Behavioural and Neuroendocrine Effects of Stress in Salmonid Fish

BY

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ABSTRACT


Stress can affect several behavioural patterns, such as food intake and the general activity level of an animal. The central monoamine neurotransmitters serotonin, dopamine, and norepinephrine are important in the mediation of both behavioural and neuroendocrine stress effects. This thesis describes studies of two salmonid fish model systems: Fish that become socially dominant or subordinate when reared in pairs, and rainbow trout (Oncorhynchus mykiss) genetically selected for high (HR) and low (LR) stress responsiveness, in terms of stress induced cortisol release. Socially subordinate individuals are often subject to chronic stress, and it was found that plasma cortisol and brain monoaminergic activity rapidly increased in subordinate fish during the initial 24 h period following fights for social dominance in pairs of rainbow trout. In pairs of Arctic char (Salvelinus alpinus), subordinate individuals were characterised by an inhibition of food intake and aggression, and low spontaneous locomotion. Appetite inhibition in subordinate fish was reversed by subsequent rearing in isolation, and this effect was probably related to a concomitant decrease in brain serotonergic activity. Furthermore, differential stress responsiveness in HR and LR rainbow trout was associated with differences in behaviour, as well as changes in brain monoaminergic activity. HR fish displayed higher locomotor activity when challenged by a conspecific intruder. This response was probably related to a larger stress induced activation of brain dopaminergic systems in these fish. Finally it was shown that the steroid 'stress-hormone' cortisol has dose- and context-dependent behavioural effects in fish, as has been described in mammals. Specifically, short-term cortisol treatment elevated the behavioural response to a territorial intruder, while long-term treatment, like chronic stress, had the opposite effect, inhibiting locomotor activity and aggression. It is concluded that the signalling systems involved in behavioural and neuroendocrine control during stress display extensive similarities between teleost fishes and mammals.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABBREVIATIONS</td>
<td>5</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>6</td>
</tr>
<tr>
<td>General overview and introduction to this thesis</td>
<td>6</td>
</tr>
<tr>
<td>The stress response</td>
<td>7</td>
</tr>
<tr>
<td>Brain monoamine neurotransmitter systems</td>
<td>10</td>
</tr>
<tr>
<td>Brain organisation in teleosts</td>
<td>15</td>
</tr>
<tr>
<td>Animal models of stress</td>
<td>16</td>
</tr>
<tr>
<td>Selection for stress responsiveness</td>
<td>17</td>
</tr>
<tr>
<td>Social dominance and social stress</td>
<td>22</td>
</tr>
<tr>
<td>OBJECTIVES</td>
<td>27</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td>28</td>
</tr>
<tr>
<td>Animals</td>
<td>28</td>
</tr>
<tr>
<td>Experimental procedures</td>
<td>27</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>36</td>
</tr>
<tr>
<td>Behaviour of dominant and subordinate fish (paper I and II)</td>
<td>36</td>
</tr>
<tr>
<td>Brain monoaminergic activity in dominant and subordinate fish (paper I and II)</td>
<td>37</td>
</tr>
<tr>
<td>Plasma cortisol in socially interacting fish (paper II)</td>
<td>42</td>
</tr>
<tr>
<td>Behaviour of high- and low-responsive rainbow trout (paper III)</td>
<td>43</td>
</tr>
<tr>
<td>Brain monoaminergic activity in high- and low-responsive rainbow trout (paper IV)</td>
<td>46</td>
</tr>
<tr>
<td>Behavioural effects of cortisol in rainbow trout (paper V)</td>
<td>50</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>54</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>57</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>58</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

AAD Aromatic L-amino acid decarboxylase
ACTH Adenocorticotropic hormone
CNS Central nervous system
COMT Catechol-O-methyl transferase
CRF Corticotrophin releasing factor
CRH Corticotrophin releasing hormone (= CRF)
DA Dopamine
DOPAC 3,4-dihydroxyphenylacetic acid
E Epinephrine
5-HIAA 5-hydroxyindoleacetic acid
HPA Hypothalamus-pituitary-adrenal (axis)
HPI Hypothalamus-pituitary-interrenal (axis)
HR High responders
5-HT 5-hydroxytryptamine, serotonin
5-HTP 5-hydroxytryptophan
HVA Homovanillic acid
LAL Long attack latency (mice)
LC Locus coeruleus
LR Low responders
L-DOPA 3,4-dihydroxyphenylalanin
MAO Monoamine oxidase
3-MT 3-methoxytyramine
NE Norepinephrine
SAL Short attack latency (mice)
TH Tyrosine hydroxylase
TRH Tryptophan hydroxylase
TRP Tryptophan
INTRODUCTION

General overview and introduction to this thesis

The behaviour of an animal is, to a large extent, determined by its physiological state. A well-studied example is provided by the behavioural changes that occur during the reproductive cycle, which are caused by the same hormones that govern reproductive physiology. Many behavioural patterns however change over considerably shorter time periods, and in these cases the connection between physiology and behaviour is perhaps less obvious. An animal that is threatened by an unexpected predator attack will abruptly stop feeding and try to escape, but even if it does escape, it will not start to eat again immediately after the predator is gone. Other forms of stress also have behavioural effects. For instance, an animal that is repeatedly subjected to agonistic acts from socially dominant conspecifics will almost inevitably show a strong behavioural inhibition, with a reduced tendency to compete for food, reproductive partners, or territorial space as one of the most prominent features. Even in the case of stressed animals, and humans for that matter, changes in behaviour and physiology coincide. A primary aim of this thesis is to explore some of the neuroendocrine mechanisms that are responsible for co-ordinating the physiology and behaviour of animals that are subjected to stress. These studies are mainly based on two different salmonid fish models: Individuals which occupy different positions in a social hierarchy, and two lines of rainbow trout that have been genetically selected for high or low stress responsiveness, in terms of post-stress cortisol levels. Particular signalling systems in the brain integrate behavioural and physiological effects of stress, and by cross-reference to the literature on similar research with other species, including mammals, I aim to show that the function of these systems share extensive similarities in different vertebrate groups. These investigations have been motivated mainly by the scientific interest in brain mechanisms that cause behavioural and physiological differences between individuals, but the project also holds some general relevance to the process of rearing animals in capture. Food produced in the Western world is being retailed in markets where concern
for the welfare of animals in capture is steadily rising. It is to be expected that farming industries capable of defining criteria of animal welfare, and incorporate these factors in the breeding process, will be at a competitive advantage. Apart from their significance in commercial aquaculture operations, salmonid fish provide an excellent model organism for the type of studies carried out in this thesis. At life stages when they are territorial in nature, salmonid fish living in small groups form distinct social hierarchies, with clear differences in behaviour and physiology between dominant and subordinate individuals. Simultaneously, these fish thrive excellent when reared in social isolation, which is necessary to obtain relevant control samples of undisturbed animals. The social behaviour of salmonids is well described, and, I believe, easier to apprehend for a human observer than that of mammals. Finally, it would appear that the brain mechanisms involved in the stress response in fish and mammals share extensive similarities, and comparative studies might therefore shed new light on how genetic, developmental, and environmental factors interact to shape individual stress responsiveness.

**The stress response**

Few terms are as popular and frequently used in biology, medicine, psychology, or sociology as "stress". The definition of “stress” as a biological term however has a long history of inconsistency and controversies (see e.g. Selye, 1950; Engel, 1985; Levine and Ursin, 1991). A consensus has now emerged where stress may be defined as a condition in which a threat to the biological functions of an organism, i.e. a stressor, is perceived by that organism, and a set of physiological and behavioural responses is mounted to counteract this challenge. Stressors, then, might be defined as any intrinsic or extrinsic stimuli that disturb the homeostatic equilibrium of the organism, or constitute some other real or perceived threat to its survival or other biological functions. In both animals and man an increasing amount of evidence suggest that psychological processes associated to how an individual assesses a given situation might be equally
important factors as the actual physical challenge in determining the severity of the stress response (reviewed by Von Holst, 1998).

Stress physiology research was long based on the work of Cannon (Cannon, 1929) and Selye (Selye, 1936, 1950, 1952). Cannon described a range of changes in bodily functions during "emotionally stimulating situations": increased heart rate, breathing rate, blood pressure, and blood sugar, as well as decreased gastric and intestinal function, which were attributed to increased activity of the sympathetic nervous system. Furthermore, Cannon concluded that all these effects served to increase the capability of an individual to react actively to a critical situation - to prepare it for 'Fight or Flight'. Selye, who introduced the terms 'stress' and 'stressor' into biomedical research (Selye, 1950), pointed out that however variable the nature of stressors, they always seemed to elicit the same pattern of physiological responses, the 'general adaptation syndrome', in which increased activity of the adrenal cortex was of particular importance. Other important contributions to the modern stress concept are the work of Mason (Mason, 1986) and Weiss (Weiss, 1972) on the effects of psychological influences like predictability and control on the general endocrine response pattern, or the distinction made by Henry between active and passive stress coping (Henry and Stephens, 1977; Henry, 1986).

The main endocrine components of the stress response are essentially similar among all vertebrate groups. These are an immediate increase in the release of catecholamines (epinephrine [E] and norepinephrine [NE]) into the circulation, accompanied by increased sympathetic tone, and a slightly delayed (within minutes) increase in the release of glucocorticoid hormones (mainly cortisol in teleost fish and man, mainly corticosterone in rodents). These steroid hormones are thought to in part function as a suppressive mechanism to dampen the acute response (Munck et al., 1984; Ursin and Olff, 1993), but they also play an important role in regulating energy homeostasis by stimulating some processes (e.g. gluconeogenesis and proteolysis), and inhibiting processes that are not immediately important for survival (e.g. digestion) (Munck et al. 1984; Pickering and Pottinger, 1995; Mommsen et al., 1999; Sapolsky et al., 2000). Increased glucocorticoid levels during stress may also serve to restrain some of
the body's own defence mechanisms (e.g. the immune system).

Both the symphatetic response and the glucocorticoid hormones are considered to serve to prepare the body for an energy-demanding attempt to counteract the effects of the perceived stressor. The catecholamines are released from the adrenal medulla in mammals, in teleost fish the main source of circulating catecholamines is the chromaffine cells embedded in the walls of the posterior cardinal vein in the region of the head kidneys (Nilsson, 1984; Reid et al., 1995). In mammals catecholamines initiate a range of physiological effects which in particular serve to optimise cardiovascular and respiratory functions, including the mobilisation of energy stores to meet increased metabolic requirements during stress. Catecholamines appear to exert similar effects on metabolism and cardiovascular function in fish and mammals (reviewed by Fabbri et al., 1998; Perry and Bernier, 1999), while the role of catecholamines in respiratory function during stress in fish is still subject to some debate (Perry and Bernier, 1999).

The release of glucocorticoids is under control of the hypothalamus-pituitary-adrenal axis (HPA-axis) in mammals, while the teleost homologue of this system is referred to as the hypothalamus-pituitary-interrenal axis (HPI-axis). The neuroendocrine control of the HPI/HPA-axis begins at the level of the hypothalamus, which has connections to the sensory systems, and also is target for several endocrine feedback loops. Thus, the communication between the hypothalamus and the pituitary is the link between the areas of the brain that is involved in sensing and interpreting a stressor, and the endocrine system. In mammals, the first step of the HPI-axis is considered to be the release of the neuropeptide corticotrophin releasing factor (CRF, or corticotrophin releasing hormone, CRH) by neurosecretory cells of the hypothalamus into the intrinsic hypophyseal circulatory network known as the portal system. However, it should be noted that other hypothalamic neuropeptides, such as arginine vasopressin or urocortin (arginine vasotocin and urotensin in fish) also have been reported to affect the next step in the axis, namely pituitary release of adenocorticotropic hormone (ACTH) into the general circulation. As opposed to mammals, teleost fish does not possess a portal system, and the corticotrophic cells of
adenohypophysis are directly innervated by axons from the hypothalamus, regulating the release of ACTH. However, even in fish CRH is generally held as the main signal substance controlling ACTH release and ACTH in turn stimulates cortisol release from the interrenal cells (reviewed by Pickering and Pottinger, 1995; Sumpter, 1997; Wendelaar-Bonga, 1997; Mommsen et al., 1999).

Brain monoamine neurotransmitter systems

The monoamines serotonin (5-hydroxytryptamin, 5-HT), dopamine (DA) and norepinephrine (NE), have been studied as neurotransmitters or neuromodulators potentially involved in the mediation of physiological as well as behavioural stress responses (for reviews see Blanchard et al., 1993, Winberg and Nilsson, 1993; Stanford, 1993). Monoamine metabolism is qualitatively identical in all vertebrates: The catecholamines NE and DA are synthesised from the amino acid precursor tyrosine, which in the brain is taken up from the blood by means of a non-specific carrier which transports large neutral amino acids across the blood-brain barrier. The hydroxylation of tyrosine to 3,4-dihydroxyphenylalanin (L-DOPA) by tyrosine hydroxylase is the first and rate-limiting step in catecholamine synthesis. L-DOPA is in turn decarboxylated to DA by aromatic L-amino acid decarboxylase (AAD), which is a non-specific decarboxylase with a wide distribution throughout the body. In NE synthesising neurones dopamine-β-hydroxylase further converts DA to NE. In E containing cells, NE is methylated by phenylethanolamine-N-methyltransferase to form E, this catecholamine has however not been identified in the teleost brain. Prior to release, both NE and DA are stored in vesicles presynaptically, and their effect is terminated by re-uptake into presynaptic nerve terminals and possibly glial cells. Following re-uptake, DA is subjected to deamination by monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetic acid (DOPAC) or methylation by catechol-O-methyl transferase (COMT) to 3-methoxytyramine (3-MT). Both 3-MT and DOPAC can be further converted to homovanilic acid (HVA), the importance of each pathway depending on species specific distribution of the metabolising enzymes. The major metabolite of CNS NE is considered to be 3-methoxy-4-
hydroxyphenylglycol (MHPG), which is formed after deamination by MAO and methylation by COMT.

5-HT is synthesised from tryptophan. The rate limiting step in 5-HT synthesis is the conversion of tryptophan (TRP) to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase, the activity of this enzyme being restricted by tryptophan availability. 5-HTP is subsequently decarboxylated to 5-HT by AAD, and free 5-HT (i.e. not stored in vesicles) is converted to 5-hydroxyindoleacetic acid (5-HIAA) by MAO. Both for 5-HT and the catecholamines, the ratio of the tissue concentration of their metabolites to that of the parent monoamine is frequently used as an index of neural activity, increased concentration of the metabolite being taken to indicate increased release and turnover of the neurotransmitter (Shannon et al., 1986; Fillenz, 1993).

The organisation of monoaminergic systems, especially that of 5-HT, appear to be remarkably constant throughout the vertebrate subphylum (Parent et al., 1984, Jacobs and Azmitia, 1992). For instance, the typical concentration of 5-HT cell bodies in the brainstem raphe region is found in all vertebrate groups, although their organisation is more variable among different species of teleost fish than in mammals (Kah and Chambolle, 1983; Ekström and Van Veen, 1984; Frankenhuys-van den Heuvel and Nieuwenhuis, 1984; Ekström and Ebbesson, 1989; Corio et al., 1991). Hypothalamic serotonergic cell bodies also exist, but appear to be more numerous in fish than mammals (Kah and Chambolle, 1983; Parent et al., 1984). Generally, the organisation of the 5-HT system could be described as diffuse and with extremely divergent projection patterns. The relatively few 5-HT cell bodies of the midbrain project their axons to a large number of regions in the CNS, where they ramify and terminate, in many cases without making definite contact on target dendrites (Törk, 1990, Jacobs and Azmitia, 1992). Thus, as well as acting as a classical neurotransmitter, 5-HT might diffuse to act on distant cellular targets as a neuromodulator, a type of transmission sometimes referred to as volume transmission (Fuxe and Agnati, 1991). The serotonergic system is considered to be a neuromodulatory system interacting with other neurotransmissions in the brain and participating in the elaboration the response of the central nervous system to external stimuli.
Serotonin is involved in a large number of physiological and psychological events, such as temperature regulation, sleep, learning and memory, behaviour, sexual function, endocrine regulation, and immune activity. Dysfunction in the serotonergic system is also implicated in stress-related pathological syndromes such as post-traumatic stress disorder, anxiety, and depression (Graeff et al., 1996; Dinan., 1996a; Hidalgo and Davidson, 2000; Nutt, 2000).

Dahlström and Fuxe (1964) provided the first detailed anatomy of the catecholaminergic system in the mammalian brain. To an even larger degree than 5-HT, NE cell bodies are concentrated in clusters of cells in the midbrain. The most important nucleus is the locus coeruleus (LC) and a nearby cluster of NE cells in the lateral tegmental area (LTA). In mammals the LC contain only a few hundred neurones, yet they send axons to almost every part of the CNS. In man, this nucleus produces about 70% of the total CNS NE content, and the LC-NE neurones project both down the spinal cord and upward to the limbic structures and the cerebral cortex, while the LTA projections are directed mainly to the hypothalamus. As 5-HT, NE has neuromodulatory actions, setting the level of activity in different CNS regions underlying different behavioural states. Like 5-HT, NE appear to exert a widespread influence over arousal, sensory perception, emotion and higher cognitive functions, as well as participating in the regulation of neuroendocrine releasing factors at the level of the hypothalamus and pituitary, and abnormal function of these systems have been implicated in mental affective illnesses, anxiety, and depression (Meltzer and Lowy, 1987; File, 1996; Dinan, 1996a; Brawman-Mintzer and Lydiard, 1997; Leonard, 1997; Coplan and Lydiard, 1998).

The brain dopaminergic systems are more complex in their organisation than 5-HT and NE systems, DA containing cell bodies being gathered in several major nuclei with more topographic projections (Sheperd, 1994). Brain DA is involved in motor control, certain aspects of behaviour, and also in endocrine regulation. The functions of some DA receptor subtypes (D3 and D4) are currently unclear, although their localisation in limbic areas of the brain suggests roles in cognitive, emotional and behavioural regulation. Studies with primates have demonstrated that the dopaminergic system is important for hedonic impact or
reward learning, as well as for reactivity to perturbation in environmental conditions, for selective information processing, and for general emotional responses, which are essential functions in the ability (or failure) to cope with the external world (reviewed by Pani et al., 2000). Excessive medial prefrontal cortex dopaminergic activity, on the other hand, has a negative impact on the cognitive functions of primates, making them unable to select and process significant environmental stimuli. Thus it appears that a critical range of DA turnover is necessary for optimal cognitive functioning during and after stress (Pani et al., 2000).

The catecholaminergic systems in the brain of teleostean fishes show a number of similarities to those of mammals. Similarities that have been demonstrated include the presence of DA-immunoreactive (ir) cells in the preoptic and hypothalamic regions (Meek et al., 1989; Ekström et al., 1990; Sas et al., 1990) and the occurrence of NE-ir and/or DBH-ir neurones in the locus coeruleus and other nuclei of the caudal brain stem (Ekström et al., 1986, 1990, Hornby and Piekut, 1990; Sas et al., 1990; Meek et al., 1993). Some striking differences are also noted, for instance mesencephalic dopaminergic cells and a dopaminergic mesostriatal pathway, which is one of the most characteristic mammalian catecholaminergic pathways, are absent in teleosts (Meek and Joosten, 1993). On the other hand, teleosts have a well-developed hypothalamic paraventricular organ with numerous DA and NE containing cerebro-spinal fluid contacting neurones (Meek et al., 1989, 1993; Ekström et al., 1990; Corio et al., 1991), a system not present in mammals.

The monoamines act on a variety of pre- and postsynaptic receptors in the brain, with the 5-HT system showing the most considerable receptor divergence. Seven 5-HT receptor families, comprising a total of 14 structurally and pharmacologically distinct subgroups (5-HT_1A-1B, 5-HT_1D-1F, 5-HT_2A-2C, 5HT_3, 5-HT_4, 5-HT_5A-B, 5-HT_6, 5-HT_7) have now been recognised in the mammalian brain on the basis of receptor binding characteristics molecular biology techniques (Mansour et al., 1998; Barnes and Sharp, 1999). The dopamine receptor family have been extended to include five distinct receptor subtypes (D_1 - D_5), which may be divided in two subfamilies whose properties resemble the original D_1- and
D₂ receptors defined biochemically (Mansour et al., 1998). Hence, the two subfamilies are often referred to as D₁-like (D₁, D₅) and D₂-like (D₂, D₃, D₄). The four types of adrenoreceptors designated α₁, α₂, β₁, and β₂ all have been identified in the mammalian brain (Fillenz, 1990). Very little is known about monoamine receptor subtypes in the teleost brain. Pharmacological evidence suggest that the stimulatory effect of NE on gonadotrophin release from goldfish pituitary cells is mediated by α₁-like receptors (Chang et al., 1991), while D₁ and D₂ like receptors have been identified in the pituitary of tilapia (Oreochromis mossambicus) (Lamers et al., 1997) and African catfish (Clarias gariepinus) (van Asselt et al., 1990) respectively. Winberg and Nilsson (1996) provided evidence for at least three different high-affinity [³H]5-HT binding sites in the brain of Arctic charr, demonstrating a considerable 5-HT receptor divergence even in teleost fish. A receptor with a pharmacological profile similar to that of the mammalian 5-HT₁₅ receptor was identified by Winberg and Nilsson (1996), and pharmacological evidence for the presence of a 5HT₂-like receptor subtype in the hypothalamus of rainbow trout has been presented by Agrawal and Omeljaniuk (2000). 5-HT₁₅ and 5-HT₁₇ receptors play an important role in the control of serotonergic function, inhibiting the evoked release of serotonin and its biosynthesis, by acting as autoreceptors localised on serotonergic neuronal terminals and cell bodies. These receptors are also post-synaptic receptors and heteroreceptors located on non-serotonergic terminals, where they affect the release of the corresponding neurotransmitters (acetylcholine, gamma-amino butyric acid, NE, etc.). For a review of the distribution and specific physiological role of each 5-HT receptor subtype, see Barnes and Sharp (1999).
Brain organisation in teleosts

A schematic overview of the salmonid brain is given in Figure 1, showing the names of structures dissected out and included in the analysis of monoaminergic activity in paper I, II, and IV. The brain organisation of salmonids shares large similarities with that of vertebrates in general, with the obvious exception that a cerebral cortex is absent. The task subdivision in vertebrate brains are thought to be as follows: The information necessary to decide on whether or not to react on a particular stimulus (i.e. food, a threat, an aggressive encounter, or a reproductive partner) is gathered and processed in the frontal brain areas, or telencephalon (in mammals areas with particular importance are the hippocampus, frontal cortex, cerebral cortex, amygdala, septum, striatum, and basal forebrain). As evolution has proceeded, considerations, expectations and appraisal based on previous experience has been given increasing impact. The final decision on whether or not to react is taken by the hypothalamus, which is influenced by the current neuroendocrine situation. How to react is decided by areas in the lower brain stem involved in motor and autonomic control (for example, the ventral tegmental area, the substantia nigra, the reticular formation, and the nucleus raphe anterior).

Figure 1. Schematic drawing of a salmonid brain. The names of structures dissected out and included in the analysis of monoaminergic activity are given in bold font.
**Animal models of stress**

Stress has been imposed on experimental animals by a variety of methods, ranging from mild stressors like exposure to a novel environment (Norcross and Newman, 1999), to for instance forced swimming (Taghzouti et al., 1999) and severe stressors like repeated inescapable electric shocks (Minor et al., 1994). This variety may reflect the long-standing concept that any noxious or homeostatically challenging stimuli will elicit a similar core physiological response pattern. Evidence is however accumulating that some aspects of the central nervous response to stress may be highly stressor specific (Bertolucci et al., 1990, Drugan et al., 1995; McBlane et al., 1994; Klauenberg et al., 1985; Lee et al., 1987). Much research is ultimately aimed to understand the pathophysiology of various stress-related psychological disorders in human, but many of these approaches can be criticised for holding little or no relevance to the natural biology of the species studied. In other words, the organism is subjected to something natural selection has not shaped it to handle, and the outcome of such manipulations can not be used to explain the biological processes involved in coping with everyday life. On the other hand it can be argued that natural selection has occurred in a fluctuating environment, and therefore gradually should have equipped living organisms with an increasing ability to cope with unprecedented and unpredictable challenges. Furthermore, the stressors faced by human in modern societies are clearly unprecedented, and finding a perfectly valid animal model is probably impossible. On the other hand, in view of the above notion that different stressors produce different response patterns in many central neuroendocrine systems involved in psychopathological changes, a naturalistic stressor would be more likely to elicit a response pattern similar to those that commonly occur in the 'real' world. In line with this view, the inclination to use so called naturalistic stressors in stress research has increased (e.g. Blanchard et al., 1998; Kramer et al., 1999). I find the above arguments valid even for work with non-mammalian species, and the use of an experimental approach that is relevant to the natural behaviour and physiology of the animal of equal importance. This thesis relies mainly on two behavioural-physiological
model systems, which hopefully meet these criteria: The behavioural and neuroendocrine effects of socially induced stress, and work on two lines of rainbow trout genetically selected for differing stress responsiveness in terms of either consistently high or low post stress cortisol levels.

**Selection for stress responsiveness**

It has long been recognised that sustained stress has a down-regulatory effect on several behaviours. Whether one measure appetite, aggression, exploration, or the general activity level, it would appear that chronically stressed animals are more passive and less willing to perform any activity than are undisturbed individuals. However, in some contexts a mild or short-term stressor might have the opposite effect, i.e. stimulate some behaviours. These two fundamentally different ways of reacting behaviourally to stress are referred to as active and passive coping, or the fight-flight and conservation-withdrawal response, respectively (Engel and Schmale, 1972; Henry and Stephens, 1977; Henry, 1993, Koolhaas et al., 1999). The transition from an active to a passive behavioural strategy with increasing duration of the stressor can be explained in adaptive terms (Haller et al., 1998): During short-term stress it is not yet certain whether an organism is able to avoid or counteract the stressor, thus an effort to actively cope with the situation (a fight or flight type of response) should be attempted. Behavioural inhibition during an initial stage of a stressor might of course also sometimes be adaptive (e.g. for animals that can not outrun a predator), but the animal then runs the risk of continuously exposing itself to adverse conditions that could in fact have been avoided if an appropriate behavioural response had been mounted. If, on the other hand, the organism is not able to escape a chronic, or repeated, stressor for a long time, it would be better of by entering a passive, energy-saving wait-and-see mode. Similar arguments can be made about the severity of the stressor: During a slight stressor, for instance if poor weather conditions temporarily restricts food availability, increased behavioural activity, responsiveness, and efficiency of cognitive processes could be advantageous (e.g. to maintain foraging). If conditions become worse (e.g. during a severe storm), the animal is better of by
shutting down behavioural activity, saving energy, and not exposing itself to danger.

Seminal observations by Henry (Henry and Stephens, 1977) and Engel (Engel and Schmale, 1972) indicated that the threshold at which the shift from an active to a passive coping strategy occurs is subject to great individual variation, and individuals with large differences in behavioural and physiological stress responsiveness coexist in a normal population. Recent studies have confirmed that individual differences in stress responsiveness are associated with differences in behaviour. In fact, behavioural and physiological traits are linked in such a way that two opposing stress coping styles, denoted active (proactive) and passive (reactive) coping, can be identified (Koolhaas et al., 1999). Koolhaas et al. (1999) defined a coping style, or coping strategy, as "a coherent set of behavioural and physiological stress responses, which is consistent over time and which is characteristic to a certain group of individuals". An active stress coping style is behaviourally characterised by a high level of active avoidance, locomotor activity, aggression, and other behavioural patterns indicating an active attempt to counteract the stressful stimulus (e.g. defensive burying in rodents) (Bohus et al., 1987; Benus et al., 1989; Driscoll et al., 1990; Sluyter et al., 1996). Passive coping, on the other hand, is characterised by immobility and low levels of aggression. Fundamental to the theory of different coping styles is that different behavioural characteristics are associated with consistent neuroendocrine and physiological differences. The active strategy is generally associated with low HPA- axis responsiveness, but high sympathetic reactivity, while the opposite is true for the passive strategy (De Boer et al., 1990; Korte et al., 1992; Fokkema et al., 1995).

Using mouse lines genetically selected for short (SAL) or long (LAL) attack latency in an intruder-resident paradigm, Benus et al. (1989, 1991) confirmed that the individual stress reaction pattern is a heritable trait. The SAL mice are characterised by vigorous offensive aggression when confronted with an intruder, which turns into flight when roles are changed, and SAL mice are confronted with a stronger resident. By contrast, LAL mice react with immobility and withdrawal in the latter situation, while being much more reluctant to attack
intruders when tested as a resident (Benus et al., 1991). SAL mice also show more active avoidance to an aversive experience (electric shock) than the non-aggressive LAL animals, which show passive behaviour during threatening conditions. The active and passive animals were simultaneously distinguished by neural and endocrine differences, the active behavioural response (fight / flight) being characterised by high sympathetic activity, but low corticosteroid levels, while passive behaviour was associated with high parasympathetic tone and elevated corticosterone (Fokkema et al., 1988, 1995; reviewed by Koolhaas et al., 1999).

Another selection model was based on the extent of stereotypic gnawing elicited by the dopamine D<sub>1</sub>/D<sub>2</sub> receptor agonist apomorphine (Cools et al., 1990). It was found that in an unselected population about 25% of the individuals were virtually lacking the gnawing response, another 25% showed a vigorous gnawing response, and the remainder of the population consisted of animals showing an intermediate response (Cools et al., 1990). Thus, the apomorphine-susceptible (apo-sus) and apomorphine-unsusceptible (apo-unsus) rats represent extremes in dopamine responsiveness. Rats from the two selected lines also show large variation in the behavioural response to stress: Apo-sus rats flee after social defeat, and show increased locomotor activity in response to novelty, as compared to apo-unsus individuals. Thus, apo-sus rats show many of the behavioural characteristics displayed by SAL mice, and susceptibility to apomorphine therefore probably was co-selected with the active behavioural strategy during stress. Apo-sus rats also showed increased TH and D<sub>1</sub> receptor mRNA levels, along with significantly higher D<sub>2</sub> receptor binding, in the nigrostriatal and tuberoinfundibular dopamine pathways (D<sub>2</sub> receptor mRNA and D<sub>1</sub> receptor binding was not different) (Rots et al., 1996a). Coherent with measures of increased tuberoinfundibular dopaminergic activity, it was later found that the prolactin response to stress was significantly lower in apo-sus than apo-unsus rats (dopamine inhibits pituitary prolactin release) (Rots et al., 1996b). Under basal morning conditions (which is at the end of the active period of the diurnal cycle in rats), apo-sus rats showed a larger number of CRH gene transcripts in the paraventricular nucleus (PVN) and higher ACTH levels in blood.
plasma, while circulating corticosterone was similar in apo-sus an apo-unsus animals (Rots et al., 1995; 1996b). Stress induced ACTH levels were also higher in apo-sus rats, but stress induced corticosterone secretion was not different between the lines. In fact, it would appear that apo-sus rats displayed an adrenal hyporesponsiveness to ACTH, since much higher ACTH levels was required to maintain similar total corticosterone levels under basal conditions. Stress-induced ACTH and total as well as free corticosterone levels however remained significantly elevated longer in apo-sus animals as compared to the apo-unsus strain, suggesting a reduced ability to terminate stress-induced ACTH release, and therefore corticosteroid feedback resistance in the apo-sus strain (Rots et al., 1995; 1996b). Thus, high susceptibility to apomorphine-induced stereotypic behaviour co-occur with increased locomotor activity in a novel environment, altered HPA-axis dynamics, and enhanced brain dopaminergic activity in the apo-sus strain.

Several other strains with genetically based differences in physiology and behaviour have been described, and they need not even be intentionally selected for. Uvnäs-Moberg et al. (1999) studied Sprague-Dawley rats from a breeder who, obeying the demand from customers for 'docile' animals, had excluded 'dominant and aggressive animals' from one strain ('Stock B'), but not from another ('Stock A'). These two stocks of animals displayed a striking difference in their ability to acquire a conditioned avoidance response. 'Stock B' animals completely failed to acquire conditioned avoidance behaviour, but 'Stock A' animals reached an asymptote of 100% avoidance within four of five training sessions (Uvnäs-Moberg et al., 1999). Stock B animals were however more reactive, as evidenced by an enhanced startle response and a shorter reaction time to auditory stimulation. By cluster analysis intercorrelations Uvnäs-Moberg et al. (1999) defined three endocrine covariating endocrine clusters in these animals: 1. Insulin, gastrin, and glucose. 2. CCK, glucagon, and somatostatin, and 3. Oxytocin and corticosterone. Subsequent calculation of cluster scores indicated a marked and statistically significant difference between the two groups in the oxytocine / corticosterone cluster, corticosterone levels being significantly higher
in the reactive (but non-learning) stock B animals, and oxytocine being significantly lower.

Thus, the tight coupling between physiology and behaviour is especially well illustrated by genetically selected strains of animals that display simultaneous differences in physiology and behaviour. In many ways, these models are superior to targeted gene knock-outs, since an entire suite of neuroendocrine mechanisms are selected for, instead of altering just one specific gene product. The systems that produce behavioural and neuroendocrine stress responses work in an integrated fashion, and selection models keep that integration intact. Limited information is however available on whether different individual stress coping styles have evolved in "lower" vertebrates. Among teleost fish consistent behavioural patterns that may reflect alternative coping styles have been observed in cichlids (Francis, 1990; Budaev et al., 1999). Pottinger and co-workers have demonstrated that rainbow trout can be segregated into high- and low-responding (HR, LR) individuals on the basis of their plasma cortisol response to a defined stressor, the magnitude of the cortisol response showing both consistency over time and a high degree of heritability (Pottinger et al., 1992; Pottinger and Carrick, 1999). Behavioural stress responses have, however, not been studied in established HR and LR strains of rainbow trout. Study III was designed to quantify several behavioural patterns in these two lines of fish following transfer to a previously unfamiliar, potentially stressful environment, thereby testing the hypothesis that differential stress responsiveness in rainbow trout is associated with differences in behaviour. Such a difference might indicate that these lines of fish represent selection for different stress coping styles, as has been described in mammals. Differences in brain monoaminergic functions also have been observed in animals displaying genetically determined variation in stress responsiveness and behaviour (e.g. Popova et al., 1991a, 1991b; Bertolucci-D'Angio et al., 1990; Nikulina et al., 1992; Rots et al., 1996a; De Kloet et al., 1996; Lepage et al., 2000). It therefore could be hypothesised that selection for stress responsiveness in rainbow trout is associated with changes in brain monoaminergic activity (study IV). The finding that HR and LR rainbow trout actually do display several differences in behaviour
(Study III, Pottinger and Carrick, 2001a) motivated paper V, in which the
behavioural effects of the steroid hormone cortisol was studied. In mammals
glucocorticoids have a range of behavioural effects, which are mediated through
genomic and non-genomic effects in the brain (see e.g. Sandi et al., 1996; Oitzl et
al., 1997; Haller et al., 1998; Marinelli et al., 1998; Orchinic, 1998; Moore and
Evans, 1999; Rose, 2000), as well as through effects on energy homeostasis
(Haller, 1995). The behavioural effects of cortisol are however poorly studied in
teleost fish, and the fact that behavioural effects of cortisol are likely to be dose-,
time-, and context-dependent (e.g. Sandi et al., 1996; Breuner and Wingfield,
2000) have not been given attention.

Social dominance and social stress

In social animals, i.e. animals that occur together and influence each other
(Tinbergen, 1953), variation in competitive ability implies that some animals will
have preferential access over others to food, or whatever commodity being in
demand. When such imbalance is a consistent feature of the interaction between
two individuals, it is referred to as a dominance / subordination relationship. In
groups of more than two individuals, dominance / subordination relationships will
often take the form of a dominance hierarchy or “pecking order”, in which the
outcome of overt or ritualised intraspecific aggressive encounters is the main
factor determining individual rank order (Huntingford and Turner, 1987). Thus,
human investigators usually reveal the structure of dominance hierarchies by
behavioural observations of who retreats and who wins during aggressive
interactions. However, social hierarchy formation potentially affects almost every
feature of animal life, so a range of other criteria also indicate hierarchical
relationships (e.g. relative food consumption, mating frequency, or non sexual
affiliative behaviour (Järvi, 1990; Hill, 1990; Winberg et al., 1993a).

Chronically elevated levels of plasma cortisol and other indicators of
sustained stress have repeatedly been observed in socially subordinate animals
(Louch and Higginbotham, 1967; Noakes and Leatherland, 1977; Golub et al.,
1979; Ejike and Schreck, 1980; Sapolsky, 1990; Blanchard et al., 1993; Albeck et
al., 1997; Shively et al., 1997a; Winberg and Lepage, 1998). This effect is
referred to as social stress since it is manifest in socially interacting animals, but not in social isolation, in the absence of external stressors. The observations referred to above concern free-living as well as captive animals, suggesting that hypersecretion of cortisol in subordinates is a rather general feature of animal life. Social stress is however probably enhanced under conditions of artificial rearing, where opportunities for social signalling and escape from fighting are limited. A number of reports also infer stress in dominant individuals, especially in free-living animals where dominants are involved in high rates of aggressive encounters (Creel et al., 1996). This discrepancy could probably be explained by taking the relative stability of social hierarchies into concern: In a changing social environment with unstable hierarchies top-ranking individuals frequently have to defend and reinforce their position (Sapolsky, 1982, 1992). Thus, dominants would tend to be frequently involved in aggressive encounters, which are of course stressful whether they are won or lost (but more so, if they are lost). In a stable social setting, on the other hand, dominant individuals are generally in a less stressful position than their subordinate fellows are.

In fish, as in other vertebrates, subordinate individuals show a general behavioural inhibition, characterised by suppressed aggressive behaviour, reduced feeding and low spontaneous locomotor activity and exploration (Abbott et al., 1985; Raab et al., 1986; Franck and Ribowski, 1993; Nakano, 1994; Winberg et al., 1993b; Winberg and Nilsson, 1993; Albonetti and Farabollini, 1994; Meerlo et al., 1997). Behavioural inhibition in subordinates can be viewed as a passive coping strategy to avoid costly interactions with dominants (Leshner, 1980; Benus et al., 1991). It would be useless to engage in competition with an individual whose competitive skills far exceed your own; thus individuals with obvious superiority should not be challenged. The result of previous aggressive encounters is probably one of the better predictors of the competitive abilities of an opponent, individual size and more or less apparent social signals are others (Abbott et al., 1985; Holtby et al., 1993; Chase et al., 1994; Zucker and Murray, 1996). However, many of the behavioural changes seen in subordinates also have been reported to occur during predator challenge (Blanchard and Blanchard, 1971; Blanchard et al., 1998), as well as in response to other types of stress
(McNaughton, 1993). Thus, the behavioural characteristics of socially subordinate animals might in part reflect a general response to chronically stressful, unpredictable and/or potentially dangerous situations where flight is not feasible.

With regard to the function of brain monoamines during stress, brain 5-HT in particular seems to play a key role in integrating behavioural, neuroendocrine, and autonomic stress responses (Blanchard et al., 1993; Chaouloff, 1993; Winberg and Nilsson, 1993). The involvement of 5-HT in the control of several fundamental behavioural patterns is well described, the overall effect of 5-HT stimulation appearing to be a general inhibition of active behavioural responses, like feeding, aggressiveness, locomotion or other behaviours (Meyerson and Malmnäs, 1978; Davis, 1980; Olivier et al., 1989; Leibowitz, 1992; Winberg et al., 1993b; Leibowitz and Alexander, 1998; De Pedro et al., 1998b). In mammals 5-HT is believed also to act stimulatory on the release of CRF and ACTH from the hypothalamus and pituitary, respectively (Chaouloff, 1993, 2000; Dinan 1996b), and in rainbow trout pharmacological stimulation with the specific 5-HT_{1A} agonist 8-OH-DPAT elevates plasma cortisol concentrations in a dose dependent manner (Winberg et al., 1997).

In fish and a range of other vertebrates social subordination and other stressors have been shown to induce a general increase in brain serotonergic activity (Dunn, 1988; Dunn and Welch, 1991; Blanchard et al., 1991; Blanchard et al., 1993; Fontenot et al., 1995; Winberg et al., 1991, 1992, 1993c, 1996; Summers et al., 1998). Increased release of 5-HT appears to be an inevitable result of social stress in animals, and occur in all major 5-HT innervated brain regions. Furthermore, serotonergic activity as indexed by the 5-HIAA / 5-HT ratio, appears to be a remarkably sensitive index of chronic stress in fish: In subordinate rainbow trout the 5-HIAA / 5-HT ratio in the hypothalamus were found to be directly correlated to the number of aggressive acts launched by their contemporary dominants the last 24 hours, as well as to plasma cortisol (Winberg and Lepage, 1998).

The other brain monoamines, DA and NE, also have been reported to mediate behavioural as well as physiological correlates of stress (Haller et al.,
Opinions are divided about the role of brain catecholamines (NE and DA) in behavioural responses to stress, but NE and DA might to some extent have behavioural effects opposite those of 5-HT (Eichelman, 1987; Winberg and Nilsson, 1992; Arregui et al., 1993; Haller et al., 1997a), and elevated DA activity has been connected to a dominant social status (Blanchard et al., 1991; Winberg et al., 1991; Winberg and Nilsson, 1992). However, the functions of these substances are generally less understood than those of 5-HT. Dopamine has been reported to stimulate, inhibit, or be without effect on the HPA-axis in mammals (reviewed by Brambilla et al., 2000), while data obtained by Höglund et al. (submitted) suggest that increasing brain DA activity by administration of the DA precursor L-DOPA lead to an inhibition of a stress induced increase in plasma cortisol release in Arctic charr. A wide variety of evidence from mammalian studies suggest that brain NE systems are involved in the activation of the HPA-axis (Plotsky et al., 1989). Electrophysiological studies on the activity of neurosecretory cells of the paraventricular nucleus suggest that medullary catecholaminergic projections exert a strong facilitatory drive to parvicellular neurons in this nucleus (Day et al., 1985). Results from catecholamine agonist or antagonist challenge studies initially seem contradictory with respect to the direction or receptor mechanisms of catecholamine action on HPA-axis function (Plotsky et al., 1989), but much of the discrepancy could be due to the possible presence of inhibitory $\alpha_2$-receptors on catecholaminergic neurons (reviewed by Stanford, 1993).

Brain catecholaminergic activity has been reported to be increased decreased, or remain unaffected by social stress (Yodyingyuad et al., 1985; Blanchard et al., 1991; Fontenot et al., 1995; Krotewicz and Romaniuk, 1995; Summers and Greenberg, 1995; Tidey and Miczek, 1996; Summers et al., 1997; Matter et al., 1998). This probably reflects the observation that stress commonly leads to a general increase of 5-HT neurotransmission in most brain regions, whereas stress can have a biphasic effect on brain catecholaminergic activity (Stanford, 1993). Alterations of brain catecholaminergic activity are also more regionally specific than those observed for 5-HT and depend more heavily on
factors such as stressor specificity, conditioning, and prior exposure to stress (Nisenbaum et al., 1991; Ge et al., 1997; Stanford, 1993).

Winberg et al. (1991) reported decreased dopaminergic activity in long-term subordinate Arctic charr, but effects of social stress on brain NE systems has not previously been explored in fish. Reports of altered brain catecholaminergic function for instance in socially stressed lizards (Summers and Greenberg, 1995; Matter et al., 1998), along with the similarities between monoaminergic function in fish and other vertebrates, however motivate further investigations on the potential effects of social stress on brain catecholaminergic activity in teleosts fish (study II). The relationship between 5-HT and appetite inhibition, which is a reliable behavioural indicator of chronic stress in many animals, is little studied in fish, and is explored in study I.
OBJECTIVES

The overall aim of the current project was to further investigate the mechanisms involved in the mediation of behavioural and neuroendocrine effects of stress in salmonid fish, focusing on the functions of brain monoamine neurotransmitters, and on the possible role of cortisol in behaviour. The following sub-tasks was identified:

I. Feeding behaviour as an index of behavioural inhibition

The objective of Study I was to evaluate the use of feed intake as a bioassay on behavioural inhibition during stress in fish, and to confirm that appetite inhibition during stress is mediated by central signal substances simultaneously responsible for the activation of the HPI-axis. The possible relationship between 5-HT and appetite inhibition in Arctic charr was explored.

II. Dynamics of HPI-axis activation and brain monoaminergic activity during the establishment of dominant-subordinate relationships

Fights for social dominance are probably stressful for both dominant and subordinate individuals, but behavioural inhibition is not seen in dominant individuals. The objective of study II was to determine if dominant individuals are able to rapidly reduce the stress reaction resulting from fights for social dominance, and also to describe the effects of social interactions on brain catecholaminergic activity in dominant and subordinate rainbow trout.

III. Behaviour of fish with genetically determined differences in stress responsiveness

The objective of study III was to determine if rainbow trout selected for high or low post-stress cortisol values also display differences in behaviour, and therefore may represent selection for different behavioural/physiological stress coping strategies.

IV. Brain monoaminergic activity in high- and low-responsive rainbow trout

Study IV aimed to explore whether HR and LR rainbow trout also display differences in stress induced brain monoaminergic activity.

V. Behavioural effects of cortisol

The objective of study V was to explore the behavioural effects of cortisol in rainbow trout. Effects of short- and long-term exposure to elevated cortisol levels on aggression and locomotor activity are reported.
MATERIAL AND METHODS

Animals

Study I was conducted at the Aquaculture Research Station in Kårvika, Tromsø, Norway, and the experimental fish were 2-year-old Arctic charr (*Salvelinus alpinus*) of the Hammerfest strain, a population which at that time had been reared for aquaculture purposes for 2 generations. Juvenile rainbow trout (*Oncorhynchus mykiss*), used in Study II and V, were obtained from commercial rearing plants in Sweden and kept in groups of approximately 200 individuals in 1 m³ holding tanks at Uppsala University for at least 2 weeks prior to the experiment. Study III and IV was performed at the Windermere Laboratory (NERC Centre For Ecology and Hydrology, UK), utilising adult females from two strains of rainbow trout selected for high (HR) or low (LR) post-stress cortisol levels. The procedures and results of the selection regime is reviewed by Pottinger and Carrick (1999). In short, two F1 lines of rainbow trout divergent for the cortisol response to a standardised stressor (confinement) were generated by individual selection for consistently high or low post-stress cortisol values within the F0 generation. HR and LR F1 families were maintained at the Windermere Laboratory in 1500 l outdoor tanks, supplied with lake water (25 l min⁻¹; stocking density approx. 20 g l⁻¹) at ambient temperature and fed three times weekly with commercial feed (Trouw Standard Expanded 40) at the manufacturers recommended rate. The experiments described in paper III and IV were carried out in April 2000, when these fish were 3 years old.

Experimental procedures

Studies of social behaviour in juvenile salmonids (paper I and II):

In the Tromsø experiment (paper I) the observation aquaria were 40 x 40 x 45 cm opaque plastic tanks with transparent bottoms, designed to allow for the assessment of spontaneous swimming activity by video recording from below. Experiments in Uppsala (study II) were conducted in glass aquaria (100 x 50 x 50 cm) which were divided into four 50 l compartments by removable PVC walls. In
both studies, experimental fish were transferred from stock tanks to experimental aquaria, or compartments of aquaria, and kept isolated for approximately 1 week prior to the actual experiment. This procedure served partly to allow acclimation to the experimental environment, and partly to diminish possible effects of prior hierarchical relations between the fish. Observations of aggressive behaviour and recordings of inter-individual variation in growth rate and food intake suggest that social hierarchical structures become less evident as group size and rearing density increases (Brown et al., 1992; Pottinger and Pickering, 1992; Siikavuopio and Jobling, 1995), but there is also some evidence of differences in the stress response of dominant and subordinate individuals in groups as large as 200 individuals, that had been reared under conditions comparable to those of commercial aquaculture operations (Øverli et al., 1999).

Typically, fish ate very little, or nothing, on the first one or two days after transfer to social isolation, but would gradually regain feed intake and eat to satiation (2-4 % body mass x day⁻¹) during less than one week. Following acclimation to the experimental aquaria, size matched pairs (<5% weight difference) of fish were formed, either by removing a separating PVC wall (study II) or by transferring two fish to an experimental aquarium which was unfamiliar to both contestants, but otherwise identical to those they had been acclimated to (study I). In both experiments, fights for social dominance started within 2 hours after pair formation, and a winner (i.e. the future dominant individual) appeared within another 2 hours in all cases (fights for social dominance are further described in the Results section of study II). During continued interaction in pairs (68 h in study I, 24 h in study II) dominant individuals were recognisable by their initiation of all aggressive acts, and by their monopolisation of food.

Study I was concerned with the possible relationship between brain serotonergic activity and two aspects of behavioural inhibition in subordinate fish: food intake and spontaneous locomotor activity. It was hypothesised that if reduced feeding in subordinate fish is a behavioural response mediated by central neuroendocrine mechanisms activated by stress (e.g. the brain serotonergic system), food intake should remain depressed in subordinates for some time after the removal of a dominant. Furthermore, the de-activation of the same
mechanisms are likely to be reflected in increased feeding during subsequent rearing in a stress-free environment. Supposing that the suppression of food intake is part of a general behavioural inhibition in subordinates, swimming activity and food intake should be regained in parallel during recovery from social stress. To assess whether behavioural inhibition in subordinates is reversible in such a manner, we observed the behaviour of dominant and subordinate individuals during pair rearing, followed by rearing in isolation. Brain tissues were sampled to analyse serotonergic activity in fish held in pairs and while in isolation, to investigate whether changes in brain serotonergic activity coincided with behavioural alterations. Thus, in Study I the following experimental groups were formed: Dominant and subordinate individuals sampled after 3 days of social interaction in pairs, and previously dominant and subordinate individuals sampled after 3 days of social interaction followed by 9 days of recovery in isolation.

Study II was aimed at describing short-term changes in blood plasma cortisol concentrations and brain serotonergic and catecholaminergic activity in rainbow trout after fights for social dominance. It was hypothesised that both winners (i.e. future dominant fish) and losers (i.e. future subordinate fish) would show a substantial stress response immediately following fights. Furthermore, it was expected that dominant fish, but not subordinate fish, would be able to suppress this response within a short period of time. In line with evidence from mammals, changes in brain monoaminergic activity and blood plasma cortisol were expected to be correlated. Thus, in study II the following experimental groups were formed: Dominant and subordinate fish sampled after 5 min, 3 h, and 24 h of social interaction following the identification of a winner or loser in staged fights for social dominance. Socially isolated fish served as unstressed controls.

In Study I individual feed intake and spontaneous swimming activity was quantified from video recordings made from a position 0.5 m below the observation aquaria, during 3 days of interaction in pairs and during 9 days of recovery following re-isolation. Behavioural recordings were made daily between 09.30 and 15.00. Observation protocols are described in detail in the Material and
Methods section of Paper I. Detailed behavioural observations were not carried out in study II, other than observing the duration and outcome of initial fights for social dominance immediately following pair formation.

Upon sampling, fish were rapidly netted and deeply anaesthetised in a high dose (500 mg/l) of ethyl m-aminobenzoate methanesulfonate. Thereafter, blood samples (study II) were collected from the caudal vasculature using a heparinized syringe and kept on ice, before being centrifuged for the separation of blood plasma. Immediately following blood sampling, fish were decapitated and brains were dissected into the different brain regions described in Figure 1. Brain samples were wrapped in aluminium foil and frozen on liquid nitrogen within 2 min of initial disturbance of the fish (i.e. the netting).

Blood plasma samples were analysed for the concentration of cortisol by a validated radioimmunoassay (RIA) originally described by Olsen et al. (1992), as modified by Winberg and Lepage (1998). Different high performance liquid chromatography systems with electrochemical detection (HPLC-EC) were utilised for the analysis of monoamines and monoamine metabolites in the two studies: In Study I quantification of 5-HIAA and 5-HT was carried out following Nilsson (1989), with a system employing an LC-3 electrochemical detector with a glassy carbon working electrode set at +750 mV vs an Ag/AgCl reference electrode (Bioanalytical Systems, USA), a U6K injector, and a 600 A solvent delivery system (Waters Assoc. Inc., USA). In study II, 5-HT, DA, and NE, and their metabolites, 5-HIAA, DOPAC, and MHPG, were analysed as described in Jobling et al. (1999). This system consisted of a new solvent delivery system (CostaMetric II, LDC, USA), an autoinjector (Midas, Spark, Holland), and an ESA 5200 Coulochem II EC-detector (ESA, USA) with two electrodes at oxidising potentials of +320 and +450 mV, and a conditioning electrode set at +40 mV. The increased sensitivity of this system allowed for the detection of comparatively low levels of catecholamine metabolites.

It should be noted that since monoamines and their metabolites are quickly degraded post mortem, a very rapid dissection was needed, and only a coarse subdivision in different brain structures, as shown in Figure 1, could be done. For instance, the brain stem contains several structures that would be of interest to
analyse separately, e.g. the raphe nuclei containing serotonergic cell bodies, or the locus coerules, which, like in mammals, contain a large portion of the noradrenergic cell bodies in the teleost brain. Also, some of the important terminal regions for monoaminergic innervation, for instance the preoptic area or the paraventricular nucleus would almost certainly be fractionated by the sectioning, and thus be divided between the regions analysed as hypothalamus, telencephalon, and brain stem.

Behaviour and neuroechemistry of rainbow trout selected for high or low stress responsiveness (paper III and IV)

The experiments described in study III and IV were carried out during April 2000. Study III aimed to test whether HR and LR rainbow trout displayed concomitant differences in behaviour and stress responsiveness, while study IV reports the analysis of brain monoaminergic activity in the same individuals. In these studies, adult female HR (n=18) and LR (n=18) rainbow trout were weighed and transferred individually from communal holding tanks to rearing in isolation in outdoor observation tanks. These polypropylene observation tanks (250 l, flow rate 15 l min\(^{-1}\)) were covered with medium plastic mesh in order to prevent escape of the fish, while allowing for behavioural observations from above.

From day 1 after transfer to rearing in isolation, fish were fed daily by hand to 1.5% of their body mass. After distribution of the food the observer stepped back and stayed motionless for 3 min while registering any food intake by the fish. All behavioural observations were carried out visually from a position 1 m away from and at an angle of approximately 45° above the tanks, thus the observer was visible to the fish during behavioural testing. Care was however taken not to disturb the fish, and to approach all individuals in a similar manner. All behavioural observations and sampling of blood and brain tissue (see below) were carried out between 10.00 and 14.00.

After being held for 6 days in isolation, locomotor activity was quantified as the time spent moving during 20 min for each fish, starting 1 h after feeding. Time spent moving was measured by triggering a stop-watch each time the fish
was in motion, and stopping it when the fish was again motionless. The definition of "moving" was restricted to active locomotion transporting the fish more than 10% of its body length, since the fish sometimes were observed to drift passively a few cm back or forth, apparently as a result of water currents rather than locomotion by the fish. Locomotor activity was quantified again the next day, this time with an intruder fish present in the observation tank. Intruder fish were smaller (< 50% body weight) group reared rainbow trout from a hatchery population, and previously unfamiliar to the test fish. The rationale for this test was that the resident fish should have acclimated to rearing isolation, and the motivation to defend the territory was tested.

Behavioural observations, which this time included observations for aggressive behaviour, started when an intruder was introduced to an observation tank, and lasted for 20 min before the intruder was removed and sacrificed. The intruder test was only carried out once with each test fish, and all intruder fish were naive to the test situation.

On the day after the intruder test, 50% of the fish from each group were transferred to 50 l confinement tanks which had previously been utilised to quantify the cortisol response during selection of HR and LR rainbow trout (Pottinger and Carrick, 1999). After 1h in the confinement tanks, fish were netted, anaesthetised in 0.5 ml/l 2-phenoxyethanol, and a blood sample was obtained from the caudal sinus into a heparinised syringe. Blood samples were kept on ice for < 1 h before being centrifuged (3000 g at 4°C for 10 min). Separated plasma was transferred to polypropylene tubes and stored frozen (-20°C) until analysed for plasma cortisol. Plasma cortisol levels were determined using the radioimmunoassay described by Pickering et al. (1987). Following blood sampling fish were killed by decapitation and brains were dissected and later transported to Uppsala on dry ice and analysed as in study II. Following brain sampling, the presence or absence of food in the stomach and / or intestines of each fish was registered.

The remaining 50% of the fish from each group were sampled directly from the observation tanks, to serve as undisturbed controls. The fish were anaesthetised and blood sampled as described above. Thus, for the analysis of
plasma cortisol and brain monoaminergic activity (study IV), four experimental
groups were established: HR controls, LR controls, HR stressed and LR stressed
(n = 9 in all groups). Both controls and stressed fish had been tested for
behavioural activity when isolated, and then in the presence of an intruder on the
day before sampling. It was however assumed that the fish had recovered from
any stress effects of the intruder test. In study II it was shown that immediately
(within 5 min) after fights for social dominance both dominant and subordinate
rainbow trout had elevated cortisol levels, but this response was abolished and
cortisol levels had decreased to control levels in fight winners (corresponding to
resident fish) within 3 h after the termination of fights. Furthermore, when pairs
of rainbow trout were split and fish left in isolation after fights for dominance,
neither dominant nor subordinate fish showed any elevation of plasma cortisol or
activation of brain monoaminergic systems when sampled 3 h or 24 h after fights
(Ø. Øverli, C. A. Harris, and S. Winberg, unpublished data).

Behavioural effects of cortisol in rainbow trout (paper V)

This study was performed with isolated juvenile rainbow trout, in similar aquaria
as was used in study II. At day 7 after transfer to rearing in isolation, 50% of the
experimental fish was given a meal of pellets that had been treated with cortisol.
Cortisol food was prepared by immersing the food in 96% ethanol containing
dissolved cortisol corresponding to 600 mg cortisol x kg\(^{-1}\) food. After evaporation
of the ethanol, cortisol remained incorporated in the diet (Gamperl et al., 1994),
and a 1% body mass meal constituted a dose of cortisol corresponding to 6 mg x
kg\(^{-1}\) fish. Controls were given the same amount of untreated food. Among the
cortisol treated rainbow trout, 50% of the fish were given cortisol food once daily
for another 2 days, corresponding to approximately 48 h of exposure to elevated
cortisol levels. In the 48 h group, the cortisol amount in the food was reduced to
75% of the original dose on day 2 of cortisol feeding, and then to 50% on day 3.
Reduced doses were given since a pilot experiment revealed that the full dose
given on 3 consecutive days would elevate cortisol to levels exceeding those
normally observed in rainbow trout even after severe stressors (>200 ng x ml\(^{-1}\)).
Video recordings were started 1 h after feeding on day 7 after transfer to rearing in isolation for controls and short-term cortisol treated fish, and on day 9 for long-term cortisol treated fish. At this point in time, intruders (rainbow trout weighing between 40% and 60% of the resident fish) were also introduced into half of the aquaria, while half of the fish were filmed when undisturbed. Video recordings continued for 30 min, before the resident fish was netted, anaesthetised in 0.5 ml x l⁻¹ 2-phenoxy ethanol, and a blood sample was obtained from the caudal. Plasma cortisol was analysed by the radio-immuno-assay used in study II. All behavioural observations and sampling was carried out between 9.00 and 14.00.

Thus, the following experimental groups were formed in study V: Controls (which was observed between 1 and 1.5h after feeding with normal food), short-term cortisol treated fish (which was observed between 1 and 1.5h after feeding with cortisol treated food), and long-term cortisol treated fish (which was observed between 48 and 48.5 h after the initial feeding with cortisol food, corresponding to 1-1.5 h after the last of three feeding bouts with cortisol food). Approximately 50% of the fish from each treatment group were subjected to the intruder test when observed, yielding in all 6 experimental groups. From the videotapes, the following parameters were recorded: For all experimental fish, swimming activity was calculated as % time spent swimming (c.f. study III) during the first 15 min from the start of filming (i.e. 60-75 min after feeding). For intruder-tested fish, latency until the resident fish attacked the intruder, and the number of aggressive acts performed against the intruder during 15 min following the first attack, was recorded. An aggressive act was scored when resident fish either a) approached the intruder rapidly, resulting in the intruder being displaced and fleeing, b) approached and bit the intruder, whether or not the bite resulted in the intruder fleeing, or c) approached and chased the intruder for at least 2 x the intruders body length, with or without biting it. If the resident fish did not attack the intruder within 30 min after it had been introduced, 0 aggressive acts were scored, and latency was set to 30 min.
RESULTS AND DISCUSSION

Behaviour of dominant and subordinate fish (paper I and II)

The observations of paper I and II confirm that strong and irreversible dominant-subordinate relationships quickly develop in pairs of juvenile salmonid fish (Abbott et al., 1985; Abbott and Dill, 1988; Holtby et al., 1993). The initial fights for social dominance following pair formation were observed in paper II: After removal of the separating wall, fish initially engaged in a series of mutual displays which after a variable (0.5 - 90 min) latency period changed to overtly aggressive behaviour consisting of attacks, biting, and circling. The duration of fights was highly variable (from 0.5-120 min), but it inevitably ended with one fish retiring from further aggression, and becoming subordinate. Even when remaining passive and having stopped to retaliate on aggressive attacks, subordinate fish would frequently be attacked, nipped, and chased by dominant fish. Thus, a phase of bi-directional aggression (fight) and a phase of unidirectional aggression (social dominance) could be distinguished in all observed pairs of rainbow trout. In paper I, behavioural observations started 24 h after the formation of pairs, thus only the interaction in established dominant-subordinate relationships were observed. In this situation, dominant individuals were recognisable by their monopolisation of food, their initialisation of all observed aggressive acts, and by higher swimming activity.

Furthermore, in paper I it was observed that during recovery in isolation after a 3 day period of social interaction feed intake gradually increased in previously subordinate fish, to approach that of dominant individuals (Figure 2). Thus, the inhibition of appetite seen in socially subordinate animals (Winberg et al., 1993a; Meerlo et al., 1997) appears to be reversible during subsequent rearing in a stress free environment. Interestingly, spontaneous swimming activity, which was also reduced in subordinate fish as compared to dominants, did not increase during recovery in isolation (paper I). This observation suggests that reduced spontaneous locomotion is either a long-lasting response to social stress, or reflects permanently different behavioural strategies of subordinate and dominant fish.
Figure 2. Daily food intake (mean + S.E.M.) of dominant and subordinate fish during pairing and during recovery in isolation. Social rank (p<0.001) as well as time in recovery (p=0.013) had significant effects on food intake (two way ANOVA) (data from paper I).

Brain monoaminergic activity in dominant and subordinate fish
(paper I and II)

When in pairs, brain serotonergic activity, as indexed by the 5-HIAA/5-HT ratio, was elevated in the hypothalamus of subordinate fish as compared to dominants (Figure 3), but this difference was abolished during subsequent rearing in isolation. These results confirm that differences in brain serotonergic activity between dominant and subordinate animals develop through social interaction, as has been observed in several other studies (Blanchard et al., 1991; Blanchard et al., 1993; Fontenot et al., 1995; Winberg et al., 1991, 1992, 1996; Summers et al., 1998).
Figure 3. Serotonergic activity, as indicated by 5-HIAA/5-HT ratios (mean + S.E.M.) in the hypothalamus of dominant and subordinate fish sampled after 3 days of pair rearing and after 9 days of recovery in isolation. Subordinate fish sampled from pair rearing displayed a significant (p<0.05) elevation in hypothalamic 5-HIAA/5-HT, while after recovery in isolation brain serotonergic activity of subordinate fish was no longer different from that of dominants (data from paper I).

Reversal of the difference in brain serotonergic activity between dominant and subordinate individuals after separation has to my knowledge not been reported previously. The reduction in brain serotonergic activity observed in reisolated subordinates was associated with increased feed intake, suggesting an inhibitory role for brain 5-HT on feed intake in Arctic char, as has been reported in mammals (Leibowitz and Alexander, 1998) and goldfish (De Pedro et al., 1998b). Unfortunately, the limited number of animals that was observed during the last phase of study I did not allow for the investigation of possible correlations between brain serotonergic activity and individual feed intake. Further studies, including pharmacological manipulation of the 5-HT system would be needed to establish a role for 5-HT in the control of food intake in salmonids. The results of paper I did not support an influence of 5-HT on spontaneous locomotor activity in Arctic charr, and other studies relating to the role of 5-HT in influencing swimming activity in fish are also ambiguous. Fingerman (1976) and Genot et al. (1984) reached opposite conclusions regarding the effect of serotonergic activity
on spontaneous swimming. The data reported by Winberg and co-workers (Winberg et al., 1993b) however clearly support an inhibitory role for 5-HT in swimming activity. These authors found that fish injected with the 5-HT synthesis inhibitor p-chlorophenylalanine (p-CPA) swam longer during 18 h test periods than did fish injected with the 5-HT re-uptake inhibitor zimeldine, as was the case for dominant vs. subordinate fish. However, the test arena used in this experiment represented a novel environment for the fish, so it could be argued that the serotonergic system suppresses exploratory behaviour, rather than spontaneous locomotor behaviour per se.

In study II, subordinate rainbow trout displayed a substantial increase in both serotonergic, noradrenergic and dopaminergic activity after 3 h and 24 h of interaction with a dominant (Figure 4). In dominant fish, on the other hand, effects on brain monoaminergic activity were observable only at 3 h following the initial fights for social dominance. Specifically, at 3 h telencephalic 5-HIAA/5-HT ratios were elevated with respect to controls in dominant as well as subordinate fish, while subordinate fish at this point in time also displayed increases in DOPAC/DA in the hypothalamus and MHPG/NE in the brain stem.

After 24 h of social interaction, brain monoaminergic activity had increased even more in subordinate fish. At this point in time, large and highly significant effects were seen on serotonergic and catecholaminergic systems of the hypothalamus and brain stem, while in the telencephalon and optic tectum only serotonergic systems appeared to be affected (paper II). Dominant fish, on the contrary, did not differ from unstressed controls after 24 h of social interaction. Thus, fights for social dominance only had limited and quickly reversible effects on brain monoaminergic activity in winners, while continued interaction in pairs induced substantial increases in the activity of catecholaminergic as well as serotonergic systems in subordinate individuals.
Figure 4. Effects of fights for social dominance and continued interaction in an established dominant-subordinate relationship on 5-HIAA/5-HT, DOPAC/DA, and MHPG/NE ratios in different brain regions of dominant and subordinate rainbow trout, as compared to non-stressed controls (mean ± S.E.M.). F, df and p-values are the result of one-way ANOVA, followed by the Tukey post-hoc test for unequal n (Spjotvoll-Stoline test). Post-hoc significance levels are indicated by asterisks, where * is used to indicate a difference to controls and [*] indicates a difference between social ranks at a given point in time (* p<0.05, ** p<0.01, *** p<0.001) (data from paper II).
The results from study II demonstrate for the first time increased brain catecholaminergic activity as a result of social subordination in fish. In an earlier study of long term (> 2 weeks) effects of social interactions on brain monoaminergic activity in Arctic charr, Winberg et al. (1991) observed increased telencephalic levels of HVA in dominant as compared to subordinate individuals, suggesting decreased dopaminergic activity in subordinates. These results are not necessarily contradictory to those of study II, since several reports have suggested a time dependent effect of stress on brain catecholaminergic activity, short term or acute stress being stimulatory but long term or repeated stress being inhibitory (Stanford, 1993).

When interpreting the results of brain monoaminergic activity, it should be kept in mind that the analysis of tissue concentrations of neurotransmitter metabolites does not reflect instantaneous neural activity, as opposed to techniques of in vivo voltammetry or microdialysis (Fillenz, 1993). The metabolites 5-HIAA, DOPAC, and MHPG are formed following re-uptake of the parent monoamines (5-HT, DA, and NE, respectively) from the synaptic cleft, and their accumulation in neural tissue is probably time dependent. In addition, a fraction of the monoamine molecules is always deaminated intraneurally prior to release, so metabolite levels are even sensitive to changes in synthesis rate and monoamine oxidase activity (Fillenz, 1993; Stanford, 1993). Brain metabolite / monoamine ratios are less sensitive than metabolite concentrations to changes in other neural processes than release rate, and are also less sensitive to variance related to tissue sampling and weight determination. Statistically significant increases in metabolite concentrations in response to stress are therefore more rarely reported than increases in metabolite/monoamine ratios, but when they are observed, increased metabolite levels are in most cases considered an indicator of increased monoamine utilisation (for review, see Fillenz, 1993; Stanford, 1993).

As in study II, utilising poikilothermic animals living at 12°C, the effects of social interaction were reflected in increased monoaminergic activity only in the telencephalon (5-HIAA/5-HT ratios) of subordinate fish 5min following the termination of fights. However, the duration of fights was highly variable (2-70 min in the case of 5min fish), and significant correlations were found between
fight duration and brain concentrations of 5-HIAA (in all brain regions), MHPG (in the brain stem), and DOPAC (in the optic tectum) at 5min (paper II). Thus, it appears that these monoaminergic systems (5-HT, NE, and DA, respectively) were activated during fights, leading to gradually increasing metabolite levels over time. Due to the variation in fighting time, however, averaged group values were in most cases not significantly affected at 5min.

**Plasma cortisol in socially interacting fish (paper II)**

In study II, plasma cortisol was drastically increased in 24 h subordinate fish, but dominant fish quickly (within 3 h) recovered to the level of unstressed controls, after an initial rise in plasma cortisol observed in both winners and losers 5 min after the termination of fights (Figure 5). Thus, fights for dominance are apparently stressful for eventual winners as well as losers, but winners are able to rapidly reduce their cortisol levels during continued interaction after dominant-subordinate relationships have been established.

Several significant correlations between blood plasma cortisol and brain 5-HIAA/5-HT ratios, notably in the brain stem, were also observed (paper II). Similar correlations were observed for brain stem MHPG/NE ratios, suggesting an influence of brain noradrenergic and serotonergic neurones on glucocorticoid release even in fish, as has been demonstrated in mammals (Plotsky et. al 1989; Chaouloff, 1993, 2000; Dinan, 1996b). Correlations were found in controls, 5min fish, and 3h subordinate fish, suggesting that brain serotonergic systems influence, or are influenced by, circulating cortisol levels under normal conditions and during moderate or short-term stress. Under severe stress (as in 24h subordinate fish) and during recovery from stress (as in 3 h and 24 h dominant fish), brain monoamines might exert similar effects, but the apparent lack of correlations suggest that plasma cortisol levels depend more heavily on other factors, like for instance the rate of clearance of cortisol from circulation (Pottinger and Moran, 1993). Cortisol also affects brain monoaminergic activity (see the discussion in paper IV), so the direction of causality is not clear for the correlations reported in paper II.
Figure 5. Effects of fights for social dominance and continued interaction in an established dominant-subordinate relationship on blood plasma cortisol in dominant and subordinate rainbow trout, as compared to non-stressed controls (mean + S.E.M.). F, df and p-values are the result of one-way ANOVA, followed by the Tukey post-hoc test for unequal n (Spjotvoll-Stoline test). Post-hoc significance levels are indicated by asterisks, where * is used to indicate a difference to controls and [✓] indicates a difference between social ranks at a given point in time (* p<0.05, **  p<0.01, ***  p<0.001) (data from paper II).

**Behaviour of high- and low-responsive rainbow trout (paper III)**

In study III it was observed that both HR and LR rainbow trout increased their activity level when a conspecific intruder was present, but HR fish reacted more than LR fish (Figure 6). There was no significant difference between the two groups when reared in isolation (Figure 6). Differential post-stress cortisol levels were later confirmed in the two groups by a standardised confinement test, while there was no significant difference in plasma cortisol between HR and LR fish sampled directly from rearing in isolation (paper III).

Contrary to our expectations, the experimental fish did not show any aggressive behaviour during intruder testing. Food intake was not observed during the 1 week acclimation period either, but examination of gut contents...
Figure 6. Locomotor activity in low- and high-responsive (LR, HR) rainbow trout, when alone and when challenged with a conspecific intruder. Post-hoc significance levels are indicated by asterisks, where * is used to indicate a difference between fish in isolation and when intruder-tested, and [*] indicates a difference between LR and HR fish (** p<0.01, *** p<0.001). Statistics: Kruskall-Wallis ANOVA followed by repeated Mann-Whitney U-tests with Bonferroni-correction (data from paper III).

Following termination of the experiment revealed that food was present in the stomach of 7 out of 18 LR fish, while this frequency was 0 out of 18 in the HR fish. Thus, ca 40% of the LR fish had eaten during the experiment (although never when an observer was watching), while HR fish did not take any food during the same period.

The difference in the frequency of feeding individuals was highly significant (two-tailed Fisher exact p=0.0076). In fish, as in mammals, stress-induced appetite reduction is in part mediated by signal substances that are simultaneously involved in central control of the neuroendocrine stress response.
(De Pedro et al., 1998a, 1998b). The resumption of feeding after a stressful experience therefore probably reflects a down-regulation of the neuroendocrine stress response (c.f. paper I). The higher incidence of feeding in LR fish thus may indicate that HR fish remained in a state of stress after transfer to a new environment for a longer time period than did fish from the LR strain.

The observations in paper III are consistent with some reported effects of cortisol in poikilotherms (decreased appetite: Gregory and Wood, 1999; increased locomotor activity: Cash and Holberton, 1999). Thus, different hormone levels may directly cause the behavioural differences between HR and LR rainbow trout. The behavioural and physiological characteristics of HR and LR rainbow trout may also be functionally linked through a number of factors which influence both endocrine and behavioural responses, as well as through direct behavioural effects of cortisol. Due to the differential role of rapidly working membrane bound receptors and of the classical intracellular type I and type II corticosteroid receptors, as well as the extensive cross-talk between cortisol and other signal systems involved in behavioural control, the behavioural effect of cortisol are likely to be time-, dose-, and context dependent (see the discussion in paper V). Both glucocorticoid- and mineralocorticoid-like receptors have been cloned in rainbow trout (Ducouret et al., 1995; Takeo et al., 1996; Colombe et al., 2000), but behavioural effects of cortisol are poorly studied in fish (which motivated study V).

It is also possible that the intruder triggered greater increases in brain CRH activity in HR than LR fish, and that CRH was involved in the increase in locomotor activity observed in HR trout. CRH antagonists injected intracerebroventricularly blocks stress-induced increases in locomotor activity in amphibia (Lowry and Moore, 1991), while CRH administration enhances locomotor activity and neuronal activity (Lowry et al., 1996). CRH administration also increases DA concentrations in dorsal medial hypothalamus of newts (Lowry et al., 2001). One of the main neurochemical differences between HR and LR rainbow trout was that HR fish respond to stress by increased DA concentrations and turnover in several brain areas, while LR fish do not (Paper IV). Increased DA synthesis and metabolism may however also be an
effect of elevated glucocorticoid concentrations (see the discussion in paper IV and V).

The concurrent differences in behaviour and HPI-axis activity observed in study III suggest that the HR and LR rainbow trout may represent selection for different physiological/behavioural stress-coping styles, as has been shown to occur in mammals (Koolhaas et al., 1999). However, the behavioural and endocrine traits of HR and LR rainbow trout do not necessarily correspond to the active (proactive) and passive (reactive) coping styles identified in mammals. For instance, the active stress coping style in mammals generally involve low HPA axis responsiveness (De Boer et al., 1990; Korte et al., 1992; Fokkema et al., 1995). In study III fish with high plasma cortisol following confinement stress (HR trout) also displayed the highest levels of swimming activity during the intruder test. Great care should however be taken when interpreting animal behaviour in terms of intentional or motivational factors. We do not know if the behavioural activity shown by rainbow trout in this experimental setting represent an active attempt to counteract a stressor, comparable to active avoidance or defensive burying in rodents, or if it reflects anxiety or a panic reaction. My personal interpretation is however that the HR trout showed anxiety-like erratic behaviour, rather than targeted attempts to actively cope with the experimental situation. If that is the case, the behavioural strategy of the LR fish (to remain passive and ignore the presence of an intruder in a low-quality territory) may actually be more equivalent to the active coping strategy that is normally associated with low HPA-axis responsiveness in rats (Koolhaas et al. 1999).

**Brain monoaminergic activity in high- and low-responsive rainbow trout (paper IV)**

Using the dissection method originally introduced in paper II, study IV showed that HR and LR rainbow trout displayed differences in tissue concentrations of monoamines and / or monoamine metabolites, or in metabolite / monoamine ratios, in all brain regions. One of the most evident findings was that HR trout reacted to stress by an increase in the tissue concentrations of both serotonin
(brain stem), dopamine (brain stem), and norepinephrine (optic tectum, telencephalon), whereas low-responsive fish did not (Figure 7). Brain stem and optic tectum concentrations of monoamine metabolites were also elevated after stress in high responders, but not in low-responsive fish. A divergent pattern was seen in the hypothalamus, were low-responsive fish displayed elevated levels of 5-HIAA and MHPG. Both populations had elevated telencephalic concentrations of these metabolites after stress (paper IV). Some differences were also seen in fish sampled directly from rearing in isolation, suggesting that the rearing environment was not entirely optimal, and the experimental fish may have experienced a mild stress even in the undisturbed condition. This notion is also in line with the lack of aggressive behaviour and low level of feed intake in these fish.

When interpreting the various data from study IV, attention should be paid to the fact that altered metabolite / monoamine ratios may be caused by changes in the concentrations of monoamine neurotransmitters as well as metabolites, or by combinations of such changes. Thus, differential patterns of activation may be assumed depending on whether concentrations or ratios are studied. For instance, brain stem 5-HIAA concentrations were significantly affected by confinement stress only in HR fish (Figure 7). Thus, it could be concluded that the brain 5-HT system was activated by confinement stress to a larger extent in HR than in LR fish. On the other hand, brain stem 5-HIAA/5-HT ratios were higher in LR than HR fish both in the control condition and after stress. The apparent contradiction is probably caused by the fact that HR fish, but not LR fish, responded to stress by an increase in 5-HT concentrations in the brain stem. Apart from that, three possible explanations can be given to the observation that 5-HIAA/5-HT ratios were elevated in LR fish: 1. The proportion of 5-HT that was actually released, and thereby exposed to the action of MAO after re-uptake from the intercellular space, was greater in LR fish. 2. MAO enzyme activity was decreased in HR fish. 3. Re-uptake of 5-HT was more effective in LR fish.
Figure 7. Tissue concentrations of monoamines and monoamine metabolites (ng/g), and corresponding [metabolite] / [monoamine] ratios (mean ± S.E.) in the brain stem (5-HT and DA) and optic tectum (NE) of HR and LR rainbow trout when reared in isolation (controls) and following 1h confinement stress. Post-hoc significance levels are indicated by asterisks, where * is used to indicate a difference between stressed fish and controls from the same population, and [*] indicates a difference between LR and HR fish (* p<0.05, ** p<0.01, *** p<0.001). Statistics are ANOVA followed by the Tukey HSD post-hoc test (data from paper IV).
Elevated 5-HIAA/5-HT ratios in LR fish due to increased 5-HT release in this group (alternative 1) would appear contradictory, since there is evidence that 5-HT act stimulatory on the HPI-axis teleost fish, like in mammals (Winberg et al., 1997). On the other hand, differential cortisol secretion in HR and LR fish might depend on differences in interrenal responsiveness (Pottinger and Carrick 2001b), rather than central control of the HPI-axis. Differential brain serotonergic activity in HR and LR fish may be attributed to differences in circulating cortisol, as well as to alterations in intrinsic properties of the neural systems. Glucocorticoid hormones may affect the activity of serotonergic neurones themselves (Chaouloff, 1993; 2000), or through interaction with other signal substances. Glucocorticoids also have been reported to have an inhibitory effect on brain MAO in mammals (Veals et al., 1977; Cvijic et al., 1995). Effects of glucocorticoids on MAO have not been reported in fish, but an inhibitory effect would be consistent with decreased 5-HIAA/5-HT ratios in HR as compared to LR fish (an effect seen both in the brain stem and telencephalon), and favour alternative 2. above. Selection for stress responsiveness may also have led to genetically determined differences in MAO. Such differences have for instance been connected to personality traits in human (reviewed by Shih and Thompson, 1999). An association between 5-HT transporter gene polymorphisms and mood alterations has also been reported in human (Lesch et al., 1996).

Increased brain stem 5-HT in HR fish as compared to LR fish after confinement points towards differential effects of stress on 5-HT synthesis in the two populations. Glucocorticoids may again be responsible, either through effects on precursor availability, or through effects on synthesising enzymes (reviewed by Chaouloff [1993, 2000]). Evidence on the possible role of cortisol in the regulation of monoamine synthesis in teleost fish is lacking, but in general it would appear that fish and mammalian monoamine systems display extensive similarities in both function and anatomy (Parent et al., 1984; Hornby and Piekut, 1990; Jacobs and Azmitia, 1992; Winberg and Nilsson, 1993; Ma, 1994). A similar influence of adrenal / interrenal steroids on monoamine functioning in these vertebrate groups therefore can not be excluded.
Similar to what was the case for 5-HT, stressed HR fish displayed increased DA levels in the brain stem, as compared to both controls from the same population and stressed LR fish (Figure 6). NE concentrations were also increased in the optic tectum and telencephalon of HR, but not LR fish, after stress (Figure 7, paper IV). Glucocorticoids may also act stimulatory on tyrosine hydroxylase (Dunn et al., 1978; Lucas et al., 1998), again suggesting that increased neurotransmitter concentrations in HR fish during stress is a consequence of increased synthesis due to elevated glucocorticoid levels, but the observed differences in catecholamine systems between HR and LR trout may also be genetically determined (e.g. Bertolucci-D'Angio et al., 1990; Rots et al., 1996a; Sallinen et al., 1999).

A stimulatory role for brain dopaminergic systems in locomotor activity has long been acknowledged, and this point of view has recently been confirmed in studies with dopamine transporter knockout mice (Spielewoy et al., 2000), while 5-HT may have an inhibitory effect on locomotor activity (Winberg et al., 1993b). It therefore seems likely that an interaction between cortisol and brain dopaminergic systems is involved in the elevation behavioural activity observed in HR fish when subjected to a conspecific intruder (paper III).

**Behavioural effects of cortisol in rainbow trout (paper V)**

In Paper V it was demonstrated that cortisol has time- and context-dependent effects on behaviour in teleost fish. Administration of cortisol through the fish food resulted in reproducible and highly significant increases in circulating plasma cortisol, with cortisol concentrations approaching those seen in severely stressed rainbow trout (100-150 ng/ml) (paper V). Short-term cortisol treatment lead to a significant increase in locomotor activity in intruder-tested fish, while being without effect in undisturbed individuals. Long-term treatment had the opposite effect, leading to a clear inhibition of locomotion in the intruder tested fish (Figure 8). Aggressive behaviour was also clearly inhibited by long-term cortisol treatment, but not by short-term exposure to cortisol (Figure 9).
The observation that cortisol treatment was without effect on locomotor activity in undisturbed fish indicates that the behavioural effects of cortisol were mediated through interaction with other signal systems activated when the resident fish was challenged by the presence of an intruder. Glucocorticoids have for instance been shown to facilitate dopamine mediated behaviours, like locomotor activity (Marinelli et al., 1998), probably through GR receptor mediated increases in dopamine release. In mammals glucocorticoids have regionally dependent effects on brain CRH gene expression (Schulkin et al., 1998), and CRH in turn has been shown to stimulate locomotor activity by direct actions on brain neurones in amphibia (Moore et al., 1984; Lowry and Moore, 1991; Lowry et al., 1996).

![Figure 8. Locomotor activity (mean + S.E.M.) in isolated and intruder-tested controls and fish subjected to short- or long-term cortisol exposure, calculated as % time spent swimming between 60 and 75 min after feed had been distributed. Experimental groups assigned different letters displayed statistically significant differences in locomotor activity (ANOVA followed by LSD post-hoc test).](image-url)
Figure 9. Number of aggressive acts registered during 15 min after the first attack in intruder-tested controls and fish subjected to short- or long-term cortisol exposure. Experimental groups assigned different letters displayed statistically significant differences in aggression (ANOVA followed by LSD post-hoc test).

The data from study V are in good agreement with those presented by Sandi et al. (1996), who found that intraperitoneally injected corticosterone at 2.5 or 5 mg / kg significantly increased activity in rats transferred to a novel environment, but not in rats that had previously been exposed to the test environment. Furthermore, the stimulatory effect of corticosterone was seen at 7.5 min and 15 min, but not 60 min, following injection (Sandi et al., 1996). This rapid effect of corticosterone was probably mediated by a non-genomic mechanism, since the effect was not abolished by either the protein synthesis inhibitor cycloheximide, or by specific glucocorticoid and mineralocorticoid receptor antagonists (Sandi et al., 1996).

Aggressive behaviour was also inhibited by long-term cortisol treatment, but a stimulatory effect of short-term treatment, as has been observed in mammals (Hayden-Hixon and Ferris, 1991; Haller et al., 1997b), could not be established. The data from study V are however in good agreement with previous studies reporting a reduction of aggressive behaviour as a result of long-term
cortisol treatment in mammals (Leshner et al., 1980), birds (Wingfield and Silverin 1986), and lizards (Tokarz, 1987; DeNardo and Licht, 1993).

In mammals, a shift in the balance between glucocorticoid (GR) and mineralocorticoid (MR) receptor activation is an important factor in mediating the time- and dose-dependent effect on corticosteroids on behaviour and neural activity (Oitzl et al., 1997; Joëls and Vreugendhil, 1998; de Kloet, 2000; Sapolsky et al., 2000). Both GR- and MR-like receptors have however been cloned in rainbow trout (Ducouret et al., 1995; Takeo et al., 1996; Colombe et al., 2000), and the distribution of GR receptors in the forebrain of rainbow trout was described by Teitsma et al. (1997). The precise role of these steroid receptors in behaviour has however not been explored in fish, but GR receptor autoregulation has been demonstrated in rainbow trout (Pottinger, 1990; Lee et al., 1992), and this mechanism may have contributed to the differential effect of long- and short-term cortisol treatment. A role for membrane-bound steroid receptors in behaviour has been described in amphibians (Orchinik et al., 1991; Moore and Orchinik, 1994), but has to our knowledge not been demonstrated in teleost fish. Other rapid effects of cortisol have however been reported in fish (Shih et al., 1990; Borski et al., 1991). For instance, prolactin release from tilapia (Oreochromis mossambicus) pituitaries in an in vitro preparation was blocked by cortisol, and this effect became significant within 20 min (Borski et al., 1991). The timing of study V was such that neither genomic nor non-genomic effects of cortisol could be excluded in the short-term cortisol group.

In conclusion, the results from study V confirm that glucocorticoids have time- and context-dependent effects on aggression and locomotor activity in rainbow trout, in much a similar manner as has been reported in mammals. Thus, glucocorticoids are probably involved in the mediation of behavioural effects of stress in salmonids, as well as contributing to the different behavioural profile of rainbow trout selected for high and low post stress cortisol concentrations (Pottinger and Carrick 2001a; study III).
SUMMARY AND CONCLUSIONS

Paper I shows that only the dominant individual will take any food in pairs of juvenile Arctic char, and there is a considerable time lag after the dominant individual has been removed, before the subordinate fish start to eat again. This observation implies that feeding inhibition in subordinate fish is not merely dependent on interference competition from the dominant individual, but is coupled to a stress reaction that persists after the stressor, i.e. the dominant fish, has been removed. This notion is further supported by the observation that brain serotonergic activity was elevated in subordinates during pair rearing, but not after 9 days of recovery in social isolation, a time point at which food intake had increased to approach that of dominant fish.

The observations in paper I is of particular relevance to paper III, where we studied the behaviour of rainbow trout genetically selected for high (HR) or low (LR) stress responsiveness. A substantially higher proportion of LR than HR rainbow trout regained feed intake within one week after transfer from a communal holding tank to rearing in isolation. This further support the notion that stress induced appetite inhibition is mediated by central signalling systems simultaneously involved in control of the physiological stress response. In paper III it was also shown that HR fish react stronger to the presence of a conspecific intruder, in terms of increased locomotor activity, than do LR fish. These observations suggest that the behavioural and physiological differences between these two lines of fish are connected, and may represent different behavioural-physiological stress coping strategies, or coping styles, as has been shown to exist in mammals.

Paper II and IV reports in greater detail differences in brain monoaminergic activity between dominant and subordinate fish during pair rearing (paper II), and between HR and LR fish (during stress as well as when undisturbed, paper IV). In paper II it was shown that cortisol concentrations was highly elevated in both fight winners (i.e. future dominant fish) and fight losers (i.e. future subordinate fish) immediately (5 min) after fights for social dominance. In dominant individuals cortisol had however decreased to control
levels within 3 h of continued interaction after the dominance-subordination relationship had been established, while continuing to increase in subordinate fish. At 3 h following fights, the brain serotonergic system was activated in both dominant fish and subordinate fish, at least in some brain regions (telencephalon). This effect was reversed in dominant individuals within 24 h of social interaction, whereas in subordinate fish a substantial activation of both serotonergic and catecholaminergic systems was manifest in all brain regions by 24 h.

In paper IV it was found that HR fish reacted to stress by an increase in the concentrations of both serotonin (brain stem), dopamine (brain stem), and norepinephrine (optic tectum, telencephalon), whereas low-responsive fish did not. Brain stem and optic tectum concentrations of monoamine metabolites were also elevated after stress in HR, but not in LR fish. The results of paper I, II, and IV together suggest that brain monoamine neurotransmitters are involved in shaping the differential behavioural and physiological profiles of HR and LR fish, as well as those seen in fish occupying different positions in a social hierarchy. For instance, higher stress-induced dopaminergic activity in HR than LR fish may well be responsible for the increased locomotor activity seen in HR fish when challenged by a conspecific intruder. Glucocorticoids are known to influence monoamine neurotransmission, so it is an open question whether the observed differences in brain monoaminergic activity are a cause or a consequence of differences in circulating glucocorticoids.

Finally, in paper V we explored the possible behavioural effects of the steroid hormone cortisol in rainbow trout. Short-term (1-1.5 h) cortisol treatment stimulated locomotor activity in rainbow trout subjected to an intruder test, while being without effect on undisturbed animals. Chronic (48 h) treatment had the opposite effect, inhibiting intruder-induced locomotion. Chronic cortisol treatment also inhibited aggressive behaviour, but a stimulatory effect of short-term treatment was not established. These results indicate that, depending on the duration and context of the stressor, cortisol may itself contribute to differences in behaviour seen in fish with differing hypothalamus-pituitary-interrenal activity.

The results of the current thesis, together with the reviewed literature from mammalian studies, suggest that the function of central signal systems involved
in the control of neuroendocrine and behavioural stress responses display extensive similarities in different vertebrate groups. These brain mechanisms seem to have been phylogenetically conserved, with the exception that the influence from anticipations and judgements based on previous experience increases with the development of the cerebral cortex. The overall conclusion I would like to state from this study is that much insight into human psychology can probably be gained from studies on animal behaviour, and vice versa, since animal brains in many ways function in a manner more similar to ours than what is usually reckoned. Thus, experimental and other animals should be treated with great care and while respecting that they are, in fact, highly useful models of the human organism.
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