Long-term effect of dietary tryptophan supplementation on the physiological stress response in Atlantic Salmon (Salmo salar).

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Ora & Labora

Abbreviations

ACTH- Adrenocorticotropic hormone

ANOVA- Analysis of variance

BDNF- brain-derived neurotropic factor

CRH- corticotropin-relasing hormone

EDTA- Ethylenediaminetetraacetic acid

FAO- Food and Agriculture Organization

HPA-Hypothalamic-pituitary-adrenal axis

HPI- Hypothalamic-pituitary-interrenal axil

LNAAs- Large neutral amino acids

SEM- Standard error of mean

SGR-Specific growth rate

SSRI-Specific Serotonin re-uptake inhibitors

STD diet - Standard diet

RIA- Radioimmunoassay

TRP- Tryptophan

5-HT- 5-hydroxytryptamine

Abstract

Several strategies to reduce stress among fish in aquaculture are currently under development. A biologically conserved feature of the stress response in vertebrates is the role of the brain serotonergic (5-hydroxytryptamine, 5-HT) signalling system in controlling the endocrine response to stress, primarily production of the steroid hormone cortisol through the hypothalamus-pituitary-adrenal axis (HPA, or HPI [interrenal] in fishes). The precursor of the monoamine neurotransmitter 5-HT is the amino acid tryptophan (TRP). Plasma levels of TRP directly affect 5-HT production in the brain. Dietary TRP treatment has previously been shown to inhibit the cortisol response to stress in both fishes and mammals. Altering monoaminergic neurotransmission and stress responsiveness may have enduring effects on neural plasticity, but it is not known whether the effect of TRP remain also after exogenous supplementation has been terminated. In the current study, three groups of juvenile Atlantic salmon (*Salmo salar*) were tagged and acclimated during 10 weeks. Thereafter, the fish were treated with three different diets (TRP 1x = standard commercial feed, and TRP 2x and 3x = 2 and 3 times standard level of TRP) during 1 week, whereupon standard food were given until the end of the experiment. In order to investigate the long-term effect of TRP on the stress response, fish were subjected to an acute stressor during 1 hour at two different occasions, 1 and 3 weeks after TRP treatment, and blood samples were obtained to analyse plasma levels of the fish corticosteroid hormone cortisol after stress. Fish fed with TRP3 showed a decrease of plasma cortisol levels during both occasions, specifically as compared to TRP 2x at sampling 1 and both TRP 2x and TRP 1x and sampling 2. Notably, fish fed with TRP 2 showed a significant elevation of plasma cortisol at first sampling, however, this effect was abated after 3 weeks. These results demonstrate that neuroendocrine effects of dietary TRP are both time- and dose-dependent. I hypothesize that long term effects of TRP on cortisol levels, are mediated by the fish brain serotonergic system. Chronic stress in fish

cause brain damages, loss of appetite, impaired growth and muscle wasting, brain effects, immunosuppression, decreased reproduction and mortality. Consequently, dietary TRP should be further evaluated in aquaculture production in order to maintain animal welfare, and limit economic losses.

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1. Introduction

1.1 Aquaculture in a historical perspective

Aquaculture refers to the cultivation of aquatic species, either plants or animals, for human consumption. This practise is not a recent innovation in human history. Finfish aquaculture (using common carp, *Cyprinus carpio*) is considered to have originated in the Chinese civilization around 2000-1000 B.C. Today, aquaculture is considered perhaps the most sustainable alternative for fish production, as an alternative to more destructive fishing techniques. In fact, a recent report by Worm et al. (2006) predicts the collapse of all species of sea fish by 2048, with the current fishing methods and development.

Currently, 50% of the global fish production is coming from aquaculture, and fish farming is the fastest growing sector within animal-derived food production. The growth rate in freshwater fish was 7.2% between 1980-2010 (FAO, 2012). Norway is the largest producer in Europe, accounting for 39,95 %, followed by Spain (10,00%), France (8,89%) and UK (7,97%). On the species level, Atlantic salmon (Salmo salar) leads the worldwide aquaculture production around 1,5 millon tonnes, followed milkfish (*chanos chanos*) by 0,8 millon tonnes and rainbow trout (Oncorhynchus mykiss) 0,7 millon tonnes (FAO, 2012).

Industrial production of salmonid fish began in Norway in the late 1970´s, spurred by research at the Norwegian University of Life Sciences. Atlantic salmon is a relatively new species in breeding, currently undergoing domestication. Consequently, this species is likely less well adapted to the rearing environment, which is very different from its natural environment, compared to most terrestrial production animals (Huntingford, 2005). This is partly countered by intensive breeding programmes and/or genetic strategies (Gjedrem, 1976; Andersen,1977; Elvingson, 1992; Hulata, 2002). Nevertheless, fish in aquaculture (salmon as

well as other species) are exposed to a range of stressors imposed by rearing in capture, which may affect both production parameters and welfare of the animals. This MSc thesis considers a dietary intervention – supplement of the essential amino acid tryptophan – as an alternative strategy to minimise stress in aquaculture. The biological principle behind the method is specifically reviewed in chapter 1.5, and further background for the study is given in the following.

1.2 Study species

The study species in this thesis is Atlantic salmon. In the wild, this species is anadromus, i.e. reproduction and early growth occurs in fresh water, whereas from the smolt stages until sexual maturation occurs fish reside in the sea. Following homing to native rivers and upstream migration, female salmon dig reds in gravel in shallow water to spawn. Mature Atlantic salmon may return to the sea but they need at least two years to reproduce again (Edwards, 1978). In their natural environment eggs will normally hatch to larvae (alevines) after two to three months, depending on the temperature. During the first phase, alevines obtain nutrition from their yolk sacs. Subsequently, as the yolk sack is consumed larvae become more active and start looking for food. This particular period is known as the "swim up" period after which follows a period of growth for typically 18 to 24 months; whereupon the fish migrates to the sea (Edwards, 1978).

Collectively, the many processes involved in the development of seawater tolerance prior to migration are known as smoltification. Several studies showed that during smoltification several physiological, behavioural and morphological changes occur (Damsgaard and Arnesen, 1998; McCormick and Saunders, 1987; Hoar, 1988). For example, hypoosmoregulatory capacity increases (McCormick and Saunders, 1987; Björnsson et al., 1989; Sigholt et al., 1995) and the fish start to swim downstream towards the sea (Eriksson,

1984; Lundqvist and Eriksson, 1985). Subsequently, salmon will live in the ocean during one to five years before reproductive maturation, whereupon they return to freshwater, typically to the river in which they were born (Edwards, 1978; Stabell 1984).

The life cycle described above concerns wild conditions. For practical and economic reasons, this life cycle is compressed and growth rates greatly increased in aquaculture production environments, which offer optimized conditions for fish development.

1.3 Welfare and Aquaculture

In line with the sharply rising public interest, fish welfare in aquaculture has become a main legislative and research issue. The welfare concept suffers from lack of a clear definition and how it should be applied to different organisms is debated (Huntingford et al., 2006). Generally, most definitions of animal are categorized into three different groups: "feeling-based" definitions, referring to subjective mental state of the animal with absence of suffering; "function-based" definitions, referring to the animal's ability to adapt to new environments; and "nature-based definitions", referring to an inherent biological nature that every animal keeps and that must be expressed (Duncan & Fraser, 1997; Fraser, 1999).

In aquaculture various stressors may occur, such as for instance accumulation of inadequate densities in the same rearing unit, poor water quality, handling and removing from water during routine husbandry procedures, unnatural light-dark regimes, and food deprivation. This type of actions can create aggressive interactions between fish, prolong adverse physiological states, and possibly increase the transmission of diseases (Huntingford et al., 2006).

Even if there are a number of differences between fish and "higher" vertebrates, stress responses are similar in both cases. Behavioural responses against threats, such as predators, poor food availability and other suboptimal environmental conditions, represent a first line of

defence (Huntingford et al., 2006). Physiological stress responses are virtually identical as in mammals (Bonga, 1997), with fish secreting cortisol from the interrenal tissue (the homologue to adrenal cortex in mammals, see details in next section) in response to adenocorticotrophic hormone under stress (Weld et al., 1987; Okawa et al., 1992; Sumpter, 1997). Therefore, even knowing that physiological stress responses alone do not correspond to welfare, monitoring stress responses might be considered as a good indicator of fish welfare (Huntingford et al., 2006)., especially when combined with other measures. Such a welfare approach is provided by Turnbull et al. (2005), including two physical (condition of body and fins) and two physiological observations (plasma concentration of glucose and cortisol).

There are several examples of research aiming to reduce stress in aquaculture. For example, Jobbling et al. (1993) trained Arctic char (*Salvelinus alpinus*) fishes by exposing them to moderate water currents for prolonged periods leads to improvements in physiological performance. Recently including the essential amino acid tryptophan (TRP) as a part of the daily diet has been shown to reduce both aggression (Winberg et al., 2001) and the physiological stress response (Lepage et al. 2002; Lepage et al., 2003) in rainbow trout. Similar results have been obtained with different species like Atlantic cod (*Gaus morhua*) (Höglund et al. 2005); *Cirrhinus mrigala* (Tejpa et al. 2009) or European sea bass (*Dicentrarchus labrax*) (Herrero et al. 2007).

1.4 The stress response

The concept of stress has been defined differently throughout history by biologists, neurologists, and psychologists. Some consensus was reached around the definition by Chrousos and Gold (1992), who stated that stress is ``a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors´´.

Barton and Iwama (1991) drew attention to the fact that during stress, which alters physiological and psychological states, animals will mount both neuroendocrine and behavioural responses. Therefore, the impact of the stressor may be measured by quantifying the amount of these responses. The most well studied physiological response to stress is an increase in cortisol and catecholamine (adrenaline and noradrenaline) concentrations in plasma. Under a stressful situation, individuals demand higher amounts of oxygen, and metabolic pathways are also affected (Barton & Iwama, 1991). Fish and mammals have comparable neuroendocrine stress responses (Wendelaar-Bonga, 1997; Donaldson, 1981; Mazeaud, and Mazeaud, 1981). In mammals, the steroid-synthesizing cells involved in the stress response form a compact mass located in the adrenal cortex. However, in teleost fishes, these cells are located in layers, cords or isolated groups along the cardinal vain and in head kidney interrenal tissue. Hence, in fish one refers to the hypothalamic-pituitary-interrenal (HPI) axis, and cortisol secretion is regulated by this axis (Milano et al, 1997; Wendelaar-Bonga, 1997).

Commonly, the physiological stress response is described as a hormonal cascade divided into several discrete steps. Initially, a release of catecholamine's is stimulated by the sympathetic nervous system. This response co-occurs with the secretion of corticotropin-relasing hormone (CRH) from the hypothalamus, which in turn promotes the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary into the general blood stream. ACTH then

activates the synthesis and secretion of cortisol (corticosterone in rodents and birds) by the steroidogenic tissues.

Secondly, these stress hormones reach their target areas and stimulate or inhibit a range of physiological processes, such as mobilization of energy substrates, heart rate, oxygen uptake and hydromineral balance, among others. In the case of acute stress, cortisol concentrations return to pre-stress levels within hours of exposure to a stressor (Pickering and Pottinger, 1989; Waring et al., 1992).

A so-called tertiary response occurs when fish cannot escape the stressor or the stressful stimulus is episodic or intermittent. In other words, the physiological stress response becomes chronic. This can result in compromised welfare and poor production, including loss of appetite, impaired growth and muscle wasting, immunosuppression and suppressed reproduction. (Bonga, 1997; Pickering, 1981; Pickering and Pottinger, 1995; Wedemeyer, Barton and McLeay, 1990).

The relevance of stress responses, in particular of the tertiary responses, for the rearing of fish in aquaculture was illustrated by Pickering and Stewart (1984), who showed that growth rate and feeding behaviour, was altered when fish density inside the rearing unit became too high. Also, the effect of stress on the reproductive ability of rainbow trout (*Salmo gairdneri*), Atlantic salmon, brown trout, (*Salmo trutta* L.) or rainbow trout (*Oncorhynchus mykiss*) has been investigated from several studies. (Pankhurst and Van Der Kraak, 2000; Campbell et al. 1994; Pickering and Pottinger, 1995; Pottinger, and Carrick, 1999; Pickering, 1987).

It is important to keep in mind that the stress responses are not always negative. According to Huntingford and Adameriwims., 2005 ``the stress response has evolved to assist the survival of the animal under demanding conditions in the natural environment´´. However, in general stress is viewed as something one wants to avoid in aquaculture.

1.5 Tryptophan, serotonin and cortisol: a close relationship.

Cortisol (hydrocortisone) is a steroid hormone, or glucocorticoid, produced by the adrenal gland. It is released in response to stress and also fluctuates in daily cycles. Plasma levels of glucocorticoids are in themselves important regulators, as there is extensive physiological feedback regulation of the production of this hormone. Its primary functions are to increase blood sugar through gluconeogenesis; suppress the immune system; and aid in fat, protein and carbohydrate metabolism (Tao Le et al, 2009).

Cortisol is considered a "universal" stress indicator because of its concentration level increases radically during exposure to all kinds of stress; compared to resting levels (Wendelaar-Bonga, 1997). After a stressor, plasma cortisol levels require some time for returning to normal levels. In an aquaculture environment, fish can be exposed to continuous stressors like for example handling, poor water quality, organic pollution, temperature and pH fluctuations and a range of other environmental perturbations. If exposure to such stressors is prolonged, the physiological stress response might become chronic and cortisol levels might remain high over longer periods (Barton and Iwama, 1991; Brown, 1993; and Donaldson, 1981). Pickering and Pottinger (1985) reported on blood plasma cortisol levels for stressed and unstressed fish of 10-100 ng/ml and < 5ng/ml, respectively. Long-term exposure to high levels of cortisol damages cells in the hippocampus in mammals (McAuley, 2009) and this damage results in impaired learning. Furthermore, it has been shown that cortisol inhibits memory retrieval of already stored information (de Quervain et al, 1998; de Quervain et al, 2000). However, recent studies showed that a low level in short-term exposure to low levels of cortisol was associated with increased brain cell proliferation (Sorensen et al, 2011; Von Krongh et al, 2010; Peterson et al, 2007). Peptide hormones controlling cortisol secretion, CRH released by the hypothalamus and ACTH released by the pituitary, are also regulated by other signalling molecules, including

the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT). In both teleost fishes and mammals there is a well-documented link between brain 5-HT neurotransmission and the regulation of the magnitude of the stress response at the levels of the HPI axis (as well as the sympathetic nervous system) (Chaouloff, 1993, Winberg and Nilsson, 1993). Serotonin is involved in the control of behavioural stress responses as well as regulation of neuroendocrine and autonomic functions (Winberg and Nilsson, 1993; Lepage et al., 2003). Stress induces increased 5-HT, which is generally associated with behavioural inhibition, recognized by for example reduced aggressive behaviour (Winberg and Nilsson, 1993), locomotor activity (Øverli et al., 1998), and feeding (De Pedro et al., 1998; Øverli et al., 1998).

In addition to its direct functions as a neurotransmitter, 5-HT has been shown to stimulate structural processes in the brain such as adult neurogenesis. This is the generation of new neurons in the adult brain from neural stem and progenitor cells (Jacobs, 2002). In mammals, adult neurogenesis is restricted to two brain regions; the olfactory bulb and the hippocampus (Duman et all, 2001). The functional significance of adult neurogenesis has for a long time remained unclear. However recently it was shown that adult-born hippocampal neurons are essential for normal expression of the endocrine and behavioural components of the stress response in mice (Snyder et al., 2011).

It has also been shown that the immediate effect of 5-HT is inhibitory on hippocampal neuronal activity. This will reduce negative feedback inhibition on the hypothalamus and thus indirectly stimulate ACTH and cortisol release (Dinan, 1996). Moreover, Mattson (2004) identified brain-derived neurotropic factor (BDNF) and 5 HT as signals regulating neural plasticity in multiple brain regions. The consequences of this are that long-term effect of chronic 5-HT activation has a trophic effect on the hippocampus, stimulating a number of developmental processes including neuronal cell division, migration, neurite outgrowth, and

synapse formation. (Patel and Zhou, 2005) The outcome of this stimulation may be enhanced hippocampal inhibitory signalling to the hypothalamic CRF neurones. In other words, the net effect of enhanced 5-HT stimulation on the magnitude of the cortisol response to stress is likely time-dependent, with short bursts of 5-HT activity being stimulatory and long-term 5-HT exposure acting inhibitory. The latter possibility has not been well researched, and non-invasive manipulation of overall brain 5-HT activity through dietary levels of the 5-HT amino acid precursor tryptophan (TRP) provides a tenable research tool.

Fish and mammals synthesize 5-HT from the essential amino acid tryptophan (TRP) (Fernstrom, 1983; Fernstrom ad Wurtman, 1997; Winberg et al., 2003). The conversion of TRP to 5-HT is catalysed by the enzyme tryptophan hydroxylase. This enzyme's saturation is linked to the brain TRP concentration and is in fact the rate-limiting step in the synthesis of 5-HT (Gessa et al. 1975). In mammals, the concentration of TRP in the brain and thus brain 5-HT levels in turn depend of the concentration of free TRP in blood plasma, and the transport mechanism though the blood-brain barrier for which TRP is competing with other large neutral amino acids (LNAAs: tyrosine, phenylalanine, leucine, isoleucine valine and methionine) and TRP. (Biggio et al., 1974; Gessa et al., 1975; Fernstrom, 1983; Fernstrom & Wurtman, 1997) This competition between TRP and LNAA for uptake into the brain is probably less significant in fish than in mammals (Aldegunde, 2000). Rozas et al. (1990) obtained results in rainbow trout which suggest that total plasma TRP directly determines brain levels, since TRP is largely found in the free state. In both fish and mammals, feed manipulations affect both plasma TRP/LNAA ratios and the brain 5-HT system. Feed manipulations have been used in studies about behaviour, mood and cognition in humans (Markus et al. 1999, 2000) and physiological responses to stress both in fish (Wimberg et al. 2001; Lepage et al 2002, 2003; Höglund et al, 2005) and in other vertebrates such as a rats

(Tanke et al., 2007); cows (Bruschetta et al., 2010) and pigs (Meunier-Salaun et al., 1991; Henry et al., 1996).

Winberg et al. (2001) demonstrated that dietary supplementation of TRP enhanced plasma and brain TRP levels in rainbow trout, with enhanced 5-HT neurotransmission and inhibition of aggressive behaviour as a result. TRP has also been given to humans and animals, in disorders involving low levels of serotonin. Such disorders include for instance depression (Sandyk, 1992), aggression (Shea et al., 1990; Hierden et al 2004) and obsessive-compulsive disorders (McDougle et al., 1999; Weld et al., 1998; Young and Leyton, 2002). After consumption of TRP-enriched diets, the subjects show elevated rates of 5-HT synthesis and metabolism (Johnston et al., 1990; Aldegunde et al., 1998, 2000; Winberg et al., 2001).

Although TRP appears to reduce aggression in fish. TRP-supplemented feed has not yet been utilised extensively by the aquaculture industry. Rather, the industry relies on alternative strategies to limit aggression like for example manipulation of rearing densities and feed distribution. Effective strategies to limit stress, apart from selection of stress resistant genotypes (Øverli et al., 2005) have however not been developed. Lepage and colleagues (2002, 2003) carried out two experiments in which the relationship between dietary intake of TRP and post stress plasma cortisol levels were investigated in rainbow trout. In both studies, these authors isolated fish in individual compartments and acclimated them for one week. During their first experiment fish were fed for 7 days with different amounts of supplemented TRP feed. After the initial seven days fish were stressed by lowering the water level in the aquaria for two hours, and blood and brains samples were taken for cortisol and brain monoamines analysis. The results showed fish fed TRP had significantly lower plasma cortisol. However in 2003, the experimental period was 3, 7 and 28 days and the same stressor was applied. At this time the results displayed significant elevation of plasma cortisol in fish fed with commercial diet and in fish fed with TRP diet during 3 and 8 days. Fish fed

with TRP diet during 7 days did not show any significant elevation of plasma cortisol after stressor. These results illustrate how the effect of TRP treatment on HPI-axis regulation is strongly dependent on time and context. Long term TRP treatment would however be very costly, and it is an open question whether continuous supplementation is necessary or whether the effect prevails after treatment has been terminated.

In the current study, in view of 5-HT's effects on brain structural plasticity referred to above, we propose that TRP enrichment fish still produce higher amount of 5-HT and lower cortisol levels after 16 and 29 days start the treatment in large juvenile groups of Atlantic salmon.

2. Material and methods

2.1 Experimental facilities and fish

The experiment was conducted at the Danish Technological University Aqua (DTU) research facility located in Hirtshals, Denmark, from the 31st of August to the 19th of December 2010.

One thousand two hundred Atlantic salmon were obtained from Fister Smolt AS/ Marine Harvest and transported to the experimental aquaculture facility. Fish were equally distributed in 12 circular tanks (Diameter 95cm and 119 cm height) (Fig 1), containing an approximate volume of 650 L. Biomass per tank corresponded to 14.13 kg on average). Fish were then left to acclimate for a period of 43 days. Salinity conditions started at ~ 15 ppt seawater, which was maintained for a period of two weeks. Followed by an increase to ~ 35 ppt, which was maintained for the rest of the experiment.

Tanks were supplied with a flow of aerated freshwater and seawater to obtain desired salinities. The freshwater was pumped from a re-circulating bio-filter system and seawater was supplied by a pipe connected to the Nordsøen Oceanarium. The total volume of the system (tanks, bio-filter, piping etc) was approximately 13.5 m^3. Water was replaced in the system at a constant rate of ~4 L min^{-1}. Following periods of oxygen uptake measurements (10 min every hour, see below), an additional volume of new water (~1 m^3) was replaced. Water quality parameters were monitored daily and did not exceed safe levels throughout the course of the study, NO_3^- < 100 mgL^{-1}, NO_2^- = 0-1mgL^{-1}, total ammonia NH_3/NH_4^+ = 0-1 mgL^{-1}, average water pH was 7.53±0.18. The high water flow through the tanks resulted in a theoretical hydraulic retention time of less than 10 min, which further eliminated any difference in water quality between tanks.

Figure 1: Holding tanks at DTU aqua, each used to house n=200 one year old Atlantic salmon smolts. A total of 12 tanks were used for the purpose of this experiment.

The tank system, modified from the system described by McKenzie et al. (2007), consisted of each tank containing an internal standpipe with a diameter of 30 cm creating a circular canal (width 35 cm) within the tank in which water could circulate. The water current drove uneaten feed and faeces into a central drain, situated below the pillar. The drain from each of the 12 tanks was fitted with a whirl separator for collecting uneaten pellets prior to returning water to the central biofilter. A three-way valve was also placed in the system in order to

maintain internal recirculation of the tank water. Water was delivered back to each tank from a central reservoir, through a vertical injection pipe (Ø 20mm, 70cm length) fixed to the wall of each tank. As the water was pumped through a series of small holes (~4 mm diameter) along the length of the pipe, a high velocity was obtained, generating water current. The velocity of the current was controlled by a valve situated at the outlet and adjusted to 0.9 body lengths s^{-1} (BL s^{-1}), when measured at the centre of the canal between the internal standpipe and the tank wall.

Each tank was equipped with diffuser supplying pure oxygen, and the injection of oxygen was controlled by a transmitter, coupled to an oxygen electrode in each tank (Oxyguard Standard, Oxyguard International A/S, Birkerød, Denmark) and an oxygen flow meter.

Fish were kept under a simulated photoperiod of 14 hours light (7.30 – 21.30) and 10 hours dark. The light system consisted of eight lamps situated two meters over the tanks and nine around the facility. Each lamp consisted of two 21w/865 fluorescent tubes, measuring 16x849 mm. Fish were fed ad libitum a diet consisting of dry pellets from Biomar at a feeding ratio of 1.75% of their body mass. Automated clock belt feeders provided by Biomar were used to distribute the feed from 8:00 to 14:30 each day. Temperature in the system was maintained between 12.3º- 13.1 ºC. Temperature control was monitored by a heater (manufactured by Billund Aqua at DTU), consisting of 100 meters of plastic pipe twisted around in a bulk, with hot water running in and laying in the sump, with flow rates controlled by a temperature-sensitive switch.

2.2 Experimental design

Fish were tagged in order to individually recognize them, by means of a pit-tag system (JOJO Automasjon, Stavanger, Norway). All fish in tanks 1, 3 and 5 were tagged (representing each of the experimental feed diets); whilst in the remaining tanks only 30% of the individuals were marked because these tanks were replicates. This was done in order to control weight gain, since individual X-ray pictures were obtained from all tagged fish two times during the experiment (see timeline in fig. 2), in order to estimate the amount of TRP ingested. All food contained ballotini beads (small glass balls), trackers placed in the feed in order to control how much feed was consumed by each individual fish by means of an X-ray picture. Marked fish in tanks 2, 4, 6, 7, 8, 9, 10, 11, 12, had their adipose fins clipped to facilitate visual recognition. During the experiment, food containing different levels of TRP was added during two different periods (first 7, then 8 days) separated by 22 days of standard food. X-ray pictures were taken from PIT-tagged fish at the end of each TRP treatment, to control whether individual feed intake was affected by TRP. For the purpose of investigating the effect of TRP treatment on overall growth rates, start weights were registered from all fish 5 days prior to the start of the initial TRP-treatment. Individual fish weights were then registered at the end of the 1st and 2nd treatment periods (c.f. fig.2, W2 and W3, respectively). Due to the short time span between W1 and W2 (12 days), in the data analysis SGR's were calculated between W1 (start) and W3 (end) only.

All tanks were initially (i.e. during the acclimation period) given a standard diet (STD) from Biomar, which contained normal levels of tryptophan and without glass beads. STD was also used during the period intervening tryptophan treatments, and as the initial of diets containing three different levels of TRP in the treatments periods (referred to as TRP1 below).

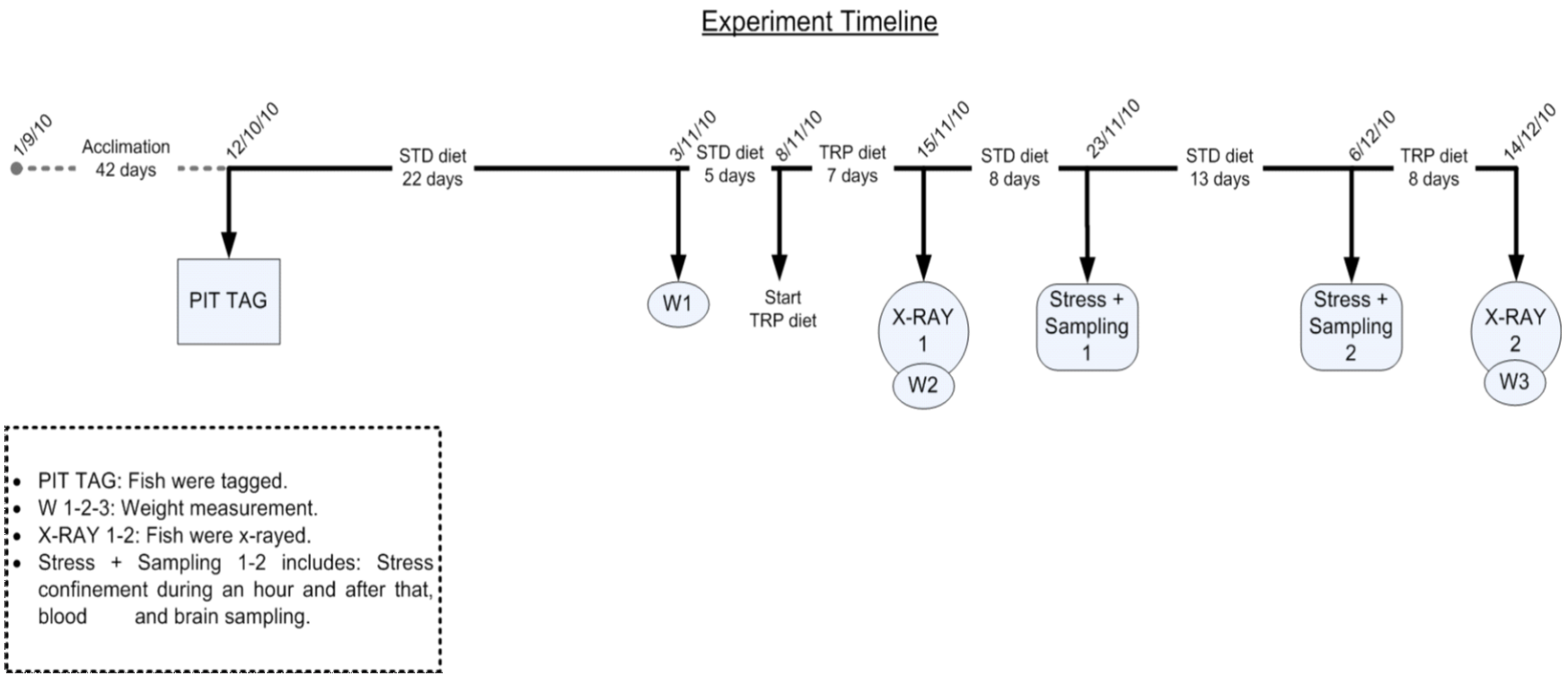

Figure 2: Experimental protocol from the fish arrival date until the last experimental day when fish were X-rayed for the last time.

Two additional diets were prepared by adding increasing quantities of crystalline tryptophan (table 1). The use of crystalline amino acids was preferred, since amino acids ingested in this form are absorbed directly into the blood stream. All feed types were provided by Biomar; the TRP1 diet corresponded to a standard feed without TRP enrichment, TRP2 was a standard feed enriched diet, representing 2 times more TRP (2x) compared to the control diet, and TRP3 represented 3 times more TRP (3x) than the control diet. All diets contained an equal concentration of glass beads for x-ray analysis. The 12 available tanks were divided into 3 groups, thus yielding 4 tanks for each diet.

These diets were maintained in a freezer at a temperature of -80^0C during the acclimation period. Meanwhile after feeding (during the experimental period), the diets were kept refrigerated at -4^0C.

Diet	Tryptophan g/100g
TRP1	0,417
TRP2	0,855
TRP3	1,241

Table 1. Concentration of tryptophan in experimental feeds. Diets were produced from the same batch of standard feed (Biomar) but the TRP 2 and TRP 3 feeds were supplemented with tryptophan to a level corresponding to two and three times the amount of tryptophan found in non-supplemented standard feed (TRP 1).

2.3 Stress treatment and sampling

In order to investigate whether TRP treatment had a lasting effect on the stress response, blood samples were obtained at two different occasions, 7 and 21 days after the termination of the TRP treatment. Before sampling, fish were exposed to a standardized acute stressor, which consisted of lowering the level of the water for a period of 60 min. Immediately after this, fish were taken from each tank (3 from tanks 2, 4, 6, 7, 8, 9, 10, 11, 12 and 9 fish each from tanks 1, 3 and 5, since in this tanks all fish were tagged allowing for individual recognition), in order to obtain blood. To serve as control, equal amounts of fish per tank were sampled right before exposure to the acute stressor.

Fish were first anesthetized with a lethal dose of ethyl-m amino benzoate methanesulphonate ($500mg*l^{-1}$) until there were no body or opercular movements observed. Immediately after this, blood was collected from the caudal vasculature using a syringe containing EDTA powder. Blood was immediately transferred to individually marked Eppendorf tubes that were kept at a temperature of -4 ºC. Tubes were then centrifuged on an Eppendorf Minispin Plus at 14.5x1000 rpm, at a temperature 4ºC for 7 min. The plasma was separated from the blood cells and transferred to previously marked Eppendorf tubes and stored at -80ºC for later analysis.

2.4 Cortisol analysis

The Eppendorf tubes with the plasma blood were transferred to the Norwegian Veterinary School in Oslo to calculate the levels of the cortisol in the plasma by conducting a radioimmunoassay (RIA). RIA buffer containing 0.05% NaN_3 (2 ratio1) was mixed with plasma and treated for 1h at $80^{O}C$. The supernatant was extracted after centrifugation and stored at $4^{O}C$ for hormone assay. Samples were assayed in duplicate, and all tubes contained 200 µl of cortisol antibody (Abcam, ab1949) and 50 µl of hydrocortisone (1, 2, 6, 7-$^{3}H(N)$) (Mayer, 1990).

2.5 Statistical methods

All values are presented as mean ± standard error of mean (SEM). Levels of cortisol and SGR between treatment and control groups were analysed by one-way analysis of variance (ANOVA) with tryptophan treatment as the categorical variable and plasma cortisol concentrations and/or SGR as independent variables. Least significance difference post hoc test was used to assert between-group differences, homogeneity of variance was confirmed by Levene's test, and normality was confirmed by the Kolmogorov-Smirnov method. All analysis was performed using Statistica software (StatSoft, Tulsa, Oklahoma).

3 Results

3.1 Statistical results by samplings

Cortisol concentrations in fish ranged from 0.4-1.5 ng/ml pre-stress 20-100 ng/ml post stress (i.e. crowding stress for 1 hour). The ANOVAs indicated that 7 days of TRP treatment had long-term effects on post stress plasma cortisol. This was observed at both 7 ($F_{(2, 49)}$ =14.83, p<0.001***) and 21 days ($F_{(2, 51)}$ =9.6, p<0.001***) after the last meal containing crystalline TRP. In sampling 1, TRP supplementation at 2x more than the standard feed (TRP2) led to a statistically significant increase in post-stress cortisol levels (post hoc probability TRP1 vs TRP2: p=0.003***). TRP supplementation at 3x standard, on the contrary, not only counteracted this effect but also led to a small, non-significant, decrease in plasma cortisol concentrations after stress (TRP1 vs TRP3, p=0.16, TRP2 vs TRP3, p<0.001***). Results from sampling 1 are graphed in figure 3.

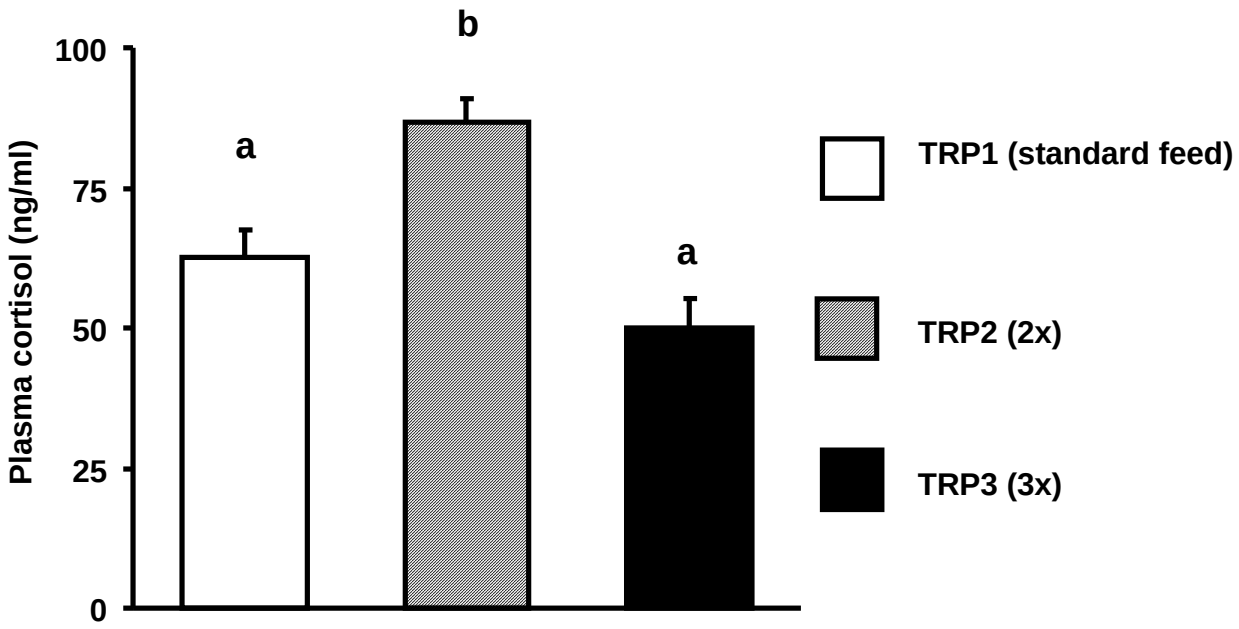

Figure 3: Plasma levels of cortisol (average ± SEM) at sampling 1 in juvenile Atlantic salmon fed diets containing 3 different levels of the serotonin precursor, TRP. See text for ANOVA statistics. The letters (a and b) indicate statistically significant differences

On the 2nd sampling occasion, i.e 21 days after the termination of TRP treatment, plasma cortisol levels were no longer significantly different between TRP1 and TRP2 (PostHoc Turkey p=0.32). However, post stress plasma cortisol levels were now significantly reduced in TRP3 groups compared to both TRP1 and TRP2, (PostHoc Turkey, TRP 1 vs TRP3 p=0.02*; TRP2 vs TRP3 p<0.001***) (Fig. 4).

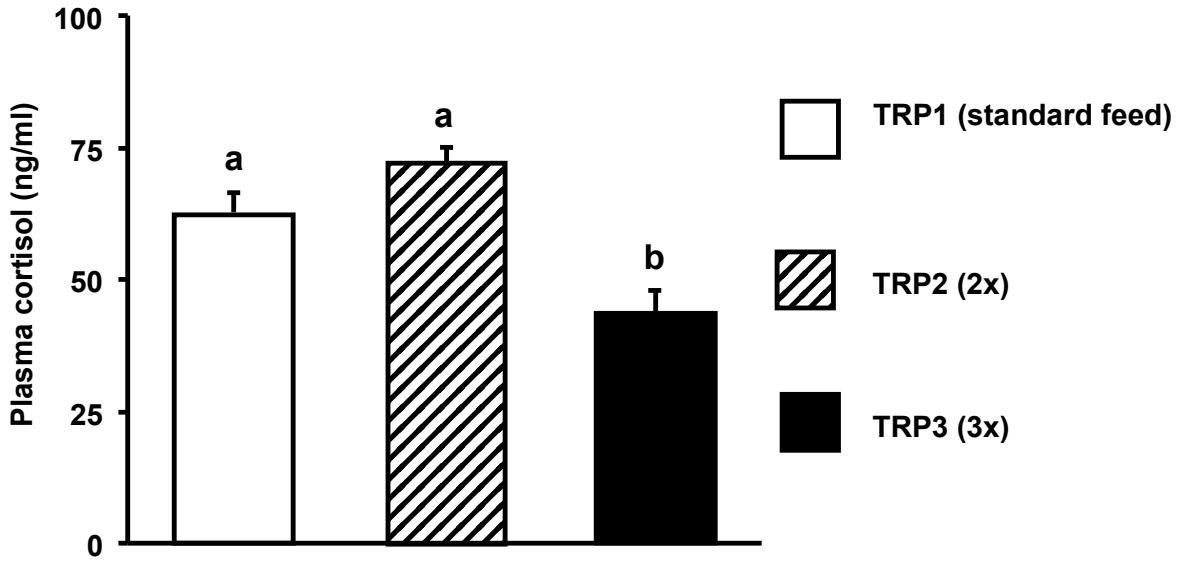

Figure 4: Plasma levels of cortisol (± SEM) in juvenile salmon fed 3 different TRP enriched diets corresponding to: a standard amount of TRP (TRP1), 2x (TRP2) and 3x (TRP3) after 21 days. TRP1-TRP2: not significant (a). TRP3-TRP1 $p<0.02$; TRP3-TRP2 $p<0.001$.

3.2 Feed intake and growth

The results from linear regression analyses performed to investigate relationships between numbers of ballotini beads in x-rayed fish, the day after tryptophan (pre-treatment) and plasma cortisol levels, were not significant ($F_{(2,44)}= 1.0$ $p= 0.15$). All fish ingested the same amount of ballotini beads and TRP. There was also no significant difference in specific growth rate (SGR) between treatment groups ($F_{(2,370)} = 0.8$, $p=0.45$)

4 Discussion

The purpose of this thesis was to investigate the possible long-term effect of TRP dietary supplementation on the physiological stress response, specifically, cortisol production. Previous studies show short term counteractive effects on stress reactivity after seven days of feeding with TRP enriched feed (Lepage et al., 2002; Lepage et al., 2003; Wimberg et al., 2001; Basic et al., 2012). This was further demonstrated in a recent study, where the stress reducing effects of TRP seemed to be limited to one day after termination of TRP treatment in cod (Basic et al. 2012). This is somewhat in contrast to the long-term effects, demonstrated in the present study. However, the above studies have all been performed in socially isolated fish, and 5-HT neurotransmission is affected by social experience, which integrates the behavioural and endocrine response to a stressor (reviewed by Winberg and Nilsson, 1993 (Summers and Winberg, 2006; Øverli et al., 1999). Thus, the social context potentially could influence the stress reducing effects of dietary TRP treatment. In the present study fish was group reared, and it is possible that the contrasts between the present study and the study performed by Basic et al. (2012) are related to differences in the social context. Moreover, the salmon where kept in sea water in the current study, a factor that may be involved in generating the contrasting results between the current study and those conducted on rainbow trout (Lepage et al., 2002; Lepage et al., 2003; Lepage et al., 2005a). For example, seawater-acclimated coho salmon (*Oncorhynchus kisutch*), have been shown to be more responsive to stress compared to fish kept in sea water. This was demonstrated in terms of mortality, ion regulatory capacity, plasma levels of cortisol and prolactin, compared to fish kept in freshwater (Avella et al., 1991; Barton et al., 1985). However, it cannot be excluded that the long term effects of TRP presented here are specific for Atlantic salmon.

The underlying mechanisms for the long-term effects observed in this study is likely related to that TRP is the main precursor for 5-HT in the brain, and that an increase in 5-HT may

lead to increased serotonergic signalling, which may in turn lead to a trophic effect on the hippocampus. Amongst these effects, there is enhanced hippocampal inhibitory signalling to the hypothalamic CRF neurones, and in consequence, a less pronounced activation of the HPA-axis (Patel and Zhou, 2005; and see overview in Introduction).The short-time inhibitory effect of TRP treatment on post-stress cortisol levels (Lepage et al., 2002; Lepage et al., 2003; Wimberg et al., 2001, Basic et al. 2012) may be related to immediate post-synaptic effects or other forms of neural plasticity such as altered 5-HT receptor expression.. Long-term effects of TRP on cortisol release, could, on the other hand, indicate trophic effect in brain parts involved in the stress response.

The current experiment addressed post-stress cortisol production, and the results show that effects of TRP on cortisol are both time- and dose-dependent. Specifically, in sampling 1 (after 7 days of continuous TRP supplementation), the lowest treatment dose (TRP2) was associated with significantly higher post-stress cortisol levels than what was seen in both untreated controls (TRP1), and in fish receiving the highest dose (TRP3). Together with ample evidence from both mammalian and fish studies (Chaouloff, 1993; Winberg et al.,1997; Lepage et al., 2002,2003; Höglund et al., 2002; Winberg and Nilsson, 1993; Dinan, 1996), this observation indicates a complex interaction between 5-HT signalling and neuroendocrine control of corticosteroid release. Other pharmacological tools which have been used to investigate serotonergic input on the HPI-axis have similarly yielded both dose- and context dependent results. For instance, 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, may have either stimulatory or inhibitory effects on HPI axis activity in rainbow trout, depending on the dose and context. In undisturbed fish 8-OH-DPAT stimulates the HPI-axis (Winberg et al., 1997; Höglund et al., 2002), whereas if administrated at low doses to stressed fish, 8-OH-DPAT suppressed stress-induced elevation of plasma ACTH and cortisol (Höglund et al., 2002).

The more novel outcome of the current study is that TRP-supplementation leaves a lasting inhibitory effect on post-stress cortisol production, even after the treatment itself has been terminated. On the final sampling, individuals fed the TRP3 diet (which contained three times more TRP than the standard diet) displayed lower levels of post-stress plasma cortisol, compared to both the other diets. This observation is in support of the suggestion that TRP-enhanced serotonergic signalling leads to changes in brain structural plasticity, which are of a long-lasting or even permanent nature. It should be noted however that other studies in rainbow trout have considered an alternative mechanism for both physiological and behavioural effects of TRP, namely enhanced production of the 5-HT derivate melatonin (Lepage et al. 2005). It is however hard to envisage that enhanced melatonin production could still be effective 21 days after the termination of the treatment (c.f. figure 4).

Lepage, et al. (2002) reported how different concentrations of enriched TRP diets affect post stress plasma cortisol levels, dependent on their dose (applied over 7 days). While fish fed with 4x TRP displayed lower cortisol levels compared to control groups, 8x TRP led to higher cortisol levels. On the other hand, Winberg et al. (2001) and Lepage et al. (2003) fed fish with TRP during 3 days but obtained no significant differences in plasma cortisol levels and/or aggressive behaviour in rainbow trout. These results suggest that feeding fish with an enriched TRP diet for less than 7 days does not yield a measurable effect in neither fish stress physiology or behaviour, another indication that some kind of modulation of brain structural plasticity is involved in mediating TRP's effect. The results of the current experiment are in accordance with this suggestion, and, in addition I observed a long-term effect of using tryptophan-enriched diets to reduce cortisol levels after stress. As far as I am aware, this is the first time periodic (as opposed to continuous) TRP treatment has been implemented and it may represent an important contribution to control stress regulation in aquaculture.

It has been previously reported that in fish high serotonergic levels in the telencephalon (which contains homologous structures to the mammalian hippocampus), diminishes their response to chronic stress, helping the organism cope better with this situation (Winberg et al. 2001; Lepage et al., 2002; Lepage et al 2002, 2003; Höglund et al, 2005). Additionally, there may be reason to believe that the direction of TRP-induced alterations to HPI-axis output changes with time. Since LNAA compete for transporters at the blood brain barrier, levels of [TRP] may be affected over time by competition with other LNAA, this may explain how during sampling 1, TRP (2x) lead to a significant increase in plasma cortisol, while in sampling 2 the effect of TRP was inhibitory (although, it should be noted that the dose used to obtain this effect was also higher (3X)). .

The delayed effect of TRP on the serotonergic system may depend on a similar mechanism as the one reported during treatment with specific serotonin re-uptake inhibitors (SSRI's). SSRI's such as fluoxetine (Prozac) are used in order to treat humans suffering from depression and the effect is only evident after 3 weeks (Mongeau et al., 1997, Nutt et all., 1999). Although the mechanism regulating this effect is not yet fully understood, it appears to be maintained across vertebrates (Winberg and Nilson, 1996). Other possibilities suggest an effect of a range of determinants of neural plasticity, such as synapse densities (Niitsu et al., 1995) or the changes of the activity of 5-HT transporter proteins (Horschitz et al., 2001).

In this experiment, there was no significant relationship between ingested ballotini beads and cortisol response. This supports the claim that it is the [TRP]/[LNAA] ratio and not the absolute ingested TRP that is important for the effects of dietary TRP. Although, its important to mention that some suggest that the competition between TRP and other LNAA's across the blood-brain barrier is less important in rainbow trout than in mammals; since TRP is largely found in a free state in the total plasma pool and is available for uptake into the brain. (Aldegude et al. 2000, Rozas et al. 1990). As brain [TRP], [LNNA] and [5-HT] were

not quantified during this experiment, it would be of particular interest to analyse the effects of these parameters in future studies.

4.1 Possible Implications for Aquaculture

The groups in this experiment showed no statistical differences in growth rate. This leads us to conclude that TRP diets did not compromise the growth rates, but have the potential to minimize stress. Tryptophan supplementation in the diet during seven days has an effect on plasma cortisol after an acute stressor at least up to 21 days after last ingested. High cortisol concentrations have a detrimental effect on cell proliferation and neurogenesis, which may lead to loss of cognitive function, memory and brain damage (Johnston et al., 1990; Aldegunde et al., 1998). TRP enriched diets may help reduce these effects in aquaculture through the reduction of post-stress plasma cortisol levels (Wimberg et al. 2001; Lepage et al., 2002; Lepage et al., 2003; Höglund et al, 2005).

This could potentially represent a solution for minimizing the stress effects caused by the different activities carried out in the aquaculture industry (e.g. vaccination, transfer, slaughtering, etc). In this way, it could be possible to reduce the occurrence of problems associated with stress, such as; disease, appetite inhibition, aggression, and death. Höglund et al. (2005, 2006) discuss how feeding fish with dietary TRP (for 7 days) attenuates stress-induced anorexia. Furthermore, they showed that in juvenile Atlantic cod fed with enriched TRP diets over 7 days reduces the amount of aggressive behaviour. Applying these measures may aid into obtaining more homogeneous fish growth rates in aquaculture tanks. In addition, the long-term effects of TRP diets possess an economical advantage, since, because of its long term effect, it is not necessary to feed fish for extended times with increased dietary TRP which is more expensive than regular feed. I have in this study showed a long-lasting effect of TRP treatment, but the extent of the duration of such effects should clearly be investigated in further studies.

5. Conclusion

The present study shows that there is a long term effect (21 days) in reduction of post stress plasma cortisol levels in juvenile salmon fed for 7 days an enriched TRP diet. This effect is believed to be mediated by the brain serotonergic system and its link to cortisol regulation. Decreased cortisol levels are associated with a decrease stress response in fish and this could aid in the avoidance of chronic stress in aquaculture systems. Since it is only necessary to feed fish for seven days in order to obtain effects for up to 3 weeks, this could become a common practice in the aquaculture industry, in order to reduce diseases, dominance hierarchies and heterogeneous growth rates.

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