

**Stressful social interaction reduces
telencephalic cell proliferation in
rainbow trout**

Master Thesis by Christina Sørensen

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Abbreviations

5-HT	5-hydroxytryptamine, serotonin
ACTH	adrenocorticotrophic hormone
BDNF	brain derived neurotrophic factor
BrdU	5-bromodeoxyuridine
BSA	bovine serum albumin
CNS	central nervous system
CRH	corticotropin-releasing hormone (=CRF, corticotropin-releasing factor)
D	area dorsalis telencephali
DA	dopamine
DAB	3,3'-diaminobenzidine
Dc	central zone of D
DI	lateral zone of D
Dld	dorsal part of lateral zone of D
Dlp	posterior part of lateral zone of D
Dlv	ventral part of lateral zone of D
Dm	medial zone of D
Dp	posterior zone of D
dpm	disintegrations per minute
E	nucleus entopeduncularis
ECL	external cellular layer of the olfactory bulb
EDTA	ethylenediaminetetraacetic acid
GC	glucocorticoid
GL	glomerular layer of the olfactory bulb
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal
HPI	hypothalamic-pituitary-interrenal
HR	high responder (rainbow trout strain)
HRP	horse radish peroxidase
ICL	internal cellular layer of the olfactory bulb
IgG	Immunoglobulin G
Ki-67	nuclear antigen present in proliferating cells
LAL	long attack latency (mouse strain)
LR	low responder (rainbow trout strain)
MR	mineralocorticoid receptor
NE	norepinephrine, noradrenaline
PAP	peroxidase anti-peroxidase
PBS	phosphate buffered saline
PBT	phosphate buffered saline with Triton X-100 and BSA
PCNA	proliferating cell nuclear antigen
PP	nucleus preopticus parvocellularis
PZ	proliferation zone
RIA	radioimmunoassay
S.E.M.	standard error of mean

SAL	short attack latency (mouse strain)
SD	standard deviation
SSRI	selective serotonin reuptake inhibitor
V	area ventralis telencephali
VBS	visual burrow system
Vc	central nucleus of V
Vd	dorsal nucleus of V
Vl	lateral nucleus of V
Vs	supracommissural nucleus of V
Vv	ventral nucleus of V

Abstract

When housed together, juvenile rainbow trout (*Oncorhynchus mykiss*) form social hierarchies in which subordinate animals show stress-related changes in behaviour, endocrine function and neurochemistry. Similar changes are observed in the subordinate animals when social hierarchies are studied in mammalian species. These animals also show a marked reduction in hippocampal cell proliferation. To determine whether this effect of stressful social interaction on cell proliferation exists in rainbow trout as well, the bromodeoxyuridine (BrdU) method of investigating proliferation was adapted for utilization in rainbow trout.

Pairs of fish were allowed to fight for dominance, and were then left to interact for 4 days. 24h before the end of the experiment, the deoxythymidine analogue BrdU was administered intraperitoneally to all fish. BrdU is incorporated into the DNA of cells going through S-phase and is thus a marker of proliferation. Proliferating cells were visualized immunohistochemically and quantified in transverse sections of the telencephalon, which is suggested to contain structures embryologically and functionally homologous to the hippocampus. Even though the method appeared to be stressful to the animals, a reduction in telencephalic cell proliferation of almost 40% was found in the subordinates compared to isolated controls. The proliferation in dominant animals did not differ significantly from any of the other groups, but there was a strong tendency of reduced proliferation in these animals as well.

These results confirm that stressful social interaction reduces brain cell proliferation in rainbow trout in a similar manner as in mammals. This change is suggested to be caused by the increased plasma cortisol levels that were found in the subordinate animals, and may be a component of the adaptive stress response.

1 Introduction

1.1 Stress and stress responses

1.1.1 Definition of stress

Although stress is a widely used term and an important concept in our daily lives, there is still considerable ambiguity regarding its definition. Here stress will be defined as the state when an intrinsic or extrinsic factor, a stressor, poses a real or imagined challenge to an animal's homeostasis, where homeostasis refers to the maintenance of a narrow range of vital physiological parameters necessary for survival (McEwen and Stellar, 1993; Chrousos, 1998). The homeostasis is attempted re-established through the animal's stress response; a complex repertoire of physiological and behavioural adaptive responses.

1.1.2 Physiological and behavioural stress responses

The organism's response to stress is mediated by the stress system, located both in the central nervous system (CNS) and in the periphery (Chrousos and Gold, 1992; Chrousos, 1998). The stress system receives and integrates a number of neurosensory and blood-borne signals, and when activated, leads to a set of time-limited behavioural and physiological changes. These changes are remarkably consistent over a wide range of stressors, and are normally adaptive, improving the individual's chances for survival (Chrousos and Gold, 1992).

Heart rate, respiration, blood pressure and blood glucose concentrations are among the physiological parameters that change during the stress response (Cannon, 1929; Seyle, 1950). These responses are at least in part mediated by the endocrinological stress response, which involves two major endocrine systems; the hypothalamic-pituitary-adrenal (HPA) system, and the sympathico-adrenomedullary system.

The sympathico-adrenomedullary system consists in mammals of preganglionic cholinergic nerves from the spinal cord triggering the release of catecholamines (adrenaline and noradrenaline) from chromaffin cells in the adrenal medulla into the

blood stream, and the neurons of the sympathetic system signalling to internal organs with noradrenaline. Since the adrenal response involves neurally stimulated release of pre-stored hormones, its activation results in an immediate increase in release of these catecholamines into the circulation (Mason, 1968). The catecholamines exercise their effect by binding to adrenergic receptors, which are distributed throughout the body.

As in mammals, stress in teleost fish is accompanied by a rapid increase in the plasma concentration of catecholamines, which are released by chromaffin cells. In fish, however, these cells are scattered throughout the kidney and the walls of the posterior cardinal vein (Sumpter, 1997). In all vertebrates, the catecholamines initiate a range of physiological effects that serve to optimise cardiovascular and respiratory functions, including the mobilisation of energy stores to meet increased metabolic requirements during stress (Chrousos, 1998; Fabbri et al., 1998; Perry and Bernier, 1999).

The mammalian HPA system, or HPA axis, as it is commonly referred to, is activated by the release of corticotropin-releasing hormone (CRH) from the hypothalamus, reaching the pituitary through the hypothalamic-hypophyseal portal system. CRH promotes release of adrenocorticotrophic hormone (ACTH) by the anterior pituitary into the circulation. ACTH in turn stimulates release of glucocorticoid hormones (GCs, mainly cortisol in humans and corticosterone in rodents) from the adrenal cortex. The endocrine character of this system leads to a slight delay (minutes) in the release of glucocorticosteroids into the circulation after the initiation of a stressful stimuli.

The main glucocorticoid effects are mediated by glucocorticoid receptors, which, like the adrenergic receptors, are found throughout the body. There are two kinds of glucocorticoid receptors, the Type I, or mineralocorticoid receptor (MR) and the Type II, or glucocorticoid receptor (GR). These receptors are often colocalized and they are functionally complementary. The MR receptor has a 10-fold higher affinity for corticosteroids than the GR, and therefore this receptor generally mediates the tonic effects on arousal caused by daily variation in glucocorticoid concentrations. The GR is thus only extensively occupied during times of high concentrations of circulating glucocorticoids, and seems to be the receptor mediating both the adaptive stress response, and the negative feedback effect of glucocorticoids on ACTH and CRH release. (DeKloet and Reul, 1987; DeKloet, 1996; De Kloet et al., 1998; Fuchs and Flugge, 2003).

The HPA-axis in mammals is paralleled in teleosts by the hypothalamo-pituitary-interrenal (HPI) axis, which also cumulates with the release of glucocorticoids, mainly cortisol, into the bloodstream (Sumpster, 1997). Unlike mammals and other higher vertebrates, fishes do not have a hypothalamic-hypophyseal portal system, and CRH reaches the ACTH producing pituitary cells via direct neuronal innervation. Fishes do not have a discrete adrenal gland, and the adrenal cortical tissue is represented by the interrenal cells which release GCs upon ACTH stimulation (Mommsen et al., 1999). Both kinds of glucocorticoid receptors have also been identified in teleosts, and they have been located to the telencephalon and other brain structures. Significantly less is, however, known about their distribution and function compared to what is known in mammals (Colombe et al., 2000; Bury et al., 2003; Greenwood et al., 2003).

The acute behavioural response to stress in mammals includes increased arousal, alertness and vigilance, improved cognition and focused attention. It also includes inhibition of vegetative functions, such as appetite, feeding and reproductive behaviour (Fuchs and Flugge, 2003). Chronic stress, on the other hand leads to a general down-regulation of behaviour, including reduction in aggression, movement and exploration (Albonetti and Farabollini, 1992; Chrousos and Gold, 1992; Chrousos, 1998; Bartolomucci et al., 2004).

In fish, similar behavioural stress responses have been identified. Chronically stressed fish show reduced aggression, activity and food intake (Winberg et al., 1991; Winberg and Nilsson, 1993a; Iwama et al., 1997; Øverli et al., 1998).

In summary, the main endocrine and behavioural components of the stress response are essentially similar in teleosts and mammals, indicating a strong conservation of these responses throughout vertebrate evolution.

1.1.3 Limbic-dependent stress

Activation of brain monoaminergic systems is an important component of the central stress response. In mammals, stressful stimuli have been reported to raise the brain concentrations of noradrenaline (norepinephrine, NE), dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) as well as their respective metabolites, indicating increased activity in these neuronal systems (Bliss et al., 1968; Puglisi-Allegra et al., 1991; Stanford, 1995). Interestingly the limbic structures of the brain, which are particularly involved in emotional and behavioural relations to stress (Maclean, 1955; Habib, 1998), receive strong monoaminergic input. These structures, and the hippocampus in particular, also have a particularly high density of both kinds of glucocorticoid receptors (Sapolsky et al., 1986). It appears that limbic circuits are activated by stressors such as restraint, fear and exposure to novel environments. Under normal circumstances limbic structures inhibit CRH release from the hypothalamus (Fuchs and Flugge, 2003), but suppression of this activity leads to activation of the HPA axis. Prior to an eventual activation of the HPA axis, integration of signals from the monoaminergic systems and information from different sensory organs takes place to determine if and to what degree the endocrinal stress response will be activated.

Although the physiological and behavioural stress responses are the same for all types of stressors, not all stressors initiate limbic processing prior to activation of the endocrinal stress response (Lopez et al., 1999; Fuchs and Flugge, 2003). Physiological stressors, such as noxious stimuli and hypothermia activate efferent pathways directly relayed to the hypothalamus, and there is no limbic integration involved in the response while responses to stressors such as predator exposure and restraint are mediated through limbic structures. Herman and Cullinan (1997) explain this dualism of the central stress system as an adaptation to the different demands on the stress response from qualitatively different stimuli. Physiological stressors require immediate reactions for survival, but no further interpretation from higher-order brain systems. Psychological challenges, on the other hand, need cortical processing and integration of previous experiences and current sensory information before an adequate response can be launched (Herman and Cullinan, 1997).

Interestingly, the neuroendocrinology of limbic-dependent chronic stress in mammals bears remarkable similarities to that of depressive disorders in humans (Yadid et al., 2000), and several animal models of this kind of stress have been developed to study biological parameters that are implicated in depression (See Yadid et al. 2000 for list). Social stress is common in many animal species and typically results from competition for resources such as space, access to reproductive partners or access to food. Some of the animal models of limbic-dependant stress utilize this tendency of different species to form social hierarchies when housed in groups, including the VBS in rats (Tamashiro et al., 2005), and the chronic psychosocial stress paradigm in tree shrews (Fuchs et al., 2004a). A key observation in this research is that socially subordinate animals are subject to chronic and unpredictable stress, which leads to characteristic changes in behaviour, physiology and neuroendocrinological parameters.

1.1.4 Social stress in fishes

Several fish species also display territorial behaviour when housed together (Andries and Nelissen, 1990; Winberg et al., 1991; Winberg et al., 1993; Oliveira and Almada, 1996; Winberg et al., 1996; Winberg et al., 1997), and both neuroendocrinology and behaviour during social stress has been investigated in these models. One species in particular has undergone very thorough investigation; the rainbow trout (*Oncorhynchus mykiss*) (Winberg et al., 1993; Winberg and Lepage, 1998; Øverli et al., 1999; Øverli et al., 2004b; Øverli et al., 2004a). Juvenile salmonids including rainbow trout show a particularly high degree of social aggression and hierarchy formation. In nature they are territorial and defend small territories consisting of patches of river-bed. A territory is a prerequisite for access to food, as they feed on drifting invertebrates, and individuals that fail to acquire a territory have reduced chances of survival (Kalleberg, 1958; Bachman, 1984; Nakano, 1995; Adams et al., 1998). In laboratory experiments, juvenile rainbow trout are kept in isolated areas with abundant access to food, in order to induce territorial behaviour. Introduction of an intruder or removing of barriers between adjoining territories will inevitably lead to a fight for social dominance, resulting in formation of a hierarchy with one dominant and one subordinate individual (Winberg and Nilsson, 1992; Øverli et al., 1999; Øverli et al., 2004b; Øverli et al., 2004a).

The social stress response has been thoroughly investigated in this system with particular regard to brain monoaminergic activity (Winberg and Nilsson, 1993a; Winberg and Nilsson, 1993b; Winberg and Lepage, 1998; Øverli et al., 1999), and so far, several similarities with the response in mammals have been described. For instance, a general elevation of 5-HT activity in stress has been found, while DA and NE activity is regional and context dependant (Bliss et al., 1968). This adds to the previous mentioned similarities within vertebrate groups in the stress response, and increases the evidence for a strong conservation in the limbic dependent stress responses throughout vertebrate evolution.

As fishes do not have a neocortex (Rose, 2002), studying psychosocial stress in these animals may give insights into the basic, cortex-independent mechanisms of this kind of stress that appear to be common among all vertebrates.

1.2 Stress and adult neurogenesis

1.2.1 Mammalian neurogenesis – Role for limbic processes

After being disregarded and forgotten for several decades after its original discovery (Hamilton, 1901), the process of adult neurogenesis in mammals, addition of new neurons to the adult CNS, was relatively recently re-discovered (Kuhn et al., 1996; Kempermann et al., 1997), following which the interest in the subject exploded. This revolution in research on neurogenesis was brought about by the introduction of the BrdU method for visualizing cell proliferation (Gratzner et al., 1975; Gratzner, 1982). 5-Bromodeoxyuridine (BrdU) is a synthetic deoxythymidine analogue with a bromine atom substituting the methyl group in the 5 position of the pyrimidine ring. When BrdU is present in a cell, it will compete with the cell's deoxythymidine for incorporation into DNA during the S-phase. BrdU will then remain in the cell's DNA throughout the cell's life (except in cells undergoing subsequent divisions whereupon the BrdU becomes diluted), and be passed on to any daughter cells. After injection of a single dose of BrdU into an animal, all the animal's cells going through S-phase during the time it is available are assumed to have BrdU incorporated into its DNA, and its presence in a cell will hence

be a marker of cell proliferation. The DNA-incorporated BrdU is then visualized immunohistochemically. This method does not give any indication of the type of the new cells. This has, however, also been investigated immunohistochemically utilizing cell-specific markers for glia and neurons in double staining experiments with BrdU, where co-localization reveals the type of the newly generated cells. It has thus been shown that generally a large part of the newly generated cells in the adult mammalian CNS are indeed neurons (Cameron et al., 1993; Kuhn et al., 1996).

The adult neuronal proliferative zones of mammals are located to discrete structures; the olfactory bulb, the subventricular zone lining the walls of the lateral ventricles and most notably the subgranular zone of the hippocampal dentate gyrus (Prickaerts et al., 2004).

The rate of neurogenesis, and particularly that of the dentate gyrus, varies with several factors. It is for instance increased by voluntary exercise (van Praag et al., 1999) and enhanced environmental complexity (Kempermann et al., 1997; Nilsson et al., 1999). Increased adult neurogenesis has been found to correlate with improved performance in hippocampal-dependent learning and memory tasks (Gould et al., 1999a; Nilsson et al., 1999; van Praag et al., 1999). This is perhaps not surprising as the hippocampus has traditionally been known for its importance for memory formation, mood regulation and neuroendocrine control (Jacobson and Sapolsky, 1991; Eichenbaum et al., 1992; Squire, 1992; Eichenbaum, 1999). The hippocampus is also important in the limbic component of the central stress system. Thus hippocampal neurogenesis has been intensively studied in regard to stress during recent years.

Several studies have found that stress reduces hippocampal neurogenesis (Fuchs and Flugge, 1998; Gould et al., 2000; McEwen, 2000; Joels et al., 2004). This effect has been suggested to be mediated directly or indirectly by increased levels of glucocorticoids, since administration of glucocorticoids inhibits neurogenesis (Gould and Tanapat, 1999), and since there are especially high densities of glucocorticoid receptors in the hippocampus (Sapolsky et al., 1986). The stress-induced reduction in hippocampal neurogenesis is counteracted by selective serotonin reuptake inhibitor (SSRI) treatment (Fuchs et al., 2004b). SSRI antidepressants seem to produce their effects partly by stimulating hippocampal neurogenesis via the 5-HT_{1A} receptor, as 5-HT_{1A}-receptor null

knock out mice are insensitive to both the neurogenic and anti-depressant effects of SSRIs (Santarelli et al., 2003). X-ray irradiation of the hippocampus also blocks the behavioural responses to these antidepressants, indicating that the behavioural effects of chronic antidepressant treatment are mediated by the stimulation of hippocampal neurogenesis. Thus, reduction in rate of hippocampal neurogenesis appears to be an element of the central stress response, and might be the mechanism behind some of the behavioural stress responses.

The hippocampus is as mentioned essential for memory and learning, and these functions are impaired by reduced rate of neurogenesis. Recently it has become evident that memory and learning are important for the integrative control of the stress response (Rodrigues et al., 2001; Blair et al., 2005). It is generally assumed that the more unpredictable a situation is, the more stressful it is to the individual. Consequently, individuals with impaired memory function or learning abilities may assess a given situation as more unpredictable than individuals with better memory or learning abilities. Thus, reduced rate of neurogenesis caused by a stressful stimuli may cause the animal to interpret the environment as unpredictable, and respond by down-regulating behaviours that otherwise could have resulted in danger to the animal's health of life.

Since psychosocial stress and reduced neurogenesis in animals mimic depressive disorders in humans, study of these phenomena comparatively may increase our understanding of the adaptive (or maladaptive) properties of depression and mood disorders.

1.2.2 Teleost neurogenesis and the teleost telencephalon

Compared to mammals, teleost fish, and all other non-mammalian vertebrates have a much higher degree of adult neurogenesis (Zupanc, 2001). The size of a teleost brain increases with age, body weight and body length throughout life, and adult proliferation has been observed within all major teleost brain structures (Birse et al., 1980; Leyhausen et al., 1987; Brandstatter and Kotrschal, 1990; Zupanc and Horschke, 1995). Adult proliferation zones have been mapped in detail in the brains of the adult stickleback (*Gasterosteus aculeatus*) (Ekstrom et al., 2001), the brown ghost knife fish

(*Apteronotus leptorhynchus*) (Zupanc and Horschke, 1995) and the gilthead sea bream (*Sparus aurata*) (Zikopoulos et al., 2000).

A comparison across vertebrate taxa suggests that continuous generation of new neurons in a large number of discrete regions in the CNS is likely to have occurred in the ancestors of today's chordates. In the course of evolution, this ability has been conserved to varying degrees in the branches leading to today's non-mammalian vertebrates but has been greatly reduced in extant mammals (Zupanc, 2001). To obtain a thorough biological understanding of adult neurogenesis it would seem essential to clarify by what mechanisms this reduction has occurred. However, very little is known about the functional regulation of neurogenesis in teleost fish.

For comparison with mammalian hippocampal neurogenesis, it would be of particular interest to investigate the proliferative zones found in proximity to structures responsible for teleostean hippocampal function. Embryological and behavioural evidence indicates that the hippocampal function of teleosts is harboured in the telencephalon (Butler and Hodos, 1996). Telencephalic ablation in goldfish has for instance produced learning deficits in the solution of spatial tasks (Salas et al., 1996; Lopez et al., 2000) in a way that is similar to the spatial deficits observed following hippocampal damage in birds and mammals (Morris et al., 1982; Bingman and Mench, 1990). Further pinpointing of the teleost hippocampal analogues can, however, be difficult, since in ray-finned fishes, and most prominently in teleosts, the topology of the telencephalon is highly distorted compared to that of all other vertebrates (Nieuwenhuys and Meek, 1990). During CNS development in ray-finned fishes, the roof plate of the embryonic telencephalon extends laterally with the effect that the paired alar plates forming the hemispheric walls roll out lateroventrally; a process called eversion (Nieuwenhuys, 1962). This results in the formation of two solid telencephalic hemispheres separated by a ventricle. The telencephalon of all other vertebrate groups are developed by paired evagination and thickening of the most rostral embryonic neural tube, and each hemisphere contains a lateral diverticulum of the ventricle centrally (Wullimann et al., 1996) (Figure 1.1).

Thus, the adult topology of the teleostean telencephalon can not alone give any immediate indication of homology with structures in other vertebrates, but the differences

between the evagination and eversion processes suggests a reversal of the medial-to-lateral topography in teleost telencephalon compared to other vertebrates (Figure 1.1), and a growing amount of connectional, immunohistochemical and behavioural data supports this idea (Reiner and Northcutt, 1992; Bradford, 1995; Butler, 2000; Rodriguez et al., 2002).

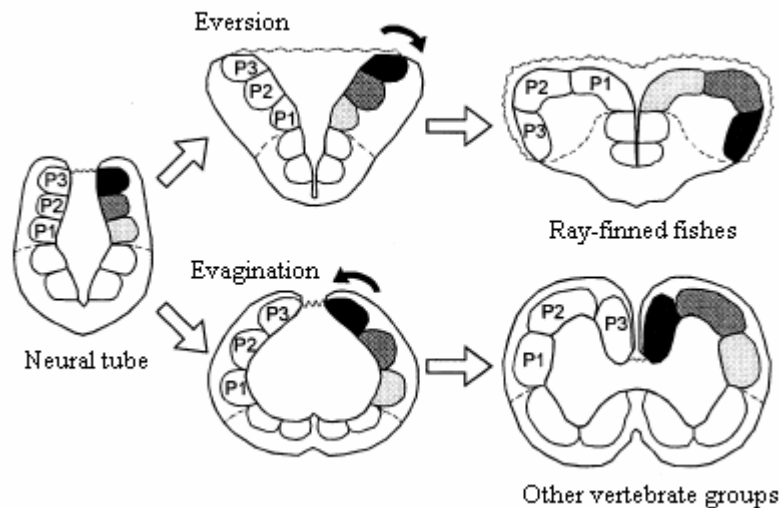


Figure 1.1 Schematic representation of the process of eversion that occurs in the telencephalon of ray-finned fishes compared with that of evagination in all other vertebrate groups. P1, P2 and P3 represent the pallia of the telencephalon, where P1 is the lateral pallium, P2 the dorsomedial pallium and P3 the medial pallium in mammals. A reversal of the medial-to-lateral topography is indicated in ray-finned fishes. Adapted from Rodríguez et al. 2002.

The hippocampus is located in the medial pallium in most vertebrate groups, which, according to the reversed topology principle is suggested to be anatomically homologous with the lateral pallium of the teleost telencephalon (Nieuwenhuys and Meek, 1990; Bradford, 1995), more precisely the posterior and lateral zones of the dorsal telencephalon (Dp and Dl) according to the terminology of Nieuwenhuys (1962) as modified by Northcutt and Davis (1983) (see list for abbreviations, and Appendix 1 for telencephalic map). Rodriguez et al. have shown that lesioning of the teleostean lateral pallium produces the same deficits in spatial tasks as ablating the whole teleostean telencephalon or lesioning of the medial pallium in other vertebrates, indicating a functional homology as well (Rodriguez et al., 2002). Thus, the focus of this thesis on the

rainbow trout telencephalon is motivated by the presence of structures both embryologically and functionally homologous to the mammalian hippocampus in the teleostean telencephalon.

All three qualitative mapping studies of the proliferation in the teleost brain has led to the identification of two distinct telencephalic proliferation zones, denoted PZ1 and PZ2 by Ekström et al. (Zupanc and Horschke, 1995; Zikopoulos et al., 2000; Ekstrom et al., 2001). PZ1 is divided into two sub-zones, PZ1a and PZ1b. PZ1a is located along the ependyma of the rostromedial aspect of the medial zone of the dorsal telencephalon (Dm), with stained cells scattered along the ventricular surface of the dorsal telencephalon (D), spreading laterally and caudally over the lateral zone of D (Dl). Aggregations of cells are found in the rostromedial pole of Dm and in the caudoventral pole of the posterior zone of D (Dp), the first within the medial pallium, and the latter within the lateral pallium, which is suggested to have hippocampal functions (Figure 1.2a-g). PZ1a is continuous with PZ1b rostrally, and this zone continues ventrally over the ependyma of the intracellular layer of the olfactory bulbs (ICL) to the ventricular midline, and further caudally into the most rostral part of the ventral nucleus of the ventral telencephalon (Vv) (Figure 1.2a-d). At this level, the most rostral level of PZ2 appears in the dorsal part of V (Vd), stretching caudally approximately to the anterior commissure (Figure 1.2c-f) (Zupanc and Horschke, 1995; Zikopoulos et al., 2000; Ekstrom et al., 2001).

The question of whether newly generated cells are neurons, and if they migrate and become functionally incorporated into the existing neuronal networks arises naturally. Studies in the zebrafish olfactory bulb and cerebellum have revealed that a portion of the newly formed cells express neuron specific proteins (Zupanc and Ott, 1999; Byrd and Brunjes, 2001). Migrational patterns of the newly born cells have also been studied in the zebrafish cerebellum (Zupanc et al., 1996; Zupanc and Clint, 2003), but little is known about these factors in the telencephalon. It will therefore be of interest to investigate not only the total proliferation in the whole telencephalon in response to stress, but also the dynamics within distinct proliferation zones, the migration patterns of new cells and the identity of newly formed cells. Due to time limitations, this study has,

however, only been concerned with the proliferation in the telencephalon as a whole, as a basis for further studies.

Ekström et al. notes that they found a certain degree of interindividual variation in the labeling of proliferating cells regardless of the method used, and that this difference was quantitative. They did not, however, investigate this further in their study, and called for a systematic exploration of this issue (Ekstrom et al., 2001). In addition to this, Marchetti and Nevitt have found significant differences in the size of the telencephalon of rainbow trout reared in the wild and in hatcheries (Marchetti and Nevitt, 2003). This taken together with the collected evidence from mammals raises the possibility that stress also reduces brain cell proliferation in rainbow trout.

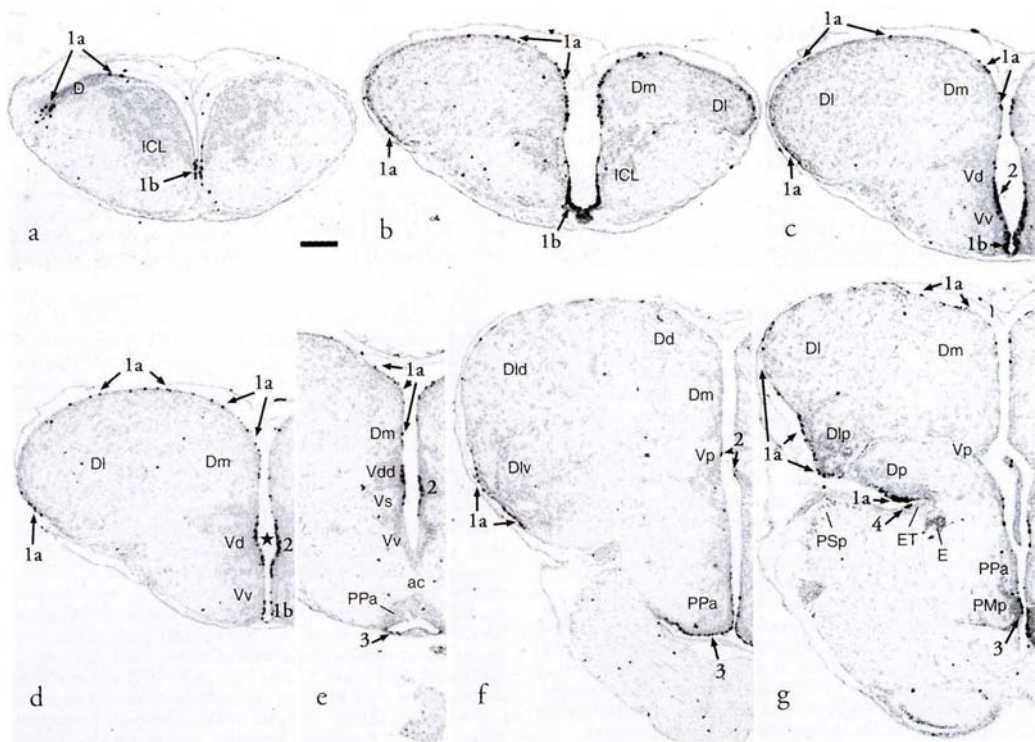


Figure 1.2 Transverse paraffin sections (5 µm) of a brain of a female stickleback, that was fixed in Methacarn 24 hours after intraperitoneal injection of ^3H -thymidine. Autoradiography and Toluidine Blue Nissl stain. Proliferations zones (PZs) are identified by Arabic numerals. For abbreviations see list. **PZ1a (a-g)**. The telencephalic PZ1a is widely spread over the ventricular surface of Dm, Dd, Dld, Dlv, Dlp, and Dp. **PZ1b (a,b)**. The telencephalic PZ1b is a small zone that appears ventral to the internal cellular layer (a,b) and extends into the rostroventral Vv (c,d). **PZ2 (c-f)**. The telencephalic PZ2 follows the Vv and Vd into Vp. **PZ3 (e-g)** and **PZ4 (g)** are respectively preoptic and thalamic, and are not considered in the current study. Adapted from Ekström et al. 2001.

1.3 Aims of the study

This study aims to develop and test a method for quantification of cell proliferation in the rainbow trout telencephalon, utilizing BrdU incorporation and immunohistochemistry. Further, the method developed will be used to investigate whether social status in a dominance hierarchy system affects the rate of telencephalic brain cell proliferation.

2 Materials and methods

2.1 Experimental animals

The experimental animals used were juvenile rainbow trout (*Oncorhynchus mykiss*) obtained from Valdres Ørretoppdrett in Valdres, Norway, March 2003. After transport to the research facilities at the University of Oslo, the fish (n = 32) were maintained for at least 6 weeks in a 750 liter holding tank that was aerated and continuously supplied with dechlorinated Oslo tap water at 7-9°C (100 L/h). Artificial lighting followed a 12h light/12 hour darkness cycle with light from 8am to 8pm, and the fish were fed daily approximately 1% of their mass with commercial pelleted food (Ewos). At the start of the experiment, the animals weighed between 84.0g and 184.5g ($126.0\text{g} \pm 5.63\text{g}$; mean \pm S.E.M.).

2.2 Experimental set-up and design

2.2.1 Observation aquaria

The experimental set-up included glass observation aquaria (50x50x100 cm = 250L) that were aerated and continuously supplied with dechlorinated Oslo tap water (2 L/h per fish). The water was held at 7-9 °C throughout the experiment. The aquaria were divided in four 50L compartments by removable opaque PVC walls and the back, sides and bottom were covered with black plastic film. The compartments were lidded with plastic mesh to keep the fish from escaping. The set-up is illustrated in Figure 2.1.

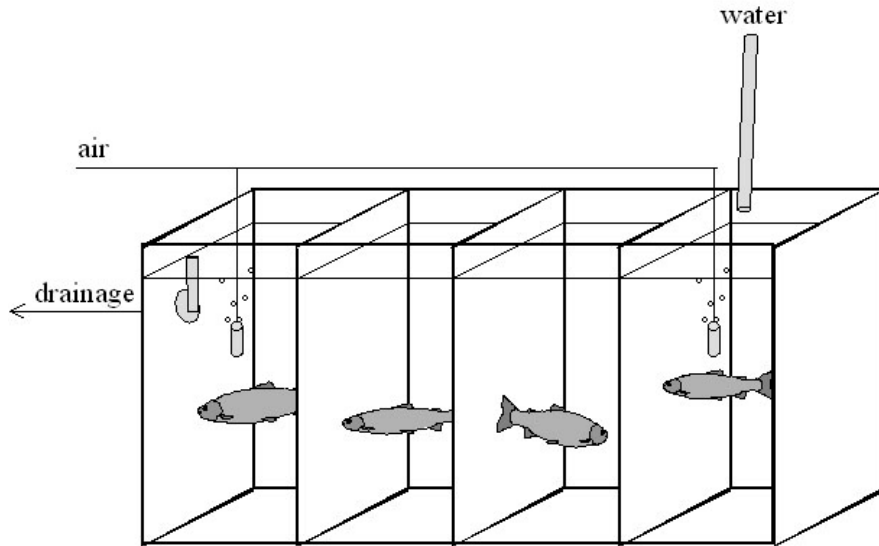


Figure 2.1 Experimental set-up consisting of a glass aquarium with three removable opaque walls.

2.2.2 The feeding test for adaptation to a new environment

Transferring a fish to a new environment involves two sources of stress; the physical handling of the fish and the uncertainty of possible dangers in the new environment. Once introduced to the new environment, a salmonid fish will typically be in a state of anorexia and immobility, which are two well characterized parameters of stress in teleost fish (Winberg et al., 1993; Winberg and Nilsson, 1993a; Øverli et al., 1998; Øverli et al., 2001). Both parameters have earlier been quantified to characterize and rate the state of stress (Winberg et al., 1993; Øverli et al., 1998; Øverli et al., 2002), the former by recording the amount of food taken by the fish when hand fed pelleted food, the latter by registering the time the fish spends moving within a fixed time interval.

In a pilot study, a test for feeding behaviour in a new environment was developed. Fish were netted and transferred to the observation aquaria. Starting 24 h after transfer, fish were hand fed daily (1% of body weight, Ewos pelleted trout food) by dropping the food items one by one or a few at a time into the aquaria. The fish were carefully observed throughout the time it took to feed them, typically 5 minutes. For all the fish, feed intake increased progressively over several days after transfer to the new environment. At first most fish took no food at all, even if the pellets were made to fall

directly (< 1 cm) in front of them, clearly within the sensory field of the fish. Some of the fish would, however, take a few of the pellets, preferably those that fell directly in front of them or they would move a short distance (less than one body length) to take falling pellets. Later, most fish would move longer distances (more than one body length) and take the majority of the pellets presented to them. They would, however, take only one pellet at a time and always return to their original location in the aquarium between each food item. Some, but not all fish would progress to appearing completely confident in the new environment, moving continuously between food items and consuming all available food. The fish would move through these stages with varying speed of progress. Relapse to an 'earlier' stage was rarely seen and when it occurred the relapse was only temporary.

Based on these observations we determined that feeding behaviour could be rated on a 4 step feeding/boldness scale (Table 2.1). There was individual variation in how quickly feed intake progressed, and the sum of the feeding points obtained by one animal throughout the period was taken as a measure of tendency to feed, or boldness, in a new environment.

Score	Behaviour
0	Fish does not respond to food
1	Fish eats only pellets falling in its immediate vicinity and does not move more than a body length to take food
2	Fish moves more than one body length to take food but returns to original position in aquarium between each food item.
3	Fish moves continuously between food item and consumes all available food

Table 2.1 Criteria for rating the feeding behaviour of individual fish held in isolation in the feeding test.

In the main experiment, a total of 32 fish were paired and matched by weight (weight difference within pair less than 5%) and put separately in neighbouring compartments in the aquaria. Starting 24h after transfer, fish were hand fed daily (1% of body weight, Ewos pelleted trout food) and observed and rated for feeding behaviour according to the described feeding test. Excess food was removed approximately 2 hours after feeding daily. All fish had resumed feeding by day 9.

2.2.3 Stressful social interaction

The main purpose of this project was to examine the effect of social subordination on brain cell proliferatin in the rainbow trout telencephalon. The social stress experiment was conducted according to earlier experiments performed by Winberg et al. and Øverli et al. (Winberg and Nilsson, 1992; Øverli et al., 1998; Øverli et al., 2004b).

After feeding on day 9, the walls between 10 randomly chosen neighbouring compartments were removed, whereupon the pairs of fish were allowed to fight to establish social hierarchies. After removal of walls between adjoining compartments in the aquaria, pairs of fish would after a latency of a few minutes begin to perform aggressive behaviour consisting of violent attacks, biting and circling. These fights for dominance would last for a variable period (0.1-14 minutes), and inevitably end with one fish retiring from further aggression, and thus becoming the subordinate animal. The subordinate fish would seek a position out of the visual field of the dominant animal, typically along the walls of the aquarium. The dominant would continually circle in most of the available space of the aquarium, and with variable time intervals attack, bite and chase the subordinate fish. The social status of all the paired animals was recorded. Fishes were recognized by individual characteristics such as skin pattern, colouring or fin shape. All times were recorded using a stop watch.

After initial establishment of the social hierarchy with one dominant and one subordinate animal the fish were left together for 4 days continuing the daily feeding and rating of feeding behaviour. 12 control fish were left in isolation in separate aquaria throughout the period of the experiment, fed daily and observed for feeding behaviour.

2.2.4. BrdU administration and sampling

24 hours before the end of the experiment (on day 12, 4h before feeding), all fish (controls, dominant and subordinate animals) were rapidly netted from the aquaria, lightly anaesthetized in a bath of 25 mg/L benzocaine and injected with a solution containing 15 mg/mL BrdU in 0.01M PBS to a dose of 100 mg/kg body mass of BrdU. Volume of injected BrdU solution was 6.62 mL/kg body mass, and ranged from 0.56 to 1.23 mL.

On day 13, 1h after feeding, all fish were rapidly netted and quickly anaesthetized in 1 g/L benzocaine. The fish were weighed and approximately 1 mL blood was removed from the caudal vein using a syringe containing EDTA (1.5 mg/mL blood). The blood was centrifuged at 3000 rpm for 3 min, and the plasma was stored at -80°C until quantification of cortisol. The fish were decapitated and the brains were rapidly dissected from the skull, then drop-fixed in 4% paraformaldehyde in 0.01M PBS. The gonads of all fish were investigated for sex determination and to control that the animals were juvenile.

An overview and time-line of the experiment is given in Figure 2.2

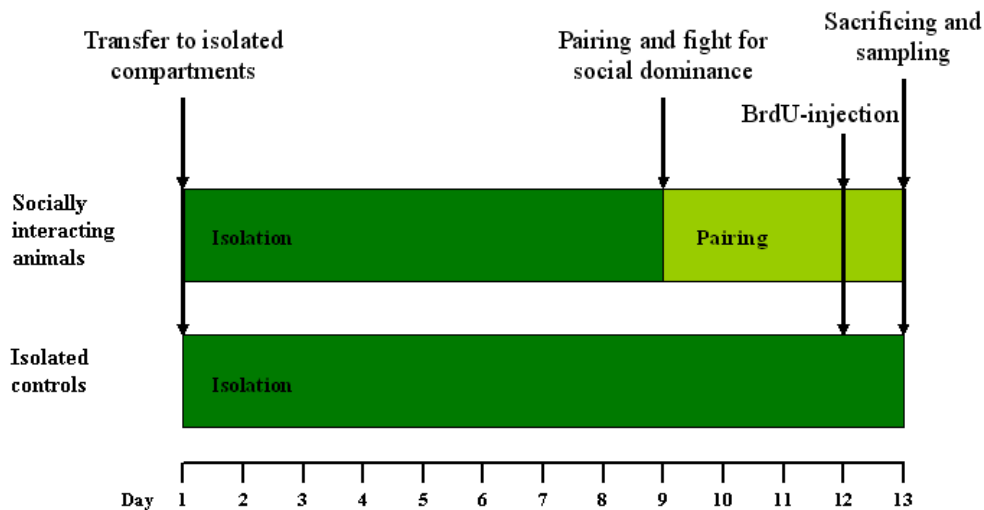


Figure 2.2 Time-line of the experiment.

2.3 The principle of BrdU immunohistochemistry

BrdU-positive cells are visualized immunohistochemically. A primary monoclonal antibody is bound to BrdU (mouse anti-BrdU), whereupon a secondary polyclonal antibody (rabbit anti-mouse IgG) is bound to the primary antibody. An aggregation of horseradish peroxidase (HRP) and mouse anti-HRP IgG (mouse PAP; peroxidase anti-peroxidase) is applied, and is bound by the secondary antibody. The peroxidase activity is utilized to polymerise DAB (3,3'-diaminobenzidine), which leaves a stable, dark brown residue easily recognized in a light microscope (Figure 2.3, 3.4 and 3.5).

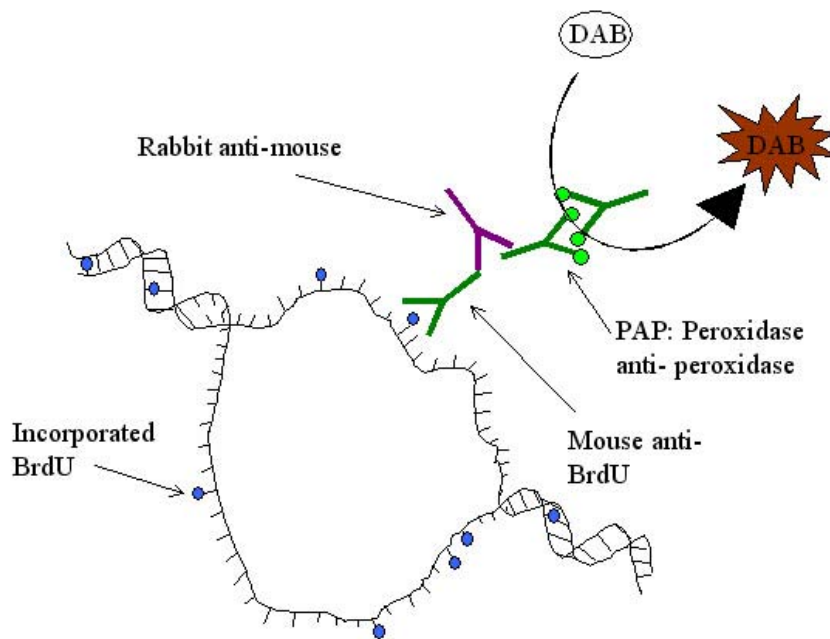


Figure 2.3 The principle for immunohistochemical visualization of BrdU incorporated into DNA. The primary antibody binds BrdU in single-stranded (denatured) DNA. The secondary antibody binds both the primary antibody and the tertiary, which is a complex of aggregated mouse anti-HRP and HRP. The peroxidase activity is used to polymerise DAB to a dark brown residue easily recognized in a light microscope.

2.3.1 Immunohistochemical visualization of BrdU

Before the experiment, a pilot study was conducted to test and optimise the method. It was found that intraperitoneal injection of 100 mg BrdU per kg body weight was sufficient to get a clear staining. The staining protocol given in the following was developed running bioassays to find the optimal conditions for antigen binding. For detailed protocol see Appendix 2.

48h following fixation in 4% paraformaldehyde, the brains were transferred to 30% sucrose in 0.01M PBS for cryoprotection. After another 48h, brains were immersed in Tissue Tek optimal cutting temperature (OCT) compound, and frozen by immersion in isopentane kept at its freezing point (-160 °C) by immersion in liquid nitrogen. The olfactory bulbs and telencephali of frozen brains were sectioned in a cryostat in the transverse plane to a thickness of 35 µm and mounted on SuperFrost Plus slides (Menzel-Gläser). Sections were dried for 12-18h at room temperature and stored at -80°C until use.

Frozen sections were thawed for 20 min at room temperature and incubated in 2.0M HCl for 60 minutes at 37 °C to denature DNA and make the incorporated BrdU more available for antibody binding. They were then incubated in 6% PBT (PBS with BSA and Triton X-100) at room temperature for 60 min. Bovine serum albumin binds to the cellular carbohydrates and blocks interference with antibodies and reduces background binding of antibodies in general. Triton X-100 is a non-denaturing, non-ionic detergent used to solubilize proteins, and thus permeabilizes membranes for easy antibody passage. Sections were then incubated with mouse anti- BrdU IgG (1:400) at 4 °C in a humidified chamber overnight. After incubation with primary antibody, sections were rinsed in PBS and treated with 1.4% H₂O₂ for 15 min to quench endogenous peroxidase activity. The sections were incubated with secondary antibody; polyclonal rabbit anti-mouse IgG (1:50) for 60 min at room temperature whereupon they were rinsed in PBS and incubated with a mouse peroxidase anti- peroxidase (PAP) complex (1:100) at room temperature for 45 min. After rinsing in PBS, a DAB solution was applied and left to work for 5-10 min (optimal staining was determined by watching the staining process in a light microscope). Sections were counterstained in haematoxylin for 45s, dried and coverslipped with DAKO permanent mounting medium.

The time limitations of the master project allowed only for investigation of half of the animals from each group (n = 15). Sections from the gastrointestinal tract were used as positive controls for BrdU incorporation. These were positive in all investigated animals. Two method controls were run; brain sections from fish not injected with BrdU, and sections containing BrdU that were incubated with 0.6% PBT instead of anti-BrdU. Both tests were negative.

2.3.2 Quantification of BrdU-positive cells

Every 9th 35 µm section throughout the telencephalon was investigated using a light microscope (Zeiss Axioplan 2 imaging) and photographed with an AxioCam HR camera (resolution 2600x2060 pixels). The area of each telencephalic section was calculated using Adobe Photoshop 7.0 software. Telencephalic areas were identified according to the rainbow trout telencephalic atlas by Northcutt and Davis (1983) (Appendix 1), and all the BrdU-positive cells within these areas were counted in each analyzed section. To estimate each animal's rate of brain cell proliferation, the total number of BrdU-positive cells counted in all sections from one telencephalon was divided by the total volume of the same sections, giving a relative cell count reflecting the telencephalon's total amount of BrdU-positive cells. This is a good approximation for finding the exact ratio of stained to unstained cells, as the total number of cells in the fish brain in juvenile animals correlates with the absolute volume of the brain (Zupanc and Horschke, 1995). The total number of BrdU-positive cells was also plotted against growth rate to investigate the relationship between whole body growth rate and telencephalic growth rate.

2.4 Radioimmunoassay (RIA) quantification of plasma cortisol

The principle for the competitive RIA is competitive binding of a known amount of a labelled antigen and an unknown amount of unmarked antigen to a fixed amount of antibody (Figure 2.4). The known amount of a labelled antigen, in this case tritiated cortisol, is mixed with a sample containing an unknown amount of the unlabelled antigen (e.g. an endogenous hormone). The fixed amount of a specific antibody with less binding sites than there are corresponding antigen molecules in the solution is added to the mix. The mix is incubated, allowing the labelled and unlabelled antigens to compete for binding with the antibody. After chemical equilibrium is reached, the proportions of labelled to unlabelled antigen both bound to antibody and free in solution will be the same as the original proportion between the two. The free antigens are then removed by binding to dextran coated charcoal, which is spun down in a centrifuge, while the antibody-antigen-complex remains in solution. The amount of labelled antigen in the solution can be measured in a scintillation counter. The count of disintegrations per min (dpm) is inversely proportional to the original concentration of unlabelled antigen in the sample, and the numerical values can be determined running standard solutions with known amounts of unlabelled antigens.

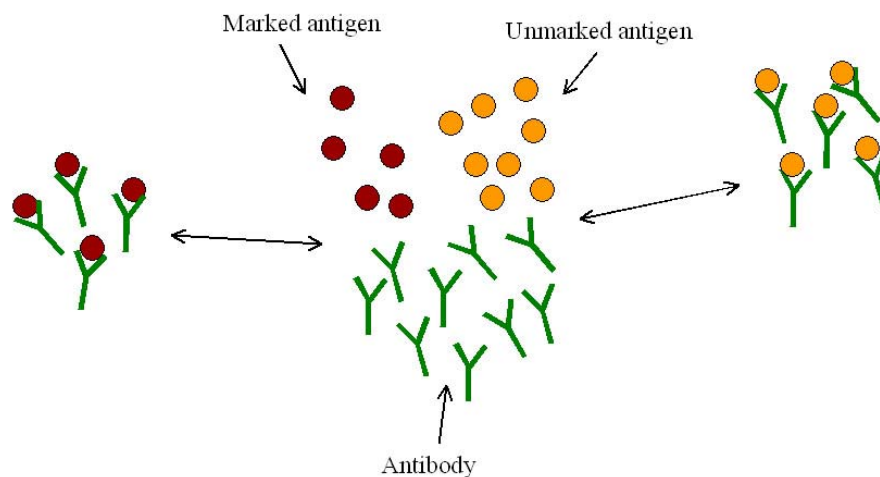


Figure 2.4 The principle of competitive RIA. A fixed amount of marked antigen is added to an unknown sample containing unmarked antigen. These are let to compete for binding to antibodies, whereupon all free antigens are removed from the solution. The amount of marked antigen bound to antibodies can be quantified and is inversely proportional to the initial amount of unmarked antibody in the sample.

Levels of cortisol in plasma were analyzed using a RIA protocol previously validated by Pickering et al. (Pickering et al., 1987). A detailed protocol for the RIA procedure is provided in Appendix 3. Frozen plasma samples were thawed slowly at 4°C to avoid clotting. For steroid extraction, ethyl acetate amounting to five times the volume of plasma was added, tubes were vortexed and centrifuged briefly. Extracted samples were stored at -20 °C until use.

Ethyl acetate containing extracted steroids was pipetted into tubes; if the unknown sample was expected to contain much cortisol (stressed fish), 20 µL was used, in the opposite case (unstressed fish), 150 µL was used. 50 µL of tritiated cortisol in ethyl acetate (previously determined to a count of approximately 20000 dpm per 50 µL) was added to each sample, and to control samples containing known amounts of hydrocortisone in ethyl acetate. Samples were dried in a vacuum exsiccator coupled to a water jet pump. Residues were dissolved in 100 µL assay buffer (0.1% BSA in PBS) and 100 µL rabbit anti-cortisol (1:10) was added. Tubes were vortexed and incubated for 1 h at room temperature, 2 h at 4 °C and finally for 15 min on ice. 100 µL of dextran coated charcoal (0.5% charcoal and 0.1% dextran in PBS) was added to all tubes before vortexing and incubation on ice for 5 min. Charcoal was spun down (3500 rpm, 4 °C, 10 min) and 200 µL of supernatant was pipetted into scintillation cocktail, mixed by inversion and analyzed in a Packard Tri-Carb Liquid Scintillation Analyzer under standard tritium conditions.

2.5 Statistical analyses

All statistical analyses were performed in STATISTICA for Windows (StatSoft, Inc., Tulsa, Oklahoma). Data on weight, plasma cortisol, and BrdU immunostaining fulfilled the criteria for parametric statistics, and were compared between groups by one-way ANOVA followed by the Tukey post-hoc test. Correlations between BrdU immunostaining and two variables, growth rate and plasma cortisol, were analysed by Pearson statistics. Homogeneity of variance and Gaussian distribution were confirmed by Levene's test and the Kolmogorov-Smirnov method, respectively.

For feeding score data, parametric ANOVA was used to compare sums of scores on day 1-9 and 10-13 between groups. To confirm the effect of the injection procedure on feeding behavior within each group, scores on the day of injection were compared to those on the previous day by non-parametric Kruskal-Wallis ANOVA followed by repeated Mann-Whitney U-tests, with Bonferroni adjustment for multiple corrections. This procedure was chosen since scores on a single day amounted to a discrete variable with only 4 possible outcomes (0, 1, 2, or 3), which renders parametric statistics inappropriate even if homogeneity of variance or normality criteria are not violated (Zar, 1999).

3 Results

3.1 Effects of social status on behaviour and cortisol

The daily frequency of fish receiving each feeding score in all of the groups is given in Figure 3.1. Total feeding score obtained on day 1-9 (isolated period) and 10-13 (interaction in pairs) were compared statistically. During the first 9 days, there was no significant difference in the mean feeding scores between the fish that were to become dominant, subordinate and controls (ANOVA; $F_{(2,29)} = 1.50$, $P=0.14$) (Figure 3.2A) There was, however, a tendency of future subordinates scoring less than future dominants, with the controls being intermediate between the other two groups.

Upon removal of walls between adjoining compartments on day 9, clear dominance-subordination relationships were observed in all pairs and in no instance did the relationship change during the experiment. In 7 out of 10 pairs, one of the fish had started feeding at an earlier point than its partner, and in all of these 7 instances, the fish that had started feeding first also became the dominant animal.

During day 10-13, when the fish were paired, subordinate fish scored significantly lower on the feeding test than both dominants and controls (ANOVA; $F_{(2,29)} = 10.57$, $P<0.001$, See Table 1, Appendix 4 for Tukey Post-hoc statistics). There was no difference between the control group and the dominant fish (Figure 3.2B).

A drop in feeding scores was observed after the BrdU-injection on day 12 (Figure 3.1). This was analyzed using Kruskal-Wallis ANOVA by ranks followed by repeated Mann-Whitney U-tests directly comparing the score on day 11 and day 12 within each group. The drop was significant (Kruskal-Wallis ANOVA; $P < 0.001$) in both the control group ($P = 0.003$), and in the dominant group ($P = 0.002$). It was not significant in the subordinate group ($P = 0.74$), though this is not surprising, since the feeding scores in this group were already rather low. For this test a Bonferroni adjustment for multiple comparisons was made, giving an adjusted alpha of 0.009).

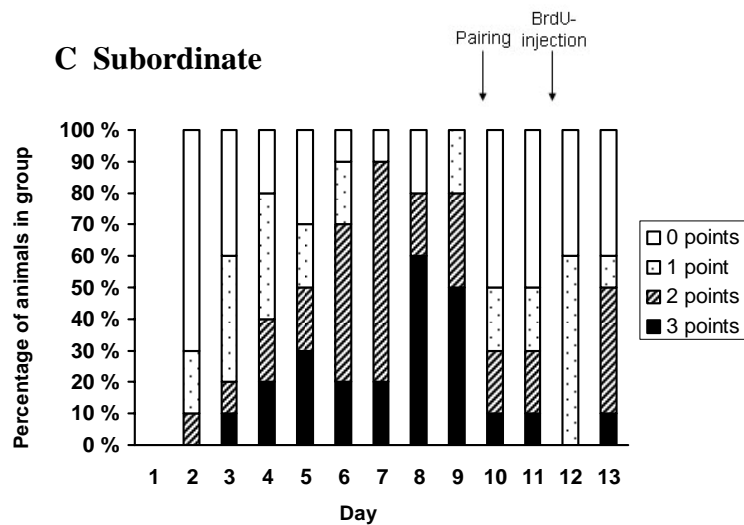
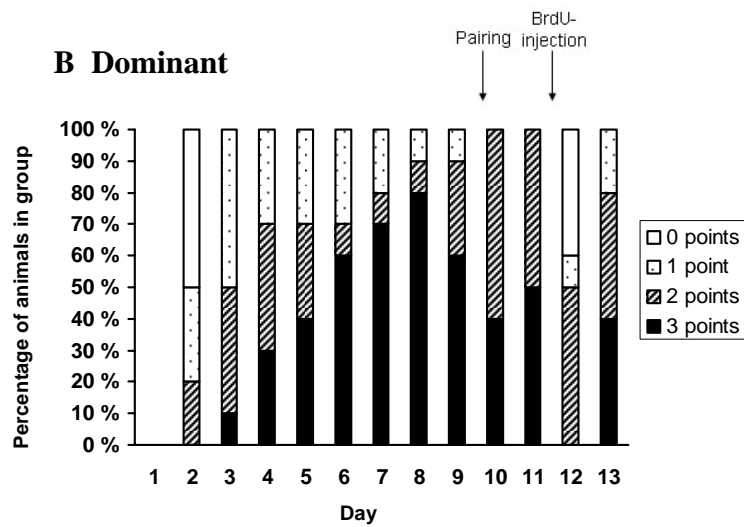
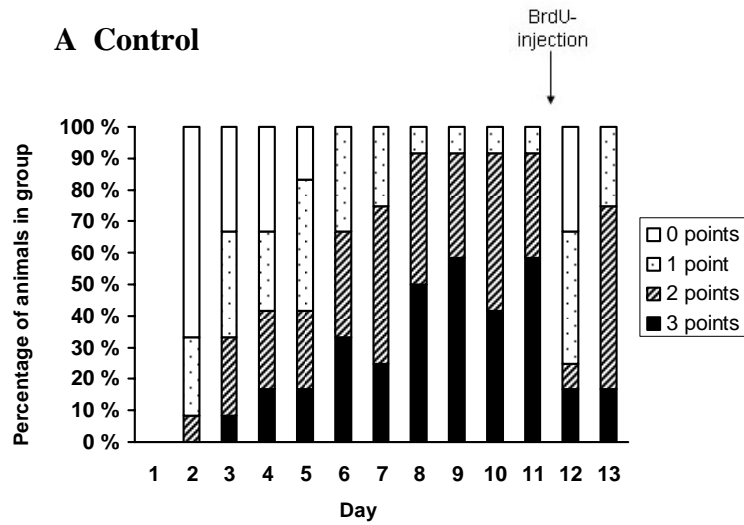
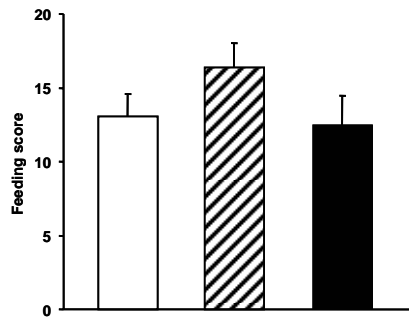


Figure 3.1 Frequency of animals that received each score on each day for controls (**A**) ($n = 12$), dominants (**B**) ($n = 10$) and subordinates (**C**) ($n = 10$).

A Feeding day 1-9



B Feeding day 10-13

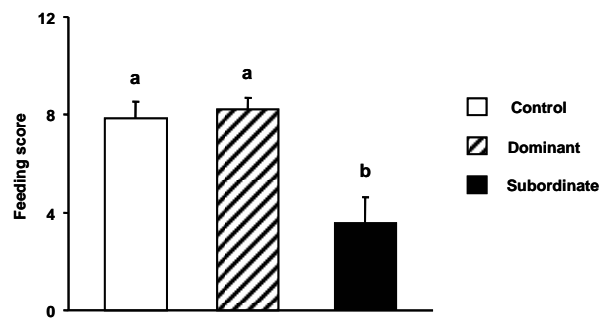
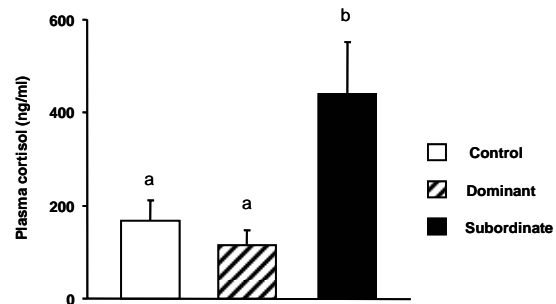


Figure 3.2 Total feeding scores (Mean + S.E.M.) (A) Feeding scores of isolated rainbow trout obtained during day 1-9 of the experiment. Bars are grouped according to later social status. ANOVA revealed no significant difference between groups ($P = 0.14$). (B) Feeding scores of socially interacting rainbow trout and isolated controls obtained during day 10-13 of the experiment. Different letters indicate a statistically significant difference between groups ($P < 0.05$, ANOVA followed by Tukey post-hoc tests). See text for ANOVA statistics and significance levels.

Mean plasma cortisol levels for all groups are shown in Figure 3.3. Subordinate animals shows a marked elevation in plasma cortisol compared to both controls and dominants (ANOVA: $F_{(2,29)} = 6.00$, $P = 0.007$, See Table 2, Appendix 4 for Tukey Post-hoc statistics).

Figure 3.3 Social modulation of plasma cortisol levels in juvenile rainbow trout. Group means + S.E.M. are graphed. Different letters indicate a statistically significant difference between groups ($p < 0.05$, ANOVA followed by Tukey post-hoc tests). See text for ANOVA statistics and significance levels.



3.2 BrdU immunohistochemistry

In 15 fish, 5 from each group (control, subordinate and dominant) sections of the telencephalon were stained for BrdU immunohistochemistry. BrdU-positive cells were easily recognized and identified by dense homogenous staining of their nuclei (Figure 3.4). Immunopositive cells were found sparsely scattered in all subdivisions of the telencephalon, but with aggregations in distinct proliferative zones. The



Figure 3.4 BrdU-positive cells

most rostral proliferation zone, PZ1b is located in the ependyma of the internal cellular layer (ICL) of the most caudal part of the olfactory bulbs. This zone extends caudally into the most rostral part of the ventral telencephalon (Vd and Vv) (Figure 3.5A,a). At this point, the most rostral part of PZ2 is located in the dorsal nuclei of V (Vd). PZ2 extends caudally through Vd approximately to the anterior commissure (Figure 3.5B-C,c,e and 3.6D,g), and to a lesser degree further caudally (3.6E,j). PZ1a extends over the whole surface of D and in the lateral V, with aggregations in lateral and medial D (Dl and Dm) (Figure 3.5A-C,b,d,f and 3.6D-F,h,i,l).

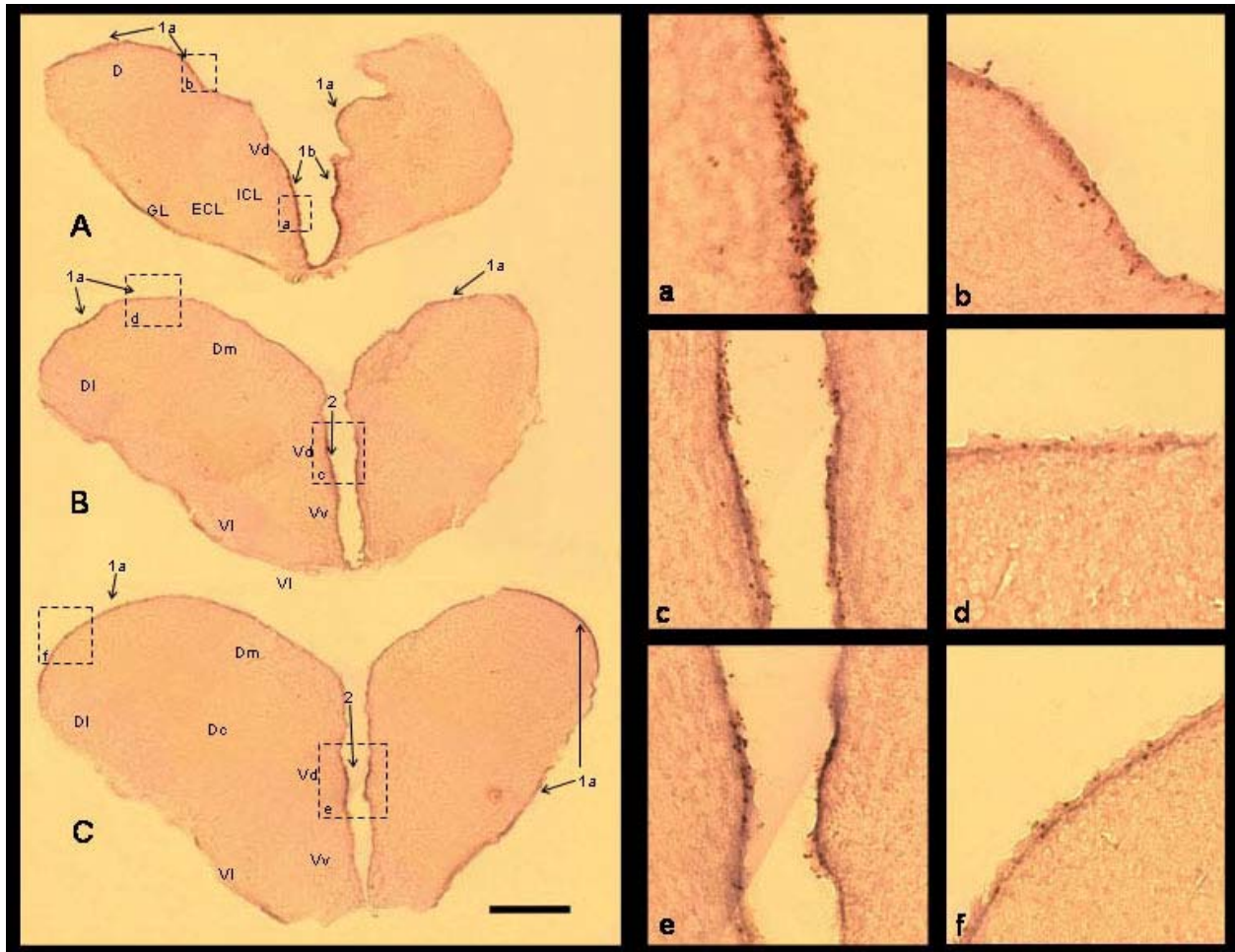


Figure 3.5 Transverse sections of the anterior part of the telencephalon of a subordinate rainbow trout, every 9th 35 μ m section. BrdU stained cells are visible in magnified sections. Proliferation zones are identified by Arabic numerals. For abbreviations see list. **PZ1a (A,B,C,b,d,f)** extends over the whole surface of D and in the lateral V, with aggregations in DI and Dm. **PZ1b (A,a)** is located in the ependyma of the ICL of the most caudal part of the olfactory bulbs. This zone extends caudally into the most rostral part of the ventral telencephalon (Vd and Vv). **PZ2 (B,C,c,e)** is located in the dorsal nuclei of V (Vd), extending caudally through Vd. Scale bar = 500 μ m.

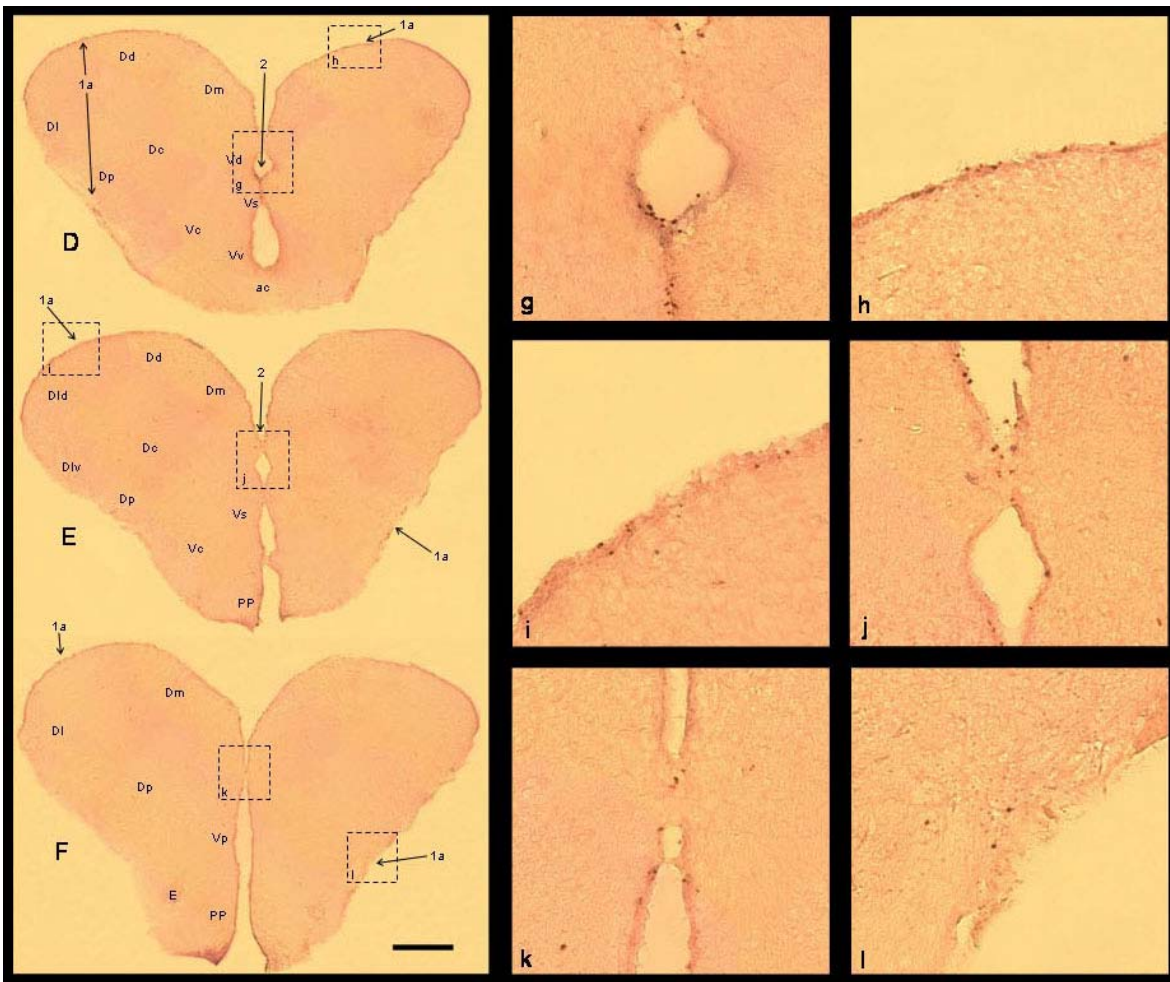


Figure 3.6 Transverse sections of the anterior part of the telencephalon of a subordinate rainbow trout. Proliferation zones are identified by Arabic numerals. For abbreviations see list. **PZ1a (D,E,F,h,i,l)** extends over the whole surface of D and in the lateral V, with aggregations in Dl and Dm. **PZ2 (D,E,g,j)** is located in the dorsal nuclei of V (Vd), extending caudally through Vd approximately to the anterior commissure, and to a lesser degree further caudally. Scale bar = 500 μ m.

Abbreviations for Figures 3.4 and 3.5

D	area dorsalis telencephali	ECL	external cellular layer of olfactory bulb
Dc	central zone of D	GL	glomerular layer of olfactory bulb
Dd	dorsal zone of D	ICL	internal cellular layer of olfactory bulb
Dl	lateral zone of D	PP	periventricular preoptic nucleus
Dld	dorsal part of Dl	V	area ventralis telencephali
Dlv	ventral part of Dl	Vc	commissural nucleus of V
Dm	medial zone of D	Vd	dorsal nucleus of V
Dp	posterior zone of D	Vl	lateral nucleus of V
E	nucleus entopeduncularis	Vv	ventral nucleus of V

3.3 Effect of social status on telencephalic cell proliferation

Quantification of BrdU immunostaining confirmed that social status affected the number of BrdU-positive cells in the telencephalon. Specifically, there was a significant difference in telencephalic cell proliferation between the subordinate fish and the control group. The dominant group was not significantly different from any other group (ANOVA: $F_{(2,12)} = 5.78$, $P = 0.017$, See Table 3, Appendix 4 for Tukey post-hoc statistics) (Figure 3.7).

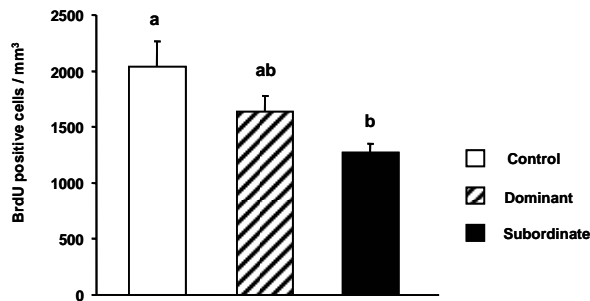


Figure 3.7 Social modulation of brain cell proliferation in juvenile rainbow trout. Values are means + S.E.M. Different letters indicate a statistically significant difference between groups ($P < 0.05$, ANOVA followed by Tukey post-hoc tests, see text for results).

3.4 Cortisol and telencephalic cell proliferation

Telencephalic brain cell counts were plotted against plasma cortisol values to elucidate an eventual effect of cortisol levels on proliferative rate. There was no significant correlation found in any of the groups (Control: Pearson $R^2 = 0.002$, $P = 0.94$; Dominant: Pearson: $R^2 = 0.002$, $P = 0.94$; Subordinate: Pearson: $R^2 = 0.002$, $P = 0.94$).

3.5 General growth rate and telencephalic cell proliferation

To investigate whether the reduction of proliferation in subordinates was due to a whole body down-regulation of cell proliferation and not a specific effect on neurogenesis, growth rate was investigated in all the groups (Table 3.1). There was no significant difference between the groups in either start weight, end weight or growth rate (ANOVA: Start weight; $F_{(2,29)} = 0.78$, $P = 0.47$; End weight; $F_{(2,29)} = 1.22$, $P = 0.31$; Growth rate; $F_{(2,29)} = 0.52$, $P = 0.60$)

Group	Start weight	End weight	Gr. rate
Control	116.9 ± 7.7	133.1 ± 7.9	1.13 ± 0.22
Dominant	132.3 ± 11.3	155.4 ± 12.9	1.38 ± 0.28
Subordinate	130.8 ± 10.8	146.7 ± 10.4	1.03 ± 0.24

Table 3.1 Weight and growth rate given as mean ± S.E.M. See text for ANOVA statistics.

Growth rate was also plotted against cell counts in all groups. There was no significant correlation found in either group (Control: Pearson $R^2 = 0.45$, $P = 0.21$; Dominant: Pearson: $R^2 = 0.003$, $P = 0.93$; Subordinate: Pearson: $R^2 = 0.003$, $P = 0.93$). Among those controls that showed a positive growth rate, however, there seemed to be a linear relationship between growth rate and brain cell proliferation (Figure 3.8, note that the correlation was only significant if one animal with negative growth rate was left out). The low n (4), presides that firm conclusions should not be drawn about whether variation in brain cell proliferation is a reflection of whole body growth rate in isolated fish.

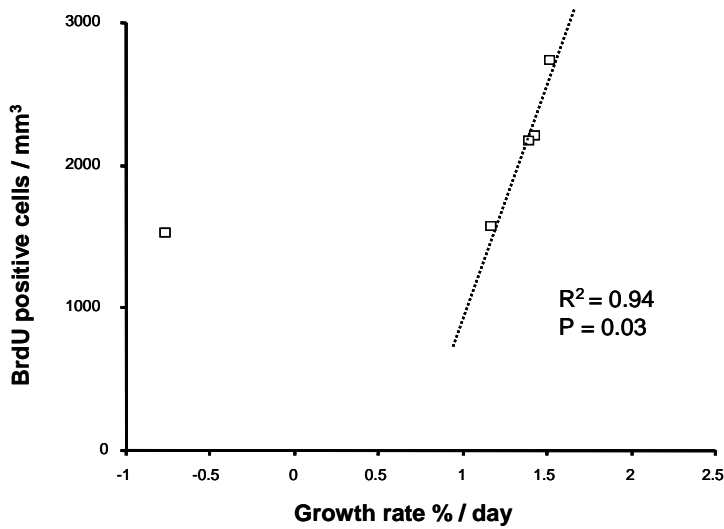


Figure 3.8 Relationship between growth rate and brain cell proliferation in those isolated controls that showed positive growth rates during the experiment. Cell count in one control fish with negative growth is shown for comparison. Pearson correlation statistics are shown.

4 Discussion

4.1 Methodological considerations

The BrdU method was chosen for its widespread use in studies of neurogenesis in mammals (Gould et al., 1999b; Fuchs and Gould, 2000), and because it has been used in most recent studies of brain cell proliferation in teleosts (Zupanc and Horschke, 1995; Zikopoulos et al., 2000; Ekstrom et al., 2001; Mueller and Wullimann, 2002).

It did appear that the intraperitoneal injection of BrdU was stressful to the animals, since a significant drop in the feeding scores of dominant and control animals was seen after the injection. The introduction of an additional stressor may have upset the behaviour and physiology of the experimental animals in a number of different ways. Since the injection affected the feeding score of both dominants and subordinates (Figure 3.1) it is very likely that it also, at least acutely, affected the levels of circulating cortisol. This taken together with the finding that acute stress influences neurogenesis in mammals (Heine et al., 2004), indicates that it is not unlikely that the injection may have had an effect on the rate of brain cell proliferation. Though mammals might be trained to tolerate injections of this kind without eliciting a stress response, this can probably not be done with teleosts. It is interesting to note that the stressor used by Veenema et al. to demonstrate differential effects on neurogenesis in different strains of rats was 3 daily intraperitoneal BrdU-injections. This stress regime resulted in a 50% reduction in neurogenesis as demonstrated by Ki-67 immunoreactivity in one of the strains (Veenema et al., 2004).

To circumvent the stressfulness of the BrdU-injection in fish, alternative methods of administration should be utilized. Cortisol (Øverli et al., 2002) has successfully been administered to fish through the food, and this could probably also be done with BrdU. Stress-induced anorexia could, however, be a problem with administering BrdU to stressed fish. A few studies have been performed by immersion of fish in baths of BrdU-solution (Brennan et al., 2001; Byrd and Brunjes, 2001). This is very suitable for small fish, but the amounts of BrdU solution needed for larger fish and the fact that BrdU is

carcinogenic and should not be released into the environment renders it an unsuitable method in most cases. BrdU-administration through intraperitoneal catheters or dorsal aorta cannulas (Sunde et al., 2003) is also an option, but this can not be performed in studies including social interaction as fighting would upset or tangle the catheters.

There are also other objections to the BrdU method. BrdU is not an internal marker, and very little is known about its uptake and movement within the body. The possibility thus exists that exposure of the animal to different treatments not only will affect the rate of proliferation, but also the availability of BrdU to different brain structures (Gould and Gross, 2002; Rakic, 2002). It is a well-known fact that blood flow in general and cerebral blood flow in particular is tightly regulated and very variable in mammals. For instance, the adrenergic response in stress causes both vasodilation and vasoconstriction in different parts of the body, and the haemodynamic response, which involves mechanisms for increased blood flow to areas of increased activity, is well documented (Attwell and Iadecola, 2002). Less is known about this phenomenon in fish, but it has been shown that the rate of cerebral blood flow is influenced by several physiological and paracrine factors including anoxia, hypercapnia, adenosine, nitric oxide and endothelin (Nilsson et al., 1994; Nilsson and Soderstrom, 1997; Soderstrom and Nilsson, 2000; Rodland and Nilsson, 2002). What is completely unknown, however, is the mechanisms of BrdU-uptake into the blood flow and further into the brain. There is thus a possibility that the treatment of experimental animals may affect factors governing the distribution of BrdU in the body, such as blood flow and blood-brain barrier permeability, resulting in changes in number of BrdU-stained cells on the basis of other factors than cell proliferation (Gould and Gross, 2002).

Another problem is that since BrdU stays in the cell permanently, the number of stained cells will, depending on the survival time after BrdU administration, reflect both rate of cell proliferation at the time of administration, subsequent rate of cell proliferation (as stem cells will continue to give rise to new cells with a diminishing amount of BrdU in their DNA for a limited time period after administration), and cell survival. This can be an advantage when investigating cell fate, but for studies focusing on proliferation, this should be taken into consideration both when determining survival time after administration, and when interpreting data.

To circumvent the problems regarding the BrdU method, it is advisable to utilize endogenous markers of cell proliferation either alone or together with BrdU to validate the use of BrdU. Proteins that are upregulated during mitosis, such as PCNA or Ki-67, are good candidates for this method, and are common in use (Wullimann and Puelles, 1999; Ekstrom et al., 2001; Mueller and Wullimann, 2003).

4.2 Effects of social status on brain cell proliferation

Though proliferation in the teleost brain has previously been mapped (Zupanc and Horschke, 1995; Zikopoulos et al., 2000; Ekstrom et al., 2001) and quantified (Zupanc and Horschke, 1995), this is the first study to document effects by external stimuli on the rate of brain cell proliferation in fish. Despite the earlier discussed methodological problems, it was found that socially subordinate rainbow trout had almost 40 % fewer BrdU-positive cells in the same analyzed volume from the telencephalon than isolated controls. This finding is as expected, and in accordance with previous studies of social stress in mammals, where the number of BrdU-stained hippocampal cells are significantly reduced after acute or chronic psychosocial stress (Gould et al., 1997; Gould et al., 1998; Kozorovitskiy and Gould, 2004). The dominant fish, however, were not found to have significant differences in numbers of BrdU-positive cells from neither subordinates nor controls.

It is therefore clear that there is an effect of social subordination on the number of telencephalic BrdU-positive cells. The question thus arises whether this is an effect of being in the social hierarchy, or whether it is an innate quality of individuals that end up becoming socially dominant or subordinate. Individual variations in stress coping styles, the strategy of coping with stressful situations (Koolhaas et al., 1999), seem to be predictive of whether an animal becomes subordinate or dominant in social hierarchies. Øverli et al. showed that fish with higher speed of reversal of stress-induced anorexia in 9 out of 10 cases became dominant in social hierarchies (Øverli et al., 2004b). In the current study, in all cases where one of the fishes in a pair started feeding before the other (7 out of 10), this individual later became the dominant of the pair. Furthermore, in a

rainbow trout breeding program where two lines of fish with divergent cortisol responsiveness (high responding, HR, showing high cortisol levels and low responding, LR, showing low cortisol levels when subjected to a standardized stressor) (Pottinger and Carrick, 1999), the LR fish showed a strong tendency of becoming dominant over HR individuals in staged fights for social dominance between pairs (Pottinger and Carrick, 2001). This indicates that social status is highly dependent on innate traits, and hence there is a possibility that the lower BrdU-staining in subordinate animals might be due to an original difference between dominant and subordinate animals. The feeding and cortisol data do, however, show that the subordinate animals are stressed, something the dominant animals do not appear to be, so the reduced amount of BrdU-positive cells in the subordinate animals could be due to stress, to innate traits or to a combination of both.

The innate trait hypothesis predicts that later dominant animals should have higher feeding scores than later subordinate animals during the first 9 days. It also predicts that the feeding scores of the controls should be intermediate between the dominants' and the subordinates'. There are, however, no significant differences between the groups, but the mean score of the dominant group is in absolute numbers higher than the mean score of subordinates with controls intermediate (Figure 3.2A). It should also be mentioned that when comparing only the dominant and subordinate groups, a one-tailed t-test shows a significant difference between them, indicating that there could be innate differences between dominant and subordinate fish.

If differences in cell proliferation preceded social interaction in pairs, control animals should therefore be intermediate between dominants and subordinates in BrdU-positive cell counts. This is not the case, as the controls have significantly higher counts than the subordinates and show a strong tendency towards having higher counts than dominants (Figure 3.7). This suggests a reducing effect of the social interaction on the amount of BrdU-positive cell, particularly in subordinate animals. The subordinate animals also show behavioural and hormonal stress responses, something the other groups do not, and as their cell counts are lower than both the other groups, it is reasonable to conclude that despite the possibility of innate differences between groups, social subordination seems to be the main factor involved in reducing the amount of telencephalic BrdU-positive cells.

Very few studies have focused on the cell proliferation of the dominant animals in social hierarchies, but Kozorvitskiy and Gould (2004) found dominant rats in a visual burrow system (VBS) to have higher numbers of hippocampal BrdU-positive cells than both control and subordinate animals. This was, however, after a survival time of 14 days compared to this experiment's 24 h, and the socially interacting animals were housed in enriched environments compared to the controls, which is known to increase neurogenesis (Kempermann et al., 1997). Therefore it is inconvenient to compare these data with the current experiment. Possible reasons for the tendency of reduced proliferation in dominant animals will be discussed in section 4.3.

To study the possibility of individual differences in cell proliferation further, it would be of interest to investigate the basal count of telencephalic BrdU-positive cells in a larger group of unstressed animals, and look for correlations with hormonal and behavioural data. It would also be of interest to do the same kind of investigation in both unstressed and stressed animals of the LR and HR lines of the fish selected for cortisol responsiveness. In mice similarly selected for different stress coping styles (short attack latency, SAL, corresponding with LR fish, and long attack latency, LAL, corresponding with HR fish), the LAL mice had a slightly, but significantly lower basal hippocampal cell proliferation rate than the SAL mice, and a reduction of almost 50% under stressful conditions (Veenema et al., 2004).

4.3 Possible mechanisms for reduction of telencephalic brain cell proliferation

Stress and elevated cortisol levels have been found to reduce food intake and growth rate in fish (McCormick et al., 1998; Gregory and Wood, 1999; Bernier and Peter, 2001). There is a possibility that the reduced amount of BrdU-positive cells in the subordinate animals can be a result of a whole body down-regulation of growth rate and not a specific effect on brain cell proliferation. Even though in this experiment subordinates did not stop eating altogether (Figure 3.2B), they showed a drastic reduction in feeding. There is still no significant difference in growth rate between the groups (Table 3.1), and there is no significant correlation between growth rate and BrdU-positive cell count. In our control group there appears to be a correlation between growth rate and cell counts of animals with positive growth rates (Figure 3.8), but there are too few observations to draw any conclusions about the relationship between basal levels of BrdU-staining and whole body growth rate.

Øverli et al. showed that subordinate fish had a higher growth rate than dominant fish when reared in isolation prior to social interaction in pairs, despite that they had lower food intake than dominants (Øverli et al., 2004b). In addition it has been found that subordinate animals have a lower growth rate than dominants while interacting socially (Abbott and Dill, 1989; Winberg et al., 1992). In this experiment the fishes were not weighted at the day of pairing, to avoid stressful disturbances of the animals. Thus, there can have been opposing differences in growth rate of the subordinate animals before and after the pairing that have cancelled each other out. Hence the reduction in brain cell proliferation found in the subordinate animals can be a reflection of a whole body down-regulation in growth rate. It is, however, of little importance for the possible cognitive consequences of the reduction in telencephalic cell proliferation whether the change was brought about by a whole body down-regulation of cell proliferation, or a specific effect on the brain.

Concerning the mechanisms of reduction in adult mammalian neurogenesis, the HPA axis is central. As mentioned earlier, increased levels of glucocorticoids reduce the rate of hippocampal neurogenesis (Gould et al., 2000), and the hippocampus has a

particularly high density of glucocorticoid receptors (Sapolsky et al., 1986). The neuronal stem cells do not appear to express these receptors, though (Gould et al., 2000), indicating that the glucocorticoid regulation of neurogenesis occurs via messengers from other hippocampal, glucocorticoid sensitive cells. A candidate for this kind of messenger is brain-derived neurotrophic factor (BDNF). BDNF mRNA levels of the hippocampus (and other brain structures) are indeed reduced in response to chronic stress and increased glucocorticoid levels (Smith et al., 1995; Tapia-Arancibia et al., 2004). There is also a close correlation between other factors that alter BDNF mRNA and those modifying neurogenesis (Falkenberg et al., 1992; Ickes et al., 2000; Marmigere et al., 2003). Chronic SSRI treatment, for instance, increases BDNF mRNA levels as well as neurogenesis (Tapia-Arancibia et al., 2004), indicating that BDNF may be one of the main mediators of neurogenesis regulation. A teleost BDNF analogue has been identified (Hashimoto and Heinrich, 1997), and the reduction of brain cell proliferation in the subordinates of the current study may thus be due to a cortisol-induced reduction in BDNF levels in the telencephalon.

The measured cortisol levels can not explain the tendency of reduction in telencephalic cell proliferation in the dominant animals. BDNF levels are, however, regulated by other factors besides glucocorticoids, including glutamate, acetylcholine, serotonin and GABA (Tapia-Arancibia et al., 2004), though it is not evident which of these may be involved in dominant animals. The measured cortisol levels are also only instantaneous values from the moment the fishes were sacrificed, and a low cortisol level at the moment of sampling does not rule out the possibility that the dominant animals can have had elevated cortisol levels at earlier times during the experiment. In particular, the BrdU injection can have been more stressful to the dominant animals than the controls due to their being in a social hierarchy. These considerations are, however, only speculations and the apparent reduction was not significant, so it may be a random effect related to low n. Further studies should therefore be carried out shed light over the findings of this study.

4.4 Conclusions

In conclusion, this study indicates that the BrdU method applying intraperitoneal administration is stressful to the animals and thus inappropriate for studying effects of social status on brain cell proliferation in fish. The use of alternative methods for administration or endogenous markers for proliferation is suggested for future experiments.

Further, social subordination in rainbow trout causes a significant reduction of cell proliferation in the telencephalon. This reduced proliferation may in part be a result of innate differences between animals that become dominant and subordinate in social hierarchies. The increased stress of the subordination is, however, likely to be the main cause of the drop in proliferation, and the increased plasma cortisol levels found in these animals is suggested to mediate the effect of the stress on proliferation.

The dominant position of social hierarchies is not associated with a significant reduction in telencephalic cell proliferation, but there was a clear tendency for this. As the dominants did not have increased plasma cortisol levels at the time of sampling, other mechanisms could be involved in causing this tendency. Further studies are, however needed to confirm and elucidate these relationships as well as details regarding the drop in proliferation seen in subordinates.

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Appendix 1 – Telencephalic organization in rainbow trout

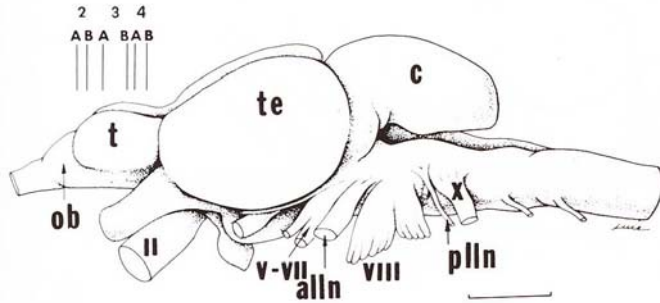
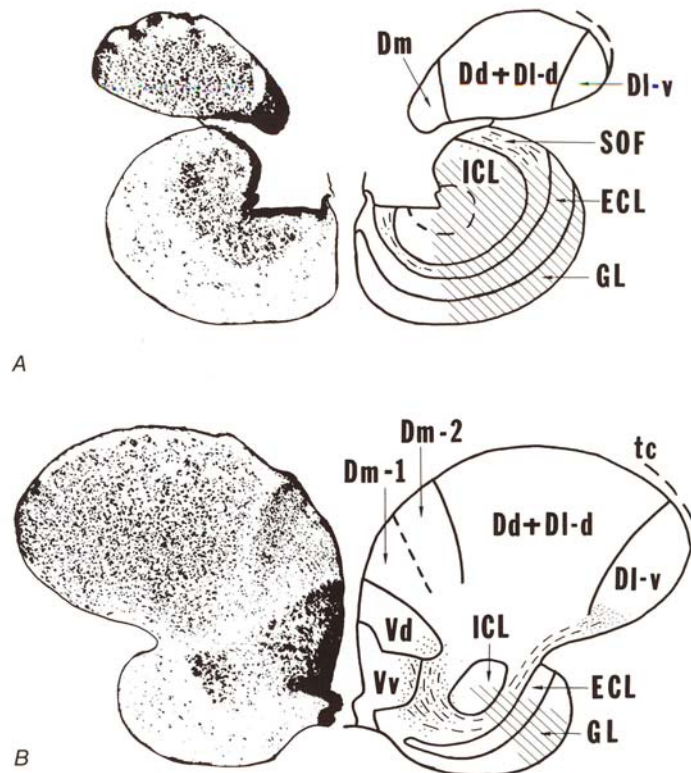


Figure 1 Lateral view of the brain of the rainbow trout (*Oncorhynchus mykiss*). Numbered lines indicate the levels of the transverse sections in figures 2-3. From Northcutt and Davis, 1983.

Figure 2 Transverse sections through the caudal olfactory bulb (A) and rostral telencephalon (B) in rainbow trout. From Northcutt and Davis, 1983.



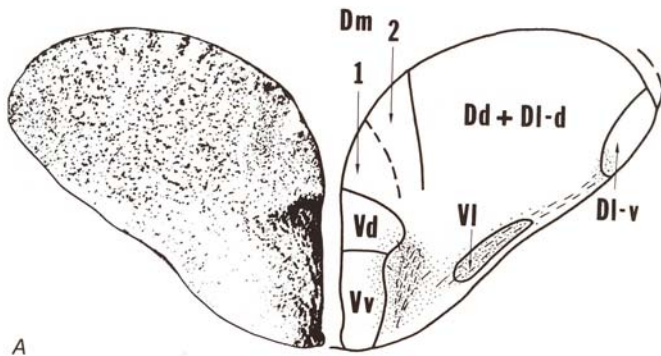


Figure 3 Transverse sections through the maximal extent of the rostral subpallial areas (A) and anterior commissure level (B) in rainbow trout. From Northcutt and Davis, 1983.

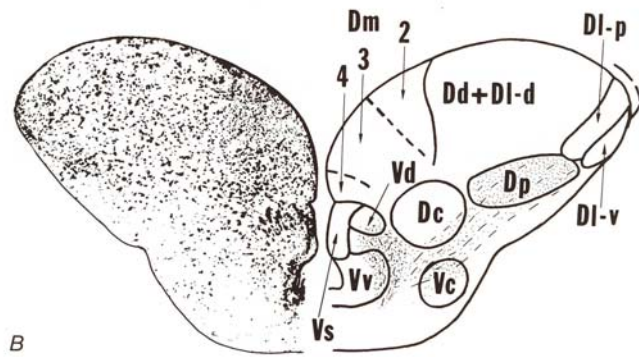
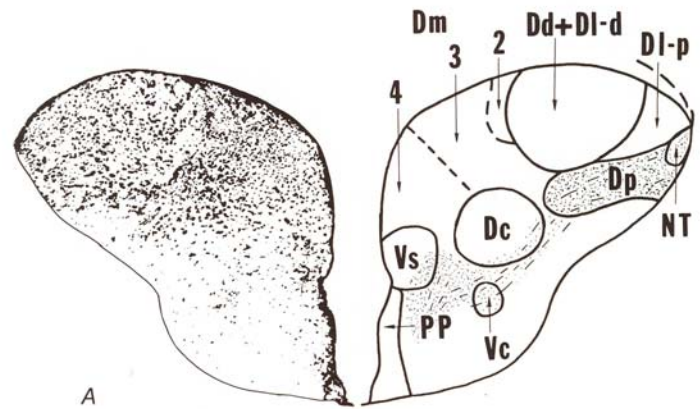
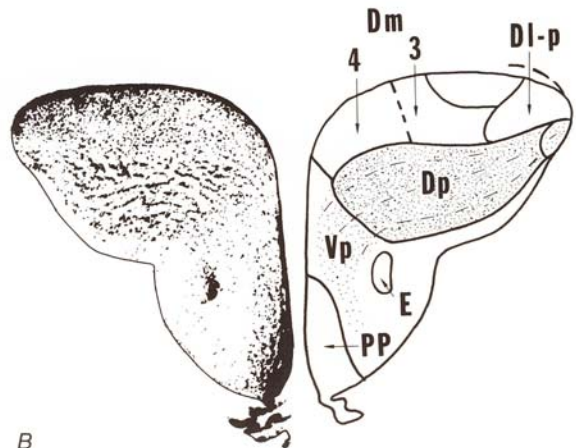


Figure 4 Transverse sections through the caudal third (A) and caudal pole (B) of the telencephalon in rainbow trout. From Northcutt and Davis, 1983.



A



B

Abbreviations for figures

c	cerebellum
D	area dorsalis telencephali
Dc	central zone of D
Dc-1,2,3,4	parts of Dc
Dc	dorsal zone of D
Dd+Dl-d	dorsal zone plus dorsal part of lateral zone of D
Dl	lateral zone of D
Dl-d	dorsal part of lateral zone of D
Dl-p	posterior part of lateral zone of D
Dl-v	ventral part of lateral zone of D
Dm	medial zone of D
Dp	posterior zone of D
E	entopeduncular nucleus
ELC	external cellular layer of olfactory bulb
GL	glomerular layer of olfactory bulb
ICL	internal cellular layer of olfactory bulb
ob	olfactory bulb
PP	periventricular preoptic nucleus
SOF	secondary olfactory fiber layer of the olfactory bulb
t	telencephalon
te	tectum
V	area ventralis telencephali
Vc	commissural nucleus of V
Vd	dorsal nucleus of V
Vl	lateral nucleus of V
Vp	postcommissural nucleus of V
Vs	supracommissural nucleus of V
Vv	ventral nucleus of V
VT	ventral thalamus

Appendix 2 - Protocol for BrdU staining

Procedure

Day 1

1. Thaw frozen slides until dry (15-20 min) in room temperature
2. Draw around sections with PAP pen (DAKO) (in fume hood), allow to dry
3. 2.0 M HCl at 37 °C for 60 min
4. 2 x 5 min rinse in PBS
5. 6% PBT for 60 min at room temp in humidified chamber
6. Anti-BrdU (1:400) in 0.6 % PBT over night at 4 °C in humidified chamber

Day 2

7. 2 x 5 min rinse in PBS
8. 1.4 % H₂O₂ at room temp for 15 min
9. 2 x 5 min rinse in PBS
10. Rabbit anti-mouse (1:50) in 0.6% PBT for 60 min
11. 2 x 5 min rinse in PBS
12. PAP (1:100) in 0.6% PBT for 45 min
13. 2 x 5 min rinse in PBS
14. DAB for 5-10 min at room temp (in fume hood)
15. 2 x 5 min rinse in dH₂O
16. Counter-staining with haematoxylin for 45 s
17. 5 min rinse in running tap water
18. Coverslipping (in fume hood) with DAKO permanent mounting medium

Reagents

0.01 M PBS (Phosphate buffered saline)

Dissolve 1 PBS-tablet (Sigma) per 200 mL distilled water. Make up 5 L at a time (25 tablets) and keep in can on lab bench.

6% PBT (Phosphate buffered saline with Triton X-100 and BSA)

10 mL	PBS
3 μ L	Triton X-100 (Sigma)
600 mg	BSA (Sigma)

Vortex until the BSA is dissolved. Can be stored at 4 °C for 7 days.

0.6 % PBT

25 mL	PBS
7.5 μ L	Triton X-100
150 mg	BSA

Vortex until the BSA is dissolved. Can be stored at 4 °C for 7 days.

1.4 % H₂O₂

9.3 mL	30% H ₂ O ₂ (Sigma)
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Add dH₂O up to 200 mL. 100 mL is enough for one cuvette (which takes 8 slides). Make fresh before each treatment.

DAB stock solution

10 mL	Autoclaved mqH ₂ O
1	DAB tablet (DAKO cytotation)

Normally the DAB tablets do not dissolve completely. The solution still works properly. Can be stored at 4 °C for up to 7 days. Be careful, very poisonous! If spilled, wash immediately with hypochlorite (commercial Klorin).

3 % H₂O₂

10 µL	30% H ₂ O ₂
90 µL	Autoclaved mqH ₂ O

Activated DAB solution

4 mL	DAB stock solution
30 µL	3 % H ₂ O ₂

Mix immediately before use. Be careful, very poisonous! If spilled, wash immediately with hypochlorite (commercial Klorin). All waste products should be inactivated with hypochlorite before thrown away in special litter bin.

Hematoxylin according to Mayer

Ready-made solution from Sigma, can be re-used. Filter before use.

Appendix 3 - RIA for cortisol

Materials

1. [1,2,6,7-³H] Cortisol 60 Ci mmol⁻¹ (Amersham)
2. Phosphate buffered saline tablets (Sigma)
3. Bovine serum albumin (Sigma)
4. Dextran, clinical grade (Sigma)
5. Activated charcoal, neutralised (Sigma)
6. Hydrocortisone (Sigma)
7. Ethyl acetate (Sigma)
8. Anti-cortisol (Sigma)
9. Scintillation cocktail (Ultima Gold, Kodak)

Reagentes

1. Labelled cortisol:

Dilute the radiocortisol solution with a solution of toluene and ethanol (9:1) in the ratio radiocortisol : toluene+ethanol 1:4. Store at -20⁰C. For working solution, add 10 µL of the stock solution to 12.5 mL of ethyl acetate. Store at -20⁰C.

To control the working solution, pipette 50 µl into 4ml scintillant cocktail and mix by inversion. Count in scintillation counter (should be approximately 20 000 dpm). Dpm will decrease over time (months) so this ratio may need to be adjusted to achieve 20 000 dpm.

2. Standard inert cortisol:

(a) Make up 1mg ml ⁻¹ Hydrocortisone in ethanol	1 mg ml ⁻¹	A
(b) Add 50 µl A to 9.95 ml ethyl acetate	5 µg ml ⁻¹	B
(c) Add 100 µl B to 9.9 ml ethyl acetate	50 ng ml ⁻¹	C
(d) Add 800 µl C to 9.2 ml ethyl acetate	4 ng ml ⁻¹	D
(e) Add 5ml D to 5ml ethyl acetate	2 ng ml ⁻¹	E
(f) Add 5ml E to 5ml ethyl acetate	1 ng ml ⁻¹	F
(g) Add 5ml F to 5ml ethyl acetate	500 pg ml ⁻¹	G
(h) Add 5ml G to 5ml ethyl acetate	250 pg ml ⁻¹	H
(i) Add 5ml H to 5ml ethyl acetate	125 pg ml ⁻¹	I
(j) Add 5ml I to 5ml ethyl acetate	62.5 pg ml ⁻¹	J

3. Assaybuffer:

To 200 ml distilled water add one PBS tablet and 200 mg BSA. Mix on magnetic stirrer. Can be kept for up to 7 days at 4 °C.

4. Dextran-coated charcoal:

To 200 ml distilled water, add one PBS tablet, 200 mg dextran and 1.0 g charcoal. Mix on magnetic stirrer. Can be kept for up to 7 days at 4 °C.

5. Antibody solution:

Dilute freeze-dried antibody as directed by supplier and dispense aliquots of 50, 100 and 250 µL. Store in freezer. For working solution, dilute the antibody 10 times in assay buffer and mix gently by inversion (do not vortex). Make up 100 µL working solution per tube.

Procedure

Standard curve

1. The standard curve consists of 200 µl of standards D-J and a null-sample. Run two duplicates, one before and one after the unknown samples.

Standard (pg)/ tube	0	12.5	25	50	100	200	400	800
200µl of stock	0	J	I	H	G	F	E	D

Extraction of cortisol

2. Steroids are removed from aqueous samples by extraction with ethyl acetate. For routine determinations, add 1.0 ml ethyl acetate to 200 µl plasma, mix well and centrifuge briefly (2min at 10 000 rpm and 4°C) to separate the two phases. The volume of sample may be varied providing the ratio of solvent to sample is 5:1. Perform extraction on ice. A quality control plasma sample (QC) should also be used, extracted at the same ratio. After extraction samples can be stored at 4°C during assay and -20°C long term.

Assay procedure

3. Pipette into assay tubes (2 ml eppendorf tubes with lid removed) the required volume of extract (e.g. 20µl for stressed fish up to 150µl for basal levels or 200µl if using dilute extract). Also pipet 20µl of extract from QC sample. An assay blank (that will contain tracer and 200 µl buffer but no antiserum) should also be included to provide a measure of non-specific binding. Also pipet out two parallels of standard series. See overview.

4. Add 50 µl of 'hot' cortisol to all tubes.

5. Dry down all tubes under vacuum but without heat. (30-60 min in a vacuum exsiccator with a water jet pump).

6. Add 100 μ l assay buffer to all tubes. To the blank add 200 μ l of buffer to bring it up to the same volume as those tubes that will receive antibody. Next add 100 μ l of antibody solution to each tube (not blank). Vortex, cover with clingfilm and incubate for 4-18 hours at 4°C or 1-2 hour on bench and 2 hours in the fridge.

7. Place racks of tubes on ice, add 100 μ l of well mixed dextran coated charcoal to each tube, vortex and incubate on ice for 5 min. (Use a repeater pipette tip that has had a couple of mm of the end cut off to avoid clogging).

8. Spin down the charcoal (3500 rpm for 10 minutes at 4°C). Dispense 4.0 ml of scintillation fluid into scintillation vials. Take tubes out of centrifuge and put back on ice. Pipette 200 μ l aliquot of the supernatant into the scintillant, mix well by inversion and count under standard tritium conditions.

Appendix 4 – Post-hoc statistics

	Control	Dominant	Subordinate
Control	-	0.938	0.001
Dominant	0.938	-	0.001
Subordinate	0.001	0.001	-

Table 1 Tukey Post-hoc statistics for feeding scores day 10-13.

	Control	Dominant	Subordinate
Control	-	0.856	0.023
Dominant	0.856	-	0.009
Subordinate	0.023	0.009	-

Table 2 Tukey Post-hoc statistics for cortisol data.

	Control	Dominant	Subordinate
Control	-	0.210	0.014
Dominant	0.210	-	0.286
Subordinate	0.014	0.286	-

Table 3 Tukey Post-hoc statistics for telencephalic cell proliferation.