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Neuroendocrine stress response in rainbow trout (Oncorhynchus mychiss): serotonergic activity and hypothalamus-pituitary-interrenal-axis

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Neuroendocrine stress response in rainbow trout (*Oncorhynchus mychiss*): serotonergic activity and Hypothalamus-Pituitary-interrenal-axis

Mémoire présenté pour l'obtention du grade
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par

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Etude de la réponse neuroendocrine au stress chez la truite arc-en-ciel (*Oncorhynchus mykiss*): activité sérotonergique et l'axe Hypothalamus-Hypophyse-Interrénal.

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Abstract

De cette étude, nous avons reporté un effet du rang social sur l'activité sérotonergique, l'expression de POMC, et la concentration de cortisol chez la truite arc-en-ciel. Les poissons subordonnés montrèrent une élévation de l'activité sérotonergique indexée par le rapport 5-HIAA/5-HT, une élévation de l'expression de POMC, et une élévation de la concentration de cortisol dans le sang. L'activité sérotonergique fut aussi corrélée à l'expression de POMC et à la concentration de cortisol.

Ces résultats nous confirment les précédentes découvertes sur l'élévation de l'activité sérotonergique chez les poissons dominés. L'augmentation de l'expression de POMC et les corrélations entre 5-HIAA/5-HT, l'expression de POMC, et la concentration de cortisol nous indiquent que le système sérotonergique pourrait comme chez les mammifères avoir unecontrôle sur la réponse endocrine au stress.

In this study, we reported a effect of social rank on serotonergic activity, POMC expression, and plasma cortisol. Subordinate fish showed higher serotonergic activity indexed by 5-HIAA/5-HT ratio, higher POMC expression, and higher plasma cortisol.

These results confirmed precedent researches on serotonergic activity in subordinate fish. Activation in POMC expression and correlations between 5-HIAA/5-HT ratio, POMC expression, and plasma cortisol indicated a control on endocrinal stress responce by serotonergic system.

Mémoire de licence en Sciences biologiques
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Promoteur: Prof. *J.-C. MICHA*
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Content

Introduction

The present work has been carried out at Uppsala University (Sweden) in fish physiology (Limnology institut). The scientific interrest in the group I worked, was essentially focused in the study of the serotonergic activity and dominance hierarchy in fish.

In a dominance hierarchy subordinate fish are subject to stress due to the aggressive acts performed by the dominants. Social stress is known to activate the Hypothalamus-Pituitary-Interrenal axis (HPI axis). The HPI is one of the two neuroendocrine stress responses. The HPI axis consists in a cascade of reactions beginning in the brain when a stressor is perceived, then corticotropine releasing hormone (CRF) is released from the hypothalamus and stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn stimulates the interrenal to release cortisol. ACTH is synthesized by proteolytic cleavage from a multifunctional proteic precursor called proopiomelanocortin (POMC), which also gives raise to other biological active peptide like α-MSH, ß-endorphin, which also is involved in the stress response. Salmonid fish are consider as diploid, consequently all salmonids possess two non-allelic copies of certain genes such as POMC. Two copies of POMC are called POMC A and POMC B.

Relationships between social rank and serotonergic activity have previously been study. The ratio 5-hydroxyindolacetic acid (5-HIAA, the major metabolate of serotonin)/serotonin (5-HT) is used as a index of serotonergic activity. Subordinate fish showed an increase in 5-HIAA/5-HT in telencephalon and brain stem. This elevation seems to be socially induced and mediate by stress. Serotonergic system might also have a inhibitory effect on the aggressive behaviour observed in subordinate fish. In mammals, serotonergic system has been connected with the HPI axis, which seems to be activated by 5-HT. In teleost fish the mechanisms responsible for modulation of neuroendocrine responses to stress are largely unknown.

In the present study, 6 groups of 3 rainbow trout were allowed to interact for a short period of 1 day to study the effects of acute social stress, and 6 other groups for a prolonged period of 7 days to study the effects of chronic social stress. After one hour, one dominant and two subordinates were recognized, one fish was kept isolated as a control in each group. The effects of subordination stress on the serotonergic system and HPI axis were studied. We analyzed the concentration of 5-HT and 5-HIAA in the brain stem, telencephalon and hypothalamus using HPLC with electrochemical detection, the pituitary POMC A and POMC B mRNA levels using *in situ* hybridisation, and the cortisol level using radio immune essay

1. Bibliographic synthesis

1.1. Dominance hierarchy

In natural ecosystems, available resources that animals need to survive and reproduce, such as food, territories and sexual partners, are not infinite. Thus, competition for such resources often occurs between individuals. In teleosts, like in many other vertebrates, social systems may often be based on a dominance hierarchy. The formation of dominance hierarchies within a group of animals generally implies that resources are unevenly distributed among individuals. Those obtaining the greater share of resources are usually denoted as high-ranking or dominant, while more unsuccessful competitors are denoted as low-ranking or subordinate.

The outcome of intraspecific aggressive encounters is probably the main factor involved in determining rank within a hierarchy. Thus, social systems evolve so that fighting ability is an important factor contributing to animal fitness (Huntingford and Turner, 1987). In fish, body weight is a major factor determining fighting ability (Huntingford and Turner, 1987). Thus, in a group of fish, the larger individuals are usually the dominant. Not surprisingly, subordinate fish show many of the physiological signs of stress. Most likely, the stress experienced by subordinate individuals initially results from loosing fights, whereas it later on probably also related to being constantly threatened and having to inhibit one's own aggression (Zayan, 1991).

1.2. The stress response

1.2.1. endocrine stress response

The vertebrate stress response is a complex spectrum of physiological changes dominated by changes in the sympathico-chromaffin system (results in release of catecholamine) hypothalamus-pituitary-interrenal axis (HPI, release of cortisol). The later system (HPI) is the best characterised in fish and the one analysed in this present study.

In classic models, interrenal corticosteroid release is stimulated by the secretion of adrenocorticotropic hormone (ACTH) from the pituitary (rostral *pars distalis*). The release of ACTH is regulated by corticotropine releasing factor (CRF), originating from the hypothalamus. Thus, glucocorticoid secretion by the interrenal (corresponding the surrenal gland in mammals) is just the final step in a cascade of events beginning in the brain. Cortisol appears to play several roles in the stress response, including energy mobilization, stimulation of ionoregulatory processes and facilitation of oxygen uptake. Further, prolonged cortisol elevation can severely consequences for disease resistence because it causes decreased immunity (Pickering, 1989), and inhibition of growth (Pickering and Pottinger, 1985) and reproduction (Donaldson, 1990), due to the inhibited release of growth hormone (GH) and gonadotropin releasing hormone (GnRH) from the pituitary.

However, other endocrine systems have been shown to be sensitive to various forms of acute and chronic stress. For exemple, the thyroidal secretion of thyroxine is stimulated by acute stress (Brown *et al.*, 1978). Acute forms of stress can suppress the levels of circulating pituitary growth hormone (GH), while more prolonged forms of stress tend to elevate plasma GH (Sumpter *et al.*, 1991).

Other hormones from the poopiomelanocortin family (see chapter 1.4), like melanocyte stimulating hormone (MSH) and β-endorphin can be secreted in response to stress (Sumpter *et al.*, 1985)

1.2.2. Nervous stress response

The other main component of the stress response is the sympathetic nervous system. This involuntary autonomic nervous system consists of two components with opposing roles.

- The parasympathetic nervous system which mediates calm and vegetative functions like digestion, growth, slow heart rate and breathing.

- The sympathetic nervous system which, in contrast to the parasympathetic system stimulates fast heart rate and breathing, and inhibits digestion and growth. It is stimulated by vigilance or emergency. Sympathetic and parasympathetic systems work in opposition, thus the parasympathetic system is inhibited by stress.

1.2.3. Social interaction and endocrine stress response in fish

Social stress resulting from the formation of dominance hierarchies is a potent form of stress. Indeed, in salmonids, subordinate fish show elevated plasma cortisol (andersen *et al.*, 1991), reduced lymphocyte counts, and increased interrenal cell body sizes (Pottinger and Pickering, 1992). This suggests a chronic activation of the interrenal stress response in these individuals. (Pickering and Pottinger, 1995). In fish exposed to a chronic social stress, the HPI axis is probably subjected to two opposing drives: negative feedback inhibition caused by the elevation of plasma cortisol, and stimulation caused by the continuous perception of the stimuli (presence of the dominant individual) (Winberg and Nilsson, 1993).

1.3. The brain serotonergic system

1.3.1. General features

Serotonin (5-hydroxytryptamine, 5-HT) discovered 50 years ago by Rapport. It got its name because of its presence in serum and its effect on blood vessels tone. 5-HT was soon recognized as neurotransmitter, and using histofluorescence technique Dahlström and Fure (1965) described the anatomy of the 5-HT system of the mammalian brains.

Monoamine neurones compose a very small fraction of the neurones in the vertebrate brain. In fact, monoamine neurones number in the thousands whereas the total quantity of neurone in the vetebrate brain number in the hundreds of millions. However, the influence of monoaminergic neurones on their target sites appears to go far beyond their numbers. In the mammalian brain, where monoaminergic neurotransmitters had been extensivly studied. The central 5-HT system is believed to be involved in the control of several behavioural patterns, notably aggression (Olivier *et al.*, 1989), mating (Meyerson and Malmnäs, 1985) and feeding (Leibowitz, 1992). Futher, the 5-HT system of mammalian brain seems to be involved in neuroendocrine regulation (Chaoulof, 1993).

In comparision to this information on monoaminergic functions in mammals, very little is known about the function of monoamine neurotransmitters system in non-mammalian vertebrates.

1.3.2. Distribution of the serotonergic system

The organisation of the 5-HT system seems to be remarkably stable throughout the vertebrate subphylum (Parent *et al.*, 1978). In mammals, 5-HT cells bodies are mainly localized in brain stem *raphe nuclei* and have axonal terminals reaching virtually all regions of the central nervous system but with a particular density in the hypothalamus and telencephalon (Jacobs and Azmitia, 1992). The distribution of 5-HT cell bodies appears to be similar in the teleost brain (Parent *et al.*, 1978, 1981, 1984). However, in teleost numerous 5-HT immunoreactive cells are localized outside the raphe region, especially in ventral malamic and hypothalamic areas (Figure 1)

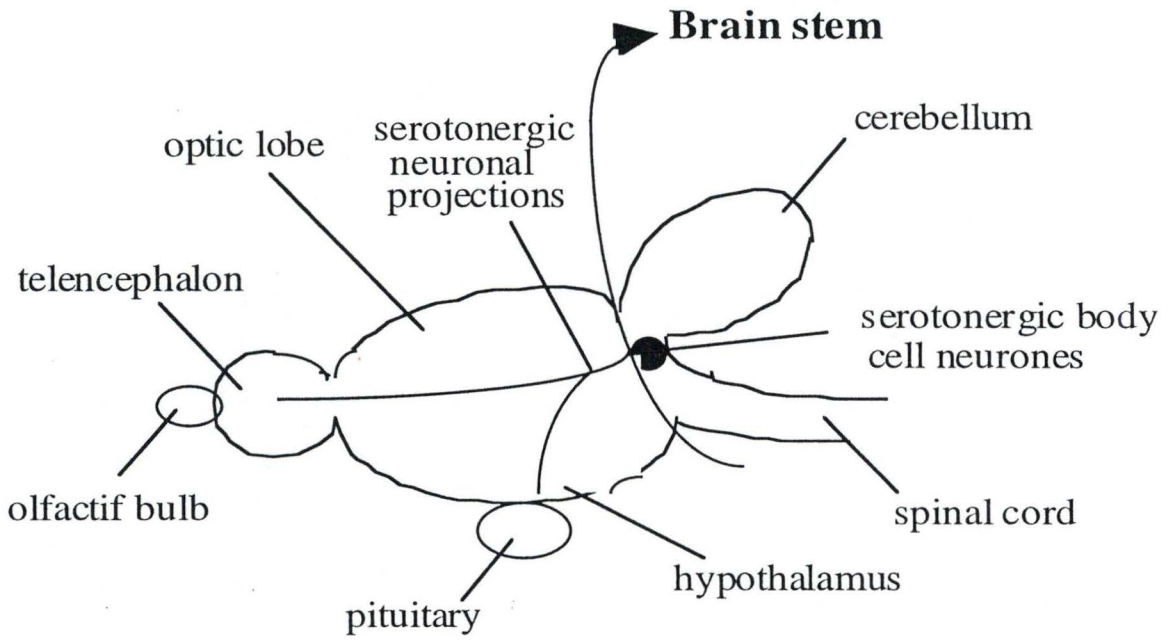


Figure 1: Rainbow trout brain and serotonergic system
the brain stem includes cerebellum, the raphe nuclei and the begenning of the spinal corde

1.3.3. Serotonergic metabolism

5-HT is synthesised from the amino acid tryptophan which is taken from the blood by a non-specific amino acid carrier. The initial step in 5-HT synthesis is the hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP), catalysed by tryptophan-5-hydroxylase. 5-HTP is subsequently decarboxylated to 5-HT by aromatic L-amino acid decarboxylase.

5-HT, like neurotransmitters in general, is concentrated and stored in vesicles which release their contents into the synaptic cleft by exocytosis when the presynaptic membrane depolarises. The effects of 5-HT are terminated by re-uptake of the neurotransmitter into presynaptic nerve terminals. Following re-uptake, 5-HT is deaminated to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase, an enzyme located on the outer membrane of mitochondria (Figure 2)

The 5-HT that is released when the serotonergic neurones are stimulated is usually replaced by synthesis in the body cell so that, the 5-HT concentration stays constant. However the concentration of 5-HIAA goes up due to the following metabolism of 5-HT into 5-HIAA. Thus both brain 5-HIAA levels and 5-HIAA/5-HT ratios are used as measures of brain serotonergic activity (Shannon *et al.*, 1986; Winberg *et al.,* 1991, 1993). However, the 5-HIAA/5-HT ratio provides a more direct index of neuronal activity than 5-HIAA levels, since it reduces variance related to tissue sampling and weight determination as well as individual variance in the total levels of 5-HT and 5-HIAA.

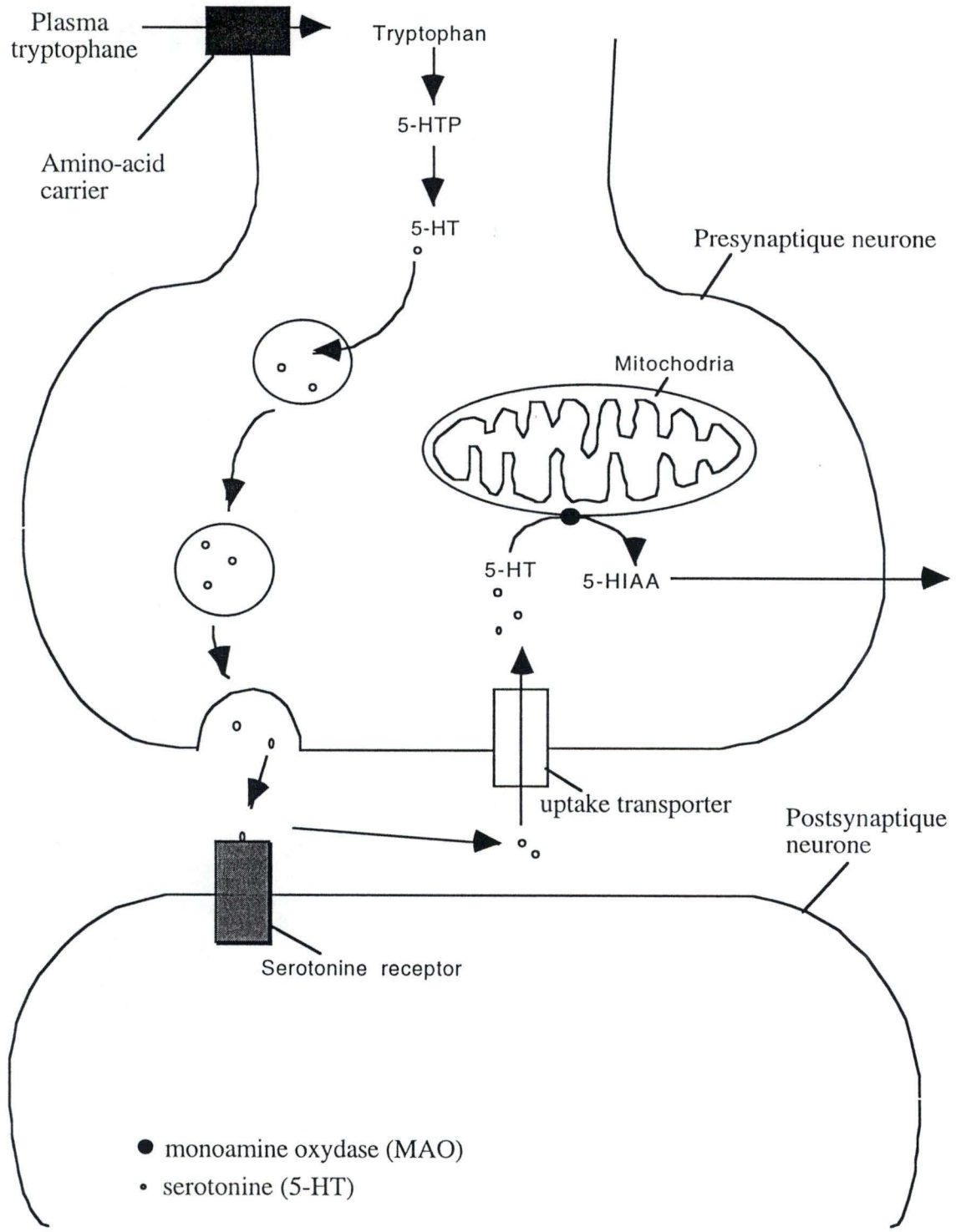


Figure 2: Serotonergic metabolism.
5-HTP is 5-hydroxytryptophan.
5-HT is serotonin.
5-HIAA is 5-hydroxyindoleacetic acid.

1.3.4. Social rank and brain serotonergic activity

Social experience has been found to greatly effect brain serotonergic activity in fish. In salmonids, subordinate fish display a general increase in of 5-HIAA concentration and 5-HIAA/5-HT ratio in the telencephalon, hypothalamus and brain stem (Winberg and Nilsson 1990, 1991). Studies on damselfish (*Pomacentrus partitus*) have shown that the number of aggressive acts received is positively correlated with 5-HIAA/5-HT ratios in the telencephalon and hypothalamus (Winberg *et al.*, 1996). In Arctic charr (*Salvelinus alpinus, Linne*), Winberg (1991) found a negative correlation between the position of the fish in a dominance hierarchy and the 5-HIAA/5-HT ratios. The elevation of central serotonergic activity in subordinate fish has a wide distribution in the brain (Winberg and Nilsson, 1993), is mediated by social interaction rather than being inherent (Winberg and Nilsson, 1992) and probably induced by stress (Winberg *et al.*, 1991).

Similarly, stress has been found to increase brain 5-HT turnover on mammals (Morgan and Rudeen, 1975; Adell *et al.*, 1988; Mitchell and Thomas, 1988). Experience of low social rank has been reported to increase brain 5-HT activity also in mammals (Blanchard *et al.*, 1991) and reptiles. Thus, this could be a phylogenetically old response to stress, which function is still not understood.

However, the apparent behavioural inhibition in subordinate fish could well be mediated by a stress-induced activation of the brain 5-HT system, as suggested by previous studies on fish (Winberg and Nilsson, 1993) as well as mammals (Davis, 1980; Soubrié, 1986).

Behavioural inhibition might be an adaptative response in a subordinate fish since it reduces the risk of initiating attacks from superior animals, and, thus probably reduces the stress in subordinates. An elevation of 5-HT activity in subordinate fish could also reflect a mechanism allowing acclimatation in these individuals. The difference in telencephalic serotonin activity between dominant and subordinate fish might be related to differences in aggressive behaviour, since telencephalon has been implicated in the regulation and integration of agnostic behaviour in fish (de Bruin, 1980).

1.3.5. 5-HT and the regulation of HPI axis

There are no studies on the effect of central 5-HT on HPI axis activity in teleost, however, monoaminergic systems of the mammalian brains have been connected with stress reactions (Dunn, 1989) as well as the central regulation of the autonomic and neuroendocrine functions (Tuomitsu and Männistö, 1985). Stress is known to activated the serotonergic system of the mammalian brain, as indicated by increase brain 5-HIAA levels. The central serotonergic system seems to play an important role in behavioural as well in neuroendocrine response in mammalian brains (Chaoulof, 1993). For instance, 5-HT is believed to stimulate the released of CRF and ACTH respectively (Chaoulof, 1993). Indeed, 5-HT immunoreactivity has been detected in the hypothalamus, and 5-HT nerves descending from the raphe area have been shown to synapse with CRF-containing fibres in the mammalian hypothalamus. Futher, 5-HT containing fibres, tryptophan hydroxylase, monoamine oxidase, 5-HT re-uptake sites, and 5-HT receptors (type 1A, 1C, and 2) have been detected as the most abundant in the hypothalamus, pituitary, and adrenal gland. Various morphological studies showed the existence of serotonin and serotonin fibres in the pituitary of some inferior vertebrates and mammals (Lamacz *et al.*, 1991). In response, cortisol might have a negative feed back effect on the serotonergic activity (Chaoulof, 1993). (Figure 3).

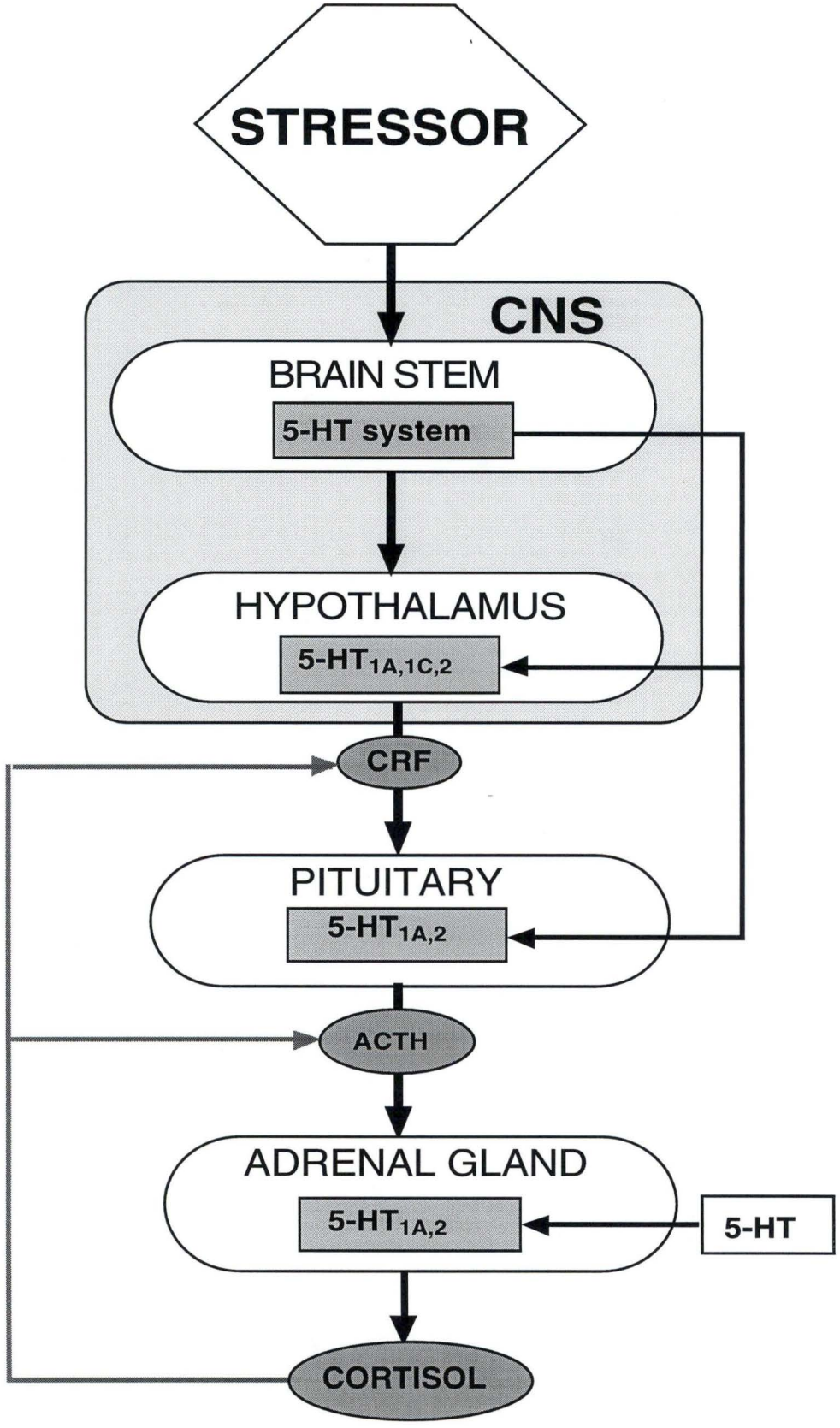


Figure 3: HPI axis and control by serotonergic system in mammal.
CNS: central nervous system
CRF: Corticotropin releasing factor.
ACTH: adrenocorticotropin hormone.
5-HT: serotonine.
$5\text{-HT}_{1A,1C,2}$: serotonergic receptor.
Stimulation is represented by black arrows.
Innibition is represented by grey arrows.

1.4. POMC gene

HPI axis is the best carrecterised endocrinal stress reponse in fish. In classic models, interrenal corticosteroid release is stimulated by the secretion of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH is synthesised from a large precursor protein, pro-opiomelanocortin (POMC). POMC is a multifunctional precursor protein which in addition to ACTH, also generates several other biologically active peptide e.g. ß-endorphin, β–lipotropine and α-melanocytestimulating hormone (α–MSH), all believed to be involved in the neuroendocrine stress response (Salbert *et al.*, 1992).

1.4.1. Synthesis pathway

Post-translational processing (proteolytic cleavage by endo- and exo-peptidase) occurs in a specific manner (Figure 4)

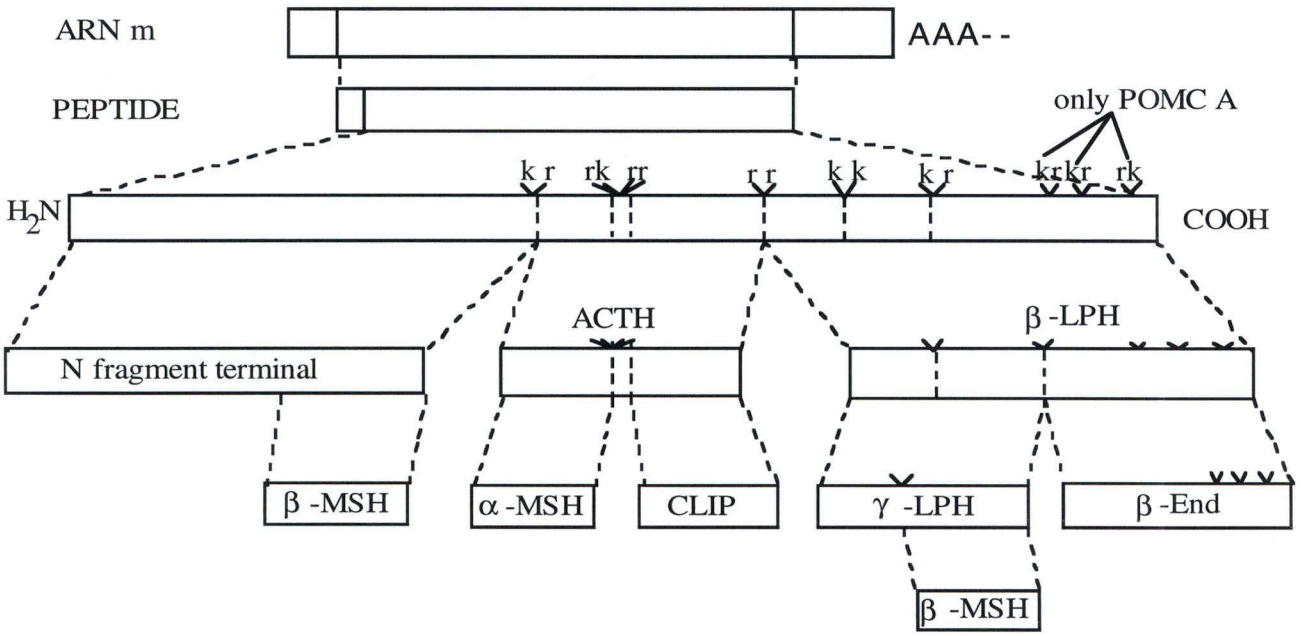


Figure 4: Syynthesis pathway of POMC derived peptides.
kr: basis amino acid; k: lysine, r: arginine.
ACTH: adrenocorticotropin hormone.
β-LPH: β-lipotropin hormone.
β -MSH: β -melanocyte stimulating hormone.
α-MSH: α -melanocyte stimulating hormone.
γ-LPH: γ -lipotropin hormone.
β-End: β-endorphine
CLIP: a inactive cleavage product of MSH secretion.

- First, the signal peptide is cleaved by the "signal peptidase".

- The following step happens in the secretion granules. The POMC molecule possesses 7 pairs of basic amino acids (lysine, arginine), wich are the recognition sites for the peptidase. POMC is synthesised in corticotrophs of the interior lobe, and in melanotrophs of the intermediate lobe, and by a population of hypothalamic neurones located in the anterior part of the nucleus tubelaris.

In corticotrops, only four cleavage sites are accessible for the peptidase, so essentially the N terminal fragment, ACTH and β-LPH are produced.

In melanotrops, all the cleavage sites are accessible, thus the N terminal fragment produces γ-MSH, ACTH is cleaved into α-MSH and CLIP (a inactive cleavage product of MSH secretion), β-LPH produces γ-LPH and β-endorphin.

The difference between these two different cleavage mechanisms has not been completely determined (Lamacz *et al.*, 1991).

1.4.2. POMC in salmonid

Because of the tetraploid status of their common ancestor, all salmonids possess two non-allelic copies of certain genes such as POMC. After gene duplication, the loci of the duplicated genes evolved independently, producing animals that must be called diploid (Salbert *et al.*, 1992)

In salmonids, for instance, those is evidence for two GH mRNAs (Pickering and Pottinger, 1995) and two melanin-concentrating hormone mRNAs (Ono *et al.*, 1988). In salmon, two different somatostatins are probably encoded by two different genes, not expressed in the same pancreatic cells (Nozaki *et al.*, 1988). Therefore, they do not exhibit the same biological activities (Marchant *et al.*, 1987).

In rainbow trout, two genes of POMC have been evidenced. Salbert *et al.* (1992) cloned these two different POMC cDNA, called POMC A and POMC B. These cDNA exhibited limited sequence homologies (44%), despite the high conservation of some peptide sequences (α-MSH, ß-MSH, ß-endorphin). The POMC A coding sequence exhibited an unusual length, generating the longest endorphin ever sequenced. The long carboxyterminal part of the ß-endorphin A contained three potential dibasic cleavage sites, allowing the occurrence of three new peptides.

Results from *in situ* hybridisation, (Salbert *et al.*, 1992) showed that the two POMC gene were expressed in the same pituitary cell. POMC A mRNA was the only one detectable in the hypothalamus of sexually inactive fish, whereas, both POMC A and POMC B genes were expressed in the hypothalamus of sexually active fish (also see plate 1 and 2). These results indicate that in POMC hypothalamic neurones the POMC B gene is likely to be under the control of sexual steroids.

1.4.3. Stress and POMC expression

1.4.3.1. In mammals

The effect of stress on pituitary POMC mRNA levels varies according to the nature of the stimulus (Aguilera, 1993). Chronic stress paradigms (e.g. repeated foot shock, swim stress, cold exposure) associated with hypersensitivity of the pituitary ACTH response to a novel stress usually result in an elevation of POMC mRNA levels, whereas other stress paradigms (e.g. osmotic stress) not associated with sensitisation of the stress response, appears to have the opposite effect on POMC mRNA concentrations (Aguilera, 1994). Larsen *et al.* (1994) showed that acute stress increased the level of POMC mRNA in the pituitary of mammals. Furthermore, POMC expression is activated in the rat pituitary, after a stress caused by cold or a novel environment. This elevation in the expression of POMC is seen in the corticotrope as well as melanotrope cells (Wu *et al.,* 1991).

1.4.3.2. In fish

There are no studies on the effects of stress on POMC mRNA levels in fish. However, stress-induced effects on plasma levels of POMC derived peptides seem to vary depending on the stimulus. Elevated plasma levels of α–MSH and β–endorphin has been reported in fish exposed to temperature shock (Sumpter *et al.,* 1985), noise (Malo-Michelle, 1980), and low environmental pH in the presence of aluminium (Balm *et al..,* 1987). In contrast, rainbow trout exposed to confinement stress display a transient increase in plasma ACTH concentrations but a reduction in plasma levels of α–MSH and β–endorphin (Balm and Pottinger, 1995).

1.5. Aim of the study

In the present study, we examined the effects of short (1 day) and prolonged (7 days) subordination stress on brain serotonergic activity and HPI activity in rainbow trout. The concentrations of 5-HT and 5-HIAA in the brain stem and in the telencephalon, the pituitary POMC A and POMC B mRNA levels, and the plasma cortisol levels were used as a measure of these effects.

Further studies of these mechanisms were primilary motivated by the fact that the functions of brain serotonergic activity appear to be essentially similar throughout the vertebrate phylum. Studies on brain serotonin activity and POMC expression in fish will do more than simply increase our knowledge of how fish function. The aim of the project is to extend our knowledge of central neurophysiological mechanisms involved in the control of aggressive behaviour and the mediation of stress responses. This will give a new perspective on the age of serotonin functions in vertebrates.

2. Materials and Method

2.1 Origin and maintenance of experimental animals

All experiments were carried out on juvenile rainbow trout (*Onchorhynchus mykiss*). The fish originated from a commercial hatchery (Färnäs Fiskodcing AB) situated at Mora (Province of Dalecarlia), and were transported by car to the holding facilities at Uppsala University, Sweden, Institute of Limnology. Here the fish were maintained in big circular tanks (800 l) in the animal facility for several weeks, each tank containing approximately 100 fish (density: 12.5 g/l). Tanks were continuously supplied with aerated Uppsala tap water (8-11 °C). Throughout the rearing (from December 1995 through to March 1996) the photo period was kept at 10L:14D. The fish were fed 1-2% of their body weight each day with commercial trout pellets.

The fish weighed before and after the experiments, and specific growth rate (SGR) was calculated :

$$SGR = 100(\ln(W_f/W_s))/t$$

Wf: weight at the end of the interaction (grammes).
Ws: weight at the begenning of the interaction (grammes).
t: duration of the interaction (days)

2.2. Experimental procedures

2.2.1. Behavioural observations

In order to identify dominant and subordinate animals, the fish were observed while they interacted with each other. This took place after the fish recovered from the stress induced by transport from the original hatchery. Behavioural observations were made in smaller glass aquaria (100 X 500 X 500 mm) continuously supplied with aerated Uppsala tap water (8-10 °C). The water level was maintained at 400 mm with a stand pipe. Light was provided by fluorescent tubes (2 X 18W, warm white) placed at 100 mm above the water surface. The photoperiod was kept at

12L:12D. The remaining sides of the aquaria were covered with black plastic. To avoid any disturbances while fish were interacting, the behavioural observations were made behind a black nylon-mesh screen attached in front of each aquaria, thus the fish could not notice the observer.

Behavioural observations were carried out according to the following protocol:

a) Experimental set-up: Individual fish were captured by dip netting in rearing tanks, and the weights were recorded. Fish were then transferred from the animal facility to the observation aquaria. Each observation aquaria was divided into four chambers of equal size by inserting 3 black PVC walls. One fish was isolated in each of the four chambers of the observation aquaria. After an acclimation period of one week, two of the three black PVC walls were removed. This created one group of three fish and one isolated control fish in each aquaria.

b) Recording of aggressive behaviour: Visual observations were carried out during three daily sessions of 5 minutes (9.30, 13.00 and 17.00 hours) through a small window cut in the black nylon-mesh screen. The fish were identified by a clip in their caudal fin. A total of twelve groups of three fish were observed, six groups for one day in order to examine the effects of acute stress, and the other six groups for seven days in order to study the effect of chronic stress due to their interractions. The aggressive acts classified according the following scale were registered in each groups:

Attack: A rapid approach towards an individual, often involving bites.
Chase: A succession of at least two approaches towards the same fleeing individual.
Nip: A bite to an individual within close proximity, without a prior approach.

2.2.2. Sampling

- At the end of each experimental period, fish in individual groups were caught simultaneously with a dip net, and anaesthetized in MS 222 (0.05% w/v).

- The body weights were first registered.

- The blood samples were collected in the tail (caudal vessel) with a syringe containing a drop of heparin (5000 IU/ml). The plasma was separated by centrifugation (10 minutes, 13000 rpm) and stored at -80°C prior to cortisol analysis.

- After blood sampling, the fish were sacrificed by decapitation. In each fish, telencephalon and brain stem were collected and immediately dipped in liquid nitrogen before being stored at -80°C for analysis of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA, the major metabolite of 5-HT). In one of the two subordinates in each group, the hypothalamus were collected for 5-HT and 5-HIAA analyzis, which was removed together with pituitaries and immediately dipped in liquid nitrogen before being stored at -80°C. All samples were rapidly collected (within 3 minutes). For the other subordinate and each dominant, the pituitaries (attached with on the hypothalamus) were collected for *in situ* hybridisation (see chapter 2.3.) within 10 minutes. Pituitaries were immediately embedded in tissue tech, frozen on dry ice and stored at -0°C.

2.3 In situ hybridisation.

The *in situ* hybridization was carried out according to Wisden W. and Morris B.

2.3.1 Dilution of oligonucleotides

Oligonucleotides arrived in a lyophilized form, from a Molecular Biology Research Institute (Scandinavian Gene Synthesis AB), in a glass vial. Our probes were a 36 mer oligonucleotide cDNA, designed to hybridize with POMC mRNA at the region coding for the beginning of β-LPH (Figure 5). The glass vial contained 0.15 µmol synthesis scales of probes. The lyophilized probes were diluted in 500 µl of distilled water to make a concentrated stock solution of 0.003 µmol/µl. Subsequently, 10 µl of the concentrated stock solution was diluted to 100 µl in sterile water (dilution 1), and frozen at -20°C for later measurement of the terminal transferase labelling reaction.

3' ctc gac ccg tga ctg ctg cgg cac atg ggg agg gac **5'**	**POMC A probe**
...atg aga cga gag ctg ggc act gac gac gcc gtg tac ccc tcc ctg gag gct ggg...	**POMC A mRNA**
...gca c·c a·g cag ctg agc agc tgg gag cag gag atg gtg gga gct ct· ·gg aac...	**POMC B mRNA**
3' gtc gac tcg tcg acc ctc gtc ctc tac cac cct cga **5'**	**POMC B probe**
--> β-LPH	

Figure 5. Sequencing of the probes.
Probe sequensing given by Scandinavian Gene Synthesis AB.
POMC sequensing found by Salbert *et al.*, 1992.

2.3.2 Terminal transferase labelling.

Labelling reaction procedure:

- The working stock solution of the probes (0.3 pmol/µl) was prepared by adding 10 µl of the dilution 1 into 1 ml of sterile water.

- The reaction solution was prepared by mixing:

a) 1.2 µl of $CoCl_2$ solution 25 mM (Boehringer-Mannheim), which is TdT cofactor.

b) 4 µl of 5 X reaction buffer (potassium cacodilate, 1M, Tris/HCl, 125mM, bovine serum albumin, 1.25 mg/ml, pH 6.6).

c) 2 µl of the Terminal Transferase (TdT, Boehringer-Mannheim, 25 U µl).

d) 2 µl of the working stock solution.

e) 10 µl of sterile water and, 3 µl of [α–^{35}S]dATP (1300 Ci mmol^{-1}, DuPont, NEN, NEG-034H).

- Afterwards, the reaction solution was incubated at 37 ^{o}C for 6 minutes. During the incubation the labelling reaction took place. To stop the reaction, 40 µl of sterile water at room temperature was added. To remove unincorporated nucleotides from the labelling reaction, the total volume from the reaction was applied on the top of a commercially prepared Biospin columns (Bio-Rad laboratories, Richmont, CA 94804, USA). The columns were centrifuged for 2 minutes at 2000 rpm. The resulting eluate was used for the application of the probes in the brain sections:

- 4 µl of the eluate were analysed by liquid scintillation counting. If the probe is in the range of 50 000 to 300 000 d.p.m /µl.of radioactivity, which means that the size of the radioactive tail is ideal, 1 µl of 1 M of dithioltheitol (DTT) was added to the eluate to preserve the probe from oxidation. The radioactivity of our probes was 151681 CPMA for POMC A, and 215996 CPMA for POMC B.

The above labelling reaction was performed to label the oligonucleotide with [α–^{35}S]dATP, using a 30:1 molar ratio of isotope to oligonuleotide. Oligonucleotides were labelled with terminal deoxynucleotidyl transferase, which is a DNA polymerase that catalyses

the synthesis of polydeoxyribonucleotides from deoxyribonucleotide triphosphates with the release of inorganic pyrophosphate. It initiates the reaction from the free terminal 3'-hydroxyl group of single-stranded DNA. Thus, if [α–^{35}S]dATP, oligonuleotide and TdT are mixed, the end result is a polydeoxyadenylic radioactive tail added to the 3' end of the nucleotide.

2.3.3. Slices preparation

Slide-mounted sections were cut on a cryostat (14 µm) from unfixed frozen brain tissue imbedded in tissue tech (Cryomold, lab-tek division) and thaw-mounted onto Superfrost Poly-lysine slides. Sections were allowed to dry at room temperature for half an hour, and then stored at -80°C, until fixation .

For the fixation, cryostat sections were dipped in 4 successive ice cold baths of:

- 4 % paraphormaldhyde solution for 5 minutes (fixation).
- PBS for several minutes (washing). PBS contained 0.13 M NaCl, 7 mM Na_2HPO4, 3 mM NaH_2PO_4, pH 7.4.
- In 70% ethanol and 95% ethanol for a few minutes (dehydration).

Brain sections were stored in 95% ethanol in the refridgerator for several hours before application of the probes.

2.3.4 Hybridization and washing

The probes were labelled, and the sections cut and stored in ethanol prior to hybridization.

The following protocol was carried out:

- The probes from the spin column eluate, were diluted in the hybridization buffer to a concentration of 1:100 (v/v). The hybridization buffer was 50% formamide, 4 times SSC (1 X SSC is 0.15 M NaCl, 0.015 M Sodium citrate, pH 7.0), 10% dextran sulphate.

- The brain slices were removed from 95% ethanol and allowed to dry.

- 100 µl of probe/hybridization buffer was applied to each brain slice. A parafilm coverslip was gently lowered over the drop of hybridization buffer.

- The brain slices were placed in a humid chamber at 37°C for the night. The humidity was maintained with 50% formamide/4 times SSC. In the humid chamber, the hybridisation took place.

- The next day the parafilm coverslips were removed, and the brain slices were directly transferred into 1 time SSC at room temperature.

- After a few minutes, slides were transferred in 1 time SSC at 60°C for 60 minutes.

- Through a brief series of ice cold baths, the slices were rinsed in 1 time SSC, 0.1 time SSC, dehydrated in 70% ethanol, 95% ethanol, and were allowed to dry.

2.3.5. Exposure and quantification

After hybridization, slides were first exposed on a phospho image plate (Bio-Imaging Systems, Equipment Products Div., Fuji Photo Film, Japan) to produce a global image and secondly dipped in a photographic emulsion bath for cellular resolution (Plate 1 and 2).

The Image Plate (IP) is a innovative technology developed for autoradiographic application (Bio-Imaging Systems, Equipment Products Div., Fuji Photo Film, Japan). It is used as a substitution for X-ray film. IP is a flexible image sensor on which very small crystals of photo-stimulable phosphor is uniformly coated. When photo-stimulable phosphor is irradiated and excited by radiation, an electron in the crystal is exited to a metastable state. If stimulated by a second light source (laser beam), the already exited electron falls to a ground state while emitting light of a shorter wavelength than that of the second stimulation light. This emitted light is termed phospho stimulated luminescence. When a radioactive source is in contact with the IP, distribution of electrons

excited to the metastable state takes place in the IP in accordance with distribution of radioisotope (RI) in the sample. Photo-stimulated luminescence emitted by the laser beam is converted to electrical signals and subsequently these signals are converted by a Bio-Imaging Analyzer to digital signals.

The Image plate is about one hundred times more sensitive than X-ray film and superior in quantification performance. Exposure time depends both on mRNA abundance and probe specific radioactivity. Sections labelled with POMC A were exposed on IP for 3 hours, and sections labelled with POMC B were exposed for only 2 hours since the specific radioactivity was stronger.

The slides were placed in a cassette and the IP placed over them. The cassette must press the IP and the sections together. After exposure time, the IP was read to get an global digitized image on a computer that is easy to quantify by densitometry. The optical density of the phospho image produced by the sections is related to the regional concentration of probes present in the sections, and thus represents the relative amount of POMC mRNA in the pituitary section. The amount of probe hybridised to individual pituitaries was measured as photo-stimulated luminescence (PSL) per m2 within the labelled area of the pituitary on 15-20 consecutive sections per fish. The average PSL value for each fish was subsequently converted to % of the control (divided by group mean of the controls) and used to calculate group means.

After exposure on the phospho image plate, the slices were dipped in photographic nuclear resolution to provide more information about POMC expression, particulary in the hypothalamus. All the following procedures were performed under safe lighting (Kodak 6B):

- The solid emulsion (Ilford K5) was melted for 30 minutes in a water bath and then mixed with a 43°C prewarmed water at in the dipping chamber (1:1 volume ratio of emulsion:water).

- Two drops of glycerol were added to the solution.

- The homogenous solution was maintained at 43°C in a water bath for half an hour to remove the air bubbles.

- The brain slices were dipped individually in this chamber and dried in complete darkness for 4 hours.

- After drying, the brain slices were transferred in a light-tight slices chamber containing a bag of silicate gel and kept at 4°C for the required time of the exposition.

- On the day of development, the slices were immersed in four different baths:

a) In D19 developer (Kodak) cooled on ice for 3 minutes.
b) In deionized water for 30 minutes.
c) In a freshly prepared solution of 30% sodium thiosulphate for 5 minutes.
d) In distilled water for washing and storage before staining.

To stain the sections:

- 0.1% solution (w/v) of thionine (Thionine/Lauth's Violet) was spread over the slices for several minutes.

- As soon as the tissue was ideally stained, the brain slices were dehydrated through a graded series of ethanol baths (1x 70%, 2x 95%, 2x 100%) and washed two times in a Xylene bath.

- Sections were mounted with glass coverslips and DPX mounting medium.

2.3.6. Specificity of the probe.

In the present study we used two different oligonucleotide probes complementary to POMC A mRNA and POMC B mRNA, respectively. To prove that there is no cross reactivity between the two probe, we compared two different *In Situ* hybridisation staining:

1) One with each radiolabelled probe separately (POMC A* and POMC B*)

2) Another with each radiolabelled probe mixed with the other non labelled probe one hundred times more concentrated (POMC A* + 100 X POMC B and, POMC B* + 100 X POMC A)

We did not see a significant difference in the mRNA levels between one probe and the same probe mixed with the other one hundred times more concentrated.

- POMC A* compared to POMC A* + 100 X POMC B: $P = 0.7208$
- POMC B* compared to POMC B* + 100 X POMC A: $P = 0.8376$

These results indicate that there were no cross reactions between POMC A probe and POMC B probes, thus we were able to quantify separately POMC A and POMC B pituitary mRNA level.

2.4. 5-HT and 5-HIAA analysis

For the analysis of serotonin and its metabolite, a homogenate of brain tissue was injected in the HPLC with electrochemical detection (HPLC-EC).

The frozen telencephalon, hypothalamus, and brain stem were homogenized according the following procedure:

- Pieces of brain were first registered and mixed with PCA solution (0.8 ml for brain stem and hypothalamus, 0.4 ml for telencephalon). PCA solution contained 4% (w/v) perchloric acid, 0.05 % (w/v) bisulphite and 0.2 % (w/v) EDTA, which have a stabilizing effect on monoamine, and 40 ng/ml of epinine, which was the internal standard. Epinine is a monoamine not present in the brain and was used as a control since the concentration was the same for each sample.

- The mixture was sonicated with a MSE 100 W Ultrasonic Desintegrator (telencephalon and hypothalamus) or homogenized with a Potter-Elvehiem homogenizer (brain stem).

- All the homogenized brain tissues were centrifuged for 10 minutes at 13 000 rpm. The supernatant containing serotonin and its metabolite was stored at -80°C until analysis.

The HPLC-BC system consisted of :

a) A ConstaMetric III Minipump set at a flow rate of 1 ml/min.

b) An Rheodine injector 7125 with a 100 µl sample loop.

c) A 4.6 mm X 120mm reversed-phase column packed with 5 µm Nucleosil-C18.

d) And a Amperometric Bioanalytical system detector set at +600 mV.

In the analysis of 5-HT and 5-HIAA, the mobile phase contained 50 mM citric acid, 12% (v/v) methanol, 45 mg/l sodium ocytil sulphate, and 0.002% (w/v) EDTA (pH 3.1).

10 µl of each homogenised samples brain tissues were injected with a microsyringue. After eluate through the HPLC column, 5-HT and 5-HIAA were separatly oxydised by the Amperometric Bioanalytical system detector, generating a electrical current, easily measurable on the chromatogram in millimetres. To enable accurate determinations of 5-HT and 5-HIAA concentrations, solutions containing known amounts of these substances were injected every tenth sample.

2.5. Cortisol analysis

To measure plasma cortisol, a Radio Immune Assay (RIA) was carried out. The analysis was performed directly on rainbow trout plasma without extraction. Since the concentration of cortisol is very high in subordinate fish, the plasma from these individuals was diluted 3 times in phosphate buffer (0.1M phosphate, pH 7.5, containing 0.2% bovine serum albumin; BSA), while dominant and control plasma were not diluted.

The following protocol was carried out:

- In 270 µl of phosphate buffer were mixted 20 µl of plasma (diluted for subordinate) or standard solution. Standards were prepared by dissolving Cortisol (Hydrocortisone, SIGMA H 4001) in ethanol (100 µg/ml), and serially diluting this solution with phosphate buffer to a concentration of 0, 1.56, 3.1, 6.25, 12.5, 25, 50 ng/ml. Samples and standards were analyzed in duplicate.

- 250 µl of NaOH/TCA solution (trichloroacetic acid 150 g/l, NaOH 45 g/l) were added to prevent protein binding.

- 250 µl of 1,2,6,7[^{3}H]cortisol (DuPont NET396, diluted in ethanol to an activity of 250 µCi) were added as a radioactive tracer.

- 250 µl of anticortisol rabbit antiserum were added (Lot 345-102280, Endocrine science products, Calabasa Hills, California, USA) as a source of antibodies. This amount of antibodies gave about 33% binding of the total radioactivity.

-The mixture was incubated overnight at 4 °C. While the night, the anticortisol antibodies bounded to the cortisol and radioactive tracer present in the solution.

- After one night of incubation, free and bound hormones were separated by centrifugation (4400 rpm at 0 ^{o}C). 500 µl of an ice cold mixture of 1% (w/v) Charcoal (Sigma C5260), 0.5% (w/v), Dextran T70 (Pharmacia Biotech 17-0280-01 and 0.1 % (w/v) of NaN_3 was added to each tube for centrifugation.

- After centrifugation, quantity of free radioactive tracer in 1 ml of the supernatant from each tube was counted by liquid scintillation spectrometry in d.p.m./µl. In order to realise the quantification, a standard curve is first established, showing a decroissance in porcentage of radioactive tracer fixed to the antibodies in presence of croissant known amount of cortisol (standard). In each samples, cortisol concentration can be deduced from this standard curve.

The-cross reactivity of the antiserum, as given by the producer is: 11-deoxycortisol 8.0%, corticosteron 4.0%, deoxycorticosterone 0.5%, progesterone 0.1%, cortisone 3.0%, 17-OH-progesterone 0.5%, predisone 3.0%, dexamethasone 0.4%.

2.6. Results analysis

- From the social interaction, we obtained 12 controls, 6 dominants 1 and 7 days interacted, 12 subordinates 1 and 7 days interacted.

- Agressive acts, plasma cortisol, brain stem and telencephalon serotonergic activity, POMC A and POMC B mRNA pituitary level, and SGR were measured, representing variables.

- Results interpretation was carried out using variance analysis test (ANOVA 1) for each variable.

- Variance homogeneity was tested using Bartlett's test.

- In case an effect of group, we realised individual comparison between dependant variables using Newman's test, and correlation using Spearman's test.

3. Results

3.1 Establishment of the dominance hierarchy

When PVC walls separating fish in each group containing 3 juvenile rainbow trouts were removed, each fish began performing agonistic behaviour within a few minutes. An initial phase of mutual displays were often followed by a series of very violent attacks and cirkling, usually lasting for 5-45 min. Fish losing this first period of encounters either swam to the surface, or took a position close to the walls in the of the aquaria (seemingly hiding). Hereafter, subordinate fish spent most of the time in a corner of the aquaria, usually with their heds at the water surface. However, even after attaining this submissive position subordinates were frequently attacked and nipped by their dominant opponent. After one or two days, the subordinates showed some bite marks on their body, damage fins, and sometimes blood spotts. Some fish were gravely hurt, floating up side down and developing some fungi decease. The latter individual was active moving freely in the tank looking perfectly healthy. In no case did the dominance-subordination relationship evident after the first period of encounters change during the experiment. Further, in none of the groups did fish losing the first encounter and becoming subordinate perform any aggressive acts during the rest of the experiment. Thus, even though the number of aggressive acts received differed between the two subordinates within a group, the individual rank of these individuals could not be determined.

The number of all aggressive acts (attack, chase, and nip) performed by dominant fish was around 25 per 5 min on the begenning on the first day of the interaction. If the fish were still allowed to interact after the first day, the agressive acts per 5 min rapidly declined with time until reaching only a few aggressive acts at the end of the 7 days period (Figure 6).

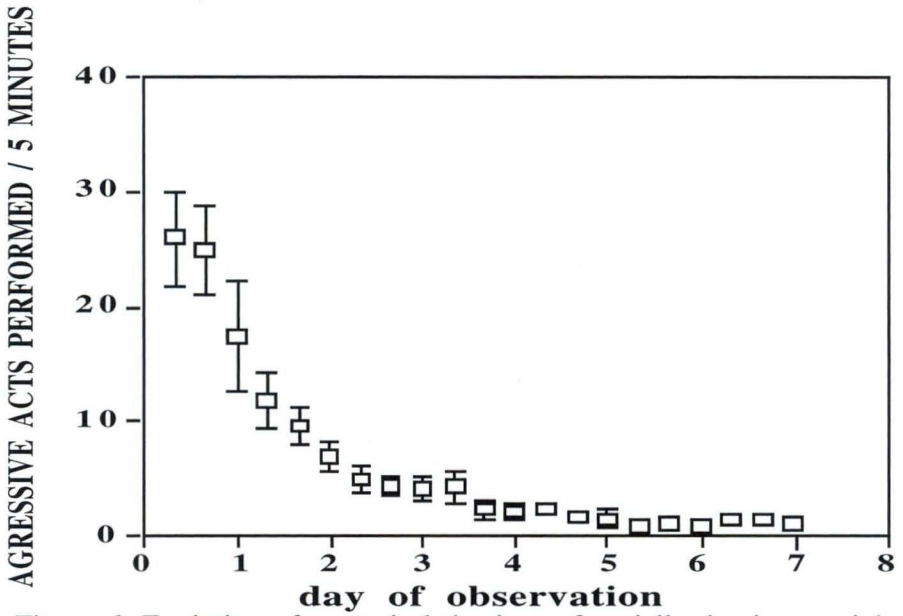


Figure 6. Evolution of agnostic behaviour of socially dominant rainbow trout. The fish were allowed to interract in group of three individuals for seven days. Values are mean and S.E.M. of aggressive acts/5 min performed by dominant fish in 12 groups. Behavioural observations were made three times a day.

Control fish and subordinate after 1 and 7 days shown a negative speciphic growth rate, indicating a lost of weight. Social rank had significant effects on SGR ($F_{4,43}$=4.388, P=0.0046; annexe 7). After 7 days of social interaction subordinate fish displayed a negative SGR significantly lower than dominant 1 day and subordinate 7 days (Figure 7).

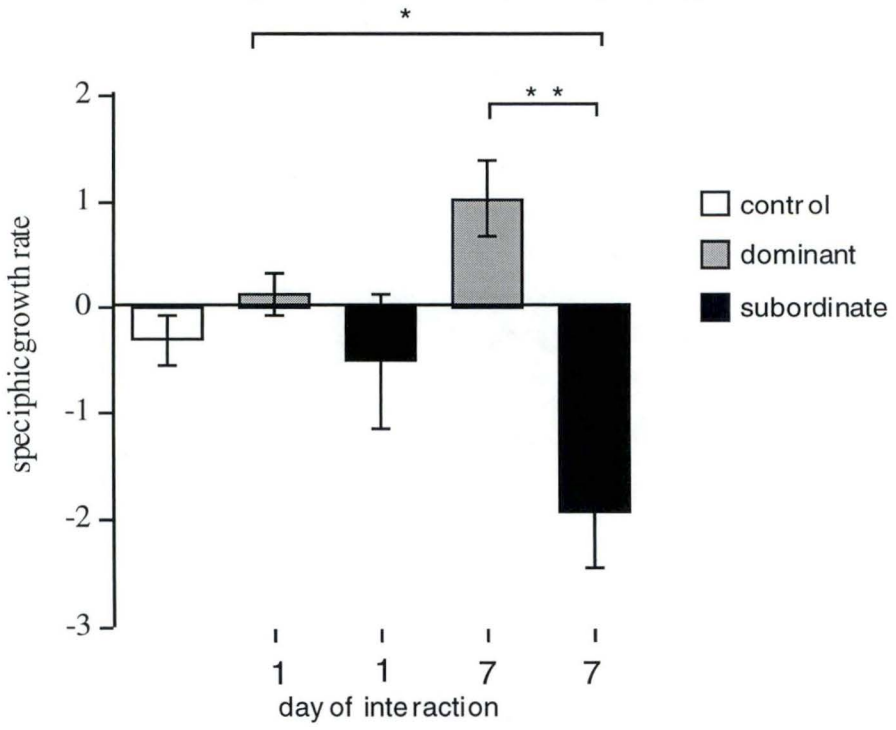


Figure 7. Specific growth rate (SGR) of control (isolated fish), dominant and subordinate rainbow trout after one and seven days of social interaction. Values are mean and S.E.M. from 12 fish (control and subordinate) and 6 fish (dominant). Significant differences between experimental groups are denoted by asterisks, *P<0.05, **P<0.01.

3.2. Serotonergic activity

Social rank had significant effects on 5-HIAA concentrations and 5-HIAA/5-HT ratios in telencephalon ($F_{4,39}=3.675$, $P=0.012$ and $F_{4,39}=6.120$, $P=0.0006$, respectively, annexe 4 and 10) and brain stem ($F_{4,41}=6.442$, $P=0.0005$ and $F_{4,42}=13.29$ $p<0.0001$, respectively, annexe 3 and 10). Specifically, subordinate fish showed a significant elevation of 5-HIAA/5-HT ratios in these brain parts after 1 as well as 7 days of social interaction, as compared to controls and dominant individuals (Figure 9). Similarly, 5-HIAA levels in telencephalon and brain stem were significantly higher in subordinate fish than in dominants and controls, both after 1 and 7 days of social interaction (Figure 8).

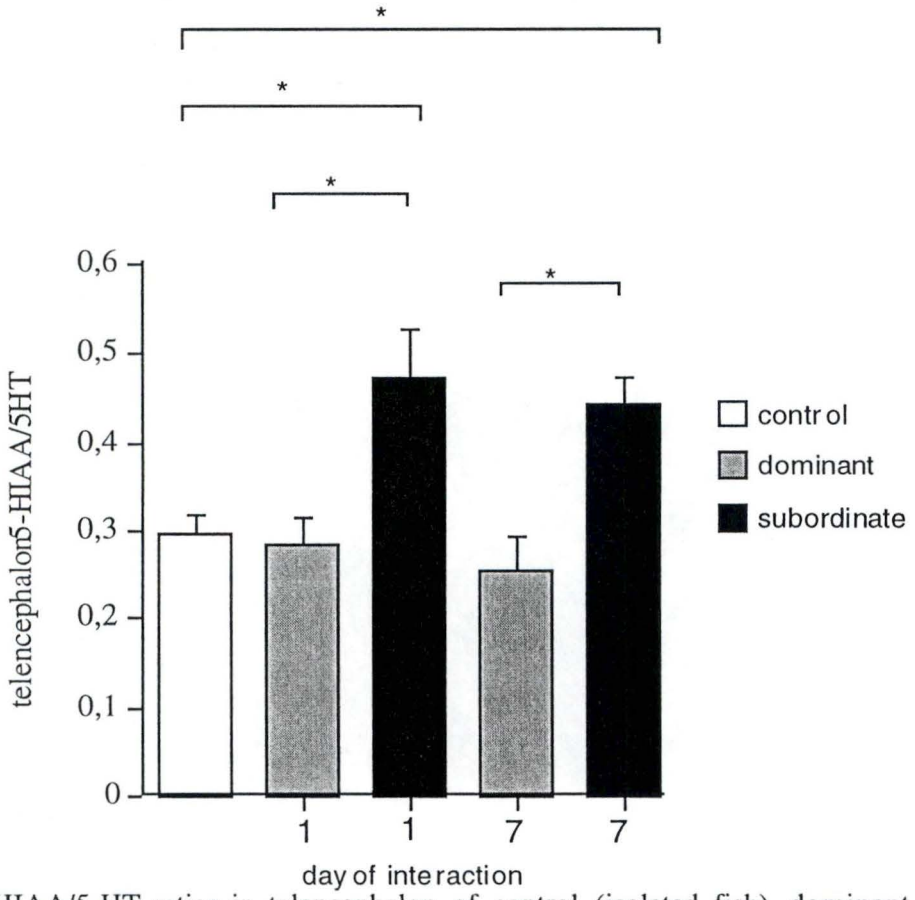


Figure 8. 5-HIAA/5-HT ratios in telencephalon of control (isolated fish), dominant subordinate rainbow trout after one and seven days of social interaction. S.E.M. from 9 fish (control), 12 fish (subordinate), 5 fish (dominant after one day of social interaction), and 6 (dominant after seven day of social interaction). Significant difference between experimental groups are denoted by asterisks, *P<0.05.

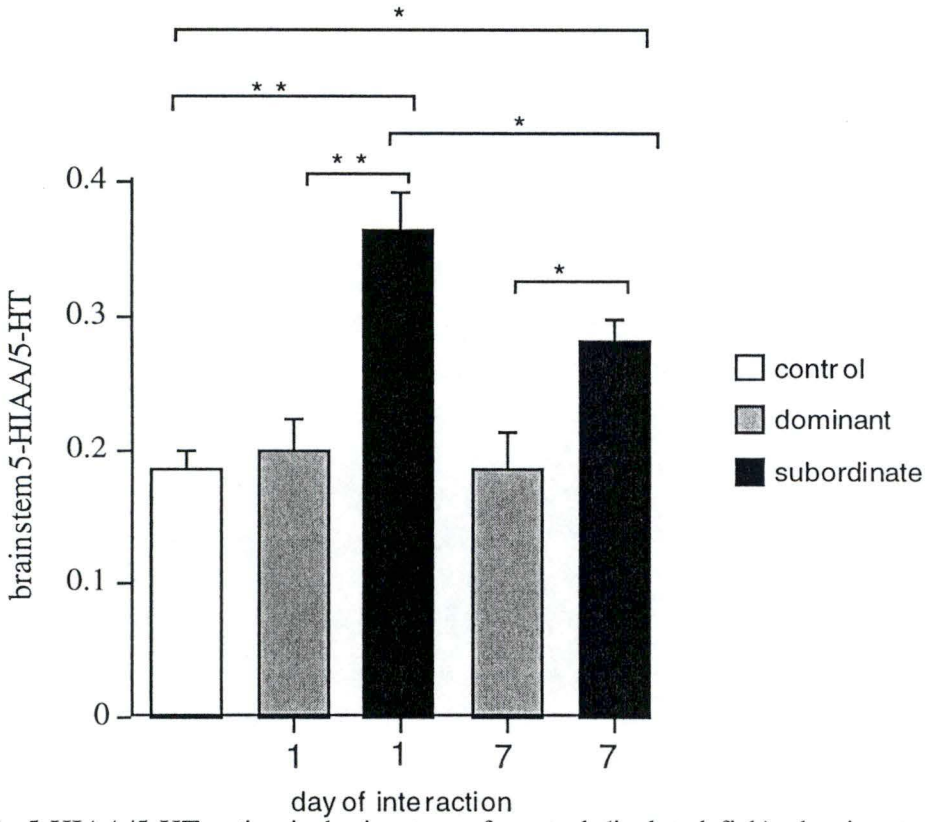


Figure 9. 5-HIAA/5-HT ratios in brain stem of control (isolated fish), dominant and subordinate rainbow trout after one and seven days of social interaction. Values are mean and S.E.M. from 11 fish (control), 12 fish (1 and 7 days subordinate) and 6 fish (1 and 7 days dominant). Significant differences between experimental groups are denoted by asterisks, *P<0.05, **P<0.01.

Brain stem 5-HIAA/5-HT ratios in subordinate fish declined with time and was significantly lower in subordinates sampled after 7 days of social interaction than in those allowed to interact for only 1 day (annexe 3; Figure 9). However, 1 and 7 day subordinates did not differ in brain stem 5-HIAA concentrations (annexe 8). Telencephalic 5-HIAA/5-HT ratios, on the other hand, did not decline with time, and did not differ between 1 and 7 days subordinates (annexe 4; Figure 8).

In dominant fish, neither 5-HIAA concentrations (annexe 8 and 10) or 5-HIAA/5-HT (annexe 3 and 4) ratios differed from that of controls in either telencephalon or brain stem.

Social position had no significant effect on 5-HT concentrations in either telencephalon or brain stem (annexe 9 and 11).

3.3. Plasma cortisol

Social rank had a significant effect on plasma cortisol concentrations ($F_{4,43}=9.621$, $p<0.0001$; annexe 2). Subordinate fish displayed significantly higher plasma cortisol than dominants and controls, after 1 as well as 7 days of social interaction (Figure 10). However, plasma cortisol levels were significantly higher in 1 day subordinates than in 7 day subordinates (Figure 10).

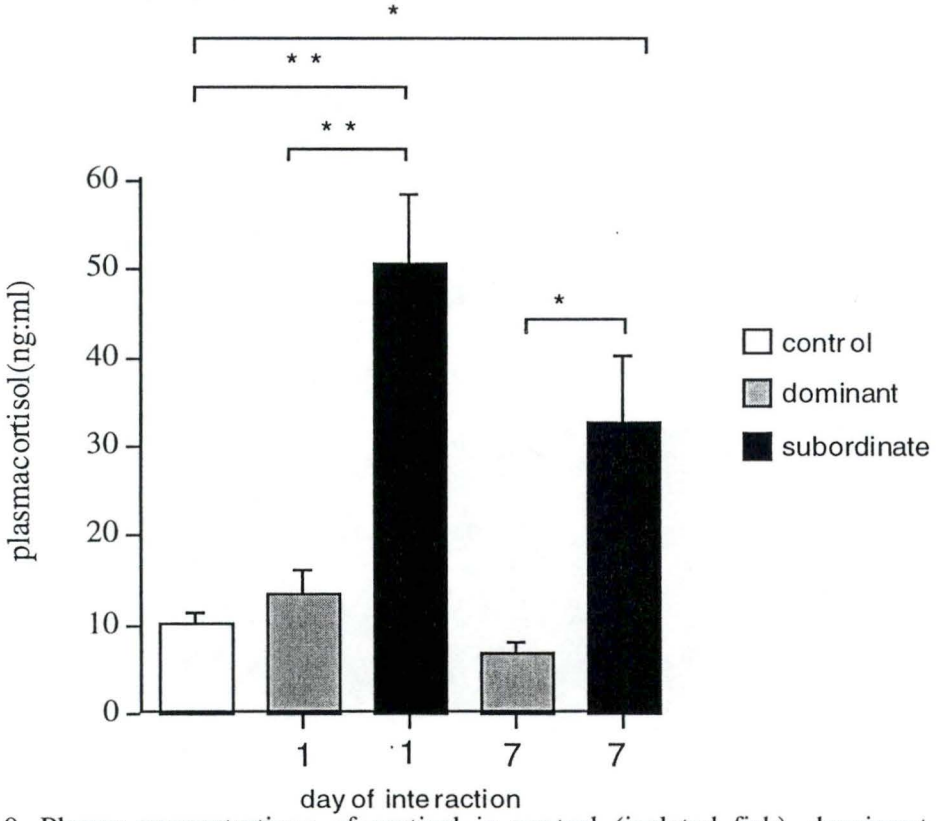


Figure 10. Plasma concentrations of cortisol in control (isolated fish), dominant and subordinate rainbow trout after one and seven days of social interaction. Values are mean and S.E.M. from 12 fish (control and subordinate) and 6 fish (dominant). Significant differences between groups are denoted by asterisks, *P<0.05, **P<0.01.

Plasma cortisol concentrations of dominant individuals did not differ from that of controls, neither after 1 nor after 7 days of social interaction (annexe 2; Figure 10).

3.4. POMC mRNA

POMC A and POMC B mRNA-labelled cells were found in *the pars intermedia* and *pars distalis* of the pituitary. In agrement with the results by Salbert *et al.* (1992), comparason of the localization of POMC A hybridisation signals with that of POMC B hybridisation signals in adjacent sections showed that POMC A and POMC B hybridisation signals in some cases were localized in the same cells. By contrast, POMC A was the only form detected in the hypothalamus.

Addition of 100-fold of the unlabelled probe to the hybridisation medium markedly reduced hybridisation signals. However, when 100-fold quantity of unlabelled mismatching probe was added to the hybridisation medium, i.e. the unlabelled POMC A probe was added to labelled POMC B probe and vice versa, hybridisation signals were not altered, confirming the specificity of the hybridisation signal.

Social rank had significant effects on pituitary POMC A ($F_{4,29}=5.174$, $P=0.0029$; annexe 6) as well as POMC B ($F_{4,26}=5.991$, $p<0.00121$; annexe 5) mRNA levels. Subordinate fish displayed a significant elevation of POMC A and POMC B mRNA levels compared to dominants and controls, after 1 as well as 7 days of social interaction (Figure 11 and 12). There was no difference in either POMC A or POMC B mRNA levels between subordinate fish sampled after 1 day and subordinates allowed to interact for 7 days (Figure 11 and 12).

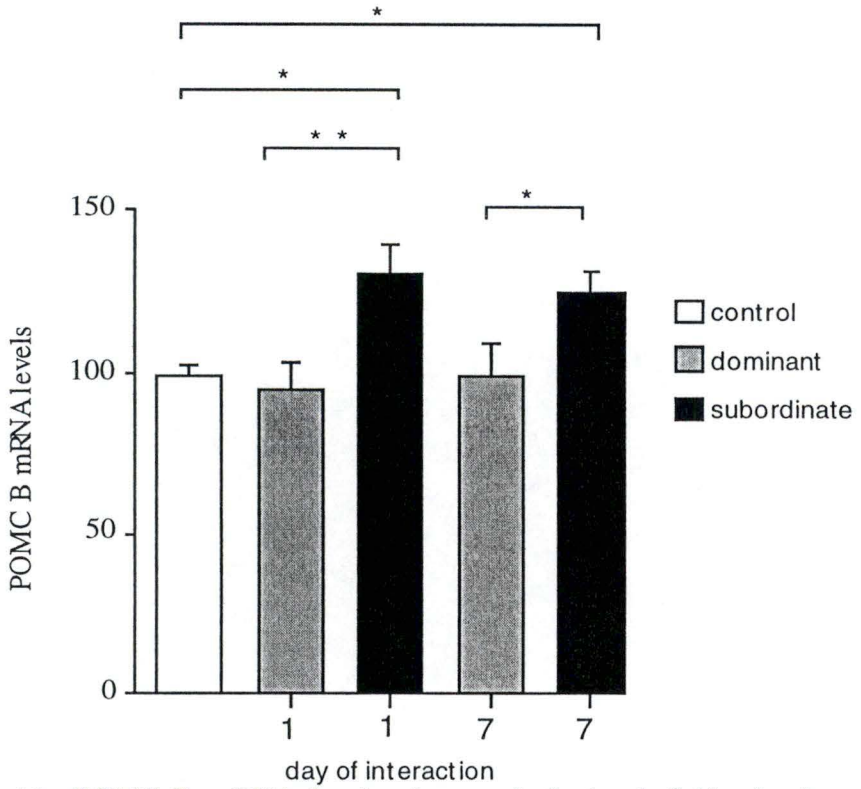


Figure 11. POMC B mRNA levels of control (isolated fish), dominant and subordinate rainbow trout after one and seven days of social interaction. Values are percent of the control and S.E.M. from 9 fish (control), 6 fish (subordinate after one day of social interaction), 8 fish (subordinate after seven days of social interaction), and 6 fish (dominant). Significant differences between experimental groups are denoted by asterisks, *P<0.05, **P<0.01.

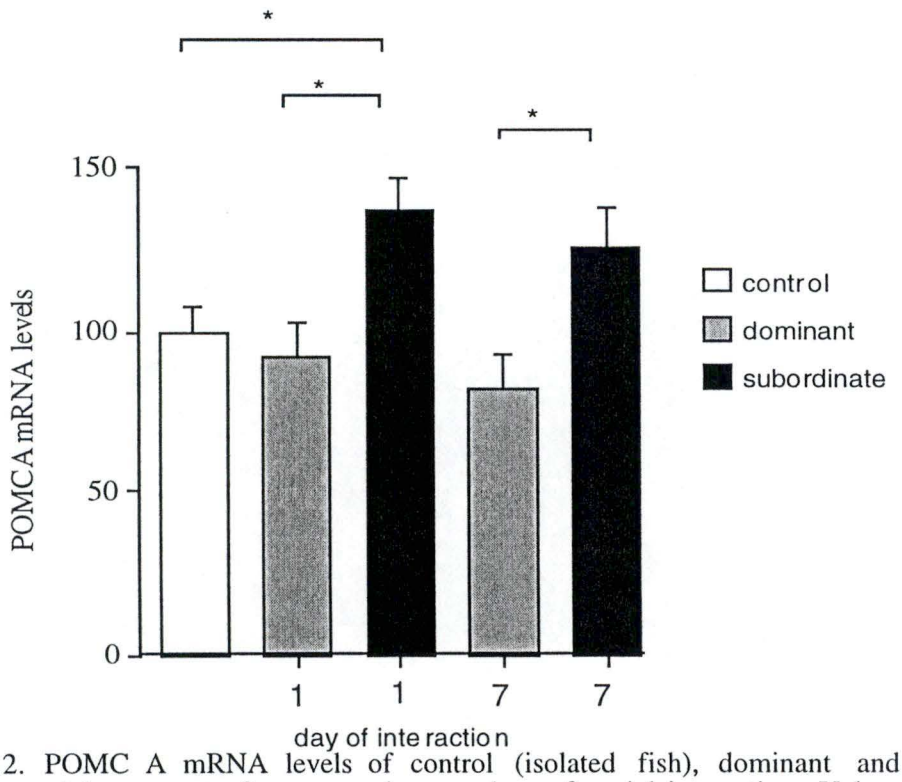
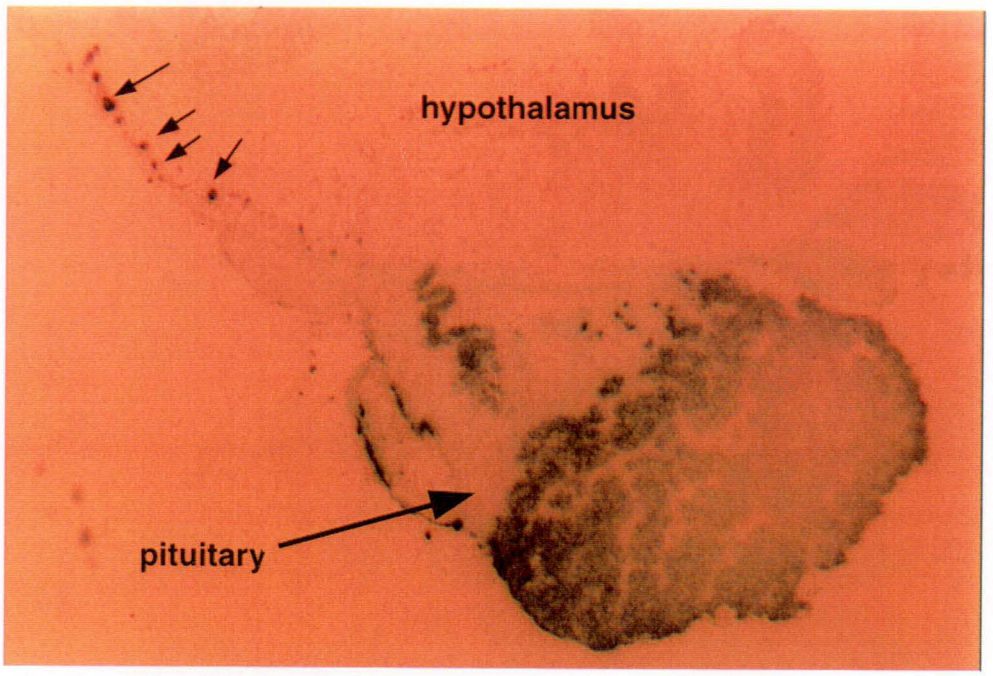


Figure 12. POMC A mRNA levels of control (isolated fish), dominant and subordinate rainbow trout after one and seven days of social interaction. Values are percent of the control and S.E.M. from 10 fish (control), 6 fish (subordinate and dominant). Significant differences between groups are denoted by asterisks, *P<0.05, **P<0.01.

1



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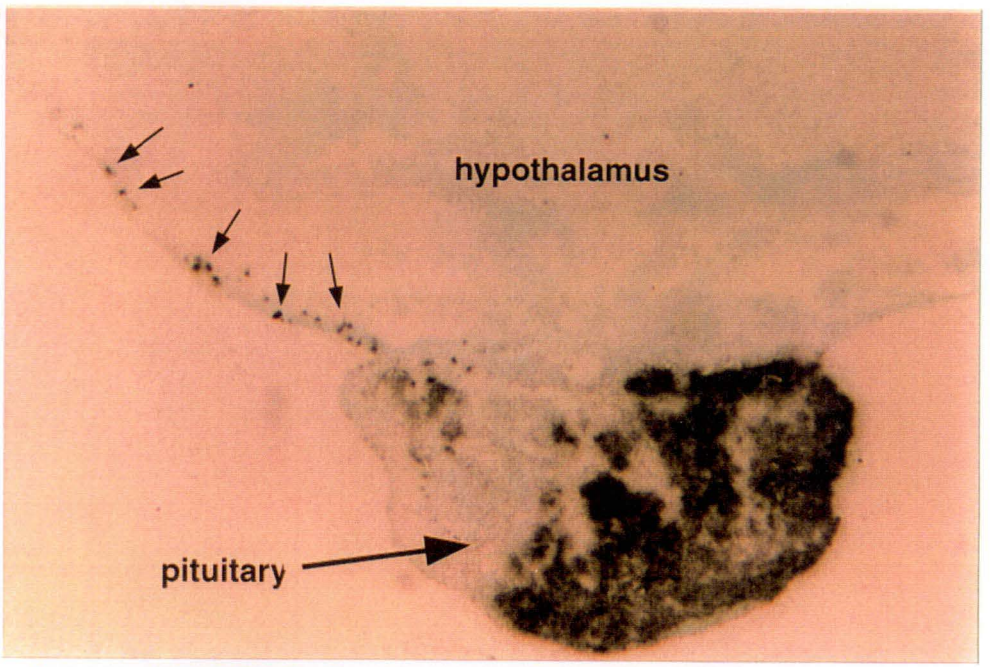
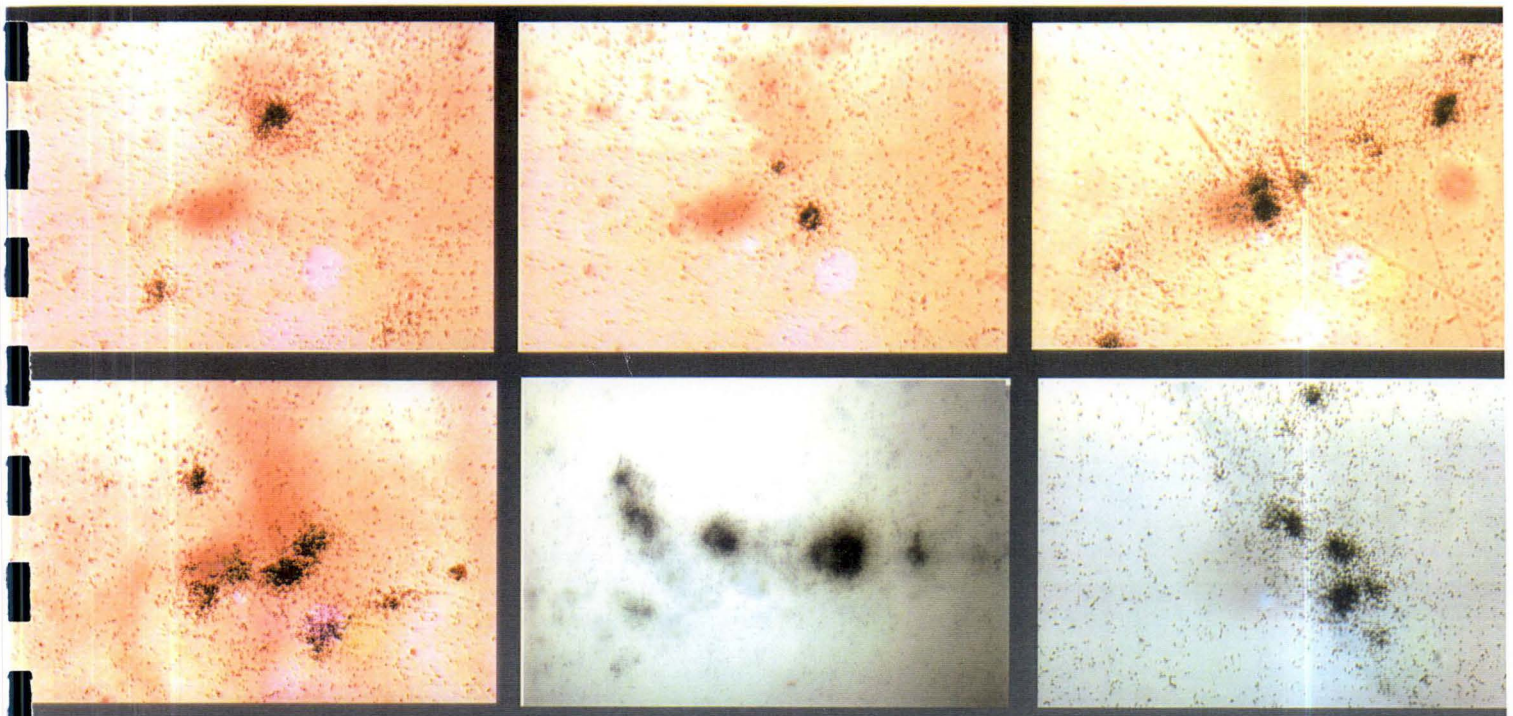
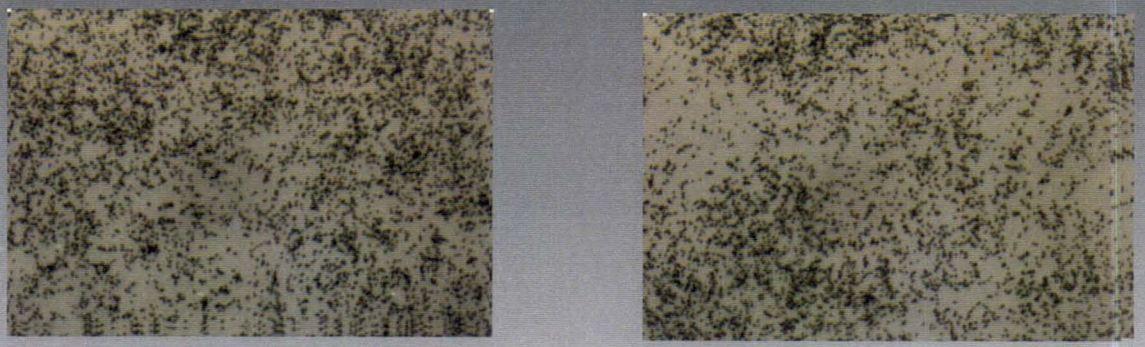


1 and 2: view of POMC A expression in pituitary and hypothalamus

Plate 1 : Hypothalamic and pituitary slices labelled by *in situ* hybridisation with POMC A cDNA probes in rainbow trout. Labelled slices were developed in photographic nuclear resolution. Silver grains represent area where POMC A cDNA probe hybridised with POMC mRNA in the tissue.



Hypothalamic neurones expressing POMC A
Magnification 40X



closed views of the silver grains in the pituitary
Magnification 100X

Plate 2 : Hypothalamic and pituitary slices labelled by *in situ* hybridisation with POMC A cDNA probes in rainbow trout. Labelled slices were developed in photographic nuclear resolution. Silver grains represent area where POMC A cDNA probe hybridised with POMC mRNA in the tissue.

3.5. Relationships between 5-HIAA/5-HT ratios, pituitary POMC mRNA levels and plasma cortisol concentrations

In the ten subordinate individuals (one from each group) where hypothalamus was analysed for 5-HT and 5-HIAA concentrations, hypothalamic and brain stem 5-HIAA/5-HT ratios were highly correlated (r^2=0.575, P=0.230) (Figure 14). Further, in these subordinate fish hypothalamic 5-HIAA/5-HT ratios were significantly correlated with plasma cortisol concentrations (r^2=0.929, P=0.0038) (Figure 13).

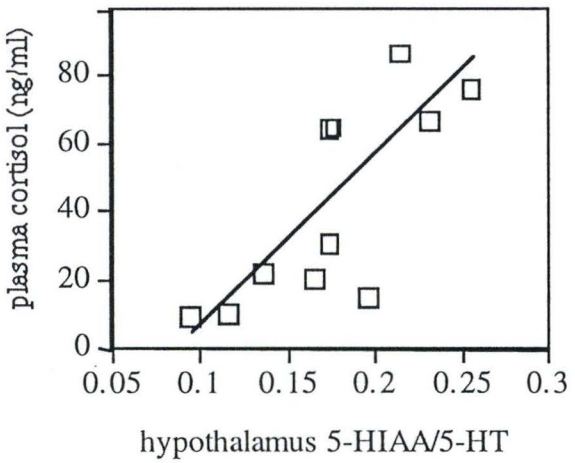


Figure 13. Relationship between 5-HIAA/5-HT in hypothalamus and plasma concentration of cortisol of rainbow trout. Cortisol concentrations are given in ng/ml. Correlation was tested Spearman's correlation test. The line is least square linear regression lines. P = 0.0038; r^2=0.929.

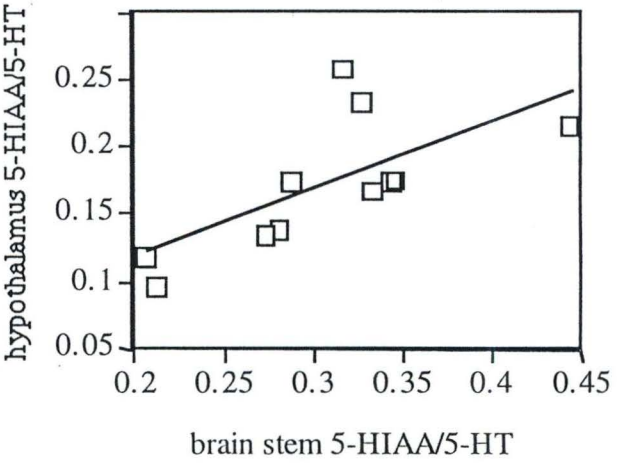


Figure 14. Relationship between 5-HIAA/5-HT ratio in brain stem and 5-HIAA/5-HT ratio in hypothalamus of rainbow trout. Correlation was tested using Spearman's correlation test. The line is least square linear regression lines. P=0.0230; r^2= 0.575.

In fish where hypothalamus was used for *in situ* hybridisation (controls, dominants and the remaining subordinates), correlations between brain stem 5-HIAA/5-HT ratios and pituitary POMC mRNA levels and plasma cortisol were tested. A significant correlation was found between brain stem 5-HIAA/5-HT ratios and and POMC B mRNA levels (r^2=0.247, P=0.0043) (Figure 15). Further, a similar, but not significant, relationship was indicated between brain stem 5-HIAA/5-HT ratios and POMC A mRNA concentrations. A significant correlation was also found between brain stem 5-HIAA/5-HT ratios and plasma cortisol concentrations (r^2=0.527, $p<0.0001$) (Figure 16).

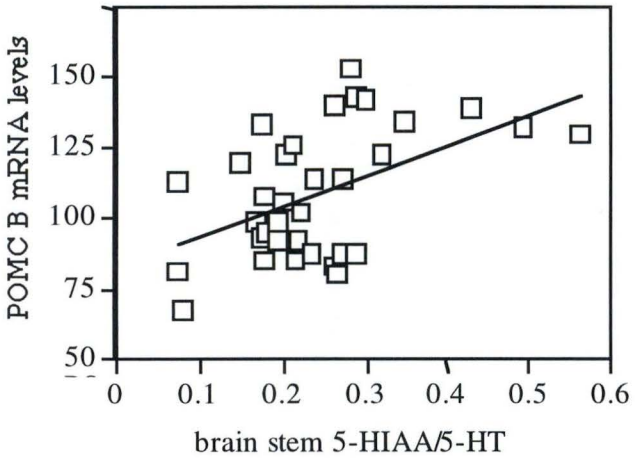


Figure 15. Relationship between 5-HIAA/5-HT ratio in brain stem and pituitary POMC B mRNA levels in rainbow trout. Values of POMC B mRNA level are transformed in procent of the control. Correlation was tested using Spearman's correlation test. Line is least square linear regression lines. P=0,004; r^2=0,247.

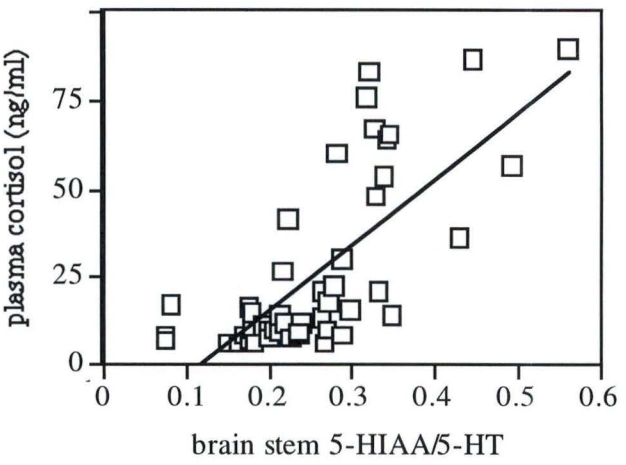


Figure 16. Relationship between 5-HIAA/5-HT ratio in brain stem and plasma concentration of cortisol. Cortisol concentrations are given in ng/ml of plasma. Correlation was tested usingSpearman's correlation test. The line is least square linear regression lines. P <0.001; r^2=0.527.

Telencephalic 5-HIAA/5-HT ratios showed a significant correlation with brain stem 5-HIAA/5-HT ratios (r^2=0.207, P=0.0028), whereas 5-HIAA/5-HT ratios in telencephalon and hypothalamus did not correlate. Still significant correlations were found between telencephalic 5-HIAA/5-HT ratios and POMC A (r^2=0.213, P=0.128), and POMC B (r^2=0.254, P=0.201) mRNA levels. Similarly, telencephalic 5-HIAA/5-HT ratios were significantly correlated with plasma cortisol concentrations (r^2=0.246, P=0.0011).

Pituitary POMC B mRNA concentrations were significantly correlated with plasma cortisol levels (r^2=0.170, P=0.0161) (Figure 17). A similar relationship was indicated betyween POMC A mRNA levels and plasma concentrations of cortisol, but in this case the correlation did not reach statistical significants.

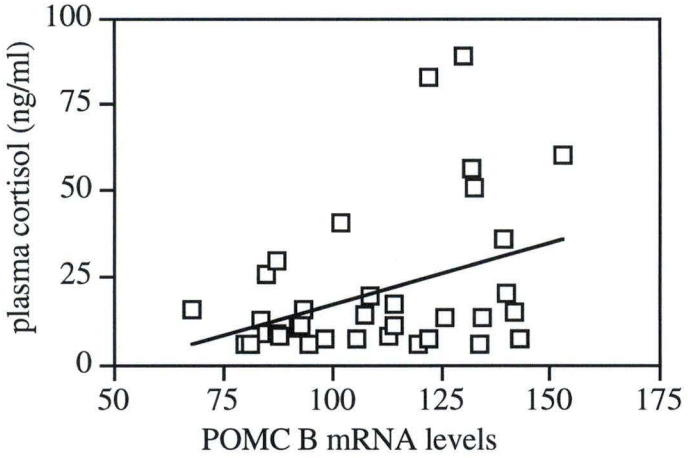


Figure 17. Relationship between plasma concentration of cortisol and pituitary POMC B mRNA levels. Cortisol concentrations are given in ng/ml of plasma. Values of POMCB mRNA levels are transformed in percent of the control. Correlation was tested using Spearman's correlation test. The line is least square linear regression lines. P = 0.0161; r^2=0.170.

Finally, there was a significant correlation between pituitary POMC A and POMC B mRNA concentrations (r^2=0.417, P=0.0.0020) (Figure 18).

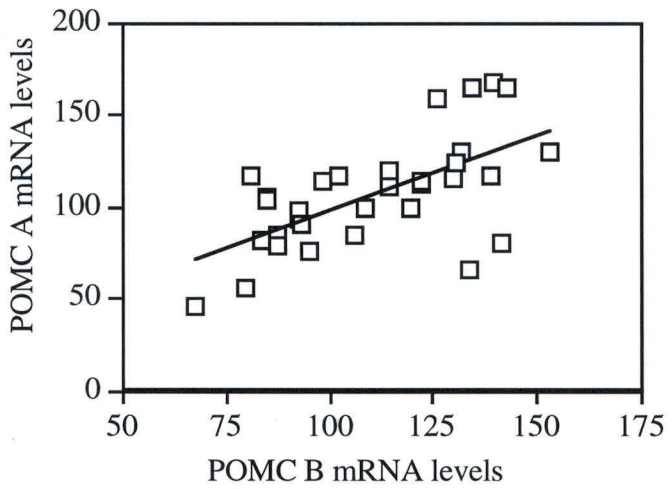


Figure 18. Relationship between pituitary POMC A mRNA levels and pituitary POMC B mRNA levels of rainbow trout. Values of POMC A and POMC B mRNA levels are transformed in percent of the control. Correlation was tested using Spearman's correlation test. The line is least square linear regression lines. P=0.0020; r^2=0.417.

4.Discussion

It is clear that prolonged subordinate experienced fish lost weight. Aggressivity is probably the major factor determining individual feeding ability (Huntingford and Turner, 1987). In a dominance hierarchy, high ranking fish are the most aggressive, thus eat more than the subordinate fish, which sometime even do not eat at all. Obviously, this must be one of the major causes of gain weight in dominants and loss weight in subordinates. However, fish suffering of food privation for one week in isolation do not lose so much weight (Huntingford and Thurner, 1987). Cortisol appears to play a role in the energy mobilisation (Pickering and Pottinger, 1995).

The switch from anabolic to catabolic in the stress experienced fish suggests that stress has a profound effect on intermediate metabolism. In the literature, non opinion yet exists about fish, either because studies have yet to be carry out, or because the results from different investigations are contradictory or conflicting. However, there can be little doubt that the effect of the above changes in the intermediary metabolism of stressed fish combined with the decrease in food intake can explain the loss of weight after prolonged stress.

The results of the present study clerly shows that short (1 day) as well as long term (7 days) subordination stress in rainbow trout is connected with an elevation in brain 5-HT activity, pituitary POMC expression and plasma cortisol concentrations. Further, the results suggest that these effects could be inter-related. The frequency of agonistic interactions decreased over time, reaching a low and constant level after 3-4 days. Thus, the stress experiensed by a subordinate individual is likely to change over time as the hierarchy gets more firmly established.

However, following a week of social interaction, plasma cortisol concentrations were still significantly elevated in subordinate fish, even though cortisol levels had declined by approximately 36%, as compared to one day subordinates. In spite of this chronic elevation of plasma cortisol, the pituitary POMC mRNA concentration remained elevated in subordinates and showed no decline in fish being subordinate for one week. Similarly, 5-HIAA/5-HT ratios in telencephalon of subordinate fish

are elevated after 1 day of social interaction and remains equally high even in fish subjected to one week of subordinate experience.

A stress-induced activation of the brain 5-HT system has been reported from mammals (Lamacz *et al.*, 1991), reptiles and several teleost species, including rainbow trout. Thus, an elevation of central 5-HT activity seems to be a phylogenetically old response to stress who´s function is still not well understod. In mammals, there are results indicating that brain 5-HT plays a coordinating role in stress reactions (Chaouloff, 1993). For instance, 5-HT is believed to stimulate the release of CRF from the hypothalamus as well as the release of ACTH from the pituitary (Chaouloff, 1993). The 5-HT system of the mammalian brain has also been implicated in the control of sympathetic nervous activity and catecholamine release from the adrenal medulla (Chaouloff, 1993). It is also believed to exert an inhibitory effect on sensory-motor reactivity to environmental stimuli (Davis, 1980; Soubrié, 1986), which might be a mechanism allowing the animal to cope with stress (Giral *et al.*, 1989).

In juvenile Arctic charr (*Salvelinus alpinus,* Linnée), subordinate individuals display elevated 5-HIAA/5-HT ratios in telencephalon, hypothalamus and brain stem already after 1 day exposure to a dominant conspecific (Winberg and Nilsson, 1993). Further, in this species 5-HIAA/5-HT ratios in these three brain parts of subordinate fish do not show any decline even after 21 days of social interaction (Winberg and Nilsson, 1993). By contrast, in the present study brain stem 5-HIAA/5-HT ratios of subordinate individuals declined after 1 week of social interaction, even though brain stem 5-HIAA/5-HT ratios of subordinates were still significantly higher than that of dominants and controls.

The juvenile rainbow trout used in the present experiment were extremely aggressive and possibly subordinate individuals were exposed to a more severe stress than the subordinate Arctic charr in the experiment by Winberg and Nilsson (1993). In fact, in the present experiment, brain stem 5-HIAA/5-HT ratios of subordinate fish were elevated by 97% after 1 day, and by 53% after 7 days of social interaction, as compared to controls. Brain stem 5-HIAA/5-HT ratios of subordinate Arctic charr were elevated by approximately 40%, as compared to controls (Winberg and Nilsson, 1993).

The effect of subordinate experience on telencephalic 5-HIAA/5-HT ratios were less pronounced (60% and 50% elevations after 1 and 7 days, respectively) but in this case it did not decline over time.

Hypothalamic 5-HIAA/5-HT ratios were highly correlated plasma cortisol concentrations, and with brain stem 5-HIAA/5-HT ratios. Similarly, telencephalic 5-HIAA/5-HT ratios showed a correlation with plasma cortisol concentrations, and brain stem 5-HIAA/5-HT ratios. Further, telencephalic as well as brain stem 5-HIAA/5-HT ratios correlated significantly with pituitary POMC mRNA levels.

In mammals, CRF is beleived to activate POMC synthesis and the 5-HT system of the mammalian brain has been found to stimulate CRF release (Chaouloff, 1993). The mammalian telencephalon and diencephalon are largely inervated by 5-HT cell bodies in the midbrain raphe area and using electron microscopic immunocytochemistry Liposits *et al.* (1987) showed that 5-HT terminals make direct synaptic contact with CRF containing neurones. The organization of the brain 5-HT system seems to be remarkebly constant throughout the vertebrate subphylum. Thus, even in teleosts, 5-HT cell bodies are mainly localized to the midbrain raphe region.

In mammals, the stimulatory effect of 5-HT on CRF release is medited by 5-HT_{1A} and 5-HT_2 receptors (Dinan, 1996). Recently, Winberg and Nilsson (1996) characterized three 5-HT receptors in the Arctic charr brain. One of these receptors shows a pharmacological profile strikingly similar to the mammalian 5-HT_{1A} receptor. Stimulation of this 5-HT_{1A}-like recceptor by 8OH-DPAT, a specific 5-HT_{1A} receptor agonist, elevates plasma cortisol concentrations in rainbow trout (Winberg *et al.*, in prep.). However, in these and other non-mammalian vertebrates 5-HT cell bodies are also found in thalamic and hypothalamic areas.

The relationships between brain 5-HIAA/5-HT ratios, pituitary POMC expression and plasma cortisol reported in the present study suggest that the brain 5-HT system acts stimulatory on the HPI axis in rainbow trout.

The correlation between POMC A and POMC B mRNA levels indicates that these no differences in control of expression in both genes.

5. Summary

In teleosts, like in many other vertebrates, social systems may often be based on a dominance hierarchy, a social structure where agonistic behaviour is an important component. Fish occupying low positions in a hierarchy are likely to be subjected to stress.

The immediate neuroendocrine changes occurring in response to stress are dominated by changes in the sympathetico-chromaffin system and the hypothalamic-pituitary-interrenal (HPI) axis for a recent review (see Pickering and Pottinger, 1995). The HPI axis consists of a series of hormonal pathways, the major components being hypothalamic corticotropin releasing factor (CRF), pituitary adrenocorticotropin (ACTH) and interrenal cortisol. Corticosteroid release is stimulated by ACTH secretion from the pituitary (rostral pars distalis), which in turn is regulated by corticotropin releasing factor (CRF). However, recent studies have shown that several other hormones/neurotransmitters are involved in the regulation of ACTH secretion from the teleost pituitary and the mechanisms responsible for modulation of neuroendocrine responses to chronic stress and acclimation, are largely unknown.

ACTH is synthesised from a large precursor protein, pro-opiomelanocortin (POMC), also giving rise to several other biologically active peptides, e.g. α-melanocytestimulating hormone (α-MSH), β-lipotropin (β-LPH) and the endogenous opiod β-endorphin, all believed to be involved in the neuroendocrine stress response. The posttranslational processing of POMC differs between different cell types. Specifically, corticotrops mainly produce ACTH, whereas the major POMC derived peptides released from melanotrops are α-MSH and β-endorphin.

As a result of the tetraploid status of their common ancestor salmonid fish possess two non-allelic copies of the POMC gene. These two forms, referred to as POMC A and POMC B, show relatively low sequence homology, despite the high conservation of some peptide sequenced (α-MSH, β-MSH, and β-endorphin), and are both expressed by melanotrops and corticotrops of the pituitary (Salbert *et al.*, 1992).

In mammals, the effect of stress on pituitary POMC mRNA levels varies according to the nature of the stimulus (Aguilera, 1993). Chronic stress paradigms (e.g. repeated foot shock, swim stress, cold exposure) associated with hypersensitivity of the pituitary ACTH response to a novel stress usually results in an elevation of POMC mRNA levels, whereas other stress paradigms (e.g. osmotic stress), not associated with sensitation of the stress response, appears to have the opposite effect on POMC mRNA concentrations (Aguilera, 1994).

There are no studies on the effect of stress on POMC mRNA levels in fish. However, stress-induced effects on plasma levels of POMC derived peptides seem to vary depending on the stimulus. Elevated plasma levels of α-MSH and β-endorphin has been reported in fish exposed to temperature shock (Sumpter *et al.*, 1985), noise (Malo-Michelle, 1980), and low environmental pH in the presence of aluminium (Balm *et al.*, 1987). In contrast, rainbow trout exposed to confinement stress displayed a transient increase in plasma ACTH concentrations but a reduction in plasma levels of α-MSH and β-endorphin (Balm and Pottinger, 1995).

Social stress is more complex than stress in general, and most likely, the stress experienced by subordinate individuals initially results from losing fights, whereas it later on probably also relates to being constantly threatened and having to inhibit one's own aggression (Zayan, 1991). In salmonids, subordinate fish display elevated plasma cortisol levels and increased interrenal cell sizes, suggesting a chronic activation of the interrenal stress response in these individuals. In a fish exposed to chronic social stress the HPI axis is probably subjected to two opposing drives: negative feedback imposed by a sustained elevation plasma cortisol concentrations, and stimulation due to the continuos perception of stressful stimuli (Balm and Pottinger, 1993). Further, as stated above, the nature of the stress is likely to change over time, as the hierarchy is established. By using two oligonucleotide probes in this present study, complementary to POMC A and POMC B mRNA, respectively, we were able to quantify each mRNA separately by *in situ* hybridisation.

Experience of a low social position results in a stress-induced increase in brain serotonergic (5-hydroxytryptamine, 5-HT) activity, as indicated by elevated brain levels of 5-hydroxyindoleacetic acid (5-HIAA, the major 5-HT metabolite) and 5-HIAA/5-HT ratios, in various teleost species, including rainbow trout.The 5-HT system of the mammalian brain seems to play an important role in behavioural as well as neuroendocrine stress responses (Chaouloff, 1993). For

instance, 5-HT is believed to act stimulatory on the release of CRF and ACTH from the mammalian hypothalamus and pituitary, respectively (Dinan, 1996).

Further, subordinate fish are characterised by a general inhibition of competitive behaviour (Abbott *et al.*, 1985; Winberg *et al.*, 1992a; McCarthy *et al.*, 1992; Winberg *et al.*, 1993), a behavioural inhibition that could well be mediated by a stress-induced increase in brain 5-HT activity (Winberg and Nilsson, 1993; Davis, 1980; Soubrié, 1986). Such an inhibition of competitive behaviour might represent a passive cooping strategy in subordinate fish, lowering the frequency of agonistic interactions with dominant individuals and, thus, possibly reducing the stress in subordinates.

In the present study we report effects of short (1 day) and long term (7 days) subordination stress on the activity of the brain 5-HT system and HPI axis in rainbow trout, as indexed by brain 5-HIAA/5-HT ratios and, pituitary POMC mRNA levels and plasma cortisol concentrations, respectively. Furthermore, brain 5-HT activity has here been directly correlated with pituitary POMC mRNA expression and plasma cortisol concentrations.

6. Conclusions and future researches

In conclusion, the results of this present study suggest that in rainbow trout, subordinate experience in a dominance hierarchy increases brain serotonergic activity, POMC A and POMC B expression, and the release of cortisol. Serotonergic system might be socially induced and has been previously hypothesised to act inhibitory on the neuronal circuities involved in aggressive behaviour. The new results suggest that the serotonergic system might have a control on the HPI by acting stimulatory in the hypothalamus or/and in the pituitary. We hypothesize that the expression of POMC gene might be induced after stimulation of the HPI, providing more ACTH which is released in the blood. ACTH, in turn act stimulatory on the release of cortisol from the interrenal.

In fish, a lot of researches need to be done about POM C expression, and relation between serotonergic system and HPI:

- Relation between serotonergic activity and CRF release is still to be discover.

- Interesting studies about distribution of 5-HT receptor in the hypothalamus and pituitary could providing more evidences that serotonergic system has a regulatory control on HPI axis.

- Future studies could be focused on the degree of control of the HPI axis by the serotonergic system wich might differ between juvenile and sexual active fish since POMC A was the only one detectable in the hypothalamus of sexually inactive fish, whereas, both POMC A and POMC B genes were expressed in the hypothalamus of sexually active fish.

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ANNEXE 1 : results table

group	cortisol (ng/ml)	brain stem 5-HIAA/5HT	telencephalon 5-HIAA/5HT	POMC B mRNA levels (percent of the control)	POMC A mRNA levels (percent of the control)	speciphic growth rate	Brain stem 5-HIAA (ng/ml)	Brain stem 5-HT (ng/ml)	Telencephalon 5-HIAA (ng/ml)	Telencephalon 5-HT (ng/ml)	hypothalamus 5-HIAA/5-HT
control	8.41	0.2	•	•	66.801	1.002514662	30.6030546	198.1652	•	•	•
control	10.37	0.196	•	92.27	94.104	0.132362693	10.7092856	54.6737213	•	•	•
control	15.7	0.177	0.388	93.2	90.632	0.138026247	13.6779218	77.1131701	58.2599072	150.1544	•
control	7.6	0.202	0.35	105.844	84.31	-0.72398506	16.0239773	79.1866718	43.4482759	124.137931	•
control	14.52	0.178	0.29	107.458	•	-0.92915883	11.2655548	50.2256	66.0390698	227.72093	•
control	5.9	0.163	0.292	•	150.964	-0.76890597	27.2265323	167.025862	38.1202671	130.54886	•
control	7.01	0.227	0.283	•	111.203	-1.67939475	19.8719034	87.6170288	19.242568	67.9949399	•
control	7.77	0.17	0.358	98.512	113.657	-0.58705809	18.382862	108.20821	12.8690749	35.9471366	•
control	9.22	0.272	0.21	87.196	85.433	0.118677437	14.5693854	53.5424914	20.2684546	98.265655	•
control	19.93	•	•	108.448	99.391	-0.90282745	•	•	•	•	•
control	7.94	0.075	0.217	112.745	•	-0.54050763	19.325565	80.358545	53.2097286	245.206123	•
control	9	0.178	0.296	84.974	103.506	1.128569228	•	•	18.5161024	62.5544	•
dom. 1 day	11.611	0.22	0.335	92.443	97.723	0.124223618	16.6256983	75.571356	13.7622053	41.0812098	•
dom. 1 day	11.42	0.24	0.365	114.264	111.512	-0.60632494	22.7239819	94.6832579	24.8739653	68.14785	•
dom. 1 day	7.85	0.237	•	87.541	79.094	0.464037956	22.6598	98.26541	•	•	•
dom. 1 day	16.28	0.083	0.299	67.684	45.166	0.554018038	15.21341	170.2235	26.8592242	89.8301812	•
dom. 1 day	25.94	0.218	0.206	84.554	104.977	0.528170242	31.1717814	120.213	18.674827	90.6545	•
dom. 1 day	7.05	0.203	0.221	122.183	113.657	-0.26536946	18.6191774	91.7200855	26.6383929	120.535714	•
sub. 1 day	29.65	0.289	0.212	87.515	•	-0.39411366	28.3669294	98.155465	37.8540563	178.556869	•
sub. 1 day	53.66	0.341	0.429	•	•	-0.42359669	27.4339751	80.45154	47.3038738	110.26544	0.17366082
sub. 1 day	75.6	0.317	0.469	•	•	0.373504001	23.982448	75.65441	28.2926501	60.32548	0.25698874
sub. 1 day	36.04	0.43	0.201	139.232	116.873	0.567889974	22.432367	52.1682952	26.3677602	131.182887	•
sub. 1 day	13.89	0.348	0.459	134.361	164.168	-0.03365305	51.266502	147.317534	74.4200167	162.135113	•
sub. 1 day	56.44	0.494	0.821	132.012	130.084	-0.1578548	47.9385039	97.0415058	202.438356	246.575342	•
sub. 1 day	89.54	0.562	0.75	130.106	116.005	0.066755796	40.925932	72.8219431	150.26545	198.498489	•
sub. 1 day	86.66	0.445	0.538	•	•	-0.30931036	79.3010691	178.20465	102.381608	190.300386	0.21542655
sub. 1 day	20.28	0.264	0.445	139.828	167.217	-1.1331566	40.9873074	155.254952	61.1141655	137.335203	•
sub. 1 day	60.17	0.282	0.417	152.9	130.431	-5.94889034	27.0503198	95.9231198	70.26854	167.2498	•
sub. 1 day	21.65	0.281	0.527	•	•	-2.36417631	30.9764691	110.236545	50.1283636	95.1202346	0.13549556
sub. 1 day	66.47	0.327	0.433	•	•	3.622647102	39.4082415	120.5145	30.4682182	70.3654	0.23171135
dom. 7 days	6.06	0.175	0.387	133.715	66.325	2.166496549	15.787702	90.21544	20.2418132	52.3044269	•
dom. 7 days	5.76	0.183	0.164	94.865	76.338	0.943199624	17.28497	91.745	18.0999256	110.3654	•
dom. 7 days	5.47	0.15	0.232	119.442	99.141	0.68336009	25.3265214	46.0663644	42.8339527	184.629107	•
dom. 7 days	5.75	0.268	0.216	79.811	55.594	0.133455626	20.720052	77.3136268	20.6614303	95.65477	•
dom. 7 days	6.05	0.075	0.328	81.005	117.152	-0.13657575	15.3265	124.32565	34.8549134	106.26498	•
dom. 7 days	12.69	0.263	0.208	83.378	82.621	2.405585475	32.4431569	123.358011	31.3384032	150.6654	•
sub. 7 days	63.79	0.344	0.36	•	•	-2.01071233	32.0311492	93.1138058	102.666286	285.184128	0.17366082
sub. 7 days	82.68	0.32	0.65	121.918	112.466	-5.27563524	21.5922729	67.4758529	48.5109652	74.6322542	•
sub. 7 days	15.32	0.3	0.524	141.648	79.821	-4.36432897	60.075795	150.26525	31.5082199	60.1301907	•
sub. 7 days	65.01	0.345	0.347	•	•	-1.95520892	52.5673911	152.36925	61.0329341	175.887418	0.1754386
sub. 7 days	9.38	0.207	0.4	•	•	-4.0650306	36.2648	180.2659	35.6195802	89.0489506	0.11645499
sub. 7 days	7.51	0.29	0.581	142.704	163.958	-1.99889077	35.8282066	123.54554	50.9318182	87.6623377	•
sub. 7 days	8.78	0.213	0.35	•	•	-0.28067139	29.41876	140.262	69.414275	198.3265	0.09517544
sub. 7 days	13.42	0.213	0.442	125.993	158.848	-0.4788956	24.2078888	113.65206	45.0623033	101.950913	•
sub. 7 days	47.67	0.33	0.284	•	•	-1.44935132	30.1419643	91.3392857	23.5587172	82.9532295	•
sub. 7 days	17.52	0.273	0.497	114.235	120.013	-0.48882796	20.542911	75.2487584	72.7242705	146.3265	•
sub. 7 days	40.92	0.222	0.464	102.149	116.326	-0.9939789	29.5700439	133.198396	74.5576827	160.684661	•
sub. 7 days	19.94	0.334	0.414	•	•	0.200856937	56.0260376	167.742628	72.3903761	174.855981	0.16549878

ANNEXE 2 : plasma cortisol

plasma cortisol (ng/ml)

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
8.41	11.611	29.65	6.06	63.79
10.37	11.42	53.66	5.76	82.68
15.7	7.85	75.6	5.47	15.32
7.6	16.28	36.04	5.75	65.01
14.52	25.94	13.89	6.05	9.38
5.9	7.05	56.44	12.69	7.51
7.01		89.54		8.78
7.77		86.66		13.42
9.22		20.28		47.67
19.93		60.17		17.52
7.94		21.65		40.92
9		66.47		19.94

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	12	123.37	10.280833	17.77989924
dom. 1 day	6	80.151	13.3585	48.7563175
sub. 1 day	12	610.05	50.8375	41.2265879
dom. 7 days	6	41.78	6.963333	7.91902666
sub. 7 days	12	391.94	32.6616666	42.1288459
Sum	48			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14033.6596	4	3508.4149	9.62098	0.00001199	2.5888340
Within Groups	15680.4960	43	364.6626			
Total	29714.15564	47				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	12	17.7798992	195.578891	31.658754
dom. 1 day	6	48.75631	243.781587	19.43417389
sub. 1 day	12	41.22658	453.492466	40.90991
dom. 7 days	6	7.9190266	39.59513	10.3463415
sub. 7 days	12	42.1288459	463.417304	41.1480595
Sum	48		1395.86538	143.497246

χ^2_{obs}= 8.614

χ^2= 9.49

Newman's test

Groups	*P-value*
control vs sub. 1 day	0.000533
control vs sub. 7 days	0.027430
control vs dom 1 day	0.739356
control vs dom 7 days	0.719898
dom. 1 day vs sub. 1 day	0.000665
dom. 7 days vs sub. 7 day	0.020127
sub. 1 day vs sub. 7 days	0.03471

ANNEXE 3 : brain stem 5-HIAA/5-HT

brain stem 5-HIAA/5-HT

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
0.2	0.22	0.289	0.175	0.344
0.196	0.24	0.341	0.183	0.32
0.177	0.237	0.317	0.15	0.3
0.202	0.083	0.43	0.268	0.345
0.178	0.218	0.348	0.075	0.207
0.163	0.203	0.494	0.263	0.29
0.227		0.562		0.213
0.17		0.445		0.213
0.272		0.264		0.33
		0.282		0.273
0.075		0.281		0.222
0.178		0.327		0.334

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	11	2.038	0.1852727	0.00229381
dom. 1 day	6	1.201	0.2001666	0.00347816
sub. 1 day	12	4.38	0.365	0.009146
dom. 7 days	6	1.114	0.1856666	0.00527986
sub. 7 days	12	3.391	0.282583	0.00304335
Sum	47			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.25428264974	4	0.0635706	13.295641	4.499E-07	2.594262
Within Groups	0.20081526515	42	0.00478131			
Total	0.45509791489	46				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	6	0.00229381	0.0114690	-30,38768761
dom. 1 day	12	0.00347816	0.0382598	-62,27374938
sub. 1 day	6	0.00914636	0.0457318	-23,47199448
dom. 7 days	12	0.0052798	0.05807853	-57,68239878
sub. 7 days	47	0.0030433	0.13999437	-266,5605426
Sum	83		0.29353	-440,3763729

χ^2_{obs}= 1.645

χ^2= 9.49

Newman's test

Groups	*P-value*
control vs sub. 1 day	0.000172
control vs sub. 7 days	0.030643
control vs dom 1 day	0.882268
control vs dom 7 days	0.956924
dom. 1 day vs sub. 1 day	0.019420
dom. 7 days vs sub. 7 days	0.019420
sub. 1 day vs sub. 7 days	0.020877

ANNEXE 4 : telencephalon 5-HIAA/5-HT

telencephalon 5-HIAA/5-HT

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
	0.335	0.212	0.387	0.36
	0.365	0.429	0.164	0.65
0.388		0.469	0.232	0.524
0.35	0.299	0.201	0.216	0.347
0.29	0.206	0.459	0.328	0.4
0.292	0.221	0.821	0.208	0.581
0.283		0.75		0.35
0.358		0.538		0.442
0.21		0.445		0.284
		0.417		0.497
0.217		0.527		0.464
0.296		0.433		0.414

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	9	2.684	0.29822	0.0036296
dom. 1 day	5	1.426	0.2852	0.00485820
sub. 1 day	12	5.701	0.4750833	0.0322449
dom. 7 days	6	1.535	0.255833	0.00705776
sub. 7 days	12	5.313	0.44275	0.0113214
Sum	44			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.35328	4	0.08832101	6.118257	0.00063	2.612303
Within Groups	0.562990	39	0.0144356			
Total	0.9162744	43				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	5	0.0036296	0.0145187	-22,474427
dom. 1 day	12	0.004858	0.05344020	-58,59796
sub. 1 day	6	0.0322449	0.16122496	-17,171963
dom. 7 days	12	0.0070577	0.077635	-54,489893
sub. 7 days	44	0.0113214	0.486823	-192,68531
Sum	79		0.79364289	-345,41955

χ^2_{obs}= 4.273

χ^2= 9.49

Newman's test

Groups	*P-value*
control vs sub. 1 day	0.016556
control vs sub. 7 days	0.022984
control vs dom 1 day	0.832190
control vs dom 7 days	0.767964
dom. 1 day vs sub. 1 day	0.017569
dom. 7 days vs sub. 7 days	0.019878
sub. 1 day vs sub. 7 days	0.599204

ANNEXE 5 : POMC B mRNA levels

POMC B mRNA levels (percent of the control)

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
	92.443	87.515	133.715	
92.27	114.264		94.865	121.918
93.2	87.541		119.442	141.648
105.844	67.684	139.232	79.811	
107.458	84.554	134.361	81.005	
	122.183	132.012	83.378	142.704
		130.106		
98.512				125.993
87.196		139.828		
108.448		152.9		114.235
112.745				102.149
84.974				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	9	890.647	98.9607	101.4735
dom. 1 day	6	568.669	94.77816	405.43592
sub. 1 day	7	915.954	130.8505	421.5363
dom. 7 days	6	592.216	98.70266	515.13180
sub. 7 days	6	748.647	124.7745	247.7641
Sum	34			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7588.969	4	1897.242	5.9917271	0.0012169	2.70139821
Within Groups	9182.66	29	316.64363			
Total	16771.6344	33				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	6	101.47351	507.3675	23.098989
dom. 1 day	7	405.43592	2432.615	36.029777
sub. 1 day	6	421.5363	2107.6816	30.219529
dom. 7 days	6	515.13180	2575.6590	31.222113
sub. 7 days	6	247.7641	1238.8206	27.56238
Sum	31		8862.1444	148.132

χ^2_{obs}= 8.297

χ^2= 9.49

Newman's test

Groups	*P-value*
control vs sub. 1 day	0.017521
control vs sub. 7 days	0.027352
control vs dom 1 day	0.679236
control vs dom 7 days	0.781255
dom. 1 day vs sub. 1 day	0.009832
dom. 7 days vs sub. 7 days	0.018856
sub. 1 day vs sub. 7 days	0.548693

ANNEXE 6 : POMC A mRNA levels

POMC A mRNA levels (percent of the control)

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
66.801	97.723		66.325	
94.104	111.512		76.338	112.466
90.632	79.094		99.141	79.821
84.31	45.166	116.873	55.594	
	104.977	164.168	117.152	
150.964	113.657	130.084	82.621	163.958
111.203		116.005		
113.657				158.848
85.433		167.217		
99.391		130.431		120.013
				116.326
103.506				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	10	1000.001	100.0001	511.6758
dom. 1 day	6	552.129	92.0215	682.17693
sub. 1 day	6	824.778	137.463	517.3500
dom. 7 days	6	497.171	82.8618	500.0893
sub. 7 days	6	751.432	125.2386	992.2845
Sum	34			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12891.2977	4	3222.824	5.17376	0.0028556	2.701398
Within Groups	18064.586	29	622.9167			
Total	30955.88456	33				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	6	511.675833	2558.3791	31.188456
dom. 1 day	6	682.1769	3410.88	32.62644
sub. 1 day	6	517.3500	2586.750	31.24359
dom. 7 days	6	500.0893	2500.4467	31.073933
sub. 7 days	34	992.2845	32745.3900	227.700
Sum	58		43801.85	353.8327

χ^2_{obs}= 7.88

χ^2= 9.49

Newman's test

Groups	*P-value*
control vs sub. 1 day	0.0298294
control vs sub. 7 days	0.078294
control vs dom 1 day	0.568345
control vs dom 7 days	0.445115
dom. 1 day vs sub. 1 day	0.013428
dom. 7 days vs sub. 7 days	0.023394
sub. 1 day vs sub. 7 days	0.383895

ANNEXE 7 : speciphic growth rate

speciphic growth rate (SGR)

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
1,002514662	0,1242236	-0,3941137	2,1664965	-2,0107123
0,132362693	-0,6063249	-0,4235967	0,9431996	-5,2756352
0,138026247	0,464038	0,373504	0,6833601	-4,364329
-0,72398506	0,554018	0,56789	0,1334556	-1,9552089
-0,92915883	0,5281702	-0,0336531	-0,1365758	-4,0650306
-0,76890597	-0,2653695	-0,1578548	2,4055855	-1,9988908
-1,67939475		0,0667558		-0,2806714
-0,58705809		-0,3093104		-0,4788956
0,118677437		-1,1331566		-1,4493513
-0,90282745		-5,9488903		-0,488828
-0,54050763		-2,3641763		-0,9939789
1,128569228		3,6226471		0,2008569

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	12	-3,6116875	-0,300974	0,6812084
dom. 1 day	6	0,7987555	0,1331259	0,89521
sub. 1 day	12	-6,1339549	-0,5111629	2,267889
dom. 7 days	6	6,1955216	1,0325869	1,0952408
sub. 7 days	12	-23,160675	-1,9300563	1,578955
Sum	48			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	41,439425	4	10,359856	4,3875313	0,0046061	2,588834
Within Groups	101,53177	43	2,361204			
Total	142,97119	47				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	6	0,6812084	3,406042	*-1,919435*
dom. 1 day	12	0,89521	9,84731	*-1,217666*
sub. 1 day	6	2,267889	11,339445	*4,094247*
dom. 7 days	12	1,0952408	12,047649	*1,000717*
sub. 7 days	48	1,578955	74,210885	*21,46787*
Sum	84		110,85133	23,425735

χ^2_{obs}= 2,66733

χ^2= 9.49

Newman's test

Groups	*P-value*
dom. 1 day vs sub. 7 days	0.0298294
dom. 7 days vs sub. 7 days	0.013428

ANNEXE 8 : brain stem 5-HIAA levels

brain stem 5-HIAA (ng/ml)

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
30.6030546	16.6256983	28.3669294	15.787702	32.0311492
10.7092856	22.7239819	27.4339751	17.28497	21.5922729
13.6779218	22.6598	23.982448	25.3265214	60.075795
16.0239773	15.21341	22.432367	20.720052	52.5673911
11.2655548	31.1717814	51.266502	15.3265	36.2648
27.2265323	18.6191774	47.9385039	32.4431569	35.8282066
19.8719034		40.925932		29.41876
18.382862		79.3010691		24.2078888
14.5693854		40.9873074		30.1419643
		27.0503198		20.542911
19.325565		30.9764691		29.5700439
		39.4082415		56.0260376

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	10	181.6560	18.16560	42.440086
dom. 1 day	6	127.0138	21.168974	33.461665
sub. 1 day	12	460.07006	38.33917	255.55867
dom. 7 days	6	126.8889	21.1481503	44.55388
sub. 7 days	12	428.2672	35.68893	179.4896
Sum	46			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3493.0248	4	873.2562	6.442294	0.0004057	2.59
Within Groups	5557.5704	41	135.5504			
Total	9050.5952	45				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	6	42.4400861	212.2004	*18.74046*
dom. 1 day	12	33.46166	368.0783	*38.61440*
sub. 1 day	6	255.5586	1277.793	*27.7172*
dom. 7 days	12	44.55388	490.0926	*41.7636*
sub. 7 days	46	179.4896	8077.035	*233.555*
Sum	82		10425.200	360.391

χ^2_{obs}= 7.95

χ^2= 9.49

Newman's test

Groups	*P-value*
control vs sub. 1 day	0.008691
control vs sub. 7 days	0.019074
control vs dom. 1 day	0.858875
control vs dom. 7 days	0.603962
dom. 1 day vs sub. 1 day	0.012220
dom. 7 days vs sub. 7 days	0.038191
sub. 1 day vs sub. 7 days	0.644758

ANNEXE 9 : brain stem 5-HT levels

brain stem 5-HT (ng/ml)

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
198.1652	75.571356	98.155465	90.21544	93.1138058
54.6737213	94.6832579	80.45154	91.745	67.4758529
77.1131701	98.26541	75.65441	46.0663644	150.26525
79.1866718	170.2235	52.1682952	77.3136268	152.36925
50.2256	120.213	147.317534	124.32565	180.2659
167.025862	91.7200855	97.0415058	123.358011	123.54554
87.6170288		72.8219431		140.262
108.20821		178.20465		113.65206
53.5424914		155.254952		91.3392857
		95.9231198		75.2487584
80.358545		110.236545		133.198396
		120.5145		167.742628

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	10	956.116	95.6116	2465.562
dom. 1 day	6	650.676	108.446	1121.69
sub. 1 day	12	1283.744	106.978	1398.124
dom. 7 days	6	553.02	92.17068	871.38716
sub. 7 days	12	1488.47	124.039	1329.329
Sum	46			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6111.0541	4	1527.763	1.0077	0.41462	2.59996
Within Groups	62157.4672	41	1516.035			
Total	68268.52143	45				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	6	2465.562	12327.811	*39.0508*
dom. 1 day	12	1121.695	12338.650	*77.2485*
sub. 1 day	6	1398.124	6990.6247	*36.2144*
dom. 7 days	12	871.3871	9585.2588	*74.4709*
sub. 7 days	46	1329.329	59819.805	*323.6593*
Sum	82		101062.151	550.644

χ^2_{obs}= 8.36

χ^2= 9.49

ANNEXE 10 : telencephalon 5-HIAA levels

telencephalon 5-HIAA (ng/ml)

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
	13.7622053	37.8540563	20.2418132	102.666286
	24.8739653	47.3038738	18.0999256	48.5109652
58.2599072		28.2926501	42.8339527	31.5082199
43.4482759	26.8592242	26.3677602	20.6614303	61.0329341
66.0390698	18.674827	74.4200167	34.8549134	35.6195802
38.1202671	26.6383929	202.438356	31.3384032	50.9318182
19.242568		150.26545		69.414275
12.8690749		102.381608		45.0623033
20.2684546		61.1141655		23.5587172
		70.26854		72.7242705
53.2097286		50.1283636		74.5576827
18.5161024		30.4682182		72.3903761

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	9	329.9734	36.663	389.88137
dom. 1 day	5	110.808	22.161	157.1555
sub. 1 day	12	881.303	73.44192	349.54869
dom. 7 days	6	168.030	28.005073	98.047262
sub. 7 days	12	687.977	57.3314523	319.7546
Sum	44			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15606.596	4	3901.6491	3.675305	0.0123891	2.61230
Within Groups	41401.816	39	1061.585			
Total	57008.4130	43				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	5	389.88137	1559.5255	23.863370
dom. 1 day	12	157.1555	1728.7105	55.6295
sub. 1 day	6	349.5486	1747.743	29.2832144
dom. 7 days	12	98.047262	1078.5198	50.4399459
sub. 7 days	44	319.7546	13749.450	248.004
Sum	79		19863.950	407.22094

χ^2_{obs}= 5.96

χ^2= 9.49

Newman's test

Groups	*P-value*
control vs sub. 1 day	0.79819
control vs sub. 7 days	0.219071
control vs dom 1 day	0.658125
control vs dom 7 days	0.603759
dom. 1 day vs sub. 1 day	0.027964
dom. 7 days vs sub. 7 days	0.192095
sub. 1 day vs sub. 7 days	0.336251

ANNEXE 11 : telencephalon 5-HT levels

telencephalon 5-HT (ng/ml)

control	dom 1 days	sub 1 day	dom 7 days	sub 7 days
	41.0812098	178.556869	52.3044269	285.184128
	68.14785	110.26544	110.3654	74.6322542
150.1544		60.32548	184.629107	60.1301907
124.137931	89.8301812	131.182887	95.65477	175.887418
227.72093	90.6545	162.135113	106.26498	89.0489506
130.54886	120.535714	246.575342	150.6654	87.6623377
67.9949399		198.498489		198.3265
35.9471366		190.300386		101.950913
98.265655		137.335203		82.9532295
		167.2498		146.3265
245.206123		95.1202346		160.684661
62.5544		70.3654		174.855981

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control	9	1142.5303	126.9478	5178.1749
dom. 1 day	5	410.249455	82.049891	4087.5469
sub. 1 day	12	1747.9106	145.65922	3075.0470
dom. 7 days	6	699.8840	116.6473	2101.3407
sub. 7 days	12	1637.643	136.47025	4362.8240
Sum	44			

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15942.56350	4	3985.640	1.1326464	0.3553378	2.6123032
Within Groups	137236.1162	39	3518.87477			
Total	153178.6797	43				

Bartlett's test

Groups	*Count*	*Variance*	*(ni-1)Si^2*	*(ni-1)lnSi^2*
control	5	5178.17497	20712.69	34.2088
dom. 1 day	12	4087.5469	44963.016	91.4727
sub. 1 day	6	3075.0470	15375.2354	40.155377
dom. 7 days	12	2101.34073	23114.7480	84.153639
sub. 7 days	44	4362.8240	187601.4332	360.377618
Sum	79		291767.1340	610.36817

χ^2_{obs}= 4.8462

χ^2= 9.49