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Physiological Causes and Consequences of Social Status in Rainbow Trout (*Oncorhynchus mykiss*)

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**PHYSIOLOGICAL CAUSES AND CONSEQUENCES OF SOCIAL STATUS IN
RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)**

By

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
in partial fulfillment of the requirements for the
M.Sc. degree in Biology
Ottawa-Carleton Institute of Biology

Thèse soumise à
Faculté des Études Supérieures et Postdoctorales
Université d'Ottawa
en vue de l'obtention de la
Maîtrise en Biologie
L'Institut de Biologie d'Ottawa-Carleton

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395 Wellington Street
Ottawa ON K1A 0N4
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395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

ISBN: 0-494-11256-5

Our file Notre référence

ISBN: 0-494-11256-5

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Master of Science (2005)
Biology

University of Ottawa
Université d'Ottawa

Title:

Physiological causes and consequences of social status in rainbow trout (*Oncorhynchus mykiss*)

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Acknowledgement

There are a number of people I would like to thank for their help throughout my stay in Ottawa. Thank you J. Thomas, M. Bell, and C. Szebedinszky for all your expert help with fish sampling, C. Doyon for teaching me the brain dissections, and J. Kulczycki for processing all the brain samples. I also want to thank Haude Levesque for showing me the proper procedure of countless numbers of biochemical assays. Comments received from Dr. Hymie Anisman and Dr. Tom Moon were also greatly appreciated and significantly improved the quality of this work. Finally, sincere thanks are extended to my family and friends for their patience as well as Dr. Katie Gilmour for her excellent counsel. Dr. Gilmour's approach taught me more than I could ever imagine learning in such a brief period, and gave me the freedom and motivation to pursue opportunities I wouldn't have otherwise had the courage to. Thanks to all.

Funding

This work was supported by NSERC of Canada research and equipment grants, as well as PREA (Ontario) funding for graduate student support to Dr. Kathleen Gilmour. Brain work was supported by Dr. Hymie Anisman, the holder of the Canada Research Chair in Neuroscience, through CIHR funding. Finally, metabolic and biochemical enzyme assays were partially supported by NSERC of Canada research and equipment grants awarded to Dr. Tom Moon.

**PHYSIOLOGICAL CAUSES AND CONSEQUENCES OF SOCIAL STATUS IN
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Abstract

A number of social and behavioural traits inherent to the individual are known to influence the outcome of social interactions in salmonid fish. In addition, recent work has raised the possibility that the prior physiological condition of a fish may predetermine its social status. Therefore, the hypothesis that elevated plasma cortisol levels influence social rank was tested, with the prediction that rainbow trout (*Oncorhynchus mykiss*) treated with cortisol would be relegated to subordinate social status in pairwise contests with an untreated conspecific. Experimental elevation of plasma cortisol significantly increased the probability that the treated fish within each pair became subordinate, an effect later identified as cortisol specific, as it was abolished by simultaneous treatment with the glucocorticoid receptor antagonist, RU486. One possible mechanism through which cortisol could exert this effect was subsequently investigated, namely that cortisol influenced social status through behavioural modifications mediated by changes in brain monoaminergic activity. Fish held in groups and given intraperitoneal implants of cortisol exhibited significant changes in brain serotonergic (5-HT) and dopaminergic (DA) activity relative to controls, suggesting a role for cortisol modulating brain monoaminergic activity.

Subsequently, brain 5-HT was experimentally elevated by feeding rainbow trout 5-HT-enriched food. However, there appeared to be no effect of 5-HT-enriched food on the outcome of social interactions between treated fish and untreated trout. Further, a number of methods were employed to raise DA activity in trout prior to social interactions. Again, however, none of the techniques used appeared to predispose a fish to dominant social status consistently, although it was unclear from the results whether

manipulation of brain monoaminergic activity was ineffective in influencing social status, or whether the delivery methods employed were ineffective in modifying brain monoaminergic activity.

Finally, one consequence of social status, specifically the characteristic low growth rates of subordinate fish and the role of cortisol in mediating this effect was investigated. Low growth rates in socially-subordinate trout reflect at least in part exclusion from food sources by dominant fish. In addition, however, the hypothesis that social status and/or cortisol affected digestive function, intermediary metabolism, or enzyme activity in rainbow trout was tested. If this were the case, the expectation was that ultimately growth would be impacted independent of competition. The findings suggested that digestive function in socially-subordinate rainbow trout was impaired, but that the negative impact of social subordination reflected in large part a lack of feeding. In addition, gluconeogenic and glycolytic enzyme activities were significantly affected by social status, and were correlated with plasma cortisol concentrations. Further, social status-induced enzymatic differences were eliminated by administration of the glucocorticoid receptor antagonist RU486, underlining a role for circulating cortisol in eliciting the differences. Therefore, this work identified factors, beyond exclusion from food, as playing a role in growth depression in subordinate rainbow trout.

Résumé

Il est reconnu que plusieurs facteurs sociaux et inhérent influencent le résultat d'interactions sociale des salmonidés. En plus, des œuvres plus récents suggèrent la possibilité que la condition physiologique précédente du poisson peut déterminer son statut social. Alors, en traitant la truite arc-en-ciel (*Oncorhynchus mykiss*) avec du cortisol et en leur permettant d'interagir avec des poissons non traités dans un environnement contrôlé, nous avons testé l'hypothèse que les niveaux élevés de plasma cortisol influencent le rang social, avec la prédiction que le traitement avec le cortisol devrait prédisposer un poisson à un statut social subordonné. Nous avons trouvé que l'élévation expérimentale du plasma cortisol a augmenté d'une façon significative la probabilité que les poissons traités dans chaque paire deviennent subordonnés, un effet que nous avons identifié ensuite comme spécifique au cortisol, puisqu'il était abolit par le traitement simultané avec le récepteur antagoniste glucocorticoïde, RU486. Un mécanisme possible pour les effets cortisol a été testé, simplement que le cortisol influence le statut social par des modifications d'agissements médié par les changements dans l'activité monoaminergique du cerveau. Des poissons dans des groupes ont été implantés de façon intrapéritonale avec du cortisol et ils ont exhibé des changements significatifs dans leur sérotonine (5-HT) cerveau et les niveaux de dopamine (DA) relatif aux contrôles, nous suggérant un rôle pour le cortisol dans les changements de monoaminergique du cerveau.

Subséquemment, le 5-HT du cerveau a été élevé de façon expérimentale dans des truites arc en ciel en leur donnant de la nourriture enrichie de 5-HT. Par contre, aucun effet a été observé dans les poissons traités suite à leurs interactions sociales

avec des poissons non traités. En plus, plusieurs méthodes ont été utilisées afin d'augmenter l'activité DA dans les poissons qui interagissent de façon sociale. Encore, aucune des techniques utilisées ne semble prédisposer un poisson à un statut social dominant de façon constante. Par contre, les résultats ne sont pas clairs si la manipulation de l'activité monoaminergique du cerveau est inefficace à influencer le statut social, ou si les méthodes de livraison utilisées sont inefficaces à modifier l'activité monoaminergique du cerveau.

Finalement, une conséquence d'un statut social, spécifiquement le bas taux de croissance des poissons subordonnés et le rôle du cortisol à médier ces effets, a été investigué. Les taux bas de croissance dans les truites arc en ciel subordonnées reflètent en parti leur exclusion de la nourriture par les poissons dominants. En plus, nous avons testé l'hypothèse que le statut social et/ou le cortisol avait un effet sur la fonction digestive, sur le métabolisme intermédiaire, ou sur l'activité enzymatique de la truite arc en ciel testée. Si cela est le cas, notre attente était que cela devrait avoir un impact sur leur croissance. Nos découvertes suggèrent que la fonction digestive des truites arc en ciel subordonnées est diminuée, mais que l'impact négatif de statut subordonné est plutôt un reflet du manque de nourriture. En plus, l'activité des enzymes glycolytiques et gluconéogenèse a été affectée de façon significative par le statut social et donc cela se corrèle avec les concentrations de plasma cortisol. Aussi, les différences enzymatiques induites par le statut social ont été éliminées par l'administration du RU486, qui suggère un rôle pour le cortisol circulant en élicitant des différences. Alors, cet oeuvre identifie des facteurs autre que l'exclusion de nourriture qui jouent un rôle dans la dépression de la croissance des subordonnés.

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Abbreviations

ACTH, adrenocorticotrophic hormone
ALT, alanine aminotransferase
AST, aspartate aminotransferase
CF, condition factor
CRF, corticotropin-releasing factor
DA, dopamine
DOPAC, 3,4-dihydroxyphenylacetic acid
GDH, glutamate dehydrogenase
GR, glucocorticoid receptors
5-HIAA, 5-hydroxyindolacetic acid
5-HT, serotonin
H_a, alternate hypothesis
H₀, null hypothesis
HPI, hypothalamic-pituitary-interrenal
HPLC, high performance liquid chromatography
HR, high responsive
HSI, hepatosomatic index
i.p., intraperitoneal
ISI, intestinal somatic index
K_D, receptor binding affinity
L-dopa, L-3,4-dihydroxyphenylalanine methyl ester hydrochloride
LR, low responsive
MR, mineralocorticoid receptor
NPY, neuropeptide Y
PCA, principal components analysis
PEPCK, phosphoenolpyruvate carboxykinase
PK, pyruvate kinase
PVC, polyvinyl chloride
RIA, radioimmunoassay
RU486, mifepristone
SGR, specific growth rate
SMR, standard metabolic rate
TRP, L-tryptophan

CHAPTER 1

GENERAL INTRODUCTION

Many of the concepts discussed in Chapter 1 are presented in a review paper accepted into the journal of *Integrative and Comparative Biology*, Gilmour et al. (2005).

Social Interactions in Salmonid Fish

Dominance hierarchies readily form in many species that live in social groups or that are confined together, especially in cases when resources are limited. Schejelderup-Ebbe (1922) first introduced the concept of a dominance hierarchy, based on observations made within groups of domestic fowl, and these “peck-order” formations have since been identified in a number of vertebrate species. For example, salmonid fish, such as rainbow trout (*Oncorhynchus mykiss*), form linear, dominance-based, social hierarchies both in the wild and in the laboratory (Noakes and Leatherland, 1977; Bachman, 1984; Abbott and Dill, 1989). These hierarchies appear to be established through agonistic interactions, with each fish being ranked according to its relative ability to out-compete all other members within a group (Adams et al., 1998). Typically, “subordinate” fish are excluded from preferential access to food and shelter (McCarthy et al., 1992), as dominants tend to monopolise available resources. Behavioural correlates of subordinate social status include decreases in feeding, locomotion, and aggression (commonly referred to as “behavioural inhibition”; (Winberg and Nilsson, 1993a; Winberg et al., 1997a; Øverli et al., 1998)), as well as the selection of positions within the environment not occupied by other fish. Given that these hierarchies are formed and/or maintained through aggressive interactions, subordinate individuals are also subjected to chronic social stress; initially due to losing aggressive encounters, but later due to the general lack of control in addition to the constant threat of attack by individuals higher in social rank (Noakes and Leatherland, 1977; Winberg and Lepage, 1998). Therefore, based on the extensive inter-individual variation in behaviour and competitive abilities of fish within these social orders, interest has focused on the behavioural aspects (*i.e.* aggression,

feeding) with evidence (reviewed by Winberg and Nilsson, 1993a) pointing towards changes in brain monoaminergic activity as an explanation for behavioural differences between fish of high and low social status. However, less attention has been paid to the physiological changes that accompany low social positions, as well as to the potential for the prior physiological condition of an individual fish to influence the outcome of social interactions. Therefore, this chapter will provide a brief overview of physiological consequences associated with low social status and the potential role of cortisol in eliciting these effects, followed by a discussion of potential contributing factors to determining social status.

A low position in a dominance hierarchy carries with it a number of physiological consequences, all capable of impacting the competitive ability of an individual fish. Subordinate fish exhibit significantly lower hepatosomatic index values (HSI = liver mass/body mass) (Sloman et al., 2001b), liver glycogen content (Ejike and Schreck, 1980), and condition factors (an index of the extent to which the total weight of a fish is high for its length) (Sloman et al., 2000a; Sloman et al., 2000b) than dominant fish; with all these factors being used as significant predictors of an individual's energy reserves (Chellappa et al., 1995). Subordinates also experience significant increases in standard metabolic rate (SMR = the minimum metabolic rate to sustain life) (Sloman et al., 2000c), which together with reduced food intake (Metcalf et al., 1989; Adams and Huntinford, 1996; Adams et al., 1998), result in decreased growth rates (Barton et al., 1987; Metcalfe et al., 1989; Winberg et al., 1992; Metcalfe et al., 1995). Finally, immuno-suppression (Peters et al., 1988; Pottinger and Pickering, 1992) and ultimately

increased mortality (Pickering and Duston, 1983; Pickering, 1993) are typical of socially subordinate fish.

In general, animals react to a stressor, social or otherwise, with a series of endocrine responses involving the catecholamine hormones adrenaline and noradrenaline in the case of acute stress and the corticosteroid stress hormones for both acute and chronic stressors (reviewed by Wendelaar Bonga, 1997; Wingfield and Ramenofsky, 1999; Barton, 2002). Subordinate fish are characterized as having plasma cortisol concentrations higher than those of dominant fish (Pottinger and Pickering, 1992; Øverli et al., 1999; Sloman et al., 2000a; Sloman et al., 2001a; Sloman et al., 2002). These marked elevations in plasma cortisol, which is the principal corticosteroid hormone in teleost fish (Mommsen et al., 1999; Barton, 2002), are thought to contribute to the poor overall physiological condition of subordinates. Cortisol secretion is regulated by the hypothalamic-pituitary-interrenal (HPI) axis (the teleost homologue of the mammalian hypothalamic-pituitary-adrenal axis): the hypothalamic neuropeptide corticotropin-releasing factor (CRF) acts directly on distinct cell clusters in the pars distalis of the pituitary gland to trigger the release of adrenocorticotrophic hormone (ACTH) (Olivereau and Olivereau, 1988; Fryer, 1989). As a result of this surge in ACTH acting directly on interrenal cells, cortisol is released into the bloodstream (Wendelaar Bonga, 1997; Mommsen et al., 1999). Secretion via this pathway is modulated by the actions of cortisol itself (*i.e.* negative feedback) at both the level of the hypothalamus and the pituitary (Mommsen et al., 1999). An acute elevation in plasma cortisol is generally considered to be adaptive, because the secretion of this hormone from the interrenal tissue causes the mobilization of energy reserves (Vijayan et al., 1991) and helps the

organism cope with the increased energy demands associated with stress (Vijayan and Moon, 1992; Bamberger et al., 1996). On the other hand, chronic elevations of plasma cortisol concentrations in response to sustained stress, as is the case with social stress (Fox et al., 1997; Sloman et al., 2001a; Corrêa et al., 2003), can compromise specific physiological response mechanisms and become detrimental to the fish's overall health (Barton, 2002).

Cortisol levels have been manipulated experimentally either using intraperitoneal implants that slowly release cortisol into the circulation, or by feeding fish cortisol-spiked food (reviewed by Gamperl et al., 1994; Gilmour et al., 2005). In either case, cortisol treatment results in a number of physiological effects which include: appetite suppression (Barton et al., 1987; Gregory and Wood, 1999), mobilisation of energy reserves as evidenced by increased plasma glucose concentrations (Barton et al., 1987; Morgan and Iwama, 1996; De Boeck et al., 2001) and reduced hepatic glycogen levels (Barton et al., 1987), morphological alterations to the digestive tract (Barton et al., 1987), reduced food conversion efficiency (Gregory and Wood, 1999), decreased condition factors (Barton et al., 1987; Gregory and Wood, 1999), increased metabolic rate (Morgan and Iwama, 1996; De Boeck et al., 2001), and a reduction in growth (Barton et al., 1987; Gregory and Wood, 1999; De Boeck et al., 2001). Increased mortality also accompanies cortisol treatment (Pickering and Pottinger, 1989; Gregory and Wood, 1999) and presumably is a function of the changes in immune cell numbers (Barton et al., 1987), which enhance the susceptibility to both bacterial infection and fungal diseases (Pickering and Pottinger, 1989). In short, chronic elevation of circulating cortisol levels associated with low social status likely contribute significantly to the physiological decline of subordinate fish.

To gain a better understanding of the physiological consequences associated with low social status and the role that cortisol plays, an experiment was conducted in which the characteristic growth suppression of subordinate fish (Metcalf, 1986; Abbott and Dill, 1989; Pottinger and Pickering, 1992; Ryer and Olla, 1996; Sloman et al., 2000b; Sloman et al., 2000c; Sloman et al., 2001b) was investigated. Even in cases where equal rations are consumed, subordinate fish do not achieve the growth rates of dominants (Abbott and Dill, 1989), indicating that factors beyond exclusion from food affect the growth of subordinates. Poor digestive function and inefficient nutrient assimilation probably contribute to the lower growth rates of subordinate fish (Olsen and Ringø, 1999; Stevens and Devlin, 2000; Earley et al., 2004), but changes in the overall metabolic capacity of the fish may also impact growth and have been less well documented. A recent study in brown trout, *Salmo trutta*, demonstrated that confinement with a conspecific resulted in an increase in the SMR of fish which became subordinate (Sloman et al., 2000c), a finding which suggests that a metabolic disadvantage is associated with subordination. To date, however, whether these differences in SMR reflect changes in enzymatic activity associated with the major metabolic pathways has not been examined. Although research on the effects of acute and/or confinement stress on intermediary metabolism (*e.g.* Vijayan et al., 1997) exists; little is known about the impact of the specific stress of social subordination on these enzyme systems. Therefore, in the interest of generating a working model to further explain the observed growth suppression of subordinate fish, an experiment was designed to test the hypothesis that subordinate social status has an impact on the digestive capacity and activity level of key enzymes involved in gluconeogenesis, glycolysis, and protein metabolism. Given the relationship

between experimentally elevated cortisol concentrations and growth suppression (Barton et al., 1987; Gregory and Wood, 1999; De Boeck et al., 2001), in addition to the presence of glucocorticoid receptors (GR) in a wide range of teleost tissues (see Mommsen et al., 1999), the role of increased plasma cortisol concentrations in subordinate growth suppression was also investigated by blocking its effects with a GR antagonist (*i.e.* RU486).

In animal contests, asymmetries between the opponents, both social (*e.g.*, prior residence, prior winning/losing experience) and/or inherent (*e.g.*, size, aggressiveness), are known to influence the outcome of social interactions (Abbott and Dill, 1985; Huntingford and Turner, 1987; Nakano, 1995; Rhodes and Quinn, 1998; Leiser et al., 2004). For example, dominance rank is often positively associated with large body size (Abbott and Dill, 1985; Nakano, 1995; Leiser et al., 2004) and level of aggression (Huntingford and Turner, 1987; Rhodes and Quinn, 1998), and relative differences in these attributes between competitors predict their relative rank within dominance hierarchies. Previous work in salmonids has also raised the possibility that the prior physiological condition of a fish, which presumably impacts an individual's initial success during competitive interactions, may predetermine its social status (Johnsson and Björnsson, 1994; Yamamoto et al., 1998; Gregory and Wood, 1999; Sloman et al., 2001a). At least three separate lines of evidence support these claims. First, salmonids with higher metabolic rates prior to social interactions tend to attain higher social status (Metcalf et al., 1995; Yamamoto et al., 1998; McCarthy, 2001). However, this effect is most likely mediated by the high levels of aggression also seen in these fish, as Cutts et al. (1998) documented significant correlations between high metabolic rate and

aggression in Atlantic salmon. Second, growth hormone treatment significantly increased the likelihood of occupying a dominant social position within pairs of juvenile rainbow trout (Johnsson and Björnsson, 1994), although Jönsson et al. (1998) suggested that growth hormone may have affected dominance by increasing feeding motivation, which could have, in turn, elevated aggression levels. Finally, higher plasma cortisol concentrations appear to be correlated with the likelihood of a fish becoming subordinate (Sloman et al., 2001a), an effect which also appears to hold true in most mammalian systems (*e.g.*, Golub et al., 1979). Sloman et al. (2001a) reported that within pairs of size-matched rainbow trout, plasma cortisol concentrations were significantly higher prior to pairing in fish which subsequently become subordinate, a finding that suggests that individuals with high plasma cortisol levels are predisposed to become subordinate. In a related study, experimental elevation of plasma cortisol concentrations reduced appetite and increased fin damage in cortisol-treated trout held in mixed groups with untreated controls, suggesting that experimentally elevated cortisol concentrations placed individuals at a physiological and/or competitive disadvantage (Gregory and Wood, 1999). Therefore, by experimentally raising plasma cortisol concentrations in selected fish and using an experimental protocol in which pairs are confined together in tanks, we tested the hypothesis that elevated cortisol levels influence social status, with the prediction that experimental elevation of circulating cortisol should predispose a fish to subordinate social status. Possible mechanisms through which cortisol might influence competitive ability and thus social status include a direct effect by reducing physiological condition (Barton et al., 1987; Barton and Iwama, 1991; Gregory and Wood, 1999), or

indirectly, through behavioural modifications mediated by changes in brain monoaminergic activity.

As previously mentioned, socially subordinate animals within a wide range of vertebrate species exhibit behavioural inhibition as a result of social defeat (Winberg and Nilsson, 1993a; Creel, 2001). Moreover, social stress-induced modifications of brain serotonergic/dopaminergic activity have been found to be, at least in part, responsible for these characteristic behavioural changes (Winberg and Nilsson, 1993a; Winberg et al., 1997a; Øverli et al., 1998). Serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) are both neurotransmitters synthesized by enzymatic modification of essential amino acids (tryptophan and tyrosine, respectively), and each is believed to play a vital role in the control and integration of behavioural and physiological stress responses in the brain (Winberg and Nilsson, 1993a). For example, in fish, 5-HT is thought to regulate HPI axis activity (Winberg et al., 1997a; Chaouloff, 2000) and behavioural responses to social stress (for review see Winberg and Nilsson, 1993a), while DA appears to facilitate aggressive interactions (Tiersch and Griffith, 1988). In general, measurements of these monoamines are reported as ratios (*i.e.* metabolite/parent monoamine), as this reflects their rate of release and turnover at the site of interest (Shannon et al., 1986; Fillenz, 1993), thus providing an estimate of overall neural activity. Further, 5-HT treatment is known to inhibit both feeding and aggression in several species of fish (Bernier and Peter, 2001; Winberg et al., 2001; Perrault et al., 2003), while subordinates generally exhibit significantly higher turnover rates of 5-HT, reflected by 5-hydroxyindolacetic acid (5-HIAA) accumulation and elevated 5-HIAA/5-HT ratios within the telencephalon, hypothalamus and brain stem relative to dominant individuals (Winberg et al., 1991;

Winberg et al., 1992; Winberg et al., 1993a; Winberg et al., 1997b). On the other hand, dominant fish are characterized by an increase in brain DA activity (McIntyre et al., 1979; Winberg et al., 1991) and injections of the DA receptor agonist apomorphine, are capable of significantly increasing aggressive behaviour in rainbow trout (Tiersch and Griffith, 1988).

5-HT and DA have also been implicated in the reversal of dominance relationships in a number of vertebrate species (Winberg and Nilsson, 1992; Sanchez and Hyttel, 1994; Deckel, 1996; Villalba et al., 1997; Larson and Summers, 2001). Work in socially interacting reptiles (Deckel, 1996; Larson and Summers, 2001) and mammals (Sanchez and Hyttel, 1994; Villalba et al., 1997) confirm that high brain 5-HT levels may prime individuals for subordination. In fish, however, this does not appear to be the case, as Winberg et al. (1992) reported that the relationship between brain 5-HT activity and social rank in Arctic charr, *Salvelinus alpinus*, developed through social interactions and was not caused by intrinsic differences in 5-HT. Although, given the highly correlative nature of this study (Winberg et al., 1992), in addition to the potential for species specific responses, we tested the hypothesis that 5-HT was not a determinant of subordinate social status by experimentally raising 5-HT activity levels in the brains of socially interacting rainbow trout. Elevation of brain 5-HT concentrations was achieved through dietary administration of L-tryptophan prior to social interaction, which, in addition to being the precursor for the first and rate limiting step of 5-HT biosynthesis (for review see Boadle-Biber, 1993), is easily taken up from the general circulation by neutral amino acid transporters in the brain (Fernstrom and Wurtman, 1972).

Finally, to further explore the idea that high circulating cortisol levels and low social status were linked through a pathway in which cortisol-induced changes in brain monoaminergic activity affect competitive ability, a means of reliably predisposing an individual fish to dominant social status was required. DA has been associated with high social status in salmonids (Winberg and Nilsson, 1992; Winberg and Nilsson, 1993a), with increased turnover rates of DA in the brains of dominant fish (McIntyre et al., 1979; Winberg et al., 1991), in addition to an inherent ability to facilitate aggression (Winberg and Nilsson, 1993a). Further, oral administration of L-dopa, the immediate precursor to DA, in Arctic charr was reported to increase the probability of the treated individual assuming dominant social status in paired encounters (Winberg and Nilsson, 1992). Therefore, treatment with a DA analogue (apomorphine) or experimental elevation of brain dopaminergic activity using L-dopa treatment, was investigated in rainbow trout as a means of inducing dominant social status in socially interacting fish.

CHAPTER 2
THE EFFECTS OF CORTISOL ADMINISTRATION ON SOCIAL STATUS AND
BRAIN MONOAMINERGIC ACTIVITY IN RAINBOW TROUT
(*ONCORHYNCHUS MYKISS*)

Chapter 2 is based on a manuscript submitted and in review with the *Journal of*
Experimental Biology.

Abstract

The hypothesis that circulating cortisol levels influence the outcome of social interactions in rainbow trout was tested. Juvenile rainbow trout, *Oncorhynchus mykiss*, were given a single intraperitoneal (i.p.) implant of coconut oil alone (sham; 0.005 ml coconut oil g⁻¹ fish), cortisol (110 mg kg⁻¹ fish) in coconut oil, or cortisol and the glucocorticoid receptor antagonist RU486 (1100 mg mifepristone kg⁻¹ fish) in cocoa butter, and sampled after five days of social interactions with either a similar sized (<1.5% difference in fork length) or smaller conspecific (>5% difference in fork length). Within size-matched pairs of fish, experimental elevation of plasma cortisol significantly increased ($\chi^2 = 5.14$, $P < 0.025$) the probability that the treated fish within each pair became subordinate, an effect that was abolished ($\chi^2 = 0.8$, $P > 0.25$) by simultaneous administration of RU486. Both sham and cortisol treatments reduced the usual tendency for the larger fish within a pair to preferentially become dominant; large treated fish became dominant in 63% of pairs within the sham group and only 40% of pairs in the cortisol-treated group, compared to 86% of pairs in the control group. These findings implicate elevated circulating cortisol concentrations as a factor that predisposes rainbow trout to low social status. To investigate one potential mechanism underlying the effect of cortisol on social interactions, fish were given an i.p. implant of cortisol (50 mg kg⁻¹ fish) or cortisol (50 mg kg⁻¹ fish) and RU486 (500 mg kg⁻¹ fish) for a period of five days, after which levels of brain monoamines (5-hydroxytryptamine [5-HT]; dopamine [DA]) and their major metabolites (5-hydroxyindolacetic acid [5-HIAA]; 3,4-dihydroxyphenylacetic acid [DOPAC]) were measured. Significant increases of serotonergic activity ([5-HIAA]/[5-HT] ratio) were detected in the telencephalon

following cortisol treatment ($P = 0.008$), an effect that was eliminated by simultaneous administration of the glucocorticoid receptor antagonist RU486. Also, cortisol treatment resulted in a significant decrease ($P = 0.021$) of dopaminergic activity in the telencephalon. Somewhat surprisingly, the observed effects of cortisol treatment on monoaminergic activity in the hypothalamus were opposite to those in the telencephalon; a significant decrease ($P < 0.001$) of [5-HIAA]/[5-HT] and an increase ($P < 0.001$) of [DOPAC]/[DA]. Moreover, in no case did administration of the glucocorticoid receptor antagonist RU486 abolish these effects. These results suggest that the effects of cortisol on social status in rainbow trout may be mediated via the modulation of central signaling systems and subsequent changes in behaviour and/or competitive ability, although the exact site of action in the brain remains uncertain.

Introduction

Salmonid fish, such as rainbow trout (*Oncorhynchus mykiss*), form linear, dominance-based, social hierarchies in both natural and artificial populations (Noakes and Leatherland, 1977; Bachman, 1984; Abbott and Dill, 1989). In the laboratory, confinement in pairs generally results in one fish becoming dominant over the other, subordinate fish, and as a result, subordinate individuals experience chronic social stress (reviewed by Sloman and Armstrong, 2002). These subordinate fish are also generally excluded from preferential access to food (McCarthy et al., 1992) and experience increased standard metabolic rates (Sloman et al., 2000c), which result in decreased growth rates (Barton et al., 1987; Metcalfe et al., 1989; Winberg et al., 1992; Metcalfe et al., 1995). Immuno-suppression (Peters et al., 1988; Pottinger and Pickering, 1992), and increased mortality (Pickering and Duston, 1983; Pickering, 1993) are also observed in subordinate individuals. Finally, subordinate fish are characterized as having plasma concentrations of the corticosteroid stress hormone, cortisol, that are higher than those of dominant fish (reviewed by Sloman and Armstrong, 2002; Gilmour et al., 2005). In fact, these marked elevations of plasma cortisol have been widely employed as an index of stress (Pottinger and Pickering, 1992) and are thought to contribute significantly to the poor overall physiological condition of subordinate fish (Gilmour et al., 2005).

A number of social (*e.g.*, prior residence, prior winning/losing experience) and inherent (*e.g.*, size, aggressiveness) factors are known to influence the outcome of social interactions in salmonids (Huntingford and Turner, 1987; Fernandes and Volpato, 1993; Rhodes and Quinn, 1998; Metcalfe, 1998). Recent work has also raised the possibility that physiological factors, more specifically the physiological condition of a fish, can

impact on an individual's initial success during competitive interactions and affect its ultimate social status (*e.g.*, Johnsson and Björnsson, 1994; Björnsson, 1997; Sloman et al., 2001a). For example, Sloman et al. (2001a) reported that plasma cortisol concentrations were significantly higher prior to pairing in size-matched rainbow trout which subsequently become subordinate, suggesting that individuals with high plasma cortisol levels are predisposed to become subordinate. In a related study, experimental elevation of plasma cortisol concentrations reduced appetite, growth rate, and condition in rainbow trout, and fin damage was greater in cortisol-treated trout held in mixed groups with untreated controls. These data suggest that elevated cortisol concentrations might in fact be symptomatic of an individual fish's poor condition, placing it at a physiological and/or competitive disadvantage (Gregory and Wood, 1999). Thus, the main objective of the present study was to test the hypothesis that circulating cortisol concentrations affect the outcome of social interactions within pairs of rainbow trout. In particular, high plasma cortisol levels were predicted to predispose a fish to low social status. In accordance with the work of Gregory and Wood (1999), two possible mechanisms through which cortisol might influence social status can be envisaged. Elevated cortisol levels, by reducing physiological condition (Barton et al., 1987; Barton and Iwama, 1991; Gregory and Wood, 1999), could impact on competitive ability directly. Alternatively, interactions between cortisol and brain monoaminergic activity could affect competitive ability indirectly by modulating behaviour.

Many of the behavioural consequences of social status are thought to be the outcome of changes in brain monoaminergic activity that accompany victory or loss in competitive interactions (reviewed by Winberg and Nilsson, 1993a). For example,

subordinate fish generally exhibit significantly higher turnover of serotonin (5-HT), reflected by 5-hydroxyindolacetic acid (5-HIAA) accumulation and elevated 5-HIAA/5-HT ratios within the telencephalon, hypothalamus, and brain stem relative to dominant individuals (Winberg et al., 1991; Winberg et al., 1992; Winberg et al., 1993a; Winberg et al., 1997b). These social stress-induced increases of brain 5-HT activity are likely, at least in part, responsible for the marked behavioural inhibition commonly observed in subordinate fish; namely decreases in feeding, aggression, and spontaneous locomotor activity (Winberg and Nilsson, 1993a; Winberg et al., 1997a; Øverli et al., 1998). In tetrapod vertebrates, experimentally elevated serotonergic activity causes a reversal of dominance relationships in a number of model systems (*e.g.*, Sanchez and Hyttel, 1994; Villalba et al., 1997; Larson and Summers, 2001), suggesting that high brain 5-HT levels in these organisms have the capacity to act as antecedents for subordination. Winberg et al. (1992) reported that the relationship between brain 5-HT turnover rate and social rank in fish developed through social interactions and was not caused by intrinsic differences in brain 5-HT activity. However, it is conceivable that high circulating cortisol levels could influence the outcome of social interactions by affecting central monoaminergic activity, specifically increasing serotonergic activity and/or decreasing dopaminergic activity. These changes, in turn, could alter behaviour (reducing aggression, locomotion, etc.) in such a way as to reduce competitive ability, resulting in low social status. Thus, in the present study the hypothesis that circulating cortisol levels influence brain monoaminergic activity was also tested as one potential mechanism underlying any observed relationship between cortisol treatment and the outcome of social interactions.

Materials and methods

Experimental animals

Juvenile female freshwater rainbow trout (weight 87.14 ± 2.3 g [mean $\pm$ SEM], $N = 217$) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). All fish were held in large 1,275-L fibreglass stock tanks for several weeks at the University of Ottawa, supplied with flowing, aerated, dechloraminated city of Ottawa tap water at a temperature of $13 \pm 1^\circ\text{C}$ and using a 12L: 12D photoperiod. Fish were then transported to Carleton University and housed in 780-L fibreglass holding tanks until use; tanks were supplied with flowing, aerated, well water ($16.5 \pm 1.5^\circ\text{C}$) and a 12L: 12D photoperiod was also used. Throughout this period, fish were hand-fed to satiation every second day with a commercial trout food diet (Purina Trout Chow).

Pilot trials to assess the efficacy of methods for elevating plasma cortisol

In preliminary trials, groups of fish (mass 100.61 ± 4.88 g; $N = 31$) were exposed to different cortisol delivery methods to determine the most reliable means of elevating plasma cortisol concentrations. Two forms of cortisol (hydrocortisone 21-hemisuccinate, sodium salt and hydrocortisone 21-hemisuccinate) and vehicle (coconut oil, cocoa butter) were tested, and attempts were made to determine the appropriate dose of cortisol (*i.e.* 110 mg kg⁻¹ fish or 220 mg kg⁻¹ fish) to be used in subsequent experiments so as to achieve the desired circulating plasma cortisol concentration of ~ 150 ng mL⁻¹, a level indicative of moderately stressed salmonids (Gamperl et al., 1994; Wendelaar Bonga, 1997). Fish were lightly anaesthetized (*i.e.* the point at which the test animal lost

equilibrium while maintaining a normal ventilation frequency) in a solution of benzocaine (0.05 g L^{-1} ethyl-*p*-aminobenzoate) and injected intraperitoneally with a pellet of the appropriate vehicle ($0.005 \text{ mL vehicle g}^{-1}$ fish was used in all cases), containing the appropriate form and dose of cortisol. The vehicle was injected as a liquid but solidified rapidly within the fish and acted as a solid implant for the remainder of the experiment. Previous work has demonstrated that both coconut oil and cocoa butter allow the delivery of a prolonged, slow-release dose of cortisol within each injected fish (reviewed by Gamperl et al., 1994). Fish were held in 780-L holding tanks in large groups (*i.e.* > 10 individuals per tank) and killed after 5 days by immersion in a lethal dose of anaesthetic (ethyl-*p*-aminobenzoate 0.5 g L^{-1}). Blood samples ($\sim 1 \text{ mL}$) were removed via caudal puncture, and separated plasma was frozen in liquid nitrogen and stored at -80°C until analysis for cortisol concentration using a commercial RIA kit (ICN pharmaceuticals).

Experiment 1: The effects of cortisol treatment on the outcome of social interactions

Fish were lightly anaesthetized (0.05 g L^{-1} ethyl-*p*-aminobenzoate) and initial masses and fork lengths were measured (mass $74.7 \pm 2.2 \text{ g}$; fork length $187.3 \pm 1.8 \text{ mm}$, $N = 152$). Abbott and Dill (1985) reported that an initial length difference of as little as 5% was sufficient to ensure dominant status to the larger individual within pairs of rainbow trout. Therefore, two separate series of experiments were carried out. In the first, fish were paired with a conspecific that was size-mismatched by 5-20% on the basis of fork length. Each pair of fish was then randomly assigned to one of three groups; control (14 pairs), cortisol treatment (11 pairs) or sham treatment (11 pairs), with the larger fish within the pair in each case receiving the treatment. In the second

experimental series, fish were size-matched by fork length ($<1.5\%$ difference), and each pair of fish was randomly assigned to one of three groups; sham treatment (16 pairs), cortisol treatment (14 pairs), or cortisol plus RU486 treatment (10 pairs), with one fish within the pair receiving the treatment.

Based on the results of the pilot trials, cortisol-treated fish received a coconut oil pellet ($0.005\text{ ml coconut oil g}^{-1}\text{ fish}$) containing dissolved cortisol ($110\text{ mg hydrocortisone 21-hemisuccinate kg}^{-1}\text{ fish}$) in the intraperitoneal cavity. Cortisol plus RU486 treatment was achieved by implanting a cocoa butter pellet ($0.01\text{ mL coconut oil g}^{-1}\text{ fish}$) containing a combination of dissolved cortisol ($110\text{ mg hydrocortisone 21-hemisuccinate kg}^{-1}\text{ fish}$) and the glucocorticoid receptor antagonist RU486 ($1100\text{ mg mifepristone kg}^{-1}\text{ fish}$; Sigma). The concentration of RU486 was chosen on the basis of previous work indicating that treatment with this compound is most effective at a dose 10-fold greater than that of cortisol (Vijayan et al., 1994b). Sham treated fish received a coconut oil injection only ($0.005\text{ ml coconut oil g}^{-1}\text{ fish}$), while control fish were untreated.

Following preparation, pairs of trout were placed in 40 L flow-through plexiglass observation tanks. The fish were separated by an opaque perforated divider for a 48 h recovery and acclimation period, and the dividers were then removed to allow pairs of fish to interact; a small piece of PVC tubing was placed within each tank to provide shelter. Behavioural observations were carried out on all paired fish twice a day for 5 days, and the fish were then terminally sampled. During the experiment, fish were hand-fed to satiation with commercial trout food pellets once a day, after all observations had been carried out. Behaviour observations were first conducted fifteen minutes after the opaque divider was removed, and then for 10 min each, once between 9:00-11:30h and

once between 15:00-17:30h. The order of tank observation was randomized to account for any observational bias.

Social status was determined by assigning points to each fish based on its food acquisition, position, aggressive behaviour and fin damage; high scores in each case were indicative of dominant behaviour or characteristics. This method has been used previously for assigning social status among salmonids (Johnsson et al., 1996; Sloman et al., 2000a; Sloman et al., 2000b; Sloman et al., 2001a). In brief, to score fish on food acquisition, one pellet of food was dropped into the tank at the beginning of each observation period and the first fish to take the pellet was given a score of one, while the other fish scored zero points. Fish that maintained their position within the water column scored ten points, whereas fish that rested on the bottom of the tank or hid within the PVC tubing scored five points, and fish that attempted to swim at the surface (a behaviour indicative of subordination; (Sloman et al., 2000a)) scored zero points. Fish directing five or more aggressive attacks towards the other individual within an observation period were given a score of two, fish performing between one and four aggressive attacks were given a score of one, and those individuals performing no aggressive attacks received a score of zero. Finally, fish were scored according to the extent of dorsal and caudal fin damage sustained during the five-day interaction period. The mean dorsal and caudal fin damage scores were calculated and then combined into a total fin score. Previous work demonstrated that the severity of fin damage is likely to reflect the social rank of the individual (Abbott and Dill, 1985; Moutou et al., 1998). Therefore, fish having no fin damage were given a score of three, minor damage (<30% of the fin missing) a score of two, severe damage (30-70% of the fin missing) a score of

one, and very severe damage (>70% of the fin missing) a score of zero. A single behaviour score was calculated from all observations by means of a principal components analysis (PCA; SPSS 10.1) (Sloman et al., 2000c). The fish with the higher overall behaviour score within each pair was assigned dominant social status, whereas that with the lower score was classified as subordinate.

Fish were rapidly killed by immersion in a lethal dose of anaesthetic solution (ethyl-*p*-aminobenzoate 0.5 g L⁻¹). Pairs were removed simultaneously from their tanks and sampled within one minute of each other; the sampling order within each pair was randomized to control for any sampling bias. Final weights and fork lengths were measured and a blood sample (~1 mL) was removed by caudal puncture. Following centrifugation (13,200 g for 3 min.), plasma was removed, immediately frozen in liquid nitrogen, and subsequently stored at -80° C until analysis. Plasma cortisol concentrations were measured using a commercially available radioimmunoassay kit (ICN pharmaceuticals). The condition factor (CF) and specific growth rate (SGR) of each fish was calculated based on the following formulae:

$$CF = 100 \cdot W/L^x$$

where W = weight of fish in grams, L = length of fish in centimeters, and x = slope of regression line for all fish of log W vs. log L (~ 3), and

$$SGR = [\ln(W_{Final}) - \ln(W_{Initial})] \cdot 100 / D$$

where W = weight of fish in grams and D = number of days elapsed.

Experiment 2: The effect of cortisol administration on brain monoamine levels

Fish were lightly anaesthetized (0.05 g L^{-1} ethyl-*p*-aminobenzoate), initial masses were measured (mass $92.5 \pm 2.3 \text{ g}$, $N = 34$) and fish were randomly placed within groups of twelve in 780-L holding tanks. Following a 5 day acclimation period, fish were injected intraperitoneally with a cocoa butter pellet (0.005 mL coconut oil g^{-1} fish) containing dissolved cortisol (50 mg hydrocortisone 21-hemisuccinate kg^{-1} fish; $N = 10$) or a combination of dissolved cortisol (50 mg hydrocortisone 21-hemisuccinate kg^{-1} fish) and RU486 (500 mg mifepristone kg^{-1} fish; $N = 12$); an additional group of untreated fish served as a control group ($N = 12$).

Fish were sampled 5 days after receiving treatment in groups of 4, to minimize disturbance of the fish remaining within each tank. Fish were killed by immersion in a lethal dose of anaesthetic (ethyl-*p*-aminobenzoate 0.5 g L^{-1}), mass was measured and the brain was rapidly removed. Two discrete brain regions were dissected out (on ice) for analysis, the telencephalon (excluding the olfactory bulbs), and the hypothalamus (excluding the pituitary gland). These brain areas were selected on the basis of earlier studies showing that monoamine activity within these regions was particularly influenced by social stress (Winberg et al., 1991; Winberg et al., 1992; Winberg et al., 1997b). Brain samples were frozen in liquid nitrogen and stored at -80°C . Blood samples ($\sim 1 \text{ mL}$) were removed via caudal puncture, and separated plasma was frozen in liquid nitrogen and stored at -80°C until analysis for plasma cortisol concentration using a commercial RIA kit (ICN pharmaceuticals). All blood and tissue samples were collected between 11:30 and 14:30 h to control for any diurnal variations in either plasma cortisol and/or brain monoamine concentrations.

Frozen brain samples were sonicated in a homogenizing solution comprising 0.1mmol L⁻¹ Na₂EDTA, 0.3 mol L⁻¹ ClCHCOOH, 10% methanol, and 12.5 pg µL⁻¹ DHBA (the internal standard). Brain monoamines were then quantified by high performance liquid chromatography (HPLC) using electrochemical detection. The HPLC consisted of a solvent-delivery system (Waters590/WaterPump), an autoinjector (Waters712WISP), a reverse-phase column (8 mm X 100 mm, Waters, NovaPak, 4 µm) kept at 30 °C and a 5100A Coulochem detector (ESA, Bedford, MA, USA) with two electrodes at oxidizing potentials of -330 mV and +350 mV. The mobile phase consisted of 1.3 g L⁻¹ heptanesulphonic acid sodium salt, 0.1 g L⁻¹ disodium ethylene tetracycline, and 7.3 mL triethyloamine adjusted to pH 2.45 with orthophosphoric acid. Sample monoamine levels were indexed to standard solutions of known concentration, corrected for recovery of the internal standard, and expressed relative to total tissue protein content.

The monoamines measured were 5-hydroxytryptamine (5-HT) and dopamine (DA), as well as their major metabolites, respectively, 5-hydroxyindolacetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC). The ratio of [metabolite]/[parent monoamine] was used as an index of brain monoaminergic activity. This index reduces variance related to tissue sampling and provides a more direct measure of brain monoaminergic activity than do levels of monoamine metabolites on their own (Shannon et al., 1986).

Statistical analysis

All data are presented as means ± 1 standard error of the mean (S.E.M.). One-sample t-tests were run on plasma cortisol concentrations from all treatment groups

within the pilot trial under the null hypothesis (H_0) that plasma [cortisol] was not significantly greater than values typically seen in unstressed fish (*i.e.* 10 ng ml⁻¹). Chi-square analysis was used to evaluate the effects of treatment group on social status and behaviour scores for all pairs of fish. One-way analyses of variance (ANOVA), followed by Bonferroni corrected *t*-tests, as appropriate, were carried out on behaviour score and plasma cortisol concentrations for each treatment group in Experiment 1, with levels being determined by the combination of social status and treatment. A two-way analysis of variance (ANOVA), followed by Bonferroni corrected *t*-tests, as appropriate, was carried out on specific growth rate and final condition factor for each treatment group in Experiment 1, using social status and treatment group as factors. The statistical significance of differences in mean brain monoamine concentrations in Experiment 2 were assessed using one-way ANOVA on ranks followed by Dunn's post hoc pairwise multiple comparisons test, as appropriate. Non-normally distributed plasma cortisol concentrations were log transformed as appropriate. The α level for significance for all tests was set at 0.05 and all statistical analyses were performed using SigmaStat v3.0 (SPSS, Inc) or SPSS v10.1 (SPSS, Inc) software.

Results

Pilot trials to assess the efficacy of methods for elevating plasma cortisol

Intraperitoneal injections of cortisol-impregnated pellets resulted in marked, dose-dependant increases in plasma cortisol concentrations within the normal physiological range in fish treated with the sodium salt form of cortisol, as measured plasma cortisol concentrations were higher than resting levels commonly seen in unstressed fish (*i.e.* ~ 10 ng mL⁻¹), although highly variable (Table 2-1). Qualitatively, the sodium salt of cortisol seemed to dissolve more completely with either vehicle than did the free form. In agreement with previous reports (reviewed by Gamperl et al., 1994), both coconut oil and cocoa butter appeared to serve as effective vehicles for delivering a slow-release dose of cortisol and so were used interchangeably throughout this study. The observations in Table 2-1 suggest that the sodium salt of hydrocortisone 21-hemisuccinate at a dose of 110 mg kg⁻¹ fish was the best choice for the desired purpose of elevating circulating cortisol concentrations to physiologically-relevant “stressed” values.

Experiment 1: The effect of cortisol treatment on the outcome of social interactions

Plasma cortisol concentrations and behaviour scores

Socially subordinate rainbow trout exhibited marked behavioural changes, including decreases in feeding and aggression as well as selection of tank positions not occupied by the other fish, during the five-day interaction period. Through the scoring scheme and PCA analysis, these differences in behaviour were translated into behaviour scores that were typically high positive values for dominant fish and low negative scores for subordinate individuals regardless of treatment group or treatment (scores ranged

from 1.5 to -2.1; Fig. 2-1A,B). Rarely, the behaviour scores for fish within a pair were not sufficiently different (*i.e.* identical behaviour scores) to enable social status to be assigned; the one pair for which this was the case was eliminated from further analysis. Interestingly, the large cortisol-treated subordinate fish from the size-mismatched trial appeared to have a reduced behaviour score in comparison to the large-cortisol treated dominant fish, as they were not significantly different from each other.

In general, plasma cortisol concentrations at the end of the interaction period were also indicative of social status in most treatment groups, with subordinate fish tending to exhibit higher circulating cortisol levels than dominant fish (Fig. 2-2A,B). The relatively low plasma cortisol concentrations of the large subordinate fish within the sham size-mismatched treatment group, the small subordinates within the cortisol-treated size-mismatched fish and the untreated subordinates of the sham size-matched fish were unexpected in this regard. Cortisol administration was generally effective in raising plasma cortisol levels, although not within the cortisol-treated dominants of the size-matched pairs, and only to a limited extent within the large dominant fish of the cortisol-treated size-mismatched pairs.

An experimental protocol in which sham-treated, cortisol-treated or cortisol + RU486 treated individuals were paired with a conspecific that was <1.5% different in fork length revealed significant differences in behaviour as a result of treatment. Chi-square analysis indicated that cortisol treatment but not sham treatment had a significant effect on the outcome of social interactions, with cortisol-treated fish becoming subordinate more often than expected by chance alone (Table 2-2). This effect of cortisol

was eliminated by simultaneous treatment with RU486; untreated and cortisol + RU486-treated fish were equally likely to be relegated to subordinate social status (Table 2-2).

In an attempt to elucidate the importance of circulating cortisol concentrations relative to a factor that is known to affect the outcome of social interactions in rainbow trout (*i.e.* body size; (Abbott and Dill, 1985)), an experiment was carried out in which untreated, cortisol-treated or sham-treated individuals were paired with a conspecific that was at least 5% (range 4.6 to 17.4%) smaller in fork length. The larger fish became dominant in 86% of size-mismatched pairs of trout in which both fish were untreated ($N = 14$ pairs), a result that was confirmed to be significantly different than that expected by chance alone via chi-square analysis (Table 2-3). This size effect was eliminated by both sham ($N = 11$ pairs) and cortisol ($N = 10$ pairs) treatments, in which 63% and 40%, respectively, of larger (treated) fish became dominant. Chi square analysis indicated that both the sham treatment ($\chi^2 = 1.63$, d.f. = 1, $P > 0.1$) as well as the cortisol treatment ($\chi^2 = 0.8$, d.f. = 1, $P > 0.25$) decreased the probability, to the point where it was not significantly different than that expected by chance, of larger fish within each pair becoming dominant (Table 2-3).

Condition factor and specific growth rate

Prior to the onset of social interactions, there were no significant differences in initial condition factor (CF_i) for either size-matched or size-mismatched pairs of rainbow trout (size-matched pairs $CF_i = 1.07 \pm 0.011$, $N = 80$; size-mismatched pairs $CF_i = 1.13 \pm 0.017$, $N = 70$). However, at the end of the 5-day interaction period, social status had a significant effect on CF_f within size-matched pairs (two-way ANOVA, $P = 0.011$). CF_f

was significantly higher in dominant trout relative to subordinate fish in the sham ($P = 0.017$) and cortisol-treated group ($P = 0.011$), but not the cortisol + RU486 group ($P = 0.868$) (Table 2-4). By contrast, significant effects of both social status (two-way ANOVA, $P = 0.002$) and treatment group (two-way ANOVA, $P = 0.027$) were present within pairs of size-mismatched fish for final condition factor (CF_F) (Table 2-5). Specifically, the CF_F of dominant fish within the control ($P < 0.001$) group was significantly higher than that of subordinate fish, while the CF_F of sham-treated fish as a whole was significantly higher ($P = 0.023$) than that of cortisol-treated fish.

Determination of specific growth rates (SGR) revealed significant effects of both social status (two-way ANOVA, $P < 0.001$) and treatment group (two-way ANOVA, $P = 0.029$) within size-matched pairs of fish (Table 2-4). There was also a significant interaction between treatment group and social status (two-way ANOVA, $P = 0.042$) within these pairs of fish. The follow-up comparisons indicated that dominant individuals demonstrated significantly higher growth rates than subordinate fish in the sham ($P < 0.001$) and cortisol treatment group ($P < 0.001$) but not the cortisol + RU486 group ($P = 0.158$). Furthermore, dominant fish from the cortisol + RU486 treated group had significantly lower SGR than dominants from the sham ($P = 0.019$) and cortisol groups ($P = 0.01$). Finally, cortisol treated fish had significantly lower SGR ($P = 0.005$) than the non-injected fish with which they were paired, while a similar analysis in the sham ($P = 0.289$) and cortisol + RU486 groups ($P = 0.72$) revealed no difference (data not shown), a finding that suggests that the observed differences in growth might be a cortisol-mediated effect. Within size-mismatched pairs of rainbow trout, social status had a significant effect (two-way ANOVA, $P < 0.001$) on SGR. Dominant fish from the

control group exhibited growth rates that were significantly higher than those of the subordinate fish with which they were paired ($P < 0.001$), as well as significantly higher than those of dominant fish within the cortisol-treated group ($P = 0.041$) (Table 2-5).

Experiment 2: The effect of cortisol administration on brain monoamine levels

To assess the impact of circulating cortisol concentrations on brain monoaminergic activity, groups of trout were injected intraperitoneally with a slow-release pellet of cortisol. Circulating plasma cortisol concentrations increased significantly (one-way ANOVA on ranks, $P < 0.001$) with cortisol administration (Fig. 2-3), and the levels attained in the plasma in both cortisol and cortisol + RU486 injected fish were similar to those associated with moderately stressed salmonids (Gamperl et al., 1994; Wendelaar Bonga, 1997) and marginally lower than those observed in previous studies that adopted similar methods (e.g., Vijayan et al., 2003; McDonald and Wood, 2004).

Brain monoaminergic activity was evaluated as a function of cortisol administration. Outlier tests were used to exclude any values that were greater than 10 standard deviations from the mean, thus identifying and accounting for HPLC measurement errors (in total, 5 data points were removed from subsequent analyses: 3 cortisol + RU486 injected 5-HT values in the telencephalon, one control DA value in the telencephalon, and one cortisol injected 5-HT value in the hypothalamus); 3 “zero” DA values were also excluded from the data set. Within the telencephalon, mean serotonergic activity was significantly increased by the cortisol implant treatment (one-way ANOVA on ranks, $P = 0.008$), an effect that was eliminated by simultaneous

administration of the glucocorticoid receptor antagonist RU486 (Fig. 2-4C). This enhanced serotonergic activity observed in trout given cortisol implants reflected the significant lowering of telencephalon 5-HT concentration (one-way ANOVA on ranks, $P < 0.001$; Fig. 2-4A) in the absence of significant change in 5-HIAA level (Fig. 2-4B). Interestingly, significantly lower levels of both 5-HT and 5-HIAA (one-way ANOVA on ranks, $P < 0.001$) were measured in the telencephalon of trout treated with implants containing both cortisol and RU486. Significant differences in telencephalon dopaminergic activity also occurred (one-way ANOVA on ranks, $P = 0.021$; Fig. 2-4F), although neither DA nor DOPAC concentrations in the telencephalon differed significantly among treatment groups (one-way ANOVA on ranks, $P = 0.086$ and 0.057 , respectively; Figs. 2-4D,E).

Surprisingly, the observed effects of cortisol treatment on serotonergic and dopaminergic activity (reflected by the [5-HIAA]/[5-HT] and [DOPAC]/[DA] ratios) in the hypothalamus were opposite to those in the telencephalon; hypothalamic serotonergic activity was significantly reduced by cortisol implants (one-way ANOVA on ranks, $P < 0.001$; Fig. 2-5C), while dopaminergic activity was significantly increased (one-way ANOVA on ranks, $P < 0.001$; Fig. 2-5F). The lower serotonergic activity occurred in the face of no significant reduction of hypothalamic 5-HT (one-way ANOVA on ranks, $P = 0.078$; Fig. 2-5A) but a significant reduction in 5-HIAA concentrations (one-way ANOVA on ranks, $P < 0.001$; Fig. 2-5B). The higher dopaminergic activity reflected significant increases of hypothalamic DOPAC levels (one-way ANOVA on ranks, $P < 0.001$; Fig. 2-5E) in combination with significantly lower dopamine concentrations (one-way ANOVA on ranks, $P < 0.001$; Fig. 2-5D). However, in no case did simultaneous

administration of RU486 with cortisol abolish these effects.

Figure 2-1. The effects of sham, cortisol, and cortisol + RU486 treatments (single dose injections) on the behaviour scores of (A) size-matched (respectively, 16, 14, and 10 pairs) and (B) size-mismatched (respectively, 14, 11, and 10 pairs) pairs of rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days. Data are presented as means values ± 1 S.E.M; the numbers appearing on the figure are the *N* values. Levels are determined by the combination of social status and treatment, with dominant treated, subordinate treated, dominant untreated, and subordinate untreated fish making up the categories. Groups that do not share a letter were significantly different from one another within each treatment group (one-way ANOVA followed by Bonferonni's post hoc multiple comparisons test, with *P* values in A of sham < 0.001, cortisol < 0.001, cortisol + RU486 < 0.001; in B, control < 0.001, sham < 0.001, cortisol-treated < 0.001).

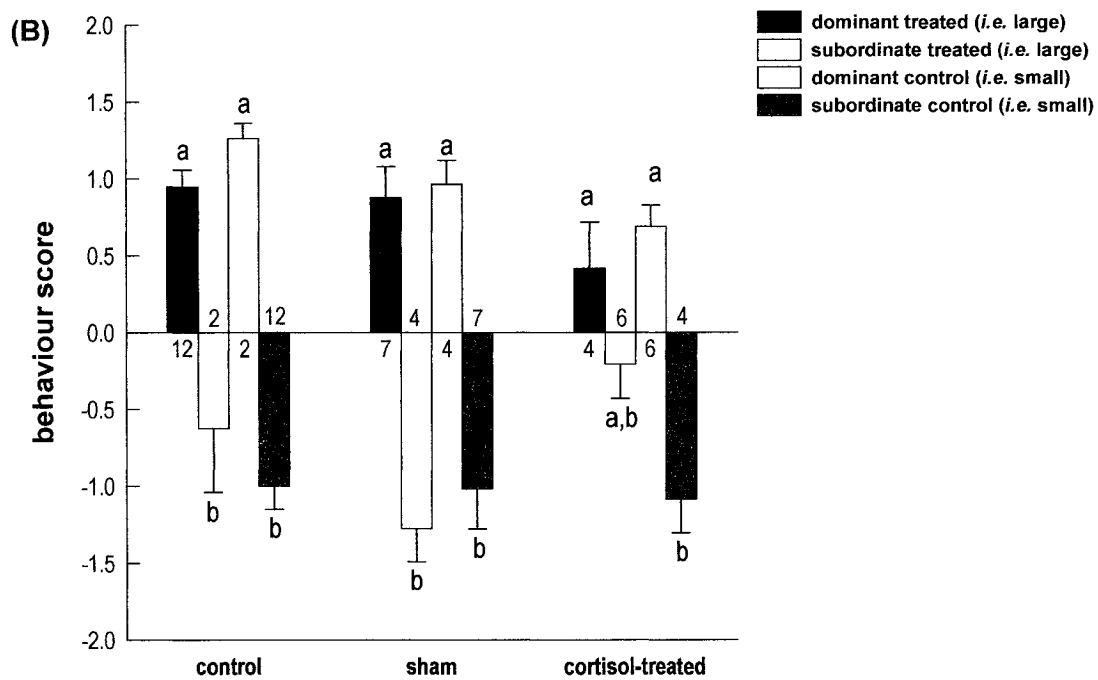
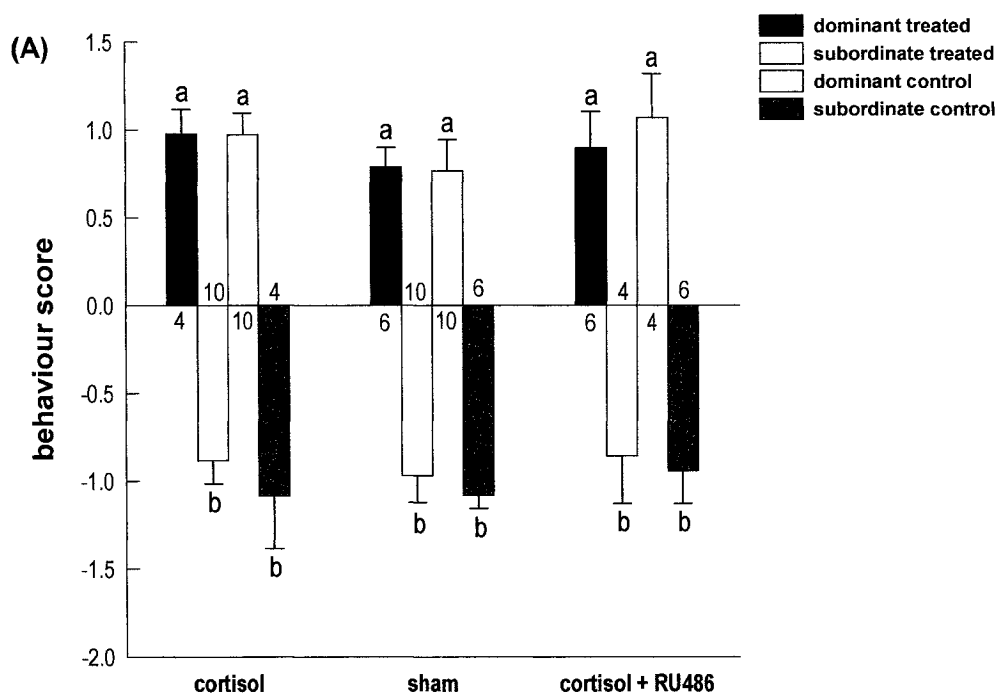


Figure 2-2. The effects of sham, cortisol, and cortisol + RU486 treatments on plasma cortisol concentrations in (A) size-matched (respectively, 16, 11, and 10 pairs) and (B) size-mismatched (respectively, 11, 11 and 10 pairs) rainbow trout (*Oncorhynchus mykiss*) confined in pairs for 5 days. Data are presented as means values $\pm$ 1 S.E.M; the numbers in parentheses below the treatment groups are the *N* values. Levels are determined by the combination of social status and treatment, with dominant treated, subordinate treated, dominant untreated, and subordinate untreated fish making up the categories. Groups that do not share a letter were significantly different from one another within each treatment group (one-way ANOVA followed by Bonferonni's post hoc multiple comparisons test, with *P* values in A of sham < 0.001, cortisol < 0.001, cortisol + RU486 0.086; in B control 0.079, sham 0.566, cortisol-treated 0.043).

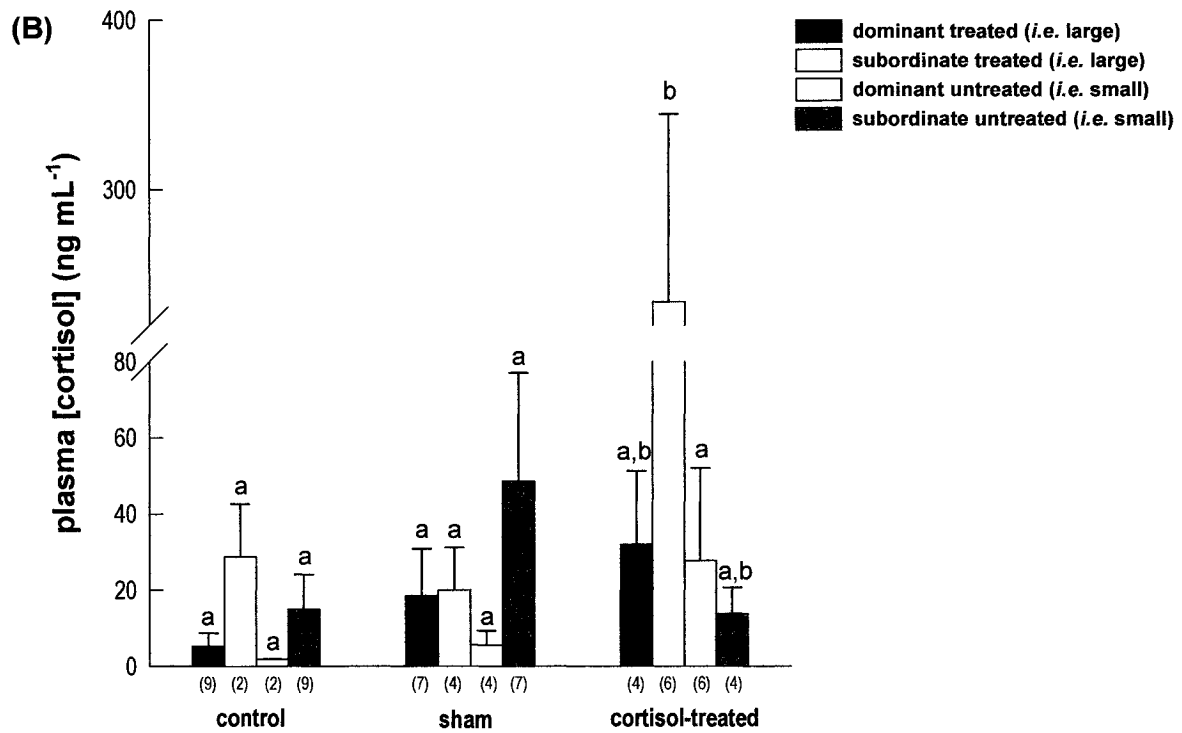
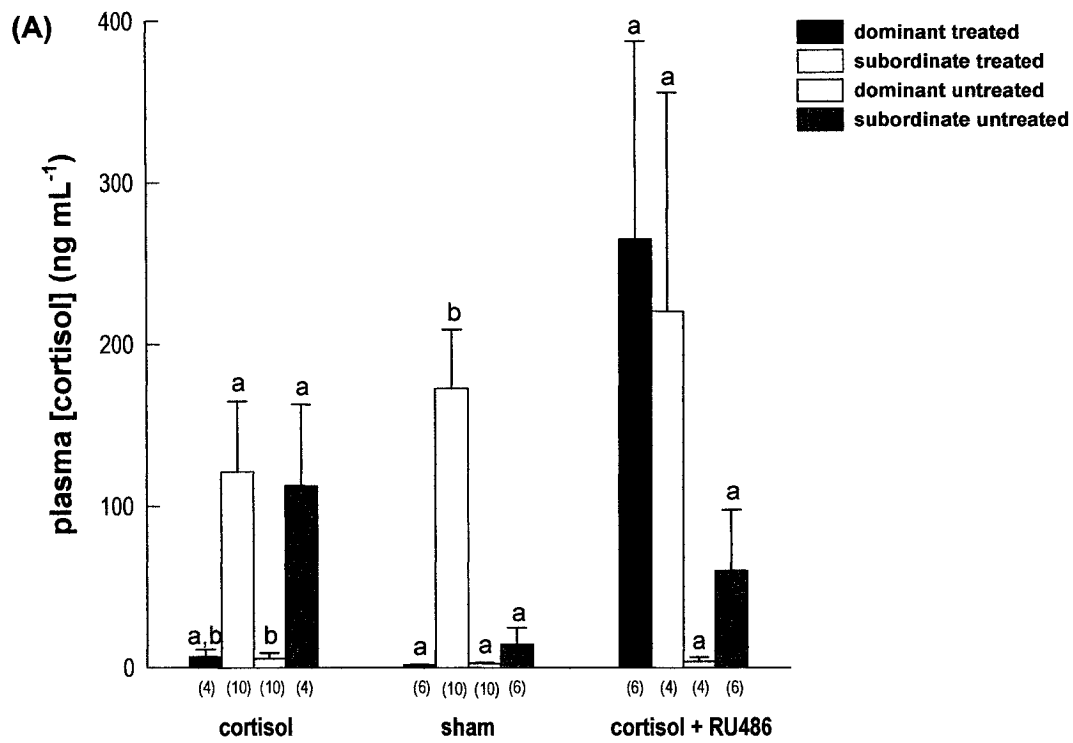


Figure 2-3. Mean plasma cortisol concentrations in rainbow trout (*Oncorhynchus mykiss*) as a function of the method of administration of cortisol; fish were either given an intraperitoneal implant containing cortisol (50 mg hydrocortisone 21-hemisuccinate kg⁻¹ fish) or cortisol + RU486 (500 mg mifepristone kg⁻¹ fish); an additional group of untreated fish served as a control group. Data are presented as mean values $\pm$ 1 S.E.M; the numbers in parentheses below the treatment groups are the *N* values. Groups that do not share a letter were significantly different from one another (one-way ANOVA on ranks followed by Dunn's post hoc multiple comparisons test, $P < 0.001$).

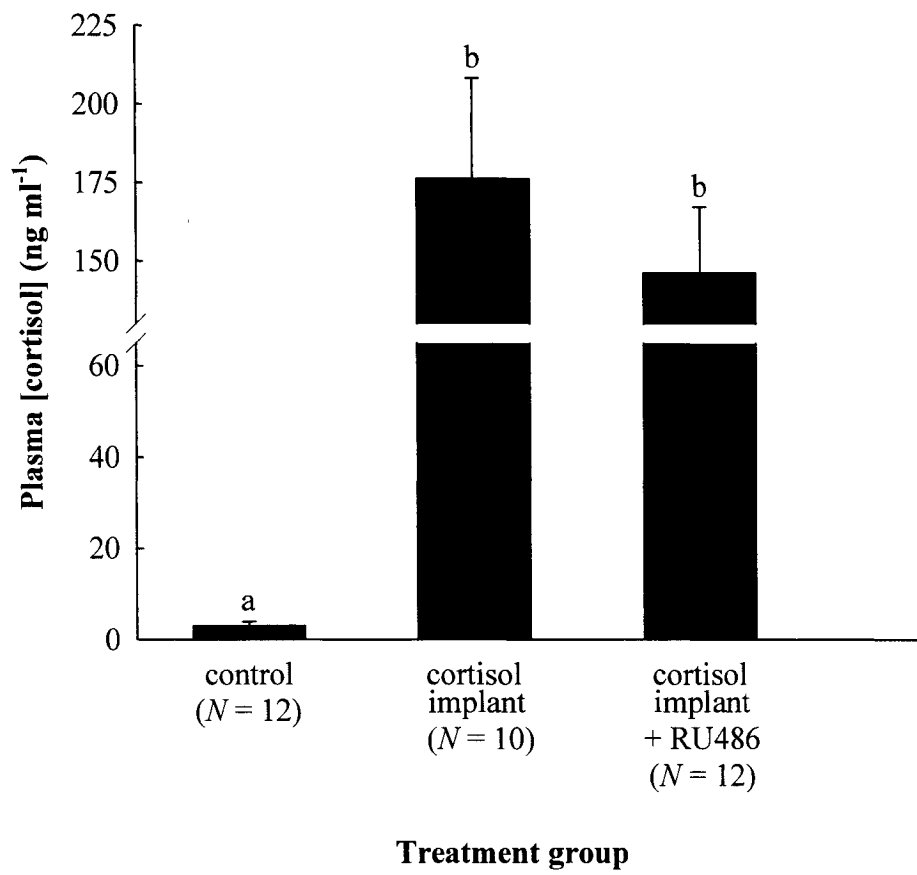


Figure 2-4. Monoamine concentrations (A, D), main monoamine metabolite concentrations (B, E) and the ratio of concentrations of main metabolite to parent monoamine (monoaminergic activity; C, F) for serotonin (A, B, C) and dopamine (D, E, F) in the telencephalon of rainbow trout (*Oncorhynchus mykiss*) treated with cortisol. Cortisol was administered either by intraperitoneal implant (“implant”, 50 mg hydrocortisone 21-hemisuccinate kg⁻¹ fish) or simultaneously with the glucocorticoid receptor blocker RU486 (500 mg mifepristone kg⁻¹ fish); an additional group of untreated fish served as a control group. Data are presented as means $\pm$ 1 S.E.M, with $N = 10-12$ for the control group, $N = 10$ for the group given cortisol implants, and $N = 9-10$ for the cortisol implant + RU486 treatment group. Treatment groups that do not share a letter were significantly different from one another (one-way ANOVA on ranks followed by Dunn’s post hoc multiple comparisons test, with P values of A < 0.001 , B 0.014, C 0.008, D 0.086, E 0.057, F 0.021).

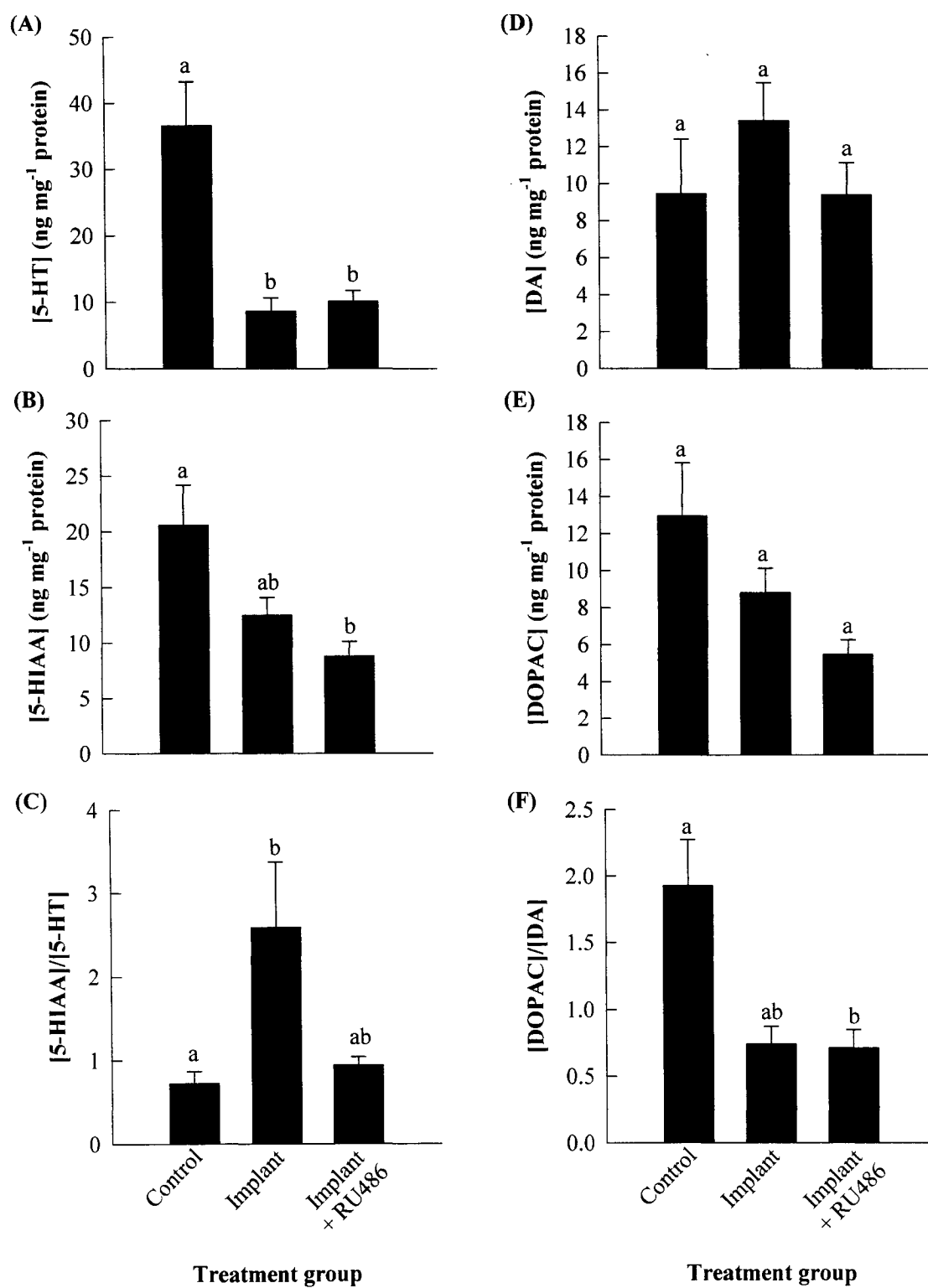


Figure 2-5. Monoamine concentrations (A, D), main monoamine metabolite concentrations (B, E) and the ratio of concentrations of main metabolite to parent monoamine (monoaminergic activity; C, F) for serotonin (A, B, C) and dopamine (D, E, F) in the hypothalamus of rainbow trout (*Oncorhynchus mykiss*) treated with cortisol. Cortisol was administered either by intraperitoneal implant (“implant”, 50 mg hydrocortisone 21-hemisuccinate kg⁻¹ fish) or simultaneously with the glucocorticoid receptor blocker RU486 (500 mg mifepristone kg⁻¹ fish); an additional group of untreated fish served as a control group. Data are presented as means $\pm$ 1 S.E.M. with $N = 12$ for the control group, $N = 9 - 10$ for the group given cortisol implants, and $N = 12$ for the cortisol implant + RU486 treatment group. Treatment groups that do not share a letter were significantly different from one another (one-way ANOVA on ranks followed by Dunn’s post hoc multiple comparisons test, with P values of A 0.078, B < 0.001 , C < 0.001 , D < 0.001 , E < 0.001 , F < 0.001).

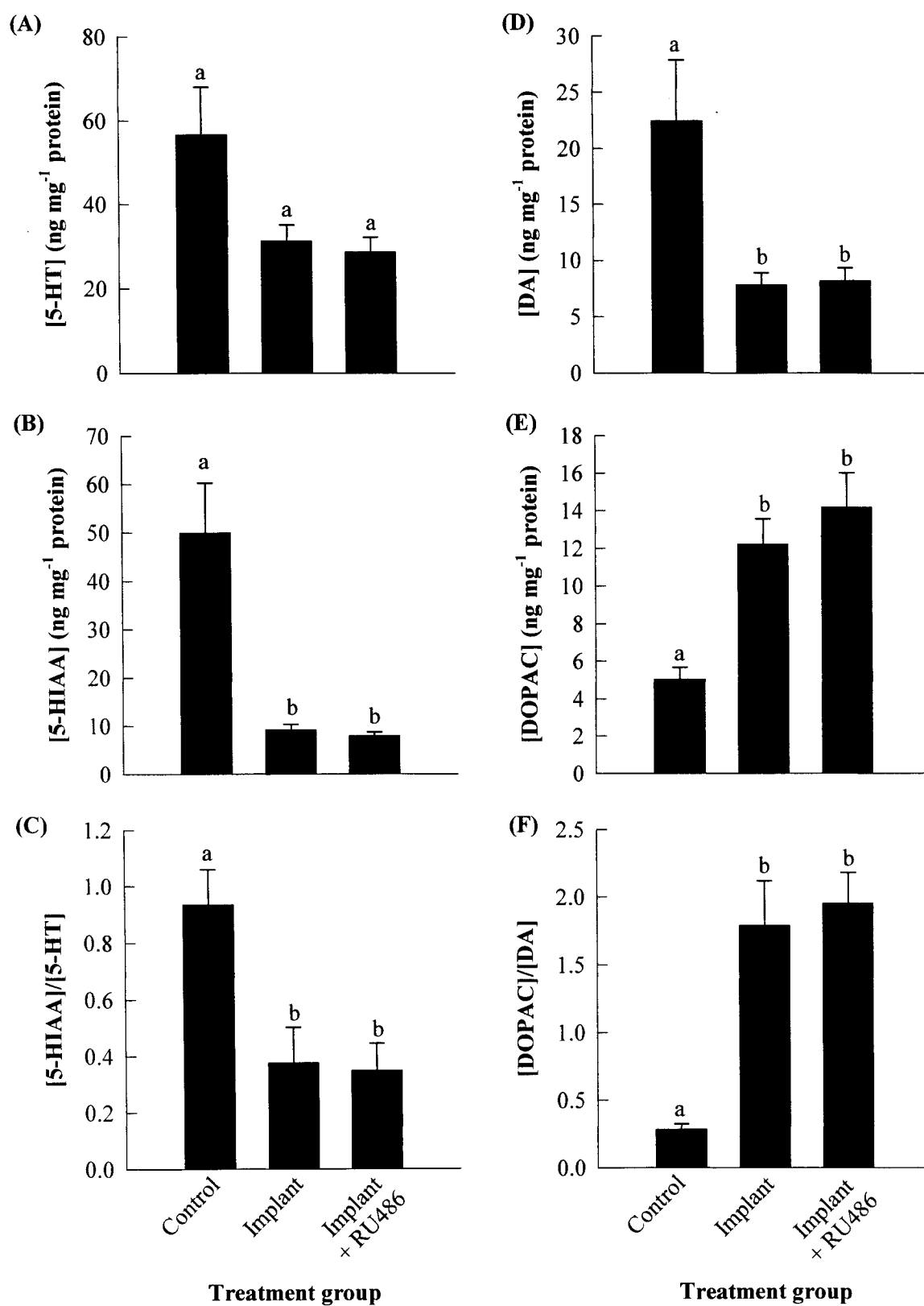


Table 2-1. Plasma cortisol concentrations after 5 days for rainbow trout (*Oncorhynchus mykiss*) given intraperitoneal implants of different forms (free form, sodium salt) and doses (110 mg kg^{-1} , 220 mg kg^{-1}) of cortisol, using different vehicles (coconut oil, cocoa butter).

Type of Cortisol	Type of Vehicle	Cortisol Dose (mg kg^{-1})	Plasma [cortisol] (ng mL^{-1})
free form	Coconut Oil	110	26.0 ± 20.3 (3)
free form	Coconut Oil	220	42.5 ± 23.4 (3)
sodium salt	Coconut Oil	110	$38.6 \pm 10.4^*$ (7)
sodium salt	Coconut Oil	220	$79.1 \pm 26.5^*$ (8)
sodium salt	Cocoa Butter	110	$284.8 \pm 112.2^*$ (5)
sodium salt	Cocoa Butter	220	$879.5 \pm 169.8^*$ (5)

Values are means $\pm$ 1 S.E.M. (*N*). An asterisk indicates a significant difference from a cortisol value of 10 ng mL^{-1} , values below which are characteristic of unstressed fish (one-sample t-test under the null hypothesis (H_0) that plasma [cortisol] was not significantly greater than 10 ng mL^{-1}).

Table 2-2. A summary of the chi-square analysis of the effects of cortisol treatment on social status in size-matched pairs of rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days.

Treatment	χ^2	$\chi^2_{0.05,1}$	Conclusion
Sham	2 (16)	3.84	Fail to reject H_0 ($P > 0.1$)
Cortisol	5.14 (14)	3.84	Reject H_0 ($P < 0.025$)
Cortisol + RU486	0.8 (10)	3.84	Fail to reject H_0 ($P > 0.25$)

Numbers in parentheses are N values. The null hypothesis (H_0) was that there was a 50% probability that the treated fish within a pair would become subordinate. The alternative (H_A) was that the treatment tested had a significant effect on this probability.

Table 2-3. A summary of the chi-square analysis of the effects of cortisol treatment on social status in size-mismatched pairs of rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days.

Treatment	χ^2	$\chi^2_{0.05,1}$	Conclusion
Control	14.28 (14)	3.84	Reject H_0 ($P < 0.001$)
Sham	1.63 (11)	3.84	Fail to reject H_0 ($P > 0.1$)
Cortisol	0.8 (10)	3.84	Fail to reject H_0 ($P > 0.25$)

Numbers in parentheses are N values. The null hypothesis (H_0) was that there was a 50% probability that the larger fish within a pair would become dominant. The alternative (H_A) was that the treatment tested had a significant effect on this probability.

Table 2-4. Specific growth rates (SGR) and final condition factors (CF_f) of dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) from sham, cortisol, and cortisol + RU486 treatment groups confined in size-matched pairs for 5 days.

Treatment	Social Status	SGR (% growth/day)	CF _f (100 x g cm ⁻³)
Sham	Dominant	0.17 ± 0.22* (16)	1.09 ± 0.02* (16)
	Subordinate	-0.72 ± 0.19 (16)	1.00 ± 0.02 (16)
Cortisol	Dominant	0.25 ± 0.12* (14)	1.09 ± 0.03* (14)
	Subordinate	-1.16 ± 0.19 (14)	0.99 ± 0.03 (14)
Cortisol +	Dominant	-0.58 ± 0.13** (10)	1.04 ± 0.03 (10)
RU486	Subordinate	-1.01 ± 0.13 (10)	1.05 ± 0.04 (10)

Values are means ± 1 S.E.M. (N). An asterisk indicates a significant difference between dominants and subordinates within the same treatment group, while a double asterisk indicates a significant difference from the corresponding social status group within the sham and cortisol treatment (two-way ANOVA with treatment group and social status as factors, for SGR, $P = 0.029$ for treatment group, $P < 0.001$ for social status, and $P = 0.042$ for interactions between these two factors; for CF_f, $P = 0.978$ for treatment group, $P = 0.011$ for social status, and $P = 0.158$ for interactions).

Table 2-5. Specific growth rates (SGR) and final condition factors (CF_f) of dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) from control, sham and cortisol treatment groups confined in size-mismatched pairs for 5 days.

Treatment	Social Status	SGR (% growth/day)	CF _f (100 x g cm ⁻³)
Control	Dominant	0.70 ± 0.28* (14)	1.08 ± 0.03* (14)
	Subordinate	-0.76 ± 0.16 (14)	0.99 ± 0.03 (14)
Sham	Dominant	0.26 ± 0.34 (11)	1.09 ± 0.03 [#] (11)
	Subordinate	-0.44 ± 0.47 (11)	0.99 ± 0.03 [#] (11)
Cortisol	Dominant	-0.30 ± 0.22** (10)	1.04 ± 0.03 (10)
	Subordinate	-1.05 ± 0.29 (10)	1.05 ± 0.04 (10)

Values are means ± 1 S.E.M. (N). An asterisk indicates a significant difference between dominants and subordinates within the same treatment group, a double asterisk indicates a significant difference from the corresponding social status group within the control treatment, while a number sign indicates a significant difference from the cortisol group as a whole (two-way ANOVA with treatment group and social status as factors. for SGR, $P = 0.084$ for treatment group, $P < 0.001$ for social status and $P = 0.344$ for interactions between these two factors; for CF_f, $P = 0.027$ for treatment group, $P = 0.002$ for social status and $P = 0.216$ for interactions).

Discussion

The findings of the present study implicate circulating cortisol concentrations as one factor that determines the outcome of competitive social interactions between pairs of rainbow trout. Specifically, high plasma cortisol levels predisposed individual trout towards low social status. Although the physiological mechanisms underlying the effect of cortisol on the outcome of agonistic encounters remain to be fully elucidated, the data support a role for interactions between circulating cortisol concentrations and brain monoaminergic activity as an indirect modulator of competitive ability.

Competitive ability is probably the key determinant of the winner of agonistic contests within groups or pairs of juvenile salmonid fish, although factors such as prior residence can also play a role (Bachman, 1984; Rhodes and Quinn, 1998; Cutts et al., 1999). Competitive ability, in turn, reflects numerous factors including innate aggressiveness (Adams et al., 1998; Cutts et al., 1999), prior experience of winning or losing social contests (Abbott and Dill, 1985; Rhodes and Quinn, 1998), body size in some cases (Abbott and Dill, 1985; Rhodes and Quinn, 1998), and, presumably, physiological condition. Physiological parameters such as abundant energy reserves, good condition and perhaps high metabolic capacity might be expected to correlate with competitive strength. For example, among several salmonid species, fish with higher metabolic rates prior to social interactions tended to achieve higher social status (Metcalf et al., 1995; Yamamoto et al., 1998; Cutts et al., 1999; McCarthy, 2001). High metabolic rate was associated with greater levels of aggression in juvenile Atlantic salmon (Cutts et al., 1998), suggesting a mechanism through which high metabolic rate could translate into competitive success, and emphasizing the complexity of factors that

determine competitive ability.

Previous work suggested that circulating cortisol levels might also be a physiological factor that affects competitive ability (Gregory and Wood, 1999; Sloman et al., 2001a). Specifically, Sloman et al. (2001a) documented significantly higher plasma cortisol levels prior to social interaction in rainbow trout that were identified as subordinate following pairing with a conspecific. Similarly, Gregory and Wood (1999) attributed the greater fin damage sustained by cortisol-treated trout held together with untreated trout to a cortisol-related competitive disadvantage. The results of the present study confirmed and extended these observations by revealing a causal relationship between high plasma cortisol concentrations prior to social interactions, and subsequent subordinate social status within pairs of rainbow trout. Experimental elevation of plasma cortisol levels was associated with a statistically significant increase in the probability of relegation to subordinate rank, an effect that was eliminated by blocking cortisol receptors using the glucocorticoid receptor antagonist RU486 (Table 2-2). Notably, the few cortisol-treated fish that did achieve dominant status failed to exhibit a significant elevation of plasma cortisol concentrations (Fig. 2-2). As cortisol levels in the plasma reflect the balance between cellular biosynthesis and secretion into the blood (*i.e.*, production), as well as clearance of the hormone from circulation (Mommensen *et al.*, 1999); the possibility also exists that these fish were able to counter the effects treatment via increases in their respective rates of cortisol clearance from the plasma, although this remains to be tested.

The observed effects in our study are in line with previous reports in which social status was closely linked with the magnitude of the cortisol response (Pottinger and Carrick, 2001). Pottinger and Carrick (2001) found that genetically maintained lines of

rainbow trout selected for high responsiveness (HR) to cortisol preferentially became subordinate when paired with size-matched, low-responsive (LR) trout in staged social settings. In addition, we found that cortisol treatment countered the effect of large size in determining dominance (Table 2-3). Larger fish became dominant in 86% of pairs in which both fish were untreated, a result that was in agreement with previous studies that reported a positive correlation between body size and dominant social status in trout (Bachman, 1984; Abbott and Dill, 1985). This significant effect of size in assuring dominant status was lost when the larger fish were given a cortisol-impregnated i.p. implant: only 40% of cortisol-treated large fish became dominant. Sham treatment also reduced the advantage normally conferred by large body size, albeit to a lesser extent than cortisol administration (63% of sham-treated large fish became dominant), even though the plasma cortisol levels measured in sham-treated fish at the end of the 5 day experimental period did not differ significantly from those of untreated fish (Fig. 2-2). It is likely that plasma cortisol concentrations were elevated in a transient fashion in sham-treated fish in response to handling stress and/or pellet implantation, but had returned to control levels by the time of sampling. Although a 2-day recovery period following pellet implantation was adopted (Gamperl et al., 1994), plasma cortisol titers may still have been high in sham-treated fish during the initial stages of social interactions, accounting for the lower than expected percentage of large fish that became dominant.

The causal relationship between high plasma cortisol levels and low social status in rainbow trout could be the result of one or more underlying physiological mechanisms. One possibility is that high circulating cortisol levels affect competitive ability directly by depressing physiological condition so that fish are not able to compete effectively.

Prolonged experimental administration of cortisol lowers growth rate and condition factor, and increases mortality (Barton et al., 1987; Pickering and Pottinger, 1989; Gregory and Wood, 1999), effects that have been attributed to appetite suppression, the mobilisation of energy reserves, changes in digestive tract morphology, reduced food conversion efficiency, increased metabolic rate, and changes in immune cell numbers and function that reduce resistance to common bacterial and fungal diseases (Barton et al., 1987; Pickering and Pottinger, 1989; Morgan and Iwama, 1996; Gregory and Wood, 1999; De Boeck et al., 2001). For example, the mean specific growth rate of cortisol-treated fish in the present study was significantly lower over the 5 day interaction period than that of the untreated fish with which they were paired, an effect that was cortisol-specific since it was eliminated by co-administration of RU486. Similarly, the chronic elevation of plasma cortisol attendant upon low social status probably accounted for, at least in part (see Gilmour et al., 2005), the significantly lower growth rates and final condition factors of subordinate fish relative to dominant fish within the control, sham, and cortisol groups of size-mismatched and size-matched pairs, respectively (Tables 2-4, 2-5). These findings are in agreement with previous reports in which lower growth rates and/or condition factor were exhibited by fish of low social status (Abbott and Dill, 1989; Sloman et al., 2000a; Sloman et al., 2000b). However, the deleterious impact of cortisol on physiological condition reflects prolonged elevation of the hormone, whereas cortisol levels were raised in the present study only 48 h prior to the initiation of social interactions. Thus, while cortisol-induced physiological depression may diminish competitive ability, and may have contributed to the association reported by Sloman et al. (2001a) between higher plasma cortisol prior to pairing and subsequent subordinate

status, it is unlikely to be the sole explanation for the results of the present study.

Alternatively, cortisol could affect competitive ability by modulating behaviour in either a direct or indirect fashion. For example, time- and context-dependent effects of cortisol administration were observed on aggressive behaviour and locomotory activity in rainbow trout (Øverli et al., 2002a). The locomotory response to an intruder was enhanced after 1 h of cortisol treatment, whereas both aggressive behaviour and activity in an intruder test were inhibited following 48 h of cortisol treatment. Locomotory activity in the absence of an intruder was unchanged by cortisol treatment, suggesting an indirect role for cortisol in modifying behaviour, through interactions with other signaling systems activated under particular circumstances (Øverli et al., 2002a). Similarly, studies involving HR and LR rainbow trout found that high-responsive trout reacted to stress-induced increased plasma cortisol concentrations by marked changes in locomotor activity, whereas low responsive trout did not (Øverli et al., 2001; Øverli et al., 2002b). With respect to social interactions in salmonid fish, brain monoamines, specifically serotonin and dopamine, represent a signaling system of particular interest because the behaviours characteristic of high or low social status are thought to result in large part from the changes in brain monoaminergic activity that accompany victory or loss in competitive encounters (reviewed by Winberg and Nilsson, 1993a). The results of the present study support, albeit not conclusively, a role for cortisol in modifying brain monoaminergic activity, and hence suggest that the causal link between high cortisol and low social status may reflect an indirect modulatory action of cortisol on behaviour mediated through brain monoaminergic systems.

Relative to control fish, serotonergic activity was markedly higher and dopaminergic activity was lower in the telencephalon of trout in which circulating cortisol levels were raised by means of an i.p. cortisol implant (Fig. 2-4). This result is consistent with work on other vertebrate groups in which corticosteroids have been found to affect brain serotonergic activity (reviewed by Chaouloff, 1993; Chaouloff, 2000). For example, intraperitoneal injection of corticosterone in male *Anolis carolinensis* lizards significantly enhanced serotonergic activity within 20 min in two separate brain regions (Summers et al., 2000), and intracortical infusion evoked transient, dose-dependent increases in serotonin overflow from neurons in the hippocampus (Summers et al., 2003). In fish, HR rainbow trout displayed significant increases in brain serotonergic activity within various brain regions in the face of increased plasma cortisol concentrations, whereas low responsive trout appeared resistant to these changes (Overli et al., 2001). Moreover, the effects of cortisol administration on telencephalon serotonergic and dopaminergic activity observed in our study seem to mimic those produced by defeat in an agonistic contest (Winberg et al., 1991; Winberg et al., 1992). Experimental treatments designed to increase brain 5-HT levels and/or serotonergic activity generally have been reported to elicit behavioural inhibition in fish (but see Stoddard et al., 2003), whereas high brain dopaminergic activity, on the other hand, seems to facilitate aggressive behaviour (Winberg and Nilsson, 1993a). For example, aggressive behaviour in rainbow trout was suppressed by dietary administration of the 5-HT precursor L-tryptophan, a treatment that also increased brain serotonergic activity (Winberg et al., 1991). Similarly, territorial aggression in a coral reef fish was depressed by intraperitoneal injection of the 5-HT selective reuptake inhibitor fluoxetine (Perrault et

al., 2003), while intracranial injection of either 5-HT or fluoxetine inhibited aggressive “chirping” behaviour in a weakly electric fish (Maler and Ellis, 1987). Aggressive behaviour in several salmonid species was increased following treatment with the DA receptor agonist apomorphine (Tiersch and Griffith, 1988) or DA itself (Nechayev and Musatov, 1992), and oral administration of the DA precursor, L-dopa, increased the probability of winning dominant social status in Arctic charr (Winberg and Nilsson, 1992). Thus, high circulating cortisol levels may be linked to low social status through a pathway in which cortisol-induced increases in brain serotonergic activity and/or decreases in dopaminergic activity result in the inhibition of the aggressive behaviour critical for success in agonistic encounters.

Within the hypothalamus, cortisol treatment resulted in a significant decrease of serotonergic activity and an increase of dopaminergic activity (Fig. 2-5), effects opposite to those observed in the telencephalon of cortisol-treated fish (Fig. 2-4), and opposite also to the impact of social defeat on hypothalamic serotonergic activity (Winberg and Nilsson, 1993a). The hypothalamus is a key component of the hypothalamic-pituitary-interrenal (HPI) stress axis in fish (Wendelaar Bonga, 1997; Mommsen et al., 1999). Hypothalamic corticotropin releasing factor acts on the pituitary to stimulate the secretion of adrenocorticotrophic hormone, which in turn elicits cortisol synthesis and mobilisation from interrenal cells. Cortisol secretion via this pathway may be modulated by the negative feedback actions of cortisol at the levels of the hypothalamus and pituitary (Mommsen et al., 1999), and several lines of evidence suggest that hypothalamic 5-HT also may be involved in the regulation of the HPI axis (*e.g.*, Winberg et al., 1997a; Hoglund et al., 2002; Lepage et al., 2002; Lepage et al., 2003). Experimental elevation of

plasma cortisol would be expected to down-regulate endogenous cortisol secretion via negative feedback. It is conceivable that the lowering of hypothalamic serotonergic activity observed in cortisol-treated fish reflected such a down-regulation of cortisol secretion pathways.

The changes of monoaminergic activity in the telencephalon and hypothalamus of rainbow trout given i.p. cortisol implants were in general not abolished by co-administration of the glucocorticoid receptor antagonist RU486 (Figs. 2-4, 2-5), except in the case of telencephalon serotonergic activity (Fig. 2-4C). While these findings raise concerns about whether the responses were cortisol-specific, at least two plausible explanations exist. First, the responses may be mediated via a mineralocorticoid receptor (MR) rather than a glucocorticoid receptor (GR). In mammals, MRs and GRs exhibit different expression patterns in the brain and play different roles in mediating the effects of corticosteroids (Chaouloff, 2000; Korte, 2001). GRs are widely distributed in the forebrain of rainbow trout (Teitsma et al., 1997; Teitsma et al., 1998) and the recently identified fish MR (Colombe et al., 2000; Greenwood et al., 2003) also appears to be present in the brain (Greenwood et al., 2003). However, the relative distributions and roles of the two corticosteroid receptors in fish brains remain to be explored. Alternatively, cortisol may exert effects in the brain via non-genomic mechanisms, a route of action that has been well documented in mammals (reviewed by Makara and Haller, 2001). For example, Mikics (2004) suggested that glucocorticoids rapidly increased aggressive behaviour in rats via non-genomic mechanisms. The non-genomic effects of corticosteroids are much more rapid than the genomic responses, and resistant to both GR and MR blockade (Makara and Haller, 2001).

In conclusion, the results of the present study revealed the causal nature of the association between high plasma cortisol concentrations and low social status, and in general supported cortisol-induced changes in brain monoaminergic activity as a potential regulatory pathway for this effect. Clearly, however, additional work is required to validate the hypothesis that high circulating cortisol levels modify brain serotonergic activity and/or dopaminergic activity in trout, resulting in the suppression of aggressive behaviour and a consequent lowering of competitive ability that increases the likelihood of relegation to subordinate social status during agonistic contests.

CHAPTER 3

AN INVESTIGATION OF FACTORS THAT CONTRIBUTE TO THE LOW GROWTH RATES OF SUBORDINATE RAINBOW TROUT (*ONCORHYNCHUS* *MYKISS*)

This chapter forms the basis of a manuscript to be submitted to an appropriate
physiological journal for publication.

Abstract

To assess the effects of subordinate social status on digestive function, intermediary metabolism, and enzyme activity in salmonid fish, juvenile rainbow trout, *Oncorhynchus mykiss*, were paired with a size-matched conspecific (<1.5% difference in fork length) for five days. Fish fasted for 5 days and fish sampled directly from the holding tank were used as control groups. Both subordinate and fasted fish experienced significant decreases in intestine mass ($P = 0.043$), and the gall bladder showed marked and significant changes in both size ($P = 0.004$) and appearance. These findings suggest that the negative impact of social subordination on digestive function reflects in large part a lack of feeding. Hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity was significantly higher in subordinate fish relative to dominants, whereas subordinate pyruvate kinase (PK) activity was significantly lower; activities of both enzymes were significantly correlated with plasma cortisol concentrations and behaviour scores. Dominant-subordinate differences in the activities of these enzymes were eliminated by administration of the glucocorticoid receptor antagonist RU486, underlining a role for circulating cortisol in eliciting the differences. Significant increases relative to control fish were also detected in red and white muscle from subordinate fish in the activity of protein catabolic enzymes (aspartate aminotransferase, alanine aminotransferase, glutamate dehydrogenase). These differences occurred in the absence of change in plasma free amino acid or ammonia concentrations, suggesting an enhanced turnover of amino acids in muscle in subordinate fish. The results support the hypothesis that changes in intermediary metabolism, beyond those elicited by low food consumption, may be responsible at least in part for the low growth rates typical of subordinate fish,

and that these changes may be related specifically to circulating cortisol levels in subordinate fish.

Introduction

Juvenile salmonid fish, like many other vertebrates, form dominance hierarchies in which subordinate individuals experience chronic social stress (reviewed by Sloman and Armstrong, 2002; Gilmour et al., 2005). A well-characterized physiological consequence of subordinate social status is a reduction in growth rate (Metcalf, 1986; Abbott and Dill, 1989; Sloman et al., 2000b; Sloman et al., 2000c). Lower growth rates reflect reduced food intake owing to the monopolisation of food sources by dominant individuals (Metcalf et al., 1989; Adams and Huntinford, 1996; Adams et al., 1998), as well as appetite suppression (see Bernier and Peter, 2001; Bernier et al., 2004). However, even in cases where equal rations are consumed, subordinate fish do not achieve the growth rates of dominants (Abbott and Dill, 1989), indicating that factors beyond exclusion from food affect the growth of subordinates. Several authors have raised the possibility that a down-regulation of digestive function could contribute to the lower growth rates of subordinate fish (Olsen and Ringø, 1999; Stevens and Devlin, 2000; Earley et al., 2004). Olsen and Ringø (1999) reported that subordinate Arctic charr, *Salvelinus alpinus* L., experienced reduced nutrient digestibility and food absorption compared to dominants and attributed these effects to impaired function of the gastrointestinal tract. In addition, chronic social subordination in convict cichlid fish resulted in bile retention and enlargement of the gall bladder (Earley et al., 2004), a physiological response to low social status that likely affects the capacity to digest food, particularly lipids (Horn, 1998).

Low growth rates in subordinate fish might also reflect changes in overall metabolic capacity. In a recent study on brown trout, *Salmo trutta*, confinement with a

conspecific resulted in an increase in the standard metabolic rate (SMR) of subordinate fish (Sloman et al., 2000c), a finding which suggests that a metabolic disadvantage is associated with subordination. Whether these differences translate into changes in the enzyme activities associated with major metabolic pathways remains to be determined. In addition, subordinate fish often display physiological changes indicative of the mobilisation of energy reserves, which can negatively impact growth. Reductions in condition factor (Sloman et al., 2000a; Sloman et al., 2000b), hepatic glycogen content (Ejike and Schreck, 1980), and liver condition (Sloman et al., 2001b), as well as increased protein catabolism (Mommensen et al., 1999) and plasma glucose levels (Peters et al., 1988; Elofsson et al., 2000) all have been associated with subordinate social status.

Many of these negative physiological changes probably can be attributed, at least in part, to the chronically high circulating cortisol levels that are characteristic of socially subordinate salmonid fish (reviewed by Gilmour et al., 2005). Several studies in fish have demonstrated that cortisol treatment results in growth suppression (Barton et al., 1987; Gregory and Wood, 1999; De Boeck et al., 2001). Cortisol treatment results in morphological alterations to the digestive tract (Barton et al., 1987), reduced food conversion efficiency (Gregory and Wood, 1999), and a chronically elevated metabolic rate (Morgan and Iwama, 1996; De Boeck et al., 2001), all of which negatively impact growth. Transient elevations of circulating cortisol levels are generally considered to be adaptive because this hormone mobilizes energy reserves and helps the organism cope with the increased energy demands normally associated with stress. However, long-term elevation of plasma cortisol, as occurs with social subordination, is associated with many

detrimental physiological consequences (reviewed by Wendelaar Bonga, 1997; Mommsen et al., 1999).

Therefore, the present study assessed the impact of subordinate social status on digestive capacity and on the activities of several key enzymes involved in gluconeogenesis, glycolysis, and amino acid metabolism. The objective was to generate a working model to account for a variety of factors that may contribute to the observed growth suppression of subordinate fish. The effects of social status *per se* and socially-induced differences in food consumption between dominant and subordinate fish were teased apart through the inclusion in the experimental design of a group of fasted fish. Moreover, the impact of elevated cortisol concentrations in subordinate fish was evaluated through the use of a glucocorticoid receptor antagonist, RU486.

Materials and Methods

Experimental animals

Juvenile female rainbow trout (weight 77.27 ± 1.92 g [mean $\pm$ SEM], $N = 114$) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). All fish were held for several weeks at the University of Ottawa in large 1,275-L fibreglass stock tanks supplied with flowing, aerated, and dechloraminated city of Ottawa tap water at a temperature of $13 \pm 1^\circ\text{C}$. A 12L: 12D photoperiod was used. Throughout this period, fish were hand-fed to satiation every second day on a commercial trout food diet (Purina Trout Chow).

Cortisol pilot trial

In preliminary trials, the cortisol response of selected fish was blocked to explore the role of circulating plasma cortisol during social interactions on growth, physiological condition, and food intake. All fish were lightly anaesthetized (*i.e.* the point at which the test animal lost equilibrium while maintaining a normal ventilation frequency) in a solution of benzocaine (0.05 g L^{-1} ethyl-*p*-aminobenzoate) and initial masses and fork lengths were measured (weight 50.97 ± 2.91 g; fork length 168.05 ± 2.49 mm, $N = 20$). Fish were paired with a conspecific that was size-matched ($<1.5\%$ difference) on the basis of fork length, and pairs of trout were then placed in individual 40-L flow-through plexiglass observation tanks. One fish within each pair received a cocoa butter pellet ($0.005 \text{ ml cocoa butter g}^{-1}$ fish) containing dissolved RU486 ($500 \text{ mg mifepristone kg}^{-1}$ fish), while the other was given a sham injection of cocoa butter only. High affinity binding of RU486 to cytosolic cortisol receptors has been reported in rainbow trout liver

preparations (Pottinger, 1990) and validated in fish fibroblast cell lines (Lee and Bols, 1989), making this an appropriate agent for selective blockade of glucocorticoid receptors. The concentration of RU486 was chosen on the basis of previous work indicating that treatment with this compound is most effective at a dose 10-fold greater than that of circulating cortisol concentrations (Vijayan et al., 1994b). All paired fish were separated by an opaque perforated divider for a 24 h recovery and acclimation period, and the dividers were then removed to allow pairs of fish to interact for 5 days; a small piece of PVC tubing was placed within each tank to provide shelter. Daily food intake was quantified (see description below) and social status was assigned based on daily behavioural observations using an integrated point system (see description below). Fish were killed after 5 days by immersion in a lethal dose of anaesthetic (ethyl-*p*-aminobenzoate 0.5 g L^{-1}). Blood samples ($\sim 1 \text{ mL}$) were removed via caudal puncture, and separated plasma was frozen in liquid nitrogen and stored at -80°C until analysis for cortisol concentration using a commercial RIA kit (ICN pharmaceuticals).

Experimental protocol

Two experiments were carried out. In the first, fish were lightly anaesthetized in a solution of benzocaine (0.05 g L^{-1} ethyl-*p*-aminobenzoate) and initial masses and fork lengths were measured (mass $88.84 \pm 2.27 \text{ g}$; fork length $197.21 \pm 1.88 \text{ mm}$, $N = 34$). Twelve randomly selected fish were placed in a 780-L holding tank and were fasted for 5 days. The remaining fish ($N = 11$ pairs) were paired with a conspecific that was size-matched ($<1.5\%$ difference) on the basis of fork length, and pairs of trout were then placed in individual 40-L flow-through plexiglass observation tanks. For the second

experiment, fish were lightly anaesthetized as above, initial measurements were taken (mass 86.05 ± 1.25 g; fork length 195.21 ± 0.99 mm, $N = 48$), and fish were then paired with a size-matched conspecific. One fish within each pair received a cocoa butter pellet (0.005 ml cocoa butter g^{-1} fish) containing dissolved RU486 (500 mg mifepristone kg^{-1} fish), while the other was given a sham injection of cocoa butter only. All paired fish were separated by an opaque perforated divider for a 24 h recovery and acclimation period, and the dividers were then removed to allow pairs of fish to interact for 5 days; a small piece of PVC tubing was placed within each tank to provide shelter. It should also be noted that in the interest of generating a sufficient number of samples in the RU486 treated subordinate category (*i.e.* $N \geq 8$), as social status could not be predicted prior to pairing and subsequent hierarchy formation, initially, a large number of pairs were set-up ($N = 24$ pairs).

Behavioural observations were carried out on all paired fish twice a day for 5 days. Observations were first conducted fifteen minutes after the opaque divider was removed, and then once between 9:00-11:30 h and once between 15:00-17:30 h each day, for 10 min. The order of tank observation was randomized to account for any observational bias. Paired fish were hand fed to satiation once a day, after all behavioural observations were completed ($\sim 17:45$ h). To accurately record the amount of food consumed by each fish, the dividers were re-inserted for a period of 15 minutes during feeding so that each fish could be fed separately. Fish were fed one pellet at a time, up to a maximum of 2% total body weight, until three consecutive pellets were rejected; all uneaten food was then removed from the tank and the dividers were once again taken away.

Social status was assessed by assigning points to each fish for position, food acquisition, aggressive behaviour, and fin damage: the fish within the pair with the higher final integrated score was then identified as the dominant, whereas that with the lower score was classified as subordinate. The point system that was employed has been used previously for assigning social status among salmonid fish (Johnsson et al., 1996; Sloman et al., 2000a; Sloman et al., 2000b; Sloman et al., 2001a). In brief, fish were scored twice daily on the basis of their position in the tank according to the following criteria: 10 points for maintenance of position within the water column, 5 points for resting on the bottom of the tank or hiding within the PVC tubing, and zero points for fish that attempted to swim at the surface (a behaviour indicative of subordination: (Sloman et al., 2000a)). Fish were also scored according to aggressive behaviour. Fish directing five or more aggressive attacks towards the other individual within an observation period were given a score of two, fish performing between one and four aggressive attacks were given a score of one, and those individuals performing no aggressive attacks received a score of zero. To score fish on food acquisition, one pellet of food was dropped into the tank at the beginning of each observation period and the fish that took the pellet scored one point. Fish that either attempted to get a food pellet but lost out to the other fish or did not attempt to feed at all were given a score of zero points. Finally, fish were scored on a 3 point scale according to the extent of dorsal and caudal fin damage sustained during the five-day interaction period (a score of 0 was given to fish with fins missing >70% of the total tissue). A single behaviour score was calculated from all observations by means of a principal components analysis (PCA; SPSS 10.1) (Sloman et al., 2000c) and used to assign social status to individual fish.

At the end of the 5-day experimental period, fish were rapidly killed by immersion in a lethal dose of anaesthetic solution (ethyl-*p*-aminobenzoate 0.5 g L⁻¹). In experiment 1, paired fish and the fasted fish were sampled at the end of 5 days together with control (*i.e.*, fed) fish ($N = 12$) that were sampled directly from the 1,275-L holding tank. In experiment 2, pairs of fish were sampled after 5 days. In both cases, the two fish within a pair were removed simultaneously from the tank and sampled within one minute of each other; the sampling order within each pair was randomized to control for any sampling bias. A blood sample (~1 mL) was removed via caudal puncture from all fish and following centrifugation (13,200 g for 3 min), plasma was removed, immediately frozen in liquid nitrogen, and stored at -80° C for subsequent cortisol and metabolite determinations (see below). Liver, red muscle, and white muscle (removed from the area below the dorsal fin and along the lateral line) tissue samples were also removed, placed in pre-chilled eppendorf tubes, and frozen immediately in liquid nitrogen. Tissue samples were stored at -80°C for the measurement of enzyme activities (see below). In experiment 2, only liver tissue samples were collected.

Cortisol, metabolite, and enzyme assays

Plasma cortisol concentrations were measured using a commercially available radioimmunoassay kit (ICN pharmaceuticals), while plasma glucose levels were determined enzymatically according to Keppler and Decker (1974). Plasma free amino acid and ammonia concentrations were measured as detailed in Troll and Cannan (1953) and Verdouw et al. (1978), respectively. It should be noted that this method of plasma amino acid determination does not allow the detection of proline.

For enzyme measurements, all reagents were prepared in 50 mM imidazole buffer (pH 7.8) and kept on ice. Tissue samples were homogenized 1:4 w/v in a buffer containing 20 mM Na₂HPO₄, 5 mM β-mercaptoethanol, 0.5 mM Na₂EDTA dihydrate, 0.2 % bovine serum albumin, and 50% [v/v] glycerol, adjusted to pH 7.4. Enzyme activities were measured kinetically at 22° C for 25 minutes using a microplate reader (Spectramax 384 plus, USD Device, Inc.) set to read at 340 nm as described by Henriksson et al. (1986). The enzyme activities assayed were, in order, phosphoenolpyruvate carboxykinase (PEPCK), pyruvate kinase (PK), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glutamate dehydrogenase (GDH). Protein concentrations for all tissue samples were measured using a commercially available kit (Bio-Rad) with bovine serum albumin as the standard. After ascertaining that tissue protein concentrations were not affected by treatment group (see below), enzyme activities were expressed as micromoles of substrate consumed per minute per g of wet tissue used.

Calculations and statistical analyses

Condition factor (CF) and specific growth rate (SGR) were calculated using the following formulae:

$$CF = 100 \cdot W/L^x$$

where W = weight of fish in grams, L = length of fish in centimeters, and x = slope of regression line of log W vs. log L (~ 3), and

$$SGR = [\ln(W_{Final}) - \ln(W_{Initial})] \cdot 100 / D$$

where W = weight of fish in grams and D = number of days elapsed.

To take into account allometric relationships between body mass and most anatomical characteristics (Earley et al., 2004), organ masses were standardized to body mass and multiplied by a factor of 100 to yield hepatosomatic index (HSI) and intestine somatic index (ISI). Based on the close relationship between the gall bladder and liver, gall bladder mass was expressed relative to liver mass (Talbot and Higgins, 1982; Earley et al., 2004).

All data are presented as means $\pm$ 1 standard error of the mean (S.E.M.). Differences in mean cumulative food intake between dominant and subordinate fish were evaluated using a Student's *t*-test. Chi-square analysis was used to evaluate the effects of treatment group on gall bladder/bile appearance in experiment 1. A one-way analysis of variance (ANOVA), followed by Bonferroni corrected *t*-tests, was used to analyse the effect of treatment group on all morphological measurements and plasma cortisol or metabolite concentrations, as well as all data from the pilot trials. Although non-transformed data are presented, non-normally distributed data were log transformed as appropriate to ensure homogeneity of variance. All enzyme activities in experiment 1 were analysed statistically using a one-way ANOVA on ranks followed by Dunn's post hoc pairwise multiple comparisons test, as were food consumption and growth data from experiment 2. Spearman rank correlation tests were used to assess the relationships between plasma cortisol concentrations or behaviour scores and hepatic PEPCK or PK activities. Finally, Student's *t*-tests were used to statistically analyse the differences between RU486 dominant and RU486 subordinate fish for plasma cortisol, plasma glucose, and liver enzyme activities in experiment 2. The α level for significance for all

tests was set at 0.05 and all statistical analyses were performed using SigmaStat v3.0 (SPSS, Inc) or SPSS v10.1 (SPSS, Inc) software.

Results

Cortisol pilot trial

In general, plasma cortisol concentrations at the end of the interaction period were indicative of social status, with subordinate fish tending to exhibit higher circulating cortisol levels than dominant fish (one-way ANOVA, $P = 0.017$) (Table 3-1). Further, reductions in specific growth rate (SGR) and condition factor (CF_f) normally associated with subordinate social status were evident in sham treated fish ($P = 0.002$), but partially relieved by RU486 treatment ($P = 0.737$). Although RU486 subordinates did not have SGR as high as dominant fish, they were not significantly different (sham dominant, $P = 0.706$; RU486 dominant, $P = 0.737$) and actually appeared to gain weight (0.17 ± 0.54 % growth day⁻¹) during the trial. A similar situation was seen with CF_f , where sham dominants had significantly higher CF_f than sham subordinates ($P = 0.009$), while this difference was abolished by RU486 treatment ($P = 0.76$). Interestingly, RU486 treatment had no effect on mean daily food intake, as the observed treatment group effect (one-way ANOVA, $P = 0.003$) was attributed to the significant differences between dominants and subordinates in both sham ($P = 0.022$) and RU486 ($P = 0.04$) treated fish.

Experiment 1: The impact of social status on physiological and metabolic parameters

Marked behavioural differences in feeding, aggression, and selection of tank positions, were observed between dominant and subordinate trout during the five-day interaction period. In general, both dominant and subordinate fish fed little in the early stages of hierarchy formation (*i.e.* day 1), whereas dominants exhibited substantial increases in feeding throughout the remainder of the interaction period (Fig. 3-1). The

mean cumulative daily food intake of dominant fish was significantly higher than that of subordinates at all time points after 24 h (48 h, $P = 0.005$; 72 h, $P < 0.001$; 96 h, $P < 0.001$). To control for possible effects of differences in food intake between dominant and subordinate fish, control (fed) and fasted groups were included in the experiment. Control fish were fed a daily ration ($6.5 \text{ mg food g}^{-1} \text{ body weight}$) comparable to the daily food intake of dominant fish ($6.90 \pm 1.71 \text{ mg food g}^{-1} \text{ body weight}$) averaged over the 5-day interaction period. The mean daily food intake of subordinate fish over 5 days was $0.95 \pm 0.47 \text{ mg food g}^{-1} \text{ body weight}$, while fasted fish received no food during this period.

Not unexpectedly, fish held under fasting conditions displayed the lowest mean specific growth rate ($-1.83 \% \text{ growth day}^{-1}$) of any group. Growth rates in subordinate fish were significantly lower than those of dominants (one-way ANOVA, $P = 0.008$), dominants having the highest growth rates of all treatment groups (Table 3-2). Neither condition factor (overall mean $CF_f = 1.07 \pm 0.014$, $N = 46$) nor hepatosomatic index (overall mean $HSI = 0.97 \pm 0.028$, $N = 46$), both of which are often used as predictors of an individual's energy reserves (Chellappa et al., 1995; Barton et al., 2002), was affected by treatment group (one-way ANOVA: CF_f , $P = 0.84$; HSI , $P = 0.47$). The differences in behaviour and growth rate between dominant and subordinate fish were accompanied by differences in plasma cortisol concentrations. As expected on the basis of previous work, subordinate fish exhibited significantly higher circulating cortisol levels than dominant fish ($P = 0.008$). Within the control and fasted groups, plasma cortisol levels were slightly above ($\sim 19 \text{ ng ml}^{-1}$) the values deemed to be representative of unstressed fish ($\sim 10 \text{ ng ml}^{-1}$; (for review see Gamperl et al., 1994)).

Intestine mass was significantly affected by treatment (one-way ANOVA, $P = 0.043$; Table 3-3), with fasted and subordinate fish exhibiting lower intestine masses than control or dominant fish. Similarly, gall bladder size (one-way ANOVA, $P = 0.003$) and appearance (Table 3-3) were influenced by fasting as well as social status. Gall bladder mass expressed relative to liver mass was significantly higher in fasted fish than in any other group (Table 3-3). As hepatosomatic index did not differ significantly among groups, the effect of treatment on gall bladder:liver mass ratio largely reflected differences in gall bladder mass. In addition, bile colour was affected by fasting and social status (Table 3-3, Fig. 3-2). Chi-square analysis indicated that, within the control group, the gall bladder was equally likely to be either dark green or colourless, whereas fasted and subordinate fish had dark green bile more often than expected by chance alone ($P < 0.001$ for both), and dominant fish usually had clear bile ($P < 0.001$). Interestingly, the only fish within the dominant group to display dark green bile fed little during the 5-day interaction period (mean daily food intake, $0.76 \text{ mg food g}^{-1} \text{ body weight}$), and the two subordinate fish that exhibited clear bile consumed uncharacteristically large quantities of feed (mean daily food intakes, 8.43 and $14.31 \text{ mg food g}^{-1} \text{ body weight}$).

Plasma glucose concentrations, which are often used as a reflection of the mobilization of energy reserves (Mommensen et al., 1999), were significantly affected by treatment group (one-way ANOVA, $P = 0.035$) (Table 3-2). While subsequent post-hoc analysis (Bonferroni's multiple comparison test) could not identify where those differences arose, the trends indicated lower plasma glucose concentrations within fasted fish and no difference between dominant and subordinate fish. Plasma ammonia (one-way ANOVA, $P = 0.074$, overall mean = $345.21 \pm 24.7 \text{ } \mu\text{mol l}^{-1}$, $N = 46$) and amino acid

concentrations (one-way ANOVA, $P = 0.274$, overall mean = $1.86 \pm 0.097 \text{ mg ml}^{-1}$, $N = 46$), both commonly used indicators of protein metabolic activity, were not significantly affected by treatment.

To assess the impact of social status on intermediary metabolism, while controlling for differences in food consumption between dominant and subordinate fish, the activities of several key enzymes involved in carbohydrate and amino acid metabolism were examined in liver and muscle. Tissue protein concentrations did not differ significantly among treatment groups in liver (one-way ANOVA on ranks, $P = 0.24$, overall mean = 87.71 ± 1.92 , $N = 34$), red muscle (one-way ANOVA on ranks, $P = 0.52$, overall mean = 57.12 ± 1.26 , $N = 34$), or white muscle (one-way ANOVA on ranks, $P = 0.072$, overall mean = 64.93 ± 1.55 , $N = 34$) and therefore enzyme activities were expressed on a per tissue weight basis, rather than being standardized to individual tissue protein content, owing to sample loss. Gluconeogenesis, the capacity to produce glucose, was evaluated by estimating the activity of liver PEPCK; while glycolytic capacity was estimated using liver PK activity. Hepatic PEPCK activity was significantly greater in subordinate fish (one-way ANOVA on ranks, $P < 0.001$) compared to all other treatment groups (Fig. 3-3A). By contrast, hepatic PK activity was significantly lower in both fasted and subordinate fish relative to dominants (one-way ANOVA on ranks, $P < 0.001$; Fig. 3-3B). To further explore the effects of differences in social status on these enzyme activities, correlations between plasma cortisol concentrations or behaviour score and hepatic PEPCK or PK activities were evaluated using the Spearman rank correlation test (Fig. 3-4). With no exception (Fig. 3-4A, $r = 0.677$, $P < 0.001$; Fig. 3-4B, $r = -0.747$, $P < 0.001$; Fig. 3-4C, $r = -0.575$, $P < 0.001$; Fig. 3-4D, $r = 0.747$, $P < 0.001$), enzyme

activities were significantly correlated with both plasma cortisol concentrations and behaviour scores. These relationships suggest that the impact of social status on hepatic enzyme activities is mediated at least in part through status-induced differences in plasma cortisol concentrations.

The activities of three aminotransferase enzymes were examined in liver, red muscle, and white muscle tissue (Table 3-4). Several interesting trends emerged from this analysis, although differences in aminotransferase activities were not consistent across enzymes or tissues. In no case did values for the fasted group differ significantly from those of the control or dominant fish, nor were any significant differences between dominant and subordinate fish apparent. However, subordinate fish exhibited elevated activities of AST in red muscle, and ALT in both red and white muscle relative to control fish, and both red and white muscle ALT activities were significantly higher also in dominant fish than in control fish. In addition, a significant difference between subordinate and fasted fish was observed for hepatic GDH activity.

Experiment 2: The role of cortisol

An experimental protocol in which size-matched sham- and RU486-treated individuals were paired was also used to investigate whether the responses to social stress observed in experiment 1 were cortisol-specific. Surprisingly, food consumption was not affected by treatment or social status (one-way ANOVA on ranks, $P = 0.117$), likely a function of the large proportion of dominant fish exhibiting low food intake values in this trial (16 out of 24 dominant fish had values < 0.5 mg food g^{-1} body weight). Further, while significant differences in SGR were apparent between dominant and subordinate

fish (one-way ANOVA on ranks, $P = 0.01$), all categories of fish (sham dominants, sham subordinates, RU486 dominants, RU486 subordinates) displayed negative growth rates and sham and RU486 subordinates were statistically similar ($P = 1.0$) (data not shown), observations seemingly in disagreement with data from our pilot trial. Therefore, in the interest of identifying cortisol specific responses from experiment 1, only RU486 treated fish from this experiment were considered. Cortisol levels in RU486-treated subordinate fish were significantly higher than those of RU486 dominants (Fig. 3-5A; Student's t-test, $P = 0.032$) but relatively low for both groups (*i.e.* $< 10 \text{ ng mL}^{-1}$). The absence of cortisol levels characteristic of stressed fish in RU486-treated subordinates was unexpected and suggested that RU486 treatment either directly or indirectly suppressed the characteristic cortisol response to low social status. In contrast, no effects were found when plasma glucose concentrations were examined (Fig. 3-5B; Student's t-test, $P = 0.549$).

As in experiment 1, hepatic protein concentrations were similar among all treatment groups ($P = 0.67$, overall mean = 109.67 ± 2.58 , $N = 22$) and, therefore, in the interest of being consistent, enzyme activities were expressed per unit mass of tissue. However, in contrast to social status associated enzymatic differences observed experiment 1, there was no significant differences between RU486-treated dominant and subordinate fish for both hepatic PEPCK (Fig. 3-6A; Student's t-test, $P = 0.189$) and PK activity (Fig. 3-6B; Student's t-test, $P = 0.268$), suggesting that these responses were abolished by RU486 treatment.

Figure 3-1. The effects of dominant and subordinate social status on mean cumulative daily food intake in pairs of rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days. Daily food intake was measured at 24 h, 48 h, 72 h and 96 h; all data are presented as mean values ± 1 S.E.M. Filled circles represents dominant fish while open circles represent subordinate fish ($N = 11$ for all data points). An asterisk indicates a significant difference between dominant and subordinate fish for the time point indicated (Student t-tests: 24 h, $P = 1.00$; 48 h, $P = 0.005$; 72h, $P < 0.001$; 96h, $P < 0.001$).

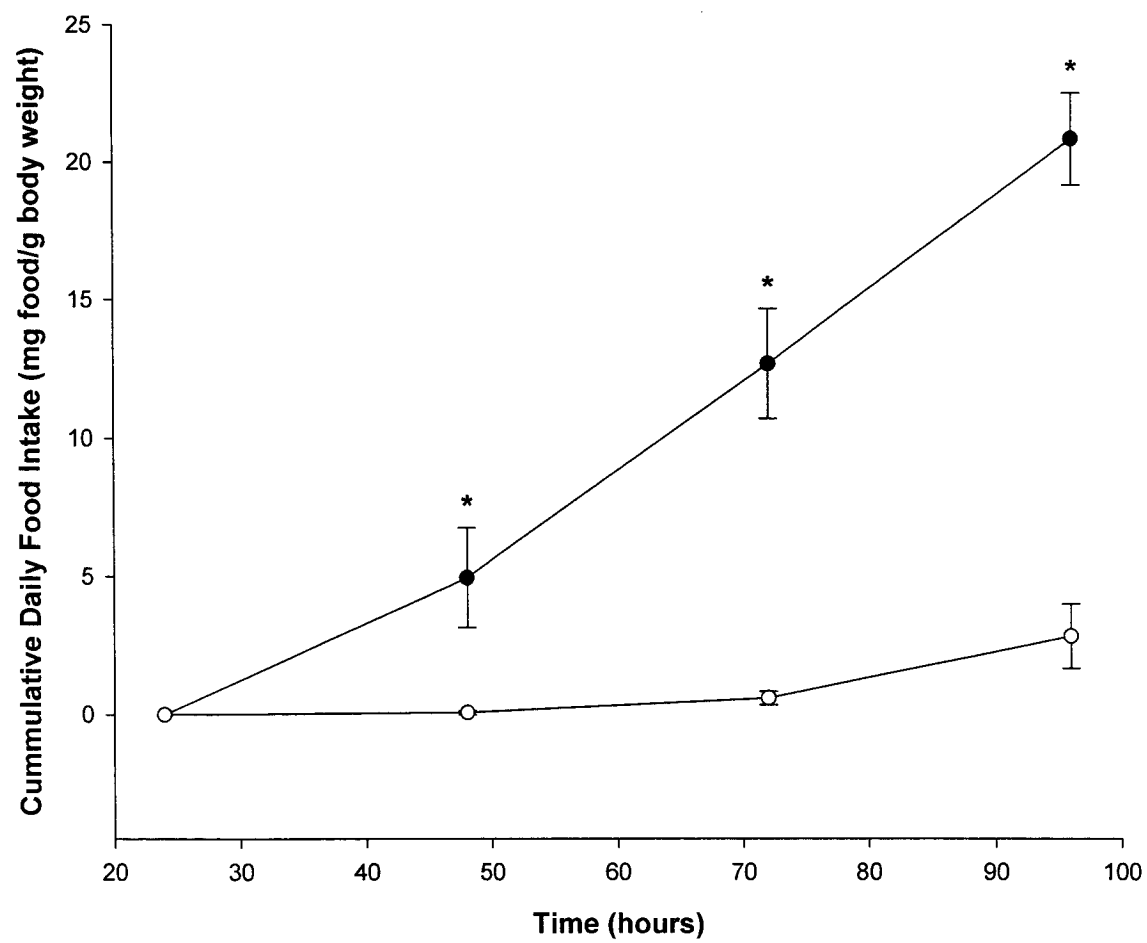


Figure 3-2. A comparison of the size and coloration of the gall bladder relative to the liver in (A) dominant versus (B) subordinate fish.

A



B

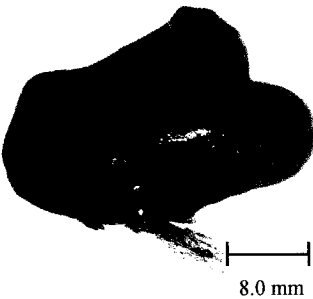


Figure 3-3. Hepatic phosphoenolpyruvate carboxykinase (PEPCK) (A) and pyruvate kinase (PK) activity (B) measured in control, 5 day fasted as well as dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days. Data are presented as mean values $\pm$ 1 S.E.M and *N* values are as follows: Control, *N* = 12; Fasted, *N* = 12; Dominant, *N* = 11; Subordinate, *N* = 11. Groups that do not share a letter were significantly different from one another (one-way ANOVA on ranks followed by Dunn's post hoc multiple comparisons test, with *P* values of A < 0.001 and B < 0.001).

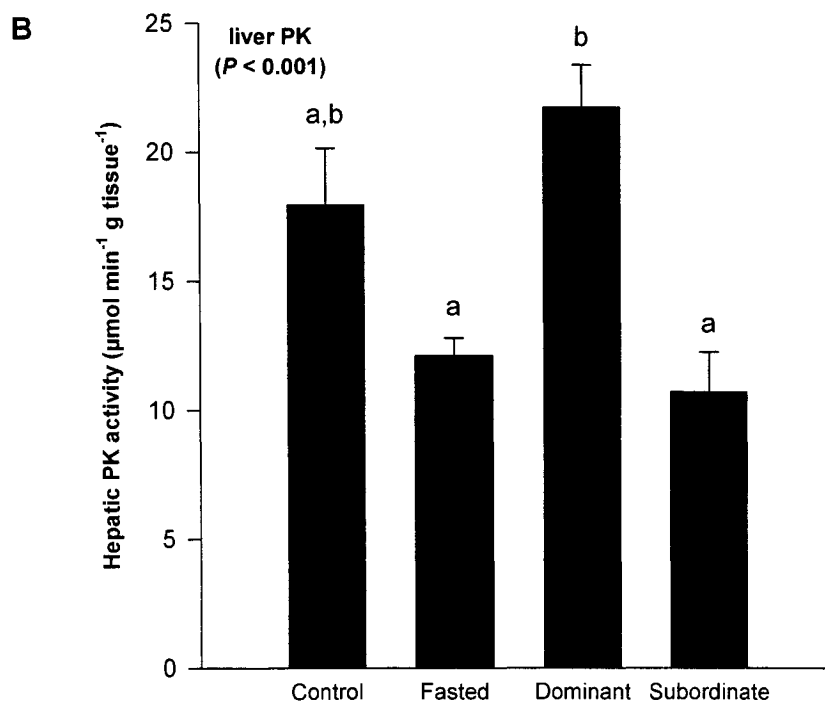
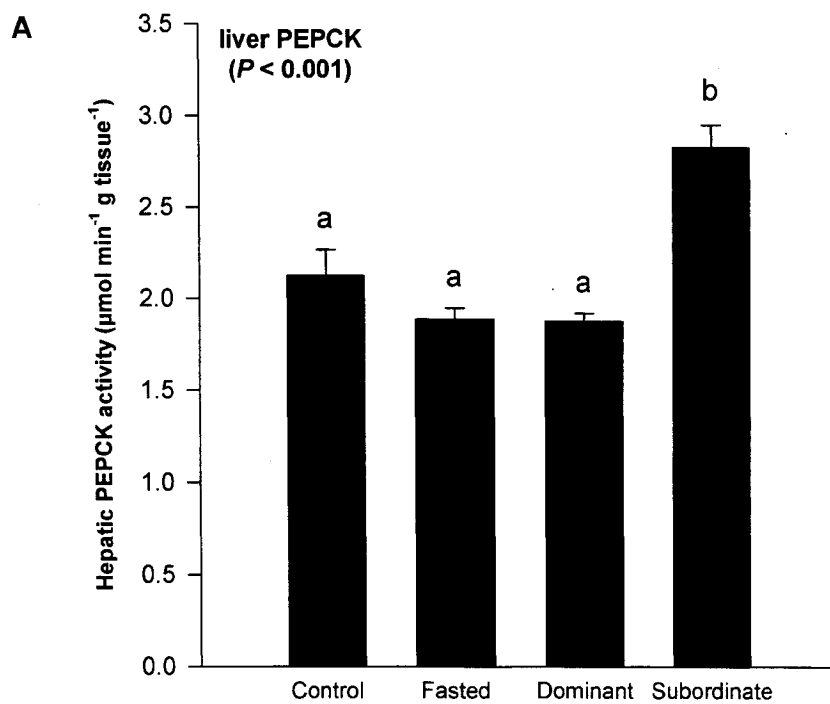


Figure 3-4. The relationship between hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity and (A) plasma cortisol concentrations as well (B) behaviour scores in dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days. Similar relationships between pyruvate kinase (PK) and (C) plasma cortisol concentrations and (D) behaviour scores are also presented. All relationships were tested using Spearman rank order correlations, and individual dominant and subordinate values were pooled for each plot ($N = 22$).

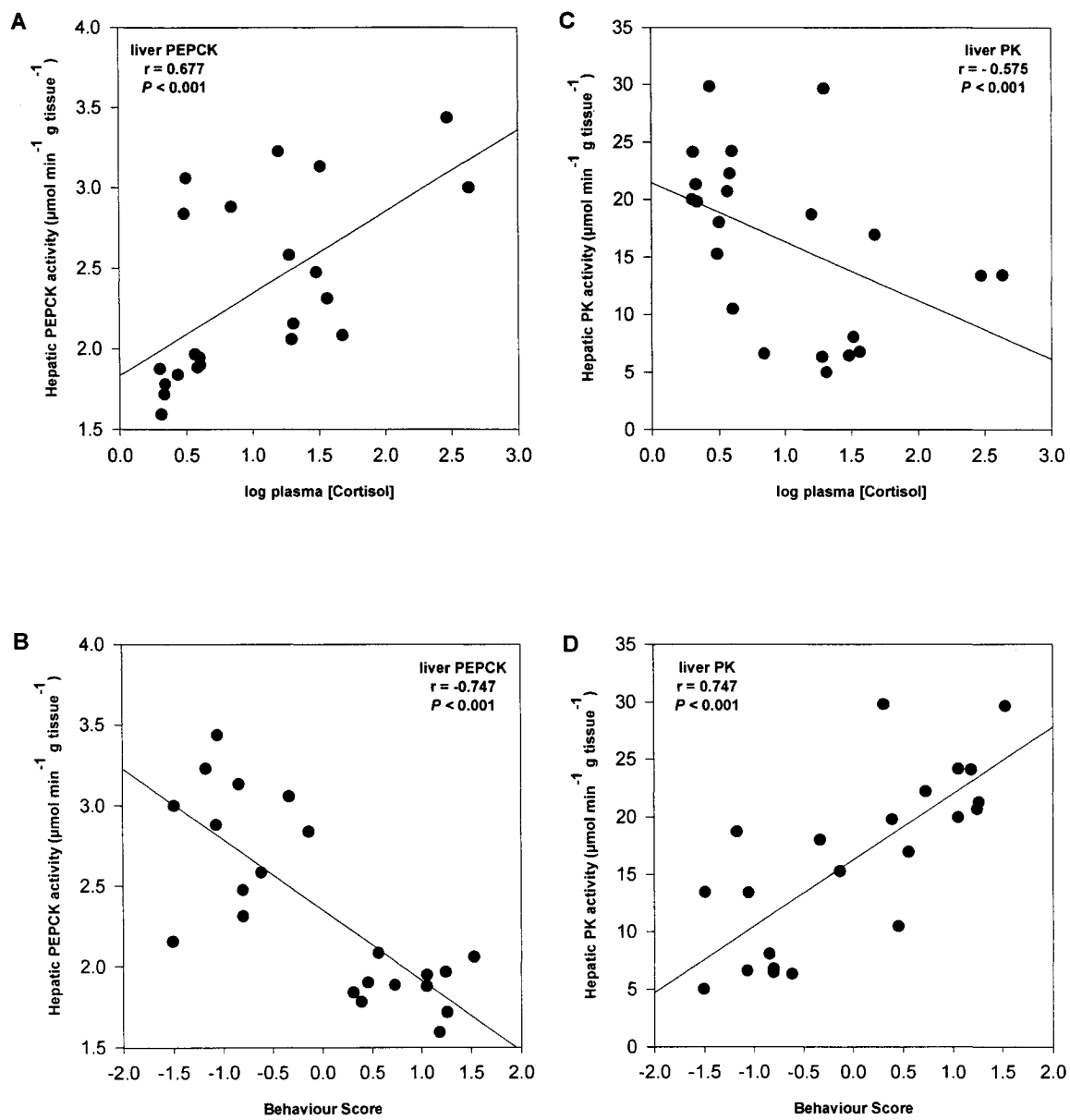


Figure 3-5. Plasma cortisol (A) and glucose (B) in RU486 (500 mg mifepristone kg⁻¹ fish) injected dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) confined with sham (0.005 ml cocoa butter g⁻¹ fish) treated fish for 5 days. Data are presented as mean values $\pm$ 1 S.E.M and *N* values are as follows: RU486/Dominants, *N* = 14; RU486/Subordinates, *N* = 8. An asterisk indicates a significant difference between dominant and subordinate RU486-treated fish (Student's t-test, *P* = 0.032).

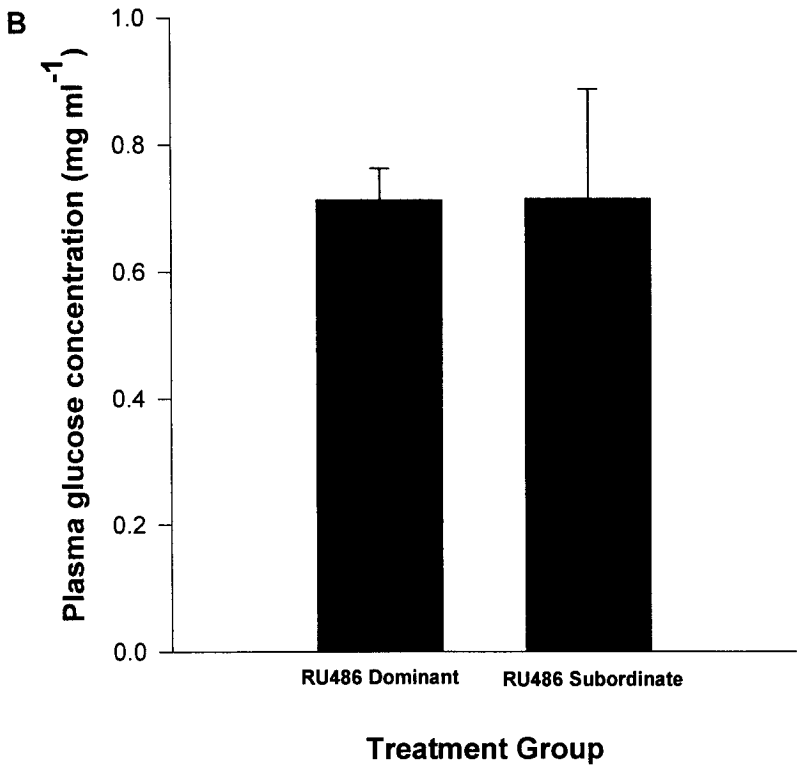
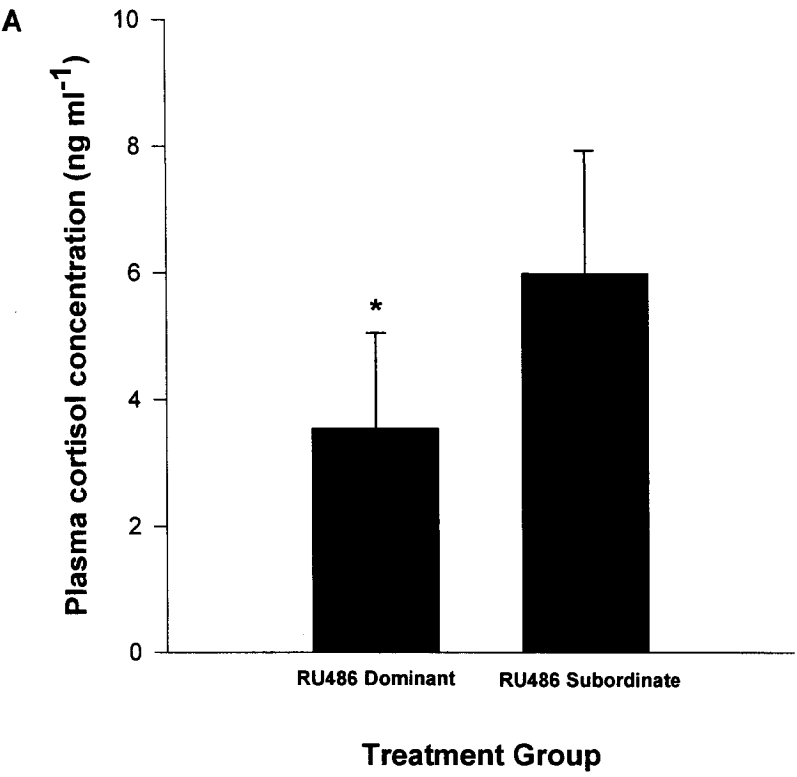


Figure 3-6. Hepatic phosphoenolpyruvate carboxykinase (PEPCK) (A) and pyruvate kinase (PK) activity (B) in RU486 (500 mg mifepristone kg⁻¹ fish) injected dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) confined with sham (0.005 ml cocoa butter g⁻¹ fish) treated fish for 5 days. Data are presented as mean values $\pm$ 1 S.E.M and *N* values are as follows: RU486/Dominants, *N* = 14; RU486/Subordinates, *N* = 8.

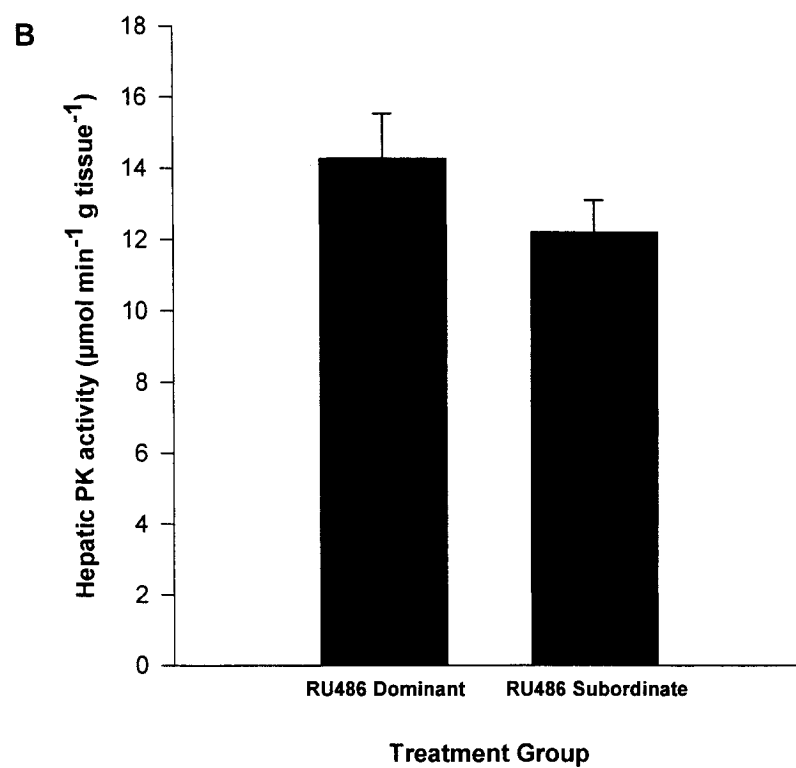
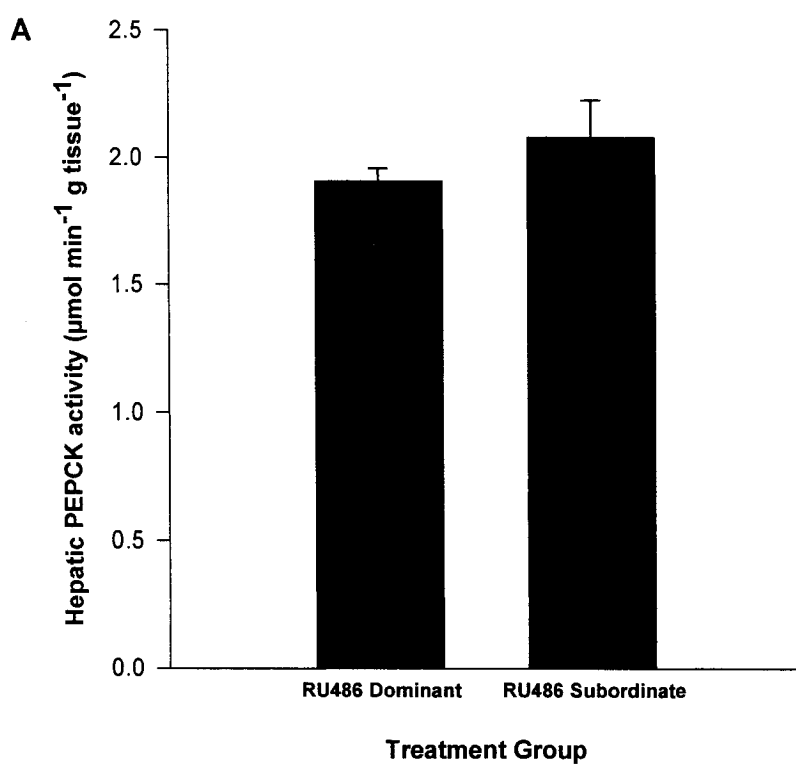


Table 3-1. Physiological measures taken from sham (0.005 ml cocoa butter g⁻¹ fish) and RU486 (500 mg mifepristone kg⁻¹ fish) injected dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days.

Treatment Group	SGR (% growth/day)	Daily Food Intake (mg food g ⁻¹ body weight)	CF _f (100 x g cm ⁻³)	Plasma [Cortisol] (ng ml ⁻¹)
Sham Dominant	1.11 ± 0.31 (5) ^a	11.35 ± 2.35 (5) ^a	1.15 ± 0.04 (5) ^a	4.62 ± 2.29 (5) ^{ab}
Sham Subordinate	-1.45 ± 0.13 (5) ^b	0.77 ± 0.67 (5) ^b	0.95 ± 0.03 (5) ^b	17.04 ± 7.99 (5) ^{ab}
RU486 Dominant	1.1 ± 0.49 (5) ^a	12.12 ± 3.25 (5) ^a	1.08 ± 0.04 (5) ^{ab}	1.94 ± 0.36 (5) ^b
RU486 Subordinate	0.17 ± 0.54 (5) ^a	2.41 ± 1.7 (5) ^b	0.99 ± 0.04 (5) ^b	50.94 ± 18.89 (5) ^a

Values are means ± 1 S.E.M. (N). Treatment groups that do not share a letter were significantly different from one another (one-way ANOVA followed by Bonferonni's post hoc multiple comparisons test). Abbreviations: specific growth rate (SGR), final condition factor (CF_f).

Table 3-2. Physiological measures taken from control, 5-day fasted, as well as dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) confined in size-matched pairs for 5 days.

Treatment Group	SGR (% growth/day)	Plasma [Cortisol] (ng ml ⁻¹)	Plasma [Glucose] ** (mg ml ⁻¹ ; <i>P</i> = 0.035)
Control	n/a	19.08 ± 6.40 (12) ^{a,b}	1.12 ± 0.064 (12)
5-Day Fasted	-1.83	18.48 ± 4.03 (6) ^{a,b}	0.94 ± 0.033 (12)
Dominant	-0.32 ± 0.24 (11) ^b	8.60 ± 4.22 (11) ^a	1.14 ± 0.051 (11)
Subordinate	-1.22 ± 0.18 (11) ^c	82.15 ± 43.58 (11) ^b	1.17 ± 0.075 (11)

Values are means ± 1 S.E.M. (*N*). Treatment groups that do not share a letter were significantly different from one another (one-way ANOVA followed by Bonferonni's post hoc multiple comparisons test). 5-day fasted SGR was calculated based on the mean initial and final body weights for the fasted group as a whole. ** A significant effect of treatment group on plasma [Glucose] was found (*P* = 0.035), however subsequent post-hoc analysis could not identify where those differences arose. Abbreviations: specific growth rate (SGR).

Table 3-3. A summary of the effects of fasting and social status on intestinal size, gall bladder size and appearance in groups of control, 5-day fasted fish as well as within size-matched pairs of rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days.

Treatment	ISI** ($P = 0.043$)	Gall Bladder/ Liver Index	Gall Bladder Appearance	χ^2 Analysis
Control	1.60 ± 0.14 (12)	0.17 ± 0.052 (12) ^a	7 clear, 5 dark	Fail to reject H_0 ($P > 0.5$)
Fasted	1.32 ± 0.063 (12)	0.29 ± 0.043 (12) ^b	12 dark	Reject H_0 ($P < 0.001$)
Dominant	1.51 ± 0.17 (11)	0.088 ± 0.014 (11) ^a	10 clear, 1 dark	Reject H_0 ($P < 0.001$)
Subordinate	1.12 ± 0.092 (12)	0.18 ± 0.035 (11) ^{a,b}	9 dark, 2 clear	Reject H_0 ($P < 0.001$)

Numbers in parentheses are N values. The null hypothesis (H_0) used in the chi-square analysis of gall bladder appearance was that there was a 50% probability of having a dark green gall bladder filled with bile. The alternative (H_A) was that the treatment tested had a significant effect on this probability. Treatment groups that do not share a letter were significantly different from one another (one-way ANOVA followed by Bonferonni's post hoc multiple comparisons test). **A significant effect of treatment group on ISI was found ($P = 0.043$), however subsequent post-hoc analysis could not identify where those differences arose. Abbreviations: intestinal somatic index (ISI).

Table 3-4. Liver, red muscle and white muscle aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutamate dehydrogenase (GDH) activity levels measured in control, 5 day fasted as well as dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) confined together for 5 days.

Tissue Type	Enzyme Type	Control Enzyme Activity ($\mu\text{mol min}^{-1} \text{g tissue}^{-1}$)	Fasted Enzyme Activity ($\mu\text{mol min}^{-1} \text{g tissue}^{-1}$)	Dominant Enzyme Activity ($\mu\text{mol min}^{-1} \text{g tissue}^{-1}$)	Subordinate Enzyme Activity ($\mu\text{mol min}^{-1} \text{g tissue}^{-1}$)
Liver	AST	40.92 $\pm$ 4.48 ^a	43.72 $\pm$ 2.21 ^a	43.22 $\pm$ 2.89 ^a	44.2 $\pm$ 2.39 ^a
	ALT	34.6 $\pm$ 2.87 ^a	41.21 $\pm$ 1.82 ^a	36.22 $\pm$ 2.21 ^a	42.01 $\pm$ 3.52 ^a
	GDH	51.07 $\pm$ 6.26 ^{ab}	34.0 $\pm$ 2.16 ^a	43.67 $\pm$ 1.86 ^{ab}	52.69 $\pm$ 4.16 ^b
Red Muscle	AST	66.0 $\pm$ 2.8 ^a	85.2 $\pm$ 4.98 ^{ab}	82.9 $\pm$ 3.22 ^{ab}	104.0 $\pm$ 4.72 ^b
	ALT	2.51 $\pm$ 0.1 ^a	3.74 $\pm$ 0.14 ^{ab}	4.52 $\pm$ 0.12 ^{bc}	5.43 $\pm$ 0.23 ^c
	GDH	10.45 $\pm$ 0.65 ^a	10.72 $\pm$ 0.65 ^a	12.35 $\pm$ 0.49 ^a	11.25 $\pm$ 0.75 ^a
White Muscle	AST	23.22 $\pm$ 1.3 ^a	23.83 $\pm$ 1.12 ^a	23.53 $\pm$ 1.24 ^a	19.45 $\pm$ 1.27 ^a
	ALT	0.82 $\pm$ 0.049 ^a	1.08 $\pm$ 0.057 ^{ab}	1.21 $\pm$ 0.058 ^{bc}	1.7 $\pm$ 0.1 ^c
	GDH	0.97 $\pm$ 0.061 ^a	1.22 $\pm$ 0.075 ^a	0.94 $\pm$ 0.072 ^a	1.4 $\pm$ 0.15 ^a

Values are means $\pm$ 1 S.E.M. (N). Treatment groups within each tissue type and for each individual enzyme that do not share a letter were significantly different from one another (one-way ANOVA on ranks followed by Dunn's post hoc multiple comparisons test, $P < 0.001$).

Discussion

The characteristically low growth rates of socially subordinate salmonid fish commonly have been attributed to reduced food intake owing to the monopolisation of resources by dominant individuals (Metcalf et al., 1989; Adams and Huntinford, 1996; Adams et al., 1998). Size-matched rainbow trout confined in pairs in the present study exhibited a similar disparity in food intake between social ranks (Fig. 3-1). However, fish were separated into individual compartments to feed, and so this difference did not reflect a monopolization of resources by the dominant individual, but rather a voluntary reduction in food intake by subordinates. Previous work has demonstrated that several different signals are integrated to regulate food intake in fish (see Bernier and Peter, 2001); however, the common theme is that increases in central serotonergic activity and/or decreases in neuropeptide Y (NPY) concentrations in the brain are the underlying cause of appetite suppression. Serotonin (5-HT) treatment has been shown to inhibit appetite not only in mammals (Dryden et al., 1996) but also in fish (De Pedro et al., 1998; Ruibal et al., 2002), while NPY appears to do the opposite (Dryden et al., 1996; Bernier and Peter, 2001) and stimulate food intake. Similarly, subordinate fish are characterized by an increase in brain 5-HT activity (Winberg et al., 1991; Winberg et al., 1992; Winberg et al., 1993a; Winberg et al., 1997b) and significant changes in NPY expression (Doyon et al., 2003), and so social stress-induced changes in 5-HT and/or NPY, possibly under the control of cortisol and the hypothalamic pituitary interrenal stress axis (see Bernier and Peter, 2001), are possibly responsible for the voluntary reduction in food intake observed in subordinate fish in this study.

Even where equal rations are consumed, however, subordinate rainbow trout do not achieve the growth rates of dominant fish (Abbott and Dill, 1989), suggesting that factors

beyond differences in food intake affect the growth of subordinates. While not directly tested in this study, past work has raised the possibility that a down-regulation of digestive function owing to social stress might contribute to growth depression in subordinate salmonids (Olsen and Ringø, 1999; Stevens and Devlin, 2000; Earley et al., 2004). Although stress-induced alterations in gastrointestinal motility (Coskun et al., 1997) and colonic transit (Mönnikes et al., 1993) in mammals have received the most attention, a recent study reported that gall bladder dysfunction accompanied social stress in fish (Earley et al., 2004).

Incomplete gall bladder emptying may hinder the capacity to assimilate energy-rich food as bile secretions influence in particular the conversion efficiency of most fatty foods (Horn, 1998; Farber et al., 2001). Subordinate rainbow trout in the present study exhibited evidence of bile retention. However, in contrast to the situation in convict cichlids, in which gall bladder hypertrophy and bile retention were associated primarily if not exclusively with subordinate social status (Earley et al., 2004), the corresponding effects in rainbow trout were likely mediated largely by the lack of feeding in subordinate fish since parallel bile retention was also observed in fasted fish (Table 3-3). Indeed, bile retention has been associated with starvation in previous work on fish (Goede and Barton, 1990) and is used as an indicator of stress in necroscopy-based health assessment schemes (Barton et al., 2002). In fact, this necroscopy method, which is based on examinations of the appearance and condition of internal tissues and organs, has been shown to be useful in detecting a lack of feeding in fish populations exposed to environmental perturbations (Adams et al., 1993; Steyermark et al., 1999).

Differences in intestine mass were also examined in the present study as a mechanism through which subordinate fish could experience significant reductions in digestive capacity. Stevens and Devlin (2000) found that growth hormone transgenic salmon possessed

enhanced gut surface area, and suggested that these fish may have enabled growth by increasing total intestinal size. The opposite may also hold, namely that subordinate fish may suffer growth depression owing to social stress-induced morphological alterations to the intestinal tract (*e.g.*, through cellular atrophy and/or necrosis) that inhibit nutrient absorption. In support of this possibility are reports that stress (Peters, 1982) and/or stress hormones (Robertson et al., 1963; Barton et al., 1987) affect the cellular integrity of the gut. For example, Peters (1982) found that eels subjected to social stress experienced a marked atrophy of gut tissue as well as degenerative changes in the gastric epithelium, while Robertson et al. (1963) found a marked necrosis of the gastric glands and submucosa in cortisol-treated rainbow trout, changes that were accompanied by a concurrent reduction in body weight. In the present study, subordinate fish exhibited the lowest intestine-somatic index (ISI). The ISI of fasted fish was also reduced relative to those of control or dominant fish, suggesting that reduced food intake as well as stress *per se* may have contributed to the lowered intestine mass in subordinate fish.

Although reduced food intake and impaired gastrointestinal function may contribute to the low growth rates of socially-subordinate trout, the available evidence also suggests that standard metabolic rate is elevated (Sloman et al., 2000c) and existing energy reserves are mobilized. Previous work has demonstrated that hepatosomatic index (HSI) and final condition factor (CF_f) are significantly, although not consistently, reduced by both subordination (Gregory and Wood, 1999; Sloman et al., 2000a; Gomez-Laplaza and Morgan, 2003) and a lack of feeding (Gregory and Wood, 1999; Peterson and Small, 2004). Indeed, both HSI and CF_f are commonly used by fisheries biologists as indirect measures of an individual's energetic status, and serve as significant predictors of overall energy reserves (lipid, protein, glycogen and total energy) (Chellappa et al., 1995). Neither HSI nor CF_f

were significantly affected by social stress or feeding status in the present study, but the short term nature of our experiment (5 days) may not have been sufficient to observe changes in these measures. Commonly used indicators of metabolism include plasma glucose, ammonia and free amino acid concentrations (Mommensen et al., 1999). Although these indicators typically increase in response to stress and/or elevation of circulating cortisol, responses can be highly variable, and additionally these concentrations provide only a snapshot of the metabolic situation without yielding information on turnover. For example, several studies have noted marked plasma glycemia in fish exposed to corticosteroids (Barton et al., 1987; Morgan and Iwama, 1996; Vijayan et al., 1997), while in others, plasma glucose concentrations decreased (Foster and Moon, 1986; Vijayan et al., 1991) and/or remained unchanged (Davis et al., 1985; Vijayan et al., 1994a). Further, amino acid metabolism was unchanged in eels, *Anguilla japonica* (Inui and Yokote, 1975), and plaice, *Pleuronectes platessa* (White and Fletcher, 1985), following stress or cortisol treatment, whereas similar methods resulted in a significant up-regulation of amino acid flux in other representative species of fish (Morales et al., 1990; Vijayan et al., 1996; Vijayan et al., 1997). Considering these results, it is therefore perhaps not surprising that neither plasma ammonia levels nor plasma amino acid concentrations were affected by social status or fasting in the present study, and that the only effect on plasma glucose levels was an apparent reduction in fasting fish (Table 3-2). A decrease in plasma glucose would be expected under extreme circumstances (*i.e.*, fasting) and was shown in a series of experiments conducted by Vijayan and Moon (1992; 1994). However, an increase in plasma glucose levels has regularly been observed in fish submitted to acute and chronic stress (Peters et al., 1980; Peters et al., 1988; Kubokawa et al., 1999) and so a possible explanation for our observed lack of change due to social status may be based on metabolite cycling. Subordinate fish might not only be

mobilizing more stored energy deposits to meet the higher energetic requirements of an increased SMR and a lack of food intake, but they may also increase their rate of glucose turnover, thus masking any immediate change in the plasma. Further, the fact that subordinate plasma glucose levels were similar to those of dominant fish (*i.e.*, opposite to the trend observed for fasted versus control fish), suggests that subordinate fish possess a means of inducing gluconeogenesis to maintain glucose levels during stress. Actions such as these are likely mediated by hormonal changes associated with subordinate social status, a prime candidate being plasma cortisol given its known ability to induce glucose production (Barton et al., 1987; Morgan and Iwama, 1996; De Boeck et al., 2001). However, our data do not support this claim as administering RU486 in this study (Fig. 3-5), effectively blocking the actions of cortisol, appeared to have no effect on plasma glucose concentrations, and so perhaps other factors are involved.

Measurements of enzyme activities may provide a more useful picture of the effects of social subordination on metabolic capacity. In fact, previous work found a strong correlation between growth rate and intermediary metabolism in the Atlantic Cod (*Gadus morhua*) (Pelletier et al., 1995), suggesting that perhaps enzymatic changes play a major role in subordinate growth depression. In the present study, both hepatic PEPCK and PK activities were affected by social status, with PEPCK being upregulated and PK downregulated in subordinate fish (Fig. 3-3). Gluconeogenesis and glycolysis are opposing metabolic pathways that play major roles in maintaining glucose homeostasis; upregulation of PEPCK activity results in an enhanced hepatic gluconeogenic potential, while the glycolytic enzyme PK has the opposite effect. Studies in which isolated tilapia (*Oreochromis mossambicus*) were subjected to confinement stress for 2- or 24-h found significant increases in the activity of both enzymes within the liver (Vijayan et al., 1997).

In addition, cortisol administration increased hepatic PEPCK activity in both brook charr (*Salvelinus fontinalis*) (Vijayan et al., 1991) as well as sea raven (*Hemitripterus americanus*) (Vijayan et al., 1996), and has been linked to changes in the activity levels of other key gluconeogenic enzymes (Vijayan et al., 1996; Vijayan et al., 1997; Vijayan et al., 2003), although this is not always the case (Andersen et al., 1991). Significant correlations between hepatic PEPCK and PK activities and behaviour scores or cortisol levels in the present study, suggest that socially induced elevations of plasma cortisol may have played a role in the observed enzyme activity differences. Moreover, given that the reductions in growth rate associated with subordinate social status were partly prevented by treatment with RU486 in pilot trials from this study (Table 3-1), a role for cortisol in metabolic control seems likely. Further use of the glucocorticoid receptor antagonist RU486 supports this hypothesis to some extent, in that liver PEPCK activities in RU486-treated dominant fish did not differ from those in the corresponding RU486-treated subordinate fish (Fig. 3-6A). Similarly, no social status differences in liver PK activities remained between RU486-treated fish (Fig. 3-6B), suggesting that elevated cortisol levels did in fact contribute to differences in liver enzyme activities observed in experiment 1.

Transaminase enzymes (aspartate aminotransferase, AST; alanine aminotransferase, ALT) also play a role in gluconeogenesis, and they, along with glutamate dehydrogenase (GDH), are closely linked with protein metabolism (Mommensen et al., 1999). Significant but relatively subtle differences in AST, ALT, or GDH activity were detected in liver, red muscle or white muscle in the present study; in general, where differences were detected the activity of the enzyme was elevated in subordinate fish relative to the control or fasted groups. Occasional differences between dominant and control groups were also observed. Although no differences between dominant and subordinate fish were found, comparisons

with the control group suggest that changes in metabolic activity may accompany social interactions, regardless of their outcome. The mobilization of protein as an energy source from muscle has been observed previously in fish submitted to chronic stress (Wendelaar Bonga, 1997; Iwama, 1997), and cortisol is thought to play a key role. For example, Vijayan et al. (1996) found that cortisol administration resulted in significantly higher AST, ALT, and GDH activity levels in the liver of the sea raven 5 days following treatment, supporting an enhanced hepatic capacity for amino acid metabolism in these fish. Therefore, the observed changes in protein metabolic activity in this study may be influenced by the fact that the initial stages of hierarchy formation require energy mobilization in both dominant and subordinate fish because of increased activity and/or elevated cortisol. Initially cortisol is increased with the onset of agonistic interactions in both fish within a pair and only returns to resting levels in fish that achieve dominant status following the cessation of most overt aggression (Øverli et al., 1999). Further, prior to the formation of a stable hierarchy, aggression levels are high and so is energy mobilization in all fish engaged in social interactions (Øverli et al., 1999). Therefore, together, these factors may provide an explanation for the observed differences (or lack thereof) in protein metabolism between dominant and subordinate groups in the present study.

In summary, the findings of the present study identify factors beyond exclusion from food or a voluntary decrease in food intake as playing roles in the growth depression characteristic of socially subordinate rainbow trout. While decreased digestive function (*i.e.*, decreased intestine size, increased bile retention) in subordinate fish appeared to be largely an effect of reduced feeding, subordinate trout displayed marked differences in glycolytic and gluconeogenic enzyme activity levels in comparison to dominants, and many of these changes were not observed in fish held under fasting conditions. Although the physiological

mechanisms underlying these changes remain unclear, the data support a role for cortisol acting via glucocorticoid receptors in hepatic tissue, possibly modulating enzymatic function in subordinate fish in response to social stress.

CHAPTER 4
GENERAL DISCUSSION

Discussion

Given the paucity of information available on factors related to physiological condition and acting as determinants of social status in fish; the overall aim of this thesis was to identify such potential factors as well as explore the role of cortisol in mediating physiological changes characteristic of subordinate social positions. My findings, along with major applications of this work, will be discussed below.

The present study recognizes the potential for the prior physiological condition of a fish, particularly circulating cortisol levels, to influence individual competitive ability and thus social status when that individual is confined in a pair or a small group of conspecifics. This is the first such study to demonstrate a causal link between elevated circulating plasma cortisol concentrations and subordinate social status in rainbow trout. Treatment with cortisol not only predisposed rainbow trout to subordinate social status when paired with a similar sized conspecific, but appeared to reduce the tendency for larger fish within a pair to become dominant when the pair consisted of individuals that were size-mismatched by 5% or more, with a 5% length difference being the point at which larger size normally ensures dominance (Abbott and Dill, 1985). Many social and inherent factors have previously been shown to influence social status in fish (Abbott and Dill, 1985; Huntingford and Turner, 1987; Nakano, 1995; Rhodes and Quinn, 1998; Leiser et al., 2004), particularly body size (although body size is not always a good predictor of social status (see Huntingford et al., 1990; Yamamoto et al., 1998)), and it appears that plasma cortisol can now join the growing list of physiological factors of importance in this regard (see Fig. 4-1). Follow-up experiments linking experimentally-elevated plasma cortisol to changes in brain monoaminergic activity provided indirect evidence that physiological factors, such as cortisol, may also modulate behaviour and reduce competitive ability, which, in turn, would

be expected to be of importance during hierarchy formation. Although paired fish held within the observation tanks of the present study were only visually isolated during acclimation, leaving open the possibility of the passage of pheromonal cues (Olivotto et al., 2002), the frequency and intensity of aggressive interactions following removal of the dividers suggested that these were crucial for hierarchy formation and influenced the outcome of individual social status.

While social interactions occurring within pairs of fish may be structurally and socially simple compared to those occurring in nature, hierarchies can and do exist in field situations, particularly in large rivers where environmental conditions are more stable (Bachman, 1984; Nakano, 1995). Further, fish held in groups within large laboratory stream tanks, which often include gravel substrate beds and flow rates comparable to natural stream systems, generally exhibit socially-mediated variations in physiology similar to those from studies using dyadic tests (reviewed by Sloman and Armstrong, 2002). Individual variability in plasma cortisol concentrations also exists in nature, as a previous study on wild spiny damselfish, *Acanthochromis polycaanthus*, showed that basal levels of plasma cortisol were quite variable (*i.e.* $< 0.5 \text{ mg mL}^{-1}$ to $> 30 \text{ ng mL}^{-1}$), with some individual fish having high levels in the plasma (Pankhurst et al., 1997; Pankhurst, 2001). Similarly, Woodward and Strange (1987) found significant differences in the stress responses of rainbow trout in the wild, noting large individual variability in the elevation of plasma cortisol concentrations in response to stressors. Although the reasons for this variability at present are unclear, it may be symptomatic of an individual fish's poor physiological condition and/or possibly impact social status in these fish. Does this mean that a fish is doomed to low social status in the wild if it happens to have been subjected to another stress (*e.g.*, predator avoidance, environmental perturbation, etc.) prior to encountering a conspecific? In most cases,

probably not, as stress-induced changes in plasma cortisol are likely transient and return to resting levels soon after the acute stressor is removed (Wendelaar Bonga, 1997). However in instances where the stressor is chronic and/or fish display individual variability in their susceptibility to a stressor, elevations in plasma cortisol could possibly have an effect on social status.

Cortisol is a well known mediator of many activities, both in the central nervous system and peripherally, and has been associated with a number of endocrine and physiological changes. It is becoming increasingly clear that behavioural and physiological stress responses are linked through common control mechanisms in the brain and cortisol appears to be a key player in this regard (see Dinan et al., 1996; Gilmour et al., 2005). Furthermore, changes in plasma cortisol concentrations could potentially have an adaptive role in hierarchy formation situations, in that cortisol could function as a “trigger” to induce a switch to submissive behavioural patterns in a fish that would be at a physiological disadvantage during subsequent competitive interactions (as a result of chronically elevated cortisol concentrations). Previous work in lizards (Tokarz, 1987) and fish (Munro and Pitcher, 1985; Øverli et al., 2002a) has shown that long-term exposure to elevated plasma stress hormone concentrations functions to increase submissive components of agonistic behaviour in both group and paired encounters. These effects also appear to be both time- and context-dependant, as acute cortisol treatment has been shown to facilitate aggressive interactions in a number of vertebrate species (Hayden-Hixson and Ferris, 1991; Haller et al., 1997). Therefore, while the data presented in Chapter 3 suggest that increased plasma cortisol concentrations impose a number of physiological costs, increased cortisol levels may pre-adapt rainbow trout to subordinate social positions, with submissive behaviour acting as a coping strategy to attenuate the behavioral consequences (*i.e.* aggression) associated with

social subordination in fish. This idea is supported by recent work in rainbow trout, in which individual differences in coping characteristics function as a stress-reducing behavioural strategy among socially interacting pairs of fish (Øverli et al., 2004).

Saturable binding sites for glucocorticoids have been found in a number of areas within the brain of salmonid fish (Knoebbl et al., 1996; Allison and Omeljaniuk, 1998; Carruth et al., 2000) and peripheral administration of the glucocorticoid analogue dexamethasone has been shown to downregulate brain cytosolic glucocorticoid receptors (GR) in rainbow trout (Lee et al., 1992). Therefore, given its widespread effects throughout the body and its ability to passively diffuse through the blood brain barrier (Gamperl et al., 1994), direct effects of increased circulating cortisol in the brain seem likely. The question that arises, however, is whether the observed elevations in plasma cortisol concentrations are physiologically relevant in terms of activation of GRs in the brain. For example, did the increased plasma cortisol concentrations associated with our cortisol treatment in Chapter 2 increase binding to and/or activation of GRs? Would the differences in plasma cortisol levels between a dominant and subordinate fish have similar effects? The identification and characterization of GRs in salmonid fish are the obvious first step in determining how the brain responds to elevations of circulating cortisol (see Bury et al., 2003; Sturm et al., 2005). Previous work in chinook salmon, *Oncorhynchus tshawytscha*, found that the receptor binding affinity (K_D) of cortisol in the brain of unstressed fish was 4.54 ± 0.06 nM (Knoebbl et al., 1996). While this is the only known study to report the dissociation constant for glucocorticoid receptors in the salmonid brain, the K_D is consistent with accepted dissociation constants for both gill (Pottinger et al., 2000) and liver (Lee et al., 1992) GR in rainbow trout, in addition to steroid hormone-receptor interactions in mammals (Sapolsky et al., 1984). These findings suggest that the increased plasma cortisol concentrations observed

in our study as a result of cortisol treatment ($\sim 150 \text{ ng mL}^{-1}$ or 405 nM) may not have impacted GR activation in the brain via increased cortisol binding, as the reported K_D values imply a saturated receptor at unstressed basal concentrations of cortisol ($< 10 \text{ ng mL}$ or 27 nM). However, studies such as these are complicated by nonspecific binding of both endogenous and introduced glucocorticoids to other cellular proteins, such as the low affinity corticosteroid binding protein previously identified in rainbow trout plasma (Caldwell et al., 1991), which may also be important in regulating target-tissue availability of cortisol. Further, given the recent identification of two distinct functional glucocorticoid receptors in teleost fish (Bury et al., 2003) and a possible MR receptor in the brain (Greenwood et al., 2003), more work is needed before any conclusions can be drawn concerning cortisol-receptor interactions.

Changes in behaviour as a result of increased serotonergic activity also play a key role in the control and integration of behavioural and neuroendocrine stress responses both in fish (Winberg and Nilsson, 1993a; Winberg et al., 1997a; Winberg and Lepage, 1998) and mammals (Chaouloff, 1993; Dinan, 1996), as stress is an intrinsic component of aggressive encounters and social interaction in a wide range of species (Sapolsky, 1990; Sloman and Armstrong, 2002; Summers, 2002). Therefore, the finding that brain serotonergic activity was responsive to peripheral cortisol treatment in the present study was not surprising, and lends supports to the proposed monoamine mechanism in Chapter 2, that cortisol may affect competitive ability via behavioural changes mediated by brain monoamines. Interactions between brain 5-HT activity and circulating plasma cortisol concentrations have important implications for our understanding of the endocrinology of the stress response. Although not examined in this study, 5-HT seems to stimulate the HPI axis and cortisol secretion in fish (Winberg et al., 1997a; Winberg and Lepage, 1998; Lepage et al., 2003). In a brief pilot trial

we performed, however, this did not appear to be the case, as dietary tryptophan (TRP) treatment resulted in no significant elevations of plasma cortisol concentrations (data not shown). While this finding is contradictory to previous reports (Winberg et al., 1997a; Lepage et al., 2002; Lepage et al., 2003), the majority of these studies reported TRP effects only in response to a stressor or by using different time periods than employed in our trial. For example, Lepage et al. (2002) found that dietary TRP resulted in only slight elevations in plasma cortisol levels (*i.e.* $< 10 \text{ ng mL}^{-1}$), whereas when subjected to stress, these same fish displayed significant reductions in the characteristic stress-induced elevation of plasma cortisol. Given, these methodological differences, perhaps elevation of brain serotonergic activity only impacts on cortisol levels when the HPI axis is activated and/or following long-term pairing; therefore, future work in this area should take these factors under consideration.

Both L-dopa and apomorphine treatment employed in the present study were ineffective in inducing social dominance in rainbow trout. As all administration routes used were employed previously in fish (Chang et al., 1985; Winberg and Nilsson, 1992; Hoglund et al., 2001), measurement of brain dopaminergic activity is an obvious next step to assess the effectiveness of these methods, as a lack of effect in the present study could simply be the result of insufficient alteration of brain neurochemistry. Moreover, a modified experimental protocol may be required to properly assess aggression and social dominance in fish treated with these drugs. The majority of studies registering monoamine associated drug-effects in fish employed resident-intruder models (Winberg et al., 2001; Hoglund et al., 2001; Øverli et al., 2002a; Øverli et al., 2002b; Perrault et al., 2003). This model consists of a resident fish being faced with much smaller intruder fish in staged encounters, with subsequent quantification of aggressive interactions; this likely gives a more accurate indication of aggression and/or dominance following drug treatment than do long-term

pairing experiments as aggression levels are usually the highest in the initial stages of hierarchy formation (Øverli et al., 1999).

Another research area addressed in this thesis was the physiological consequences associated with low social positions. While physiological costs associated with dominant social status have been identified both in mammals (Drickramer et al., 1996) and in fish (Cutts et al., 1998; Harwood et al., 2003; Buchner et al., 2004), they are relatively rare, as they tend to manifest themselves only in situations when the dominance hierarchy is unstable (Drickramer et al., 1996; Buchner et al., 2004). By contrast, physiological changes associated with subordinate social status are well documented and include reductions in condition factor (Sloman et al., 2000a; Sloman et al., 2000b), hepatic glycogen content (Ejike and Schreck, 1980), liver condition (Sloman et al., 2001b), and growth rate (Barton et al., 1987; Metcalfe et al., 1989; Winberg et al., 1992; Metcalfe et al., 1995), as well as increased protein catabolism (Mommensen et al., 1999) and plasma glucose levels (Peters et al., 1988; Elofsson et al., 2000). In this thesis, factors contributing to one cost of low social status, reduced growth rate, were investigated. We found that subordinate trout experience marked changes in metabolic capacity, as the activity of several enzymes associated with intermediary metabolism were altered as a result of chronic social stress. These changes were correlated with plasma cortisol concentrations and alleviated by treatment with RU486, suggesting that social stress-induced changes in circulating cortisol levels may have led to changes in metabolic activity, ultimately compromising growth. Although modified digestive capacity in subordinate fish could possibly impact growth, both indices examined (gall bladder:liver index and intestinal somatic index) in Chapter 3 appeared to change as a result of reduced food intake as well as social stress *per se*. These indices only scratch the surface of the hypothesis that digestive impairment suppresses growth in subordinate fish,

however, digestive capacity and food conversion efficiency appear to be impacted by a wide range of factors in salmonids (for review see Mommensen, 2001). One area which shows promise as a possible research direction in fish is that of digestive enzyme function. Activity of the enzyme trypsin, which is involved in protein digestion, was found to be significantly correlated with food conversion efficiency in the Atlantic Cod, *Gadus morhua*, and was suspected to potentially limit growth rate (Lemieux et al., 1999). Other enzymes of interest are pepsin, the main digestive enzyme in the stomach (Lauff and Hofer, 1984), as well as the protease chymotrypsin which is synthesized by the pancreas and causes selective hydrolysis within the intestinal lumen (Tietz, 2004).

Future Applications

Data from these experiments have and/or may be extended to apply to the improvement of the health and welfare of farmed fish in aquaculture. For example, in an aquaculture environment, fish typically are held in large numbers under artificial conditions (*i.e.*, holding tank volume, lighting regime, water quality, sorting procedures, etc.) that would not normally be encountered in the wild. These conditions may be stressful, affecting fish health and thus production (Barton et al., 1987). Competitive interactions for food are also a major source of differences in growth among social rank within hatchery facilities, as they result in aggressive dominant individuals consuming a disproportionate amount of food and growing fast relative to subordinates (Metcalf et al., 1989; Adams and Huntingford, 1996; Adams et al., 1998). While earlier studies have identified precautions that can be taken to reduce interactions and growth depensation in aquaculture facilities, such as scatter feeding food (McCarthy et al., 1999) and employing high stocking densities (Davis et al., 2002), findings from this thesis, particularly data on the determinants of social status and/or

subordinate growth suppression, could prove useful and offer some insight. As plasma cortisol was identified as a potential determinant of social status in the present study, blocking this response in fish held together in groups might eliminate this apparent physiological disadvantage. Further, given that RU486 treatment appeared to partially alleviate growth suppression and a low condition factor in subordinate fish during a pilot trial from Chapter 3, perhaps similar treatment compounds should be investigated for possible use within hatcheries.

Identification of individual traits which are consistently predictive of behavioural and/or physiological characteristics, such as those predisposing fish to subordinate social status, could also be useful as criteria for selective breeding programs. Steps have already been made in this regard, with genetically maintained lines of rainbow trout, selected for high or low responsiveness to cortisol, having been developed in aquaculture facilities (Pottinger and Carrick, 2001). A low responsive fish may be advantageous in terms of production in that it would be less prone to the detrimental effects of cortisol on social status and physiological condition. However, caution should be observed, as the consequences of low social status are due in part to high circulating cortisol levels, but also reflect other factors such as behaviour changes and/or as yet unidentified links between physiology and behaviour.

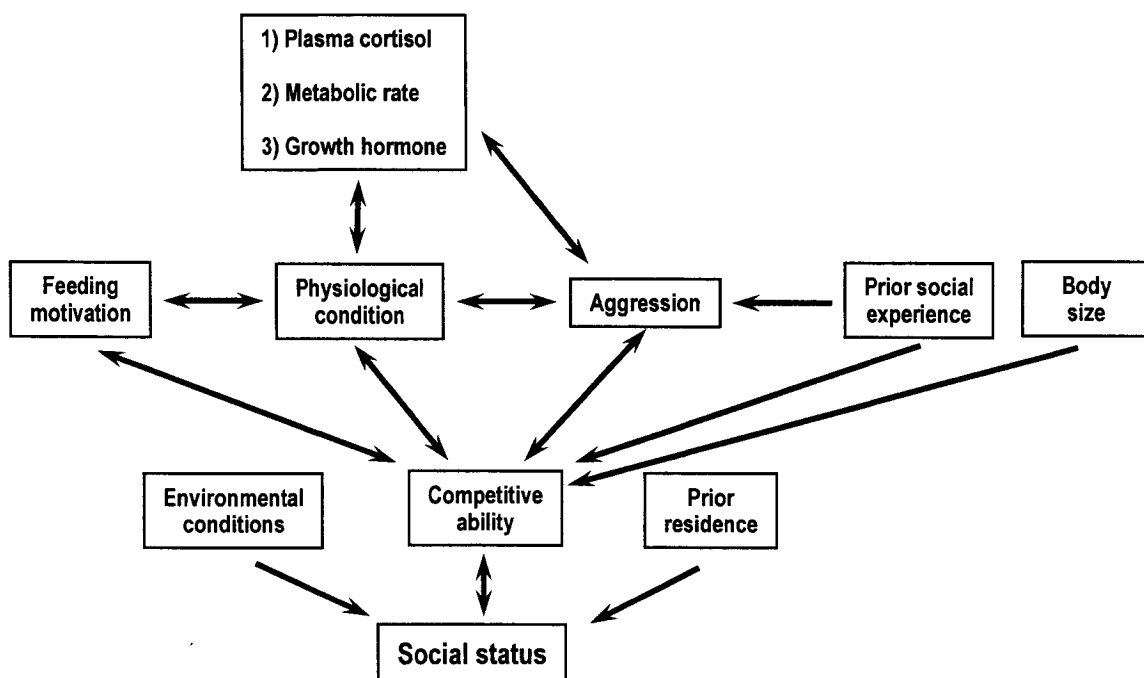
Finally, given the uncertainty of social interactions between hatchery-reared and native fish upon the restocking of target streams, information regarding the factors that affect competitive ability and thus physiological status are crucial. Hatchery fish tend to be much larger and more aggressive than native fish (Peery and Bjornn, 2004), often displacing them from territories, and so ways to minimize these effects should be examined; hopefully information contained in this thesis can be applied in this regard. Specifically, means of

reducing aggression in hatchery reared fish prior to their release would be a boon to current restocking programs and changes in brain monoaminergic activity through dietary treatment appears to be a potential solution warranting further research.

Conclusions

In conclusion, regulation of the complex behaviour exhibited by socially interacting pairs of rainbow trout clearly does not rely on a single mechanism, however, it is likely that critical mechanisms exist that influence all other components of the regulatory system. Circulating cortisol levels appear to play a key role, with widespread effects throughout the body, impacting not only physiology but behaviour and social position as well. Monoaminergic activity was also explored as a means by which the effects of cortisol on social status could be expressed behaviourally, ultimately impacting competitive ability, and was found to change significantly with cortisol treatment. Finally, this work identified enzyme activity as a potential limit on the growth rate of fish occupying low social positions, with cortisol again playing a large role, and so this appears to be an area of research which merits further investigation.

Figure 4-1. A schematic diagram of some of the factors that influence the outcome of social interactions in salmonid fish (modified from Gilmour et al. (2005))



APPENDIX A

A PRELIMINARY INVESTIGATION OF THE EFFECTS OF MANIPULATING BRAIN NEUROTRANSMITTERS ON SOCIAL STATUS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Abstract

Serotonin (5-HT) and dopamine (DA) have been implicated as possible determinants of social status and in the regulation of aggressive behaviour in salmonid fish, but it has remained challenging to separate these effects from the suite of behavioural and physiological changes associated with hierarchy formation. The objective of this study was to determine whether manipulation of 5-HT and DA levels impacts the outcome of social interactions in rainbow trout (*Oncorhynchus mykiss*). Dietary administration of the amino acid L-tryptophan (TRP) was employed as a non-invasive means of raising serotonergic activity within the brains of juvenile rainbow trout. TRP-fed trout were paired with a size-matched conspecific to allow social hierarchies to form. Experimental elevation of dietary TRP did not increase the probability that a treated fish became subordinate when paired with an untreated conspecific ($\chi^2 = 1.33$, $0.25 > P > 0.1$). Further, behaviour scores for TRP-treated fish were not significantly lower than those for the untreated fish with which they were paired ($P = 0.542$). To investigate the effects of elevated brain dopaminergic activity on social status and aggression, one randomly selected individual from size-matched pairs of fish was treated with either L-dopa, the precursor to DA, or apomorphine, a DA receptor agonist, using a variety of methods (oral gavage, whole body immersion, intraperitoneal [i.p.] injection) and doses (10, 20, 100 mg kg⁻¹ fish). None of the methods employed to elevate brain dopaminergic activity appeared to predispose a fish to dominant social status. It is unclear from the results of this study whether manipulation of brain monoaminergic activity is ineffective in influencing the outcome of social interactions, or whether the delivery methods employed were ineffective in modifying brain monoaminergic activity.

Introduction

When held in small groups, juvenile rainbow trout, like other salmonid fish, form dominance hierarchies, with each fish being ranked according to its relative ability to out-compete all other members within a group (Adams et al., 1998). Typically, subordinate fish are excluded from preferential access to food and shelter (McCarthy et al., 1992) as dominants tend to monopolise all of the available resources. Given that this type of social order is maintained through aggressive interactions, subordinate individuals are also subjected to chronic social stress as a result of losing aggressive encounters as well as the constant threat of attack by dominant individuals (Noakes and Leatherland, 1977; Winberg and Lepage, 1998). Sustained social stress leads to a number of physiological changes, including chronic activation of the hypothalamic-pituitary-interrenal (HPI) axis and resultant maintenance of elevated circulating cortisol concentrations (for review see Wendelaar Bonga, 1997).

Social experience also greatly modifies the behaviour of fish. Behavioural correlates of subordinate social status include decreases in feeding, locomotion, and aggression (referred to as “behavioural inhibition”; (Winberg and Nilsson, 1993a; Winberg et al., 1997a; Øverli et al., 1998)), as well as the selection of positions within the environment not occupied by other, more dominant, fish. These behavioural modifications have been attributed to the changes in brain monoaminergic activity that accompany victory or loss in competitive interactions (reviewed by Winberg and Nilsson, 1993a). For example, social subordination typically results in significantly higher turnover rates of serotonin (5-hydroxytryptamine, 5-HT), reflected by 5-hydroxyindolacetic acid (5-HIAA) accumulation and elevated 5-HIAA/5-HT ratios within the telencephalon, hypothalamus, and brain stem relative to dominant individuals (Winberg et al., 1991; Winberg et al., 1992; Winberg et al.,

1993a; Winberg et al., 1997b; Winberg and Lepage, 1998). Further, experimentally elevated 5-HT has been reported to inhibit aggressive behaviour in a wide range of vertebrate species (Adams et al., 1996; Edwards and Kravitz, 1997; Larson and Summers, 2001), as well as to negatively impact feeding motivation (De Pedro et al., 1998; Ruibal et al., 2002) and locomotory activity (Genot et al., 1984; Winberg et al., 1993b) in fish.

Previous work has also shown that experimental manipulations of serotonergic activity can cause a reversal of dominance relationships in a number of vertebrate systems (e.g., Sanchez and Hyttel, 1994; Deckel, 1996; Villalba et al., 1997; Larson and Summers, 2001), suggesting that high brain 5-HT levels prime the individual for subordination. Artificially increasing serotonin levels in *Anolis carolinensis*, for example, turned aggressive, dominant lizards into subordinates (Larson and Summers, 2001), and similar results were reported for socially interacting rodents (Villalba et al., 1997). In fish, however, Winberg et al. (1992) reported that the relationship between brain 5-HT turnover rate and social status developed through social interactions and was not caused by intrinsic differences in brain 5-HT activity. One objective of the present study was to confirm the findings of Winberg et al. (1992), which were for Arctic charr (*Salvelinus alpinus* L.), as subsequent studies involving elevations of brain serotonergic activity, social status, and stress in salmonids (Winberg and Nilsson, 1993b; Winberg et al., 1997b; Øverli et al., 1998; Winberg and Lepage, 1998; Øverli et al., 1999) are largely based on this single report. In the present study, the hypothesis that elevated brain serotonergic activity is a consequence and not a cause of subordinate social status in rainbow trout, *Oncorhynchus mykiss*, was tested by raising central serotonergic activity levels in select socially interacting fish. Dietary administration of the amino acid L-tryptophan (TRP) was employed as a non-invasive means of raising brain serotonergic activity, as TRP is the precursor of 5-HT and its hydroxylation to 5-

hydroxytryptophan is the first and rate-limiting step in 5-HT biosynthesis (for review see Boadle-Biber, 1993).

Dominant social status in fish is generally associated with an increase in dopaminergic activity (McIntyre et al., 1979; Winberg et al., 1991). For example, in juvenile Arctic charr, socially dominant individuals demonstrated significantly higher dopaminergic activity than subordinate fish in the telencephalon (Winberg et al., 1991), while dopamine (DA) was significantly higher in whole brain homogenates of dominant rainbow trout fingerlings when compared to their submissive group members (McIntyre et al., 1979). DA also appears to facilitate intraspecific aggressive behaviour in fish (reviewed by Winberg and Nilsson, 1993a). Aggressive interactions in several salmonid species were increased following treatment with the DA receptor agonist apomorphine (Tiersch and Griffith, 1988) or with DA itself (Nechayev and Musatov, 1992), and oral administration of the DA precursor, L-dopa, in Arctic charr was reported to increase the probability of the treated individual winning dominant social status when subjected to dyadic encounters (Winberg and Nilsson, 1992). Therefore, given the reported links between high brain dopaminergic activity and high social status, treatment with a DA analogue (apomorphine) or experimental elevation of brain dopaminergic activity using L-dopa treatment were investigated as means of inducing dominant social status.

These experiments were carried out with the objective of identifying a means of manipulating social status and brain monoaminergic activity, so as to test in greater detail the hypothesis proposed in Chapter 2, that high cortisol levels are linked with low social status through a pathway in which cortisol-induced elevation of brain serotonergic activity and/or lowering of dopaminergic activity impair competitive ability.

Materials and Methods

Experimental Animals

Juvenile female freshwater rainbow trout (weight 75.56 ± 3.01 g [mean $\pm$ SEM], $N = 64$) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). All fish were held in large 1,275-L fibreglass stock tanks for several weeks at the University of Ottawa, supplied with flowing, aerated, dechloraminated city of Ottawa tap water at a temperature of $13 \pm 1^\circ\text{C}$ and using a 12L: 12D photoperiod. Fish used in experiment 1 were transported to Carleton University prior to experimentation and housed in 780-L fibreglass holding tanks until further use; tanks were supplied with flowing, aerated, well water ($16.5 \pm 1.5^\circ\text{C}$) and a 12L: 12D photoperiod was also used. Throughout this period, fish were hand-fed to satiation every second day with a commercial trout food diet (Purina Trout Chow).

Experiment 1: The effects of tryptophan treatment on the outcome of social interactions

Fish were lightly anaesthetized (*i.e.* the point at which the test animal lost equilibrium while maintaining a normal ventilation frequency) in a solution of benzocaine (0.05 mg ethyl-*p*-aminobenzoate mL^{-1} water; Sigma) and initial weights and fork lengths were measured (weight 46.46 ± 1.95 g; fork length 164.9 ± 2.0 mm [mean $\pm$ SEM], $N = 12$). Fish were subsequently paired with a size-matched (on the basis of fork length; $< 1.5\%$ difference) conspecific, and placed in 40-L plexiglass experimental tanks for behaviour observations. Initially, the fish within a pair were visually isolated from one other by an opaque perforated divider. Following a 48 h acclimation period, all fish were fed separately, to satiation (denoted by the rejection of three food pellets in a row) on commercial trout food pellets once a day for a period of five days; food consumption was quantified. For the next

seven days, one randomly selected individual from each pair received an 8X tryptophan (TRP) supplemented feed (Finfish gold/4.72% Tryptophan, Zeigler Brothers Inc.), while the other fish continued to receive the regular diet. Both fish were fed to satiation or to a maximum of 2% body weight. The TRP diet was identical to the regular diet in every respect except that it had been enriched with the essential amino acid tryptophan to eight times its normal content. Previous work demonstrated this to be an effective, non-invasive means of raising brain serotonergic activity (Winberg et al., 2001; Lepage et al., 2002; Lepage et al., 2003). Food consumption was again quantified.

Following the treatment period, dividers were removed to allow the fish within a pair to interact, and a small piece of PVC tubing was placed within each tank to provide shelter. Behavioural observations were carried out on all paired fish twice a day for 5 days, and the fish were then terminally sampled. During the experiment, fish were hand-fed to satiation with commercial trout food pellets (*i.e.*, regular feed only) once a day, after all observations had been carried out. The dividers were re-inserted for 15 minutes during feeding so that each fish could be fed separately; all uneaten food was then removed from the tank and the dividers were once again taken away. Behaviour observations were first conducted fifteen minutes after the opaque divider was removed, and then for 10 min each, once between 9:00-11:30 h and once between 15:00-17:30 h. The order of tank observation was randomized to account for any observational bias.

Social status was determined by assigning points to each fish based on its food acquisition, position, aggressive behaviour, and fin damage as described in Chapters 2 and 3. A single behaviour score was calculated from all observations by means of a principal components analysis (PCA; SPSS 10.1) (Sloman et al., 2000c) and the fish with the higher

overall behaviour score within each pair was assigned dominant social status, whereas that with the lower score was classified as subordinate.

At the end of the experimental period, fish were rapidly killed by immersion in a lethal dose of anaesthetic solution (ethyl-*p*-aminobenzoate 0.5 g L⁻¹); final weights and fork lengths were measured.

Experiment 2: The effects of manipulating dopaminergic activity on the outcome of social interactions

L-dopa trials

Fish were lightly anaesthetized in a solution of benzocaine (0.05 mg ethyl-*p*-aminobenzoate mL⁻¹ water) and initial weights and fork lengths were measured (weight 83.71 ± 1.77 g; fork length 193.82 ± 1.30 mm [mean $\pm$ SEM], $N = 40$). Fish were subsequently placed in pairs, size-matched on the basis of fork length ($< 1.5\%$ difference), in 40-L plexiglass experimental tanks for behaviour observations. Initially, fish were kept visually isolated from one another by an opaque perforated divider. Following a 24 h acclimation period, one randomly selected fish from a pair ($N = 10$) was lightly anaesthetized (0.01 mg ethyl-*p*-aminobenzoate mL⁻¹ water) and “gavaged” with 10 mg kg⁻¹ fish L-dopa (L-3,4-dihydroxyphenylalanine methyl ester hydrochloride; Sigma) dissolved in a 0.02 M HCL solution (1 ml vehicle mg⁻¹ L-dopa). The other fish from each of these pairs received a comparable sham gavage, which consisted of an equal volume of 0.02 M HCL alone. As per Winberg and Nilsson (1992) and Höglund et al. (2001), gavage treatment involved feeding polyethylene tubing (PE-160, Clay-Adams) into the stomach of the fish for delivery of the dose. 30 minutes following treatment, dividers were removed to allow the

fish within a pair to interact, and a small piece of PVC tubing was placed within each tank to provide shelter. Alternatively, one randomly selected fish from each pair received an intraperitoneal (i.p.) injection of either 20 mg kg⁻¹ fish ($N = 4$) or 100 mg kg⁻¹ fish ($N = 6$) L-dopa dissolved in a 0.9 % saline solution (1 ml vehicle mg⁻¹ L-dopa). Fish were given 24 hours to recover from this treatment prior to removal of the dividers.

Behaviour observations were carried out on all paired fish twice a day for 3 days, as described above. In addition, however, aggressive acts in the form of attacks, nips, or chases were recorded separately (for a description see Noakes, 1980), where an attack was defined as a rapid approach towards an individual that finished with a bite, a chase consisted of two or more successive attacks toward the same fleeing individual, and a nip was defined as a bite at a closely located individual without prior approach. After 3 days of social interactions, fish were rapidly killed by immersion in a lethal dose of anaesthetic solution (ethyl-*p*-aminobenzoate 0.5 g L⁻¹); final weights and fork lengths were measured.

Apomorphine Trials

Fish were lightly anaesthetized in a solution of benzocaine (0.05 mg ethyl-*p*-aminobenzoate mL⁻¹ water; Sigma) and initial weights and fork lengths were measured (weight 95.64 ± 3.41 g; fork length 198.32 ± 2.99 mm [mean $\pm$ SEM], $N = 12$). Fish were subsequently placed in pairs, size-matched on the basis of fork length (< 1.5% difference), in 40-L plexiglass experimental tanks for behaviour observations. Initially, fish were visually isolated from one another by an opaque perforated divider. Following a 24 h acclimation period, one randomly selected fish from a pair ($N = 6$) was transferred to a 20-L aquarium containing apomorphine (4 mg apomorphine hydrochloride hemihydrate L⁻¹ water; Sigma) for a period of 30 minutes, while the other fish from the pair was placed in an identical tank

containing only water. Fish were then replaced together in the original experimental tank, and dividers were removed to allow interaction while a small piece of PVC tubing was placed within each tank to provide shelter. Both the immersion time and apomorphine concentration used in this experiment were modified from previous work in fish that employed similar methods (Mok and Munro, 1998; Dietrich et al., 2002).

Behaviour observations were carried out on all paired fish twice a day for 3 days, as described above. During the experiment, fish were hand-fed to satiation with commercial trout food pellets once a day, after all observations had been carried out. Following the interaction period, fish were rapidly killed by immersion in a lethal dose of anaesthetic solution (ethyl-*p*-aminobenzoate 0.5 g L⁻¹); final weights and fork lengths were measured.

Statistical analyses

All data are presented as means $\pm$ 1 standard error of the mean (S.E.M.). Chi-square analysis was used to evaluate the effects of treatment on social status for all pairs of fish. A two-way ANOVA followed by Bonferroni's post hoc pairwise multiple comparisons test, as appropriate, was used to compare mean daily feed intake with both time and treatment as factors (*i.e.*, Day 1-12; acclimation and treatment period), while treatment and social status were used as factors to assess the differences in behaviour score and mean daily feed intake during the interaction period in experiment 1. Similarly, one-way ANOVA on ranks followed by Dunn's post hoc multiple comparisons were used to compare aggressive acts for fish separated by both treatment (L-dopa treated, untreated) and social status (*i.e.*, dominant, subordinate) from experiment 2. The α level for significance for all tests was set at 0.05 and

all statistical analyses were performed using SigmaStat v3.0 (SPSS, Inc) or SPSS v10.1 (SPSS, Inc) software.

Results

Experiment 1: The effects of tryptophan treatment on the outcome of social interactions

Although fish tended not to feed on day 1 of the experiment, food intake past day 1 increased progressively during the pre-treatment and treatment feeding periods, peaking just prior to divider removal for social interactions (Fig. A-1; two-way ANOVA with treatment group and time as factors, $P = 0.203$ for treatment group, $P < 0.001$ for time, and $P = 0.441$ for interactions between the factors). Interestingly, feed intake in TRP-fed fish decreased significantly in comparison to fish fed normal feed on day 9 (two-way ANOVA followed by Bonferroni's post hoc multiple comparisons test, $P = 0.027$) and day 10 (two-way ANOVA followed by Bonferroni's post hoc multiple comparisons test, $P = 0.048$) of the treatment period. During the social interaction period, however, a decline in food intake occurred in fish identified as subordinate (Fig. A-2) as evidenced by the significantly lower mean daily food intake values, averaged out over the entire 5-day interaction period, in subordinate relative to dominant fish within both the TRP-treated (two-way ANOVA, $P = 0.028$) and untreated groups (two-way ANOVA, $P = 0.028$). Interestingly, there was an apparent trend for TRP-fed subordinates to have even lower food intake values than untreated subordinates, although this difference was not found to be significant (two-way ANOVA, $P = 0.35$), owing to the small samples sizes in these groups ($N = 2$ and $N = 4$, respectively).

Consumption of a TRP-enhanced diet did not appear to affect behaviour, as 2 of 6 TRP-fed fish became subordinate (not significantly different from that expected by chance, chi-square analysis, $\chi^2 = 1.33$, $0.25 > P > 0.1$). Through the scoring scheme and PCA analysis, differences in behaviour were translated into behaviour scores that were typically high positive values for dominant fish and low negative scores for subordinate individuals (Fig. A-3). Behaviour scores for TRP-treated fish were not significantly lower than those for

the untreated fish with which they were paired (two-way ANOVA on treatment group: $F = 0.405$, $P = 0.542$), while there was a significant difference between dominants and subordinates (two-way ANOVA on social status: $F = 26.383$ $P < 0.001$), with no interaction (two-way ANOVA interactions: $F = 26.383$ $P = 0.516$).

Experiment 2: The effects of manipulating dopaminergic activity on the outcome of social interactions

Although dominant fish were typically aggressive and subordinates submissive (in no case did a subordinate fish attack, chase or nip a dominant fish), the mean number of aggressive acts (*i.e.*, a combined count of attacks, nips, and chases) performed by dominant fish declined over the 3-day interaction period (Fig. A-4). Untreated dominant fish performed significantly more aggressive acts than untreated subordinates on Days 1 and 2 (one-way ANOVA on ranks followed by Dunn's post hoc multiple comparisons test, $P < 0.001$ for both), but not Day 3 (one-way ANOVA on ranks, $P = 0.089$). Surprisingly, L-dopa treated fish did not perform significantly more aggressive acts than untreated fish with which they were paired on any day tested, in fact, they consistently performed fewer aggressive attacks than untreated dominants throughout the trial (Fig. A-4).

None of the various methods used to increase dopaminergic activity in trout prior to social interaction appeared to predispose a fish to dominant social status (Table A-1). Chi-square analysis indicated that L-dopa treatment had no significant effect on the outcome of social interactions, with L-dopa (10 mg kg^{-1} fish) gavaged, low dose (20 mg kg^{-1} fish), and high dose (100 mg kg^{-1} fish) L-dopa i.p. injected fish becoming dominant in 50%, 50%, and 67 % of the pairs respectively. Similarly, 50% of fish subjected to apomorphine immersion treatment became dominant (Table A-1).

Figure A-1. Mean daily feed intake, standardized to individual body mass, in tryptophan-treated ($N = 6$) versus untreated ($N = 6$) rainbow trout (*Oncorhynchus mykiss*) confined in pairs for 17 days. All pre-treatment (*i.e.* acclimation feeding; Day 1-5) and treatment (Day 6-12) values are included in this figure. Data are presented as mean values ± 1 S.E.M. An asterisk indicates a significant difference between tryptophan-treated and untreated fish (two-way ANOVA followed by Bonferroni's post hoc multiple comparisons test, with P values of Day 9, $P = 0.027$; Day 10, $P = 0.048$). *Filled diamonds* represent tryptophan-treated fish, whereas *open diamonds* represent untreated fish.

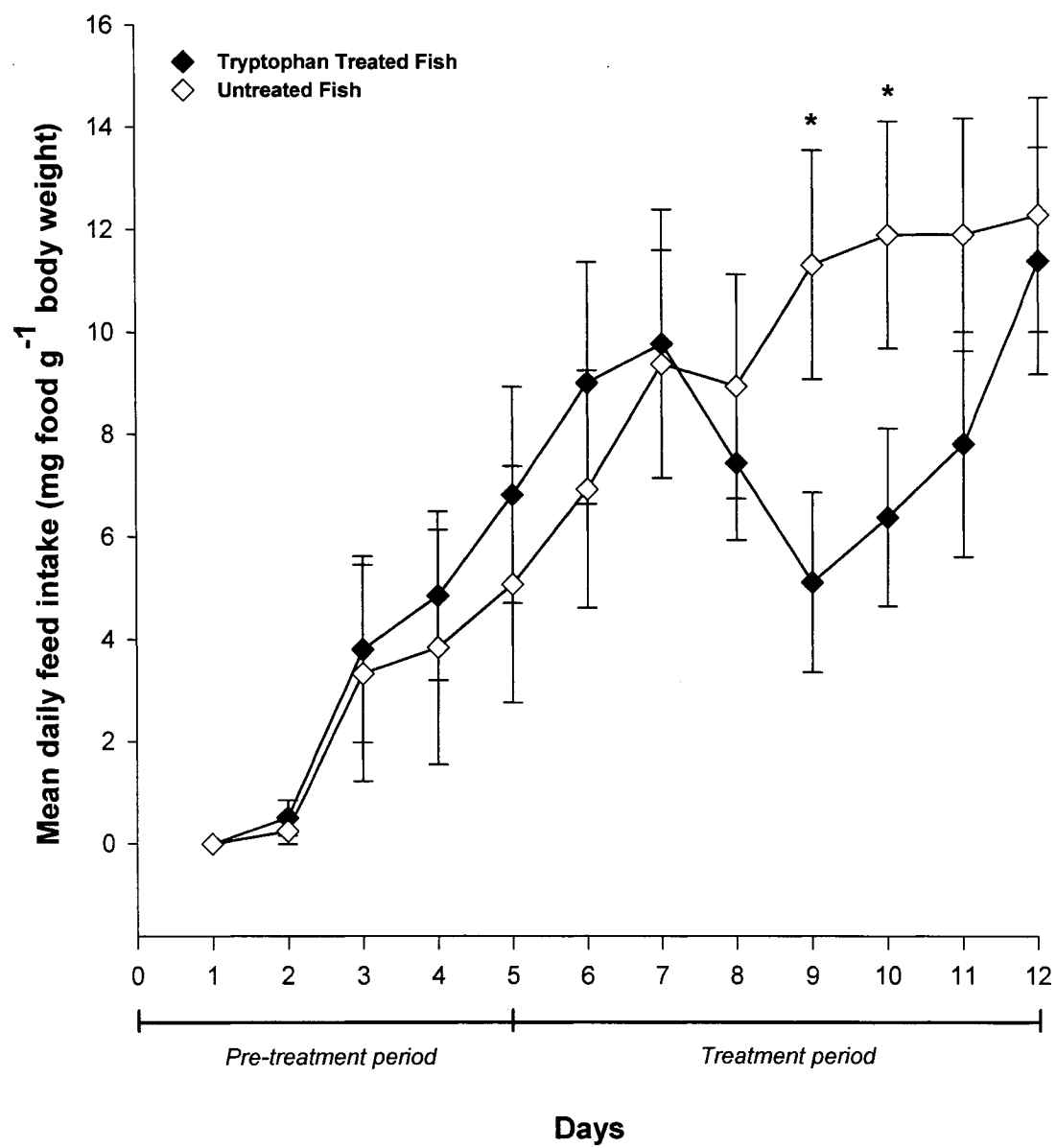


Figure A-2. Mean daily feed intake, standardized to individual body mass and averaged out over the entire 5 day interaction period, of tryptophan-fed versus untreated rainbow trout (*Oncorhynchus mykiss*) separated by social status (*i.e.* dominant and subordinate). Data are presented as mean values $\pm$ 1 S.E.M and *N* values are as follows: Tryptophan-fed/Dominant, *N* = 4; Tryptophan-fed/Subordinate, *N* = 2; Untreated/Dominant, *N* = 2; Untreated/Subordinate, *N* = 4. An asterisk indicates a significant difference between dominants and subordinates within the same treatment group (two-way ANOVA with treatment group and social status as factors, *P* = 0.199 for treatment group, *P* = 0.005 for social status, and *P* = 0.996 for interactions between these two factors).

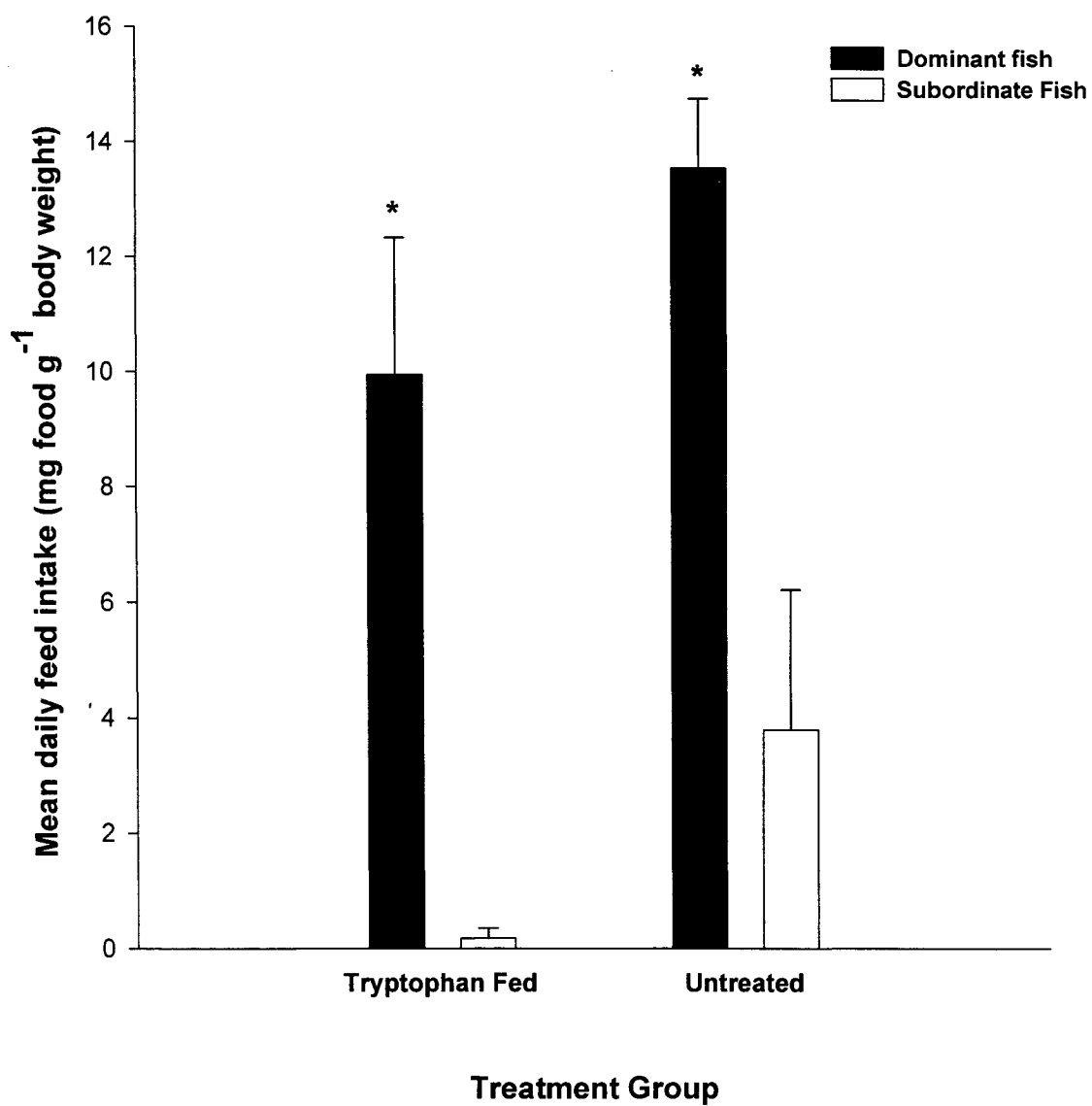


Figure A-3. Behaviour scores of tryptophan-fed versus untreated rainbow trout (*Oncorhynchus mykiss*) separated by social status (*i.e.* dominant and subordinate) and confined together in pairs for 5 days of social interactions. Data are presented as mean values $\pm$ 1 S.E.M and *N* values are as follows: Tryptophan-fed/Dominant, *N* = 4; Tryptophan-fed/Subordinate, *N* = 2; Untreated/Dominant, *N* = 2; Untreated/Subordinate, *N* = 4. An asterisk indicates a significant difference between dominants and subordinates within the same treatment group (two-way ANOVA with treatment group and social status as factors, *P* = 0.542 for treatment group, *P* < 0.001 for social status, and *P* = 0.516 for interactions between these two factors).

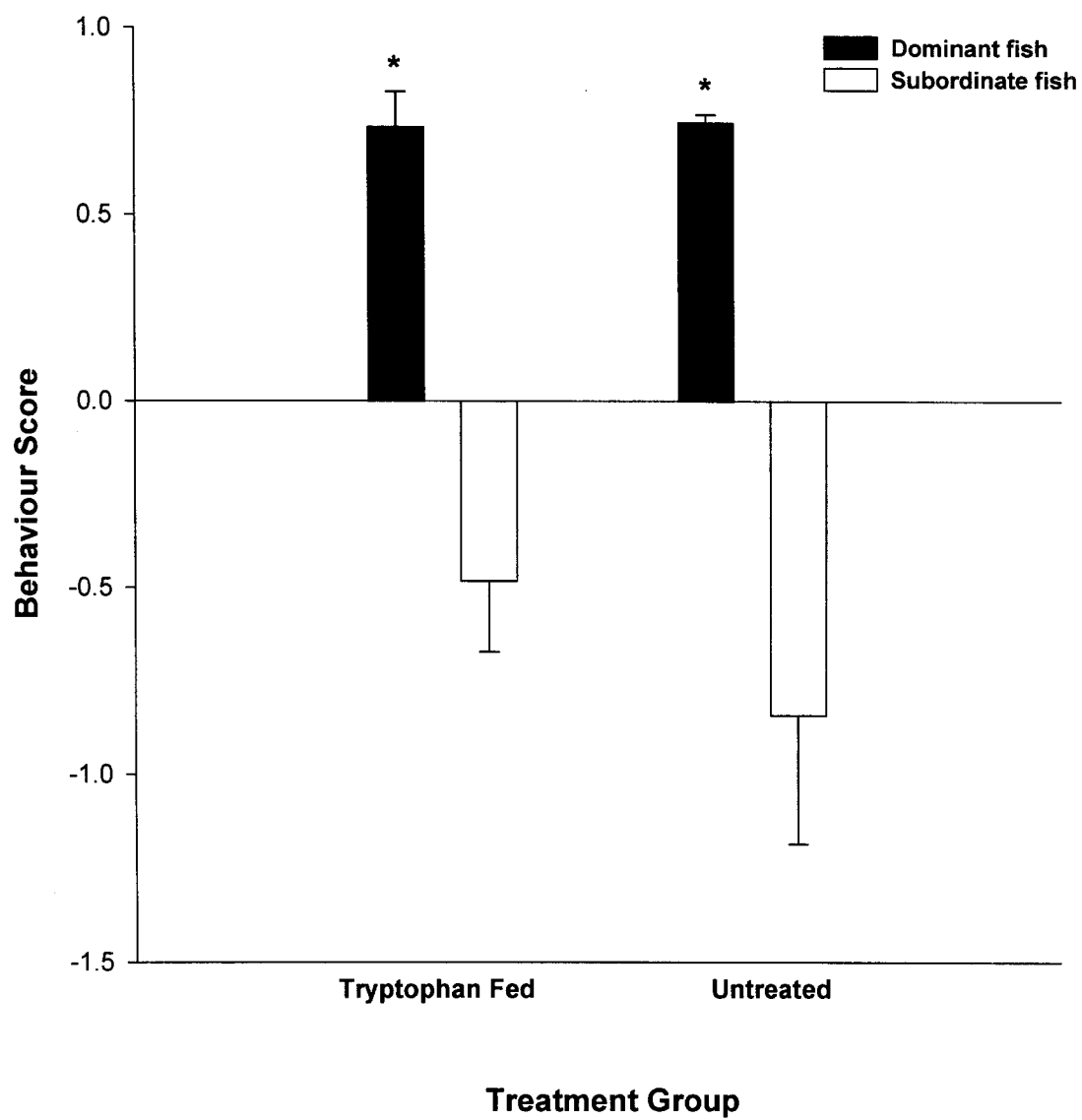


Figure A-4. Mean number of aggressive acts (*i.e.* the combined count of attacks, nips, and chases) performed during individual 15 minute observation periods by L-dopa treated versus untreated rainbow trout (*Oncorhynchus mykiss*) separated by social status (*i.e.* dominant and subordinate) and confined together in pairs for 3 days of social interactions. Data are presented as mean values $\pm$ 1 S.E.M. and *N* values are as follows: L-dopa treated/Dominant, *N* = 11; L-dopa treated/Subordinate, *N* = 9; Untreated/Dominant, *N* = 9; Untreated/Subordinate, *N* = 11. One fish from each pair was either L-dopa “gavaged” (10 mg kg⁻¹) or injected intraperitoneally with L-dopa (low dose, 20 mg kg⁻¹; high dose, 100 mg kg⁻¹) and allowed to interact with untreated fish for a total of 3 days, with 2 observation periods each day; data was pooled from all L-dopa trials. An asterisk indicates a significant difference from subordinate fish within the corresponding treatment group (one-way ANOVA on ranks followed by Dunn’s post hoc multiple comparisons test, with *P* values of Day 1, *P* < 0.001; Day 2, *P* < 0.001).

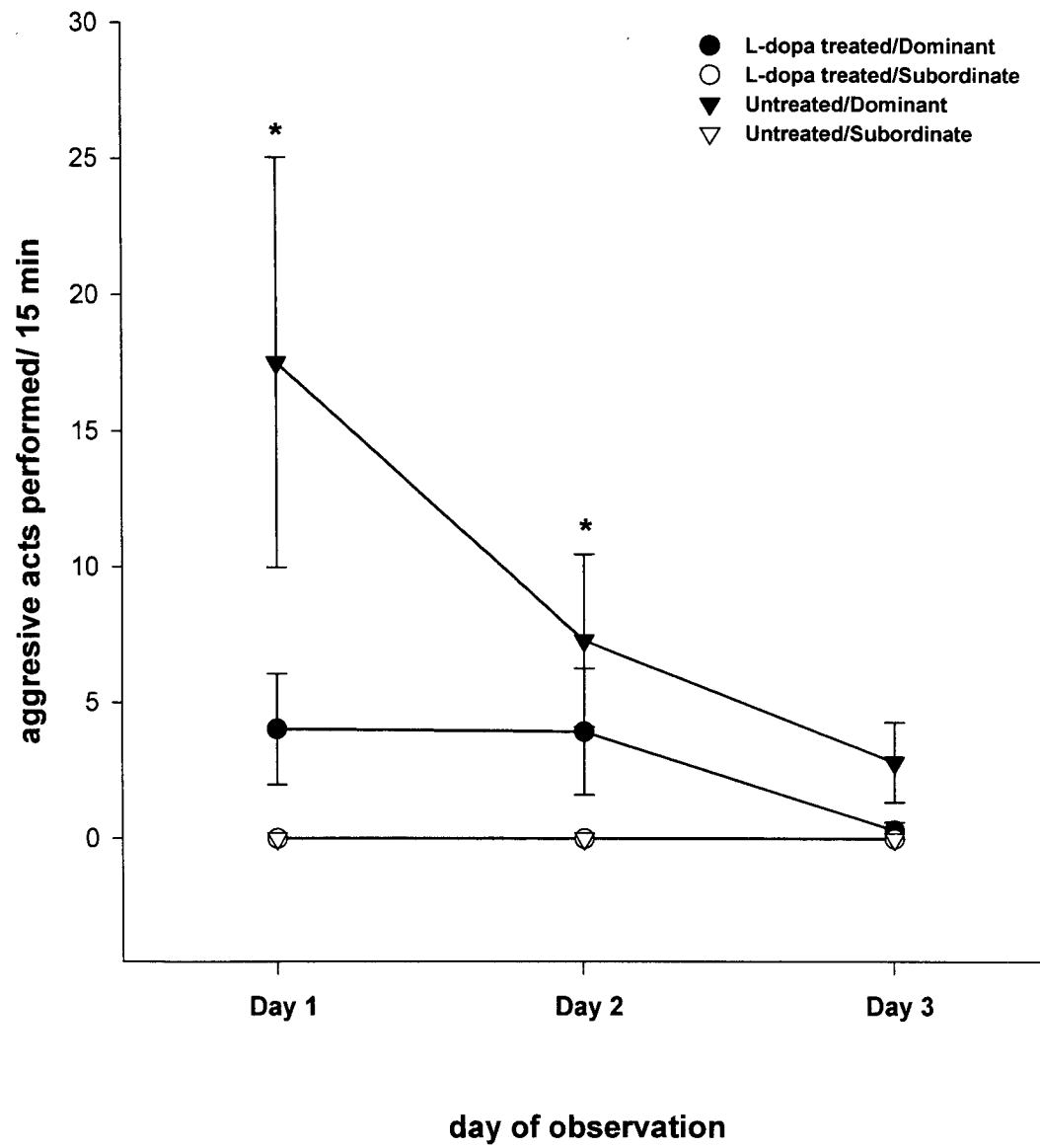


Table A-1. A summary of the chi-square analysis of the effects of L-dopa (gavage or intraperitoneal [i.p.] injections) and apomorphine (whole body immersion) treatment on social status in size-matched pairs of rainbow trout (*Oncorhynchus mykiss*) confined together for 3 days.

Treatment	Type of Vehicle	χ^2	$\chi^2_{0.05,1}$	Conclusion
L-dopa gavage (10 mg kg ⁻¹ fish)	0.02 M HCL	0 (10)	3.84	Fail to reject H ₀ ($P > 0.5$)
L-dopa i.p. injection (20 mg kg ⁻¹ fish)	0.9% Saline	0 (4)	3.84	Fail to reject H ₀ ($P > 0.5$)
L-dopa i.p. injection (100 mg kg ⁻¹ fish)	0.9 % Saline	1.33 (6)	3.84	Fail to reject H ₀ ($0.25 > P > 0.1$)
Apomorphine immersion (4 mg L ⁻¹ water)	n/a	0 (4)	3.84	Fail to reject H ₀ ($P > 0.5$)

Numbers in parentheses are *N* values. The null hypothesis (H₀) was that there was a 50% probability that the treated fish within a pair would become dominant. The alternative (H_A) was that the treatment tested had a significant effect on this probability.

Discussion

Although serotonin (5-HT) and dopamine (DA) have been implicated in the regulation of social status in salmonid fish, it has remained challenging to separate causality from consequence with respect to the suite of behavioural and physiological changes associated with hierarchy formation. The objective of the present experiment was to manipulate brain serotonergic or dopaminergic activity experimentally to determine whether, respectively, low or high social status could be induced. The results of the experiment suggest that manipulation of brain monoaminergic activity does not affect the outcome of social interactions.

Tryptophan (TRP) feeding was employed as a non-invasive means of stimulating brain serotonergic activity, as recent studies have used this method successfully in rainbow trout (Winberg et al., 2001; Lepage et al., 2003). Previous work has shown that TRP injection (Aldegunde et al., 2000), exposure (Koutoku et al., 2003), as well as incorporation into the diet (Winberg et al., 2001; Lepage et al., 2002; Lepage et al., 2003) results in significant and sustained elevations of brain serotonergic activity in all fish. 5-HT does not regulate its own biosynthesis via negative feedback and so the elevation of brain TRP levels results in an immediate increase in 5-HT synthesis/turnover in the brain (*e.g.*, Aldegunde et al., 1998; Aldegunde et al., 2000; Winberg et al., 2001; Lepage et al., 2002). Further, it appears that brain TRP levels are highly sensitive to the supply of this neutral amino acid in circulation (Fernstrom and Wurtman, 1972) and so TRP incorporation into the diet remains a favourable option. However, TRP treatment appeared to be without effect on the social status of fish following social interactions with a size-matched conspecific because only 33% of fish provided with elevated dietary TRP intake became subordinate. Differences in behaviour were also translated into behaviour scores that were typically high positive values

for dominant fish and low negative scores for subordinate individuals, and based on this, behaviour appeared to be largely unaffected by treatment. Behaviour scores of TRP treated fish were not significantly lower than those of untreated fish, while there was a significant difference between dominants and subordinates (Fig. A-3). Behavioural differences among social positions are to be expected, as our observation criteria are scaled to differentiate between dominant and subordinate fish (*i.e.*, low scores for behaviours indicative of subordination versus high scores for dominance correlates), however, a lack of effect of TRP treatment suggests that perhaps brain serotonin levels were not being elevated. Winberg et al. (2001) found that dietary TRP supplementation resulted in a suppression of aggressive behaviour in rainbow trout, with the presumed mechanism being an increase in brain 5-HT metabolism, an effect that was not obvious in the present study. However, Winberg et al. (2001) also noted that a lag occurred before TRP-dependant effects on aggression were observed (*i.e.*, significant effects occurred at seven but not three days of TRP supplementation) and it is therefore possible that the feeding and/or interaction periods of the present study were too brief to register such changes. This conclusion is supported by work in mammals, in which the effects on direct measures of behaviour of stimulating serotonergic activity, through the use of selective 5-HT reuptake inhibitors such as Prozac, only occurred after long-term treatment (Mongeau et al., 1997).

The disparity in food intake between dominant and subordinate fish observed in the present study was similar to that reported previously in salmonid fish (Metcalf et al., 1989; Adams and Huntinford, 1996; Adams et al., 1998) and confirms findings in Chapter 3 of this thesis. Once allowed to interact, subordinate fish ate significantly less relative to dominants during the interaction period (Fig. A-2). In fact, this reduction in feeding occurred in the absence of dominant individuals (*i.e.* fish were separated briefly during feeding) and so does

not necessarily reflect a monopolization of resources but rather a reduction in voluntary food intake in subordinates. Stress and/or stressful situations have been shown to inhibit food intake in both fish (Bernier and Peter, 2001) and mammals (Chaouloff, 2000), possibly via stress-induced increases in brain 5-HT activity (see Bernier and Peter, 2001), and so this remains the most likely explanation for the observed differences in feed consumption in this study. In support of this hypothesis, TRP-treated subordinates appeared to feed even less throughout the entire interaction period in comparison to subordinate fish given normal feed (Fig. A-2), which suggests a TRP and/or serotonergic based mechanism of feed inhibition. Further, TRP-fed fish consumed less food than fish fed normal feed three days after the treatment regimen was introduced (Fig. A-1; Day 9 and 10 of the treatment period), which suggests that these changes were not simply a function of acclimation to the treatment feed and/or procedure, but rather a transient response to elevated dietary TRP levels themselves.

Recent work by Gilmour et al. (2005) also raised the possibility that high circulating cortisol levels are linked to low social status through a pathway in which cortisol-induced increases in brain serotonergic activity and/or decreases in dopaminergic activity affect competitive ability, and in fact, data presented in this thesis support this claim (see Chapter 2). Cortisol treatment not only predisposed fish to subordinate social status following dyadic encounters, but had a significant affect on both serotonergic and dopaminergic activity in the brains of rainbow trout. Given the reported links between high brain dopaminergic activity and high social status (McIntyre et al., 1979; Winberg et al., 1991; Winberg and Nilsson, 1992), treatment with apomorphine, a DA receptor agonist, and experimental elevation of brain dopaminergic activity using L-dopa treatment were investigated in the present study as a means of inducing dominant social status. Surprisingly, none of the methods employed in our study predisposed a fish to dominant social status (Table A-1). The dose and method of

administration of apomorphine used in the present study were based on the methods employed by Dietrich et al. (2002) and Mok and Munro (1998). In these studies, treated fish responded to apomorphine treatment with increases in spontaneous eye movements and locomotor activity, findings which were considered to be a standard physiological response to altered DA brain neurochemistry. In addition, previous work in goldfish, *Carassius auratus* (Chang et al., 1985; Wong et al., 1993) and other fish species (Lin et al., 1993; Lescroart et al., 1998) have reported significant increases in growth hormone levels following intraperitoneal injection of apomorphine, and in turn, growth hormone treatment has been associated with the induction of dominant social status (see Johnsson and Björnsson, 1994). In the present study, however, apomorphine treatment was not effective in inducing dominant social status. Several possible explanations could account for this result, including inappropriate dose and/or timing of apomorphine treatment, or lack of a causal relationship between manipulation of brain dopaminergic activity and the outcome of social interactions.

Previous work has found that treatment with the DA precursor L-dopa is capable of predisposing fish to dominant social status (Winberg and Nilsson, 1992), as well as counteracting stress-induced elevation of plasma cortisol and brain 5-HT activity in Arctic charr, *Salvelinus alpinus* (Höglund et al., 2001). Using the gavage technique and L-dopa doses employed in the present study, Winberg and Nilsson (1992) reported that 18 of 22 L-dopa treated Arctic charr became dominant when paired with an untreated size-matched conspecific. Moreover, Höglund et al. (2001) found that L-dopa treatment resulted in fish becoming more resistant to stress-induced physiological changes and behavioural inhibition. It was therefore surprising that all L-dopa treatments employed in the present study were ineffective at inducing social dominance in rainbow trout, particularly as all administration

routes used in the present study were employed previously in fish (Chang et al., 1985; Winberg and Nilsson, 1992; Hoglund et al., 2001). Measurement of brain dopaminergic activity to directly assess the effectiveness of the L-dopa treatment in the present experiment is an obvious next step.

Aggressive interactions were quantified as an indirect index of the effectiveness of L-dopa treatment. L-dopa treatment was expected to have a significant impact in increasing aggression. For example, in Arctic charr, L-dopa treatment facilitated aggressive behaviour in terms of increasing the raw number of aggressive attacks performed (Hoglund et al., 2001). Surprisingly, L-dopa treated fish in the present study failed to perform significantly more aggressive acts than untreated fish with which they were paired on any single day of the interaction period (Fig. A-4). In fact, a lack of increased aggression in L-dopa treated fish over the entire interaction period, coupled with no effect of L-dopa on social status in any of the trials, brings into the question the effectiveness of our DA delivery methods and whether it in fact increased dopaminergic activity in the brains of treated fish.

In conclusion, methods used previously to induce changes in brain monoaminergic activity were ineffective in affecting the outcome of social interactions in the present study. Although we were unable to independently verify that the techniques used were effective in eliciting changes in brain monoaminergic activity, previous studies have shown them to be effective. As social status was not affected by either TRP, apomorphine, or L-dopa treatment, we believe that, in addition to the possibility that changes in brain monoaminergic activity post-interactions are solely the result of the interactions themselves, either the delivery was ineffective at altering brain monoaminergic activity or both need to be manipulated together. Regardless, we were unable to come up with a suitable means of

manipulating social status and brain monoaminergic activity, and so were unable to further probe the hypothesis resulting from Chapter 2. Perhaps our approach was naïve, in that this system appears to integrate many different inputs from several different sources, and so to expect the answer to be this simple may have been unwise.

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