UPPSALA
UNIVERSITET

Neural activation of winners and losers

Effect of stress and social interaction on immediate early genes c-Fos and EGR-1

CHINMAYA SADANGI

Degree project in biology 45 hp, 2013

Department of Neuroscience

Supervisor: Professor Svante Winberg

Table of Contents

LIST OF FIGURES

ABBREVIATIONS

GC - *Glucocorticoid*

HSPs - *Heat shock proteins*

HPI - *Hypothalamic pituitary interrenal axis*

HPA - *Hypothalamic pituitary adrenal axis*

CRH - *corticotrophin-releasing hormone*

CRF - *corticotrophin-releasing hormone*

ACTH - *Adrenocorticotropic releasing hormone*

CAs - *Catecholamine*

O_2 - *Oxygen*

POMC - *Proopiomelanocortin*

α-MSH – *alpha melanocyte stimulating hormone*

AVT - *Arginine vasotocin*

MCH - *Melanocorticotropic releasing hormone*

GH - *Growth hormone*

PRL - *Prolactin*

5-HT - *Serotonin*

NE - *Norepinephrine*

DA - *Dopamine*

L-DOPA - *(S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid*

DOPAC - *2-(3,4-Dihydroxyphenyl) acetic acid*

IEG - *Immediate early genes*

EGR-1 - *Early growth response*

qPCR - *quantitive polymerase chain reaction*

PF - *Paraformaldehyde*

HPLC - *High performance liquid chromatography*

DHBA - *2,3-Dihydroxybenzoic acid*

PCA - *Perchloric acid*

ABSTRACT

Social interaction has an impact on survival of an animal. There is a tendency of winners winning and losers losing in animals. Juvenile rainbow trout (*Oncorhynchus mykiss)* were used in these experiments that were allowed to socially interact for five days. For five minutes, one fish was allowed to see the other fish through a see through mirror and then they were allowed to interact socially. In the control group, fish were kept isolated for a period of 1 hour daily for five continuous days. After the period of social interaction the fish were sacrificed to collect blood plasma for cortisol analysis and brains were collected for qPCR and HPLC analysis for gene expression and brain monoamines respectively. Another subset of fish was cardially perfused and brains were collected for in-situ hybridization to check for gen expression of c-Fos and EGR-1 in different brain regions. Plasma cortisol levels were analyzed by radioimmunoassay (RIA) to determine the stress level in the fish. The number of aggressive attacks made by fish determined winners and losers.

INTRODUCTION

1.1 STRESS AND STRESSORS

"Everybody knows what stress is and nobody knows what it is" – Hans Selye [1]

Stress can simply be defined as the organism's response to environmental stressors or a stimulus. In other words, stress is a response by the body to an external stimulus such as fear or pain, which disturbs the normal physiological equilibrium. The body responds to stressful events by the sympathetic nervous system resulting in a fight or flight response. According to Hans Selye, who coined the term stress, stress can be defined as the 'nonspecific response of the body to any demand for change'. During stress neurotransmitters like adrenaline (epinephrine), nor-adrenaline (nor-epinephrine), dopamine and hormones like cortisol and glucocorticoids get secreted by the body. Stress can be considered as a state of threatened homeostasis that is re-established by a complex suite of adaptive responses [2].

A stressor can be considered to be any stimulus that produces a physiological stress and is measured by an increased in the Glucocorticoid (GC) concentration. The basic needs of most animals are similar in the animal kingdom therefore many types of stressors are universal. Examples of universal stressors include deviations from optimal ranges for environmental parameters (e.g., ambient temperature, oxygen supply), insufficient food availability, inadequate refuge from sunlight or predators, and the demands of social interactions such as territorial disputes [3].

Animals respond to stressors by releasing endocrine responses thereby increasing the immediate availability of energy and inhibiting processes that are not required for survival of the animal. The primary response to stress is achieved by activation of the HPA (Hypothalamic-pituitary-adrenal) axis in case of mammals and HPI (Hypothalamic-pitituary-interrenal) axis in case of vertebrates. This in turn, increases the GC concentration in the body. If the stressor is not eliminated then it may lead to harmful consequences such as loss of immune response, loss of muscle mass and reproductive loss.

Studying stress in fish species is advantageous because they are easy to keep in the laboratory environment and cheap. It could also provide advantage in the aquaculture industry by improving the welfare of the fish by reducing the amount of stress in the fish. Stressors in fish includes fluctuations in water salinity, pH, hardness, alkalinity, dissolved solids, water level or current, exposure to waterborne pathogens or toxicants,[3] maintaining a social hierarchy and anthropogenic sounds. In terms of behavior, an obvious example is the instinctive urge to fight or flee when faced with an adverse stressor such as a predator [3].

1.2 FISH RESPONSE TO STRESS

The fish response to stress can be divided into three categories: primary, secondary and tertiary [4]. In the primary phase the animal releases catecholamines secreted from the chromaffin and the HPI (hypothalamic-pituitary-interrenal) axis and is stimulated in releasing cortisol into circulation in response to the stress. The secondary response includes changes in the plasma and tissue ion levels together with an increase of stress proteins (HSPs). These are associated with adjustments in physiological levels such as metabolism, respiration. The tertiary response involves changes in the complete characteristics of the fish like growth and development, the immune system and ultimately the survival. The first two responses are considered adaptive and enable fish to adjust to stressors and maintain homeostasis [3].

1.3 THE HPI AXIS

In humans and other mammals, the HPA axis is responsible for stress response. The homologous neuroendocrine system in fish is called as the brain-pituitary-interrenal axis[5] or the HPI axis.

The cells in the hypothalamus produce corticotrophin-releasing hormone (CRF) in response to stressors and these CRF binds to specific receptors in the pituitary gland to produce adrenocorticotropic hormone (ACTH). ACTH is transported to the adrenal glands thereby producing adrenal hormones. The adrenal glands increase the secretion of cortisol and metabolic effects are initiated to decline the harmful effect of stress by using the negative feedback mechanism to both the hypothalamus and anterior pituitary decreasing the concentration of ACTH and cortisol in the blood once stress is reduced. The involvement of the hypothalamus and pituitary glands in corticosteroid secretion is well known. The CRH and ACTH are very important secretagogues. Cortisol is the end product of the HPI axis.

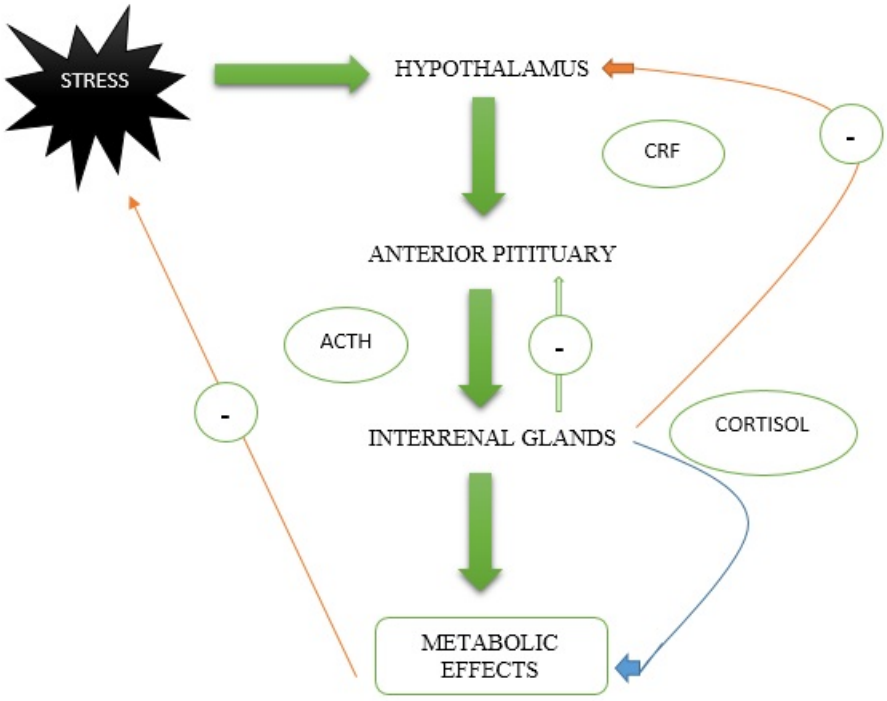


Figure 1: The hypothalamic-pituitary-interrenal (HPI) axis in teleost

The pathway for cortisol release begins in the HPI axis with the release of CRH, or factor (CRF), chiefly from the hypothalamus in the brain, which stimulates the corticotrophin cells of the anterior pituitary to secrete adrenocorticotropin[6]. The ACTH stimulates the interrenal cells, which is the homologue of the adrenal cortex to synthesize and release corticosteroids into circulation for distribution to the cells and other tissues. Cortisol is the principle corticosteroid in actinopterygian (*i.e.*, teleostean, other neopterygian and chondrostean) fishes whereas 1α-hydroxycorticosterone is the major corticosteroid in elasmobranchs [6]. The control of cortisol is mediated through the negative feedback mechanism at all levels of the HPI axis.

1.4 EFFECT ON CORTISOL

The major targets for cortisol are the gills, intestine and liver. The elevation of cortisol level is a reliable indicator of stress in fish. The plasma cortisol level rises after being exposed to a stressor. When the stressor is chronic, the cortisol level may still remain elevated, although well below peak level [5].

Higher level of cortisol up to 50 ng/ml can be explained by differences in assay specificity, husbandry conditions, capture procedures, or natural factors [7]. According to Barry et al, [8] netting induced cortisol secretion could be demonstrated from up to 2 weeks after hatching. Cortisol level also varies with feeding. In farmed Coho salmon, elevated level of cortisol can be seen after they reach the ocean under free-living condition. Thus not all elevated levels of cortisol can be ascribed to stressors. Conversely, the absence of clearly elevated levels does not always guarantee the absence of stressors. In salmonids, there is a slight increase in cortisol level, which can be considered chronic in case of depression of immune function and disease resistance.

After being exposed to stressors, fish produces stress proteins and they function in cellular restoration of affected functions, cellular protection and increasing the stress tolerance of the affected fish. According to Sanders, the best-known stress proteins are metallothioneins (metal-binding molecules produced in response to toxic metals) and heat shock proteins (chaperone-like molecules induced by rapid temperature changes and many other stressors) [9].

1.5 EFFECT OF CATECHOLAMINES

The release of CAs (adrenaline and nor-adrenaline) is an integral part of the physiological response to stressors in all vertebrates [10]. The function of CA includes respiratory and cardiovascular effects, blood oxygen (O_2) transport capacity, blood glucose and free fatty acid levels [5]. Because of the increase in the ventilation rate the stress response in turn increases O_2 uptake rate of the gills. In the stimulation of the ventilation rate, the role of circulating CA is unclear.

During chronic stress, there is high CA level, which could lead to desensitization of target cells by down regulation of these cells. But the effect of chronic stress is not just limited to moderation of CA release. Due to stressors there is a rapid rise in muscle and plasma lactate combined with decreased blood pH and O_2 content [5]. These results in immense CA release from the chromaffin cells and rise in ventilation, branchial blood flow, gas exchange and blood glucose level.

The stress is related mainly to the CAs on glucose release from the liver and facilitates hyperglycemia in a lot of teleost, which is the main carbohydrate storage in fish. The hyperglycemia observed during hypoxia in rainbow trout can be prevented by infusion of adrenoceptor antagonists[5].

In rainbow trout, plasma adrenaline and nor-adrenaline levels vary from <1 nM to > 200 nM during active stress [11]. In case of Antarctic teleost *Trematomus bernacchii* stress did not affect circulating CAs but the heart rate, ventral aortic blood pressure, and haematocrit raised. These fish likely depend on increased sympathetic nerve activity during acute stress [12].

1.6 EFFECT ON GROWTH AND APPETITE

Stress in fish is also characterized by reduction in food intake. Along with suppressed appetite, many other features such as searching for food, hunting for food can also be affected by stress. The effect of

stressors influence growth rates and is reflected on amount of food consumed, intestinal uptake and metabolic rates to name a few. Reduction in food intake is one of the major causes of growth suppression in brown trout under crowded conditions. In aquaculture, stocking density also affects food intake and varies from species to species. Stress also affects metabolic rates. According to Bonga et al, instead of showing a stress response, some fish species respond to hypoxia with down regulation of metabolic activity well below the standard metabolic rate, an adaptive reaction called metabolic depression [5]. The capacity of metabolic depression also varies with species wherein salmon showing the poorest and eels and carp show the best performance in terms of growth and food consumption.

1.7 NEUROTRANSMITTERS, NEUROPEPTIDES AND HORMONES

A. NEUROTRANSMITTERS

Brain structures like cholinergic, adrenergic and serotonergic systems are triggered due to stress.
According to Winberg et al [13], increase in serotonin rate has been reported in brains of Arctic char (*Salvelinus alpinus*) due to stress by netting or having a subordinate position in the hierarchy. It also affected the locomotor activity of these stressed animals

B. NEUROPEPTIDES

Neurons secreting CRH are present in the paraventricular nuclei of the hypothalamus. During stress, CRH is an important neuropeptide in growth and reproduction. POMC derived peptides like α-MSH and ACTH-like transmitters have been found in fish. There are two POMC genes in the hypothalamus of the rainbow trout [14]. Sexually active fish expressed both the genes whereas sexually inactive fish expressed only one gene. Arginine vasotocin (AVT) belongs to the vasopressin family and is expressed in fish. In Goldfish, it has been shown that AVT is related to nonspecific integrated stress response. This is comparable to the hypothalamic vasopressin release in mammals where it triggers the release of ACTH by CRH. MCH (Melanophore concentrating hormone) is secreted from the hypothalamus to the circulation and antagonize the effect of α-MSH at the pituitary and at the target cell level. When administered to rainbow trout, it depressed the release of CRH and ACTH in addition to α-MSH [15, 16]. According to Bird et al opioid factors like enkephalin and endorphin-like substances have been shown in the fish brain but their role is unknown [17].

C. HORMONES

In teleost fish, the growth hormone (GH) level is related to growth and hydro mineral control in seawater and it is also influenced by stressors. Prolactin (PRL) functions by the inhibitory control of the permeability of the integument to water and ions into the freshwater environment. A reduction in plasma prolactin in confined rainbow trout has also been reported [18]. However, plasma PRL level didn't elevate after lowering water level and chasing, which elevated plasma cortisol, Somatolactin and growth hormone level. Somatolactin belongs to the PRL/GH family and its function is related to gonadal maturation and spawning, hydro mineral and acid base regulation and energy mobilization. A rapid increase in Somatolactin level in rainbow trout is observed during chasing and low water levels.

1.8 EFFECT ON GROWTH AND ENERGY BALANCE DUE TO STRESS

There is a reduction in growth during stress, which is expressed as changes in body weight or other parameters such as condition factors of food conversion efficiency. These are reliable indicator of stress and have been applied to fish.
Reduced appetite has been mentioned in stressed fish. It has been shown that, 2 minutes of handling stress in brown trout (*Salmo trutta)* leads to reduced feeding activity. Stress also affects the capacity of food assimilation. There is an increase in metabolic rate due to acute and chronic stress. Brief

disturbances of steelhead trout (*Oncorhynchus mykiss)* increased metabolism up to 25% above standard metabolism [19]. Not every stressor increases the metabolic rate however instead of showing a stress response, some fish species respond to hypoxia with down regulation of the metabolic activity well below the standard metabolic rate, an adaptive reaction called metabolic depression [5]. Growth hormone plays the major role in growth under multiple endocrine controls. CAs and cortisol are the major hormones with growth-inhibiting effects [20]. Reports suggest that, in goldfish (*Carassius auratus auratus*) there is an increase in plasma growth hormones after injecting stress but remains unaffected in chum salmon (*Oncorhynchus keta*) after handling stress. But, in rainbow trout a decrease has been observed under acute handling and confinement tests and also an increase in plasma cortisol and ACTH level.

1.9 ROLE OF SEROTONIN AND DOPAMINE IN AGGRESSION AND MOTIVATION

Using Microdialysis studies in rats have shown a decrease in 5-HT release in the pre-frontal cortex during aggression, but not nucleus accumbens. Other studies have shown increased level of 5-HT during aggressive interaction in the brain regions believed to involved in regulating aggression when inhibition of aggression would be dysfunctional. In rainbow trout, during fights for social dominance, there is an initial rise of 5-HT and cortisol in both the winners and losers. Long-term elevation of brain 5-HT activity results in decrease in aggressive behaviour. Early activation of brain NE and DA systems have an effect on inhibitory effects of 5-HT on aggressive behaviour. Long-term stress induced elevation in the brain serotonergic activity is important in mediating behavioural inhibition in subordinate animals. DA is involved in aggressive behaviour and DA agonist and antagonist (Apomorphine and chlorpromazine respectively) caused reduced aggression. Even high level of 5-HT can reduce agonistic interaction before it starts.

1.10 DOMINANCE AND SUBORDINATION

The DA and NA are involved in regulation of agonistic behaviour. Experience effects aggressive behaviour either by learning or by status changes which would in turn affect subsequent competitions. This can be brought on by changes in brain neuromodulator activity and hormonal reaction to social interactions.

Social subordination can be characterized by rise in plasma cortisol and increased serotonergic activity. In aquaculture, with increasing stocking densities there is a decrease in formation of social hierarchies but a lower growth rate is common because the fish would be socially subordinate. Differences in behavior, hormone levels and neurochemistry associated with social status in various teleost appear mainly as a result of chronic stress in subordinates.

During social interaction fish undergo a lot of stress and results in an elevated level of cortisol. Social dominance in a hierarchy is prejudiced by previous interactions and capacity to react to stressful conditions, whereas social subordination results in lower number of attacks and longer attack latencies. Social standing in the hierarchy is also influenced by previous social interaction and capacity to respond to stressful conditions. A defeat in social interaction is considered as a powerful stressor, which leads to drastic alteration in physiology and behavior of the fish. Zayan highlights that social stress is generally measured as a physiological response that reflects both physical (aggression) and psychological stressors [21]. There is change in behavior after social defeat in teleost like reduced aggression, decrease in reproductive behavior and there is an increase in submissive and defensive behaviors towards agonist. During primary response, there is release of catecholamine from chromaffin tissues and the stimulation of the HPI axis releases corticosteroid hormones into circulation.

1.11 IMMEDIATE EARLY GENES (IEG)

Neurons interact in a time span of milliseconds creating an output commonly known as the action potential but there is transmission of neurons at a much slower pace, transmitting gene expression. According to Clayton, its function, however, is not directed toward immediate transmission of a synaptic signal but rather toward the experience-dependent modification of the underlying synaptic circuitry [22]. This is termed as immediate early gene (IEG) response and is assumed to be a part of consolidated memory. Following stimulation, IEG undergo regulation of gene expression but they do no require de novo synthesis of proteins. IEG are transient genes and their induction occurs within minutes. All cells in all tissues presumably have the capacity to mount a rapid genomic response to stimulation, and this general phenomenon is commonly referred to as "Immediate Early Gene" (IEG) activation [23-25].

IEG expression matches the activation of some specific circuitry of the brain, which are related to perception, and integration of primary stimuli in addition to behavioral, neuroendocrine and autonomic responses. Intense expression of c-Fos and other IEG in areas such as alocortex, neocortex, and parts of hypothalamus have been found due to immobilization stress. Two of the most widely expressed immediate early genes are c-Fos and EGR-1[22] which have been used as genomic markers. c-Fos is an indicator of immediate neural activity. c-Fos expression as a neuronal marker offers advantage because c-Fos is a delayed maker of brain activation and slows the process of immediate early gene activation.

The Zif-268 or EGR-1 genes respond to nerve growth factor treatment resulting in neuronal differentiation and response to serum treatment giving rise to mitosis. Studies have shown that EGR-1 is regulated by seizure activity, electroconclusive shock, neurodegeneration, apoptosis and drug-induced stress and so on. EGR-1 is believed to be involved in formation of associative memory but it is not known which aspect of learning, memory, storage or retrieval are implicated and whether EGR-1 plays any role in these.

The aim of the current report is to study the effect of stress and aggressive behavior due to social interaction on activation of immediate early genes 'c-Fos' and 'EGR-1' in juvenile rainbow trouts. The plasma cortisol levels have been directly correlated with the socially dominant and socially subordinate fish. We measured IEG expression by using qPCR to measure mRNA expression in the telencephalon region of the brain in addition to measuring brain monoamines and their metabolites from the brain stem using HPLC.

MATERIALS AND METHODS

2.1 Animals

Experiments were carried out at Uppsala University, Biomedical center (BMC) using the juvenile rainbow trout as model organism. These trout were acquired from a hatchery in Sweden and were kept in holding tanks for more than 3 weeks before starting the experiments. Uppsala tap water was supplied and a 12 hours day: 12 hours night light – dark regime was conserved. The juvenile trout were fed pellets according to 1.5% of their body weight. The size difference between a big and a small fish was 15-20 g. The mean S.E.M. for small fish was 104.8 ± 4.4 g (n=32) and the mean ± S.E.M. for large fish was 113.5 ± 4.6 g (n=32). The small fish were 8 % smaller than the larger fish.

2.2 Social Interaction

The fish were transferred to a glass aquarium for experiment and were separated from each other by PVC walls. Also, a one – sided see through mirror was placed along with the PVC wall. The trout were transferred from the holding tank, fin tagged or pit tagged, weighed and fork length was measured and were transferred to the glass aquarium. The fish were kept separated for 5 days before allowing them to interact. Pairs of fish were placed into aquaria and separated by a one-way mirror for 5 minutes prior to recording interactions. Set ups were such that either only the big fish could see the small fish, or vice versa (fig 1). The fish were allowed to interact for one hour every day for five consecutive days and recordings were made for 30 minutes. Attacks made by the fish were counted. Behaviors were recorded simultaneously in these pairs. In case of the controls, the big fish were allowed to see the small fish for one hour in two runs and the small fish were allowed to see the big fish for one hour in two runs. The social interaction and sampling were carried out between 11.00 and 17.00.

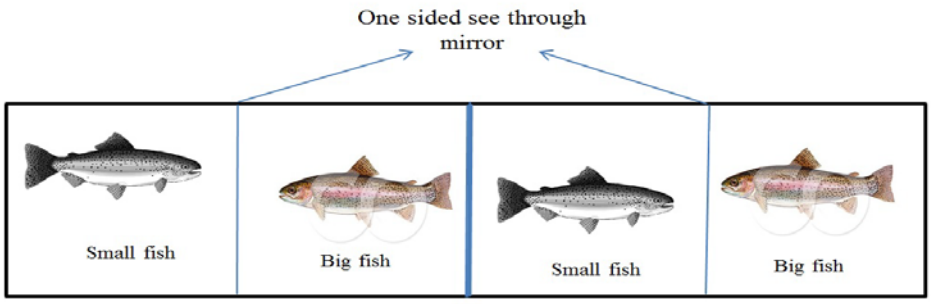


Figure 2: Experimental setup - Pairs of fish were plaed into aquaria and seperated by a one-way mirror for 5 minutes prior to recording interactions. Set ups were such that either only the big fish could see the small fish or vice versa

2.3 Brain and Blood collection

Upon sampling, the pair of fish were allowed to see through the one sided see through mirror for 30 minutes. The fish were netted and anaesthetized using benzocaine and blood (500 µL) was collected from near the tail fin that was subsequently centrifuged at 5000 g for 5 minutes at 4 degrees. Plasma was collected and stored at –80 degrees until further analysis using radioimmunoassay (RIA). Furthermore, the brain was collected and stored in tissue tek at -80 degrees.

2.4 Cardial Perfusion

Sixteen pair of fish and eight control fish were cardially perfused for In-Situ Hybridization. Upon perfusion, the big fish were allowed to see the small one for 30 minutes in one round and vice-versa in the second round through the one sided see through mirror. After social interaction for five days, the

chest of the fish was opened and the heart was punctured through the ventricle. Paraformaldehyde (PF) (4%) in phosphate buffer was pumped into the blood circulatory system until all blood had been washed out of the system and replaced by the PF. Thereafter, the brain of the fish was removed and transferred to the same fixative solution and kept overnight at 4 º C. The following day the brain was washed in phosphate buffer twice for one hour each followed by transfer to 30% sucrose solution overnight. The next day the brain had sunk to the bottom and was taken out of the sucrose solution. The brain was stored in tissue tek at -80 degrees.

2.5 RNA extraction

Micro-dissection of different brain region telencephalon (qPCR) and brain stem (HPLC) was performed by microRNA extraction using punches. RNA was extracted from the punches using Invitrogen Pure link Micro RNA Kit (12183-016) according to manufacturer's instruction. For quality and quantity measures, the total RNA was measured by using spectrophotometry (Nanodrop, Thermo Scientific).

2.6 qPCR

cDNA was prepared from 0,6 µg total RNA (Maxima First Strand cDNA Synthesis Kit for RT-qPCR, K1641, Fermentas) according to manufacturer instructions. After successful cDNA synthesis the reaction volume of 20 µl was diluted to 800 µl and further aliquot. 4 µl of diluted cDNA was used in each qPCR reaction. Primers were 19-25 nucleotides in length with melting point around 60 º C and formed products in the range of 82-219 bp. From an original set of five reference genes, one gene that displayed the smallest variation across treatment was selected (His (Histone)) and used for subsequent normalization of qPCR data of the genes of interest, c-Fos and EGR-1. qPCR was run using the ABI biosystem 7900 HT fast real time PCR system.

2.7 HPLC

The frozen brain samples (brain stem) were homogenized in 4% (wt/vol) icecold perchloric acid (PCA) containing 40 ng/ml DHBA (Dihydroxybenzoic acid, used as an internal standard) using a sonicator. Samples were centrifuged at 10,000 *g* for 10 min at 4 °C and the supernatants were analyzed for the following monoamines and monoamine metabolites using high performance liquid chromatography (HPLC) with electrochemical detection (HPLC-EC): Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), serotonin (5-hydroxytryptamine, 5-HT), and 5-hydroxyindoleacetic acid (5-HIAA). The mobile phase was delivered at 1 ml/min and consisted of 75 m*M* sodium phosphate, 0.7 m*M* octane sulfonic acid, in deionized water containing 10% methanol and brought to pH 3.1. Samples were analyzed for concentrations of monoamines (5-HT, NE and DA) and monoamine metabolites (5-HIAA, and DOPAC, respectively) by comparing it with standard solutions of known concentrations.

2.8 In-Situ Hybridization

In-situ was done using a *Onchorynhus mykiss* probe for c-Fos (278 bp) and EGR-1 probe. The probe was prepared by running a PCR using c-Fos primers designed from *Onchorynhus nerka* (AB111053.1) c-Fos mRNA sequence. The PCR product was cloned into the plasmid pCRII-TOPO and sequenced (See appendix). Digoxigenin was used to label the probe and was visualized using NBT/BCIP.

Tissues was taken out from – 80 °C and allowed to air dry at room temperature for one hour and then transferred to 65 °C for ten minutes. Slides were then rehydrated once in 2XSSC for one minute. Slides were treated with 10 µg/mL Proteinase K for five minutes followed by post fixation with 4 % Paraformaldehyde for five minutes and washing with 1 X KPBS twice for two minutes each. Freshly made 0.25 % acetic anhydride & 0.1 M TEA (pH 8) buffer was added onto the slides and left at room

temperature for 10 minutes. The slides were then washed with 2XSSC and were air dried for one hour. The probes were diluted in hybridization buffer (25 mL Formamide, 50 % dextran sulfate, 50 µL blocking solution and 100 µL tween 20) and was applied on the slides, covered with hybrid slips and incubated overnight at 65 (degrees) Celsius.

The following day, the slides were washed twice with 2XSSC for 30 minutes each and subsequently washed with formamide for 30 minutes and at the end again washed twice with 2XSSC for 10 minutes. This was followed by washing in RNase A for 20 minutes at37 °C and RNase buffer for 20 minutes at 65 °C. The slides were then washed with a combination of 2XSSC, 0.05 triton X -100 and 2% blocking reagent for two hours and 100 µL of FAB fragment DIG alkaline phosphate was added and kept overnight.

On the subsequent day, the slides were washed twice in maleate buffer for 10 minutes and once in visualization buffer (20 mL 1mM Tris-Hcl, 1M 1mL MgCl, 17g 100mM NaCl and 175 mL distilled water) for 10 minutes. The slides were incubated with 200 µL of chromogen substrate mixed with 2.4 mg of levamisole overnight. As the color started to develop, the slides were washed with stop buffer and visualized using a light microscope.

2.9 Statistics

Statistical significance was determined by using paired one-way analysis of variance (ANOVA) with fisher's test using Graphpad Prism version 6.00 [26]. P value was ≤ 0.05. The data is presented as mean ± S.E.M.

RESULTS AND DISCUSSION

3.1 Agonistic behavior

Juvenile trout form a dominant-subordinate relationship minutes after social interaction amongst them. Once, the PVC wall and the mirror were removed there was a phase where they showed mutual displays such as circling but after a few minutes they turned out to be very aggressive which was characterized by attacks and biting. At the end there was a socially dominant fish which circled at the bottom of the aquarium whereas there was a subordinate fish which moved on to the surface of the tank and stayed there. Although the fish attained subordinate status the dominant fish still made attacks and chased the subordinate fish around the tank.

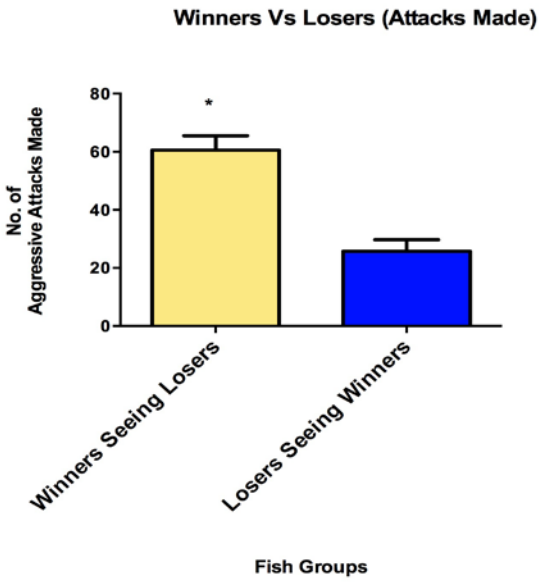


Figure 3: Number of aggresive acts made by winners and losers. The difference in number of aggresive acts were statistically significant with $p<0.05$

The dominant fish made a larger number of aggressive attacks in comparison to the subordinate fish. Thus, at the end of the experiment, the number of aggressive attacks explained which were the socially dominant and socially subordinate fish.

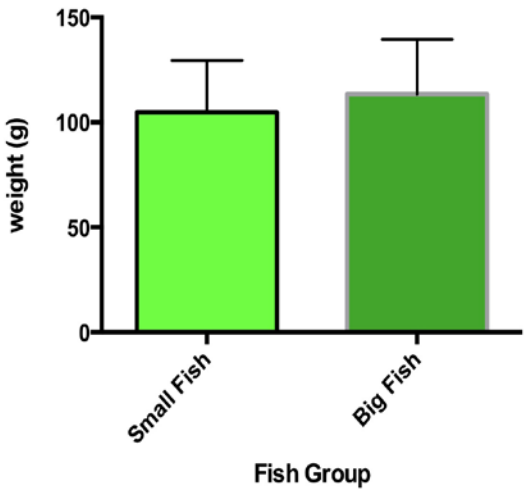


Figure 4: The mean ± SEM weight for small fish was 104.8 4 ± 4 g whereas for the big fish was 109.5 ± 4.6 g. The small fish were 4% smaller than the large fish.

In all pairs of fish, the larger fish ended up being dominant whereas the smaller fish ended up being subordinate. After transfer from the holding tank into the aquarium, where they were socially isolated the fish were reluctant to eat. After a period of 3-4 days the fish started eating again and ate to satiation.

During social interaction, the feed intake in dominant fish was high after each round of aggressive attacks but after 3 days of social interaction period the feed intake in subordinate fish also increased. In case of the controls, the big fish seeing the small fish consumed more food in comparison to small fish seeing the big fish. This leads to the conclusion that the small fish seeing the big fish for one hour daily were more stressed and consumed less food. Also, the socially dominant fish showed more spontaneous swimming activity as compared to socially subordinate fish during isolation which was observed during video analysis of the social interactions.

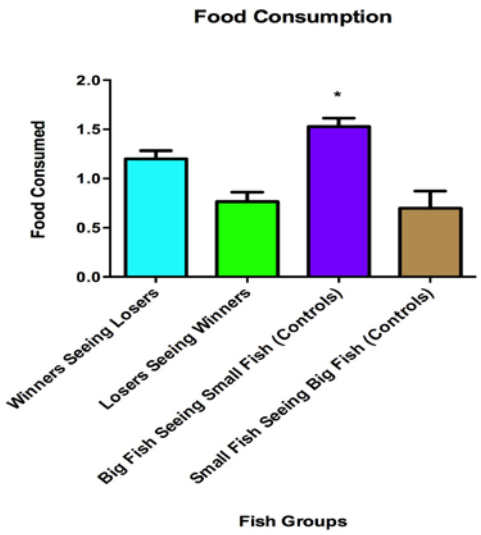


Figure 5: Amount of food consumed by winners, losers and control was statistically significant with p<0.05

3.2 Plasma Cortisol level in socially interacting fish

Cortisol was measured in all the four cases i.e. winners seeing losers and losers seeing winners, 5 minutes before the fight and for two groups of controls i.e. big fish who saw the small fish and small

fish who saw the big fish for one hour each day for five days. On the day of the sampling, all the fishes were allowed to see their counterparts for 30 minutes and were simultaneously netted. It is evident from the graphs that plasma cortisol level was drastically increased in socially subordinate fish but dominant fish returned to normal cortisol level after an initial rise of plasma cortisol. Also, in the case of controls the big fish that was seeing the small fish for one hour daily had low level of cortisol as compared to the small fish seeing the big fish. However, the cortisol levels in winners seeing losers and losers seeing winners are not statistically significant which may be due to the fact that the fish were not allowed to interact on the day of sampling but just allowed to see their previous antagonist for 30 minutes. In the case of controls, the small fish seeing the big fish was more stressed. From the above data, we could conclude that a big fish seeing the small fish or vice – versa has an effect on the social interaction capability of the fish.

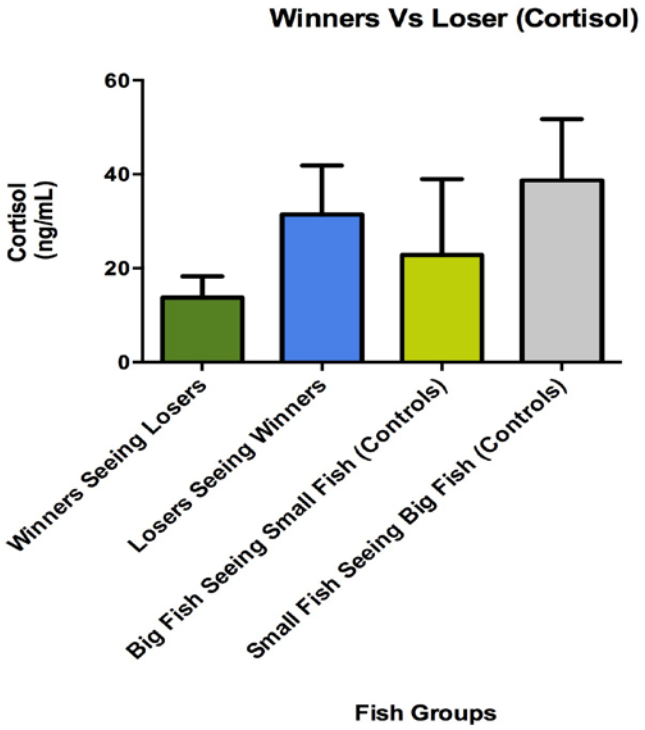


Figure 6: The winners had low cortisol level as compared to winners. This suggests that the losers were more stressed after social interaction.

3.3 Gene expression using quantitative PCR

Gene expression studies were conducted using qPCR to differentiate between expression of c-Fos and EGR-1 in stressed and aggressive fish. The level of EGR-1 and c-Fos was different between the two groups. c-Fos was down regulated in winners while both of them were up regulated in case of losers. Thus, it can be concluded that due to stress and aggressive behavior the losing fish have more expression of both c-Fos and EGR-1.

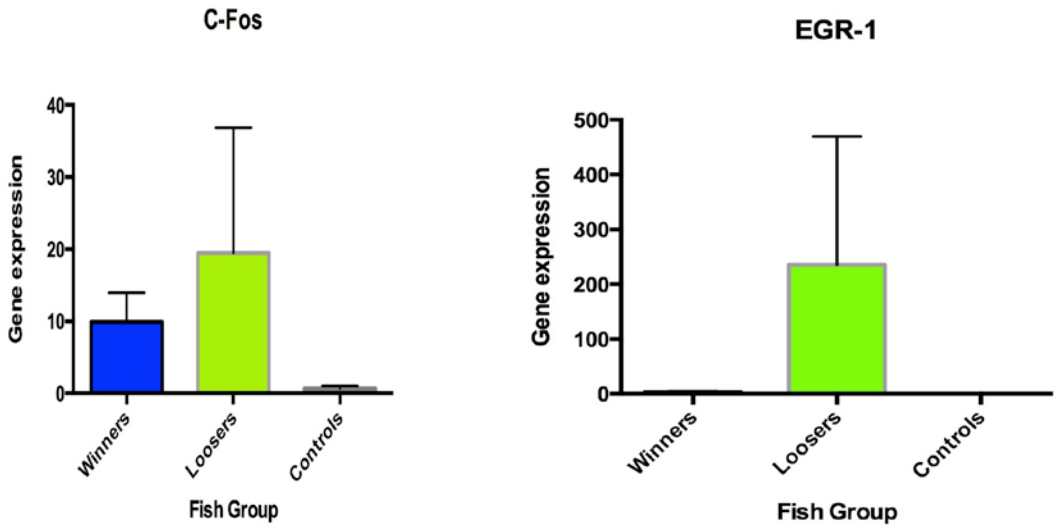


Figure 7: Gene expression of (a) c-Fos and (b) EGR-1

3.4 Brain monoaminergic activity

The monoaminergic neurons comprise a very small fraction of the vertebrate brain and they have been linked to stress reaction and control of the autonomic and neuroendocrine systems. Serotonin (5-HT) is synthesized from tryptophan and it is transported by blood using non-specific carrier that transports large neutral amino acids across the blood-brain barrier.

The subordinate fish had an increase in serotonin which can be deduced from the 5-HIAA level and the 5-HIAA/5-HT ratios. The brain 5-HT plays an important role in the activation of the HPI axis and copes well with chronic stress by acting through individual neuronal pathways. Social interaction has an effect on the fish brain affecting the 5-HT system. The subordinate fish have a high level of 5-HIAA concentrations as compared to dominant fish. The brain serotonergic activity is characterized by the 5-HIAA/S-HT ratio. Stress and food deprivation increases the brain 5-HIAA level. Fish occupying low positions in a social hierarchy are likely to be subjected to both food deprivation and stress, caused by repeated attacks from high-ranking fish as well as by the constant threat imposed by the sheer presence of dominant individuals [13].

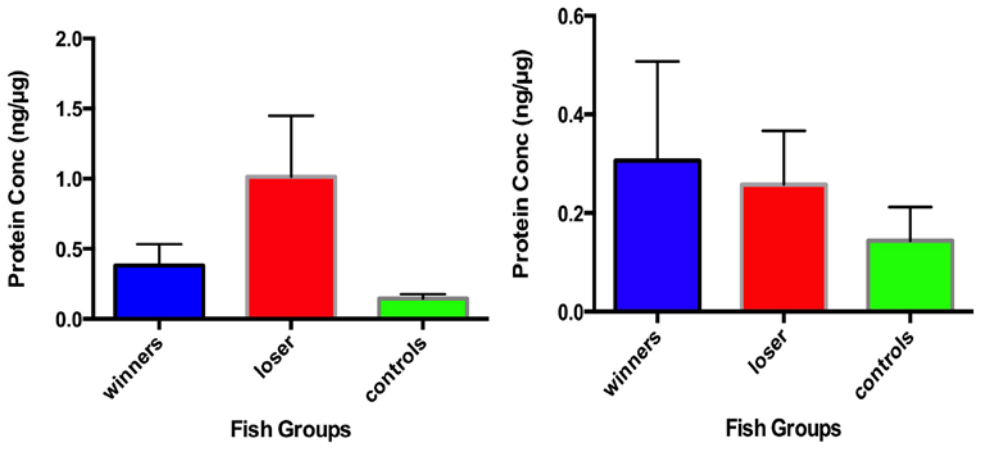
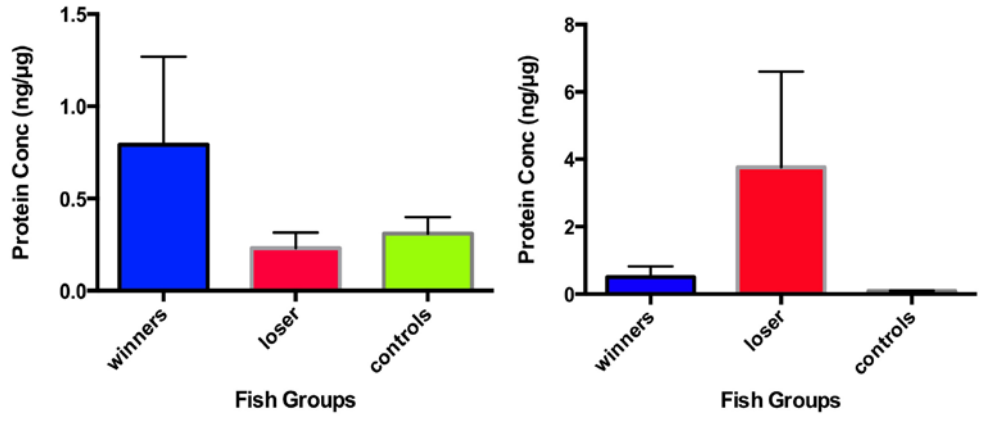


Figure 8: (a) 5-HT was higher in losers as compared to winners with p=0.18 & (b) 5-HIAA was higher in winners with p=0.8

The DA system is believed to be involved in regulation of agonistic behavior. There is no foremost DA metabolite in fish but Nilsson reported that HVA is the major DA metabolite in crucian carps (*Carassius carassius*) whereas DOPAC is the major metabolite in goldfish (*Carassius auratus auratus*). According to Saligaut et al [27] 3-methoxytyramine (3-MT) is the major DA metabolite in rainbow trout but saligaut et al [28] says that DOPAC is dominant in the hypothalamus and pitituary region of the rainbow trout and exceeds the 3-MT level. The DA was higher in the winners in comparison to the losers suggesting that they were more aggressive as compared to the losers. The DOPAC/DA ratio was higher in the winners.



(a) (b)

Figure 9: (a) DA was higher in losers as compared to winners with p = 0.57 and (b) DOPAC was higher in losers with p = 0.37

The NE level was higher in case of losers as compared to the winners. McIntyre et al. reported that there is an effect of social rank on the catecholamine levels in the fish brain. They found that dominant rainbow trout had lower NE and higher DA concentration in brain than the subordinate fish [29]. The increased DA level indicates more aggressive behavior in the dominant fish while the increased NE level in the subordinate fish indicates increase in stress. DA is the precursor for NE so treatment with L-DOPA will increase the synthesis of NE.

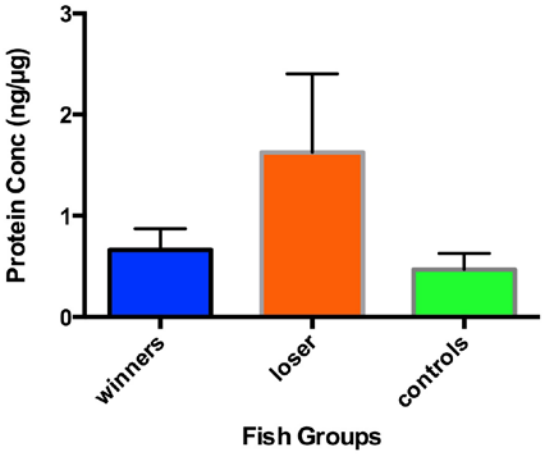


Figure 10: Brainstem NE level with p=0.32

Stress increases brain 5-HIAA levels without affecting the 5-HT concentration. It has been demonstrated in damselfish that stress increases brain 5-HT activity. Thus, the 5-HIAA/5-HT ratio,

which can been used as an index of serotonergic activity [30], was elevated in stressed fish. Therefore, it can be concluded that the fish seeing the other fish before social interaction has considerable amount of effect on the brain monoaminergic activity. There is an elevation of NE and 5-HT in the losing fish due to excitement and stress respectively whereas there is an elevation of DA in winners.

3.5 Gene expression using In-Situ Hybridization

Using the c-Fos & EGR-1 probes it was observed that both the genes were expressed in the telencephalon and the optic-tectum of a winner, loser and a big fish seeing a small fish (control) in the pilot experiment. Furthermore, in-situ hybridization would be done on the brains to check the expression of EGR-1 and c-Fos in the telencephalon, optic-tectum and cerebellum.

ACKNOWLEDGMENTS

I would like to thank Svante Winberg for sharing his invaluable experience with stress physiology in fish. I would also like to thank Per-Ove Thornqvist for the discussions and sharing his expertise. A big thanks to Lars Ebbesson for the opportunity to do the in-situ hybridization in Bergen.

REFERENCES

1. Selye, H. The evolution of the stress concept. *Am Sci* **61**, 692-9 (1973).
2. Chrousos, G.P. Stressors, stress, and neuroendocrine integration of the adaptive response - The 1997 Hans Selye Memorial Lecture. *Stress of Life* **851**, 311-335 (1998).
3. Harper, C. & Wolf, J.C. Morphologic Effects of the Stress Response in Fish. *Ilar Journal* **50**, 387-396 (2009).
4. Barton, B.A. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr Comp Biol* **42**, 517-25 (2002).
5. Wendelaar Bonga, S.E. The stress response in fish. *Physiol Rev* **77**, 591-625 (1997).
6. Barton, B.A. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* **42**, 517-525 (2002).
7. Barton, B.A. & Iwama, G.K. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* **1**, 3-26 (1991).
8. Barry, T.P., Ochiai, M. & Malison, J.A. In vitro effects of ACTH on interrenal corticosteroidogenesis during early larval development in rainbow trout. *Gen Comp Endocrinol* **99**, 382-7 (1995).
9. Sanders, B.M. Stress proteins in aquatic organisms: an environmental perspective. *Crit Rev Toxicol* **23**, 49-75 (1993).
10. Hart, B.B., Stanford, G.G., Ziegler, M.G., Lake, C.R. & Chernow, B. Catecholamines - Study of Interspecies Variation. *Critical Care Medicine* **17**, 1203-1222 (1989).
11. Nikinmaa, M. Membrane-Transport and Control of Hemoglobin-Oxygen Affinity in Nucleated Erythrocytes. *Physiological Reviews* **72**, 301-321 (1992).
12. Davison, W., Axelsson, M., Forster, M. & Nilsson, S. Cardiovascular responses to acute handling stress in the Antarctic fish Trematomus bernacchii are not mediated by circulatory catecholamines. *Fish Physiology and Biochemistry* **14**, 253-257 (1995).
13. Winberg, S. & Nilsson, G.E. Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **106**, 597-614 (1993).
14. Salbert, G., Chauveau, I., Bonnec, G., Valotaire, Y. & Jego, P. One of the two trout proopiomelanocortin messenger RNAs potentially encodes new peptides. *Mol Endocrinol* **6**, 1605-13 (1992).
15. Baker, B.I. in International Review of Cytology (eds. Jeon, K.W. & Friedlander, M.) 1-47 (Academic Press, 1991).
16. Baker, B.I. Melanin-concentration hormone updated functional considerations. *Trends in Endocrinology & Metabolism* **5**, 120-126 (1994).
17. Bird, D.J., Buckingham, J.C., Baker, B.I. & Mukherjee, S. Hypothalamo-pituitary-interrenal responses to opioid substances in the trout: I. Effects of morphine on the release in vitro of corticotrophin-releasing activity from the hypothalamus and corticotrophin from the pituitary gland. *General and Comparative Endocrinology* **68**, 33-39 (1987).
18. Pottinger, T.G., Prunet, P. & Pickering, A.D. The effects of confinement stress on circulating prolactin levels in rainbow trout (Oncorhynchus mykiss) in fresh water. *Gen Comp Endocrinol* **88**, 454-60 (1992).
19. Barton, B.A. & Schreck, C.B. Metabolic Cost of Acute Physical Stress in Juvenile Steelhead. *Transactions of the American Fisheries Society* **116**, 257-263 (1987).
20. Pickering, A.D. Stress and the Suppression of Somatic Growth in Teleost Fish. *Progress in Comparative Endocrinology* **342**, 473-479 (1990).
21. Zayan, R. The Specificity of Social Stress. *Behavioural Processes* **25**, 81-93 (1991).
22. Clayton, D.F. The genomic action potential. *Neurobiol Learn Mem* **74**, 185-216 (2000).
23. Goelet, P., Castellucci, V.F., Schacher, S. & Kandel, E.R. The long and the short of long-term memory--a molecular framework. *Nature* **322**, 419-22 (1986).
24. Morgan, J.I. & Curran, T. Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. *Trends Neurosci* **12**, 459-62 (1989).
25. Sheng, M. & Greenberg, M.E. The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* **4**, 477-85 (1990).
26. windows, P.f. (Prism 5 for windows, San Diego, California, United States Of America, 2010).
27. Saligaut, C., Bailhache, T., Salbert, G., Breton, B. & Jego, P. Dynamic characteristics of serotonin and dopamine metabolism in the rainbow trout brain: a regional study using liquid chromatography with electrochemical detection. *Fish Physiology and Biochemistry* **8**, 199-205 (1990).

28. Saligaut, C., Salbert, G., Bailhache, T., Bennani, S. & Jego, P. Serotonin and dopamine turnover in the female rainbow trout (Oncorhynchus mykiss) brain and pituitary: Changes during the annual reproductive cycle. *General and Comparative Endocrinology* **85**, 261-268 (1992).
29. McIntyre D. C., H.L.M.a.S.M. Intraspeties aggression and monoamine levels in rainbow trout (Salmo gairdneri) fingerlings. *Behav. Neural. Biol* **25**, 90-98 (1979).
30. Winberg, S., Nilsson, G. & Olsén, K.H. Social rank and brain levels of monoamines and monoamine metabolites in Arctic charr, Salvelinus alpinus (L.). *Journal of Comparative Physiology A* **168**, 241-246 (1991).

APPENDIX

Sequences:

Oncorhynchus mykiss c-Fos 278bp (Plasmid pCRII-TOPO 412 ng/µl) (Oncorhynchus masou 444-167)

CTCAAGCTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGT
GCTGGAATTCGCCCTT**GCCCAGAGCTGTGGCCCTTG**TTCCTCATGGCTCTAGTGTAGG
TTGGGGGGCTGGCACTGTAGGGATGGGCTCTGTAAGAAGGTGCCACAGAGGATAGC
GGCTGGACCAACCACTGCAGGCCTGGGCTGGCAGAGATGGCGGTAACAGTAGGGAT
GAAGGACGGACCACTGGACACTGAATTCAGGTCTGTGAAGTCCTGAGATTGGGGAG
AGCCCATGCTGGAGTAGGATCCCTCAGGAGAGTTGAAGTAAACCAGCTTGTTGCCG
GATGGAGAAGCTAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCA
TGCATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTT
TTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACAT
CCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAG
TTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGT
GTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTT

⇦ 167

> dbj|AB111054.1| Oncorhynchus masou mRNA for c-Fos protein, complete cds
Length=1864

Score = 439 bits (486), Expect = 6e-123
Identities = 264/278 (95%), Gaps = 0/278 (0%)
Strand=Plus/Minus

Primer pair c-Fos from O. nerka

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGCTTCTCCATCCGGCAACAAGC	Plus	23	85	107	59.75	56.52	4.00	2.00
Reverse primer	GCCCAGAGCTGTGGCCCTTG	Minus	20	374	355	59.97	70.00	8.00	2.00
Product length	290								

Products on intended target

AB111053.1 Oncorhynchus nerka mRNA for c-Fos type1 protein, complete cds

product length = 290

Forward primer 1 AGCTTCTCCATCCGGCAACAAGC 23
Template 85 107

Reverse primer 1 GCCCAGAGCTGTGGCCCTTG 20

Template 374 355