentrations associated with acclimation to ion-deficient water is believed to be associated with the proliferation of chloride cells, which also occurs during soft water acclimation (Bindon *et al.* 1994a). Greco *et al.* (1996) demonstrated that acclimation of rainbow trout to soft water caused a doubling of the gill epithelial surface area occupied by chloride cells. Chloride cells are involved in the transport of chloride ions across the gill epithelia (Perry *et al.* 1992) and have also been implicated as the site of branchial calcium uptake (Perry and Laurent 1993). Therefore, under ion-deficient conditions, the proliferation of chloride cells appears beneficial to the fish in terms of maintaining ionic homeostasis. However, associated with chloride cell proliferation is an increase in the thickness of the gill epithelia (Bindon *et al.* 1994a; Greco *et al.* 1996), resulting in a decrease in respiratory efficiency (Bindon *et al.* 1994b; Greco *et al.* 1996). Despite several studies that have highlighted an apparent association between elevation of plasma cortisol concentrations in freshwater fish and chloride cell proliferation, the proximate stimulus for chloride cell proliferation remains poorly understood.

Thus, the aims of the present study were to investigate further the role of cortisol in chloride cell proliferation in soft water by monitoring plasma cortisol concentrations over time during acclimation to ion-deficient water, and by blocking cortisol receptors using both a recognised glucocorticoid receptor blocker in teleosts (RU486; Vijayan and Leatherland 1992) and a compound (spironolactone) that is known to block mineralocorticoid receptors in amphibians and higher vertebrates (Verrey *et al.* 1987).

## 6.3 Materials and Methods

Rainbow trout (Experiment 1: weight 38.10 ± 0.69 g; length 15.72 ± 0.10 cm (mean ± S.E.M.) n = 96; Experiment 2: weight 37.85 ± 0.60 g; length 15.48 ± 0.009 cm (mean ± S.E.M.) n = 48) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). The fish were held in large stock tanks supplied with aerated, flowing, dechlorinated City of Ottawa tap water at 13 °C, under a light regime of 12L:12D.

### *6.3.1 Experiment 1 - Cortisol and chloride cell proliferation*

After two weeks acclimation to the laboratory conditions, fish were allocated to an experimental group (n = 12 for each treatment); (i) control (untreated) soft-water, (ii) RU486-implanted, soft-water, (iii) sham-implanted, soft-water, (iv) spironolactone-implanted, soft-water, (v) control (untreated), normal water, (vi) RU486-implanted, normal water, (vii) sham-implanted, normal water, or (viii) spironolactone-implanted, normal water. Fish were anaesthetised in a solution of benzocaine (0.05 mg benzocaine $ml^{-1}$ water), and fin clipped and/or marked with alcian blue dye injected into their fins (Kelly 1967) according to the experimental group to which they were allocated. Fish treated with RU486 were given intraperitoneal implants consisting of RU486 suspended in coconut oil (0.005ml vehicle $g^{-1}$ body weight; 0.5mg RU486 $g^{-1}$ body weight). Similarly, spironolactone-treated fish were given intraperitoneal implants consisting of spironolactone suspended in coconut oil (0.005ml vehicle $g^{-1}$ body weight; 0.1mg spironolactone $g^{-1}$ body weight). Sham-implanted fish received only coconut oil (0.005ml $g^{-1}$ body weight). Fish were held in tanks supplied with aerated, flowing dechlorinated City

of Ottawa tap water ('normal' water) or a mix of tap water and water that had been passed through a reverse osmosis system to lower water ion levels ('soft' water; Table 6a). Fish were acclimated to soft water over a 24 h period and ionic concentrations were monitored daily. Soft water fish were fed to excess once daily on trout chow and the same amount of food was given to control tanks (average amount of food per fish per day = 1 g).

Table 6a: Water ion concentrations for soft water and control tanks (mean ± S.E.M.).

| | Soft Water Concentration (mM) | Control Water Concentration (mM) |
|---|---|---|
| Sodium ($Na^+$) | 0.032 ± 0.002 | 0.163 ± 0.003 |
| Potassium ($K^+$) | 0.007 ± 0.0004 | 0.021 ± 0.002 |
| Calcium ($Ca^{2+}$) | 0.096 ± 0.006 | 0.397 ± 0.009 |
| Chloride ($Cl^-$) | 0.051 ± 0.005 | 0.184 ± 0.008 |

Seven days after receiving the implants, all fish were killed by a lethal dose of benzocaine (0.5g $ml^{-1}$ water) and final fork lengths and weights were recorded. Blood samples were withdrawn by caudal venipuncture from half of the fish (n = 6), for later analysis of plasma cortisol using a commercial radioimmunoassay (ICN Pharmaceuticals) and gill samples were removed for light microscopy. In the remainder of the fish (n = 6), blood samples were withdrawn by caudal venipuncture for later analysis of plasma ion concentrations. Blood samples (0.5-1.0ml) were centrifuged (13,000 g) and the plasma was removed, frozen in liquid nitrogen and stored at –70 °C for later analysis.

For light microscopy, the second left gill arch was excised and rinsed in 0.9 % saline. Small samples of filaments were removed and fixed in buffered glutaraldehyde (5 %

glutaraldehyde in phosphate buffer; 1 h; 4 °C). The gill tissue was stained with an osmium-zinc iodide preparation (Garcia-Romeu and Masoni 1970) (1 part 2 % $OsO_4$ to 4 parts 3 % $ZnI_2$; 18 h; 20 °C) which causes a reduction of osmic acid to osmium, blackening the phospholipids. Following staining, the tissues were dehydrated in an ethanol series (30 %, 50 %, 75 %, 95 %, 100 % x 2, 20 minutes in each; 20 °C), rinsed in Histochoice® (2 x 20 minute rinses; 20 °C) and Paraplast® (2 x 20 minute rinses; 55 °C), embedded in Paraplast® and sectioned at 7 µm using a microtome (Spencer).

Eight slides, with eight sections per slide, were prepared for each fish, producing a total of 64 sections per fish. Slides were viewed using a light microscope (25 x objective; Leitz Dialux® microscope) and digital pictures were acquired (JVC TK1381 1/2"CCD digital video colour camera, 470 lines resolution PCI Image Capture card; Viglen P460 PC). Six pictures each were produced for four fish from each treatment from randomly selected sections; the slide was positioned so that the field of view contained approximately seven lamellae and a small portion of the filament at the bases of the lamellae. For quantification, the gill tissue area in a picture was recorded using Scion Image software and the number of chloride cells per unit tissue area was calculated by visually counting the intensely stained cells. The thickness of the lamellae was measured (two measurements per image) and also the interlamellar distances (two measurements per image). The value for each fish was taken as the mean of the values for the six images.

*6.3.2 Experiment 2 - Time course of cortisol concentrations during soft water acclimation*

After acclimation to the laboratory conditions 60 fish were transferred to an experimental tank. Over a 24 h period fish were gradually acclimated to ion-deficient water by reducing the flow of normal dechlorinated tap water to the tank and initiating a flow of dechlorinated tap water that had been treated using a reverse osmosis system to remove ions. Ion concentrations were monitored daily as in experiment 1 (Table 6a). Fish were fed to excess once daily on pellets. After 24 h acclimation to soft water 6 fish were removed and sampled for blood plasma cortisol as in experiment 1. The sampling process was repeated at 48, 72, 96, 120, 144 and 168 h.

*6.3.3 Analytical Methods*

Sodium, potassium and calcium ions were measured using an atomic absorption spectrophotometer (Varian SpectraAA). Chloride ions were measured using a colorimetric assay (Zall 1956). Cortisol concentrations were measured using a commercial RIA.

*6.3.4 Statistical methods*

Plasma cortisol concentrations, plasma ion levels and gill morphometric variables were compared among the eight experimental groups using analysis of variance (ANOVA) models. Data are presented as means ± S.E.M. The fiducial limit of significance in all analyses was 5 %.

## 6.4 Results

### *6.4.1 Experiment 1 - Cortisol and chloride cell proliferation*

Among the eight experimental treatments, there were no significant differences in plasma cortisol concentrations at the end of the seven day period (ANOVA: $F_{7,40} = 0.844$, $P > 0.5$; Table 6b) and cortisol concentrations were all characteristic of unstressed fish (Pickering and Pottinger 1989) ($3.00 \pm 0.84$ ng $ml^{-1}$ (mean $\pm$ S.E.M.) $n = 48$). There were also no significant differences among the groups in plasma ion concentrations (Table 6c; ANOVA: $Na^+$ $F_{7,38} = 0.925$, $P = 0.498$; $K^+$ $F_{7,38} = 1.859$, $P = 0.104$; $Ca^{2+}$ $F_{7,38} = 1.943$, $P = 0.089$; $Cl^-$ $F_{7,38} = 1.577$, $P = 0.172$).

Table 6b: Plasma cortisol concentrations for each treatment group (mean $\pm$ S.E.M.) ($n = 6$ fish per treatment).

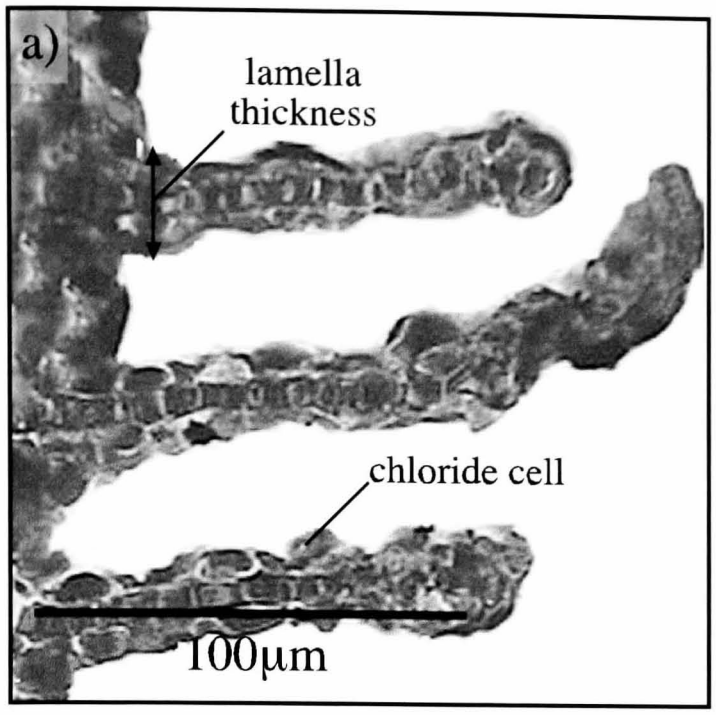
| Treatment | Control Water Plasma Cortisol (ng $ml^{-1}$) | Soft Water Plasma Cortisol (ng $ml^{-1}$) |
|---|---|---|
| Untreated Fish | $1.32 \pm 0.17$ | $1.14 \pm 0.07$ |
| Sham-Implanted | $3.48 \pm 1.13$ | $1.93 \pm 0.25$ |
| RU486-Implanted | $7.82 \pm 6.46$ | $3.46 \pm 1.11$ |
| Spironolactone-Implanted | $1.64 \pm 0.23$ | $3.47 \pm 1.13$ |

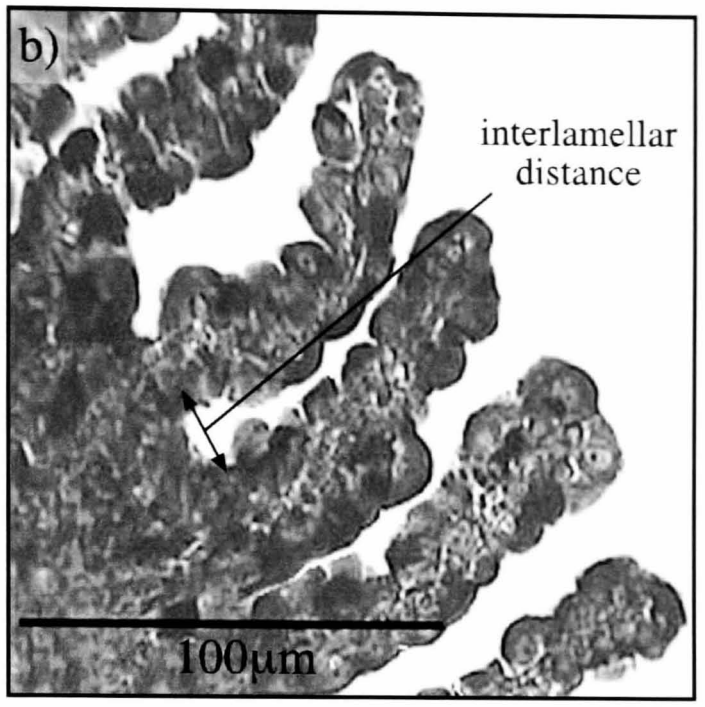
Table 6c: Plasma ion concentrations for each treatment group (mean ± S.E.M.) (n = 6 fish per treatment).

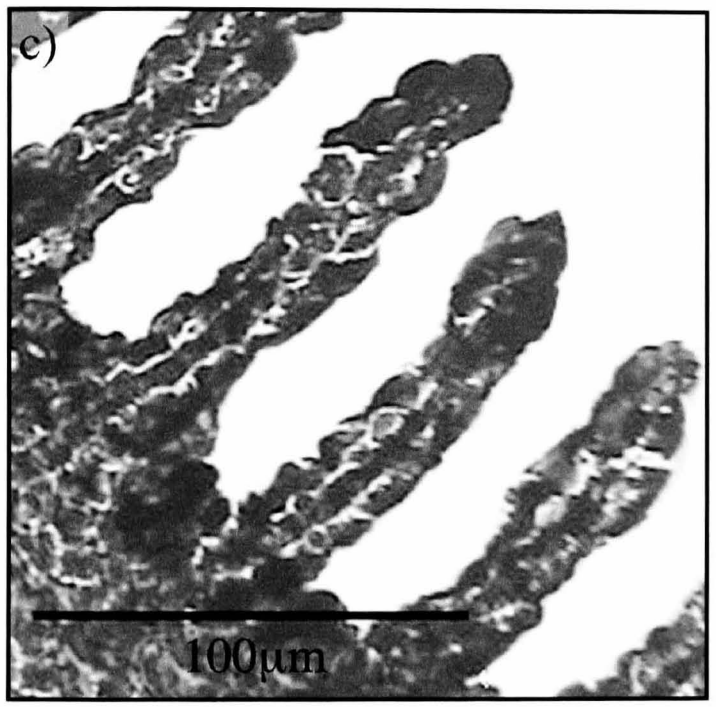
| Treatment | $Na^+$ (mM) | $K^+$ (mM) | $Ca^{2+}$ (mM) | $Cl^-$ (mM) |
|---|---|---|---|---|
| Control Water | 161.5 ± 3.0 | 3.31 ± 0.2 | 3.35 ± 0.1 | 161.1 ± 2.9 |
| Soft Water | 151.1 ± 11.0 | 3.51 ± 0.2 | 3.33 ± 0.1 | 170.8 ± 4.2 |
| Sham Implanted Control Water | 159.3 ± 4.0 | 2.87 ± 0.2 | 3.16 ± 0.1 | 160.9 ± 1.5 |
| Sham Implanted Soft Water | 168.3 ± 4.5 | 3.39 ± 0.1 | 3.30 ± 0.0 | 168.1 ± 3.3 |
| RU486 Implanted Control Water | 158.4 ± 3.4 | 3.01 ± 0.3 | 3.26 ± 0.1 | 157.6 ± 4.0 |
| RU486 Implanted Soft Water | 161.9 ± 2.5 | 3.19 ± 0.1 | 3.05 ± 0.1 | 167.5 ± 5.7 |
| Spironolactone Implanted Control Water | 160.4 ± 2.4 | 3.19 ± 0.2 | 3.30 ± 0.1 | 167.5 ± 6.5 |
| Spironolactone Implanted Soft Water | 157.9 ± 2.6 | 3.21 ± 0.1 | 3.01 ± 0.14 | 156.3 ± 3.8 |

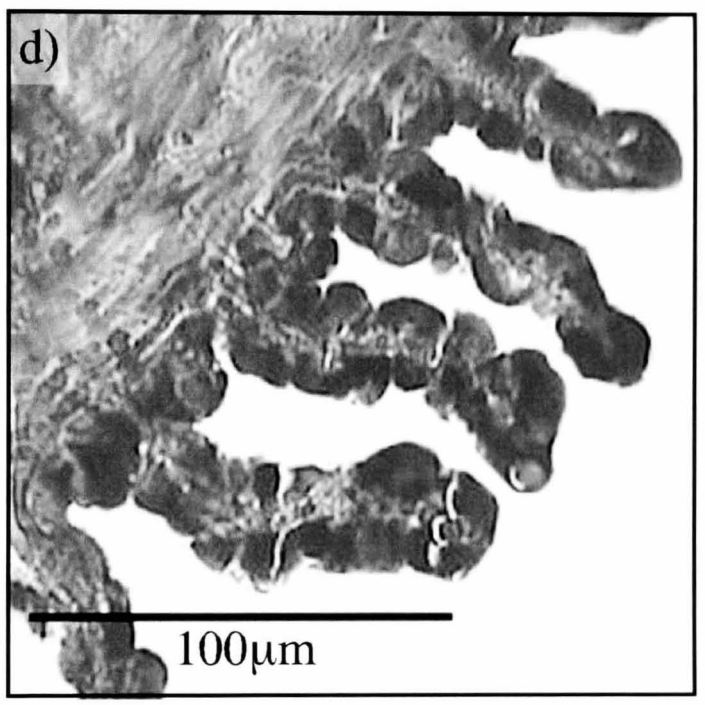
A different picture emerged, however, when gill morphology was examined. Gill sections from each of the eight treatment groups are shown in Fig. 6.1. Significant differences were detected for lamellae thickness (ANOVA: $F_{7,183} = 56.08$, $P < 0.001$; Fig. 6.2a), interlamellar distances (ANOVA: $F_{7,183} = 81.72$, $P < 0.001$; Fig. 6.2b) and chloride cell densities (ANOVA: $F_{7,183} = 34.52$, $P < 0.001$; Fig. 6.2c). In general, those fish held in soft water had significantly higher chloride cell densities, associated with increased lamellae thickness and decreased interlamellar distances, than those fish held in normal water. However, the exception was those fish implanted with spironolactone, where no significant differences in gill structure were seen between fish held in soft water and those held in normal water.

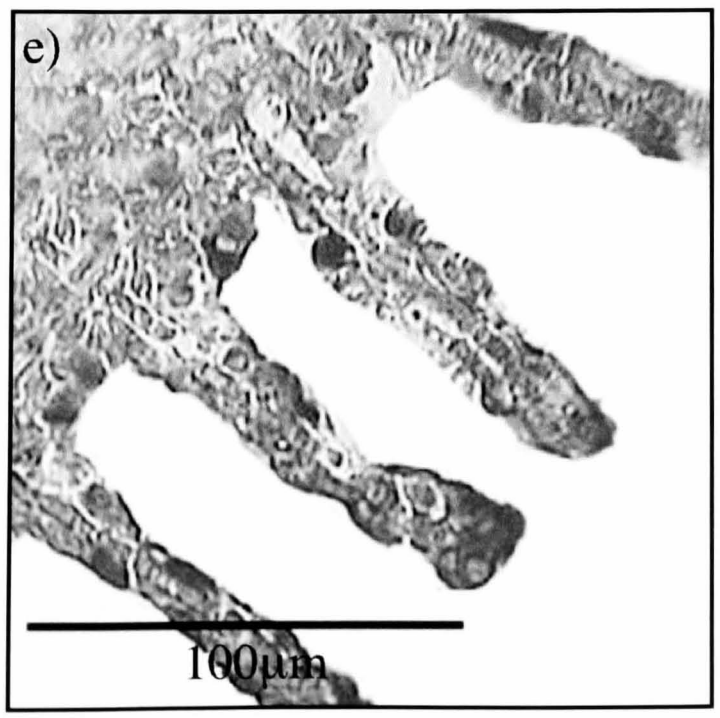
Figure 6.1: Images of gill sections showing (a) control water, (b) soft water, (c) RU486 implanted, control water, (d) RU486 implanted, soft water, (e) spironolactone implanted, control water, and (f) spironolactone implanted, soft water.

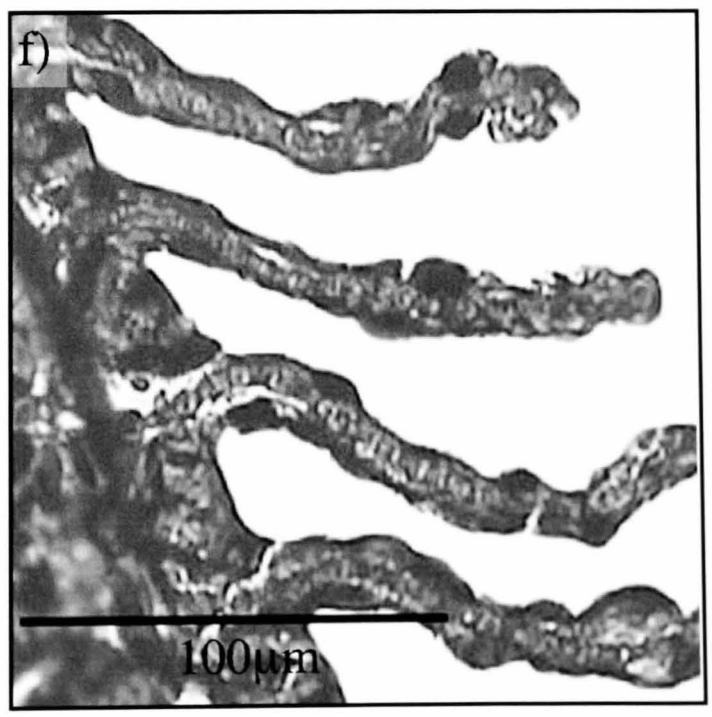

a)
lamella
thickness
chloride cell
100µm


b)
interlamellar
distance
100µm


c)
100µm


d)
100µm


e)
100µm


f)
100µm

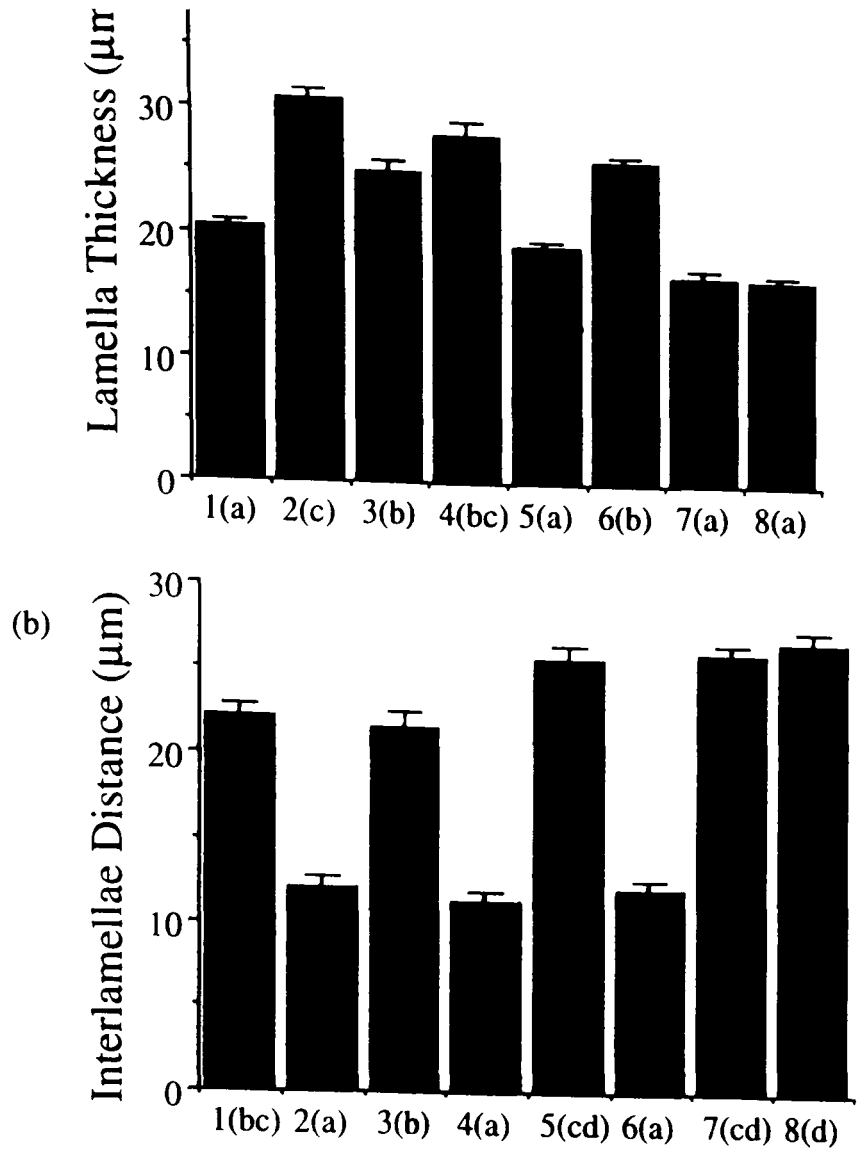



Figure 6.2: Lamella thickness (a), interlamellar distance (b) and chloride cell density (c) for each treatment group; control water (1), soft water (2), sham implanted, control water (3), sham implanted, soft water (4), RU486 implanted, control water (5), RU486 implanted, soft water (6), spironolactone implanted, control water (7) and spironolactone implanted, soft water (8). Data are expressed as mean ± S.E.M. Treatments sharing the same letter are not statistically different (see text for statistical analysis).

*6.4.2 Experiment 2 - Time course of cortisol concentrations during soft water acclimation*

No significant differences were found in plasma cortisol concentrations among all of the sampling times (Table 6d) and the mean concentrations of cortisol in all of the groups was characteristic of unstressed fish (Pickering and Pottinger 1989) (2.03 ± 0.20 ng $ml^{-1}$ (mean ± S.E.M.) n = 48).

Table 6d: Plasma cortisol concentrations of fish acclimated to soft water over a one week period (mean ± S.E.M.) (n = 6 fish per time).

| Time (hours) | Plasma Cortisol Concentration (ng $ml^{-1}$) |
|---|---|
| 24 | 2.32 ± 0.80 |
| 48 | 2.29 ± 0.4 |
| 72 | 1.62 ± 0.26 |
| 96 | 1.50 ± 0.25 |
| 120 | 1.43 ± 0.27 |
| 144 | 2.58 ± 0.75 |
| 168 | 2.36 ± 0.61 |

## 6.5 Discussion

Determining the exact mechanisms of action of cortisol is made difficult by the inability to remove easily endogenous sources of cortisol. Some surgical procedures that enable tight control of endogenous production of cortisol include hypophysectomy (Donaldson and McBride 1967) and adrenalectomy (Butler *et al.* 1969). However, both of these procedures involve the stress associated with major surgery and the former results in the removal of all pituitary hormones, while the latter can result in electrolyte imbalance, neither of which is desirable (Vijayan *et al.* 1994). An alternative to controlling

endogenous concentrations of plasma cortisol, blocking cortisol receptors, was utilised in the present study. The steroid analogue RU486 is a known glucocorticoid receptor blocker in mammals (Moguilewsky and Philibert 1984) and has been used previously to inhibit cortisol-mediated conversion of $T_3$ to $T_4$ in the liver of rainbow trout (Vijayan and Leatherland 1992). Vijayan *et al.* (1994) demonstrated that RU486 significantly increased *in vitro* hepatocyte glycogen breakdown and blocked the cortisol-elicited increases in alanine-glyconeogenesis and glycogen utilisation for endogenous use in the rainbow trout. RU486 has also been used to demonstrate that both the cortisol-induced apoptosis of ß cells and the inhibition of neutrophil apoptosis by cortisol in the immune system of the common carp, *Cyprinus carpio* are receptor mediated (Weyts *et al.* 1998a, b). The compound, spironolactone, a known mineralocorticoid blocker in mammals (Maguire *et al.* 1999), does not appear to have been used previously in teleost fish. Further research into the usefulness of spironolactone as a mineralocorticoid receptor blocker in teleosts is clearly required, but it appears to be a potentially useful tool for future research into the control of ionic homeostasis in fish.

Acclimation of rainbow trout to soft water induced chloride cell proliferation in the gill epithelia in comparison with those fish held in normal water, a well-characterised response to the ionoregulatory challenge provided by ion-deficient water (Bindon *et al.* 1994a; Greco *et al.* 1996). Chloride cell proliferation in soft water was abolished however, by the treatment of fish with the mammalian mineralocorticoid receptor blocker, spironolactone (Fig. 6.2). By contrast, chloride cell proliferation in soft-water acclimated fish was unaffected by treatment of the fish with the glucocorticoid receptor blocker RU486. As

the presence of the mammalian mineralocorticosteroid, aldosterone, in fish is undetermined (Matty 1985) it has been suggested that cortisol assumes both mineralocorticoid and glucocorticoid roles in fish. Until recently, it was thought that, despite the dual role of cortisol in fish, only one type of cortisol-binding receptor occurred, glucocorticoid receptors (GR) (Sandor *et al.* 1984; Kloas *et al.* 1998). However, a recent study has demonstrated the presence of a mineralocorticoid-like receptor in the rainbow trout (rtMR) (Colombe *et al.* 2000). Colombe *et al.* (2000) cloned an rtMR from rainbow trout testis that demonstrated a clear homology with various mineralocorticoid receptor (MR) cDNA sequences of higher vertebrates, and had a high affinity for cortisol ($K_a$ = 0.53 ± 0.03 nM, $K_d$ = 1.9 nM).

The results of the present study support the hypothesis that two different hormone-receptor systems may exist in rainbow trout, one with a glucocorticoid role and the other with a mineralocorticoid role. Blocking mineralocorticoid receptors with spironolactone inhibited chloride cell proliferation in rainbow trout acclimated to ion-poor water, but blocking glucocorticoid receptors with RU486 was without effect. As the presence of glucocorticoid receptor mRNA has already been established in the chloride cells of chum salmon, *Oncorhynchus keta*, (Uchida *et al.* 1998) rtMRs that are involved in branchial ion transport are probably coexpressed with glucocorticoid receptors in the chloride cells (Colombe *et al.* 2000). Thus, cortisol may be acting as both a glucocorticoid and a mineralocorticoid hormone with the dual roles mediated by different receptors. Furthermore, it is likely that the abundance of each type of cortisol receptor is modified

according to the type of stress encountered by the fish. An alternative hypothesis would postulate the existence of aldosterone, or an aldosterone substitute in fish.

Interestingly, no significant differences in the concentrations of the different plasma ions were noted among the eight experimental groups. Other studies involving exposure of fish to soft water have achieved varying results. Greco *et al.* (1995) demonstrated decreased plasma ion concentrations of fish acclimated to soft water whereas Perry and Wood (1985) found no significant differences in plasma ions in fish acclimated to water deficient in calcium. Laurent and Perry (1989) demonstrated significantly depressed $Na^+$ and $Cl^-$ concentrations in rainbow trout after four days exposure to water deficient in NaCl. No changes in $K^+$ or $Ca^{2+}$ concentrations were seen. Acclimation of freshwater fish to ion-poor water results in an initial net loss of NaCl , predominantly across the gills. This rate of loss decreases with time, and, coupled with increases in ion uptake, enables ionic homeostasis to be regained, although the initial ion balance is often not restored (Laurent and Perry 1989). McDonald and Rogano (1986) demonstrated that prolonged exposure of rainbow trout to ion-poor water resulted in increased loss of NaCl but not $Ca^{2+}$, and although rate of loss decreased with time, the initial ion balance was not restored.

In mammals, administration of RU486 has been found to result in a dose-dependent increase in plasma cortisol concentrations, presumably owing to negative feedback mechanisms (Healy *et al.* 1983). However, no significant differences in cortisol concentrations were detected among the treatment groups in the present study seven days after implantation. Previous research using RU486 in teleosts has been carried out with

similar results (Vijayan *et* al. 1994; Reddy *et al.* 1995). However, Bernier *et al.* (1999) demonstrated an increase in plasma cortisol concentrations 24 h after implantation of goldfish, *Carassius auratus*, with RU486. It appears that the cortisol elevation associated with RU486 administration is short-lived and that by seven days after implantation cortisol concentrations would have returned to basal concentrations (Bernier *et al.* 1999). As spironolactone has not been used previously in teleosts, its effects on the HPI axis remain undetermined. It is possible that administration of spironolactone also results in a peak in plasma cortisol concentrations, with a return to basal concentrations after seven days.

A transient elevation in cortisol concentrations during acclimation to ion-deficient water has been found to occur in previous studies (Perry and Wood 1985; Perry and Laurent 1989) and is one of the main reasons behind the hypothesis that cortisol is involved in the proliferation of chloride cells in the gill epithelia. However, in the present study no peak in cortisol concentrations was seen during acclimation to ion poor water (experiment 2); indeed, all cortisol concentrations were low throughout the experiment and indicative of unstressed fish (Pickering and Pottinger 1989). It is possible that in the present study there was a transient increase in cortisol concentrations occurring within the first 24 hours of exposure to ion deficient water and that cortisol concentrations had returned to basal levels by the first sampling time. A rapid recovery of cortisol to basal levels is not unusual in response to an acute stressor (Pickering *et al.* 1982). Plasma cortisol concentrations in the present study were also much lower than in the study of Perry and Laurent (1989), where even control cortisol concentrations were above $5ngml^{-1}$. Therefore, it is also

possible that in the present study, fish were less stressed by the holding conditions and adapted more quickly to the ion deficient water.

In conclusion, the present study has demonstrated that cortisol does indeed play a role in chloride cell proliferation in the gill epithelia of trout acclimated to ion-deficient water. The data support the hypothesis that cortisol functions as a mineralocorticoid rather than a glucocorticoid hormone in this context, and that its effects are mediated by a population of mineralocorticoid receptors. Clearly, the hypothesis that two types of hormone-receptor systems exist in fish gills and that the abundance and type of cortisol receptors available control the physiological effects of cortisol warrants further investigation.

# CHAPTER 7

## Chapter 7: Effects of Social Interaction on the Stress Response in Rainbow Trout, *Oncorhynchus mykiss.*

A version of this chapter will be submitted for publication with co-authors: Colin J. Montpetit and Kathleen M. Gilmour.

### 7.1 Abstract

The aim of the present study was to investigate in rainbow trout the effect of social status on the ability to secrete cortisol and release catecholamines using a perfused posterior cardinal vein preparation (PCV), the first use of this preparation to investigate cortisol secretion of the interrenal cells *in situ*. Subordinate fish showed a characteristic elevation of circulating plasma cortisol concentrations compared with dominant fish. No significant effect of subordination was seen on the release of the catecholamines adrenaline and noradrenaline upon stimulation of the chromaffin cells in the *in situ* perfused PCV preparation with acetylcholine. However, interrenal cells of the subordinate fish demonstrated a decreased sensitivity compared to those of dominant fish when stimulated with adrenocorticotropin (ACTH) in the *in situ* perfused PCV preparation. The chronic elevation of plasma cortisol associated with subordination appeared to result in a negative feedback mechanism that affected cortisol secretion.

## 7.2 Introduction

The behavioural interactions occurring when two fish are confined together result in the formation of a dominance hierarchy, one fish becoming dominant over the other, subordinate, fish. The subordinate fish will often display characteristic physiological changes associated with subordinance, including elevated plasma cortisol concentrations (Peters *et al.* 1980; Laidley and Leatherland 1988), increased plasma glucose concentrations (Peters *et al.* 1988), decreased disease resistance (Peters *et al.* 1988), growth rate and condition. Plasma cortisol concentrations in the subordinate fish of paired rainbow trout are rapidly elevated upon confinement, being significantly higher than those of the dominant fish 4 h after pairing, and remain high for extended periods of time (1 week or more) (Sloman *et al.* unpublished (Chapter 2)). These physiological changes are similar to those elicited by stressors such as crowding, that are considered to be chronic stressors (Pickering and Pottinger 1989) and therefore, it has been suggested that the social stress of subordinance under these artificial confinement conditions is that of a chronic stressor (Øverli *et al.* 1999; Sloman *et al.* unpublished (Chapter 2)).

It has previously been shown that chronic elevation of cortisol may affect the physiological response of the fish to acute stressors. For example, juvenile rainbow trout fed food containing cortisol for a 10 week period showed a reduction in cortisol elevation after acute handling (Barton *et al.* 1987). Vijayan and Leatherland (1990) demonstrated that stimulation of the interrenal cells of brook charr, *Salvelinus fontinalis*, by adrenocorticotropic hormone (ACTH) *in vitro* produced a lower response in fish stocked

at high density compared to those of a low density. It was suggested that the interrenal cells of fish reared at a high stocking density (and presumably subjected to chronic stress in consequence) were maximally active and therefore unable to respond to stimulation with ACTH. Therefore, the possibility exists that subordinate fish, with chronically elevated plasma cortisol concentrations, may be less able or even unable to respond to acute stressors (such as handling) by the production of plasma cortisol if the interrenal cells are already stimulated to their maximum capacity.

Catecholamines, also released as a primary and intermediate response to stress (review by Randall and Perry 1984), are stored ready for release in the chromaffin cells (Reid *et al.* 1998). Acute environmental stressors such as decreased oxygen concentration (hypoxia; Perry and Reid 1992) and increased carbon dioxide concentration (hypercapnia; Perry *et al.* 1989) have been demonstrated to result in the release of catecholamines. Physiological effects induced by catecholamine release include changes in physiology designed to alleviate the consequences of short term stressors, e.g. enhanced cardio-respiratory function and mobilisation of energy stores (Reid *et al.* 1998).

Although the separate effects of both acute and chronic stressors on the release of catecholamines and cortisol have been intensively researched in teleost fish (see review by Pickering and Pottinger 1995), the interactions between the adrenergic (catecholamines) and hypothalamo-pituitary-interrenal (cortisol) stress response systems, and particularly the effects of chronic stressors on the ability of fish to respond to acute stress, are less well understood. In teleosts, the chromaffin (catecholamine-releasing) and interrenal (cortisol-

secreting) cells line the walls of the posterior cardinal vein in the region of the head kidney and hence are in close proximity to one another (Nandi 1961). This physical arrangement lends itself to the possibility of paracrine control in the release of the stress hormones, a possibility for which there exists some experimental support. For example, Montpetit and Perry (1998) demonstrated that a five-day exposure to hypoxia (*i.e.* a chronic stress) significantly increased the responsiveness of chromaffin cells to cholinergic stimulation whilst Reid *et al.* (1994) found that rainbow trout subjected to chronic physical stress (twice daily chasing to exhaustion for five days) displayed a decreased responsiveness to the cholinoceptor agonist carbachol. These results suggest that the physiological effects of chronic stress may affect the primary response to an acute stress (*i.e.* catecholamine release).

Thus, the aim of the present study was to investigate whether the chronic stress endured by a subordinate fish (*i.e.* a behavioural stressor) would likewise affect the physiological capacity of the fish to respond to acute stressors. An *in situ* perfused posterior cardinal vein preparation (Fritsche *et al.* 1993) was used to make it possible a direct comparison of the primary stress response (*i.e.* the ability to release catecholamines and cortisol, the primary stress hormones) between dominant and subordinate rainbow trout.

## 7.3 Methods

Rainbow trout (weight 252.22 ± 7.97 g; length 27.38 ± 0.23 cm (mean ± S.E.M.) n = 40) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario), and held in

large (1250 L) stock tanks in aerated, flowing, dechlorinated, City of Ottawa tap water. After at least a month of acclimation to the aquaria conditions fish were anaesthetised in a solution of benzocaine anaesthetic (0.05g $ml^{-1}$ water) and marked for identification with alcian blue dye injected into their fins (Kelly 1967). Initial fork lengths and weights were recorded. Following a 24 h recovery period, the fish were placed in 20 size matched pairs (mean size difference = 0.3 ± 0.07 cm (mean ± S.E.M.)) and were held in separate experimental chambers (33 L). Twice daily fish were fed to excess on commercial trout pellets and behavioural observations were carried out in order to determine the dominance relations within the pair. After six days of confinement in pairs, cortisol or catecholamine secretion rates in response to ACTH or acetylcholine, respectively, of dominant and subordinate fish were assessed using an *in situ* perfused posterior cardinal vein (PCV) preparation.

### *7.3.1 Behavioural observations*

Dominance was measured by assigning points during the six day experimental period. The points system that was utilised has been used previously for determining social status among salmonids (e.g. Metcalfe *et al.* 1989; Johnsson *et al.* 1996; Sloman *et al.* 2000a (Chapter 3), c (Chapter 4)). Fish were scored twice daily according to their position in the tank. Fish that maintained their position in the water column scored 10 points, fish that rested on the bottom of the tank scored five points and those fish that swam at the water surface (indicative of subordinance, Sloman *et al.* 2000a (Chapter 3)) scored zero points. Fish were also scored according to food acquisition. Twice daily, a single item of food was introduced to the tank and the fish that took the food scored one point. The other fish

scored zero points. Fish were then fed to excess to ensure that both fish were able to obtain a full ration during the course of the experiment.

### *7.3.2 In Situ Posterior Cardinal Vein Preparation*

After six days confinement in pairs, the two fish of a pair were killed simultaneously (within 20 s) by a blow to the head. A blood sample (0.5 - 1.0 ml) was immediately withdrawn by caudal venipuncture. Following centrifugation (13,000g), the plasma was removed, frozen in liquid nitrogen and stored for later analysis of plasma cortisol concentrations using a radioimmunoassay (ICN Pharmaceuticals). The fish were then used in the *in situ* saline-perfused PCV preparation (Fritsche *et al.* 1993). The fish were placed ventral side up on ice, and a ventral incision was made along the length of the fish beginning at the anus and ending just anterior to the pectoral girdle. The tissue overlying the heart was removed by blunt dissection to expose the ventricle and bulbus arteriosus. A cannula (Clay-Adams PE160 polyethylene tubing) was inserted into the ventricle via an incision in the bulbus and was secured with a ligature; this cannula formed the outflow for the perfusion system. The PCV was cannulated in the anterograde direction to act as an inflow. The preparation was then perfused with Cortland saline (Wolf, 1963) at a flow rate of approximately 1ml $min^{-1}$. A positive pressure difference between the surface of the saline and the outflow cannula was used to drive the perfusion. Preparations were perfused with saline for 15 min to allow stabilisation of catecholamine or cortisol secretion, and were then perfused with saline containing either acetylcholine or ACTH for the measurement of catecholamine or cortisol secretion rates, respectively. Ten pairs of fish were then used for plasma catecholamine analysis and ten for analysis of cortisol.

*7.3.3 Series 1: Catecholamines*

Following the 15 min stabilisation period, a sample of outflowing perfusate ('pre' sample) was taken to quantify the basal *in situ* catecholamine secretion rates. Acetylcholine (initial dose $10^{-11}$ mol $kg^{-1}$) was then administered via a three way valve into the inflow cannula. After a period of one minute to allow delivery of the acetylcholine to the chromaffin tissue, five outflowing perfusate samples were collected in pre-weighed microcentrifuge tubes at one minute intervals. This procedure (including collection of a 'pre' sample to quantify the basal catecholamine secretion rate) was repeated with a 15 minute interval between each successive dose for doses of 5 *· $10^{-10}$, $10^{-10}$, $10^{-9}$, $10^{-8}$, 5 *· $10^{-7}$, $10^{-7}$, $10^{-6}$ and finally $10^{-7}$ mol $kg^{-1}$ acetylcholine. The last dose of $10^{-7}$ mol $kg^{-1}$ was used to confirm that the PCV preparation was still responding the series of consecutive acetylcholine doses. All perfusate samples were frozen immediately in liquid nitrogen and stored at -86 °C until later analysis of catecholamines.

Before analysis, perfusate samples were re-weighed to allow for subsequent calculation of perfusion flow rates and therefore catecholamine secretion rates. Plasma catecholamine concentrations were determined on alumina-extracted samples using high-pressure liquid chromatography (HPLC) with electrochemical detection (Woodward 1982). 3,4-Dihydroxybenzylamine hydrobromide was used as an internal standard.

*7.3.4 Series 2: Cortisol*

Following the 15 min stabilisation period, a sample of outflowing perfusate ('pre' sample) was taken to quantify the basal *in situ* cortisol secretion rate. Adrenocorticotropin hormone (ACTH; initial dose 500 mU $ml^{-1}$) was then administered via a three way valve into the inflow cannula. The preparation was perfused with ACTH for a period of 15 minutes, after which time the perfusate was switched back to saline. A sample of perfusate was collected into a pre-weighed micro-centrifuge vial after 7.5 minutes, 15 minutes and there after every 15 minutes for a period of one and a half hours. This procedure (including the collection of the 'pre' sample) was then repeated for a second dose (1000 mU $ml^{-1}$) of ACTH. All perfusate samples were frozen immediately in liquid nitrogen and stored at -86 °C until later analysis of cortisol concentrations using a commercial radioimmunoassay (ICN Pharmaceuticals). Before analysis, perfusate samples were re-weighed to allow for calculation of perfusion flow rates and therefore cortisol secretion rates.

*7.3.5 Statistical analysis*

The position and feeding scores for each pair of fish were processed using a principal components analysis (PCA), which combined the different measurements (weighting them according to the extent to which they correlated with the derived principal axis) so as to generate an overall behaviour score for each fish (Sloman *et al.* 2000a (Chapter 3), b (Chapter 8), c (Chapter 4)). The PCA results allowed each fish to be classified as either dominant or subordinate; the fish with the higher score within each pair being defined as the dominant and the fish with the lower score being the subordinate fish. Circulating

plasma cortisol concentrations were compared for dominant versus subordinate fish using analysis of variance (ANOVA) models. Basal ('pre') and maximum catecholamine secretion rates for each acetylcholine dose were compared using Wilcoxon paired ranks test to confirm that catecholamine release had occurred. Those doses which did not elicit release were omitted from further statistical analyses. Doses that evoked catecholamine release and maximum secretion rates were compared between dominant and subordinate fish using repeated measures ANOVA and t-test models. Maximum cortisol secretion rates were compared between dominant and subordinate fish using repeated measures ANOVA and *t*-test models. Data are presented as means ± S.E.M. The fiducial limit of significance in all analyses was 5 %.

**7.4 Results**

Subordinate fish had significantly elevated plasma cortisol concentrations after six days confinement in pairs compared with dominant fish (Unpaired t-test: $F = 14.67$, $P < 0.001$; Fig. 7.1). In addition, a significant negative correlation was detected between behaviour (PCA) score and plasma cortisol concentration (Linear Regression: $F_{1,39} = 6.016$, $P < 0.05$; Fig. 7.2), such that the more subordinate the behaviour of the fish, *i.e.* the lower its behaviour score, the higher was its plasma cortisol concentration.

All doses of acetylcholine except $5^{*}10^{-11}$ mol $kg^{-1}$ elicited significant catecholamine release. There was no significant statistical difference between the catecholamine secretion rates for the first and second $10^{-7}$ mol $kg^{-1}$ doses of acetylcholine (Paired t-test:

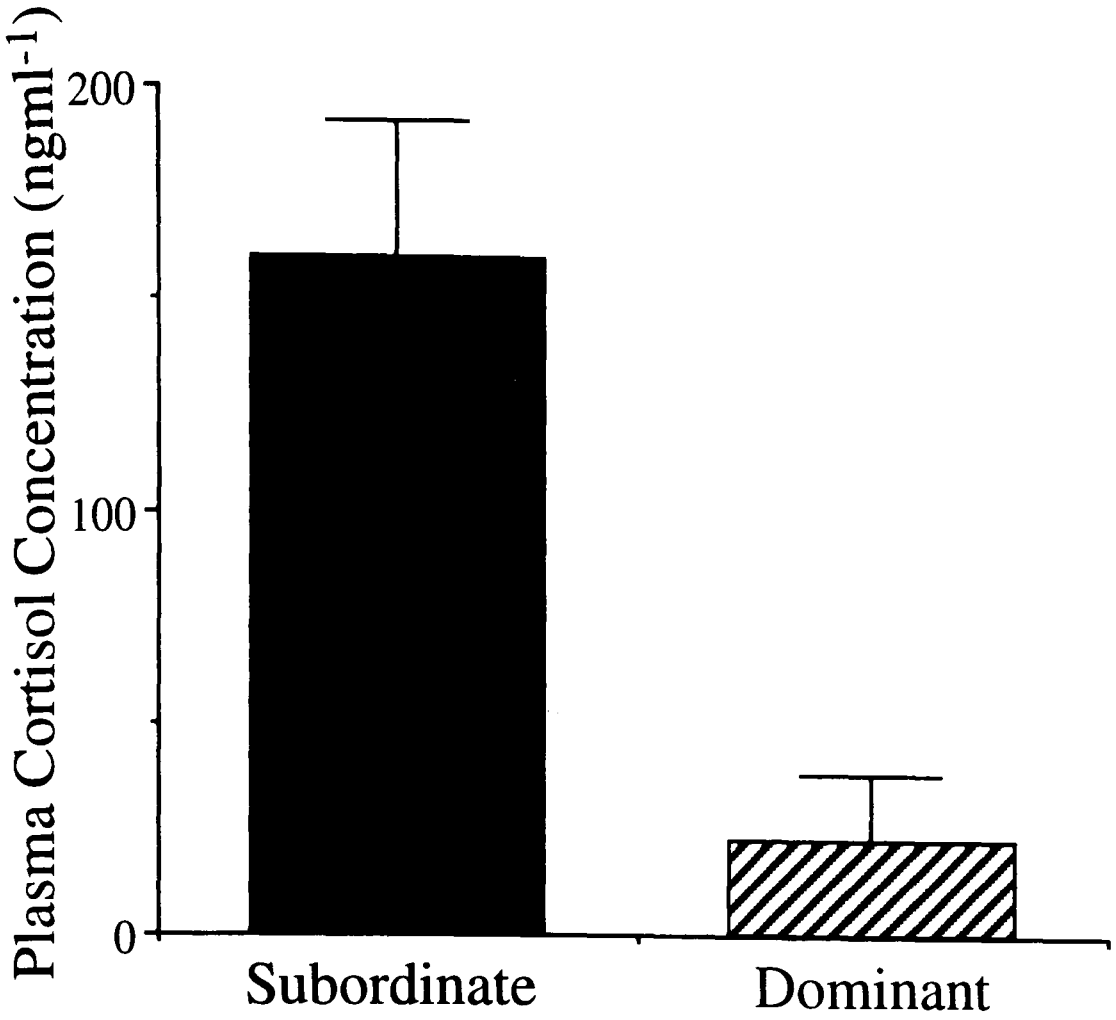



Figure 7.1: Plasma cortisol concentrations for subordinate (■, n = 20) and dominant (□, n = 20) fish after six days confinement in pairs (Unpaired t-test: $F = 14.67$, $P < 0.001$).

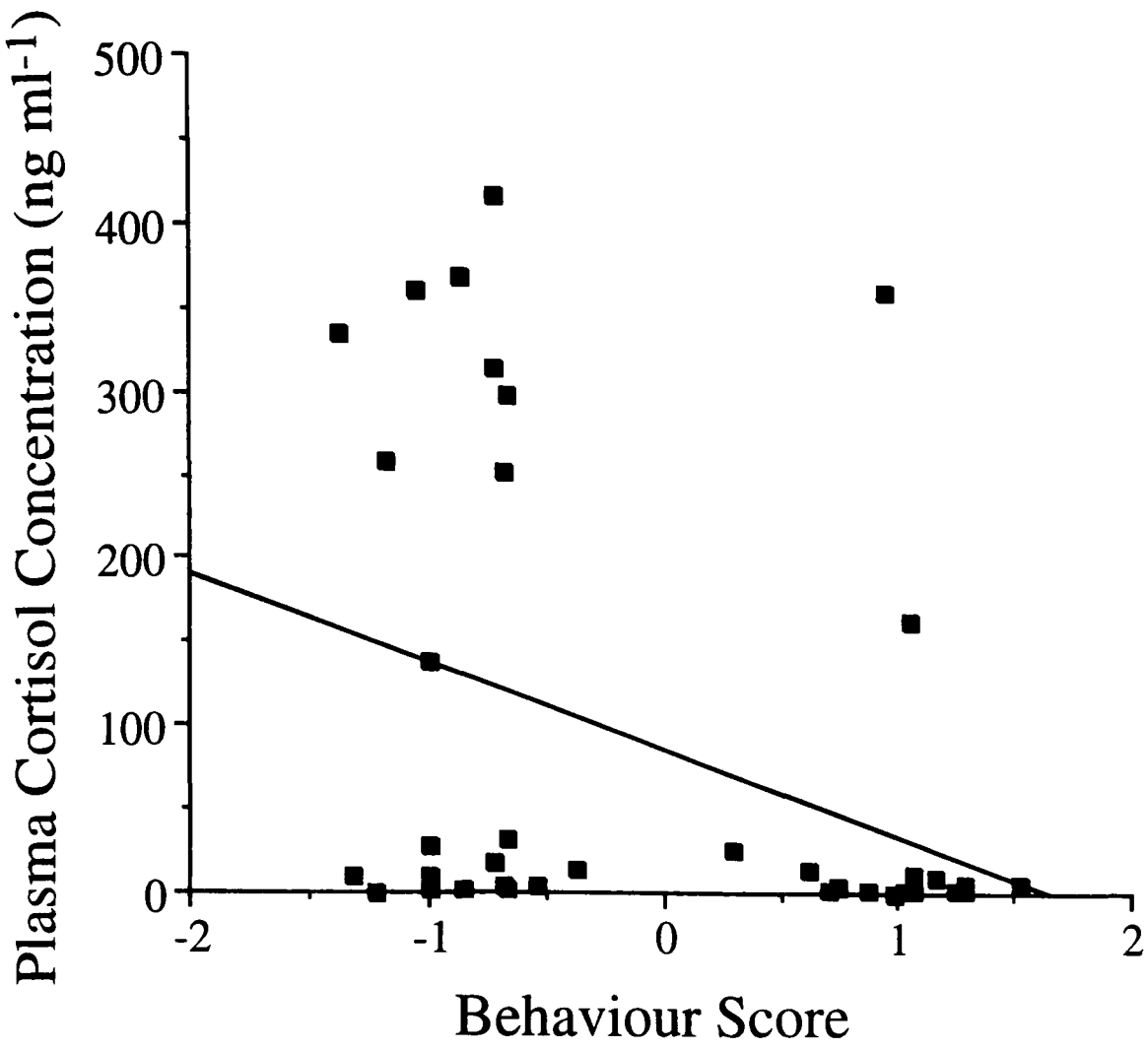



Figure 7.2: Behaviour score (generated from PCA analysis) against plasma cortisol concentration. The higher the behaviour score the more dominant the fish. The regression equation is also plotted (y = -66.156x + 82.909). See text for statistical analysis.

Noradrenaline: Subordinate T = 1.654, P > 0.1; Dominant T = 1.515, P > 0.1; Adrenaline: Subordinate T = -0.657, P > 0.1; Dominant T = 1.110, P > 0.1) despite the apparently lower secretion rates with the second dose of $10^{-7}$ mol $kg^{-1}$, indicating that the preparation remained capable of catecholamine secretion throughout the perfusion period. However, there was no significant effect of acetylcholine dose on either noradrenaline (Repeated measures ANOVA: within-subject effect of dose, $F_{8,112} = 1.611$, P > 0.1) or adrenaline release (Repeated measures ANOVA: within-subject effect of dose, $F_{8,128} = 0.466$, P > 0.1). Finally, the dominance status of the fish appeared to have no effect on acetylcholine-evoked catecholamine release in the perfused PCV preparation - there was no significant effect of dominance status on the secretion rates for either catecholamine (Repeated measures ANOVA: between-subject effect of status Noradrenaline; $F_{1,14} = 0.221$, P > 0.1; Adrenaline; $F_{1,16} = 0.008$, P > 0.1) (Fig. 7.3).

By contrast, ACTH-evoked cortisol secretion in the perfused PCV preparation was affected by the dominance status of the fish. Figure 7.4 indicates that, while cortisol secretion rates increased following perfusion with ACTH in dominant fish, the increase was negligible in subordinates. Statistical analyses indicated that there was a significant relationship between time after initiation of ACTH administration and plasma cortisol concentrations for the 500mU $ml^{-1}$ dose (Repeated measures ANOVA: within-subject effect of time, $F_{7,133} = 2.983$, P < 0.01; Fig. 7.4) and there was also a significant overall effect of dominance status and plasma cortisol secretion (Repeated measures ANOVA: between-subject effect of status, $F_{1,19} = 4.704$, P < 0.05). However, there was no significant interaction of time with dominance status indicating that the pattern of cortisol

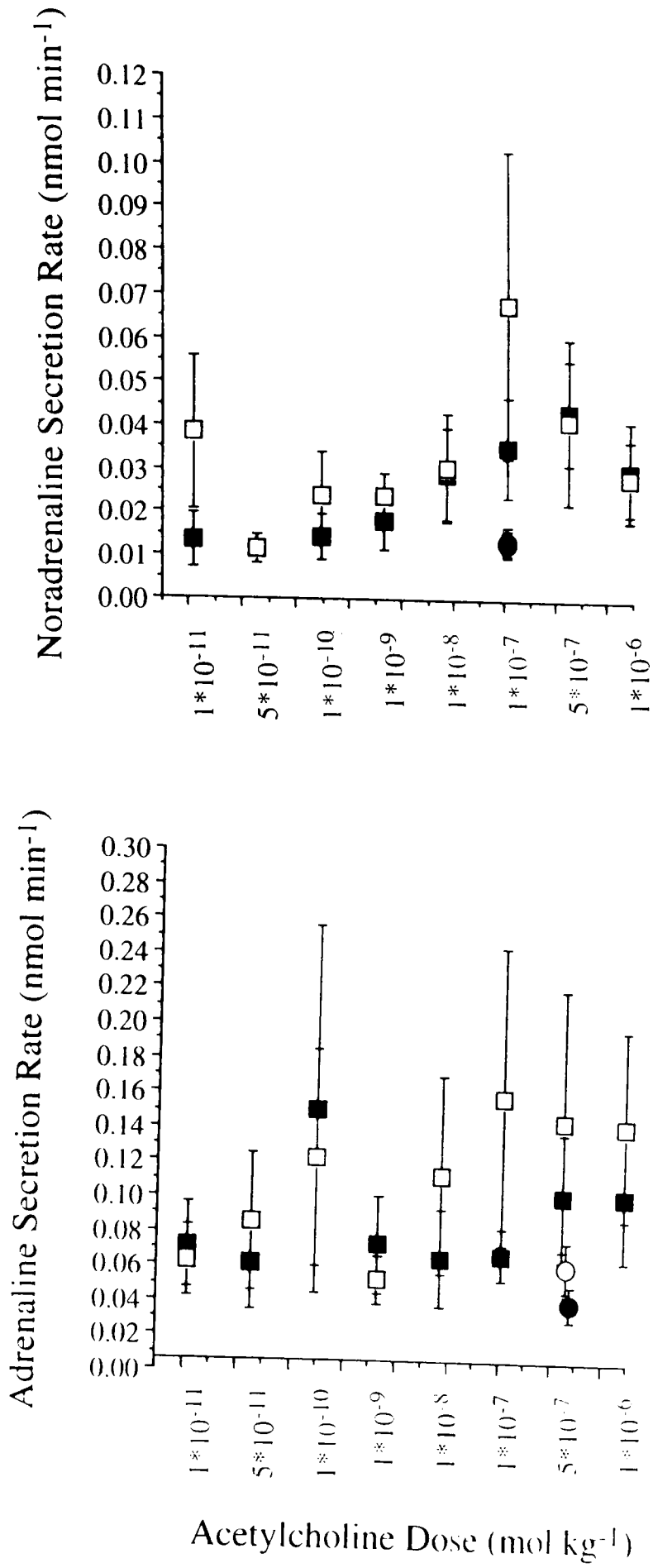



Figure 7.3: Catecholamine secretion rates for subordinate (■, n = 10) and dominant (□, n = 10) fish at given doses of acetylcholine. The secretion rates for the second dose of $10^{-7}$ mol $kg^{-1}$ acetylcholine administered are given as circles. See text for statistical analysis.

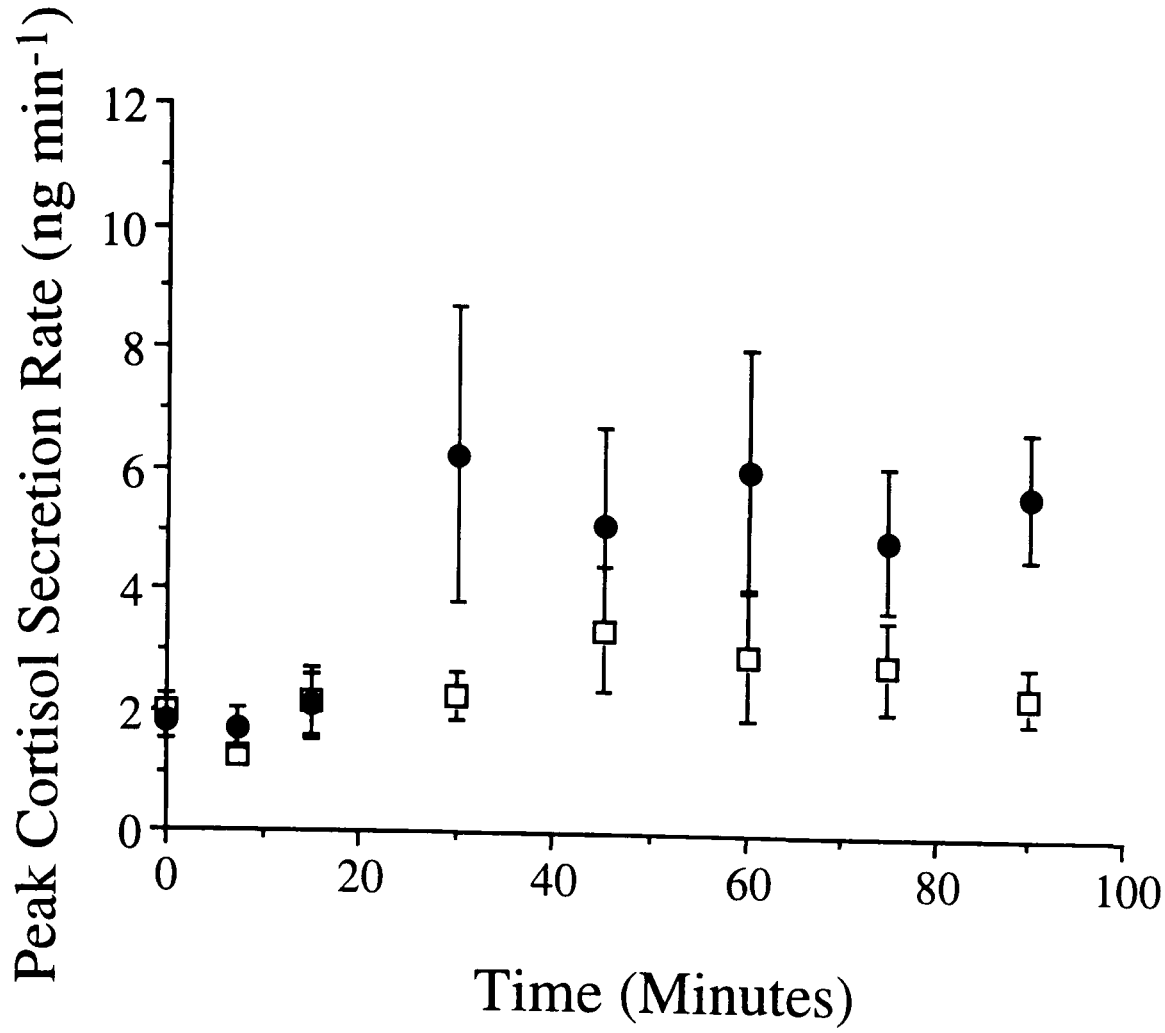



Figure 7.4: Cortisol secretion rates (ng $min^{-1}$) over time from initiation of administration of 500mU $ml^{-1}$ ACTH at time 0 for subordinate (□, n = 10) and dominant (●, n = 10) fish.

release remained different between dominants and subordinates throughout the whole experiment (Repeated measures ANOVA: time*status interaction, $F_{7,133} = 1.261$, $P > 0.05$). No significant relationship between time after intiation of ACTH administration and plasma cortisol concentration existed, however, for the 1000mU $ml^{-1}$ dose (Repeated measures ANOVA: effect of time, $F_{7,112} = 0.353$, $P > 0.1$). Therefore, it appears likely that desensitisation of the preparation occurred with time and/or additional ACTH perfusion, and so only data from the 500mU $ml^{-1}$ dose were used in further statistical analysis. Subordinate fish showed significantly lower peak cortisol secretions than did dominant fish, when administered a 500mU $ml^{-1}$ dose of ACTH (Unpaired t-test: $F = 4.523$, $P < 0.05$; Fig. 7.5).

## 7.5 Discussion

In the present study, the secretion of the catecholamines, noradrenaline and adrenaline, and the corticosteroid hormone cortisol were assessed in relation to social status in salmonid fish using an *in situ* perfused posterior cardinal vein preparation. In addition to being one of only a few studies to address the interactions between the adrenergic and hypothalamo-pituitary-interrenal axes as well as the interactions between behavioural and physiological stressors, the present study is the first to measure cortisol secretion using the *in situ* PCV preparation. The key findings of the present study are that subordinate fish demonstrated a decreased ability to secrete cortisol in response to ACTH perfusion, and that dominant and subordinate fish did not appear to differ significantly in their secretion

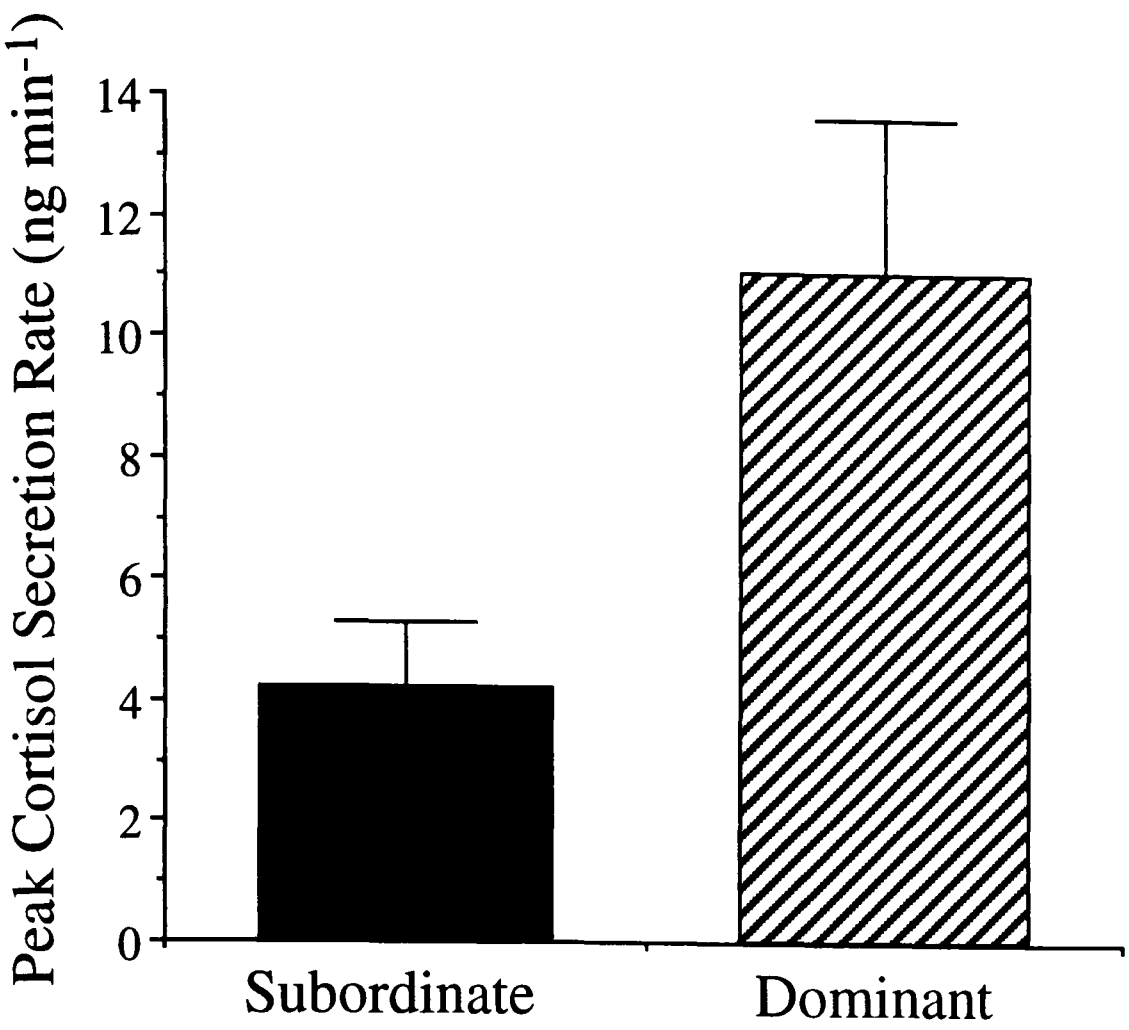



Figure 7.5: Peak cortisol secretion rates (ng $min^{-1}$) for subordinate (■, n = 10) and dominant (□, n = 10) fish after administration of 500mU $ml^{-1}$ ACTH. (Unpaired t-test: $F = 4.523$, $P < 0.05$).

of catecholamines in response to acetylcholine perfusion, where dominant and subordinate status were assigned on the basis of behavioural observations.

In the present study, as in previous studies that have investigated the secretion of cortisol from the interrenal cells of the head kidney *in vitro*, ACTH was used as a stimulant (Vijayan and Leatherland 1990; Balm and Pottinger 1995). Two consecutive doses of ACTH were administered to the perfused PCV preparation, 500mU $ml^{-1}$ and 1000mU $ml^{-1}$ respectively. Although owing to the probability of desensitisation, data for only the first dose were considered reliable, the perfused PCV clearly holds great promise for studies of cortisol secretion. Problems with *in vitro* techniques of assessing cortisol secretion may arise because static systems have been used, where cortisol can build up in the medium and may affect interrenal sensitivity (Mommsen *et al.* 1999). Superfusion *in vitro* systems largely overcome this problem, but *in situ* PCV preparations have the added advantage that the head kidney can remain in the fish and therefore the tissue is not disturbed.

While the present study was the first to our knowledge to use the perfused PCV preparation to study cortisol secretion, PCV preparations have been extensively used previously to study the release of catecholamines. In general, previous studies have not used more than one dose of secretagogue per preparation due to the potential problem of desensitisation. In the present study, some evidence for desensitisation of cholinergic receptors was detected, in that no dose-dependent relationship between acetylcholine and catecholamine release was exhibited in either dominant or subordinate fish. However, complete desensitisation did not appear to occur since significant release of both

adrenaline and noradrenaline was recorded for the majority of doses used, nor was there any significant difference in catecholamine release in response to repeating the dose of $10^{-7}$ mol $kg^{-1}$ acetylcholine.

The decreased cortisol secretion in subordinate fish in the *in situ* perfused PCV preparation upon stimulation with ACTH is likely to be related to the social stress that the fish had previously experienced. A significantly higher concentration of circulating plasma cortisol was found in subordinate fish compared to dominant fish. This characteristic elevation of plasma cortisol concentrations in subordinate salmonid fish has been well documented (e.g. Pottinger and Pickering 1992; Øverli *et al.* 1999; Sloman *et al.* 2000a (Chapter 3)). In the present study, as in previous work (Sloman *et al.* 2000a (Chapter 3)) there was also a significant correlation between behaviour score and circulating plasma cortisol concentrations, with more subordinate fish having higher elevations of plasma cortisol.

The secretion of cortisol is under the control of the hypothalamo-pituitary-interrenal axis (HPI axis; see reviews by Donaldson 1981; Mommsen *et al.* 1999). Classically, corticotropin releasing factor (CRF) from the hypothalamus acts on the anterior pituitary to stimulate the secretion of adrenocorticotropic hormone (ACTH), which is the main secretagogue for cortisol. ACTH secretion, and consequently cortisol secretion, can be modulated by a variety of factors, including hormones, stress and negative feedback of cortisol at the level of the hypothalamus and pituitary (see review by Mommsen *et al.* 1999). In the present study, it is hypothesised that the chronically elevated circulating

cortisol concentrations associated with the social stress of subordinance decreased the sensitivity of the interrenal cells to ACTH stimulation either by downregulation or desensitisation of interrenal cell ACTH receptors (Mommsen *et al.* 1999). A similar explanation has been proposed to account for the effects of confinement stress on cortisol secretion from rainbow trout interrenal cells in an *in vivo* superfusion system (Balm and Pottinger 1995). In contrast to these effects of stress on interrenal cell sensitivity to ACTH, administration of exogenous cortisol in otherwise unstressed fish does not appear to reduce interrenal sensitivity to ACTH (Balm and Pottinger 1995) suggesting that the secretion of cortisol by interrenal cells is being mediated via the hypothalamus. Therefore, it appears that the chronic elevation of plasma cortisol that occurs in subordinate fish acts through negative feedback loops that suppress the interrenal cell response (Bradford *et al.* 1992).

It is possible, even likely, that more than one negative feedback loop is occurring in response to the chronically-elevated plasma cortisol concentrations in subordinate fish. A direct effect of elevated cortisol on the interrenal cells themselves is unlikely given the lack of effect of exogenous cortisol administration on interrenal cell sensitivity to ACTH reported by Balm and Pottinger (1995). However, effects of elevated circulating cortisol concentrations at the level of the pituitary and/ or hypothalamus remain possible. Cortisol is known to have a direct inhibitory effect on ACTH secretion from the pituitary in goldfish (Fryer *et al.* 1984), and recent research by Bernier *et al.* (1999) has demonstrated that cortisol may act directly on the expression of CRF genes in the goldfish brain. Intraperitoneal implants of cortisol reduced the level of CRF mRNA mediated by

glucocorticoid receptors in the telencephalon-preoptic region of the brain, thus controlling cortisol secretion at the hypothalamus level. While in the present study only the responsiveness of the interrenal cells to stimulation by ACTH was examined, further investigation of the hypothalamo-pituitary-interrenal axis response in fish stressed chronically by behavioural interactions is clearly warranted.

In contrast to the effect of social status on cortisol secretion rates in the *in situ* perfused PCV preparation, no significant differences between dominant and subordinate fish were detected with respect to catecholamine secretion rates. While it is not known whether social interactions have any impact on circulating catecholamine concentrations in teleost fish, it seems likely that any elevation of circulating catecholamines would be transient, occurring in response to the initial period of aggression following confinement in pairs (~ 4 h) in which social status is largely determined. Thus, desensitisation of the adrenergic stress system in response to prolonged elevation of circulating catecholamine concentrations seemed unlikely. However, an effect of the elevated plasma cortisol concentrations of subordinate fish on catecholamine storage and/ or release remained a possibility, particularly given previous work in this regard.

For example, Reid *et al.* (1994) demonstrated that repeated physical stress (twice daily chasing to exhaustion for 5 days) in rainbow trout caused a decrease in the responsiveness of chromaffin cells to the cholinergic agonist carbachol. This regimen of repeated chasing bouts probably caused catecholamine release into the circulation during each chasing period, as well as an elevation of circulating cortisol concentrations (Reid *et al.* 1994) and

therefore the proximate cause of the decreased responsiveness of the chromaffin cells could not be distinguished. In a subsequent study, Reid *et al.* (1996) found that chromaffin cells from rainbow trout given an intraperitoneal implant of cortisol for seven days exhibited both higher basal rates of adrenaline secretion and enhanced sensitivity of adrenaline and noradrenaline release in response to carbachol in an *in situ* perfused PCV preparation. Furthermore, cortisol-implanted trout showed higher levels of stored adrenaline and noradrenaline in the kidney and PCV.

Given these two previous studies in which stress effects on catecholamine release *in situ* perfused PCV preparation occurred, albeit in opposite directions, it was perhaps somewhat surprising that no effect of social stress on catecholamine secretion rates in the *in situ* perfused PCV preparation was detected in the present study. The cortisol implants used by Reid *et al.* (1996) raised circulating cortisol concentrations to approximately 30 ng $ml^{-1}$, a level substantially lower than that experienced by subordinate fish in the present study (~ 160 ng $ml^{-1}$). Thus, differences in circulating cortisol concentrations may account for the different responses of the chromaffin tissue in the two studies. In addition, marked differences in the effects elicited by stress-induced cortisol elevations and cortisol administration in unstressed fish have been reported previously (e.g. Balm and Pottinger 1995). Investigation of catecholamine storage levels following social stress is warranted. Clearly, the interactions between the adrenergic and hypothalamo-pituitary-interrenal axis stress responses are complex.

The apparent lack of interaction between the adrenergic and HPI stress response, at least with respect to catecholamine secretion *in situ*, demonstrated in the present study may be beneficial to the subordinate fish in coping with the detrimental effects of social stress. Down-regulation of the ACTH response of interrenal cells mediated via the hypothalamus will serve to minimise the release of cortisol in response to additional stressors, potentially reducing the detrimental physiological consequences associated with long-term elevation of plasma cortisol concentrations (Pickering 1992). However, the apparent lack of action of circulating cortisol concentrations on the catecholamine response means that the fish can still respond to acute, potentially life threatening, stressors in the classical 'fight-or-flight' response.

In conclusion, the chronic elevation of plasma cortisol concentrations characteristically associated with subordination, produced a down-regulation of the response of interrenal cells to ACTH, implying a decreased ability to secrete cortisol upon further stimulation of the interrenal cells. However, subordinate fish retained the ability to secrete the catecholamines noradrenaline and adrenaline.

# CHAPTER 8

## Chapter 8: Physiological effects of dominance hierarchies within groups of brown trout, *Salmo trutta*, held under simulated natural conditions.

A version of this chapter has been published in Fish Physiology and Biochemistry, 22: 11-20 with co-authors Kathleen M. Gilmour, Alan C. Taylor and Neil B. Metcalfe.

### 8.1 Abstract

While the existence of dominance hierarchies within natural populations of salmonids is well known, little is known about the physiological consequences of these social interactions. To investigate such physiological effects, replicate groups of four brown trout (*Salmo trutta*) were held under simulated natural conditions in an artificial stream tank. Behavioural observations allowed the fish to be ranked for dominance. After two weeks, physiological status was assessed through measurements of specific growth rate, condition factor, plasma cortisol and ion concentrations, haematocrit, leucocrit, hepatosomatic index, hepatic glycogen concentration, interrenal cell nuclear area and gill epithelium chloride cell density. Weight gain in the first-ranking (dominant) fish was significantly higher than in the second-ranking fish. In addition, the condition factor of the second-ranking fish decreased over the experimental period while those of the first- and third- ranking fish increased, resulting in significant differences among the three groups. The only other physiological parameter which varied significantly among the ranked fish was chloride cell density, which was significantly higher in the second-ranking fish than in the dominant fish. Cortisol concentrations were low in all fish and did not vary

significantly with dominance status. Overall, the least beneficial position, in physiological terms, appears to be the second rank in the dominance hierarchy.

## 8.2 Introduction

Most stream-dwelling salmonids are known to be strongly territorial, owing to the need to compete for finite resources and space (Allen 1969). The potential of territoriality as a factor in limiting production of natural salmonid populations has led to research into the competitive social interactions occurring within natural salmonid fry and parr populations, with particular reference to stocking density (McFadden 1969; Fraser 1969; Grant 1993; Armstrong 1997). The formation of linear dominance hierarchies within artificial and natural fish populations (Noakes and Leatherland 1977; Bachman 1984; Fausch 1984) is believed to be associated with competition for food (Kalleberg 1958; Chapman 1966). Grant *et al.* (1989) demonstrated that aggressive acts in wild brook charr (*Salvelinus fontinalis*) tended to be directed upstream, as fish defended access to drift food by excluding other fish from the area upstream of their resting position. Similarly, Fausch (1984) found that in laboratory stream aquaria, coho salmon (*Oncorhynchus kisutch*), brook charr, and brown trout (*Salmo trutta*) established intraspecific hierarchies in which the dominant fish held the position with the maximum profit in terms of net energy gain. Modelling of dominance hierarchies (Gurney and Nisbet 1979) indicates that such hierarchies are an important factor in population stability, provided that the rewards of dominance are not too extreme.

The benefits of dominance are well documented for fish confined in small tanks. Increases in plasma cortisol concentrations in the subordinate of paired rainbow trout (*Oncorhynchus mykiss*) were noted by Laidley and Leatherland (1988), and Pottinger and Pickering (1992), while Ejike and Schreck (1980) made similar observations for the subordinates of groups of six coho salmon parr. Ejike and Schreck (1980) also observed that hepatic glycogen content decreased with increasing subordinance. Other physiological alterations associated with subordinance include increased leucocrit (Peters *et al.* 1980), and plasma glucose concentrations, and decreased disease resistance (Peters *et al.* 1988). Finally, Abbott and Dill (1989) found that subordinates of paired juvenile steelhead trout had slower growth rates, even when fed on the same food rations as dominant fish, thus suggesting that subordinates were at a metabolic disadvantage.

However, the rewards of dominance for fish in natural or semi-natural populations have received little attention. In one of the few studies of this kind, Noakes and Leatherland (1977), studying rainbow trout held under simulated natural conditions, recorded an increase in interrenal cell activity (indicative of activation of the pituitary-interrenal axis (McLeay 1975)) with increasing subordinance, the exception being the top ranking fish which had a higher than expected interrenal cell activity. Li and Brocksen (1977) also examined the effects of dominance in rainbow trout held under simulated natural conditions and found that at low stocking densities dominant fish grew faster and had a higher body lipid content than subordinate fish.

The extent to which social interactions cause physiological stress in fish in their natural environment is therefore not clear. In the present study, small groups of brown trout were held under simulated natural conditions in artificial stream tanks and the effects of social interactions were examined for a wide range of physiological parameters for individual fish in relation to their position in a social hierarchy. The physiological parameters chosen included primary (plasma cortisol concentration), secondary (e.g. hepatic glycogen content, leucocrit, haematocrit, plasma ion concentrations) and tertiary (specific growth rate, condition factor) indicators of stress (Barton and Iwama 1991). Secondary effects of stress are very varied and include metabolic, haematological, structural and hydromineral changes (Barton and Iwama 1991). Tertiary or 'whole animal' responses to stress include growth and condition indices and can also be used to assess stress (Barton and Iwama 1991). Condition factors are ratios between morphological and anatomical features of fish (e.g. body weight divided by the cube of body length) and a decline in condition factor is often indicative of a depletion of energy stores (Goede and Barton 1990). In addition, the novel possibility that gill epithelium morphology may be affected by socially-induced stress was examined.

## 8.3 Materials and Methods

### *8.3.1 Experimental animals*

Brown trout (weight 88.76 ± 4.27 g, length 20.0 ± 0.3 cm (mean ± S.E.M), n = 44) were obtained from Howietoun trout farm, Stirling (Scotland) and held in a stock tank at the

University Field Station, Rowardennan. After a two week acclimatisation period, the fish were anaesthetised using a solution of benzocaine (0.05 mg $ml^{-1}$) and unique combinations of alcian blue dye marks (Kelly 1967) were injected into their fins. Initial fork lengths and weights were recorded. Four size-matched fish were then allocated to replicate sections of an artificial stream tank (flume), each section being 5 m in length by 0.6 m wide with an average water depth of 0.28 m. The flume is described in more detail in Valdimarsson *et al.* (1997), and has the advantage of having glass side walls to allow behavioural observations. The topography of the artificial stream tank was designed to be as close to the natural environment of a stream as possible, with a gravel and cobble substratum and both riffle and pool areas that differed in flow rate (the average flow rate at 60% of water depth ranged from 0.138 to 0.063 m $s^{-1}$, respectively). The artificial stream tank was supplied with a continuous turnover of untreated water from Loch Lomond kept under ambient temperature conditions (12.6 ± 0.7 °C). The outdoor location of the artificial stream tank ensured that the fish received an ambient photoperiod which varied between 16.75 h and 18.25 h during the period of the experiments (May to July). Fish were fed to excess on pellets (BOCM Pauls Ltd Keystart; oil 16.0%; protein 55.0%) by automatic feeders, located at the upstream end of each section and dispensing the food at a trickle rate throughout the 24 h. Although the method of dispensing food was spatially predictable, and therefore not necessarily representative of a natural environment, the feeding method was designed to allow identification of social hierarchies.

Twenty four sets of behavioural observations were made over the two-week experimental period. Each group of fish was kept in the experimental tank for two weeks, during which

time 10 min behavioural observations were conducted (3 per day, separated by at least half an hour, on four days each week). Dominance was measured by assigning points using a scoring system (Table 8a) based on previous studies of salmonid behaviour (Metcalfe *et al.* 1989; Johnsson *et al.* 1996).

Table 8a: Each fish was given a score for concealment, activity and location at the beginning of each 10 minute observation period and then obtained a point for each attempt at feeding during the 10 minute period. Higher scores are indicative of more dominant behaviour.

| Behaviour | | Score at each observation |
|---|---|---|
| Feeding: | Fish that took introduced food item | 1 |
| Concealment: | Hiding | 0 |
| | Not Hiding | 1 |
| Activity: | Resting on the substrate | 0 |
| | Swimming in the water column | 1 |
| Location: | Nearest to food source | 4 |
| | Second nearest to food source | 3 |
| | Third nearest to food source | 2 |
| | Furthest away from food source | 1 |

The mean scores for food intake and the three measurements of position in the tank were calculated for each fish across the 24 observation periods, and the four types of score were then combined into a single overall dominance score for each fish using a principal components analysis (PCA). Due to limitations in tank availability, the experiment was carried out over a six week period as not all the replicates could be run simultaneously.

*8.3.2 Physiological measurements*

After two weeks in the flume tank, the four fish within a replicate section were caught simultaneously, using hand held nets, and killed within 60 s with a lethal dose of benzocaine (0.5 mg $ml^{-1}$). Final fork lengths and weights were recorded, and blood samples (0.5-1.0 ml) were withdrawn by caudal venipuncture. Blood haematocrit and leucocrit were measured as the height of the red cell column or buffy coat column, respectively, against the height of the entire sample, following centrifugation of capillary tubes at 13,000 g for 6 minutes. The remainder of the blood sample was centrifuged (13,000 g), and plasma samples were frozen in liquid nitrogen for later analysis of plasma cortisol and ion concentrations.

Gill, liver and head kidney tissue were also sampled for later analysis. The second gill arch was removed and washed in 0.9% saline, following which pairs of filaments were fixed in buffered glutaraldehyde (5% glutaraldehyde in phosphate buffer; 1 h; 4 °C) and stored in phosphate buffer at 4 °C for 24 h. The liver from each fish was placed in a pre-weighed Eppendorf tube, weighed, frozen in liquid nitrogen and stored at -70 °C for later analysis of glycogen content. Finally the head kidney lobes were removed from each fish and fixed in buffered formalin (10%) for several days.

*8.3.3 Analytical techniques*

Plasma samples were analysed for plasma cortisol, $Na^+$, $K^+$, $Cl^-$ and $Ca^{2+}$ concentrations. A radioimmunoassay (ICN pharmaceuticals Ltd) was used for measurement of plasma [cortisol] (Gamperl *et al.* 1994). Plasma [$Na^+$] and [$K^+$] were measured by flame

photometry, [$Cl^-$] by colorimetric analysis using the method of Zall *et al.* (1956) and [$Ca^{2+}$] by atomic absorption spectrophotometry (Philips PU9200), following addition of lanthanum chloride to the plasma samples (1 part $LaCl_3$ : 50 parts sample) to prevent the interference of any other ions present. Liver glycogen content was assessed using the anthrone method of Wedemeyer and Yasutake (1977).

For analysis of chloride cell density, the fixed gill tissue was stained with an osmium-zinc iodide preparation (1 part 2% $OsO_4$ : 4 parts 3% $ZnI_2$; 18 h; 20 °C) (Garcia-Romeu and Masoni, 1970), dehydrated through graded ethanols (30%, 50%, 75%, 95%, 100% x 2, 20 minutes in each; 20 °C), rinsed in Histoclear® (2 x 20 minute rinses; 20 °C) and paraffin wax (2 x 20 minute rinses; 55 °C), embedded in paraffin wax and sectioned at 7µm using a microtome (Leitz 1512). Sections were viewed using a light microscope (40 x objective; Leitz Dialux® microscope) and photographs were taken with an attached camera (Wild Leitz MPS51 camera; Wild MPS45 photoautomat). Twenty slides, with eight sections per slide, were prepared to produce a total of 160 sections per fish. In general, 11 photographs (minimum = 10) of gill tissue were taken per fish from randomly selected sections. For each photograph, the slide was positioned so that the field of view contained approximately seven lamellae and a small portion of the filament at the bases of the lamellae. Chloride cell numbers were quantified by analysing the 11 photographs taken for each fish. For quantification, the gill tissue area in a photograph was digitised using a digitising tablet (BBC Cherry A3 Graphics Tablet). The number of chloride cells per unit tissue area was calculated for each photograph by visually counting the intensely stained

cells, and the value for each fish was taken as the mean of the values for the 11 photographs.

For histological analysis of head kidney tissue, the fixed tissue was dehydrated through graded alcohols (70%, 2 x 3 h rinses; 90%, 3 h rinse; 8% phenol in 95% ethanol, 2 h rinse; 100% 2 x 2 h rinse; 20 °C), rinsed in Histoclear® (1 x 2 h rinse and 1 h rinse; 20 °C) and paraffin wax (2 x 3 h; 55 °C), embedded in paraffin wax and sectioned at 7 μm using a microtome. Slides were stained with Mayer's haematoxylin and eosin (Mayer 1903). Slides were made of the whole head kidney of each fish with eight sections per slide and viewed as above. In general, 11 photographs (minimum = 10) were taken per fish, at 1000 x magnification, from randomly selected sections. For each photograph, the slide was positioned so that the field of view contained as many interrenal cells as possible. Due to the breakdown of cell membranes, only nuclear areas could be measured. The method of Noakes and Leatherland (1977) was used; the nuclei are taken to be either perfect spheres or perfect ellipses. The value for each fish was taken as the mean of the nuclear areas determined for approximately 10 interrenal cells from each of the 11 photographs, resulting in a total of 110 measurements per fish.

*8.3.4 Statistical methods*

A total of 11 replicate trials were run during the experiment producing a sample size of 44 fish in all subsequent analyses. Specific growth rates were calculated as % weight change per day (Ricker 1979). Condition factor was calculated as (weight/fork length$^{2.88}$) x 100, where the constant 2.88 was the slope of the regression of log (weight) on log (fork

length) ($r^2 = 0.863$; $n = 44$, $P < 0.01$) (Bolger and Connolly 1989). Hepatosomatic index (HSI) was calculated as (liver weight (g)/ fish weight (g)) x 100. Data are presented as means ± 1 standard error of the mean (S.E.M.). Comparisons of physiological parameters among the four ranks were accomplished using a one way analysis of variance (ANOVA) followed by Scheffé's tests for multiple comparisons. Actual values for specific growth rate and change in condition factor for each rank of fish were compared to zero using a 1 sample Student's t-test. The limit of significance in all analyses was 5%.

## 8.4 Results

### *8.4.1 Behaviour*

The principal components analysis carried out on the behavioural data indicated that there was close agreement among the different scores and allowed fish to be ranked in order of dominance within their experimental groups. Little aggression was observed during the two week experimental period. The greatest difference in behavioural score was between the first and second ranks of fish, which were easily distinguished by their behaviour (Fig. 8.1). Dominant fish were generally closest to the food source and scored significantly higher than the other three ranks of fish for food intake ($P < 0.01$). As fish were scored according to which fish took each food item introduced into the tank during the observational period, the percentage of times that each rank of fish took food items could be calculated (Fig. 8.2). Although food intake was not measured directly the behavioural observations allowed an indirect estimation of food consumption to be made. All ranks of fish below the dominant had a tendency to hide ($P < 0.05$) and occupy positions less

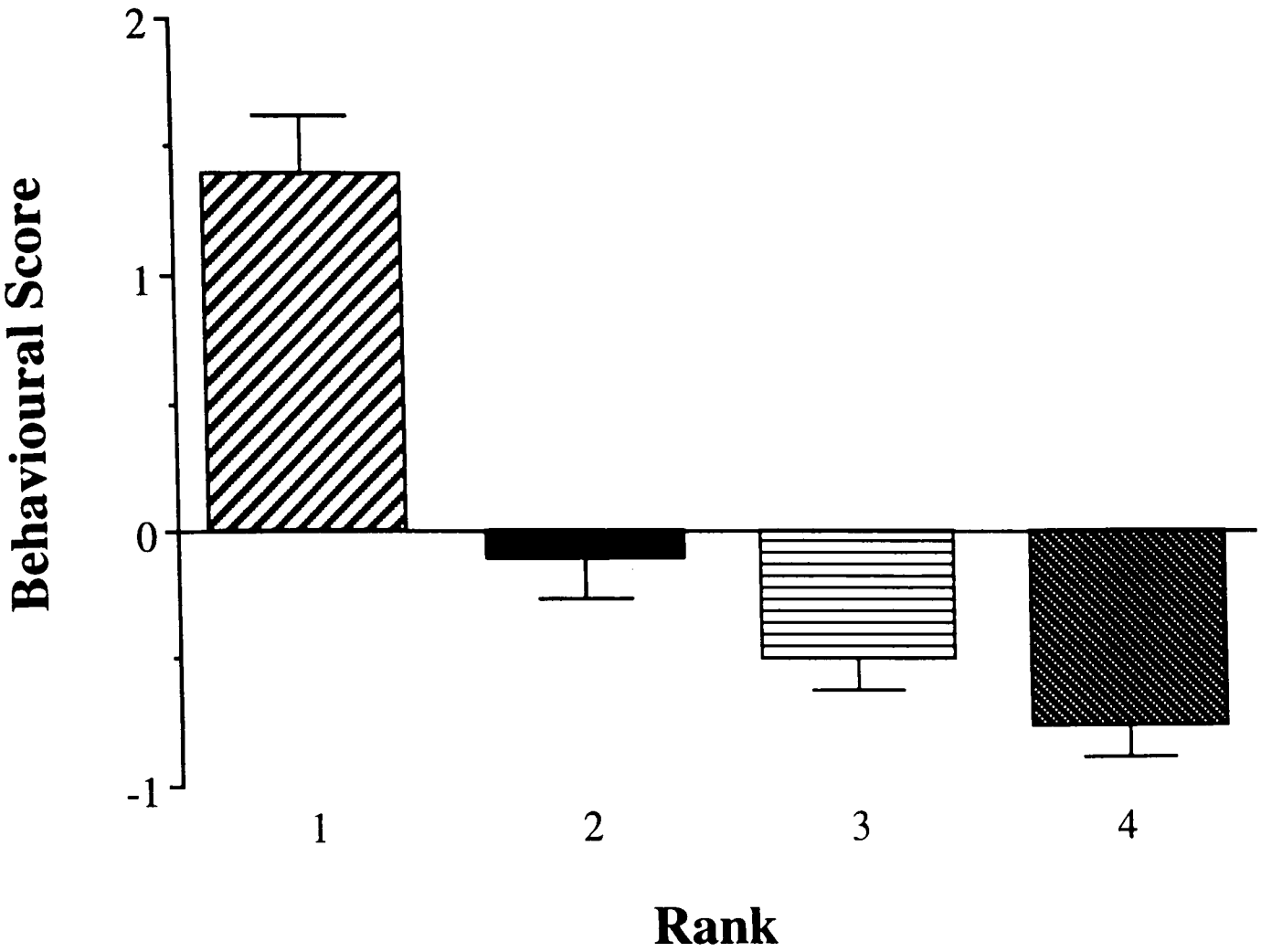



Figure 8.1: Behavioural scores derived from a principal components analysis are presented according to the dominance rank subsequently assigned to brown trout held in groups of four. Rank 1 is the most dominant. Data are presented as means ± S.E.M. (n = 44).

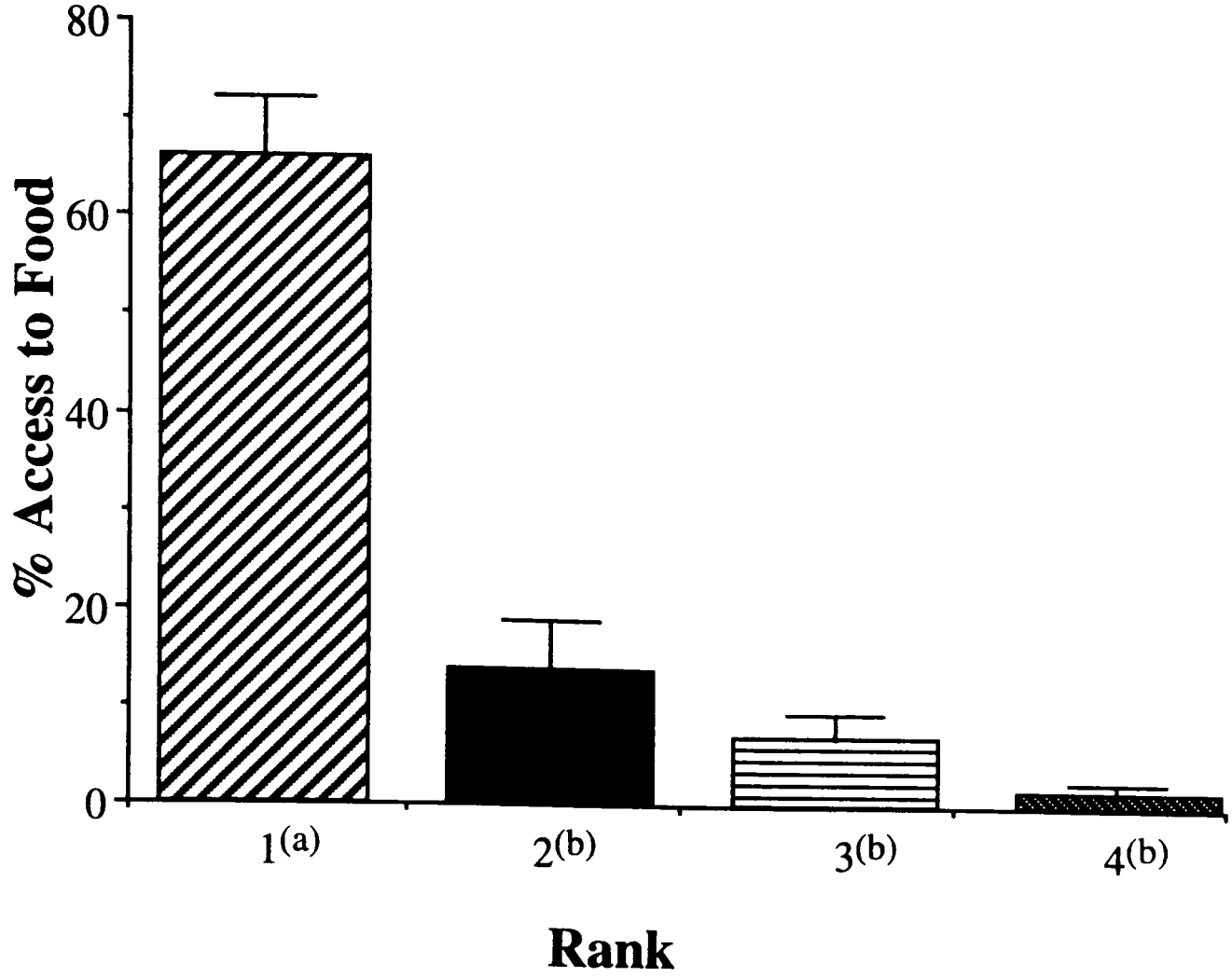



Figure 8.2: Percentage of times that each rank of fish took food items first where rank 1 is the most dominant. Data are presented as means ± S.E.M. Statistical differences (one way ANOVA followed by Scheffé multiple comparisons test, $P < 0.01$) are indicated by the letters; groups sharing the same letter are not significantly different from one another.

profitable in terms of food intake than dominant fish ($P < 0.05$), and also spent less time cruising in the water column ($P < 0.01$).

*8.4.2 Physiology*

Physiological measurements were analysed according to rank in the dominance hierarchy. However, it was necessary to control for slight environmental variation among the replicate sections of the artificial stream tank arising from the desire to simulate natural conditions. For instance, the pumped water from Loch Lomond that entered the tank at one point would include a small amount of natural planktonic food and there were also slight differences in the degree of overhead shade. Therefore, the parameters are expressed as residual values, where the mean for each group of four fish was calculated and then subtracted from the corresponding value for each individual fish. Therefore a large residual value represents a substantial deviation from the trend for a group of four fish.

Specific growth rate varied among the four ranks (Fig. 8.3). Dominant fish (i.e. the fish with the highest dominance rank) exhibited a weight gain significantly greater than that of second-ranking ('subdominant') fish ($P < 0.01$). Similarly, a difference in change in condition factor was also observed, the first-ranking fish showing an increase in condition factor whereas the subdominant fish showed a decrease over the two week period (Fig. 8.4; $P < 0.05$). Although there was a significant difference in residual specific growth rates among the ranks of fish, the actual mean values recorded for each rank of fish were not significantly different from zero. The mean actual change in condition factor values for each rank of fish showed that only the subdominant fish had a decrease in condition factor

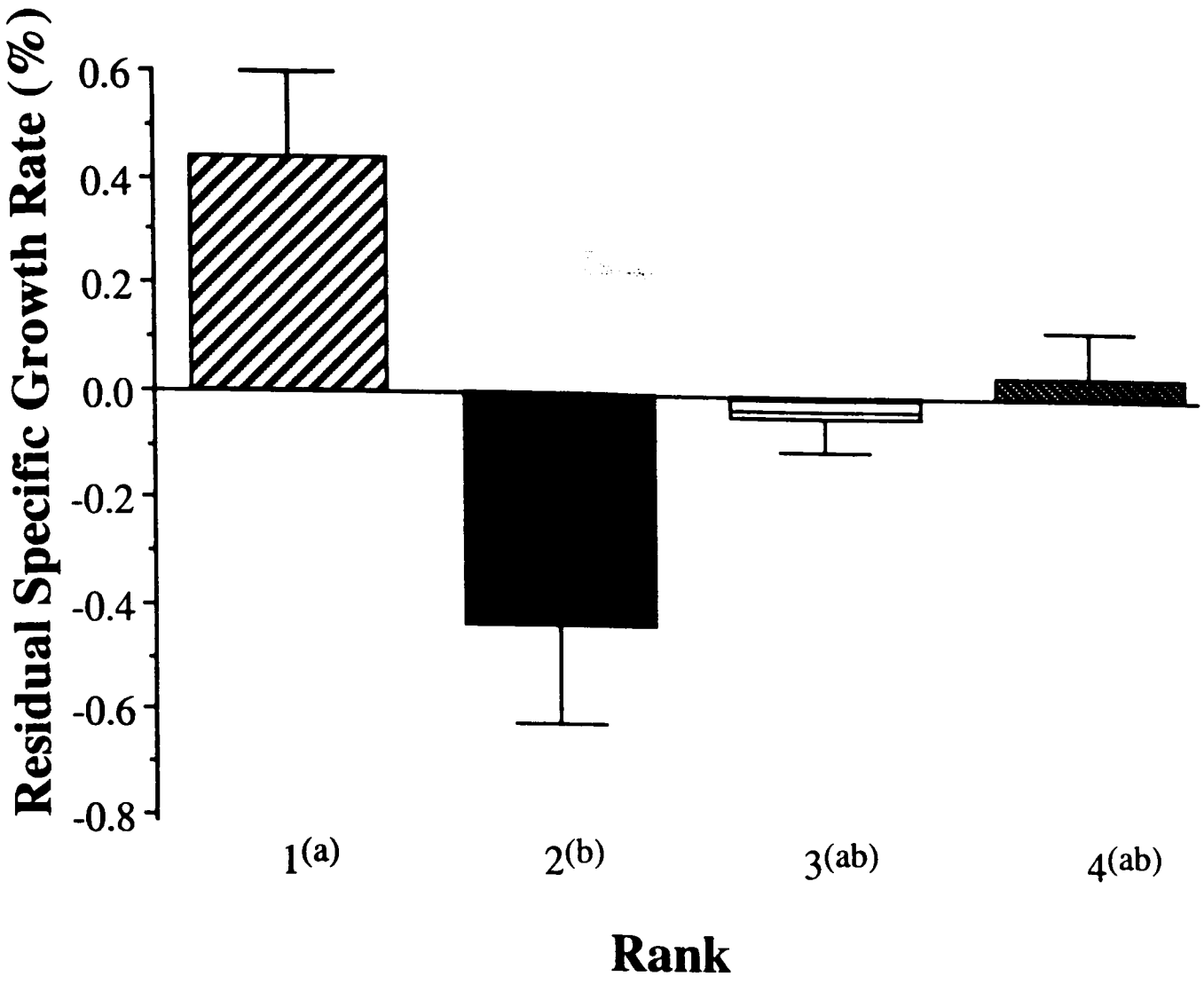



Figure 8.3: Specific growth rates (% change in weight per day) according to rank of fish where rank 1 is the most dominant. Data are presented as mean residual values ± S.E.M. Statistical differences (one way ANOVA followed by Scheffé multiple comparisons test, $P < 0.01$) are indicated by the letters; groups sharing the same letter are not significantly different from one another. The mean actual values of specific growth rate for each rank of fish were not significantly different from zero (1-sample Student's t-test).

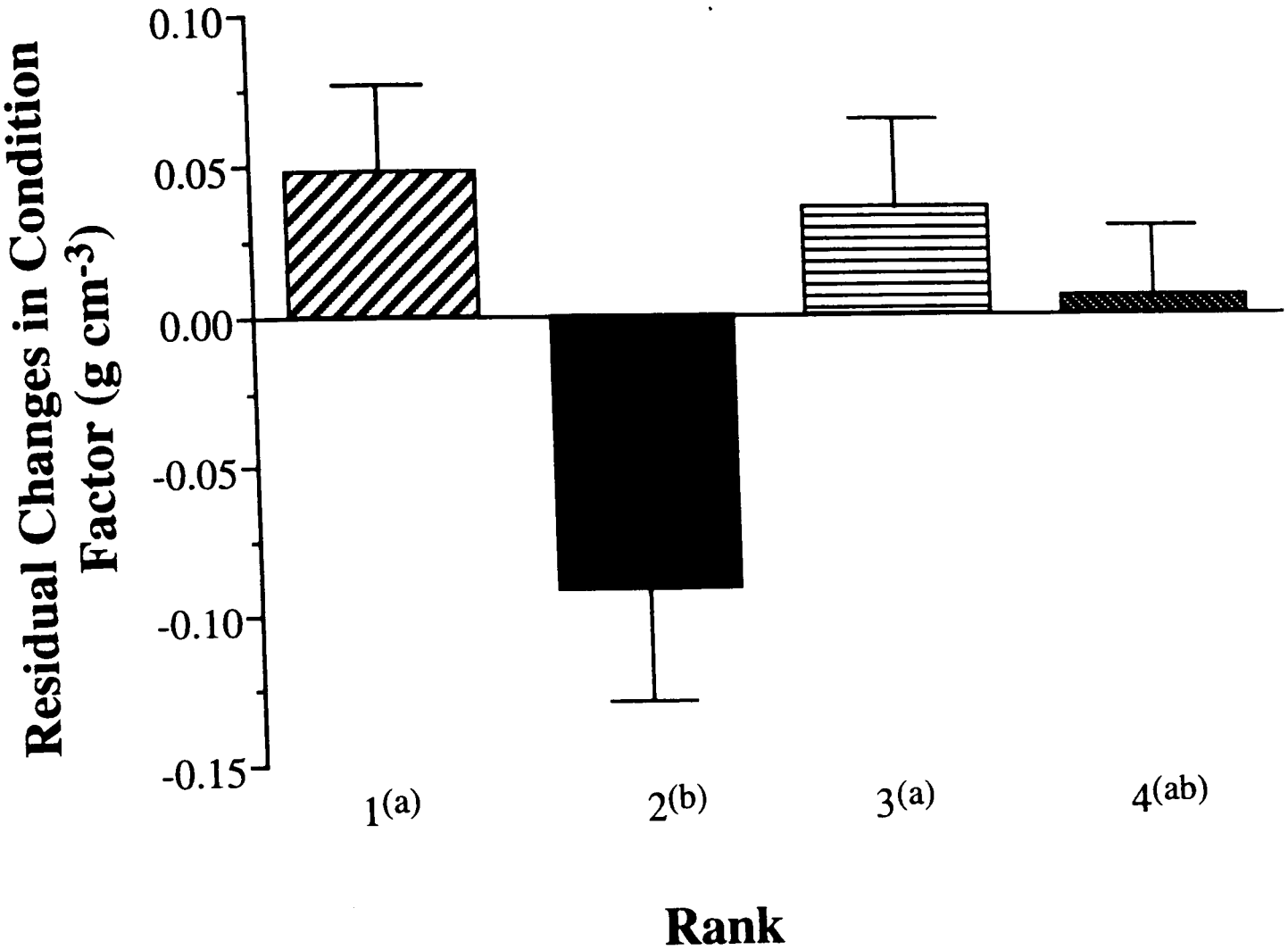



Figure 8.4: Changes in condition factor during the two week trial are presented for the four ranks of fish as mean residual values ± S.E.M. Rank 1 is the most dominant. Statistical differences (one way ANOVA followed by Scheffé multiple comparisons test, $P < 0.01$) are indicated by the letters; groups sharing the same letter are not significantly different from one another. The mean actual change in condition factor for the second-ranking fish was significantly lower than zero (1 sample Student's t-test: $P < 0.05$).

significantly different from zero ($P < 0.05$). No significant differences among the ranks of fish were detected for plasma cortisol concentrations, haematocrit, leucocrit, hepatosomatic index, hepatic glycogen concentrations, interrenal cell nuclear areas or plasma ion concentrations (Table 8b).

Table 8b: Plasma cortisol concentrations, hepatic glycogen content, hepatosomatic index, haematocrit and leucocrit values for each rank of fish (1 = most dominant; n = 11). Data are presented as actual values as opposed to residual values and are presented as means ± S.E.M; statistical analyses are based on the residual measurements.

| Physiological parameters | Rank | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | F-value (df = 3, 40 ) |
| Plasma cortisol (ng $ml^{-1}$) | 10.08 ± 5.06 | 2.86 ± 0.83 | 4.17 ± 1.33 | 4.11 ± 1.15 | 0.623 (NS) |
| Haematocrit (%) | 31.21 ± 2.04 | 34.07 ± 1.49 | 31.17 ± 0.92 | 31.13 ± 1.46 | 1.907 (NS) |
| Leucocrit (%) | 1.61 ± 0.24 | 1.68 ± 0.24 | 1.89 ± 0.22 | 1.52 ± 0.20 | 0.955 (NS) |
| Hepatosomatic index (%) | 1.11 ± 0.08 | 0.99 ± 0.08 | 0.95 ± 0.06 | 1.04 ± 0.09 | 1.865 (NS) |
| Hepatic Glycogen Content (mg 100$mg^{-1}$ dry weight of liver tissue) | 1.51 ± 0.46 | 1.13 ± 0.42 | 1.38 ± 0.37 | 1.44 ± 0.43 | 2.074 (NS) |

Interestingly, a significant difference in chloride cell densities was apparent between dominant and subdominant fish, with the subdominants having the higher chloride cell density (Fig. 8.5; $P < 0.05$).

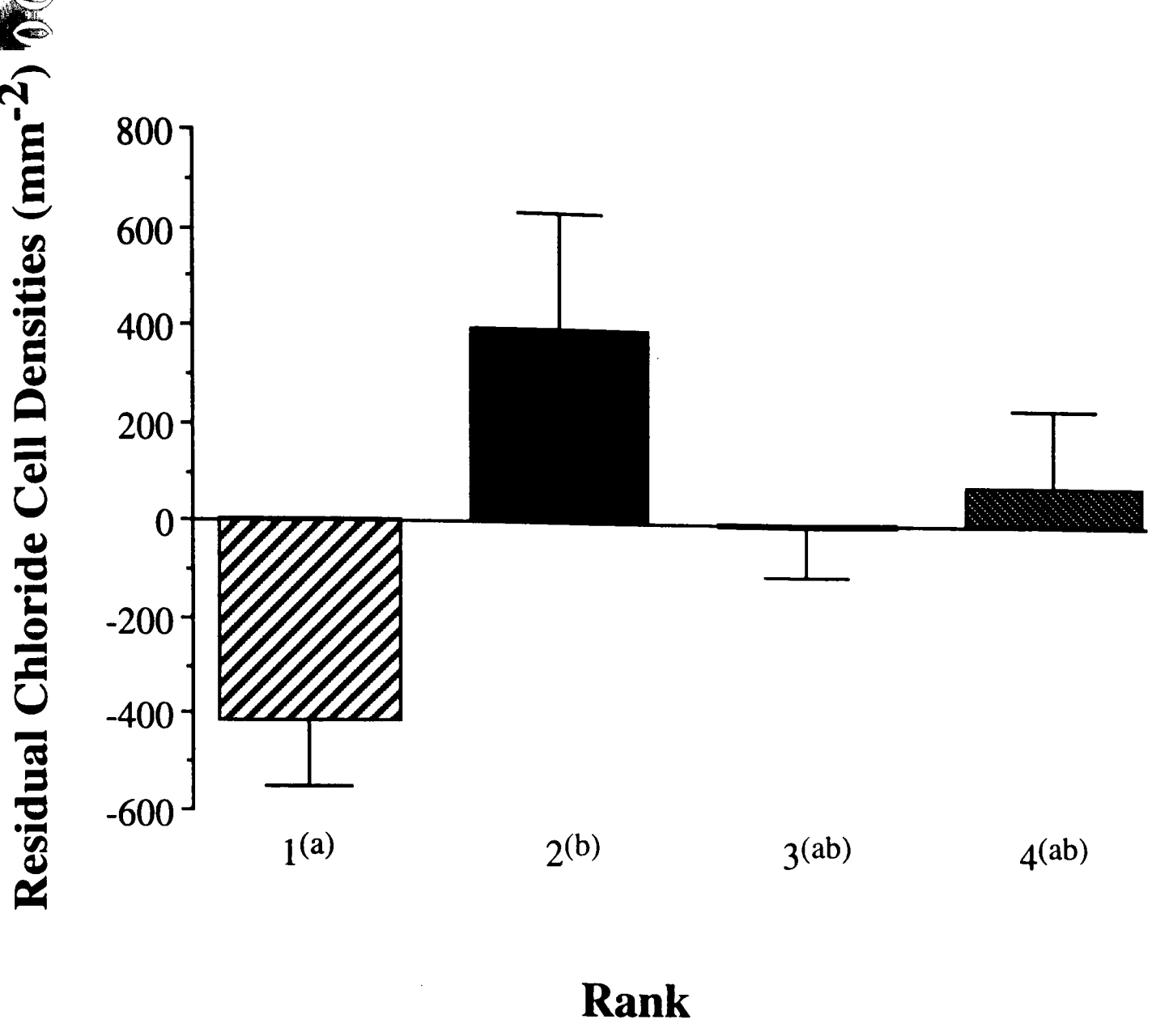



Figure 8.5: Chloride cell densities according to rank of fish are presented as mean residual values ± S.E.M. Rank 1 is the most dominant. Statistical differences (one way ANOVA followed by Scheffé multiple comparisons test, $P < 0.01$) are indicated by the letters; groups sharing the same letter are not significantly different from one another.

## 8.5 Discussion

Behavioural observations on groups of four brown trout held under simulated natural conditions in an artificial stream tank demonstrated that the fish established a linear dominance hierarchy within the two week experimental period, and that the greatest difference in behaviour was between the first- and second-ranking fish. Dominant fish maintained a position in the stream tank nearest to the food source and usually obtained food first. Consequently, they exhibited the highest food score and specific growth rate, as well as an increase in condition factor over the experimental period. The subdominant fish was nearer to the food source than the third- or fourth-ranking (subordinate) fish and competed with the dominant fish most often for food. Although there were no significant differences among the food scores for the second-, third- and fourth-ranking fish there was a decreasing trend in the amount of food obtained by each rank. Furthermore, subdominants lost weight over the experimental period and exhibited a decline in condition factor, whereas little change in either parameter was observed for subordinate fish. Little aggression occurred, probably because disputes had already been resolved by the time the first behavioural observations were made and the hierarchy was maintained without the need for further conflict.

The present study indicates that it is most beneficial to be the dominant fish in the hierarchy with regard to food intake and growth rate, a finding in agreement with the results of Li and Brocksen (1977) and Fausch (1984) in semi-natural environments and Abbott and Dill (1989) and Pottinger and Pickering (1992) in small bare tanks. Li and

Brocksen (1977) reported that dominant fish grew faster and had a higher body lipid content than subordinate fish. Re-evaluation of their data by Metcalfe (1986) allowed two distinct subordinate strategies to be identified, a high energetic return/ high energetic cost strategy in which subordinates maintained a feeding station in the fastest flowing water and encountered significant aggression from dominant fish, and a low energetic return/ low energetic cost strategy in which subordinates opted out of the competition and avoided conflict by remaining predominantly in shelters. A subordinate with the high return/ high cost strategy competed for the optimal food source and thus expended a large amount of energy in competitive interactions, incurring a high cost. The energetic return, however, was also high. The low return/ low cost strategy, on the other hand, hinged on minimising metabolic costs. Metcalfe (1986) concluded that subordinates adopting a high return/ high cost strategy expended more energy than they obtained, while those adopting the low return/ low cost strategy continued to grow despite receiving a low level of food intake.

It would appear that the second-ranked fish in the present study were adopting a high return/ high cost strategy, since they took up positions nearest to the dominant and obtained the second-highest food intake, but in doing so apparently incurred extra energetic costs. Neither food intake nor activity were measured directly but the loss of weight and reduced body condition would suggest that the second-ranking fish was energetically disadvantaged. In contrast, the third- and fourth-ranked fish appeared to adopt the low return/ low cost strategy and hence exhibited little change in either weight or condition factor. A strategy that results in higher energy expenditure than intake is not

sustainable in the long-term as survival depends on at least minimal growth. It seems likely that the hierarchies in the present study may not have been completely stabilised by the end of the two week period; subdominants would presumably have reduced their energy expenditure in the longer term, and would possibly have emigrated if the option were available.

While there were clear advantages and disadvantages in terms of food acquisition, specific growth rate and condition factor to the dominant and subdominant fish, respectively, these differences were not reflected in the differences in the other physiological parameters monitored. Notably, plasma cortisol concentrations were unaffected by dominance rank in the social hierarchy. The glucocorticosteroid hormone cortisol is synthesised and released from the interrenal cells of the head kidney in fish as a primary stress response to a wide variety of stressors, including social stressors (Pickering 1993a). The absence of cortisol differences related to social status in the present study contrasts with the results of several studies on paired fish, in which subordinate fish were found to have elevated cortisol concentrations (Peters *et al.* 1980; Pottinger and Pickering 1992; Sloman *et al.* 2000a (Chapter 3)). The differences between the results of such paired confrontations under artificial conditions and the present study emphasise the importance of examining behaviour/ physiology interactions under simulated natural conditions.

Perhaps unsurprisingly, given the absence of differences in plasma cortisol concentrations among the different ranks of fish, no significant differences were found in a variety of secondary physiological indicators of stress, including plasma ion concentrations,

haematocrit, leucocrit, interrenal cell nuclear area, hepatic glycogen content or hepatosomatic index. It should not, however, be concluded from the lack of response of these variables to social status that the formation of dominance hierarchies does not involve social stress (Barton and Iwama 1991). Indeed, the significant differences in specific growth rate and condition factor, variables that constitute tertiary responses to stress (Barton and Iwama 1991), suggest that subdominant and subordinate fish experienced some degree of stress. It is possible that changes occurred in the secondary stress response variables during the initial establishment of the dominance hierarchy, but that the values returned to control levels during the two week experimental period. For example, haematocrit and hepatic glycogen content, although not significantly different among the ranks of fish, exhibited a trend similar to that for weight change and condition factor. The high haematocrit values and low hepatic glycogen content of the subdominant fish are also indicative of stress (Ejike and Schreck 1980; Barton *et al.* 1987).

Interestingly, no differences in interrenal cell nuclear area were observed in the present study. By contrast, Noakes and Leatherland (1977) found that interrenal activity correlated inversely with dominance rank in groups of 6 rainbow trout with the exception of dominant fish, which exhibited a higher-than-expected activity. Interrenal cell activity, measured by morphometric analysis, has been demonstrated to be a reasonable indicator of pituitary-interrenal activity in teleost fish (McLeay 1975). The inverse correlation with dominance rank is thought to reflect increasing social stress as subordination increases, probably owing to subordinate fish being on the receiving end of aggression from fish higher in the social hierarchy (Noakes and Leatherland 1977). The interrenal activity of

dominant fish was higher than expected from the correlation, a result that may reflect the higher levels of activity and involvement in agonistic encounters associated with maintaining the top-ranked position in the dominance hierarchy (Noakes and Leatherland 1977). The lack of any correlation between interrenal cell nuclear area and dominance rank in the present study is consistent with the lack of plasma cortisol concentration differences and may reflect the smaller group size used in the present study (4 vs. 6) and/ or species differences in behaviour and aggression between brown and rainbow trout.

The chloride cells of the branchial epithelium have been shown to proliferate when teleost fish are exposed to ion-poor water, an up-regulation of branchial ionoregulatory mechanisms that is designed to minimise ionoregulatory disturbance (reviewed by Perry 1997, 1998). Proliferation of chloride cells in response to ionoregulatory challenges is thought to be cortisol-mediated, as transient increases in plasma cortisol levels have been documented during acclimation to ion-deficient conditions (Perry and Wood 1985; Perry and Laurent 1989) and injections of cortisol induce chloride cell proliferation (Doyle and Epstein 1972; Laurent and Perry 1990; Madsen 1990b; Bindon *et al.* 1994a; Laurent *et al.* 1994). The potential for elevated plasma cortisol concentrations to induce chloride cell proliferation in the absence of an ionoregulatory challenge was recently examined in rainbow trout, using the social stress of confinement in pairs in an artificial environment to elevate plasma cortisol concentrations (Sloman *et al.* 2000a (Chapter 3)). Although confinement with a dominant resulted in elevated plasma cortisol concentrations in subordinate fish, branchial chloride cells were unaffected (Sloman *et al.* 2000a (Chapter 3)). Interestingly, however, chloride cell densities in dominant fish in the present study

were significantly lower than in the subdominants. A possible explanation of this observation is that the high chloride cell density of the subdominant fish resulted from a stress-induced, transient, elevation of the plasma cortisol concentration.

The first-ranking fish in the present study had an average chloride cell density of approximately 2100 $mm^{-2}$ and the second ranking fish approximately 2900 $mm^{-2}$ (38% higher). Greco *et al.* (1996) found that rainbow trout acclimated for two weeks to ion-deficient water had chloride cell densities around 5000 $mm^{-2}$, 25% higher than in controls. Among these fish there was a significant difference in blood-water gas diffusion distance across the gills with a consequent effect on respiration. Bindon *et al.* (1994a) demonstrated that injections of a combination of cortisol and ovine growth hormone caused increases of 250% in chloride cell densities in rainbow trout, which also coincided with an impairment of respiratory gas transfer. Although blood-water gas diffusion distances or other parameters of respiratory ability were not measured in the present study, there may have been consequential variations in respiratory function between first- and second-ranking fish owing to their different chloride cell densities. Differences in chloride cell densities between the results of the present study and previous studies can be attributed to the different species of fish, used as well as differences in water ion levels.

In conclusion, the present study is one of the first that has attempted to examine the interactions between behaviour and physiology in groups of fish held under simulated natural conditions. The results of the present study indicate that significant advantages are accrued by the top-ranked fish in terms of food intake and consequently specific growth

rate and condition factor. Second-ranked fish appeared to adopt a high energetic return/ high energetic cost behavioural strategy that was clearly disadvantageous, as shown by the loss of weight and loss of condition factor experienced by these fish over the two week experimental period. The lower-ranking subordinate fish, on the other hand, may have adopted a low energetic return/ low energetic cost behavioural strategy. Despite the differences in specific growth rate and condition factor, dominance rank-linked effects on primary (cortisol) and secondary (haematocrit, leucocrit, hepatic glycogen content, hepatosomatic index, plasma ion concentrations) stress responses were not apparent. However, differences in branchial chloride cell density were found between dominant and second-ranked fish and these differences warrant further investigation. The aim of the present experiment was to investigate the physiological effects of established dominance hierarchies, rather than the physiological effects incurred by fish during hierarchy establishment. Thus it is not surprising that the majority of physiological changes observed were tertiary responses to stress. The effect of actual establishment of social hierarchies on fish physiology remains to be clarified.

# CHAPTER 9

# Chapter 9: Effects of an Environmental Perturbation on the Behaviour and Physiological Function of Brown Trout, *Salmo trutta*.

A version of this chapter has been published in Animal Behaviour, with co-authors: Alan C. Taylor, Neil B. Metcalfe and Kathleen M. Gilmour

## 9.1 Abstract

The effect of an environmental perturbation on dominance hierarchies established among groups of brown trout, *Salmo trutta*, was investigated. Hierarchies were established over a one week period under constant simulated natural conditions in artificial stream tanks. In the perturbation treatment water levels were then lowered for a week to simulate a drought, whereas conditions remained the same in the control tanks. Behavioural interactions were recorded before and after the environmental perturbation. Following the two week experimental period, the fish were sacrificed for measurement of specific growth rate, blood plasma cortisol concentrations, hepatic glycogen content, hepatosomatic index, gill epithelial chloride cell densities and interrenal nuclear areas. Social interactions showed a non-significant tendency to increase in the drought tanks when the water level was lowered, and the behaviour and social ranking of the fish were significantly affected by the environmental perturbation. Furthermore, the pronounced benefits of dominance in terms of growth rate that were observed in the control tanks were not apparent in the drought tanks. However, the plasma cortisol concentrations of the drought fish were not significantly higher than those of fish from the control tank at the end of the experimental

period, suggesting that the environmental change itself was not physiologically stressful in the long term. Given that a stable social system (and its physiological consequences) were only observed in a constant environment, it is suggested that misleading conclusions may be drawn if environmental perturbations are not incorporated into experiments studying the behaviour of stream-living fish in simulated natural conditions.

## 9.2 Introduction

Simulated natural environments have been widely used in the study of the behaviour of freshwater fish (Kalleberg 1958; Mason and Chapman 1965; Li and Brocksen 1977). The use of stream tanks provides an intermediate approach between studies carried out in the natural environment, which is not always possible, and those conducted under artificial laboratory conditions where behaviour may be affected by the unnatural holding environment. Within artificial stream tanks it has been demonstrated that species such as salmonids will establish apparently natural dominance hierarchies (Kalleberg 1958; Mason and Chapman 1965; Noakes and Leatherland 1977; Fausch 1984).

The primary response to stress in teleosts is the release of 'stress hormones', adrenaline, nor-adrenaline and cortisol (Sumpter 1997). Adrenaline and nor-adrenaline, both catecholamines, are released in what is commonly termed the adrenergic response (Mazeaud and Mazeaud 1981) and cortisol is secreted in the hypothalmo-pituitary-interrenal (HPI) response, (Donaldson 1981). These stress hormones, in turn, produce the secondary responses to stress. Elevated plasma cortisol concentrations elicit physiological

changes including increased oxygen consumption rate and a depression in the respiratory quotient (Chan and Woo 1978), an increase in interrenal cell activity (Noakes and Leatherland 1977), and changes in carbohydrate metabolism (Pickering and Pottinger 1995). Tertiary physiological changes may also occur, including decreases in growth and condition (Redding *et al.* 1986).

Using simulations that are designed to resemble the natural environment of the fish as much as possible, it has been found that there are physiological consequences of being a subordinate fish (Sloman *et al.* 2000b (Chapter 8)). While dominant fish tend to acquire the most profitable position in streams in terms of energy intake (Fausch 1984) and so may grow fastest (Sloman *et al.* 2000b (Chapter 8)), subordinate fish may experience high levels of stress. Noakes and Leatherland (1977) showed that interrenal activity was inversely correlated with dominance rank, with the exception of the most dominant fish, and increased interrenal activity is known to be associated with the release of the stress hormone cortisol (McLeay 1975).

However, studies using artificial stream tanks to simulate a natural environment generally maintain relatively constant environmental parameters during a given experiment. In contrast, social groups living under completely natural conditions would be exposed to environmental perturbations. Thus, the aim of the present study was to test by means of a perturbation experiment how the social behaviour and resulting physiological status of stream-living fish are affected by environmental fluctuations. The effects were studied in brown trout, *Salmo trutta*, and the chosen environmental perturbation was drought,

simulated by the lowering of water levels. Drought conditions are a natural intermittent occurrence in brown trout streams (Elliott 1987; Titus and Mosegaard 1989). However, a reduction in water levels may itself be stressful: Einarsdóttir and Nilssen (1996) demonstrated that lowering water levels in rearing tanks from 40 to 10 cm caused a significant elevation of plasma cortisol concentrations in Atlantic salmon, *Salmo salar*. It is therefore possible that drought may be a potential stressor among natural salmonid populations, because it forces fish to use shallow habitats that they would normally avoid and/or it increases local densities of fish (and hence interaction rates).

The physiological parameters measured in the present study included plasma cortisol concentrations, specific growth rate, hepatic glycogen content and hepatosomatic index (HSI). Recent work (Sloman *et al.* 2000b (Chapter 8)) suggests that the formation of dominance hierarchies within simulated natural conditions may not significantly affect plasma cortisol concentrations of salmonid fish but cortisol concentrations are known to be affected by physical stressors (Barton and Iwama 1991; Pickering 1992). Gill epithelial chloride cell densities and interrenal cell nuclear areas were also measured, since chloride cell densities have previously been shown to increase in subdominant fish (Sloman *et al.* 2000b (Chapter 8)) and a relationship between interrenal nuclear areas and dominance has previously been demonstrated (Noakes and Leatherland 1977). Gill epithelia chloride cells are involved in the transport of chloride ions across the gills (Perry and Laurent 1989) and proliferation of these cells has been demonstrated by the artificial elevation of plasma cortisol concentrations (Laurent and Perry 1990). The association between chloride cell proliferation and social stress however, remains unclear.

Thus, the aims of the present study were to observe the behaviour and physiological consequences of dominance hierarchies formed under constant conditions, as in previous studies, and then to investigate how these are affected by an environmental perturbation.

## 9.3 Methods

### *9.3.1 Experimental protocol*

Brown trout were obtained from the University Field Station, Rowardennan (Scotland). They were the offspring of wild adults from Loch Awe, western Scotland, but had been reared under hatchery conditions. The fish were held in a 70 L stock tank in aerated, recirculating, dechlorinated tap-water at ambient temperature (12.77 ± 0.11 °C mean ± S.E.M.). The artificial lighting system was set to mimic ambient conditions; it provided 12 hours of full daylight plus 30 minutes subdued dawn and dusk lighting at the beginning and end of the day. Dim 'night-time' lighting was used in addition for the whole 24 h period. After several months acclimation to the laboratory conditions, the fish were anaesthetised using a solution of benzocaine (0.05ml $ml^{-1}$) and unique combinations of alcian blue dye marks (Kelly 1967) were injected into their fins. No adverse effects of either the anaesthetic nor the marking were seen and all fish recovered quickly. Initial fork lengths and weights were recorded (weight 9.76 ± 0.79 g, length 9.76 ± 0.18 cm (mean ± S.E.M.), n = 40). The fish were then allocated to groups of four size-matched fish (mean size difference ± S.E.M. = 0.00 ± 0.04 cm). All work was carried out under home office animal care licences.

The experimental set-up consisted of replicate artificial stream tanks designed to simulate the natural environment in a small stream. The layout of the tanks is shown in Fig. 9.1. In each tank the white marble chip substrate, designed to allow the fish to be seen easily, was arranged to create variation in initial water depth from 13 cm to 19 cm. Water was pumped in an anti-clockwise direction by a pump located between zones one and four. Fish were fed to excess on pellets (BOCM Pauls Ltd Keystart; oil 16.0%; protein 55.0%) by automatic feeders which dispensed the food at a trickle rate throughout the 24 h. The fish were only able to access the food source from one end, the pump preventing access from the other direction. Food was provided to excess, but as the food source was located in one position, competition for food still occurred.

During the first week of the experiment, initial behavioural observations were made on five out of the seven days. The groups of fish were observed for a 30 minute period in the morning (between 0900 and 1030 a.m.), at noon (between 1200 and 1330 p.m.) and in the evening (between 1530 and 1700 p.m.), all behavioural observations being made during the day-light period. Dominance was measured by assigning points using a weighted scoring system (Table 9a) based on previous studies of salmonid behaviour (Metcalfe *et al.* 1989; Johnsson *et al.* 1996). Each fish was scored according to its position in the tank, its consumption of food and its social interactions. At the beginning of each 30 minute observation period a food item was introduced into the tank and the fish that took the food item scored one point. Fish that either attempted to get a food item but lost out to another fish or did not attempt to feed at all scored zero points. Fish were then scored

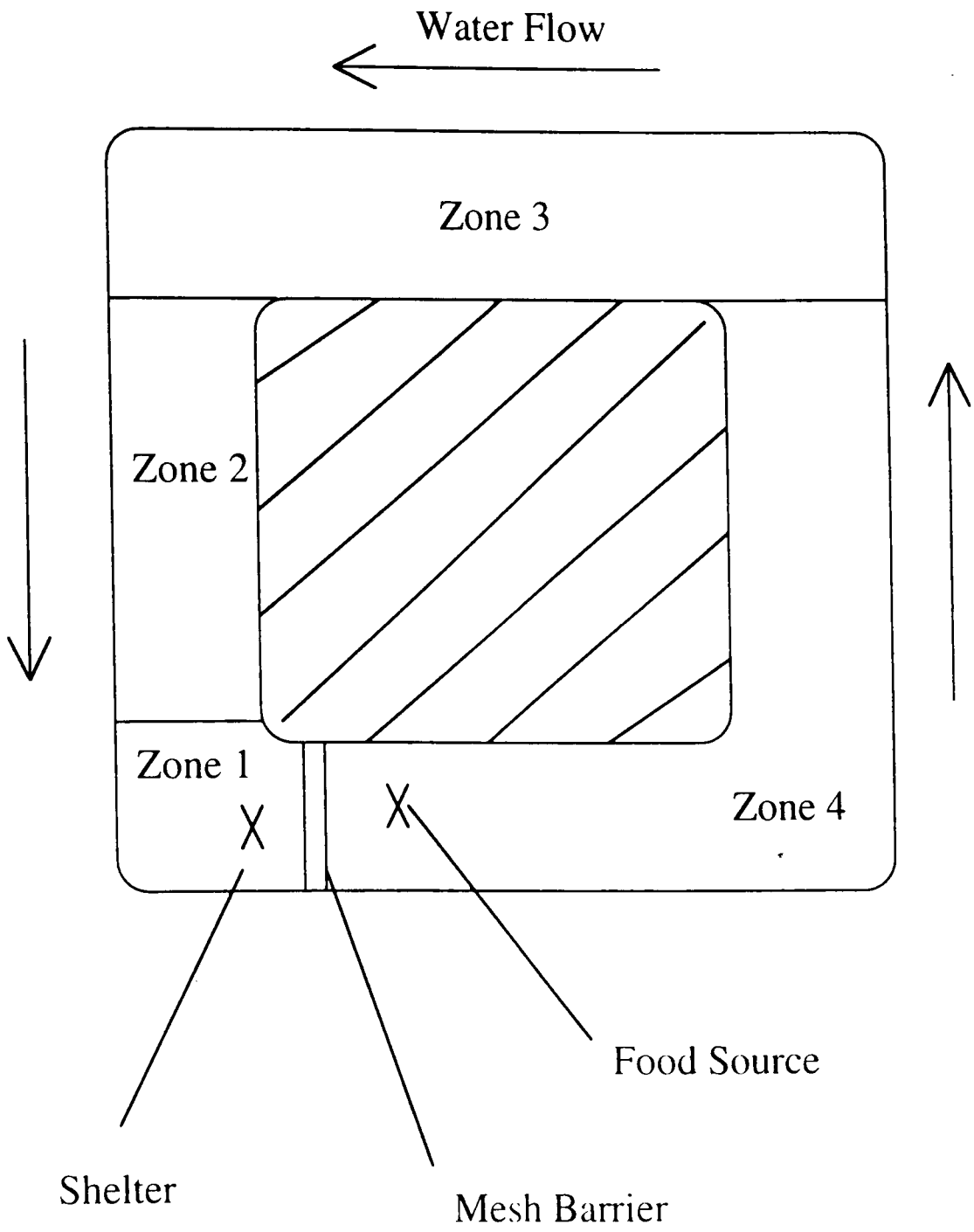



Figure 9.1: Plan of experimental tanks, showing the mesh barrier and the zones used to record fish position. Water flowed continuously around the tank in the direction indicated by the arrows, being driven by a pump with an outflow at the mesh barrier. Fish were prevented from entering the hatched area by barriers.

every five minutes during the 30 minute period according to their position in the tank zones shown in Fig. 9.1. A principal components analysis (PCA, based on the correlation matrix) was then carried out on the data using each fish's mean scores (average across the week) for food intake and position in the tank and its total scores for social interactions as variables. This produced an overall dominance score for each fish for the initial week of the experiment (Sloman *et al.* 2000a (Chapter 3)). Fish could then be ranked in order of descending dominance score, where rank one was classed as the dominant fish, rank two the subdominant, and ranks three and four as subordinate fish.

Table 9a: Each fish was given a score for feeding at the beginning of each 30 minute observation period, and then scored every five minutes for position in the tank according to the zonation of the tank (Fig. 9.1). Fish were also scored for any social interactions during the observational period. Higher scores are indicative of more dominant behaviour. The maximum that one fish could score within a 30 minute period was one point for food and 20 points for position.

| Behaviour | | Score at each observation |
|---|---|---|
| Feeding: | Fish that took introduced food item | 1 |
| | Fish that failed to get food item | 0 |
| Position: | Zone 4 | 4 |
| | Zone 3 | 3 |
| | Zone 2 | 2 |
| | Zone 1 | 1 |
| Social Interaction: | Avoiding another fish | 0 |
| | Chasing another fish | 1 |
| | Bitten by another fish | 0 |
| | Biting another fish | 1 |

After the first week, the water level was lowered in tanks allocated to the experimental treatment (n = 5), which were chosen at random and designated drought tanks; those in which the water level was not altered were designated control tanks (n = 5). The water level in the drought tanks was lowered to a mean depth of 7 cm in the deepest areas, (*i.e.* 37% of the original depth), a reduction sufficient to magnify the differences between the shallowest and deepest areas and to alter the topography and microhabitat structure of the stream, but small enough to minimise stress to the fish. No distress appeared to be caused by this manipulation; indeed no physiological changes were observed (see results) as a result of the drought conditions. A PVC tube shelter was also added after the first week to all tanks at the point furthest from the food source as a refuge for subordinates to ensure that no unnecessary stress was caused. Although aggressive interactions were seen amongst the fish, the PVC tube provided enough of a refuge to ensure that the extent of aggression encountered by the fish was not severe. No injuries were seen. However, no effect of the PVC tube was seen on the behaviour of the fish and therefore the dominance score did not appear to be altered by the addition of the tube. Behavioural observations were made on five out of the subsequent seven days as before, in both drought and control tanks. These behavioural data were used in a second PCA to give a dominance score for each fish for the final week of the experiment. A third, overall, dominance score for the entire experimental period (*i.e.* for both weeks combined) was then generated by combining the initial and final behavioural scores for each fish in a separate PCA analysis (variable: eigenvalue = 2.103, % variance = 35.05). Behavioural observations were carried out for only one week after the lowering of the water level as the aim of the experiment was to investigate the short term effects of the environmental perturbation.

*9.3.2 Physiological measurements*

At the termination of the two week experimental period, the four fish in each tank were caught simultaneously, using hand held nets, and killed within 60 s using a lethal overdose of benzocaine anaesthetic (0.5 mg $ml^{-1}$). Final fork lengths and weights were recorded, and blood samples (0.5-1.0 ml) were withdrawn by caudal venipuncture. The blood samples were centrifuged (13,000 g), and plasma samples were frozen in liquid nitrogen for later analysis of plasma cortisol.

Gill, liver and head kidney tissue were also sampled for later analysis. The second gill arch was removed and washed in 0.9 % saline, following which pairs of filaments were fixed in buffered glutaraldehyde (5% glutaraldehyde in phosphate buffer; 1 h; 4 °C) and stored in phosphate buffer at 4 °C for 24 h. The liver from each fish was placed in a pre-weighed Eppendorf tube, weighed, frozen in liquid nitrogen and stored at -70 °C for later analysis of glycogen content. Finally, the head kidney lobes were removed from each fish and fixed in buffered formalin (10%) for several days.

Plasma samples were analysed for plasma cortisol concentrations using a radioimmunoassay (ICN Pharmaceuticals Ltd) (Gamperl *et al.* 1994). Liver glycogen content was assessed using the anthrone method of Wedemeyer and Yasutake (1977). For the analysis of chloride cell density, the fixed gill tissue was stained with an osmium-zinc iodide preparation (1 part 2% $OsO_4$: 4 parts 3% $ZnI_2$; 18 h; 20 °C) (Garcia-Romeu and Masoni 1970), a procedure which causes the chloride cells to stain an intense black colour

and which can therefore be used to quantify chloride cell numbers. The fixed, stained tissue was dehydrated through graded ethanols (30%, 50%, 75%, 95%, 100% x 2, 20 minutes in each; 20 °C), rinsed in Histoclear® (2 x 20 minute rinses; 20 °C) and paraffin wax (2 x 20 minute rinses; 55 °C), embedded in paraffin wax and sectioned at 7 μm using a microtome (Leitz 1512). Sections were viewed using a light microscope (40 x objective; Leitz Dialux® microscope) and photographs were taken with an attached camera (Wild Leitz MPS51 camera; Wild MPS45 photoautomat). Ten slides, with sixteen sections per slide, were prepared to produce a total of 160 sections per fish. Eight photographs of gill tissue were taken per fish from randomly selected sections. For each photograph, the slide was positioned so that the field of view contained approximately 10 lamellae and a small portion of the filament at the bases of the lamellae. Chloride cell numbers were quantified by scanning the 8 photographs for each fish and quantifying the gill tissue area in a photograph using Scion Image software. The number of chloride cells per unit tissue area was calculated for each photograph by visually counting the intensely stained cells, and the value for each fish was taken as the mean of the values for the 8 photographs.

For histological analysis of head kidney tissue, the fixed tissue was dehydrated through graded alcohols, rinsed in Histoclear® and embedded in paraffin wax using the procedure detailed above for gill tissue. The entire head kidney tissue sample was then sectioned at 7 μm. The sections were mounted on slides, which were then stained with Mayer's haematoxylin and eosin (Mayer 1903) and viewed as above. Seven photographs were taken per fish, at 1000 x magnification, from randomly selected sections. For each photograph, the slide was positioned so that the field of view contained as many interrenal

cells as possible. Due to the breakdown of cell membranes, only nuclear areas could be measured. Measurements were carried out using the Scion Image software as above. The value for each fish was taken as the mean of the nuclear areas determined for 10 interrenal cells from each of the seven photographs, resulting in a total of 70 measurements per fish.

### *9.3.3 Statistical methods*

A total of five drought and five control replicate trials were conducted. Specific growth rates were calculated as % weight change per day (Ricker 1979). Repeated measures analysis of variance (ANOVA) analyses were used, with tanks as subjects, ranks of fish as the within-subject effect and treatment as the between-subject effect, to determine whether social rank and/or treatment influenced physiological parameters. The data for hepatosomatic index (liver weight as a percentage of body weight) and hepatic glycogen data were combined using a PCA analysis (correlation matrix) to produce an overall value expressed in the results as 'liver condition' (HSI: eigenvalue = 1.682, % variance = 84.11). Analyses of covariance and linear regressions were used to assess relationships between physiological and behavioural data. Except in the comparisons of behaviour between the first and second week, overall behaviour scores and ranks were used in statistical comparisons. The fiducial limit of significance in all analyses was 5%.

## 9.4 Results

During the first week of the experimental period dominant individuals were evident amongst the fish in both drought and control tanks. During the two days following the

lowering of the water level the number of aggressive interactions in the drought tanks appeared to increase, although the change in aggression was not statistically significant due to the high inter-tank variability in the number of aggressive interactions (Fig. 9.2). As expected, there was a relationship between the initial (first week) and final (second week) dominance scores but the relationship was also dependent on treatment (analysis of covariance: interaction between initial score and treatment; $F_{2,37} = 14.75$, $P = 0.05$). Figure 9.3 indicates that the dominance relationships were stable in the control tanks (linear regression: $F_{1,18} = 26.11$, $r^2 = 0.592$, $P < 0.001$), whereas there was no relationship between the initial and final dominance scores for fish subjected to a lowering of water levels (linear Regression: $F_{1,18} = 3.63$, $r^2 = 0.168$, $P > 0.05$). Although the dominance hierarchies in the control tanks appeared more stable than those in the drought tanks, overall changes in specific ranks varied and there was no consistent pattern in the changes in status.

At the end of the two week experimental period, no significant differences were found among ranks of fish in either the drought or control treatment for plasma cortisol (repeated measures ANOVA: $F_{3,24} = 1.193$, NS; Fig. 9.4), gill epithelial chloride cell densities (repeated measures ANOVA: $F_{3,24} = 0.297$, NS; Fig. 9.5) or interrenal nuclear areas (repeated measures ANOVA: $F_{3,24} = 0.485$, NS; Fig. 9.6). Plasma cortisol concentrations were low in all ranks of fish in the drought treatment, but were more variable in the fish in the control tanks.

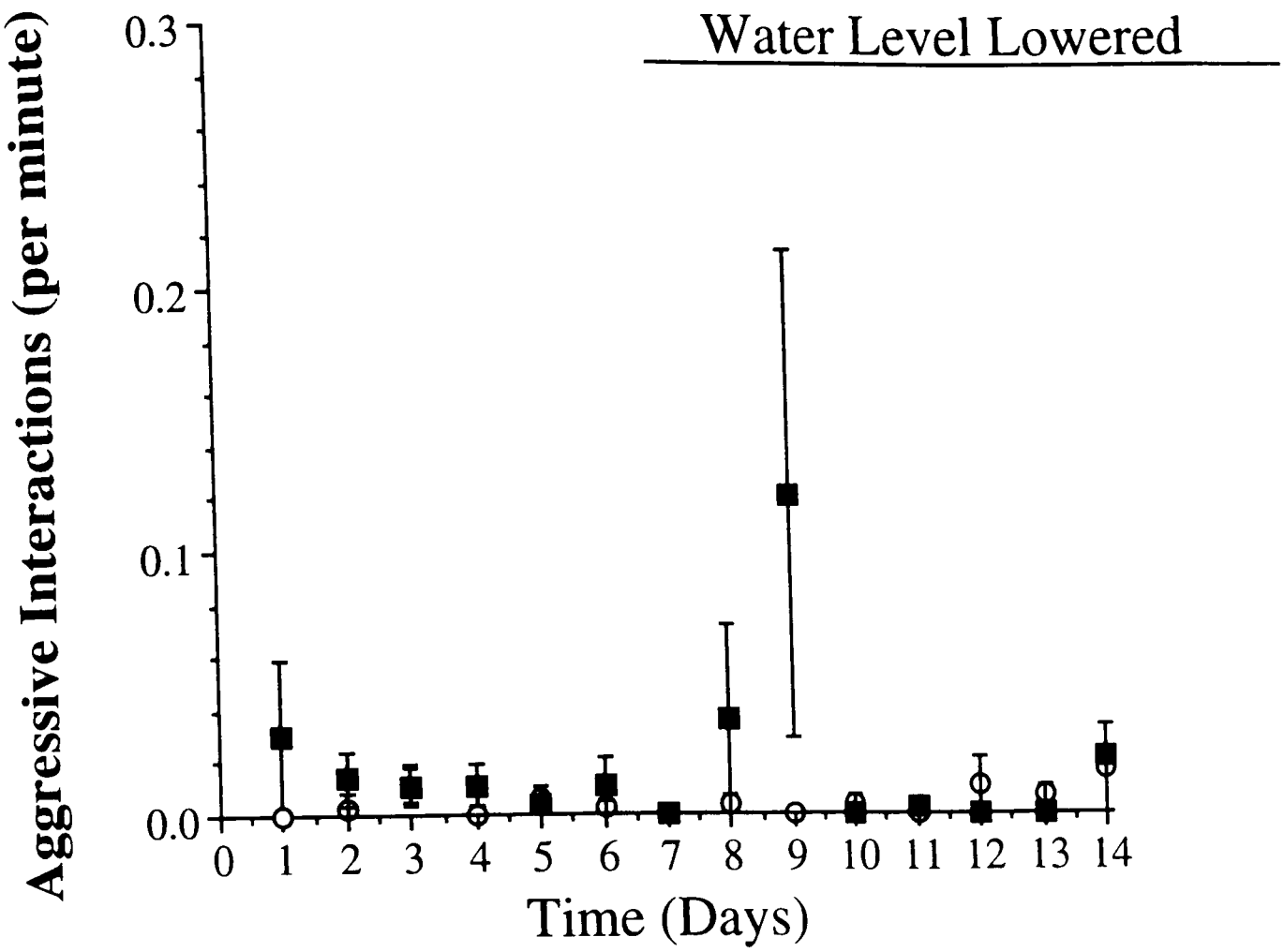
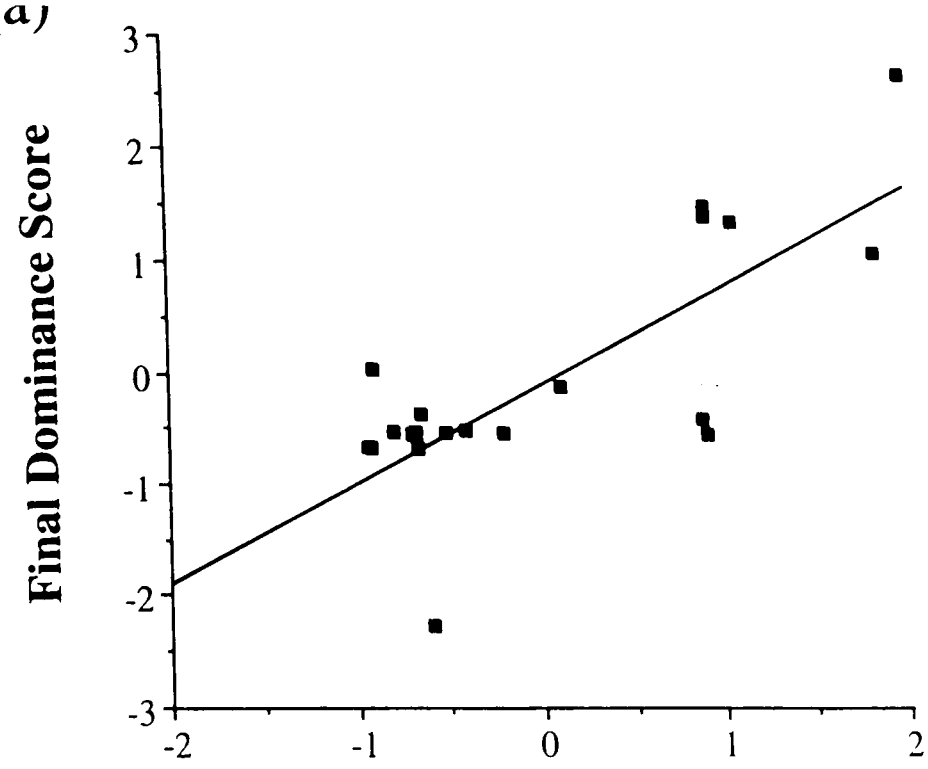



Figure 9.2: Aggression levels per fish per minute in drought (■) and control (O) tanks (n = 20 fish per treatment). Data are presented as means ± S.E.M. Water levels were lowered in experimental tanks at the end of day seven.

(a)




(b)

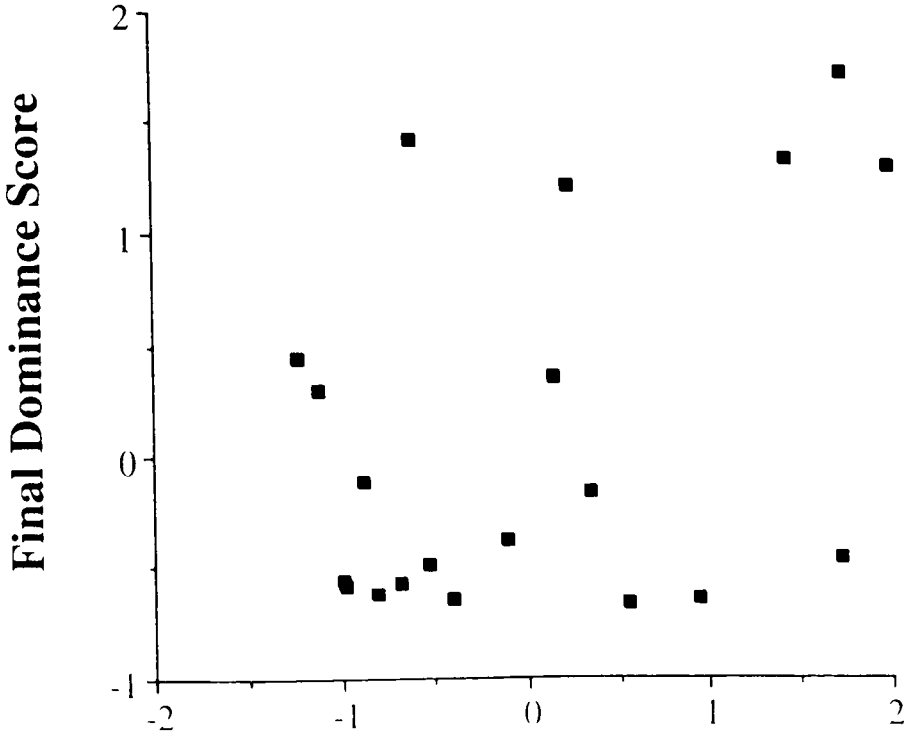



Figure 9.3: Relationship between initial and final dominance scores in the (a) control (n = 20) and (b) drought treatments (n = 20). The regression line for the control data is also plotted; the regression equation was: final dominance score = 0.904 initial dominance score + 0.0836, $r^2 = 0.592$, n = 20, $p < 0.05$. The relationship was not significant for the drought treatment (see text).

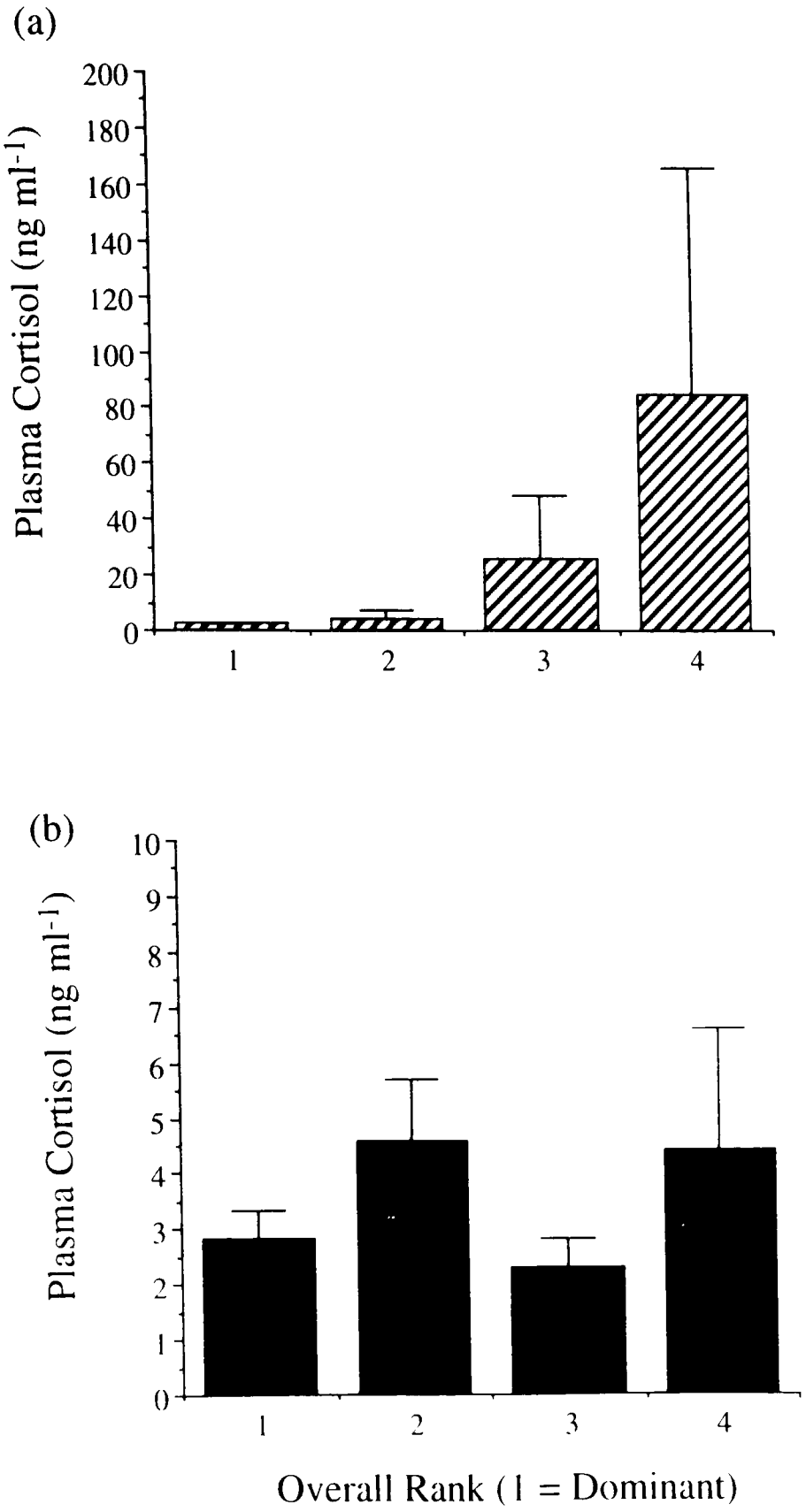



Figure 9.4: Plasma cortisol concentrations according to overall (2 week) dominance rank of fish for (a) control (n = 20) and (b) drought treatments (n = 20). Data are presented as means ± S.E.M. (see text for statistical analysis).

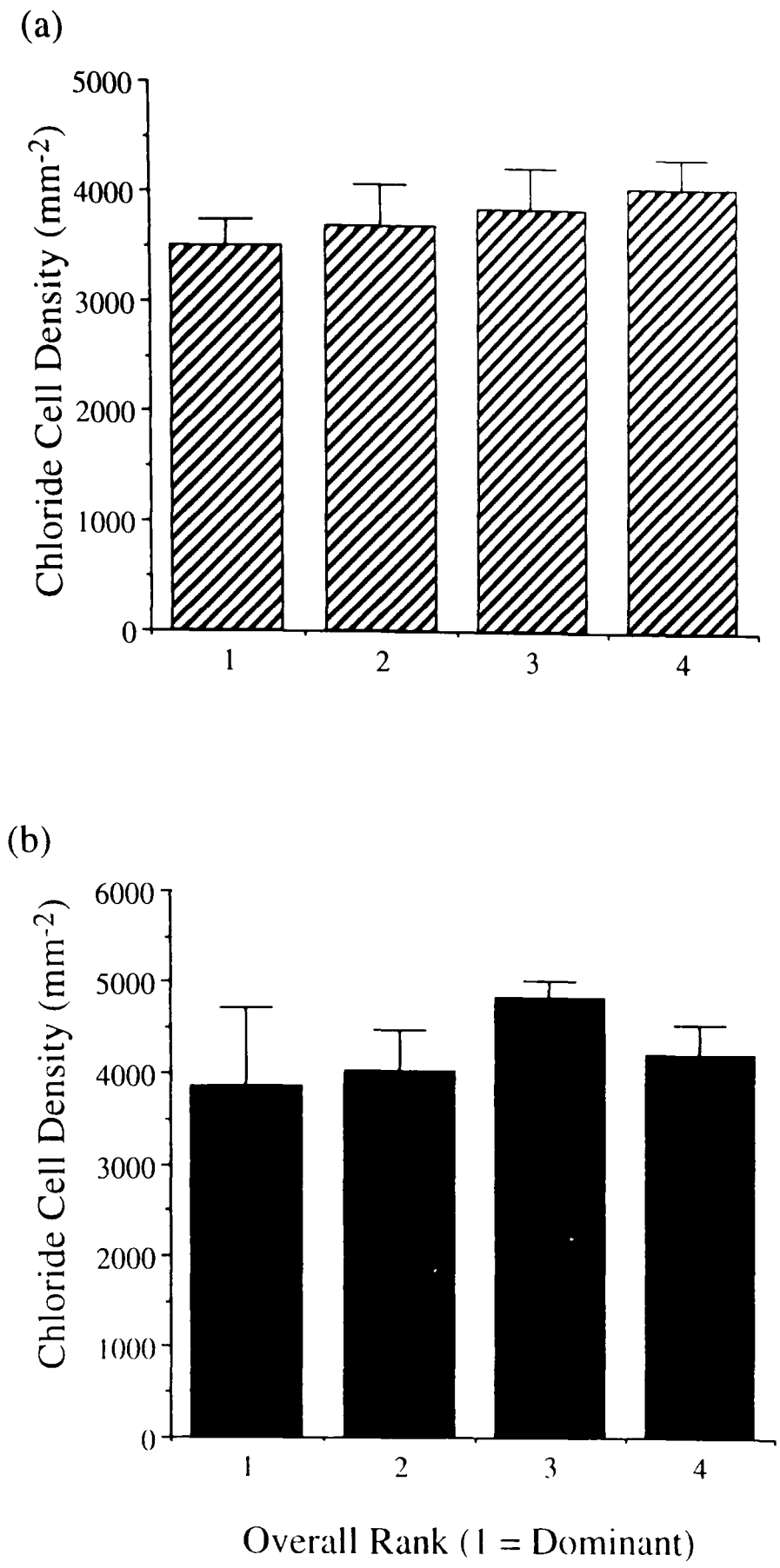



Figure 9.5: Gill epithelia chloride cell densities according to overall dominance rank of fish for (a) control (n = 20) and (b) drought (n = 20) treatments. Data are presented as means ± S.E.M. (see text for statistical analysis).

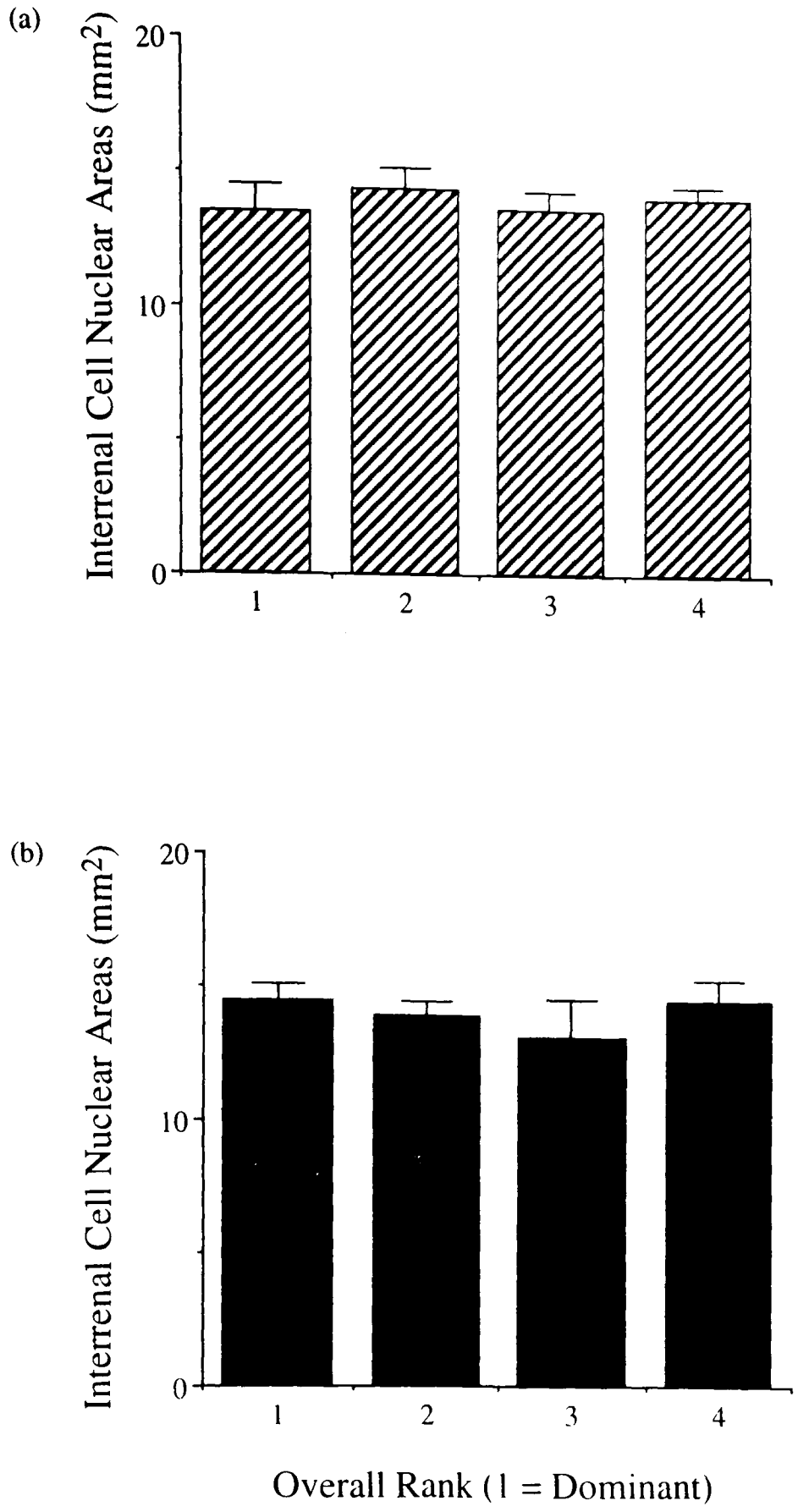
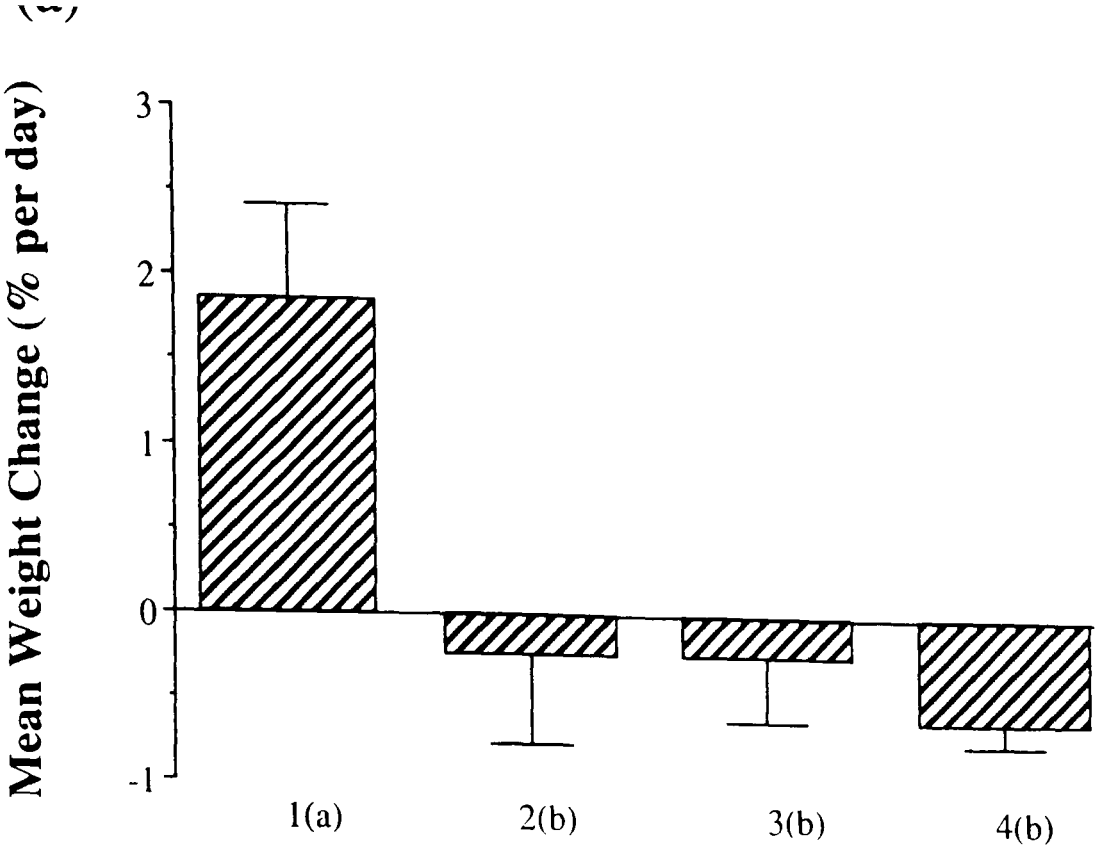



Figure 9.6: Interrenal cell nuclear areas according to overall dominance rank of fish for (a) control (n = 20) and (b) drought treatments (n = 20). Data are presented as means ± S.E.M. (see text for statistical analysis).

Growth rates varied significantly with social rank (repeated measures ANOVA: $F_{3,24}$ = 4.627, P = 0.011), but this relationship was also dependent on treatment (repeated measures ANOVA: interaction between rank and treatment, $F_{3,24}$ = 3.413, P = 0.034). Under stable (control) conditions, the most dominant fish over the two week period grew faster than those more subordinate, but there was no clear relationship between dominance and growth in the tanks where water level fluctuated (Fig. 9.7). Since all top ranked trout did not dominate the other fish within their groups to a similar degree, the relationship between growth and overall dominance score (a more sensitive measure of interactions than the four categories of social rank) was examined. Covariance analysis revealed that the relationship between overall dominance score and growth rate differed between the treatments (treatment*dominance score interaction; $F_{2,37}$ = 8.173, P = 0.05). In the control tanks there was a significant relationship between overall dominance score and specific growth rate (linear regression: $F_{1,18}$ = 18.22, $r^2$ = 0.503, P < 0.001) but this was not the case among the ranks of drought fish (linear regression: $F_{1,18}$ = 0.439, $r^2$ = 0.024, NS).

There was a significant effect of social rank on liver condition (repeated measures ANOVA: $F_{3,24}$ = 21.572, P = 0.01). Figure 9.8 indicates that the relationship with rank appears strongest among ranks of fish in the control tanks although the relationship was not significantly dependent upon treatment (repeated measures ANOVA: interaction between rank and treatment, $F_{3,24}$ = 0.778, NS).




(b)

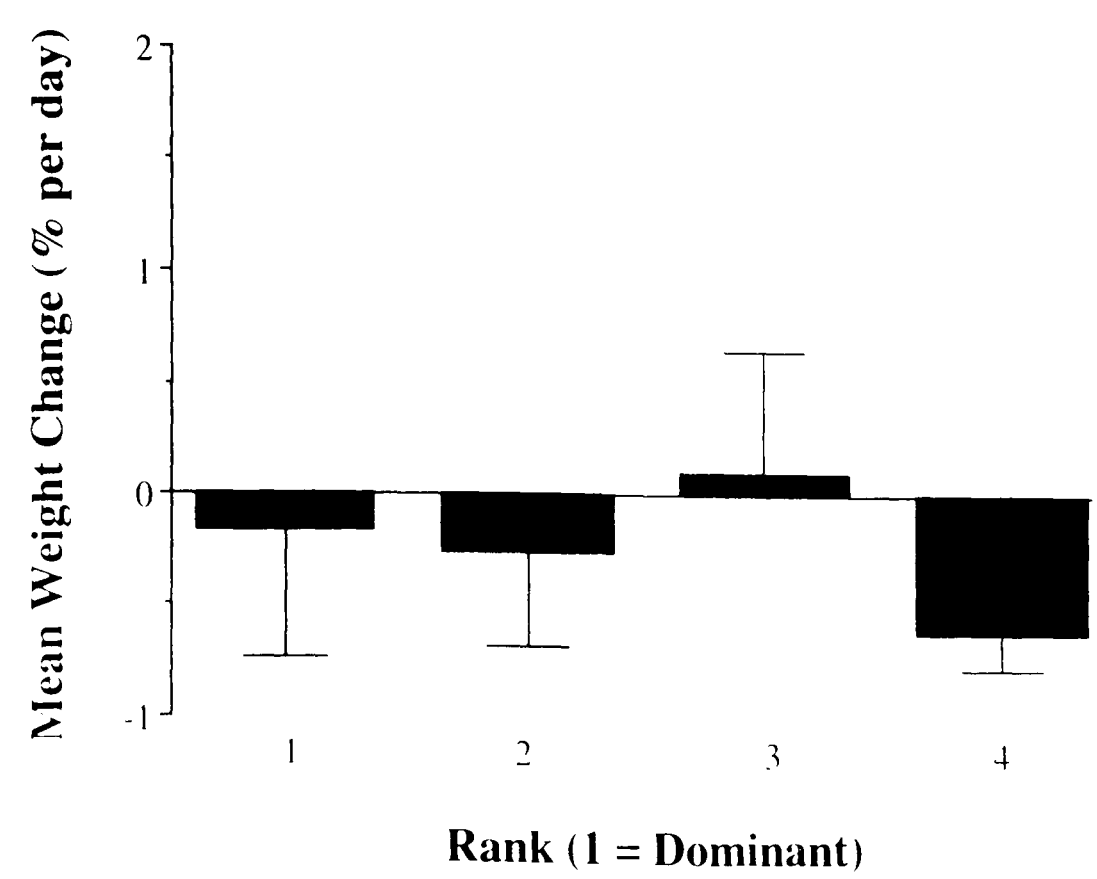
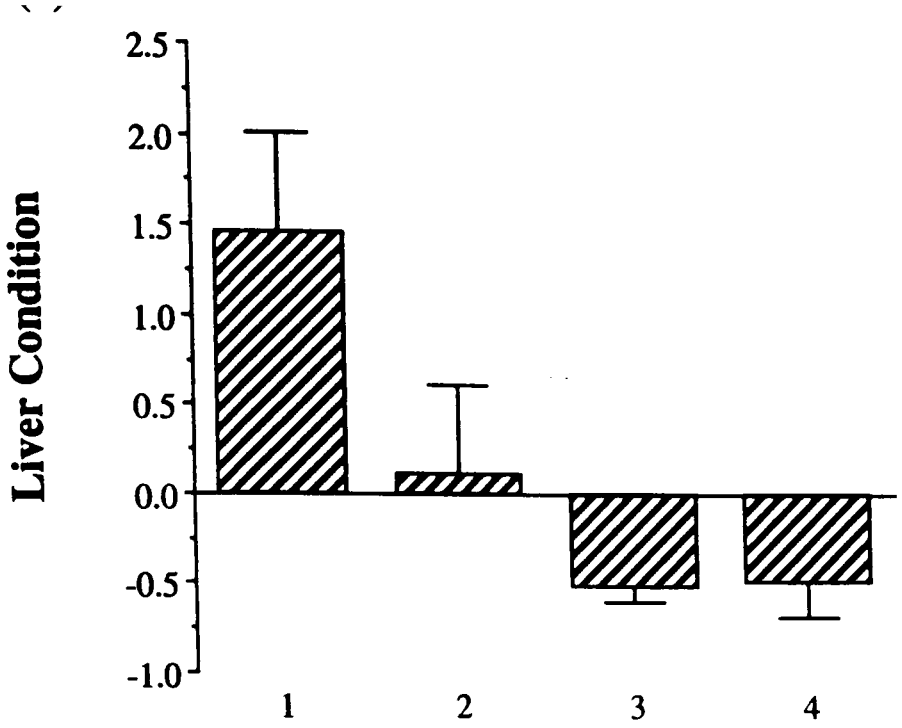



Figure 9.7: Specific growth rates (% change in weight per day) according to overall (2 week) dominance rank of fish for (a) control (n = 20) and (b) drought treatments (n = 20). Data are presented as means ± S.E.M. (see text for statistical analysis).




(b)

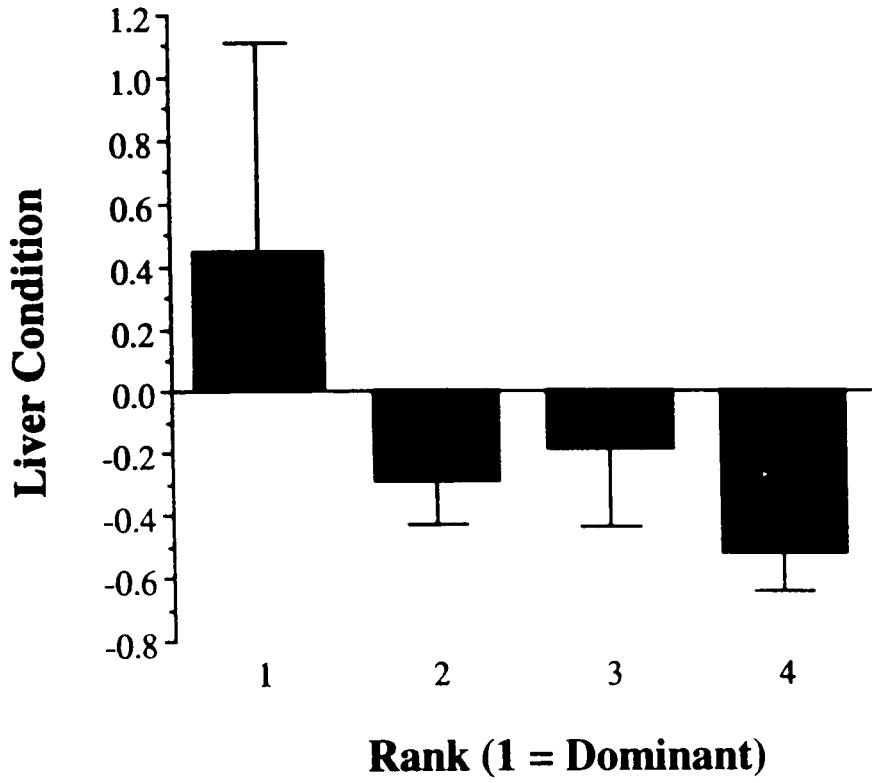



Figure 9.8: Liver condition values according to overall dominance rank of fish for (a) control (n = 20) and (b) drought treatments (n = 20). Data are presented as mean ± S.E.M. Liver condition varied with social rank but there was no significant effect of treatment (see text for statistical analysis).

## 9.5 Discussion

Lowering the water level in the drought tanks appeared to affect both the behaviour of individual fish and the structure of the hierarchy. In the control treatments, there was a strong positive correlation between original dominance score, based on observations during the first week of the experimental period, and the final dominance score, based on observations from the second week of the experiment. The correlation between these scores would suggest that the behaviour of individual fish and the social structure in these tanks did not change appreciably over the two week period. It has previously been observed that within natural populations of brown trout living in a large river (and presumably not subject to large fluctuations in water level), hierarchies may remain stable over long periods of time (Bachman 1984). However, in the drought tanks there was no relationship between the initial and final dominance scores, suggesting that both the behaviour of individual fish and the hierarchy structure in these tanks were altered by the imposition of 'drought' conditions. The trend for an increase in aggressive interactions in the drought tanks during the first two days after lowering the water level, while not statistically significant, was also suggestive of a period of behavioural change elicited by the environmental perturbation. The change observed in both the behaviour of individual fish and the social hierarchy could have arisen because fish were forced to move out of areas that had become too shallow and therefore had moved into territories already occupied by other fish. It is also possible that the change in topography and mean water depth favoured different kinds of fish, resulting in a change in dominance relationships.

Despite the behavioural evidence for the impact of environmental fluctuations on social structure, only minor differences were found in indices of physiological condition. At the end of the experimental period, no differences in plasma cortisol concentrations were found among ranks of fish in either the control or drought tanks. Lowering of the water level in the drought tanks appeared to have no long term stress effects, since plasma cortisol concentrations measured at the end of the two week experimental period were all characteristic of unstressed fish (*i.e.* plasma cortisol concentrations < 5 ng $ml^{-1}$) (Pickering and Pottinger 1989). While reductions of water levels have previously been shown to induce stress (Einarsdóttir and Nilssen 1996) such experiments were carried out under conditions intended to increase stress levels rather than simulate natural environmental changes. It is possible, however, that in the present experiment there was a transient increase in plasma cortisol concentrations in response to the lowering of the water level, but that the concentrations returned to basal levels before the fish were sampled (*i.e.* within seven days), since cortisol concentrations can return to basal concentrations within 24-48 h after an acute stressor (Pickering and Pottinger 1989). It would have been interesting to measure cortisol during the two days after the onset of drought conditions when the rate of aggression occurred amongst the fish was highest. However, blood sampling from the fish at this time would have in itself disturbed the fish and most likely disrupted the hierarchy and so this procedure was not carried out.

The pattern of growth rates was significantly affected by the drought treatment. In the control tanks the dominant fish had significantly higher growth rates than the other ranks

of fish and dominance score and specific growth rate were positively correlated. Similar effects of social status on growth rate have been found in previous experiments (e.g. Fausch 1984; Abbott and Dill 1989; Sloman *et al.* 2000b (Chapter 8)). A high specific growth rate in the dominant fish is not surprising since dominant fish are known to occupy the most profitable positions in streams (Fausch 1984) and the rewards of becoming a dominant fish have been illustrated in many previous studies and include in particular the monopolisation of food sources leading to a higher food intake and specific growth rate (Li and Brocksen 1977; Metcalfe *et al.* 1992).

However, while the dominant fish in the control treatments had higher specific growth rates than the lower ranking fish, there was no apparent effect of rank on specific growth rate among the ranks of fish in the drought treatments, and dominance scores were not correlated with growth rates. It appears that in the drought tanks, the breakdown in hierarchy structure after the environmental perturbation was imposed meant that no single fish obtained the pronounced rewards of dominance that were seen in the control tanks.

The metabolic benefits of dominance and consequences of environmental perturbation could also be detected in the measurements of liver condition. Liver condition was measured in terms of both hepatosomatic index (HSI) and liver glycogen, both of which are thought to be influenced by stress (Goede and Barton 1990; Pickering and Pottinger 1995). Davis *et al.* (1985) showed that catfish (*Ictalurus punctatus*) given supplementary cortisol demonstrated a decrease in liver size (i.e. HSI), while Barton *et al.* (1987) demonstrated that daily stressing of juvenile rainbow trout caused a decrease in HSI.

There was a significant effect of rank on liver condition that appeared to be greatest in the control tank. Higher liver condition in the dominant fish would be indicative of a profitable position. The subdominant and subordinate fish had a significantly lower liver condition than the dominant fish suggesting that they had smaller livers and smaller glycogen reserves. No significant differences were seen amongst the ranks of fish for gill epithelia chloride cell densities or interrenal cell nuclear diameters. The lack of significant differences among the ranks of fish for these parameters is perhaps not surprising as there were no significant changes in plasma cortisol concentrations.

The use of simulated natural environments is widespread in behavioural research on stream-living fish, particularly in studies of salmonid dominance hierarchies (Kalleberg 1958; Mason and Chapman 1965; Li and Brocksen 1977; Noakes and Leatherland 1977; Fausch 1984; Huntingford and Garcia de Leaniz 1997; Huntingford *et al.* 1998a, b; Cutts *et al.* 1999; Sloman *et al.* 2000b (Chapter 8)). Previous studies have focused almost exclusively on environmental conditions that are constant throughout the course of the experiment, and have in general concluded that, under such conditions, salmonids form very stable dominance hierarchies in which the dominant fish accrues significant benefits. Huntingford *et al.* (1998b) demonstrated that an environmental perturbation such as drought can cause a displacement of juvenile Atlantic salmon, *Salmo salar*, from their home sites and the results of the present experiment suggest that the benefits of dominance in small streams (where water levels often fluctuate markedly) may not be as pronounced as previous experiments have indicated. Certainly, the lack of a markedly dominant fish in the drought treatment tanks appeared to be due to behavioural changes caused by the

environmental perturbation, rather than the environmental parameter itself. It would therefore appear that the drought treatment only affected the physiological condition of the fish, indirectly, through its effects on behaviour and structure of the dominance hierarchy.

In conclusion, the results of the present experiment reinforces the importance of carrying out behavioural studies under conditions that are as representative of those in the natural environment as possible given experimental constraints. Although hierarchies formed under simulated stable natural conditions may resemble those formed within natural populations, the rewards of dominance may appear more extreme due to the absence of environmental fluctuations that the fish would normally encounter.

# CHAPTER 10

# Chapter 10: The Effects of Increased Flow Rates on Linear Dominance Hierarchies and Physiological Function in Brown Trout, *Salmo trutta*.

## 10.1 Abstract

Semi-natural stream tanks have been widely used to determine the behaviour of salmonid fish, and the formation of dominance hierarchies among salmonid populations has been well documented. However, the present study illustrates that environmental perturbations can play an important role in determining salmonid behaviour. Under constant semi-natural conditions, hierarchies were formed among groups of four brown trout and the dominant fish displayed physiological advantages over the subordinate fish, in terms of both higher growth rate and higher hepatic glycogen content. When the environmental perturbation of increased water flow, simulating spates, was imposed on these constant conditions, however, the social behaviour of the fish was altered and the physiological advantages of dominance were lost. It is concluded that environmental changes can affect the behaviour, and consequently the physiology, of salmonid fish. These results highlight the importance of taking environmental disturbances into consideration in studies of social behaviour.

## 10.2 Introduction

Numerous studies have been carried out on the behaviour of salmonid fish to investigate both the formation of dominance hierarchies and the consequent effects on their physiology (e.g. Li and Brocksen 1977; Fausch 1984; Huntingford and Garcia de Leaniz 1997; Sloman *et al.* 2000a (Chapter 3), b (Chapter 8)). Hierarchies form among salmonid populations through competition over limited resources (Newman 1956), with dominant fish acquiring the most profitable positions in streams whilst subordinate fish are excluded (Fausch 1984). The resultant social stress induced in subordinate fish can be physiologically disadvantageous, particularly in confined environments. Increases in plasma cortisol (e.g. Laidley and Leatherland 1988; Pottinger and Pickering 1992) and plasma glucose concentrations (Peters *et al.* 1988), decreases in weight, and condition (e.g. Pottinger and Pickering 1992; Sloman *et al.* 2000a (Chapter 3)) and lowered disease resistance (Peters *et al.* 1988) are among previously documented physiological consequences of subordinance.

Two commonly used approaches to study salmonid behaviour have been to pair fish in a confined, artificial environment (Pottinger and Pickering 1992; Sloman *et al.* 2000a (Chapter 3)), or to examine small groups of fish under semi-natural conditions in stream tanks (Noakes and Leatherland 1977; Fausch 1984). Stream tanks have been widely used to observe salmonid behaviour under conditions that are considered to simulate the natural environment of the fish (Kalleberg 1958; Fausch 1984; Valdimarsson *et al.* 1997). Social stress within stream tank environments is generally less severe than in artificial

environments, but it is still characteristic of the dominant fish to have a significantly higher growth rate (Li and Brocksen 1977; Sloman *et al.* 2000b (Chapter 8)). Subdominant (second-ranking) fish that adopt a high-cost/ high-return strategy by competing with the dominant fish may lose weight, while subordinate fish that adopt a low-cost/low-return strategy through avoiding aggressive interaction tend to avoid the most severe consequences of subordinance (Metcalfe 1986; Sloman *et al.* 2000b (Chapter 8)).

Although stream tanks are considered to simulate the natural environment of the fish quite successfully, they are generally used under constant environmental conditions. In their natural environment, however, a variety of environmental perturbations may be experienced by fish including changes in temperature, water levels and flow rates (e.g. Elliott 1987; Titus and Mosegaard 1989; Jensen and Johnsen 1999). Recently, Sloman *et al.* (2000d (Chapter 9)) demonstrated that the imposition of an environmental perturbation (lowered water levels) on a constant stream tank causes changes in the behaviour of the fish within previously established hierarchies. Growth and other physiological advantages enjoyed by dominant fish under constant semi-natural conditions were no longer apparent. Thus, the behaviour of salmonid fish in constant stream environments may not be truly representative of those in the natural environment.

Lowering of the water levels was used to simulate periods of drought that the fish may experience in their natural habitat (Sloman *et al.* 2000d (Chapter 9)). Another environmental perturbation that is known to occur within stream environments is variation in water flow. During spates, water flow within streams increases and this change may

affect the distribution of food and the characteristics of the stream environment. Increased energy expenditure may be required by the fish to maintain the same position in faster-flowing streams, and so new positions may become more favourable (Pert and Erman 1994; Vehanen *et al.* 2000). In the present study, the hypothesis that increased water flow, an important environmental parameter, would alter previously established dominance hierarchies was tested, and the effects of such a change on the physiological advantages of the dominant fish were investigated.

## 10.3 Materials and Methods

Brown trout were obtained from Howietoun fish farm, Stirling (Scotland) and held in 70 L stock tanks in recirculating, dechlorinated tap water at ambient temperature (12.3 ± 0.09 °C (mean ± S.E.M.)). After four weeks acclimation to laboratory conditions, fish were anaesthetised using a solution of benzocaine anaesthetic (0.05 ml $ml^{-1}$ water), initial fork lengths and weights were recorded (weight 35.47 ± 1.36 g, length 14.98 ± 0.18 cm (mean ± S.E.M.) (n = 40)) and each fish was given a unique alcian blue dye mark injected into its fins (Kelly 1967). The fish were then allocated to groups of four size-matched fish (mean size difference 0.04 ± 0.06 cm (mean ± S.E.M.)).

Groups of fish were placed in artificial stream tanks. Characteristics of these artificial stream tanks, which were described in detail by Sloman *et al.* (2000d (Chapter 9)), included; a water depth of 13 - 19 cm, white marble substrate to allow the fish to be observed, artificial lighting designed to mimic ambient conditions (12 h full daylight, 30

minutes dawn and dusk lighting and 24 h dim 'night-time' lighting), and a unidirectional water flow ($0.49 \pm 0.05$ $ms^{-1}$). Fish were fed to excess on pellets (BOCM Pauls Ltd Keystart; oil 16.0%; protein 55.0%) by automatic feeders which allowed food to enter the tank at the upstream end at a slow rate throughout the 24 h period. Fish were only able to access the food from one direction, as the pump prevented access from the other direction.

*10.3.1 Behavioural Observations*

For the first week of the experiment, behavioural observations were carried out on the fish for three 5 minute periods on 10 of 14 days. Each five minute period was separated from the next by at least 5 minutes. Dominance was measured by assigning points based on previous studies of salmonid behaviour (Metcalfe *et al.* 1989; Johnsson *et al.* 1996; Sloman *et al.* 2000d (Chapter 9)). Fish were scored according to their position in the tank. The tank was divided into zones, with zone four being closest to the food source and zone one being furthest away, and fish scored points equivalent to the zone that they occupied. Fish were also scored on the basis of food acquisition and social interaction, according to the method of Sloman *et al.* (2000d (Chapter 9)). Fish taking an item of food introduced to the tank scored one point and those fish either attempting to feed but being out-competed by another fish, or not attempting to feed at all scored zero points. Fish exhibiting dominant behaviours e.g. chasing or biting another fish scored one point, whereas fish exhibiting subordinate behaviours e.g. avoiding or being bitten by another fish, lost points. For each fish a mean behaviour score for position, food and social interaction during the first week of the experiment was therefore obtained.

After one week, in only those tanks allocated to the experimental (spate) treatment (n = 5), a second pump was switched on. The additional pump increased the water flow to 0.84 $\pm$ 0.05 $ms^{-1}$ (mean $\pm$ S.E.M.) in the spate tanks but did not significantly affect the water level. Conditions in the remaining tanks (control, n = 5) were not changed. Behavioural observations were then repeated for a further week in both the control and spate treatments. Again, mean behaviour scores for each fish for position, food and social interaction were obtained for the second week of the experiment.

A principal components analysis (PCA) was then carried out on the mean position, food and social interaction scores for each fish for the first week of the experiment (initial scores), the second week of the experiment (final scores) and the first and second weeks combined (total scores). The PCA weights and combines the data according to their importance. The principal component was clearly related to dominance, with strongly positive values being associated with more aggressive and assertive behaviours. An overall behaviour score was therefore obtained for each of the three sets of data; initial, final and total scores. These behaviour scores were then used to rank the fish. Dominant fish (rank one) were defined as the fish with the highest behaviour scores, sub-dominants (rank two) had the second highest scores and subordinate fish (ranks three and four) exhibited the lowest behavioural scores.

*10.3.2 Physiological measurements*

At the end of the two-week experimental period, the four fish in each tank were caught simultaneously, using hand held nets, and killed within 60 s using a lethal dose of benzocaine (0.5 mg $ml^{-1}$). Final fork lengths and weights were recorded. Blood samples (0.5-1.0 ml) were withdrawn by caudal venipuncture, and after centrifuging (13,000 g), plasma samples were frozen in liquid nitrogen for later analysis of plasma cortisol concentrations using a commercial radioimmunoassy (ICN Pharmaceuticals Ltd). The liver from each fish was placed in a pre-weighed Eppendorf tube, weighed, frozen in liquid nitrogen and stored at -70 °C for later analysis of glycogen content using the anthrone method of Wedemeyer and Yasutake (1977).

*10.3.3 Statistical methods*

A total of five experimental and five control replicate trials were conducted, producing a sample size of 20 spate and 20 control fish for all subsequent analyses. Specific growth rates were calculated as % weight change per day (Ricker 1979). Linear regression analyses were used to assess relationships between physiological and behavioural data. One way analysis of variance (ANOVA) was used to determine whether statistically significant differences in physiological parameters occurred among the different ranks of fish within treatment groups. Except for comparisons of behaviour scores from the first and second week, overall ranks and scores were used in all analyses. The fiducial limit of significance in all analyses was 5%.

## 10.4 Results

### *10.4.1 Behaviour*

While few aggressive interactions were noted among the fish, the fish were observed to form dominance hierarchies, in that behaviour scores ranged from 3.89 to 0.05 for dominant fish, versus 0.05 to -1.09 for subordinate fish. There was no significant correlation between initial and final behaviour scores in either the control (Linear regression: $F_{1,18} = 1.997$, $r^2 = 0.100$, $P = 0.175$) or spate conditions (Linear regression: $F_{1,18} = 0.218$, $r^2 = 0.012$, $P = 0.646$) indicating that the hierarchies were not stable in either treatment from the first to the second week. However, the much lower $r^2$ values for the spate treatment in comparison to that for the control group suggest that the change was greatest in the spate conditions. In the control tanks, 60 % of fish that were dominant in the first week remained dominant in the second week, whereas only 40 % of fish remained dominant between the first and second week in the spate conditions. There was no significant difference in number of aggressive acts between control and experimental tanks (ANOVA: $F_{1,36} = 0.012$, $P = 0.914$).

### *10.4.2 Physiology*

At the end of the two week experimental period, there was a significant difference in specific growth rate among the overall ranks (generated from the total scores across the two weeks) of fish in the control treatments (ANOVA: $F_{3,16} = 3.686$, $P < 0.05$), with dominant fish (rank one) having a significantly higher growth rate than subordinate fish

the experiment; the remainder of the ranks lost weight (Fig. 10.1). However, there were no significant differences in specific growth rate among the ranks of fish in the spate conditions (ANOVA: $F_{3,16} = 0.523$, $P > 0.1$). Therefore, under constant environmental conditions, the dominant fish in the control tanks exhibited growth advantages, but there was no similar relationship between dominance and growth rate under spate conditions.

There was also a significant difference in hepatic glycogen content among the ranks of control fish (ANOVA: $F_{3,16} = 3.361$, $P = 0.045$; Fig. 10.2), with ranks one and four again being significantly different from each other. Dominant fish had the highest hepatic glycogen content, while the remaining three ranks of fish exhibited lower hepatic glycogen contents. No significant difference in hepatic glycogen content was apparent among the fish subjected to the spate treatment (ANOVA: $F_{3,16} = 0.336$, $P = 0.799$), nor was a relationship between dominance and hepatic glycogen content found.

No significant differences among the ranks of fish in either control or spate conditions existed for plasma cortisol concentrations (ANOVA: control: $F_{3,16} = 1.391$, $P = 0.282$; spate fish: $F_{3,16} = 0.58$, $P = 0.981$), or hepatosomatic indices (ANOVA: control: $F_{3,16} = 2.098$, $P = 0.141$; spate: $F_{3,16} = 0.586$, $P = 0.633$; data not shown). Cortisol concentrations for both groups were slightly higher than those typical of unstressed fish (5 $ngml^{-1}$; Pickering and Pottinger 1989) but at $13.52 \pm 4.72$ ng $ml^{-1}$ (mean $\pm$ S.E.M., $n = 40$), the concentrations were not greatly elevated.

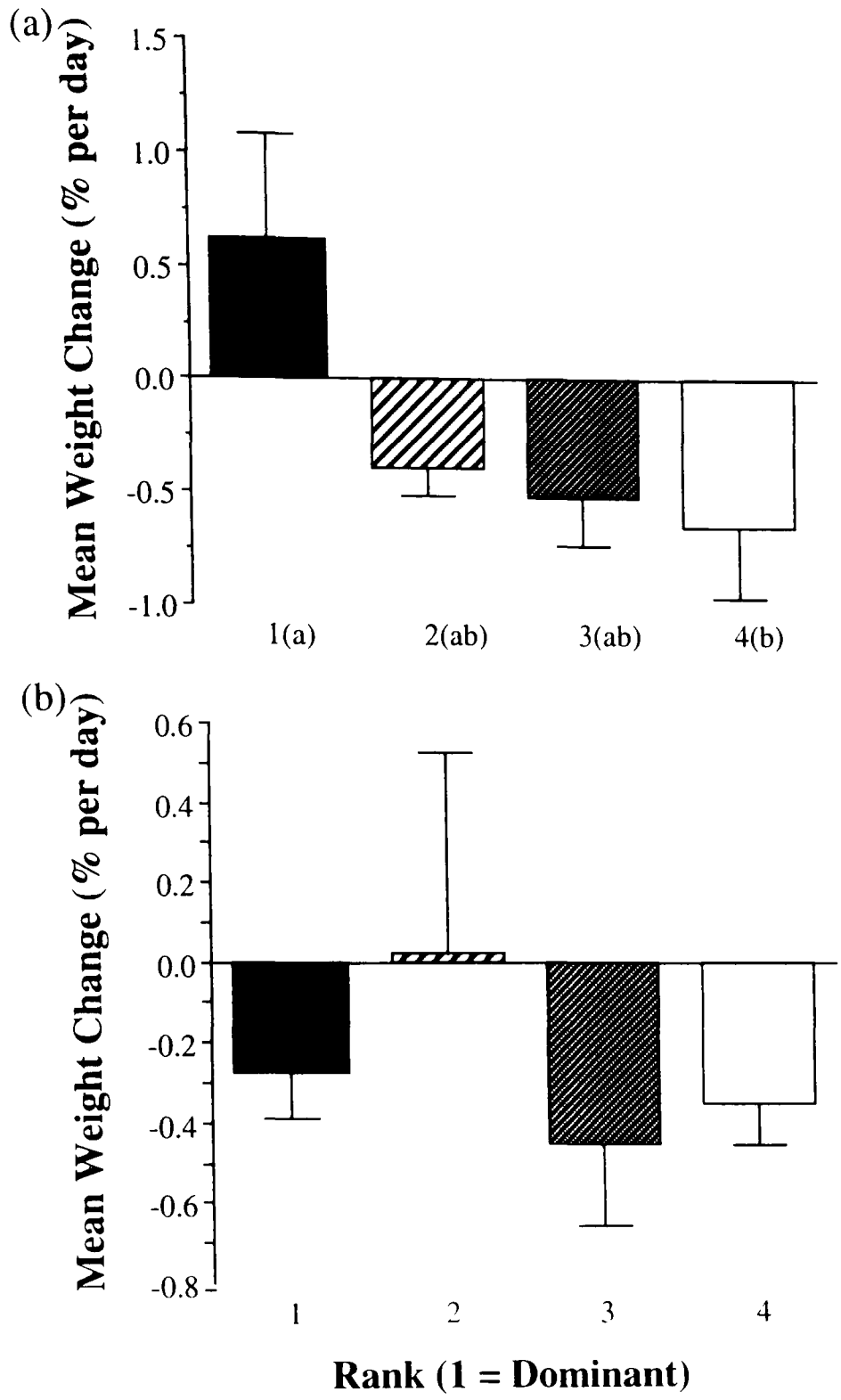



Figure 10.1: Specific growth rates (% change in weight per day) according to overall (2 week) dominance rank of fish for (a) control and (b) spate treatments. Data are presented as means ± S.E.M. (see text for statistical analysis).

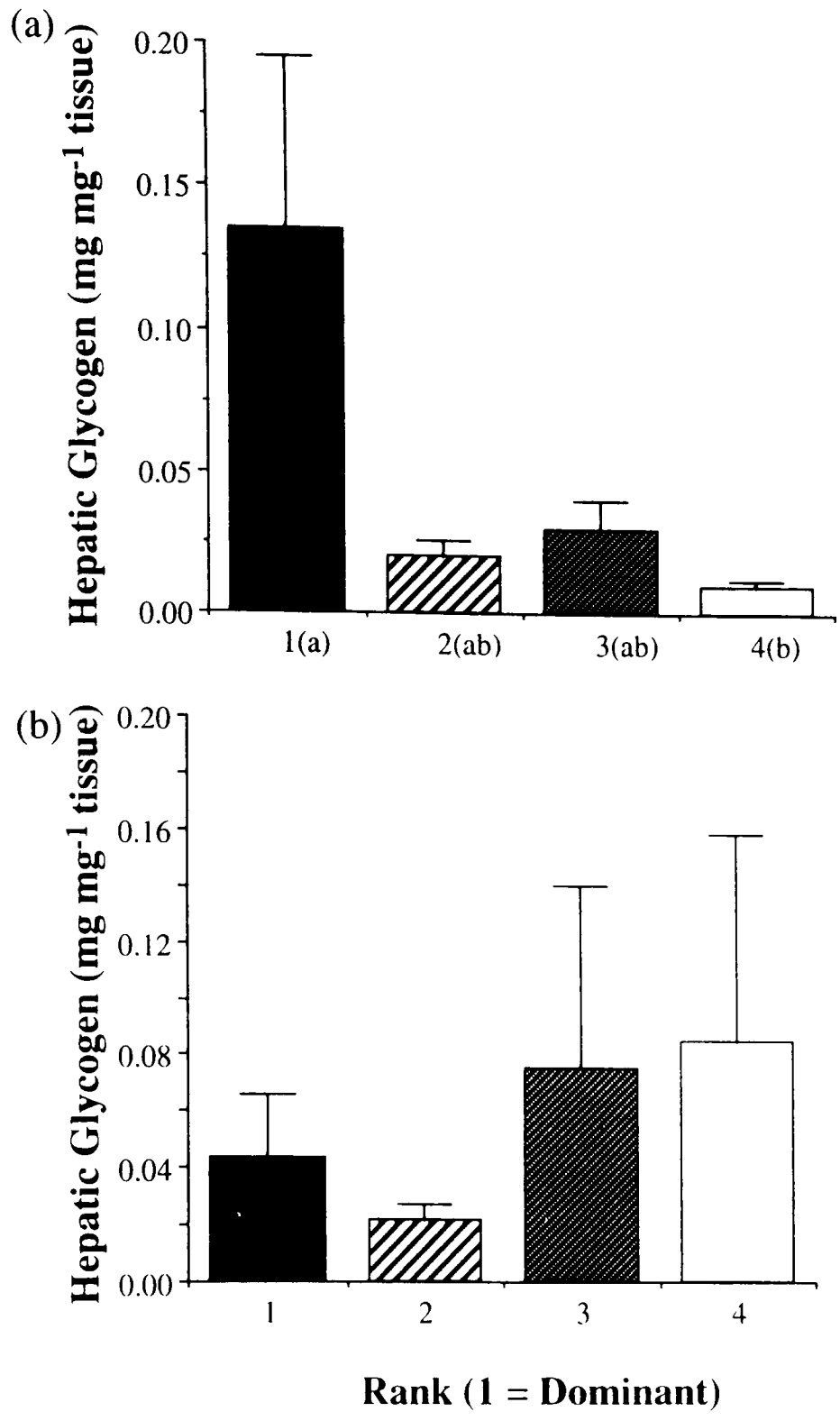



Figure 10.2: Hepatic glycogen content according to overall dominance rank of fish for (a) control and (b) drought treatments. Data are presented as in Fig. 10.1 (see text for statistical analysis).

## 10.5 Discussion

Under constant (semi-natural) conditions (control treatments), dominance hierarchies were formed among the four fish in a group and these social interactions had a significant effect on growth, with dominant fish exhibiting the highest specific growth rate. The significant effect of rank on growth rate under these conditions is a common finding (Li and Brocksen 1977; Sloman *et al.* 2000b (Chapter 8), d (Chapter 9)). Dominant fish generally occupy the most energetically-profitable positions in streams (Fausch 1984) and consequently tend to exhibit significantly higher growth rates. In addition, Abbott and Dill (1989) demonstrated that dominant fish grow faster than subordinate fish even when dominant and subordinate fish are fed equal rations of food.

Another indicator of the benefits enjoyed by dominant fish was illustrated by hepatic glycogen content, which was significantly affected by rank under constant conditions. Hepatic glycogen is believed to be influenced by stress, although the precise effect of stress on hepatic glycogen deposits is unclear (Pickering and Pottinger 1995). In some instances, hepatic glycogen content has been shown to decrease in response to elevated cortisol concentrations (indicative of stress) (e.g. Swallow and Fleming 1970; De la Higuera and Cardenas 1986), while in others it has increased (e.g. Barton *et al.* 1987; Andersen *et al.* 1991). It has been suggested that the glycogenolytic role of catecholamines, combined with an undetermined role of cortisol in carbohydrate metabolism, may account for the discrepancies in the effect of stress on hepatic glycogen

(Pickering and Pottinger 1995). In the present study, the dominant fish in the control tanks exhibited a high hepatic glycogen content. A high hepatic glycogen content would provide the fish with larger glycogen reserves and should therefore be considered advantageous. Therefore, under constant conditions the dominant fish had a combined physiological advantage of both higher growth rates and greater short-term energy reserves.

Despite the effect of rank on hepatic glycogen content, there was no effect of rank on hepatosomatic index (HSI) under constant conditions. Hepatosomatic index is also believed to be influenced by stress (Goede and Barton 1990). For example, Sloman *et al.* (2000d (Chapter 9)) found a significant effect of rank on HSI rather than hepatic glycogen. Clearly, both HSI and hepatic glycogen content have the potential to be affected by stress, but the expression of such effects appears to be quite variable.

In the present study, the hierarchies formed under constant conditions were not as stable as demonstrated in previous studies, (Sloman *et al.* 2000b (Chapter 8), d (Chapter 9)), with some dominant fish from the first week dropping in rank in the second week. However, 60 % of dominant fish in the first week remained dominant throughout the experimental period. While reasons for the instability were not clear, it was still evident that dominant hierarchies were formed, and that under constant conditions dominant fish gained advantages due to their social rank.

A relationship between growth and dominance was not apparent in groups of fish subjected to the environmental disturbance of spate conditions; there were no significant

differences in growth rate among the ranks of fish. It is likely that the increased water flow applied to the spate tanks disrupted the dominance hierarchy and consequently the physiological benefits of dominance found under constant conditions were lost. Similarly, Sloman *et al.* (2000d (Chapter 9)) demonstrated that a reduction in water level had a disruptive effect on established social hierarchies, removing the growth advantages that were apparent under constant conditions for dominant fish. No relationship existed in the spate conditions between rank of fish and hepatic glycogen, again indicating that the metabolic advantages displayed by dominant fish under constant conditions were lost.

Increased or fluctuating flow rates have been found to affect the behaviour of brown trout in the present and previous studies, but the present study is the first to indicate that these changes in behaviour may affect the physiology of the fish. Vehanen *et al.* (2000) found that higher water velocities displaced juvenile brown trout, particularly at low temperatures, although the fish did not change position rapidly as a result of altered water flow. Rate of aggressive interactions was also noted to decrease during periods of increased water flow (Vehanen *et al.* 2000). In the present study, no significant change in the number of aggressive acts occurred when the environmental perturbation was applied. The environmental perturbation of increased water flow would presumably increase the energy required by fish to maintain position in the water column, thereby reducing the amount of energy available for aggressive interactions and accounting for the reduction in aggression noted by Vehanen *et al.* (2000). Swimming-induced inhibition of aggressive interactions has also been noted in other studies (Adams *et al.* 1995). By contrast Sloman *et al.* (2000d (Chapter 9)) found that an increase in aggressive interactions occurring

among brown trout when water levels were lowered. Lowering of water levels would reduce the size of habitat available to the fish and result in competition for resources. Despite the lack of change in aggressive behaviour in the present study, the change in water flow clearly influenced the behaviour of the fish in terms of the social hierarchy, and these changes in turn, appeared to affect the physiology of the fish.

In conclusion, the present study highlights the effects of environmental perturbations on both the physiology and social behaviour of brown trout. The use of stream tanks to simulate the natural environment of salmonid fish has been widespread, but it is clear that such simulations need to take into account the effects of environmental perturbations. Many different environmental disturbances can occur within the natural stream environment, (e.g. changes in temperature, water levels, water flow rate, substrate), and these may also occur simultaneously. It is clear from the present study that the behaviour of salmonid fish will be affected by environmental perturbations, and that these behavioural changes can have physiological consequences.

# CHAPTER 11

## Chapter 11: General Conclusion

Salmonid fish are known to interact socially with each other and these social interactions and competitions for limited resources, such as food and shelter, can be physiologically stressful for the fish. The stress imposed by such social encounters among conspecifics has been termed 'social stress'. The effects of social stress can vary depending on the environment of the fish; the social stress occurring between paired fish in a laboratory tank is markedly different from that occurring in semi-natural environments. Whereas, the stress of social encounters between a pair of fish will be manifested totally in the submissive animal (the subordinate), among larger groups of salmonids dominance hierarchies may form with more than one subordinate fish (Newman 1956; Fausch 1984). The aims of the present study were to investigate further the effects of social stress by manipulating pairs of fish under laboratory conditions, in particular to look at the effects on metabolic rates (as estimated via measurements of rates of oxygen consumption), the stress response (the release of catecholamines and cortisol in response to further stimulation of the head kidney) and plasma cortisol concentrations over time. Also, the impact of the environment on the physiological effects of stress within hierarchies of salmonids was investigated in fish held under semi-natural conditions and exposed to environmental perturbations. In addition, the potential for chloride cell proliferation in response to stressors other than ion-deficient water was investigated. The results will be discussed here in terms of social stress between pairs of fish in artificial conditions, chloride cell proliferation, and social stress among groups of fish in semi-natural environments.

## 11.1 Social stress between pairs of fish

In an artificial environment, where two fish are confined as a pair, it has been demonstrated that one fish will become dominant over the other, subordinate, fish (Peters *et al.* 1980; Laidley and Leatherland 1988; Pottinger and Pickering 1992). Subordinate fish characteristically show an increase in circulating concentrations of the stress hormone, cortisol, in the blood plasma. Following the confinement of rainbow trout in pairs, Øverli *et al.* (1999) demonstrated that blood cortisol concentrations increased in both dominant and subordinate fish after aggressive interactions occurred. After three hours, although the cortisol concentrations of the dominant fish had returned to basal levels, the cortisol concentrations in subordinate fish remained elevated. Chapter 2 demonstrated that in both rainbow and brown trout, aggressive interactions occurred when fish were paired with size-matched conspecifics. Plasma cortisol concentrations were significantly elevated in the subordinate fish, compared with the dominant fish, where cortisol levels were low and typical of unstressed fish, and cortisol remained elevated in the subordinate from four hours after confinement in pairs for a period of one week. The continuous elevation of circulating plasma cortisol concentrations in the subordinate fish is characteristic of exposure to a chronic stressor and suggests that under these artificial conditions no alleviation from social stress occurs. Similarily, Laidley and Leatherland (1988) noted elevated cortisol concentrations in subordinate rainbow trout after two weeks and Pottinger and Pickering (1992) found elevated concentrations even after six weeks of confinement.

The factors determining the rank in a social hierarchy that an individual fish achieves are not well understood. It has been suggested that size may not be a good indicator of dominance, as physiological condition can play an important role (Beaugrand *et al.* 1996). Previous research into the mechanisms that result in fish becoming subordinate have found that, in juvenile Atlantic salmon, high standard metabolic rate (SMR) is a possible indicator of dominance (Metcalfe *et al.* 1995). However, until the present study, it was not known whether cortisol, so characteristically elevated in subordinate fish as a result of social stress, played a role in the actual determination of social status. Higher plasma cortisol concentrations were seen in subordinate fish, than in their dominant partners before confinement in pairs (Chapter 2), suggesting that elevated cortisol may indeed be a predictor of subordinance. However, it is not known whether the elevated cortisol concentrations seen in the subordinate fish were themselves responsible for determining social status, or whether they were indicative of poor physiological condition or previous social experience.

Following confinement in pairs, other physiological changes (secondary and tertiary responses) may be associated with the elevation of plasma cortisol (primary response) in subordinate fish. Pottinger and Pickering (1992) demonstrated that subordinate rainbow trout show a reduction in weight and condition. Subordinate fish have also been shown to have decreased disease resistance and increased blood plasma glucose levels (Peters *et al.* 1988). In the present study other secondary and tertiary responses were examined

including SMR, the ability to respond to additional stressors and chloride cell proliferation.

Although standard metabolic rate (SMR) has been examined previously as an indicator of dominance (Metcalfe *et al.* 1995), the present study demonstrated that changes in the physiology of subordinate fish after confinement also include an increase in SMR (Chapter 4). Furthermore, the change in SMR was found to be correlated to the behaviour scores of the pairs of fish, *i.e.* the greater the aggression of the dominant fish, the greater the effect on the SMR of the subordinate. Therefore, it is believed that not only does social stress have physiological consequences for the subordinate fish but that the significance of these consequences is dependent upon the polarity of the pair (the more pronounced the dominant-subordinate relationship, the greater the stress response in the subordinate).

Another physiological consequence of social stress for the subordinate fish demonstrated in the present study is the reduced ability to respond to further stressors (Chapter 7). Stimulation of the head kidney tissue in an *in situ* perfused head kidney preparation with acetylcholine (to stimulate adrenaline and nor-adrenaline release), and ACTH (to stimulate cortisol release), had differential effects in subordinate versus dominant fish. In subordinate fish, cortisol secretion was significantly lower than in dominant fish, although catecholamine secretion did not differ between dominants and subordinates. It is concluded that the elevated cortisol concentrations so characteristic of subordinate fish result in the stimulation of negative feedback mechanisms that decrease the ability of subordinates to further release cortisol. The results of other workers suggest that the elevated cortisol levels are also likely to promote catecholamine storage and release,

counteracting any desensitisation caused by repeated stimulation of cholinergic receptors elicited by the continuous presence of a dominant fish (Reid *et al.* 1996). Therefore, the catecholamine response of the subordinate fish remains similar to that of the dominant fish.

The present study also considered the possibility that social stress may induce chloride cell proliferation. In general, acclimation to ion-deficient water is associated with proliferation of chloride cells in the gill epithelium. During the acclimation period, a transient increase in plasma cortisol has also been noted (Perry and Laurent 1989), and injection of cortisol at supraphysiological concentrations has been demonstrated to elicit chloride cell proliferation (Bindon *et al.* 1994a). Therefore, the possibility that chloride cell proliferation is related to circulating cortisol concentrations has been proposed. In an ion-deficient environment, the increase in ion transport efficiency mediated by the increase in chloride cells is beneficial to the fish, but is also associated with a thickening of the gill epithelia, resulting in decreased respiratory efficiency. If chloride cell proliferation were to occur in response to elevated cortisol concentrations in the absence of an ionoregulatory challenge, any increase in cell numbers would be likely to be detrimental to the fish. It was demonstrated in the present study that proliferation of chloride cells was not induced by elevated cortisol concentrations resulting from social stress (Chapter 3). Although elevation of plasma cortisol was noted in the subordinate of paired rainbow trout, there was no significant difference between the chloride cell densities of dominant and subordinate trout.

There are questions that have arisen from the present study, concerning the physiological effects of the social stress experienced by paired fish, that need to be addressed in the future to further our understanding of social interactions in these animals. As standard metabolic rate of the subordinate fish is affected by social stress it could be hypothesised that performance in a swim respirometer would also be dramatically affected. It would also be interesting to investigate the causes of subordination further. Manipulation of cortisol concentrations could be used to determine whether fish with artificially elevated cortisol concentrations are predisposed to subordination.

## 11.2 Chloride Cell Proliferation

To explore the relationship between chloride cell proliferation and cortisol further, the effect of an acute stress, in the absence of an ionoregulatory stressor, was investigated. However, as for the chronic social stress (Chapter 3), the acute stress of air emersion (Chapter 5) did not induce chloride cell proliferation, even though elevated cortisol concentrations were produced. Therefore, it appears that proliferation of chloride cells induced solely by administration of cortisol occurs only at supraphysiological concentrations. Under these circumstances, it would appear that the transient peak in plasma cortisol seen in fish during acclimation to soft water (Perry and Laurent 1989) may not be directly related to chloride cell proliferation. The present study has demonstrated that elevations in cortisol concentrations alone are not enough to cause chloride cell proliferation. It has also been demonstrated that an ionoregulatory challenge by itself, achieved by blocking cortisol receptors, appears to be insufficient to elicit proliferation of

chloride cells. Therefore, there remains the possibility that the elevation of plasma cortisol concentrations combined with an ionoregulatory challenge results in chloride cell proliferation.

The significance of the transient cortisol peak seen by Perry and Laurent (1989) was investigated further by using the glucocorticoid blocker, RU486 (Vijayan and Leatherland 1992) and the mineralocorticoid blocker, spironolactone (Verrey *et al.* 1987), to block the actions of cortisol, and by carrying out a time course study of plasma cortisol concentrations during acclimation to soft water. It was hypothesised that if cortisol was responsible for proliferation of chloride cells, then the acclimation to soft water of fish implanted with RU486 and/or spironolactone should not elicit chloride cell proliferation in the gill epithelia. It was found that, whereas RU486 did not block chloride cell proliferation, implantation of spironolactone did inhibit proliferation of chloride cells during acclimation to soft water (Chapter 6). The ability of cortisol to act as a mineralocorticoid hormone in fish has long been recognised (Madsen 1990a) and it has recently been suggested that fish possess mineralocorticoid-like receptors, that have the ability to bind with cortisol (Colombe *et al.* 2000). The results of the present study support the hypothesis that cortisol acts as a mineralocorticoid in fish and it is tentatively hypothesised that, during ionoregulatory stress, up-regulation of mineralocorticoid receptors allows the glucocorticosteroid and mineralocorticosteroid functions of cortisol to be distinguished.

Clearly, further experiments are needed to confirm the presence of two types of corticoid receptor in fish. Although mineralocortcoid-like receptors have been cloned in rainbow trout, they were cloned from the testis. Cloning of these receptors from other tissues, in particular the gill tissue, would help to confirm these results. In addition, the actions of spironolactone need to be considered in more detail to confirm that it has similar actions in teleosts as in mammals. It would also be interesting to consider the possibility that an unidentified mineralocorticoid hormone exists in fish. The effects of spironolactone and RU486 on the $Na^+/K^+$-ATPase activity in the gills, kidney and gut of rainbow trout are currently under investigation.

### 11.3 Social stress in semi-natural environments

Within artificial environments, the difference in the physiology of dominant and subordinate fish is quite profound but within dominance hierarchies formed under semi-natural conditions, physiological differences appear much less extreme. Semi-natural environments, most usually stream tanks, have been widely used in the study of fish behaviour, in particular in studying salmonid dominance hierarchies (Kalleberg 1958; Mason and Chapman 1965; Li and Brocksen 1977). Behavioural studies have shown that, among groups of salmonid fish, linear dominance hierarchies form. Dominant fish will generally acquire the most profitable position in the stream in terms of energy resources

(Fausch 1984) and therefore display the highest growth rates (Chapters 8, 9 and 10). In artificial conditions, subordinate fish characteristically demonstrate elevated cortisol concentrations. However, within semi-natural environments there is generally no difference in cortisol concentrations among ranks of fish (Chapters 8, 9 and 10).

Metcalfe (1986) demonstrated that under certain circumstances subdominant (second-ranking) fish may exhibit lower growth rates than other subordinate fish. The experiment described in chapter 8 also produced the result that subdominant fish displayed the lowest growth rates. It is believed that a low growth rate in subdominant fish may occur because they adopt a high cost/ high return strategy. Subdominant fish expend a large amount of energy competing with the dominant fish for the best food resources, but are out-competed by the dominant fish and hence experience an energy deficit. However, subordinate fish adopting a low cost/ low return strategy do not expend as much energy competing for resources and so exhibit a higher growth rate. Chapter 8 also reported that subdominant fish displayed significantly higher chloride cell densities in this experiment. It is not clear why higher chloride cell densities occured in the subdominant fish, as work on social stress in paired fish was not consistent with this finding (Chapter 3). However, as the factors eliciting chloride cell proliferation in the gill epithelia appear more complex than previously hypothesised (Chapter 6), it is not impossible that the high chloride cell densities recorded in the subdominant fish are a physiological change induced by the dominance hierarchy.

The majority of work using semi-natural environments to study the behaviour and physiology of salmonid fish has concentrated on the use of constant environmental conditions (Kalleberg 1958, Noakes and Leatherland 1977; Huntingford and Garcia de Leaniz 1997). Although the stream tank conditions have been designed to mimic the natural environment of the fish as much as possible, the environmental perturbations encountered by the fish such as drought, spates, hypoxia, have been excluded. It was demonstrated in chapters 9 and 10 that these environmental perturbations can have profound effects on both the behaviour and physiology of the fish. Chapter 9 demonstrated that lowered water levels, simulating drought, affected previously established hierarchies. Drought is an environmental perturbation that is periodically encountered by fish in their natural habitat (Elliott 1987; Titus and Mosegaard 1989). The imposition of lowered water levels disrupted the hierarchy and appeared to cause an increase in aggressive interactions among the fish. In control tanks, in the absence of the environmental perturbation, growth rate was highest in the dominant fish. However, after the reduction of water levels in the experimental treatments, there was no significant difference in growth rates between the ranks of fish and the physiological advantage displayed by the dominant fish in the control tanks was lost.

A similar result was demonstrated in chapter 10 using the environmental perturbation of increased water flow to simulate spates. Although no increase in aggressive interaction was seen among the fish after the application of increased water flow, there appeared to be a change in hierarchy structure. The lack of increased aggression was probably due to displacement of the fish and an increased requirement of energy to maintain position in the

water column (Adams *et al.* 1995). Therefore, energy would have been diverted from aggressive interactions to swimming. Again, whereas in the control tanks (where water velocity remained constant) the dominant fish exhibited the highest growth rate, in the experimental treatments there was no significant difference in growth rates among the ranks of fish. It was therefore concluded that hierarchies formed under constant semi-natural conditions may not be truly representative of those formed in the natural environment, due to the absence of environmental perturbations which have a profound affect on both the behaviour and physiology of the fish.

In conclusion, the subordinate of paired salmonid fish held under artificial conditions will characteristically display severe physiological changes resulting in a decrease in condition and health. These include increases in blood plasma cortisol, SMR, blood plasma glucose, decreases in weight, condition, disease resistance and in the ability to respond to further stress and are all characteristic responses to a chronic stress. Subordinates within groups of salmonid fish held in semi-natural conditions do not display such extreme physiological disadvantages, although the dominant fish will generally have the highest growth rate. However, the effects of environmental perturbations on both the physiology and behaviour of salmonid fish suggest that results from experiments carried out under constant semi-natural conditions should be interpreted with caution. The present study has also shed some light on the mechanisms involved in proliferation of the gill epithelial chloride cells. It is suggested that the existence of both glucocorticoid and mineralocorticoid receptors allows the fish to respond differently to different types of stressors and eliminate ionoregulatory changes from the stress associated with social interaction and air emersion.

This study has enhanced our understanding of the role of cortisol in both the determination and consequences of social stress and also in the proliferation of chloride cells in the gill epithelia of salmonid fish.

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