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Neuroendocrinology of Agonostic Interaction and Social Signalling in Artic charr (*Salvelinus alpinus*)

Studies on the Neuroendocrine Regulation of Aggressive Behaviour, Stress Responses and Skin Colour

BY

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Abstract

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This thesis shows that socially subordinate Arctic charr (*Salvelinus alpinus*) display elevated brain serotonergic (5-HT) and norepinephric activity along with a chronic activation of the hypothalamic-pituitary-interrenal (HPI) axis, including elevated plasma concentrations of α-MSH. Furthermore, subordinate fish showed an inhibition of aggressive behaviour and darker body coloration, skin darkness being positively correlated with plasma α-MSH. Fish kept on dark background, and thus being darker in body colour, were less aggressive than conspecifics interacting on white background, supporting the hypothesis that skin darkening could signal social submission. The 5-HT_{1A} -receptor agonist 8-OH-DPAT stimulated HPI axis activity in non-stressed fish, but if administrated to stressed fish it inhibited HPI axis activity, suggesting that 5-HT_{1A} receptors may act as both post- and pre-synaptic receptors. 8-OH-DPAT also induced skin darkening in both non-stressed and stressed fish. Stimulation of brain dopaminergic activity by L-dopa treatment counteracted the stress-induced inhibition of aggressive behaviour, and stress related effects on brain 5-HT activity and plasma levels of cortisol. In conclusion, social subordination in Arctic charr results in skin darkening and an inhibition of aggressive behaviour. Stress-induced effects, that could be mediated by elevated brain 5-HT activity, and serve as a way of signalling social position and coping with stress.

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ABBREVIATIONS

ACTH - Adrenocorticotropic hormone
α-MSH - α-melanocyte-stimulating hormone
CRF - Corticotropin-releasing factor
DA - Dopamine
DOPAC – 3,4-dihydroxyphenylacetic acid
E - Epinephrine
HPA axis - Hypothalamic-pituitary-adrenocortical axis
HPI axis - Hypothalamic-pituitary-interrenal axis
5-HIAA - 5-Hydroxyindoleacetic acid
5-HT - Serotonin, 5-Hydroxytryptamine
HPLC – High-performance liquid chromatography
MCH - Melanin-concentrating hormone
MHPG - 3-metoxy-4-hydroxyphenylglycol
NE – Noradrenaline
8-OH-DPAT – 8-hydroxy-2-(di-*N*-propylamino)tetralin
POMC – Pro-opiomelanocortin
RIA-Radioimmunoassay

INTRODUCTION

Social animals are often organised in dominance-based hierarchies, or peck orders, where the social position of an animal mainly depends on its fighting ability (Huntingford and Turner, 1987). A subordinate individual, occupying a low position in a dominance-based hierarchy, is subjected to social stress due to repeated attacks and threats from more dominant individuals as well as to limited access to resources in demand, such as food, mating partners and territories. Social stress leads to profound behavioural and physiological changes in subordinate animals. They often show a general behavioural inhibition, including reduced food intake, suppressed aggression, and lowered locomotor activity. Stress-induced alterations in brain monoaminergic neurotransmission play an important role in mediating such behavioural effects. Furthermore, the brain monoaminergic systems also take part in the regulation of endocrine stress responses. Interestingly, in fish some of the hormones released during stress may also affect body coloration (Fujii and Oshima, 1986). Visual cues seem to play an important role in controlling agonistic behaviour in fish (Huntingford and Turner, 1987), and stress-induced changes in body coloration may serve as social signals during agonistic interactions in fish.

This thesis focuses on the role of central monoaminergic neurotransmitters in the control of aggressive behaviour, endocrine stress responses, and skin colour changes occurring in juvenile Arctic charr (*Salvelinus alpinus*) during agonistic interactions.

Stress

The concept of stress

Seley (1936) originally introduced the term stress and proposed that stress is a non-specific response of the body to any demand made on it. Since then several definitions of the concept of stress has been proposed. The fact that the stimuli eliciting the response as well as the physiological response itself have both been referred to as stress has created a lot of confusion. Now it seems to be generally agreed that the stimuli eliciting the physiological stress response should be referred to as a stressor whereas the physiological response of the animal is referred to as stress. A stressor is any stimuli that represents a threat to the survival or homeostatic power of an organism, and stress is the attempts of the organism to counteract the effects of the stressor and to re-establish homeostasis (Chrous *et al*., 1988). In the present discussion the term stress will be used for "physiological and behavioural responses induced by actual or impeding aversive stimuli" (Anisman and Zacharako, 1982)

The physiological stress response

When an animal is confronted with a stressor, such as a predator or a conspecific intruding on its territory or challenging its social position, the body prepares for "fight or flight". In fact, the physiological stress response has also been referred to as the fight or flight reaction. This is an adaptive response that serves to make stored energy available for immediate use, and to save energy by shutting down all processes not immediately necessary to survival, no matter how important these processes might be for long-term survival (Sapolsky, 1992). Reproduction, feeding, the immune system, growth, tissue repair, and the perception of pain and inflammatory responses are all

examples of functions inhibited by stress. Inhibition of these functions may be functional and improve the possibilities for an animal to fight or flee from a threat to its survival. However, if the animal survives but is unable to flee from or eliminate the stressor, the stress response may become chronically activated. For obvious reasons following long-term activation, the stress response ceases to be adaptive and becomes maladaptive resulting in adverse effects on growth, reproduction and immuno-competence.

The physiological stress response is controlled mainly by two neuroendocrine systems: the sympathetic-adrenomedullar (SA) system and the hypothalamic-pituitary-adrenocortical (HPA) axis, the teleostean homologues being the sympathetico-chrommafin (SC) system and the hypothalamic-pituitary-interrenal (HPI) axis, respectively.

An activation of the SA/SC system results in elevated sympathetic nervous activity, and release of norepinephrine (NE) and epinephrine (E) into the bloodstream from the adrenal medulla/head kidney chromaffin cells. These humoral and neuronal events are responsible for most of the acute stress response, elevation of heart rate, increase in blood pressure, redistribution of blood flow, and glycogenolysis resulting in increased blood glucose levels.

The stress-induced activation of the HPA/HPI-axis has a slower onset and a more prolonged time-course. Activation of this neuroendocrine axis results in the secretion of glucocorticoids, in teleost fish and humans mainly cortisol and in rodents mainly corticosterone, from the adrenal cortex, or the teleostean homologue, the interrenal tissue. Glucocorticoids have multiple effects. For instance they act to promote proteolysis and the utilisation of amino acids for gluconeogenesis. Moreover, they are also involved in mediating stress-induced inhibition of many functions such as reproduction and immune responses. By freely passing the blood-brain barrier and enter the central nervous system glucocorticoids may interact with glucocorticoid receptors as well as have effects the synthesis and release of certain neurotransmitters and expression of various receptor subtypes.

The HPI/HPA-axis consists of a series of hormonal pathways, the major components being hypothalamic corticotropin-releasing factor (CRF), pituitary adrenocorticotropic hormone (ACTH) and adrenal/interrenal cortisol. In mammals the CRF released from the hypothalamus reaches the cells of the pituitary gland via a portal blood system. Teleost fish lack this portal system, and CRF reaches pituitary cells via direct neural contact. CRF stimulates the release of ACTH from the pituitary, which in turn is transported by the blood stream to reach the adrenal cortex, or in fish the interrenal cells of the head kidney, where it stimulates synthesis and release of cortisol. This is a classical but simplified description of the adrenocortical stress response. However, the HPI/HPA-axis is subjected to feedback control at multiple levels and the HPI/HPA-axis also interacts with the sympathetic nervous system. Furthermore, other hormones are involved in the regulation of cortisol release, and some of them will be discussed further down, and are presented in Fig 1.

Pro-opiomelanocortin -derived peptides

In the corticotrop cells in the frontal lobe of the pituitary ACTH is synthesised from a pre-hormone known as pro-opiomelanocortin (POMC). However, POMC is also expressed in melanotrops in the pituitary intermediate lobe. In these cells POMC processing results in the production of α-melanocyte-stimulating-hormone (α-MSH) and β-endorphin (Fig 2). α-MSH has also been reported to stimulate cortisol release in fish (Balm *et al*., 1995; Lamers *et al*., 1992) as well as in foetal and new born mammals (Glickman *et al*., 1979; Llanos *et al*., 1979 and Challis and Torosis, 1977). Furthermore, β-endorphin has been reported to act in synergy with α-MSH inducing cortisol release in fish (Balm *et al*., 1995). An elevation of circulating plasma levels of ACTH appears to be a general response to all stressors (Sumpter, 1997), whereas stress-induced effects on the release of α-MSH and β-endorphine seems to depend on the nature and/or the intensity of the stressor (Wendelaar Bonga *et al*., 1995).

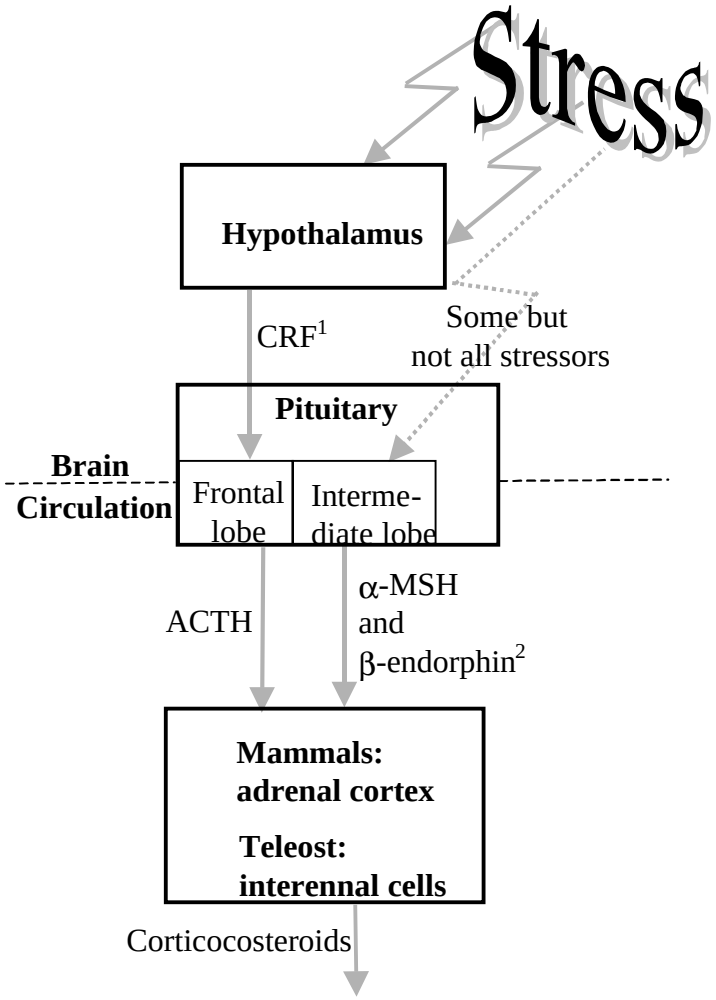

Fig 1. *Neuroendocrine control of glucocorticoid release by the mammalian adrenal and the teleostean interrenal tissue. CRF, corticotropin-releasing-factor; ACTH, adrenocortocotropic-hormone; α-MSH, α-melanocyte-stimulating-hormone.*

[1] In mammals CRF is released into the circulation and transported to the frontal lobe of the pituitary via the portal system. In fish the frontal lobe is directly innervated by CRF-neurones.

[2] In fish, β-endorphin have been reported to act in synergism with α-MSH, stimulating cortisol release (Balm et al., 1995).

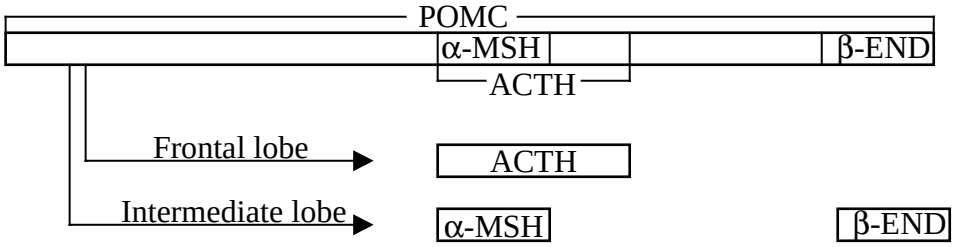

Fig 2. *Tissue specific processing of pro-opiomelanocortin (POMC). In the frontal lobe POMC processing results in the formation of adrenocorticotropic hormonne (ACTH), whereas in the intermediate lobe it results in the formation of α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (β-END).*

Stress and skin colour changes

In ectotherm vertebrates skin darkening is generated by dispersion of pigment containing granules (melanosomes) found in special cells known as chromatophores. The dispersion and aggregation of melanosomes within the chromatophores are controlled by humoral factors, direct innervation, or in some cases by a combination of these two mechanisms (Bentley, 1998). The hormone α-MSH is well known for its role in the control of skin colour, and actually got its name because from its skin darkening effect. ACTH may also induce skin darkening, an effect which might be related to the structural similarity between ACTH and α-MSH (Fig 2). Another hormone released from the pituitary effecting skin colour is melanin-concentrating-hormone (MCH), which lightens the skin. One important function for skin colour changes, except for its role in social behaviour, is background adaptation, and some studies have shown that environmental background colour may affect stress repose in fish. Baker and Rance (1981) observed elevated plasma content of cortisol in eel and trout kept in a dark environment as compared to conspecifics kept in a bright environment. Furthermore, MCH is released during adaptation to bright background, and have been suggested to exert an inhibitory effect on the HPI-axis by suppressing CRF or ACTH release (Suzuki *et al.*, 1995). The chromatophores are also innervated by the sympathetic nervous system (Fujii and Oshima, 1986), which is activated during an acute stress, such as social confrontations. Furthermore, circulating catecholamines are also able to induce colour changes.

Brain monoamines

The monoamines consist of the indoleamine, serotonin (5-hydroxytryptamine, 5-HT) and the catecholamines, dopamine (DA), epinephrine (E), and norepinephrine (NE). In fish, as in other vertebrates, concentrations of E in the brain is however very low, and the role of E in the brain is still unknown (Pennypacker *et al.*, 1985; Hornby and Piekut, 1988; Nilsson, 1989).

Monoamines are believed to act as neurotransmitters and neuromodulators. Monoaminergic neurones compose a very small fraction of the total number of neurones in the brain, but they have been suggested to be involved in mediating several behaviour patterns, such as aggression (e.g. Mason, 1984; Miczek and Donat, 1989; Olivier *et al.*,1989), feeding (e.g. Leibowitz, 1992) and mating (e.g. Meyerson

and Malmnäs, 1978). Moreover, they have a regulative role in many endocrine processes.

The brain monoamines act on a great variety of receptor subtypes, through which they could gain specificity in different brain areas. The receptors activated by 5-HT shows the greatest diversity among the monoamine receptors. In the mammalian brain no less than 14 different subtypes of 5-HT receptors, belonging to seven 5-HT receptor "families"(5-HT_{1-7}), have been described (Mansour *et al*., 1998). Furthermore, the DA receptors consist of five subtypes, which can be divided into D1- and D2-like subfamilies (Mansour *et al*., 1998). The adrenergic receptors α_1, α_2, β_1 and β_2, which are activated by NE and E have been found in the mammalian brain (Fillenz, 1990). Very little is known about monoamine receptors in the fish brain. At present, two different 5-HT receptor subtypes, with pharmacological profiles similar to the mammalian 5-HT_1 and 5-HT_2 receptor families, have been described in salmonid fish (Winberg and Nilsson, 1996; Agrawal and Omeljanuk, 2000). Furthermore, pharmacological evidence suggests that D1, D2 and α_1 like receptors are present in the brain of the goldfish (*Carrassius auratus*; Chang *et al*., 1991; Otto *et al*., 1999).

Involvement of brain monoamines in the stress response

The involvement of the central 5-HT system in the regulation of HPA-axis activity in mammals has been a target for ample investigation, and a growing body of evidence suggests a stimulatory role of the central serotonergic system on the HPA-axis. The main hypothesis, reviewed by Dinan (1996), is that 5-HT neurones within the raphe nuclei, the major 5-HT cell-body-containing area in the brain, project to the paraventricular hypothalamus where they make direct synaptic contact with neurones expressing CRF. In addition, 5-HT has also been suggested to act at the level of the pituitary, stimulating the release of ACTH (Dinan, 1996). A stimulatory role of brain 5-HT on the HPI-axis has been suggested also in fish (Winberg *et al*., 1997; Winberg and Lepage, 1998; Øverli *et al*., 1999).

The role of brain 5-HT in the regulation of the HPA-axis is still under debate however, and contradictory results have been reported (Welch *et al*., 1993; Saphier *et al*., 1995). One of the reasons for these contradictory results could be that some 5-HT receptors (i.e. 5-HT_{1A} and 5-HT_{1B}) may act as inhibitory pre-synaptic autoreceptors, (Blier and Montigny, 1999). Still, these same 5-HT receptor subtypes are present also as post-synaptic receptors, and in this case at least 5-HT_{1A} receptors have been suggested to act stimulatory on the HPA axis (e.g. Gilbert, *et al*., 1988; Haleem *et al*., 1989; Korte *et al*., 1991) (Fig 3).

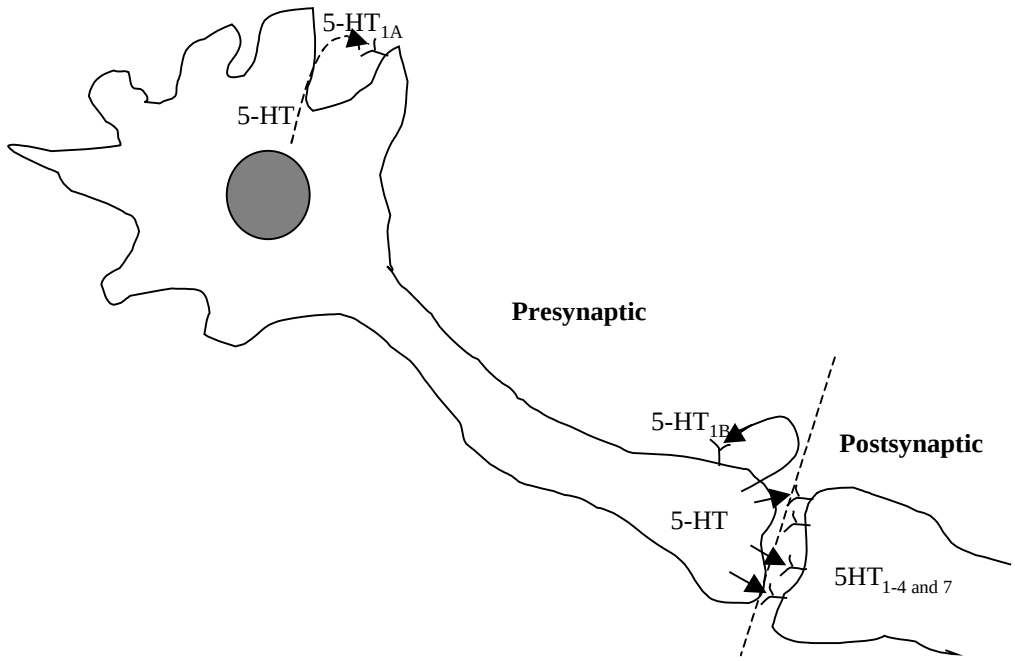

Fig 3. A serotonergic neurone and a synapse. *Presynaptic 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors inhibit 5-HT release by negative feedback control. Moreover, these same receptors are present as postsynaptic receptors, where at least 5-HT$_{1A}$ has been suggested to act stimulatory on the HPA-axis (e.g. Gilbert, et al., 1988; Haleem et al., 1989; Korte et al., 1991). 5-HT: serotonin. The receptor types and families depicted are those that have been electrophysiologically identified in unitary recordings (Blier and Montigny, 1999).*

There is evidence of the presence of a 5-HT receptor with a pharmacological profile strikingly similar to the mammalian 5-HT$_{1A}$ receptor, in the salmonid brain (*Salvelinus alpinus;* Winberg and Nilsson, 1996). Furthermore, Winberg *et al*. (1997) showed that administration of the selective 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylamid) (8-OH-DPAT) resulted in a dose dependent increase in plasma cortisol concentrations, suggesting that the 5-HT$_{1A}$ receptor is involved in the regulation on the HPI-axis. Still, it is not known if 5-HT$_{1A}$ receptors may act as autoreceptors also in the teleost brain.

In mammals, catecholeaminergic neurones, mainly NE and DA, whit the CRF expressing neurones in the hypothalamus, suggesting that these catecholeamines are also involved in the regulation of the HPA-axis (Liposists and Paull, 1989; Plotsky *et al*., 1989). Stress is known to activate the NE system of the mammalian brain (reviewed by Stanford, 1993), and NE have been suggested to facilitate hypothalamic CRF secretion (reviewed by, Plosky *et al*., 1989). Øverli *et al*. (1999) reported a strong correlation between brain stem NE activity and plasma cortisol levels in social stressed rainbow trout (*Oncorhyncus myskiss*), suggesting a stimulatory role for NE even in fish.

The role of DA in regulation of the HPA-axis is still controversial, and in mammals central DA has been suggested to act stimulatory, inhibitory or not to take part in the regulation of the HPA-axis (Boden *et al*., 1972; Wilcox *et al*., 1975; Chambers and Brown, 1976; Frisina *et al*., 1983; Hagan and Brooks, 1996; Reid *et al*., 1986; Jezova and Vigas, 1988; Borowsky and Kuhn, 1991; Borowsky and Kuhn,

1993; Surman and Havemann-Rienecke, 1995; Matthews *et al*., 1996; Brambilla, 2000). Still less is known about the involvement of brain DA in the regulation HPI-axis activity in teleost fish. In rainbow trout interacting in pairs for 24 h the subordinate fish shows an elevation of central DA activity (Øverli *et al*., 1999) along with a concomitant rise in plasma cortisol levels. On the other hand, Winberg *et al*. (1991) reported that central DA was activated in dominant Arctic charr following long-term social interaction in groups consisting of four fish. In the study by Winberg *et al*. (1991) the plasma content of cortisol was not quantified. However, following long-term social interaction in stable dominance hierarchies, dominant fish usually show low levels of plasma cortisol (Winberg and Lepage, 1998; Øverli *et al*., 1999).

The brain monoamines have also been suggested to be involved in the regulation of α-MSH and β-endorphine release. 5-HT is believed to induce α-MSH release in both mammals (Goudreau *et al*., 1994) and other vertebrates, such as goldfish, frogs (*Xenopus laevis*, Olivereau *et al*., 1980), and lizards (Olivereau *et al*., 1980; Levitin, 1980). Both NE and DA, on the other hand, are well known to inhibit α-MSH release from the pituitary (Bentley, 1998).

Skin colour patterns as a social signal

In fish skin colour patterns seem to act as visual cues. In cichlides single components of the colour pattern has been associated with agonistic behaviour, as for example in *Haplocromis burtoni* where the presence of a black bar behind the eye has been shown to increase aggression (Hieligenberg, 1976). Furthermore, in the shinspot cichlid (*Sarotherodon melanotheron*) the size of a black spot on the shin is correlated with dominance (Denight and Ward, 1982). In the cichlid, *Nannacara anomala*, colour pattern changes have been suggested to co-ordinate different agonistic actions during an escalating fight (Hurd, 1997). Single components of colour pattern effecting agonstic behaviour have even been shown in the lizard, *Anolis carolinensis*, where the speed with which the black spot behind the eye becomes visible predicts the outcome of a dyadic fight for social dominance (Larson and Summers, 2001; Summers and Greenberg, 1994). In juvenile Atlantic salmon (*Salmo salar*) darkening of the sclera around the eye has been observed in fish losing a fight (O´Connor *et al*., 1999). Moreover, in salmonids a darker body colour has been coupled to social subordination (*Oncorhynchus mykiss*, Abbot *et al*., 1985; *Salvelinus fontinalis*, Newman, 1956; *Salmo salar*, O´Connor *et al*., 1999). Furthermore, it has been suggested that darker body coloration of subordinates may act as a social signal in salmonids, and that darker body colour of subordinates may reduce aggression from dominant individuals (Abbot *et al*., 1985; O´Connor *et al*., 1999).

The involvement of brain monoamines in socially induced effects on behaviour and skin colour

Social subordination, in teleost fish as well as in other vertebrates, often results in a general behavioural inhibition, including a suppression of aggressive behaviour, reduced feeding and lowered spontaneous locomotor activity (Abbott *et al*., 1985; Nielsen and Andries, 1988; Winberg *et al* 1993 a,b; Blanchard *et al*., 1993; Meerlo *et al*., 1997; Øverli *et al*., 1998). Behavioural inhibition as well as darkening of the body coloration may be ways of announcing fighting ability, and could serve to avoid

unnecessary fights and energy loss. Behavioural inhibition in subordinate animals could, at least in part, be mediated by a stress-induced activation of the central 5-HT system (Winberg *et al*., 1993b; Øverli *et al*., 1998). Moreover, a stress related elevation of brain 5-HT activity may also mediate skin darkening, by stimulating the release of ACTH and α-MSH.

Elevation of brain DA activity, on the other hand, has been suggested stimulate aggressive behaviour in mammals (Pohto, 1979; Kramarcy *et al*., 1984), and to induce social dominance in fish (Winberg and Nilsson, 1992). NE has also been reported to stimulate aggressive behaviour in the weakly electric fish, *Apteronotus leptorhyncus* (Maler and Ellis, 1987). Furthermore, these catecholeamines are believed to have an inhibitory effect on pituitary α-MSH release.

AIMS OF THE THESIS

Social position greatly affects the behaviour and physiology of an individual and subordinate fish have been shown to display a general behavioural inhibition along with a chronic activation of the HPI axis and the central serotonergic system. Brain monoaminergic systems play an important role in the central control of behavioural and physiological stress responses, and behavioural inhibition in subordinates could be mediated by a stress-induced activation of the 5-HT system. In addition to its behavioural effects, central 5-HT is also believed to stimulate HPI axis activity. Another characteristic of socially subordinate salmonids is their dark body colour. Darkening of the body coloration in subordinate fish may act as a social signal announcing submission, provide a cryptic coloration, making the subordinate less visible, or be a side effect of HPI axis activation. Skin darkening could be mediated by a stress-induced elevation of α-MSH secretion. Serotonin may stimulate pituitary α-MSH secretion, whereas DA and NE appear to have the opposite effect. The central catecholaminergic systems, NE and DA, may also have effects on behaviour opposing those of 5-HT. In the light of this the following aims of the thesis were formulated:

1) To study the effects of pharmacologically induced alterations in brain monoaminergic activity on aggressive behaviour, skin colour, and neuroendocrine stress responses (studies I and IV).

2) To study the relationships between social rank, brain monoamine utilisation, plasma levels of POMC-derived peptides, and skin colour (study II).

3) To study the effect of environmental background colour on aggressive behaviour, and socially induced effects on skin colour, brain monoaminergic activity and plasma levels of POMC-derived peptides and cortisol (study III).

MATERIALS AND METHODS

Fish

Juvenile Arctic charr, *Salvelinus alpinus* L., were used in the experiments. Prior to the experiments the fish were kept indoors at the Evolutionary Biology Centre, Uppsala University, at a density of 200-400 fish/m^3 in a light grey-coloured (study I), or dark green-coloured holding tank (study II, III and IV). The holding tanks were continuously supplied with aerated Uppsala tap water (8-10 °C, 1.5 l/min). The fish were kept in the holding tanks for more than 1 year before the experiment in a light/dark regime that was continuously and automatically adjusted to conditions at latitude 51 ^{o}N. The fish were hand-fed with commercial trout pellets (Ewos ST40) at 1-2 % of the body mass per day.

Experimental protocol

The studies were performed in glass aquaria, measuring 1000 x 500 x 500 mm (study I) or 1000 mm x 300 mm x 500 mm (study II-IV), continuously supplied with aerated tap water. Light was provided by fluorescent tubes (2x20 W, warm white) placed 250 mm above the water surface. Each aquarium was divided into three 33 l chambers (study II), or four 50 l (study I) or 25 l (study II-IV) chambers by removable black plastic walls. In study III, half of these chambers had black bottoms and backsides, whereas the other half of the chambers had white backsides and bottoms. Fish were moved from the holding tanks and placed within individual chambers in the glass aquaria. In this way, the fish were kept visually isolated for three weeks before the experiment in the purpose to reducing the effects of previous tank colour and social experience (study II-IV). In study I, the fish were acclimatised in the individual chambers for two days. In study I-III fish were tagged by small clips in the caudal fin before placing them in the individual chambers. Following acclimation, the plastic walls separating the fish were gently removed, and the fish were allowed to interact in groups of three for five days (study II), or in pairs for two (study I) or five days (study III). In study III pairs of interacting fish consisted of fish that had been acclimated to the same background colour. During social interaction aggressive behaviour was recorded.

Study I

In study I, the effect of pharmacological stimulation of the brain DA system, using L-dopa, the immediate precursor of DA, on the stress responses and aggressive behaviour in subordinate and dominant fish was investigated. To study how social interaction affected attack latency and aggression, the fish were subjected to intruder tests (described below) before and after two days of social interaction in pairs. During social interaction aggressive acts were recorded and based on the number of aggressive acts performed and received the fish were ranked as dominant or subordinate (see below). Following the second intruder test 6 of the dominant and 6 of the subordinate fish were given L-DOPA, whereas the remaining 6 dominant and 6 subordinate fish were given vehicle and served as controls. One hour after treatment fish were subjected to third intruder test, to measure the effect of L-dopa on attack latency and aggression. The fish were sacrificed immediately after the last intruder test and brain

tissue and blood plasma were sampled for analysis of brain concentrations monoamines and monoamine metabolites, and plasma levels of cortisol.

Study II

This study aimed at clarifying relationships between social rank, skin colour, brain monoaminergic activity, and plasma levels of POMC-derived peptides and cortisol. Skin colour was quantified before and after five days of social interaction. The fish interacted in groups consisting of three fish. Aggressive behaviour was recorded during social interaction and the fish were ranked as 1 (dominant), 2 or 3 (the most subordinate, based on the number of aggressive acts performed and received (for further details see below). Following the second skin colour measurement the fish were anaesthetised and brain tissue and blood plasma were sampled for analysis of brain concentrations of monoamines and monoamine metabolites, and plasma levels of POMC-derived peptides and cortisol. Nine fish remained visually isolated throughout the experiment and served as undisturbed controls.

Study III

This study aimed at investigating the effects of environmental background colour on aggressive behaviour, socially induced skin colour changes, brain monoaminergic activity and plasma levels of POMC-derived peptides and cortisol. The experimental protocol followed that of study II, except that the fish were acclimated to, and allowed to interact in pairs on either black or white background. Fish kept visually isolated on white or black background served as controls.

Study IV

This study was designed to investigate the effects of pharmacological stimulation of 5-HT_{1A} receptors, using the specific 5-HT_{1A} receptor agonist 8-OH-DPAT, on skin colour and plasma levels of cortisol and POMC-derived peptides in stressed and non-stressed fish. In order to study the effects of 8-OH-DPAT in non-stressed fish, 8-OH-DPAT or saline (vehicle control) were administrated through a permanent i.p. implanted catheter (see below). Three weeks prior to the experiment, the fish were transferred from the holding tank to individual chambers in the experimental aquaria and implanted with an i.p. catheter. Controls received saline administrated through the catheter, whereas 8 fish were left uninjected and served as undisturbed controls.

In order to study the effects of 8-OH-DPAT in stressed fish the drug (or vihicle alone) was administrated through standard i.p. injections without anaesthesia, the i.p. injection serving as the stressor. In this case the fish was netted, lifted up from the aquaria, held in a moist paper towel, and injected i.p. using a syringe. Following injection they were rapidly returned to their respective chamber in the aquaria. Fish receiving neither drug nor vehicle served as undisturbed controls. Skin colour measurements were performed 24 h before and 1 h after drug treatment. Blood samples were taken immediately after the last skin colour measurement.

Behavioural observations (study I-III)

Social ranking

In study I, II and III, aggressive acts performed and received by individual fish were counted during two daily observation sessions of 5 min each, at 10:00 and 16:00 hours. Three types of aggressive acts were counted (for a description see Fabricus, 1953; Fernö *et al*. 1976; Noakes 1980):

Attack: A rapid approach towards an individual often finished with a bite.
Bite: A bite at a closely located individual without a prior approach.
Charge: A direct but slow approach towards another individual. The charging fish may have extended fins.

The first observation was performed 30 min after placing the fish in-groups of three (study II) or in pairs (study I and III) and the last on the day before terminating the experiment. In study II the fish were ranked as 1 (dominant), 2 and 3 based on the number of aggressive acts performed and received, using a dominance matrix (Martin and Bateson, 1986). In studies I and III, using pairs of fish, the fish were ranked as dominant or subordinate due to the number of aggressive acts performed and received.

Intruder test

In study I, the level of aggression (number of attacks), and the latency to first attack (attack latency), of individual fish was determined by introducing a small conspecific (approximately 50% of the body weight of the resident fish) into the isolated experimental fish. The behaviour of such pairs was recorded on video during 10 min, starting from the time of the first recorded attack. Following these 10 min the intruder was removed. Each intruder was only used once. From video recordings, the attack latency, and the number of attacks performed by the resident fish during the 10 min test was registered. If the resident fish did not attack the smaller intruding conspecific within 30 min, the intruder was removed and the latency to first attack was set to 1 800 sec and aggression to 0.

Drug treatment (study I and IV)

In study I, L-dopa (Sigma Chemical Co.), the immediate precursor of DA, was given orally at a dose of 10 mg/kg (a 1 mg/ml L-dopa solution in 0.02 M HCl was fed into the stomach via a plastic catheter). Controls were given 0.02 M HCl (vehicle).

In study IV, two solutions, containing 0.1 and 0.2 mg/ml of 8-OH-DPAT ((±)-8-hydroxy-2-(di-*N*-propylamino)tetralin hydrobromide, Sigma no. H-8520) in saline (0.09% NaCl), respectively, were freshly prepared and used for injections. The 0.1-mg/ml solution of 8-OH-DPAT was used for administrating 62.5 or 125 μg/kg of 8-OH-DPAT, whereas the solution containing 0.2 mg/ml of 8-OH-DPAT was used to administer 250 and 500 μg/kg to the fish. The injection volume was in the range of 0.054-0.16 ml/ fish. Control fish received a saline injection (vehicle, 0.1 ml/fish).

Implantation of intra peritoneal (i.p.) catheter (study IV)

The fish was anaesthetised (ethyl-*m*-aminobenzoate methanesulphonate, 100 mg/l) and a small incision (ca 10 mm), about 5 mm in front of the pelvic fins, was made using a scalpel. A small piece of silicon tubing with an inner diameter slightly smaller than the outer diameter (o.d.) of the catheter, and an o.d. of 10 mm was fitted on the tip of the catheter in order to prevent it from slipping out of the fish during the experiment. The catheter (the end fitted with silicon tubing) was inserted into the body cavity after which the incision was closed and the catheter secured using sutures. The catheter was filled with sterile saline solution, sealed by melting and subsequently checked daily during feeding. The fish rapidly acclimated to the presence and handling of the catheter and at the end of the 3-week isolation period they no longer reacted to the experimenter gently picking up and handling the catheter.

Skin pigmentation measurements (studies II, III and IV)

Skin pigmentation was quantified as the darkness of the skin, and measured by placing the fish in a plastic box with a transparent cover. The box was filled with foam rubber, which immobilised the fish against the transparent cover. The fish were filmed with a video camera through the plastic cover under constant light conditions. Thereafter, the filmed fish was analysed using an image analysis program (Scion Image, based on NIH image for Macintosh, modified for Windows, by Wayne Rasband, NIH, Betheseda, MD, USA). Skin darkness was measured on a linear black-white scale on which 0 corresponds to white and 255 to black. A grey-scale with eleven standard measure points, ranging from 0 to 250 with a step value of 25, was attached to the transparent cover and used for calibration between measurements. The time taken for the pigment measuring procedure, from netting to the completion of measuring, was approximately 40 seconds.

Blood and brain tissue sampling

Blood (approximately 1 ml) was collected from the caudal vasculature, using a syringe pre-treated with EDTA. Blood samples were rapidly transferred to Eppendorf tubes containing aprotinin (Sigma, A1153, 3000 KIU/ ml blood) and were centrifuged at 1 500g for 10 min at 4°C. Thereafter the blood plasma was separated, frozen on dry ice and stored at –80 ^{o}C. In studies I, II and III, the fish were killed by decapitation following blood sampling, and the brain was rapidly removed (within 2 min) and divided into telencephalon (excluding olfactory bulbs), hypothalamus (excluding the pituitary gland), optic tectum, cerebellum, and brain stem (including the medulla and part of the spinal cord). Each brain part was wrapped into aluminium foil, frozen in liquid nitrogen and stored at –80 ^{o}C.

Assays

In studies II, III and IV the frozen brain samples were homogenised in 4% (w/v) ice-cold perchloric acid (PCA) containing 0.2% EDTA and 40 ng/ml epinine (deoxyepinephrine, the internal standard), using a Potter-Elvehjem homogeniser (optic lobes, cerebellum and brain stem) or an MSE 100 W ultrasonic disintegrator (telencephalon and hypothalamus).

5-HT, 5-HIAA , DA, 3,4-dihydroxyphenylacetic acid (DOPAC, a major DA metabolite), NE and 3-methoxy-4-hydroxyphenylglycol (MHPG, a major NE metabolite) were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection, following Øverli *et al.* (1999). The HPLC system consisted of a solvent-delivery system (CostaMetric II, LDC, USA), an autoinjector (Midas, Spark, Holland), a reverse-phase column (4.6 mm x 100 mm, Hichrom, C18, 3.5 μm) kept at 40 ºC, and an ESA 5200 Coulochem II EC detector (ESA, Bedford, MA, USA) with two electrodes at oxidising potentials of +320mV and +450 mV. A conditioning electrode with a potential of +40 mV was employed before the analytical electrodes to oxidise any contaminants. The ratio of [metabolite]/[parent monoamine] was used as an index of brain monoaminergic activity. This is a more direct index of monoaminergic activity than brain levels of monoamine metabolites per se, since variance related to tissue sampling, and differences related to total levels of the parent monoamine and its metabolite, are reduced (Shannon *et al*., 1986)

Blood plasma samples were assayed for cortisol (study I, II, III and IV), and for ACTH (studies II, III and IV), α-MSH (studies II, II and IV) and β-endorphin (study II and IV). Cortisol analysis was performed directly on Arctic charr plasma without extraction, using a validated radioimmunoassay (RIA) modified from Olsen *et al.* (1992) as described by Winberg and Lepage (1998). Plasma concentrations of ACTH were determined by RIA as described by Balm and Pottinger (1993) and Balm *et al*. (1994). α-MSH and β-endorphin (*N*-acetyl-β-endorphin) concentrations in the plasma samples were quantified by validated RIAs following Balm *et al*. (1995).

RESULTS AND DISCUSSION

Brain monoamines, POMC-derived peptides and the control of HPI-axis activity

In mammals, 5-HT, NE and DA neurones make direct synaptic contact with the CRF-neurones in hypothalamus (Dinan, 1986; Liposists and Paull, 1989; Plotsky *et al*., 1989), which could suggest that they all interfere with the HPA-axis activity. Among the brain monoamines especially NE and 5-HT have been shown to have excitatory effects on HPA-axis activity in mammals.

Serotonin

The results from study I-III are in accordance with previous studies (Winberg and Lepage, 1998; Øverli *et al*., 1999) showing that social subordination results in an activation of the brain 5-HT system along with elevated plasma levels of cortisol. In study II, there was a significant correlation between brain 5-HIAA/5-HT ratios and plasma levels of ACTH, lending further support to the hypothesis that the brain 5-HT system may act stimulatory on HPI axis activity.

Pharmacological stimulation of the 5-HT system using the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, has previously been reported to generate a dose dependent rise in plasma concentration of cortisol in dorsal aorta cannulated rainbow trout (Winberg *et al*., 1997). However, the stimulatory effect of 8-OH-DPAT on HPA-axis activity in mammals are still under debate, even though there are multiple studies suggesting that 5-HT_{1A} agonists act stimulatory on the HPA-axis, and that this effect is mediated by stimulation of central postsynapitic 5-HT_{1A} receptors (e.g. Gilbert *et al*., 1988; Haleem *et al*., 1989; Korte *et al*., 1991). The reason for contradictory results on the effect of 5-HT_{1A} receptor stimulation on HPA axis activity in mammals has mainly been claimed on the fact that 5-HT_{1A} receptors also occur as pre-synaptic autoreceptors, inhibiting 5-HT neurotransmission and thus possibly HPA-axis activity.

In order to further clarify the role of 5-HT_{1A} receptors in the control of the HPI axis in Arctic charr, the acute effects of several doses of the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, on plasma levels of cortisol, ACTH, α-MSH and *N*-acetyl-β-endorphin were studied in both stressed and non-stressed animals (study IV). The hypothesis being that an inhibitory effect of 8-OH-DPAT on HPI-axis activity would be more easily detected in stressed fish, where the HPI-axis is already activated, whereas a stimulatory effect of this drug on HPI-axis activity would be more obvious in non-stressed fish.

The results of study IV show that 8-OH-DPAT has an inhibitory effect on HPI-axis activity if administered to stressed fish (the drug administrated through i.p. injections), an effect that was most pronounced at low doses (62.5 and 125 μg/kg) of 8-OH-DPAT (Fig 4). On the contrary, if administrated during relatively stress free conditions (permanent i.p. catheter), 8-OH-DPAT had a dose dependent stimulatory effect on HPI-axis activity (Fig 4). Moreover, ACTH followed the same general pattern as cortisol in both unstressed and stressed fish, further suggesting that 8-OH-DPAT could act either stimulatory or inhibitory on the HPI-axis activity, depending on the state of the fish. The stimulatory effect of 8-OH-DPAT on HPI axis activity in non-stressed Arctic charr is likely to be mediated by post-synaptic 5-HT_{1A} receptors,

whereas inhibitory effects of this drug on HPI axis activity may be mediated by activation of pre-synaptic autoreceptors.

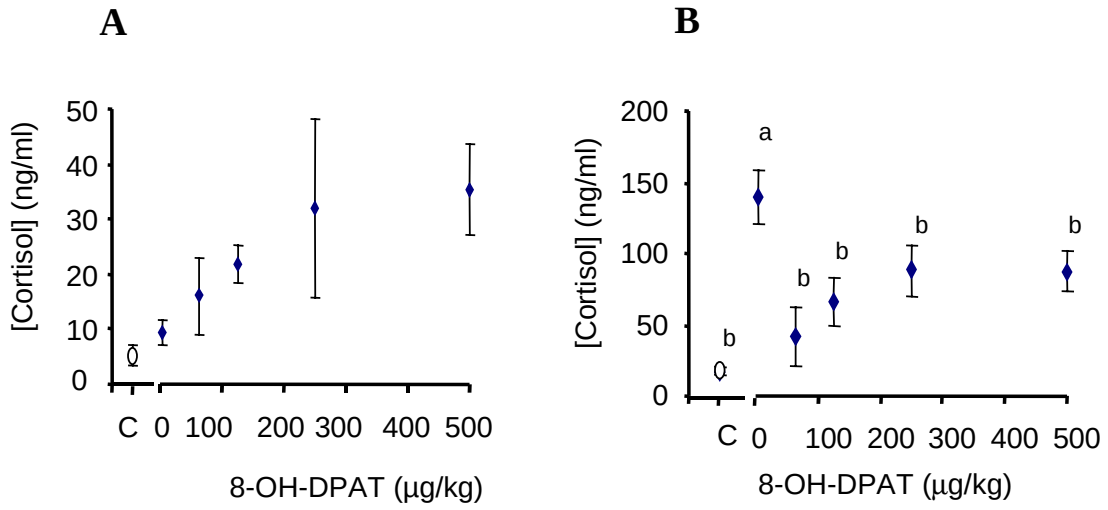

Fig. 4. *The effect of 8-OH-DPAT, administrated under relatively stress free conditions through a permanent i.p. catheter (A) or by standard i.p. injections without anaesthesia, on plasma levels of cortisol (B). C: undisturbed controls. Different letters indicate significant differences at the level of P<0.05.*

Korte *et al*. (1990) suggested that the inhibitory effects on the HPA-axis observed in response to low doses of the 5-HT_{1A} agonist, ipsapirone, could be explained by somatodendritic autoreceptors having a higher affinity for ipsapirone than post-synaptic 5-HT_{1A} receptors. Thus, low doses of ipsapirone would act preferentially on pre-synaptic 5-HT_{1A} receptors, inhibiting 5-HT neurotransmission and HPA axis activity, whereas higher doses would act on both pre- and post-synaptic 5-HT_{1A} receptors, resulting in a stimulation of the HPA-axis. On the other hand, Whelch *et al*. (1993) suggested that central $5HT_{1A}$ receptors only take part in the inhibition of the HPA-axis, and that the stimulatory effects on HPA-axis activity reported after administration of 5-HT_{1A} agonists is mediated by a cardiovacular/sympathomedulary reflex response, which in turn stimulates ACTH release via an adrenergic effect. However, Gilbert *et al*. (1988) observed that pre-treatment of rats with the 5-HT synthesis inhibitor, *p*-chlorophenylalanin, does not affect the 8-OH-DPAT induced elevation in plasma ACTH concentrations, suggesting the presence of post-synaptic 5-HT_{1A} receptors acting stimulatory on HPA-axis activity. Moreover, injections of 8-OH-DPAT directly into the hypothalamic paraventricular nucleus generates an elevation of plasma levels of ACTH and corticosterone (Haleem *et al*., 1989; Korte *et al*., 1991), whereas lesions of the parventricular nucleus completely blocks 5-HT_{1A} induced corticosterone response in rats (Bagdy and Makara, 1994).

Winberg and Lepage (1998) reported a sustained up-regulation of pituitary POMC mRNA expression in subordinate rainbow trout, an effect that appeared to be caused mainly by elevated POMC mRNA expression in melanotropes of the pituitary neurointermediate lobe. In study II, fish of social rank 3 (the most subordinate fish), in addition to increased brain 5-HT activity and elevated plasma levels of ACTH and

cortisol, also showed increased plasma concentrations of α-MSH (Fig 5). Thus, 5-HT could be one of the factors stimulating the production and release of POMC-derived peptides of both corticotrope and melanotrope origin in subordinate fish. However, in study II there was no significant correlation between brain 5-HIAA/5-HT ratios and plasma levels of α-MSH.

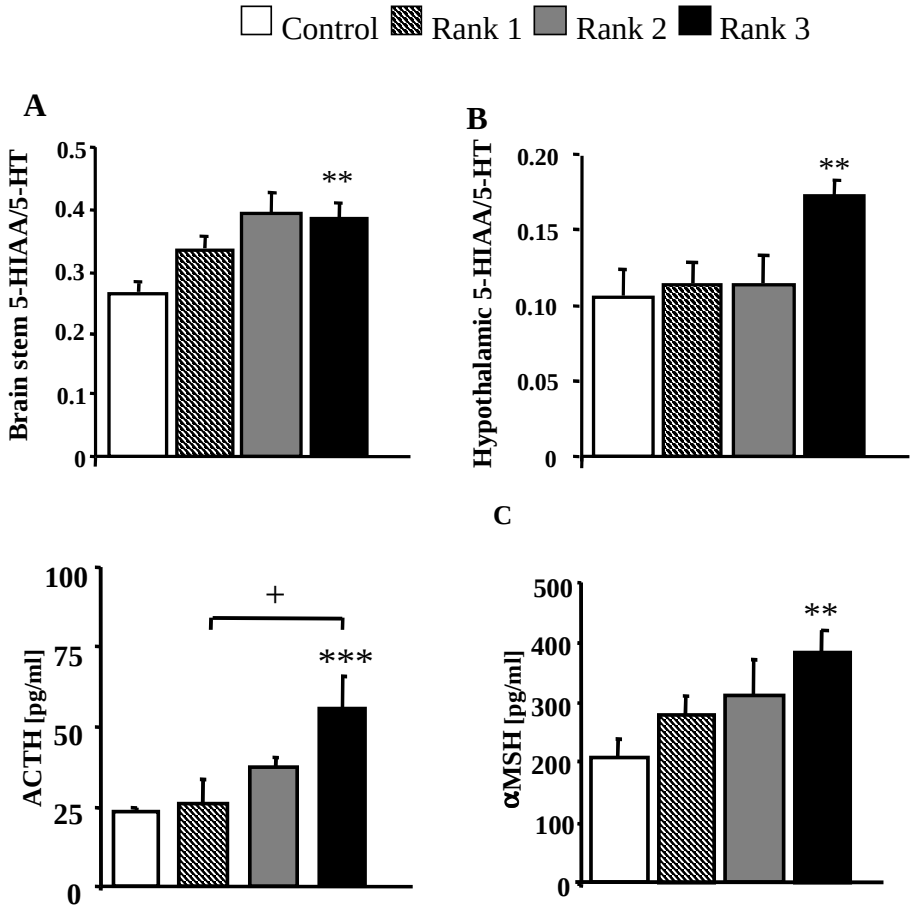

Fig 5. *The 5-HT activity, measured as 5-HIAA/5-HT ratios, in the brain stem (A) and hypothalamus (B), and the plasma levels of ACTH (C) and α-MSH (D), in Arctic charr occupying different positions in a dominance hierarchy developed over five days. Fish ranked as 1 being the most dominant and fish ranked as 3 the most subordinate. Controls are fish that were kept visually isolated. Values are mean + S.E.M. from nine fish of social rank 1, four fish of social rank 2, 14 fish of social rank 3 and nine controls. An asterisk indicates a significant difference from visually isolated controls, and a plus sign indicates a significant difference between social ranks. +P<0.05, **P<0.01, ***P<0.001. 5 HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; ACTH, adrenocoricotropic-hormone; α-MSH, α-melanocyte-stimulating-hormone.*

In rats, 5-HT has also been shown to induce release of POMC-derived peptides other than ACTH (Sapun-Malcom *et al*., 1983), and treatment with MK-212, a non-selective 5-HT_1/5-HT_2 receptor agonist, generated an elevation of plasma levels of both α-MSH and β-endorphin in rats (Carr *et al*., 1991). The central 5-HT system has been suggested to act stimulatory on α-MSH secretion also in non-mammalian vertebrates, such as lizards (*Anolis carolinensis*, Levtin, 1980) and goldfish (*Carassius auratus*, Olivereau *et al*., 1980). However, the results from study IV do not support a

stimulatory role of the 5-HT_{1A} receptor in the regulation pituitary release of α-MSH and β-endorphin in the Arctic charr. Neither in stressed nor in non-stressed fish did 8-OH-DPAT significantly affect plasma levels of α-MSH or β-endorphin. In the studies on serotonergic regulation of α-MSH secretion in lizards and goldfish (Levtin, 1980; Olivereau *et al*., 1980), only the effects 5-HT or 5-hydroxytryptophan were tested, and no selective 5-HT receptor agonist or antagonists were utilised. Thus, it is not known which 5-HT receptor subtype that is responsible for the stimulatory effect of 5-HT on α-MSH release observed in these species.

The results from the stepwise regression analysis performed in study II imply that α-MSH and ACTH both are involved in the control of cortisol release (Table 1). Interestingly, Lamers *et al*. (1992) demonstrated that α-MSH stimulates cortisol release *in vitro* in tilapia. Furthermore β-endorphin, appears to act in synergism with α-MSH, stimulating cortisol release in tilapia (*Oreochromis mossambicus*) (Balm *et al.*, 1995). Activation of the pituitary corticotropes and an elevation of circulating plasma levels of ACTH seems to be a general response to all stressors (Sumpter, 1997). In contrast, effects on pituitary melanotropes, and circulating plasma levels of α-MSH and β-endorphin, seem to depend on the nature and/or the intensity of the stressor (Wendelaar Bonga *et al.,* 1995). For instance, handling and confinement stress in combination with a thermal shock induced a rise in plasma concentrations of β-endorphin and α-MSH in brown trout (*Salmo trutta*) (Sumpter *et al*., 1985). Similarly, restraint stress caused an elevation of α-MSH in rainbow trout (Sumpter *et al*., 1986), as did exposure to acidified water in tilapia (Lamers *et al*., 1991). However, other types and/or combinations of stressors may not effect plasma levels of α-MSH and β-endorphin or may even reduce plasma concentrations of these peptides (Balm and Pottinger, 1995)

Table 1. *Results of step-wise multiple linear regression analysis with plasma concentrations of cortisol and skin darkness as dependent variables and plasma concentrations of α-MSH, N-ac-β-endorphin and ACTH as independent variables.*

dependent varable	independent variable	*F*	d.f.	*adjusted r2*	*P*	*β*
log cortisol		**22.24**	**2,31**	**0.56**	**0.00001**	
	[α-MSH]				0.015	0.40
	[N–ac-β-endorphin]				n.s.	
	[log ACTH]				0.0002	0.64
skin darkness		**6.62**	**2,31**	**0.25**	**0.00403**	
	[α-MSH]				0.019	0.41
	[N–ac-β-endorphin]				n.s	
	[log ACTH]				n.s	

The P values given in bold denote the total probability of the model. Other P values denote the contribution of each independent variable to the model. n.s. means not significant.
α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone.

Norepinephrine

In mammals, stress is known to activate the brain NE system (reviewed by Stanford, 1993), and Øverli *et al*. (1999) reported elevated brain MHPG/NE ratios and a positive correlation between brain MHPG/NE ratios and plasma cortisol levels, in subordinate rainbow trout. In study II fish of social rank 3 (the most subordinate) showed elevated MHPG/NE ratios in the optic tectum as compared to isolated controls. Moreover, there was a strong positive correlation between MHPG/NE and plasma levels of ACTH, suggesting that the brain NE system is activated by social stress and that central NE may act stimulatory on the HPI axis in Arctic charr.

This suggestion is further supported by the results from study III, where fish were allowed to interact in pairs on white or black background. In this study, subordinate fish on white background received a higher number of attacks from the dominant fish than subordinates on black background, implying that subordinates interacting on a white background colour were exposed to a more intense social stress. Subordinate fish on white background showed elevated brain MHPG/NE ratios, an effect not seen in subordinates on black background. Together these results strongly suggest that the central NE system is activated by social stress and plays a role in the regulation of the teleost HPI-axis.

Dopamine

In study I, juvenile Arctic charr having a two days experience of being dominant or subordinate in a pair were given L-dopa (10 mg/kg, orally). L-dopa treated fish showed a significant elevation of brain DOPAC/DA ratios, as compared to vehicle controls, confirming an L-dopa induced stimulation of brain DA activity. However, L-dopa treatment had no significant effects on brain NE activity, as measured by the MHPG/NE ratio.

Drug administration was a stressful treatment, involving netting, air exposure and feeding the solution into the stomach via a catheter fed through the mouth and esophagus of the fish. However, L-dopa appeared to have a stress dampening effect, and fish receiving L-dopa showed significantly lower plasma cortisol levels than vehicle treated fish. The suggestion that L-dopa had a dampening effect on stress responses is further supported by the fact that L-dopa treated fish displayed lower brain stem 5-HIAA/5-HT ratios than vehicle controls, and that a similar but non-significant (P=0.055) trend was observed in hypothalamic 5-HIAA/5-HT ratios.

Contradictory results have been reported as regarding the influence of brain DA on the HPA axis activity in mammals. There are results showing that L-dopa decreases plasma levels of cortisol and ACTH (Frisina *et al*., 1983; Reid *et al*., 1986) but there are also reports suggesting that L-dopa treatment has no effect on plasma cortisol levels (Boden *et al*., 1972; Chambers and Brown, 1976). Similarly, the DA agonist bromocriptine has been reported to decrease plasma levels of ACTH, but surprisingly not cortisol, in ovine fetus (Hagan and Brooks, 1996), whereas APO, another DA agonist, has been reported to stimulate ACTH release in rats (Jezova and Vigas, 1988) and humans (Surman and Hevemann-Reinecke, 1995). Interestingly, Kramarcy *et al*. (1984) reported that L-dopa (12.5 mg/kg, oral) lowered brain 5-HT activity in rats, and also had behavioural effects similar to those observed in study I (see below)

The involvement of brain monoamines in social behaviour and skin colour changes

The results from study II show that fish of social rank 3 (the most subordinate fish) turned darker following five days of social interaction (Fig 6).

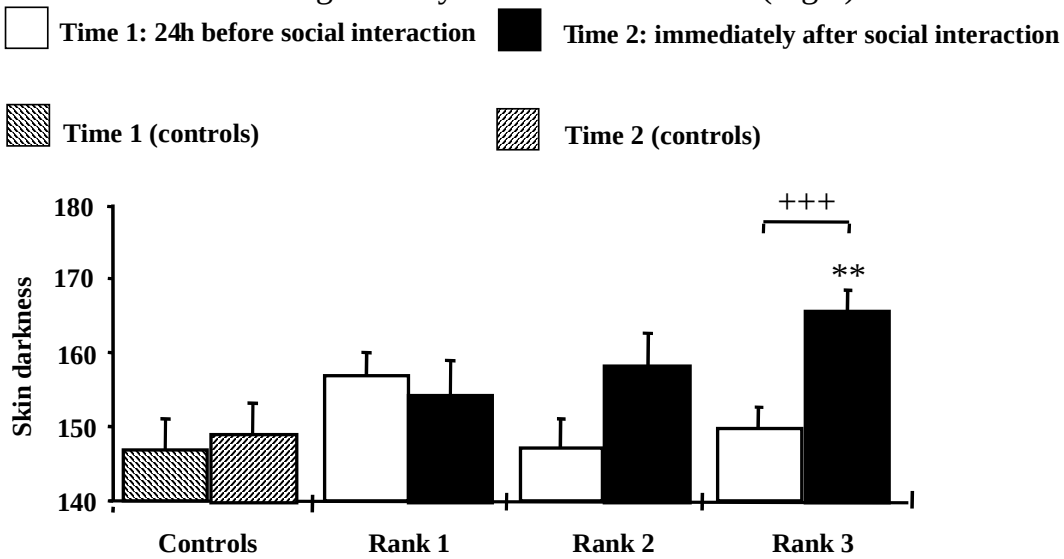

Fig 6. *Skin darkness of Arctic charr occupying different positions in a dominance hierarchy developed over 5 days, social rank 1 being the most dominant and social rank 3 the most subordinate fish in a group. Measurements were performed with a image analysing system on a linear grey scale, on which 0 is white and 255 is black, before and after five days of social interaction. Controls are fish that were visually isolated. Values are mean + S.E.M. from nine fish of social rank 1, four fish of social rank 2, 14 fish of social rank 3. An asterisk indicates a significant difference from visually isolated controls at time 2, and a plus sign indicates a difference between time 1 and time 2. **P<0.01; ++P<0.001.*

Moreover, a stepwise multiple regression analysis with plasma levels of β-endorphin, ACTH and α-MSH as dependent variables showed that of these variables only plasma levels of α-MSH had a significant effect on skin darkening, suggesting that α-MSH is involved in socially induced skin darkening in Arctic charr (Table 1).

Skin darkening has been observed following injections of 5-HT or 5-hydroxytryptophan (5-HTP), the precursor of 5-HT in goldfish (*Carassius auratus*, Olivereau *et al*., 1980), lizards (*Anolis caroliensis*, Levitin, 1980) and frogs (*Xenopus laevis*, Olivereau *et al*., 1980). The finding that treatment with 5-HT, as well as with 5-HTP, caused degranulation of pars intermedia cells in goldfish (Olivereau *et al*., 1980) further supports a role for 5-HT as an α-MSH releasing factor.

In study IV, high doses of 8-OH-DPAT induced skin darkening in the fish without having any significant effect on plasma levels of α-MSH. Thus, factors other than α-MSH, may be responsible for the skin darkening effect observed after high doses of 8-OH-DPAT in study IV. For instance, 8-OH-DPAT has been shown to induce a rise in plasma levels of epinephrine in rats (Korte *et al*., 1995), and catecholeamines are known to affect chromatophore dispersal in fish (Fujii and Oshima, 1986). It is also possible that 8-OH-DPAT has effects on the neural regulation of chromatophore dispersal. Furthermore, the presence of 5-HT receptors have been demonstrated on chromatophores in frogs (*Xenopus laevis*, Potenza and Lerner, 1994),

and a direct action of 8-OH-DPAT on skin chromatophores can not be excluded in study IV.

In addition, to its effects on pituitary release on POMC-derived peptides and HPI axis activity, the brain 5-HT system may also be involved in mediating behavioural effects of social subordination (Winberg and Nilsson, 1993; Øverli *et al*., 1998). Interestingly, brain DA seems to have effects opposing those of 5-HT, both on behaviour, pituitary α-MSH release, and possibly also HPI-axis activity.

The results of study I clearly show that experience of social subordination suppresses aggressive behaviour in juvenile Arctic charr. Following a 2 day experience of being subordinate, the fish showed a decrease in the number of attacks launched against the intruder as well as a longer attack latency, as compared to the intruder test performed immediately prior to social interaction.

Serotonin is believed to have effects inhibiting active behavioural responses, including aggressive behaviour, and behavioural inhibition in subordinate animals seems to be connected to a stress-induced activation of the central 5-HT system (Olivier *et al*., 1989; Winberg *et al*., 1993a,b; Leibowitz and Alexander, 1998; Øverli *et al*., 1998). In study I subordinate fish showed elevated hypothalamic 5-HIAA/5-HT ratios.

Following dyadic interaction for 2 days, dominant and subordinate fish were given an oral administration of L-dopa (10 mg/kg) or vehicle, and tested again for aggressive behaviour one hour later using the intruder test. Fish receiving L-dopa showed shorter attack latency than fish receiving vehicle. However, L-dopa treatment did not appear to affect aggressive motivation per se, since L-dopa treated fish did not show shorter attack latency after receiving L-dopa as compared to the latency time recorded in the intruder test performed immediately prior to drug treatment. Neither did L-dopa treatment affect the total number of attacks performed against the intruder during the test. It is tempting to suggest that the behavioural effects of L-dopa observed in the present study were related to an L-dopa mediated suppression of the neuroendocrine stress response induced by handling in connection with drug treatment (see discussion above). Vehicle controls showed an increase in attack latency, as compared to the latency recorded prior to treatment. This increase in latency to first attack, which may well have been an effect of stress, was abolished in fish receiving L-dopa, administrated in the same way.

As stated above, stress is known to activate the brain 5-HT system and behavioural inhibition in subordinate animals could at least in part be mediated by a stress-induced elevation of brain 5-HT activity. In study I, L-dopa appeared to have a suppressive effect on brain 5-HT activity (discussed above) and at the same time increasing behavioural responsiveness (shortening attack latency). Behavioural effects of L-dopa are however unlikely to have been directly mediated by a dopaminergic suppression of brain 5-HT activity, since the effect of L-dopa on brain 5-HT activity was most obvious in subordinate fish, whereas the behavioural effects of L-dopa were mainly observed in dominant fish.

In addation to size (Cutts *et al*., 1999), attack latency may be an important factor determining the outcome of dyadic fights for social dominance in fish. It has been shown that attack latency, and not the number of bites, as determined by introducing a conspecific enclosed in a glass jar to a territory holder, predicts the

outcome of a dominant/subordinate relationship in pairs of male sticklebacks (*Gasterosteus aculetus*) (Fitzgerald and Kedny, 1987). Winberg and Nilsson (1992) reported that L-dopa (10 mg/kg), administrated orally in the same way as in study I, increased the likelihood of a juvenile Arctic charr becomming dominant over a size-matched conspecific in staged fights. In that study, plasma cortisol levels and brain 5-HT activity were not reported. However, it could not be excluded that the competitive advantage of the L-dopa treated fish in the study by Winberg and Nilsson (1992) were also related to suppressive effects of L-dopa on neuroendocrine and behavioural stress responses induced by handling a administration of L-dopa or vehicle.

The effect of skin colour on aggression

In study III background colour was used to manipulate skin colour in pairs of interacting fish. Pairs of fish interacting on white background were brighter and performed a higher number of aggressive acts than fish interacting on black background (Fig 7).

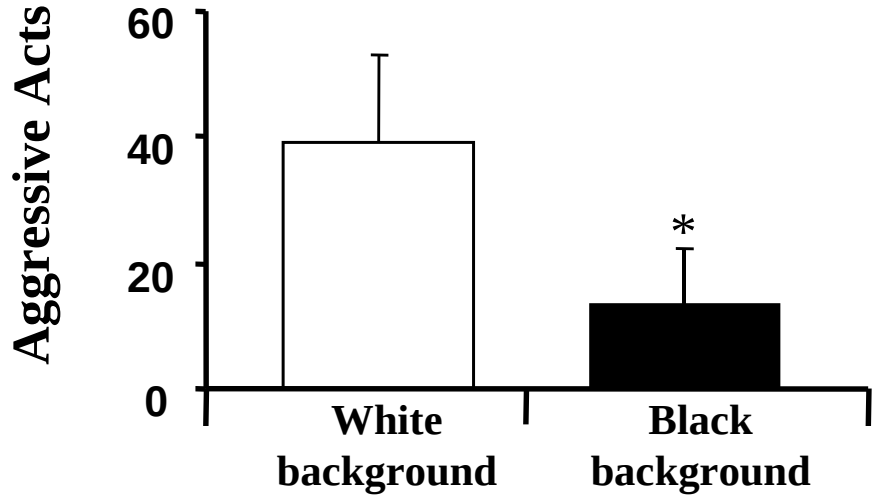

Fig. 7. *Total number of aggressive acts by the socially dominant fish observed during five days of social interaction in size-matched pairs of Arctic charr interacting on white or black background colour. Values are mean ± S.E.M, for 7 pairs on white and 7 pairs on black background colour. * Indicates a significant difference at the level of P<0.05 (Mann-Whitney U-test).*

Assuming that dark body coloration signals social subordination, a darker fish may represent less of a threat and elicit less aggression than a conspecific displaying paler body coloration. The results from study III seem to support this hypothesis. On white background both pair members were initially pale in coloration and showed a higher level of aggressive behaviour, than fish interacting on black background. However, on white background the frequency of aggressive interactions declined over time and it is suggestive that this decline in aggressive behaviour was related to the fact that the fish becoming subordinate took on a darker body coloration. In pairs of fish interacting on dark background both members were dark in coloration from the start, and the subordinate fish did not show any additional darkening of its body colour. In these

pairs the decline in aggressive interaction over time was less obvious than in pairs interacting on white background.

In Atlantic salmon (*Salmo salar*) parr interacting on a light-coloured substrate social subordination results in darkening of the body and sclera (O´Connor *et al*., 1999). Salmon parr becoming socially subordinate showed a sudden change to a darker colour, occurring at the moment a fish loses an aggressive interaction. In response to this darkening of the subordinate fish, the behaviour of its dominant opponent immediately changed, and as a result the number of attacks on the subordinate rapidly declined (O´Connor *et al*., 1999). Thus, darkening of the body colour appears to act as a social signal announcing defeat and/or subordinate social status in both Atlantic salmon (O´Connor *et al*., 1999) and Arctic charr (study II and III). However, it has to be acknowledged that the higher number of aggressive acts observed in dominant fish on white background in study III could also be related to the fact that subordinate fish are more visible against a pale background colour.

Concluding discussion

Social subordination greatly affects both the physiology and behaviour of an animal. The effects of social subordination on HPI axis activity, aggressive behaviour and body colour appear to be interrelated, and the overall aim of the thesis was to further explore the role of the brain monoaminergic systems in mediating these effects. The results confirm that social experience is reflected in brain monoaminergic activity of Arctic charr. Socially subordinate fish display elevated brain 5-HT and NE activity along with a chronic activation of the HPI axis, also including elevated plasma concentrations of α-MSH. In addition, the results show that social experience affects the body colour and behaviour of Arctic charr, subordinate fish showing darker body coloration and an inhibition of aggressive behaviour.

Plasma levels of ACTH and cortstisol were positively correlated with brain 5-HT and NE activity, as indicated by 5-HIAA/5-HT and MHPG/NE ratios, respectively. This shows that the brain 5-HT and NE systems may stimulate HPI axis activity. Moreover, 5-HT has previously been suggested to act inhibitory an aggressive behaviour, and there are also results suggesting that 5-HT may stimulate pituitary release of α-MSH. Brain catecholaminergic systems may to some extent have effects antagonistic to those of 5-HT, on aggressive behaviour and plasma α-MSH concentrations.

The results from study I suggest that stimulation of brain DA activity by L-dopa treatment may counteract the stress-induced behavioural inhibition as well as stress-related effects on the activity of the HPI axis and the brain 5-HT system. These results seem to support the hypothesis that DA and 5-HT have antagonistic effects on aggressive behaviour and HPI axis activity. However, the effects of L-dopa on brain 5-HT activity were most pronounced in subordinate fish, whereas behavioural effects of L-dopa were only observed in dominant fish. Thus, the behavioural effects of L-dopa observed in study I do not seem to be directly related to a DA mediated suppression of brain 5-HT activity.

The results from study II suggests that skin darkening in socially subordinate fish could be mediated by a stress-induced elevation of plasma α-MSH levels. The results of study III show that fish kept on dark background, and thus being darker in

body colour, are less aggressive than conspecifics interacting on white background, supporting the hypothesis that skin darkening could act as a social signal, announcing social submission. The reduced aggression on dark background was also reflected in lower brain NE activity and a tendency towards lower plasma levels of cortisol and ACTH in subordinate fish on dark, as compared to white background.

The results from study IV show that stimulation of 5-HT_{1A} receptors, using the selective 5-HT_{1A} receptor agonist 8-OH-DPAT, elevates HPI axis activity in non-stressed fish but inhibits HPI axis activity in stressed fish. This provides additional support for the suggestion that 5-HT_{1A} receptors are involved in the control of the HPI axis in salmonids. A tentative explanation to the observation that the effect of 5-HT_{1A} stimulation varies depending on the state of the fish could be that 5-HT_{1A} receptors are present at both post- and pre-synaptic sites in the salmonid brain, in the latter case acting as autoreceptors.

In conclusion, the results of the present studies show that socially induced effects on brain monoaminergic activity are important in mediating effects of social subordination on behaviour, endocrine stress responses, and social signalling in Arctic charr. Subordinate Arctic charr show a darker body coloration and an inhibition of aggressive behaviour, stress-induced effects that could be mediated by elevated brain 5-HT activity, and serve as a way of signalling social position and coping with stress. Moreover, the results suggest that the brain NE system may also play a role in the activation of the HPI axis, in response to social stress. Brain DA, on the other hand, may have effects opposing those of 5-HT on HPI axis activity and behaviour.

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-Go fishing!
-What do you like to eat?
-Fish!
-What's your job?
-Studying fish!
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