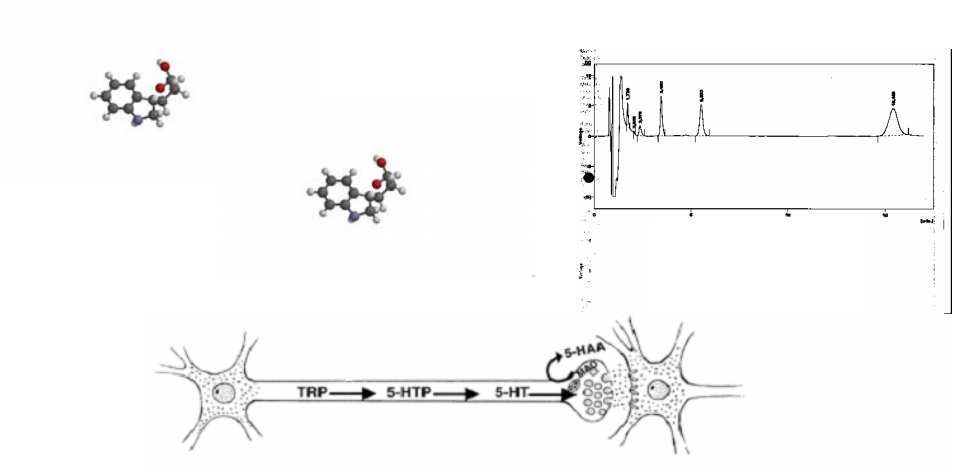
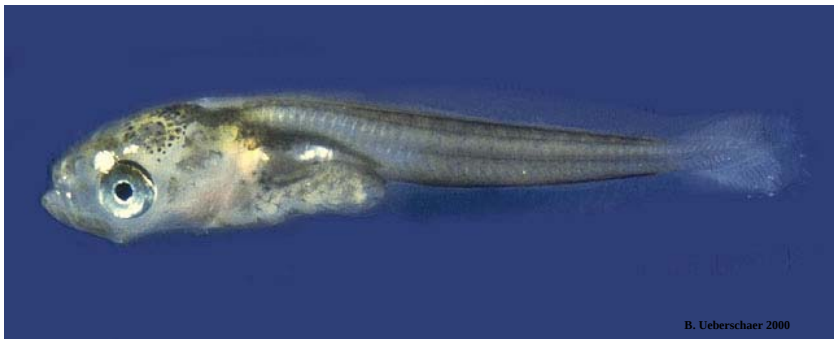
Stimulation of the serotonergic activity and suppression of aggression by L-tryptophan treatment in juvenile Atlantic cod (*Gadus morhua*)





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Cand. Scient. Thesis
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2004

Acknowledgements

First of all, I would like to thank Erik Höglund, who has patiently helped me and supported me through the project. Thank you for trying to teach me the art of science! I would also like to thank Professor Göran Nilsson for encouragement and guidance. Øyvind Øverli, your support and enthusiasm has been important, thank you for good advice and proofreading of this manuscript.

I would like to thank Havlandet Marin Yngel AS (Florø, Norway), represented by Halvard Hovland, for generously donating juvenile Atlantic cod to this project, and EWOS Innovation (Dirdal, Norway) for providing experimental pellets.

In Uppsala (Sweden), Svante Winberg and his group guided me through the HPLC analysis, and I would like to thank Olivier Lepage and Joachim Schjolden especially for valuable help.

Aina and Maria, your smiles have lightened up my day many times. Thank you both for being my friends and for letting me be a part of your lives.

One can not work at the programme for physiology without having fun. For good times and many laughs at work, and during “torsdagspils” and other social events, I would like to thank everybody in Göran Nilsson’s group and the physiology programme.

Oslo, May 2004

Marit Jørgensen Bakke

Abstract

The interest for commercial rearing of Atlantic cod (*Gadus morhua*) has grown over the last decades. However, heterogeneous growth and cannibalism during early juvenile stages causes serious financial losses. Generally in fishes, dominance based hierarchies leads to heterogeneous growth. Furthermore, a close relationship between cannibalism and heterogeneous growth has been observed. Moreover, it has been demonstrated that an increase in serotonergic activity is associated with suppressed aggressive behaviour in various vertebrate species. For instance, dietary feed supplemented with the serotonin precursor tryptophan (TRP) has been shown to suppress aggressive behaviour in rainbow trout. However, little is known about the social organization, aggressive behaviour and the underlying neural mechanisms of intraspesific competition in juvenile cod.

To develop a method for quantifying aggressive behaviour in juvenile cod, two different protocols were tested, and the effects of dietary TRP supplementation on aggression were investigated. The study also included an analysis of the effects of dietary TRP supplementation on brain serotonergic activity (indexed by the ratio between the serotonin metabolite 5-hydroxyindoleacetic acid, and serotonin).

Following an initial control period, fish were given TRP supplemented feed for seven days. This resulted in a decrease in aggressive behaviour when it was quantified with daily pairwise interactions (experiment 1). Furthermore, TRP treatment resulted in an increase in serotonergic activity (experiment 2). However, when aggression was quantified by a resident-intruder test protocol (experiment 2), no effects on aggression of dietary TRP supplementation was observed. The absence of a measurable suppression of aggression in experiment 2 could be related to stress induced by the resident-intruder test, suggesting that this protocol is less suitable for detecting changes in aggressive behaviour in juvenile cod.

In conclusion, this study shows that juvenile cod are highly aggressive, and that dietary TRP supplementation can suppress this behaviour. Thus, TRP supplementation may offer a presumptive strategy for decreasing aggression and associated problems, such as size heterogeneity and cannibalism, in cod rearing.

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Abbreviations

AAD	Amino Acid Decarboxylase
ALDH	Aldehyde Dehydrogenase
ALRD	Aldehyde Reductase
BH_4	Tetrahydrobiopterin
CNS	Central Nervous System
DA	Dopamine
5-HIAA	5-Hydroxyindoleacetic Acid
5-HIAL	5-Hydroxyindoleacetaldehyd
HPLC-EC	High Performance Liquid Chromatography with Electrochemical Detection
5-HT	5-Hydroxytryptamine, Serotonin
5-HTOH	5-Hydroxytryptophol
5-HTP	5-Hydroxytryptophan
LNAA	Large Neutral Amino Acid
LNAAc	Carrier for Large Neutral Amino Acids
MAO	Monoamine Oxydase
NE	Norepinephrine
PCA	Perchloric Acid
S.E.M.	Standard Error of Mean
SSRI	Selective Serotonin Reuptake Inhibitor
SD	Standard Deviation
TH	Tryptophan Hydroxylase
TRP	Tryptophan
VMAT	Vesicular Membrane Transporter

1 Introduction

1.1 Heterogeneous growth and cannibalism in commercial rearing of juvenile Atlantic cod

The decrease in cod stocks and resultant fishing restrictions has led to a rising interest for rearing Atlantic cod (*Gadus morhua*) commercially. A general problem in aquaculture is that intraspesific aggression leads to heterogeneous growth and cannibalism in larvae and juvenile fish (Jobling and Wandsvik, 1983; Baras and Jobling, 2002; Kestemont et al., 2003). This has also been observed in juvenile cod, where mortality due to cannibalism has been reported to be especially high from the time the larvae reach the length of 12-15 mm, and undergo metamorphosis, until the digestive system is fully developed and the fish are 40-50 mm (van der Meeren, 2002). During this time frame, allometric changes in mouth size, body height and length have been suggested to make juvenile cod more vulnerable to cannibalism (Otterå and Folkvord, 1993). Moreover, cannibalistic individuals have been suggested to have a greater growth rate than individuals eating only commercial dry feed (Folkvord and Otterå, 1993), implying that cannibalism could increase size heterogeneity. This has also been observed in other species, and Baras and Jobling (2002) stated that size heterogeneity facilitates cannibalism, and that cannibalism itself also affects size heterogeneity. In other words, cannibalism can be both a cause and an effect of size heterogeneity.

Strategies employed to reduce this problem in cod rearing are: continuous distribution of feed, optimalisation of feed size, good feed access throughout the tank (Van der Meeren 2002), optimal stocking densities (Hecht and Pinienaar, 1993), and size grading (Otterå and Folkvord, 1993). However, these methods are both time and recourse consuming, and more cost effective methods of reducing size heterogeneity and cannibalism are desirable.

1.2 Aggressive behaviour, dominance based hierarchies and size heterogeneity

Many vertebrates are socially organized in dominance based hierarchies, where aggressive behaviour is an important component. In fish, as in other vertebrates, the establishment of such hierarchies is usually based on repeated encounters between two individuals, and the outcome of these pairwise encounters mainly depends on individual fighting ability (Huntingford and Turner, 1987).

An individual occupying a low position in a dominance based hierarchy often shows physiological signs of chronic stress, following constant threats and attacks from individuals occupying higher social positions. This leads to a general behavioural inhibition, including reduced feed intake, suppressed aggressive behaviour and reduced locomotor activity (Noakes and Letherland, 1977; Ejike and Schreck, 1980; Scott and Currie, 1980). Dominant individuals, on the other hand, show aggressive behaviour towards subordinate fish, ensuring that they consume a larger part of the feed offered (Jobling and Wandsvik, 1983; Metcalfe, 1986; Winberg et al., 1993; Alanara et al., 1998). Since one major factor determining the fighting ability in fish is the body size, the dominant individuals constantly consolidate their high position in the hierarchy by growing faster (Abbott and Dill, 1989).

Heterogeneous growth is also common in juvenile cod (Folkvord and Otterå, 1993), but information on the social organization of the cod is poor. However, it has been demonstrated that cod also show aggressive behaviour, and may be organized in dominance based hierarchies (Brawn, 1961). This suggests that suppression of aggression could result in less heterogeneous growth and a reduced incidence of cannibalism in commercial rearing of cod.

1.3 Involvement of the central serotonergic system in aggressive behaviour and social hierarchies

A connection between aggressive behaviour and the activity of the brain serotonergic system(s) has been suggested for a variety of vertebrate species, from primates (Raleigh and McGuire, 1991), rodents (Chen et al., 1994; Saudou et al., 1994; Cases et al., 1995; Hen, 1996), and reptiles (Deckel, 1996; Korzan et al., 2001; Summers et al., 2003) to teleost fish (Winberg and Nilsson, 1993; Perreault et al., 2003). The anatomical organization of the serotonergic system(s) in the brain seems to be remarkably constant throughout the vertebrate subphylum (Parent et el., 1984). Along the midline of the brainstem lies clusters of serotonin (5-hydroxytryptamine, or 5-HT) containing cell bodies, including the dorsal and medial raphe nuclei from which the whole central nervous system (CNS) is innervated in mammals (Takeuchi, 1988; Törk, 1990; Jacobs and Azmitra, 1992). The same seems to be true for teleost fish (Kah and Chambolle, 1983; Yoshida et al., 1983; Parent et al., 1984; Bolliet and Ali, 1992; Ekström et al., 1992).

Social subordination and other stressors result in a general activation of the brain serotonergic system(s) in fish (Winberg and Nilsson, 1993; Winberg and Lepage, 1998; Øverli et al., 1999; Höglund et al., 2000) as in other vertebrates (Dinan, 1996; Summers et al., 2003). A stress-induced increase in 5-HT release in fish is often associated with a general behavioural inhibition, observed as reduced aggressive behaviour (Winberg and Nilsson, 1993), feeding (De Pedro et al., 1998; Øverli et al., 1998) and spontaneous locomotor activity (Winberg et al., 1993; Øverli et al., 1998). This has also been shown in mammals (Nelson and Chiavegatto, 2000; 2001), and Nelson and Chiavegatto (2001) concluded that the central 5-HT system play a key role in the expression of aggressive behaviour. However, the underlying neural mechanisms in this type of behaviour is complex, and other neurotransmitters, such as dopamine (DA) and norepinephrine (NE), have been suggested to be involved in the mediation of aggressive behaviour in fish (Winberg and Nilsson, 1993, Øverli et al., 1999; Höglund et al., 2001).

Pharmacological manipulations of the central 5-HT system with 5-HT precursors, selective 5-HT reuptake inhibitors (SSRI), and 5-HT_{1A} and 5-HT_{1B} receptor agonists,

mainly suggest an inhibitory role for this system on aggression in mammals (Miczek et al., 1998; Fish et al., 1999; Lyons et al., 1999; Nelson and Chiavegatto et al., 2001). This has also been demonstrated in a reptile, *Anolis carolinesis*, where treatment with sertraline, a SSRI, reduces aggressive displays and attacks, and reverses social status (Larson and Summers, 2001). Furthermore, treatment with fluoxetine, another SSRI, has been shown to suppress aggressive behaviour in a teleost, the bluehead wrasse (*Thalassoma bifasciatum*) (Perreault et al., 2003). This suggests that the inhibitory role of the serotonergic system(s) on aggression is an evolutionary old mechanism and that this system plays a key role in the development and maintenance of social hierarchies in the vertebrate subphylum.

1.4 Serotonin synthesis and release

The neurotransmitter and modulator 5-HT belongs to a group of signal substances called monoamines, which also includes the catecholamines dopamine (DA) and norepinephrine (NE). DA and NE are synthesized from tyrosine, whereas 5-HT is synthesized from tryptophan (TRP), Fig. 1.1(A).

Generally, serotonin synthesis is limited by TRP availability (Fernstrom, 1983). When TRP reaches the brain, it is hydroxylated by tryptophan hydroxylase (TH) to 5-hydroxytryptophan (5-HTP). TH is only found in serotonin synthesizing cells, and its activity is restricted by TRP availability. This enzyme is not saturated under normal conditions (Jequier et al., 1967, Jequier et al., 1969, McGeer et al., 1968), and synthesis of 5-HTP is considered the rate limiting step in the serotonin synthesis (Green and Sawyer, 1966). TH also depends on the availability of oxygen and the cofactor tetrahydrobiopterin (BH_4), but the enzyme is unsaturated by these two substrates in intact neurons (Fernstrom and Wurtman, 1971; Carlsson and Lindquist, 1978). 5-HTP is directly decarboxylated to serotonin (5-hydroxytryptamine, 5-HT) by aromatic L-amino acid decarboxylase (AAD), which is widely distributed throughout the body. Decarboxylation apparently occurs immediately following TRP hydroxylation, since

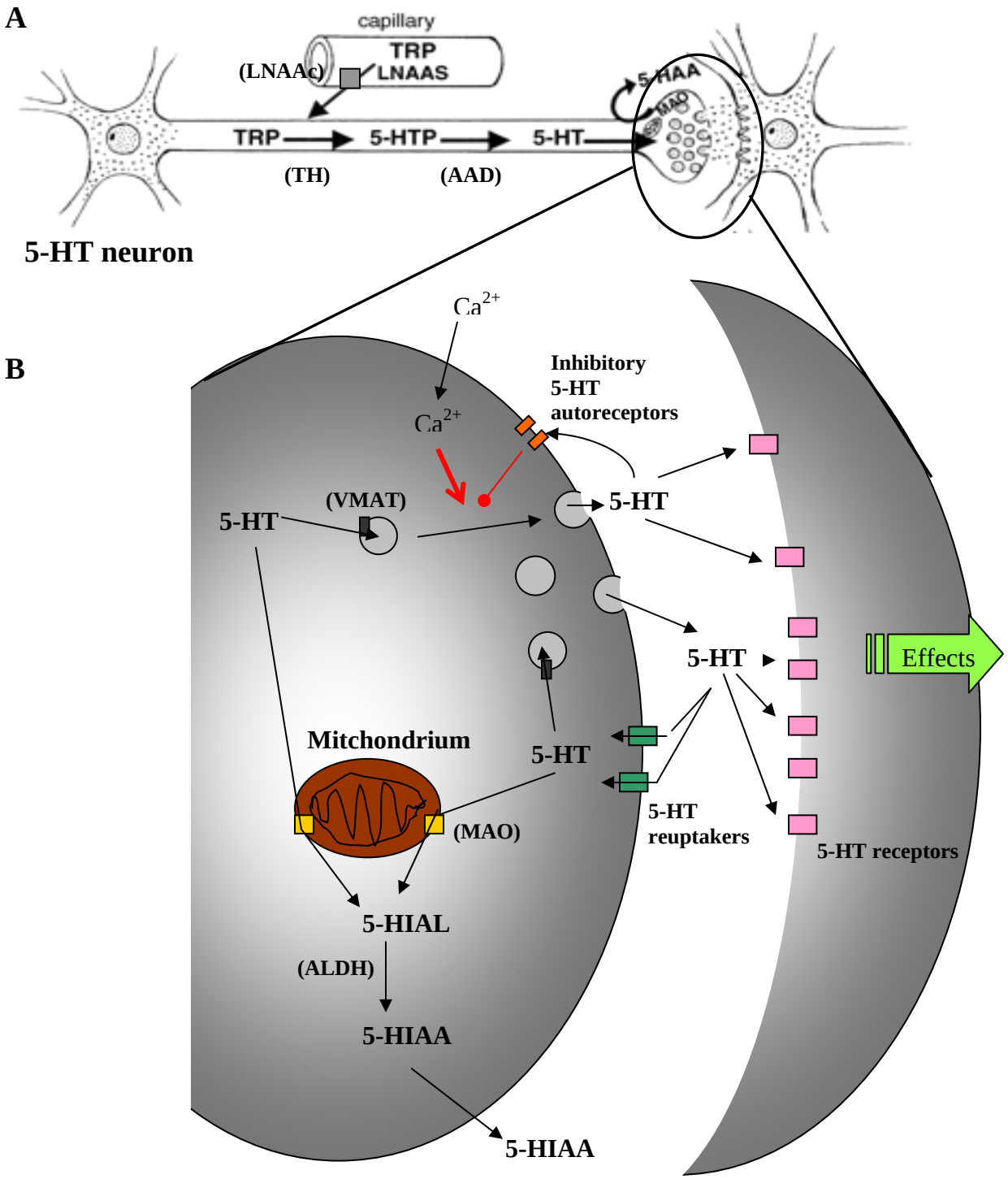

Figure 1.1 Pathways for synthesis and catabolism of serotonin in the brain. See text for details. AAD=amino acid decarboxylase; ALDH=aldehyde dehydrogenase; 5-HIAL=5-hydroxyindoleacetaldehyde; 5-HIAA=5-hydroxyindoleacetic acid; 5-HT=5-hydroxytryptamine, serotonin; LNAA=large, neutral amino acids; LNAAc=carrier for LNAA; MAO=monoamine oxydase; SERT=serotonin transporter; TH=tryptophan hydroxylase; TRP=L-tryptophan; VMAT=vesicular membrane transporter
(Figure A is modified from Fadda, 2000)

5-HTP levels are very low and difficult to detect (Long et al., 1982).

As most neurotransmitters, serotonin is stored in vesicles in the nerve terminals (Tamir and Gershon, 1990). Cytosolic 5-HT is actively transported into the vesicles through vesicular membrane transporters (VMAT). The non-vesicular pool of cytosolic 5-HT is metabolized by monoamineoxydase (MAO) (Boadle-Biber, 1993) as described below. As much as 70% of intraneuronal 5-HT can be metabolized without being released (Lookingland et al., 1986). When an action potential reaches the terminal and the membrane depolarizes, 5-HT is released into the synaptic cleft by exocytose. After release, the serotonergic signal is terminated by presynaptic reuptake of 5-HT (Blakely et al., 1991; Hoffman et al., 1991). Following uptake, 5-HT is either recycled into vesicles, or deaminated to 5-hydroxyindoleacetaldehyd (5-HIAL) by MAO which is located in the outer membrane of mitochondria. 5-HIAL is either reduced to 5-hydroxytryptophol (5-HTOH) by aldehyd reductase (ALRD) or oxidized to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase (ALDH). Whether reduction or oxidation takes place, depends on the ratio of NADH and NAD^+ in the tissue, since ALRD is NADH dependent and ALDH is NAD^+ dependent. However, the main metabolite of serotonin in the brain is 5-HIAA (for review, see Boadle-Biber, 1982; 1993). Metabolism of 5-HT in the brain is presented in figure 1.1(B).

1.5 Quantification of serotonergic activity

Since 5-HT is not deaminated by MAO when stored in vesicles, the ratio between the concentrations of 5-HIAA and 5-HT (5-HIAA/5-HT) is commonly used to quantify central serotonergic activity. Shannon et al. (1986) showed that electrical stimulation of dorsal raphe nucleus in rats resulted in increased levels of 5-HIAA and also in the 5-HIAA/5-HT ratio in various regions of the forebrain in rats. The ratio was suggested to be a more valid index of serotonergic neuronal activity than the metabolite concentration *per se*, because variances related to tissue sampling and total levels of 5-HT and 5-HIAA are reduced. The reability of 5-HIAA for quantification of 5-HT activity has been discussed,

mostly because the formation of 5-HIAA not necessarily is related to functional 5-HT release. As mentioned in section 1.4, 5-HT can be oxidized by MAO directly, if it is not stored in vesicles (Boadle-Biber, 1993). Thus, increased levels of 5-HIAA may also reflect a rise in the intraneural 5-HT pool available for catabolism rather than functional release of serotonin (Cumming et al., 1992). However, the 5-HIAA/5-HT ratio is widely used as an index of serotonergic activity, and presently, there are no practical alternatives to this method. Therefore, this approach has been adopted in the present study.

1.6 Manipulation of serotonin by dietary amino acid composition

TRP is an essential amino acid for teleost fish, as well as for other vertebrates, and availability of TRP limits the synthesis of serotonin (Fernstrom, 1983). An unspecific carrier for large neutral amino acids (LNAAc), transports TRP over the blood-brain barrier. In addition to TRP, this carrier also transports tyrosine, phenylalanine, leucine, isoleucine and valine (Fernstrom and Wurtman, 1972; Fernstrom 1983). Thus, the amount of TRP entering the brain depends not only on plasma levels of TRP, but also on the plasma levels of the other amino acids transported by the LNAAc. In particular, the relation between TRP and tyrosine uptake is of interest, since tyrosine is the precursor for DA and NE. These catecholamines are, in addition to 5-HT, known to be involved in the modulation of aggressive behaviour in fish (for review, see Winberg and Nilsson, 1993; Øverli et al., 1999; Höglund et al., 2001). However, Lepage et al. (2002; 2003) showed that TRP supplemented feed given for seven days, increased brain levels of TRP without decreasing brain levels of other LNAA in rainbow trout (*Oncorhyncus mykiss*). This was accompanied by an increase in serotonergic activity, as indicated by measurements of the 5-HIAA/5-HT ratio. Moreover, acute i.p. administration of TRP has been shown to increase TRP hydroxylation in rainbow trout (Aldegunde et al., 1999), lending further support to a positive relationship between the circulating TRP level and 5-HT activity in fish.

1.7 Possible effects of dietary TRP supplementation on size heterogeneity and cannibalism

In line with a stimulatory effect of 5-HT synthesis and release, TRP treatment has been shown to suppress aggressive behaviour in vertebrates including mammals (DeNapoli et al., 2000), birds (Shea et al., 1990) and teleost fish (Winberg et al., 2001). Moreover, this effect of TRP treatment has been demonstrated to suppress the tendency to develop dominance based hierarchies in male chicken of domesticated hen (Shea et al., 1990). Effects related to suppression of aggressive behaviour and the tendency to develop dominance based hierarchies by TRP treatment, have also been observed in the juvenile grouper *Epinephelus coioides,* where reductions in size heterogeneity and cannibalism were reported in groups of fish given feed supplemented with TRP (Hseu et al., 2002).

Shea et al. (1990) reported that the suppressive effect of TRP treatment on aggression was highest in the dominant chicken. Moreover, they suggested that this effect results in a more even distribution of feed within a group. This effect of TRP has also been suggested for fish forming dominance based hierarchies, since a TRP induced suppression of aggression will be most pronounced in dominant individuals who consume the larger part of the feed offered (Winberg et al., 2001). Thus, TRP treatment offers a presumptive tool to decrease size heterogeneity and cannibalism during intensive rearing of juvenile cod.

1.8 Aims

This study aims to develop a protocol suitable for quantification of aggressive behaviour in juvenile Atlantic cod by comparing two different protocols, and to investigate if the central serotonergic system(s) is involved in the mediation of this behaviour. Moreover, effects of dietary TRP supplementation on aggressive behaviour and serotonergic activity are investigated.

2 Materials and Methods

In order to find a suitable method to quantify aggression in pharmacological studies of juvenile cod, this study includes two experiments with different approaches to measure aggression. In experiment 1, aggression was quantified in pairs of cod that were allowed to interact in daily sessions, whereupon the fish were re-isolated (further described in section 2.3.1.). In experiment 2, a daily resident-intruder test was used to quantify aggression (further described in section 2.3.2.). To investigate the effects of dietary TRP supplementation and the resident-intruder tests on the central serotonergic system, the [5 HIAA]/ [5-HT] ratio was quantified in the brains originating from the individuals in experiment 2.

2.1 Animals

Juvenile Atlantic cod (*Gadus morhua*) was donated by Havlandet Marin Yngel AS (Florø, Norway), and brought to the University of Oslo at six weeks post-hatching, weighing 0.1-0.2 grams. Fish were kept indoors in a 400 liters holding tank, continuously supplied with aerated saltwater, at approximate density of 0.5 fish kg/m3. During the experiment the water temperature varied between 9 and 14°C. The water in the holding tank was bubbled with air to ensure saturated oxygen levels. Light regime was set to 12 hours light and 12 hours dark (lights on form 0800 to 2000). The fish were handfed daily to satiation with commercial pellets (granular size 0.6 and 0.8 mm) designed for marine juvenile fish (AlgoNorse, Ewos, Norway).

Fish were kept in the holding tank for at least 1 week prior to the experiments, and selected according to size to obtain as homogeneous experimental groups as possible. Each fish was weighed before it was transferred to experimental aquaria. Prior to experiment 1, fish were marked for individual recognition of pair members by a small cut in the lower or upper part of the tail. In experiment 1, fish weighed 0.36 ± 0.08 g, and the difference within a pair did not exceed 20%. Resident fish in experiment 2 weighed 0.44 ± 0.12 g, whereas intruders weighed 0.27 ± 0.06 g (all weights are mean ± SD).

2.2 Experimental setup

2.2.1 Observation aquaria

Behavioural studies were conducted in 32cmx23cmx20.5cm (10 l) transparent plastic observation aquaria divided in two chambers with a removable wall of grey PVC. Each chamber had separate water-supply, drain and air-supply. Three sides of the tanks were covered with black plastic film, with only the front left open to allow observations. The top of the tanks were covered with transparent PVC lids.

2.2.2 Feed

In experiment 1, a wet feed was prepared as described by Winberg et al. (2001): 1 kg of herring fillets and 1 kg of shrimps were ground and divided in two 1 kg portions. 0.5 liter of water with gelatin was added to each portion. One of the portions served as control feed, while the other was supplied with 5 g TRP, mixed in the water with a magnetic stirrer. The feed was evenly distributed to a 3 mm thick sheet on tin foil, and frozen at -20°C. Before use, the frozen feed was pressed through a steel mesh, giving granules of approximately 0.8 mm, and mixed with water to a concentration of 1g food pr. 25 ml water. Thus 1ml food-solution corresponds to approximately 10% of the body weight of the largest fish.

In experiment 2, EWOS Innovation (Dirdal, Norway) provided both control and tryptophan coated pellets (0.9mm).

Control feed and TRP supplemented feed from both experiments were analyzed for TRP levels with HPLC-EC (see section 2.4). In experiment 1, the TRP content of the supplemented feed was 28.1 mg/g dry weight while that of control feed was 0.10 mg/g dry weight. In experiment 2, the supplemented feed contained 27.6 mg/g dry weight and the control feed 0.22 mg/g dry weight.

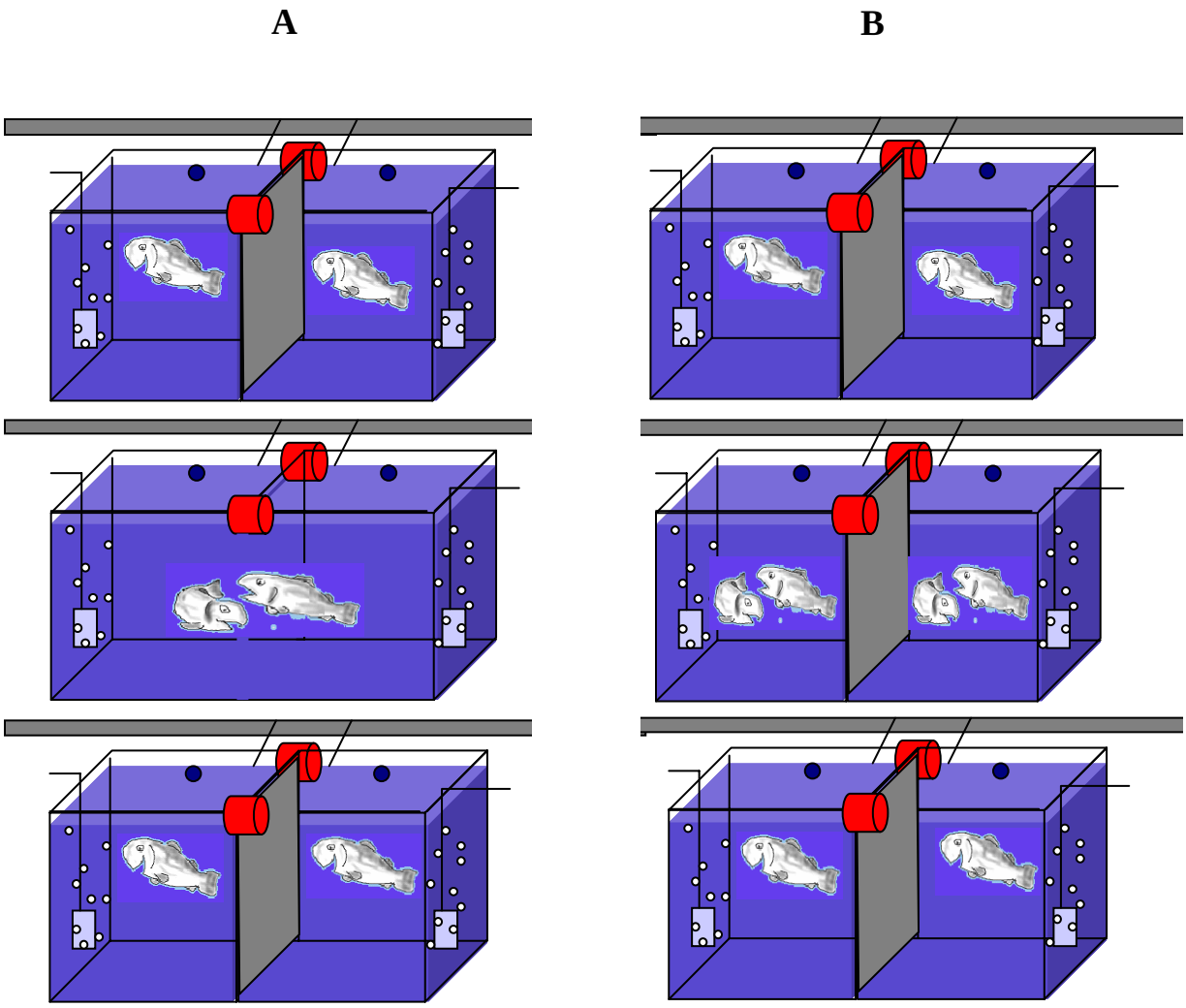

Fig. 2.1 Experimental setup for experiment 1 (A) and experiment 2 (B). Fish were isolated in individual compartments, and aggressive behaviour was observed daily by lifting the removable wall between the compartments (A) or by resident-intruder tests (B). After the observations, the fish were reisolated until the next day.

2.3 Quantification of aggressive behaviour

The following aggressive behaviour patterns were observed: Approach, chase and nip/bite. These are patterns previously described as aggressive behaviour for other fish species (Baerends and Baerens-van Roon, 1950; Fabricius, 1953; Noble, 1934), and also for larger juvenile cod (>5cm) (Brawn, 1961). Approach: A fast approach towards the other fish with dilation of the mouth bottom and operculum. A pectoral fin is usually the target, but sometimes the approaches are aimed at the caudal (tail) fin. This was the most common of the aggressive acts observed, occurring during the whole experiment. Chase: An approach that elicits an escape response and the attacking fish follows the escaping fish for more than three body lengths. This act was most pronounced the first days of the experiment. Nip/ bite: A fish nips or bites the other. This was usually seen after an approach or a chase.

2.3.1 Experiment 1

Following transfer from the holding tank to observations aquaria, fish were isolated and allowed to acclimate for three days before the behavioural studies started. During this period, all fish were given TRP free wet feed to satiation daily.

After the acclimation period, aggression was quantified daily. In each experimental tank, the PVC wall was gently lifted up, and the two fish sharing an aquarium were allowed to interact (Fig. 2.1A). The attack latency was quantified as the time from the wall was removed to the first aggressive attack was observed. During ten minutes following the first attack, the number of aggressive acts was quantified, whereupon fish were re-isolated until the next day. If no aggressive acts were observed during ten minutes, the number of aggressive acts was set to zero, and the attack latency to 10 minutes.

Baseline aggression was quantified for three days, whereupon TRP supplemented feed was introduced to 7 of the 14 pairs. The remaining 7 pairs were fed TRP free wet feed throughout the experiment and served as controls. Control and TRP fed pairs were observed on a daily basis for 7 days. One control fish died during this period,

consequently the control group was reduced to 6 pairs at the end of the experiment. During the whole experiment, fish were given feed at least two hours prior to the observations.

2.3.2 Experiment 2

As in experiment 1, fish in experiment 2 were allowed to acclimate for three days before behavioural studies were initiated. During this period, all fish were given TRP free experimental pellets to satiation daily. On day four, baseline aggression was quantified in 20 fish by a resident-intruder test, while 16 fish were kept isolated and served as controls. Aggression was quantified daily the following seven days by a resident-intruder test, whereupon the experiment was terminated on day eleven.

In this resident-intruder test, quantification of aggressive behaviour was based on the same aggressive acts and attack latency as described in experiment 1. However, instead of removing the wall in each tank and let the two fish interact, a smaller intruder was introduced to a resident fish. 20 intruders were kept in a holding tank, and were used repeatedly during the experiment. Intruders were gently moved from the holding tank to the experimental tank with a net. Attack latency was measured as the time it took from the intruder entered the experimental tank to the resident fish attacked the intruder. Thereupon the total number of aggressive acts was counted for ten minutes. After quantification of baseline aggression on day four, TRP supplemented feed was introduced to 10 fish subjected to the resident-intruder test, and 8 of the isolated controls.

Mortality occurred in all groups, and the final numbers of replicates were 6 for fish fed TRP supplemented feed exposed to resident-intruder tests, 7 for isolated control fish fed TRP supplemented feed, 8 for fish fed control feed and exposed to resident-intruder tests, and 5 for isolated control fish fed control feed.

Directly following the last quantification of aggression, fish were sacrificed by decapitation, and the brains were rapidly dissected out. Brains and bodies were placed in Eppendorf tubes and frozen in liquid nitrogen, within three minutes after netting. The brains and fish bodies were stored at -80°C.

2.4 Quantification of TRP, 5-HT and 5-HIAA with High Performance Liquid Chromatography (HPLC)

Samples of experimental feed and fish bodies were homogenized in 4% (w/v) ice-cold perchloric acid (PCA) containing 0.2% EDTA, and whole brains were homogenized in 4% (w/v) PCA containing 0.2% EDTA and 40 ng ml^{-1} epinine (deoxyepinephrine, internal standard). The samples were subsequently centrifuged at 13000 rpm for 10 minutes at 4°C and the supernatants were stored at -80°C until analyzation.

[5-HT] and [5-HIAA] from whole brains were quantified using a reversed phase HPLC with electrochemical detection (HPLC-ED), modified form Øverli et al. (1999). The system consists of a solvent delivery system (Coulochem, W/Multiole, P'STATS, USA), an auto injector (Midas, Spark, Holland), a reverse phase column (4.6 x 100 mm, Hichrom, C18, 3.5μm) kept at 40°C, and an ESA 5200 Coulochem II EC-detector (ESA, Bedford, Ma., USA) with two electrodes at oxidizing potentials of +320 and -40mV, respectively. A conditioning electrode with a potential of +40mV was employed before the analytical electrodes to oxidize potential contaminants. The mobile phase was delivered at 1ml/min and consisted of 75mM sodium phosphate, 0.3 mM octane sulfonic acid, in demonized (18.2 MΩ) water containing 4% acetonitril and brought to a pH 3.1. Samples were analyzed by comparison with standard solutions of known concentrations, and corrected for recovery of the internal standard (epinine) using HPLC software (CSW, DataApex Ltd., the Czech Republic).

To quantify [TRP] in all samples (feed, bodies and brains), the same HPLC-ED system was used as for quantification of [5-HT] and [5-HIAA], except that the oxidizing potential was set to +600mV and the mobile phase consisted of 75mM sodium phosphate in demonized water containing 10% methanol (Lepage et al., 2001).

The ratio [5-HIAA]/ [5-HT] was used as an index of serotonergic activity.

2.5 Statistical analyses

Weights are given as means±SD, while all other values are means ± standard error of mean (S.E.M.). For the statistical analysis, the aggressive acts and latency to first attack observed during the three days before treatment in experiment 1 (day 1-3) were pooled. Values were subjected to a repeated-measures multiple analysis of variance (MANOVA) with TRP and control treatment as independent variables. Furthermore, data on body [TRP], and brain [TRP], [5-HIAA], [5-HT] and [5-HIAA]/[5-HT] were subjected to a two-way ANOVA, with TRP or control treatment, and resident-intruder test or isolated controls as independent variables. The Tukey post hoc test (Tukey honest significant difference test for unequal N) was applied to compare means between different groups. To fulfill the assumption of normal distribution, data on aggressive behaviour, body [TRP], brain [TRP], [5-HIAA], [5-HT] were subjected to log-transformation, whereas the [5-HIAA]/[5-HT] ratio was subjected to arcsine transformation. Data on body and brain [TRP] were subjected to Pearsons correlation test. Furthermore, the relationship between brain [TRP] and brain serotonergic activity were investigated using nonlinear curve fit with GraphPad Prism 4. All other statistical analyses were performed using Statisca 5.1 (StatSoft Inc., Tulsa, USA).

3 Results

3.1 Effect of dietary TRP supplementation on aggression

In experiment 1, where aggression was quantified in repeated pairwise interactions, a significant interactive effect of TRP supplemented feed and time on the number of aggressive acts was evident, Fig. 3.1A; ($F_{7,70}$=2.65, P=0.017, repeated measures MANOVA). This resulted in a significant decrease in the number of aggressive acts in the TRP treated group on day six and seven compared to the initial control period (P=0.001 and P=0.0001 respectively). Further, a reduced number of aggressive acts was seen in the group receiving TRP supplemented feed compared to the group receiving control feed on day six and seven was observed (P=0.027 and P=0.0005 respectively). No significant effect of time on the number of aggressive acts was observed in the control group (P=0.95). There was a non-significant trend towards an interactive effect of TRP treatment and time on attack latency Fig. 3.1B; ($F_{7,70}$=2.1, P=0.052).

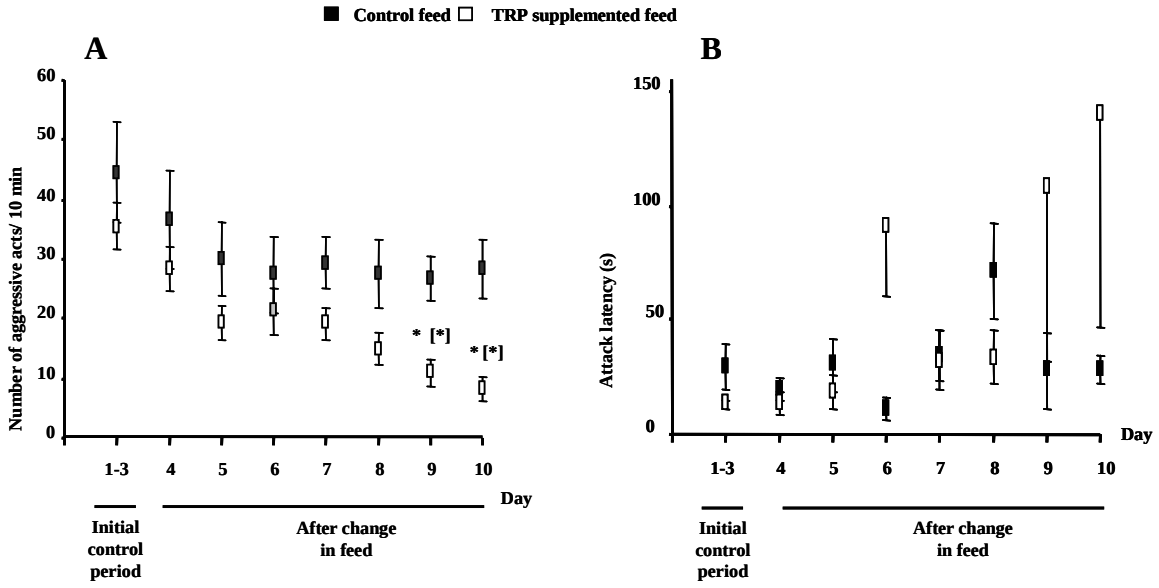

Fig. 3.1 Experiment 1: Results from daily measurements of aggressive behaviour in juvenile cod fed control or TRP supplemented feed (repeated pairing of equal sized individuals). **A** shows the number of aggressive acts and **B** shows attack latency. Asterisks, *, indicates a significant difference between experimental groups (control *vs* TRP supplemented) and asterisks in brackets, [*], indicates a difference compared to the initial control period. n=6 in both groups. Values are daily means±S.E.M. The level of significance was set to P<0.05.

In experiment 2, where aggression was quantified by resident-intruder tests, there was no interactive effect of TRP supplemented feed and time on the number of aggressive acts, Fig. 3.2A; ($F_{7,98}$=0.9107, P=0.501566), or attack latency, Fig. 3.2B; ($F_{7,98}$=0.41, P=0.89). In this experiment, a resident-intruder test was used and the data indicates a much larger variance in the measurements compared to experiment 1.

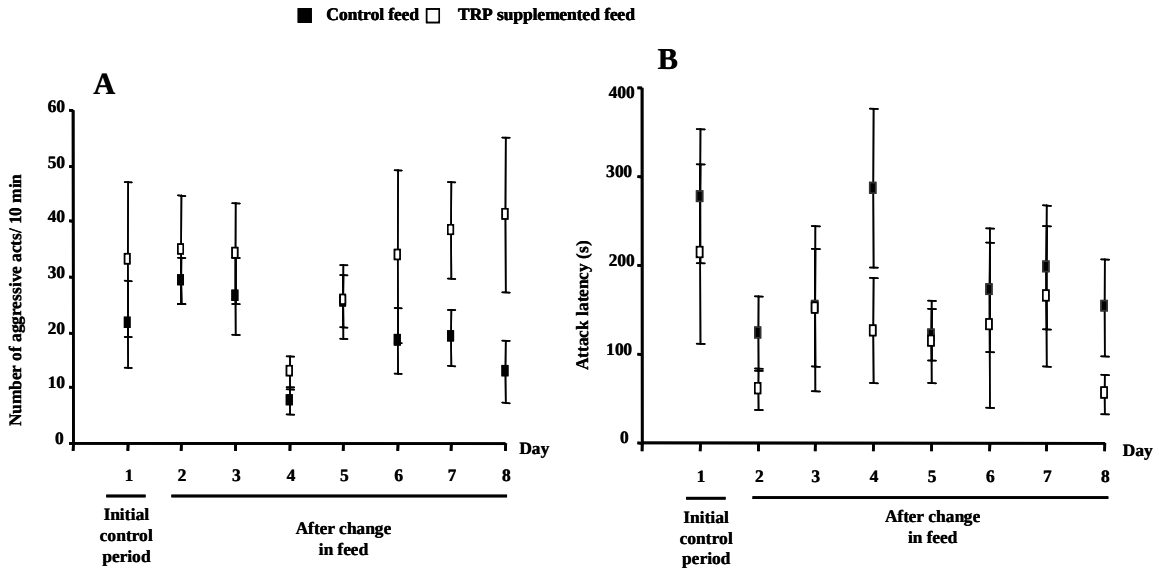

Fig. 3.2 Experiment 2: Results from daily measurements of aggressive behaviour in juvenile cod fed control or TRP supplemented feed (repeated resident-intruder tests). **A** shows the number of aggressive acts and **B** shows attack latency. n=10 in the control group, and n=6 in the TRP treated group. Values are daily means±S.E.M. The level of significance was set to P<0.05.

3.2 Effects of dietary TRP supplementation and resident-intruder test on body and brain [TRP]

Cod having received TRP supplemented feed for seven days showed significantly increased body [TRP] compared to controls (P=0.0026), Fig. 3.3(A), and a similar, non-significant trend was observed for brain [TRP] (P=0.16), Fig. 3.3(B).

There were no significant effects of subjecting the fish to resident-intruder tests compared to isolated controls on either on body [TRP] or brain [TRP] (table 3.1). Furthermore, there were no combined effects of the two variables (Table 3.1).

A significant linear relationship between body and brain [TRP] was observed (r^2=0.84, P<0.001), Fig. 3.4.

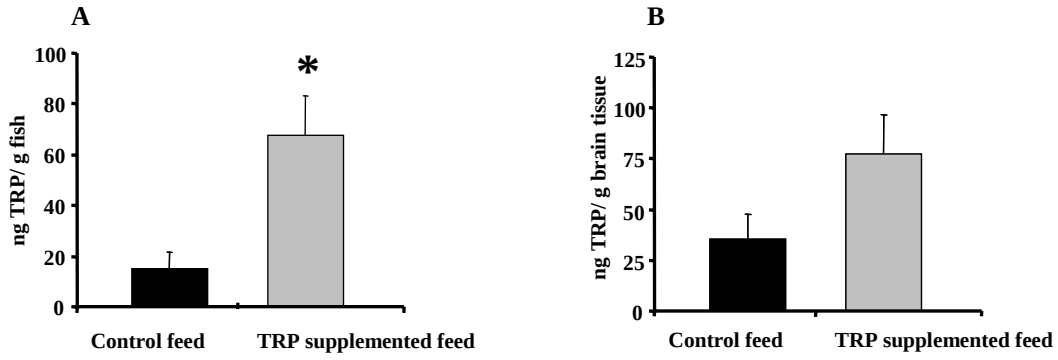

Fig 3.3 Effects of TRP treatment on body (**A**) and brain (**B**) [TRP]. n=12 in both groups. *P<0.05. See Table 3.1 for ANOVA values. Values are means+S.E.M.

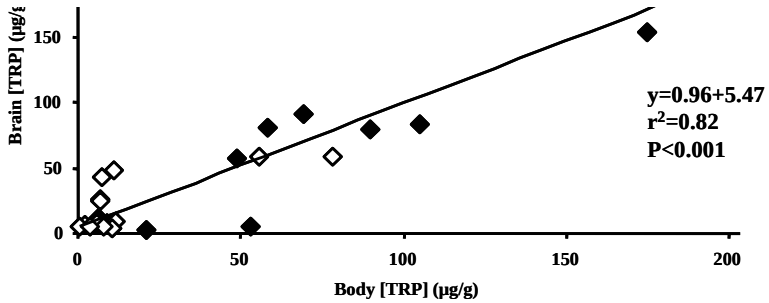

Fig 3.4 The relationship between body and brain [TRP] in fish receiving feed supplemented with TRP (27.6 mg TRP/g dry weight) (n=12; 5 isolated fish and 7 resident-intruder tested fish), or control feed (n=12; 5 isolated fish and 7 resident-intruder tested fish) during seven days.

3.3 Effect of dietary TRP supplementation and resident-intruder test on brain [5-HT], [5-HIAA] and the [5-HIAA]/ [5-HT] ratio

Receiving TRP supplemented feed for seven days did not affect brain [5-HT] when the TRP treated group was compared to the group receiving control feed throughout the experiment (Table 3.1). However, brain [5-HIAA] was significantly increased by TRP treatment ($F_{1,22}$= 0.87, P=0.024), Fig. 3.5(A). Serotonergic activity, quantified as the [5-HIAA]/ [5-HT] ratio, was significantly increased in the group receiving TRP supplemented feed compared to the control group ($F_{1,22}$=4.48, P=0.046), Fig. 3.5(B). For all three variables, data on isolated fish and fish exposed to resident-intruder tests were pooled for each treatment group.

Subjecting the fish to resident-intruder tests did not affect brain [5-HT], [5-HIAA] or [5-HIAA]/[5-HT] (Table 3.1). Furthermore, there were no combined effects of the two variables.

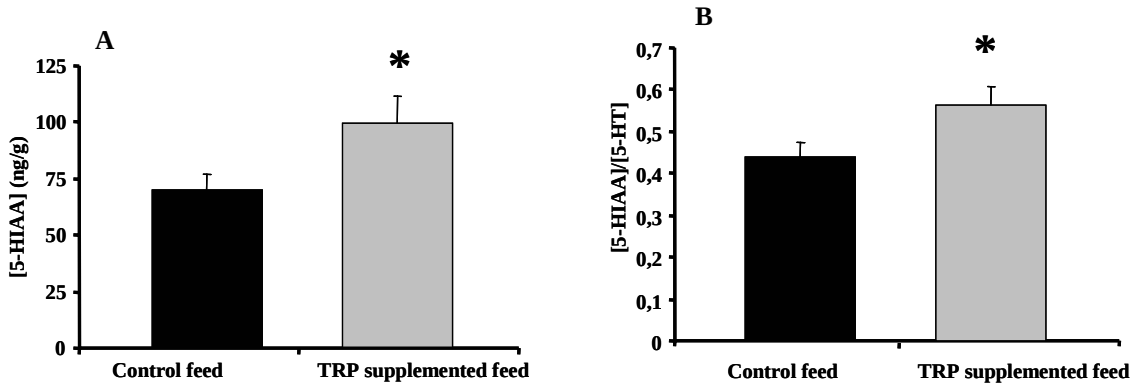

Fig. 3.5 Brain [5-HIAA] (**A**) and the [5-HIAA]/[5-HT] ratio (**B**) in fish given control feed (n=14) or TRP supplemented feed (n=12) for seven days. *P<0.05. See Table 3.1 for ANOVA values. Values are means+S.E.M.

Table 3.1 Effects of dietary TRP supplementation and resident-intruder test on body [TRP] and brain [TRP], [5-HIAA], [5-HT] and [5-HIAA]/[5-HT] in juvenile Atlantic cod.

Effect	TRP treatment	Resident-intruder test	TRP treatment and resident-intruder test				
				isolated controls		resident-intruder tested	
	ANOVA	**ANOVA**	**ANOVA**	control	TRP	control	TRP
Body [TRP]	**$F_{(1,20)}$=11.805, P=0.003**	$F_{(1,20)}$=0.707, *P*=0.410	$F_{(1,20)}$=0.560, *P*=0.448	19.82±14.77	85.13±22.65	12.66±5.43	42.54±17.67
Brain [TRP]	$F_{(1,20)}$=2.131, *P*=0.160	$F_{(1,20)}$=0.624, *P*=0.439	$F_{(1,20)}$=0.799, *P*=0.382	21.38±10.01	87.28±28.15	42.68±19.31	63.52±26.13
[5-HIAA]	**$F_{(1,22)}$=5.874, *P*=0.024**	$F_{(1,22)}$=0.391, *P*=0.538	$F_{(1,22)}$=0.005, *P*=0.945	68.87±18.01	97.08±17.42	70.57±6.57	103.28±18.14
[5-HT]	$F_{(1,22)}$=0.613, *P*=0.442	$F_{(1,22)}$=0.703, *P*=0.411	$F_{(1,22)}$=0.633, *P*=0.435	166.97±28.63	173.59±31.90	159.87±11.09	200.23±17.15
[5-HIAA]/[5-HT]	**$F_{(1,22)}$=4.485, *P*=0.046**	$F_{(1,22)}$=0.078, *P*=0.782	$F_{(1,22)}$=0.758, *P*=0.394	0.42±0.06	0.59±0.05	0.45±0.04	0.52±0.08

Values are means±S.E.M. (n=5-8).
Significant effects are indicated by bold font.
Values are presented as ng/g wet mass.

3.4 Relationship between brain [TRP] and [5-HIAA]/[5-HT]

There was a significant non-linear regression between the brain [TRP] and brain [5-HIAA]/[5-HT] ratio (y=0.67x/(6.0x)), as indicated by a r^2=0.58 and P<0.01, Fig. 3.6.

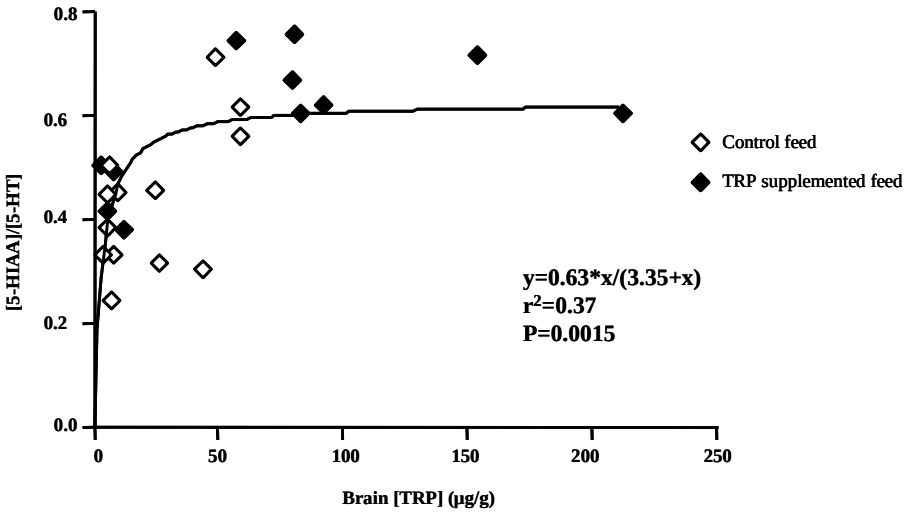

Fig. 3.6 The relationship between brain [TRP] [5-HIAA]/[5-HT] ratio in fish receiving TRP supplemented feed (27.6 mg TRP/g dry weight) (n=12; 5 isolated fish and 7 resident-intruder tested fish), or control feed (n=12; 5 isolated fish and 7 resident-intruder tested fish) during seven days.

4 Discussion

This study shows that aggression is part of the behavioural repertoire of juvenile Atlantic cod, and the results indicate that feed supplemented with the 5-HT precursor TRP suppresses this behaviour. Furthermore, TRP treatment resulted in elevated body and brain TRP content and increased brain serotonergic activity, suggesting that this neurotransmitter is involved in the mediation of aggressive behaviour in cod.

4.1 Effects of dietary TRP supplementation and different protocols on aggressive behaviour

The results from experiment 1 show that receiving TRP supplemented feed for six days reduces aggressive behaviour, quantified as the number of aggressive acts, in juvenile cod. Further, in experiment 2, juvenile cod fed TRP supplemented feed for seven days was shown to have increased brain serotonergic activity. Similar results have been obtained in chickens (Shea et al., 1990), dogs (DeNapoli et al., 2000), and rainbow trout (Winberg et al., 2001), where suppression of aggressive behaviour and increased serotonergic activity followed TRP administration. This supports the general consensus that an activation of the serotonergic system(s) suppresses aggressive behaviour.

When aggression was quantified by a resident-intruder test (experiment 2), effects of TRP treatment on aggressive behaviour were not detectable. Two different formulated feeds were used in the two experiments. However, there was no difference in the energy content of the two feeds (Winberg et al., 2001), and differences in feed composition seem unlikely to be responsible for contrasting behavioural effects (further discussed in section 4.2). Thus, it appears that the two different protocols for quantification of aggressive behaviour caused the apparent conflicting result. As further discussed below, it seems that the protocol used to quantify aggressive behaviour in experiment 1 was better suited for juvenile cod (<0.5 g) than the resident-intruder test employed in experiment 2.

Differences between the two protocols include, for instance, that the lid had to be removed before aggression quantification, in order to introduce the intruder in experiment 2, whereas just the separating wall was gently removed in experiment 1. Moreover, in experiment 2, fish were confronted with unfamiliar individuals during each resident-intruder test, and it is possible that this also contributed to a higher disturbance level. Higher levels of disturbance in the resident-intruder test could result in more intense acute stress, affecting the behaviour of the resident fish.

A positive relationship between acute stress and aggressive behaviour has been demonstrated in rainbow trout by Øverli et al. (2004), and it is possible that suppression of aggression following TRP treatment in experiment 2 was masked by a stress-related increase in aggression. Furthermore, the variation in the number of aggressive acts measured within the same treatment-groups appeared to be much larger in the data obtained with the resident-intruder test compared to the pairwise interaction test. This indicates that the resident-intruder test was less suitable for quantifying aggression in juvenile cod. Thus, repeated confrontations with the same individual for ten minutes per day is probably a suitable method for measureing the level of aggression in juvenile cod.

In juvenile rainbow trout (>90g), the resident-intruder test is the established protocol to quantify aggressive behaviour. However, the present study suggests that this quantification protocol is not suitable for juvenile cod (<0.5g).

4.2 Effects of dietary TRP supplementation on brain TRP availability

The size of the cod in the present study did not allow analysis of circulating TRP levels. However, individuals given TRP supplemented feed showed increased body [TRP], and a trend towards the same pattern was seen in brain tissue. This resulted in a linear relationship between body and brain [TRP], suggesting that circulating TRP availability for uptake in the brain was increased. This has also been demonstrated in rainbow trout, where dietary TRP supplementation induced elevated levels of plasma and brain [TRP] (Winberg et al., 2001). These results from studies on fish are also in line

with mammalian studies, where brain TRP availability depends on the relative plasma concentrations of TRP to other LNAA (Fernstrom and Wurtman, 1972).

Plasma content of insulin has also been shown to affect brain [TRP] in mammals (Fernstrom and Wurtman, 1972), suggesting that circulating carbohydrate concentrations affect the serotonergic system. Normally, high plasma levels of glucose are followed by increased circulating insulin in mammals (Zimmerman et al., 1972) and fish (Cowey et al., 1977; Wilson 1994; Blasco et al., 1996; Baños et al., 1998), inducing glucose and amino acid uptake in muscles. In mammals, as much as 75-80% of the circulating TRP is bound to albumin (Tagliamonte et al., 1973), and thereby protected from uptake in muscles compeared to other amino acids (Pozefsky et al., 1969; Fernstrom and Wurtman, 1972). Thus, hyperinsulinemia increases TRP availability to the brain, resulting in increased serotonin synthesis (Fernstrom and Wurtman, 1972). Rainbow trout albumin, on the other hand, lacks the binding site for TRP (Fuller and Roush, 1973; McMenamy, 1977), and the relationship between brain serotonergic activity and hyperglycemia appears to be different in teleost fish and mammals. In an experiment with rainbow trout, Ruibal et al. (2002) observed no effect of hyperglycemia on serotonergic activity in the hypothalamus, and a trend for a diminished serotonergic activity in telencephalon. These authors conclude that hyperglycemia does not modify brain TRP availability in rainbow trout.

Differences in feed carbohydrate contents can not be ruled out as a cause for the contrasting effects of dietary TRP supplementation on aggressive behaviour in experiment 1 and 2. However, the notion that the relative concentrations of TRP to other LNAA is unaffected by elevated insulin levels in fish (Ruibal et al., 2002), argues against this.

4.3 Effects on the brain serotonergic system(s)

In experiment 1, a significant effect of dietary TRP supplementation on aggressive behaviour was observed. A stimulating effect of dietary TRP supplementation on serotonergic activity was later shown in experiment 2, which lends support to the view

that increased brain serotonergic activity suppresses aggressive behaviour (Winberg and Nilsson, 1993; Winberg et al., 2001; Perreault et al., 2003). Furthermore in experiment 2, the relationship between brain TRP and serotonergic activity (Fig 3) suggests that the serotonin synthesis and activity is elevated to saturation in fish given TRP supplemented feed (28 mg/g). Thus, it is not unlikely that even a lower dose of TRP could generate increased serotonergic activity, and thereby decreased aggressive behaviour.

The effect of TRP supplementation on aggression in experiment 1 was observed after six and seven days (day 9 and 10). Other studies of the effects of TRP treatment demonstrate a seven days delay before behavioural and endocrine effects can be detected (Winberg et al., 2001). This time course suggests that mechanisms other than a direct effect of increased 5-HT synthesis and release are involved. Interestingly, the delay of effects resembles the one seen after administration of selective 5-HT reuptake inhibitors (SSRI) in primates (Mongeau et al., 1997) and reptiles (Larson and Summers, 2001). Generally, the underlying mechanism for SSRI is suggested to be regulation of receptor sensitization, both pre- and postsynaptically (Mongeau et al., 1997; Nutt et al., 1999).

In fish, little is known about the diversity of 5-HT receptors, but affinity assays and pharmacological studies demonstrate the presence of a 5-HT_{1A}-like receptor in salmoid fish (Winberg and Nilsson, 1996; Winberg et al., 1998; Höglund et al., 2002). In Arctic charr (*Salvelinus alpinus*), effects of 5-HT_{1A} receptor activation are dose and context dependent, suggesting that 5-HT_{1A} receptors are expressed both pre- and postsynaptically also in fish (Höglund et al., 2002). Activation of presynaptic 5-HT_{1A} autoreceptors in mammals has been associated with a decrease in aggression (de Boer et al., 2000), whereas postsynaptical 5-HT_{1A} receptors have been suggested to be involved in increased aggression (van der Vegt et al., 2003). Actions at both pre- and postsynaptic 5-HT_{1A} receptors appear to play a role in the therapeutic properties of 5-HT_{1A} ligands, although their relative contributions have to be clarified more precisely. To better understand the underlying mechanisms for a TRP induced suppression of aggression, further studies on the effect of TRP treatment on density and sensitization of the 5-HT_{1A} receptor as well as other 5-HT receptors are necessary.

4.4 Possible applications of dietary TRP supplementation in aquaculture

A serious problem observed during rearing of larvae and juvenile fish is that heterogeneous growth leads to cannibalism (Baras and Jobling, 2002). The heterogeneous growth is likely to be related to the development of dominance based hierarchies. In such a social organization, aggressive interactions between individuals are an important factor determining the social position of an individual (Huntingford and Turner, 1987). In aquaculture adverse effects of aggressive behaviour and social interactions include injury, unequal distribution of food and poor growth in subordinates.

In the present study it was shown that aggression is an important component of the behavioural repertoire in juvenile cod. This supports an early study by Brawn (1961), which suggested that the cod are organized in social dominance based hierarchies, where aggressive behaviour is an important factor for establishing the social position of an individual. Furthermore, dietary TRP supplementation was in the present study shown to suppress aggressive behaviour in the cod. Under rearing conditions, the most pronounced effects of dietary TRP supplementation are expected in dominant individuals, because they consume a larger part of the feed offered (Jobling and Wandsvik, 1983; Metcalfe, 1986; Winberg and Lepage, 1993; Alanara et al., 1998). Presumably, suppressed aggression in dominant juvenile cod could give subordinate individuals more access to feed, leading to a more homogeneous growth. Moreover, this would decrease other adverse effect of social subordination and sustained stress, such as decreased appetite (Øverli et al., 1998) and increased susceptibility to disease (Barton, 1997).

In line with the above argument, (Hseu et al., 2003) reported that feed supplemented with TRP induced decreased size heterogeneity and cannibalism in juvenile grouper (*Epinephelus coioides*). The direct behavioural effect of TRP observed in the present study and in the study performed by (Winberg et al., 2001) lends support to suppressed aggression as a main factor for decreased cannibalism and size heterogeneity reported by Hseu et al. (2003). However, Hseu et al. (2003) observed that groups of fish treated with L-TRP had lower growth rate, and suggested that this could be an effect of increased brain serotonergic activity and decreased appetite. In the present study, feed

intake was not studied, but feed was offered *ad libitum*, and other studies have reported that TRP supplementation was without effect on feed intake in chickens (Shea et al., 1991) and rainbow trout (Lepage et al., 2002; Lepage et al., 2003; Winberg et al., 2001).

4.5 Conclusions

In conclusion, aggression is an important part of the behavioural repertoire of juvenile cod, but results on quantifications of this behaviour depends on the protocol. A test where the same two individuals are exposed to each other in daily sessions in an environment familiar to both individuals is suggested to be more sensitive in detecting changes in aggressive behaviour, whereas a resident-intruder test induces a higher level of short term stress to juvenile cod, and thereby masks the original aggressive behaviour with stress-related aggression.

Further, feed supplemented with TRP suppresses aggressive behaviour in juvenil cod, an effect which seems to be mediated by the central serotonergic system.

If used in commercial rearing, a TRP induced decrease in aggression is likely to be most pronounced in dominant individuals, who consume the larger part of the feed offered. Thus, decreased size heterogeneity and cannibalism could be expected in association with TRP supplementation. This effect could be even more pronounced when combined with other methods for decreasing cannibalism, such as optimal feed dispersal and optimal stocking densities (Hecht and Pinienaar, 1993). However, by changing the behaviour of the fish instead of altering the social and/or physical environment, TRP supplemented feed could be more cost effective and less time consuming than other manipulations aimed at decreasing cannibalism, such as size grading.

References

Abbott, J. C., Dill, L. M. (1989). The relative growth of dominant and subordinate juvenile steelhead trout (*Salmo gairdneri*) fed equal rations. *Behav.* **108,** 104-113.

Alanara, A., Winberg, S., Brannas, E., Kiessling, A., Hoglund, E., Elofsson, U. (1998). Feeding behaviour, brain serotonergic activity levels, and energy reserves of Arctic char (*Salvelinus alpinus*) within a dominance hierarchy. *Can. J. Zool.* **76**, 212-220.

Aldegunde, M., Soengas, J. L., Rozas, G. (1999). Acute effects of L-tryptophan hydroxylation rate in brain regions (hypothalamus and medulla) of rainbow trout (*Oncorhyncus mykiss*). *J. Exp. Zool.* **286,** 131-135.

Baños, N., Baro, J., Castejon, C., Navarro, I., Gutierrez, J. (1998). Influence of high-carbohydrate enriched diets on plasma insulin levels and insulin and IGF-I receptors in trout. *Regul. Pept.* **77,** 55-62.

Baras, E., Jobling, M. (2002). Dynamics of intracohort cannibalism in cultured fish. *Aquacult. Res.* **33**, 461-479.

Barton, B. A. (1997). Stress in finfish: Past, present and future - A historical perspective. In *Fish stress and health in aquaculture*, pp. 1-33, (eds. G. K. Iwama A. D. Pickering J. P. Sumpter and C. B. Schreck), Cambridge (UK): Cambridge University Press.

Baskerville-Bridges, B., Kling, L. J. (2000). Larval culture of Atlantic cod (*Gadus morhua*) at high stocking densities. *Aquaculture* **181**, 61-69.

Blakely, R. D., Berson, H. E., Fremeau, R. T., Caron, M. G., Peek, M. M., Prince, H. K., Bradley, C. C. (1991). Cloning and expression of of a functional serotonin transporter from rat-brain. *Nature* **354 (6348),** 66-70.

Blasco, J., Fernández-Borrás, J., Marimon, I. (1996). Plasma glucose kinetics and tissue uptake in brown trout in vivo: effect of an intravascular glucose load. *J. Comp. Physiol. B.* **165,** 534-541.

Boadle-Biber, M. C. (1982). Biosynthesis of serotonin. In *Biology of serotonergic transmission,* pp 63-94, (ed. N. N. Osborne), John Wiley.

Boadle-Biber, M. C. (1993). Regulation of serotonin synthesis. *Prog. Biophys. Molec. Biol.* **60,** 1-15.

Bolliet, V., Ali, M. A. (1992). Immunohistochemical study of the development of serotonergic neurons in the brain of the brook trout *Salvelinus fontinalis. Brain Behav. Evol.* **40,** 234-249.

Brawn, V. M. (1961). Aggressive behaviour in the cod (*Gadus callarias* L.). *Behaviour* **18**, 108-127.

Carlsson, A., Lindquist, M. (1978). Dependence of 5-HT and catecholamine synthesis on concentrations of precursor amino acids in rat brain. *Naunyn Schmiedberg's Arch. Pharmacol.* **303,** 157-164.

Cases, O., Seif, I., Grimsby, J., Gaspar, P., Chen, K., Pournin, S., Muller, U., Aguet, M., Babinet, C., Shih, J. C., Demaeyer, E. (1995). Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. *Science* **268,** 1763-1766.

Chen, C. Rainnie, D. G., Greene, R. W., Tonegawa, S. (1994). Abnormal fear response and aggressive behvaiour in mutant mice deficient for α-calcium-calmodulin-kinase II. *Science* **266,** 291-294

Cowey, C. B., Knox, D., Walton, M. J., Adron, W. (1977). The regulation of gluconeogenesis by diet and insulin in rainbow trout (*Salmo gairdneri*). *Br. J. Nutr.* **38,** 463-470.

Cumming, P., Brown, E., Damsma, G., Fibiger, H. (1992). Formation and clearance of interstitial metabolites of dopamine and serotonin in the rat striatum: An in vivo microdialysis study. *J. Neurochem.* **59 (5),** 1905-1914.

de Boer, S. F., Lesourd, M., Mocaer, E., Koolhaas, J. M. (2000). Somatodendritic 5-HT1A autoreceptors mediate the anti-aggressive actions of 5-HT_{1A} receptor agonists in rats: An ethopharmacological study with S-15535, alnespirone, and WAY-100635. *Neuropsycopharmacol.* **23 (1),** 20-33.

Deckel, A. W., (1996). Behavioural changes in *Anolis carolinensis* following injection with fluoxetine. *Behav. Brain Res.* **78,** 175-182.

De Pedro, N., Piñillos, M. L., Valenciano, A. I., Alonso-Bedate, M., Delgado, M. J. (1998). Inhibitory effect of serotonin on feeding behavior in goldfish: Involvement of CRF. *Peptides* **19**, 505-511.

DeNapoli, J. S., Dodman, N. H., Shuster, L., Rand, W. M., Gross, K. L. (2000). Effect of dietary protein content and tryptophan supplementation on dominance aggression, territorial aggression, and hyperactivity in dogs. *J. Am. Vet. Med. Assoc.* **217 (4),** 504-508.

Dinan, T. G. (1996). Serotonin and the regulation of hypothalamic-pituitary-adrenal axis function. *Life Sci* **58**, 1683-94.

Ejike, C., Schreck, C. B., (1980). Stress and social hierarchy rank in coho salmon. *Trans. Am. Fish. Soc.* **109,** 423-426.

Ekström, P., Östholm, T., Ebbesson, S. O. E. (1992). A morphometric study of age-relatet changes in serotonin-immunereactive cell groups in the brain of coho salmon, *Oncorhynchus kisutch* Walbaum. *Exp. Neurol.* **116,** 204-209.

Fadda, F. (2000). Tryptophan-free diets: A physiological tool to study brain serotonin function. *News Physiol. Sci.* **15,** 260-264.

Fernstrom, J. D. (1983). Role of precursor availability in control of monoamine biosynthesis in brain. *Physiol. Rev.* **63**, 484-546.

Fernstrom, J. D., Wurtman, R. J. (1971). Brain serotonin content : physiological dependence on plasma tryptophan levels. *Science* **173,** 149-152.

Fernstrom, J. D., **Wurtman, R. J.** (1972). Elevation of plasma tryptophan by insulin in rat. *Metabolism* **21 (4),** 337-342.

Fish, E. W., Faccidomo, S., Miczek, K. A. (1999). Aggression heightened by alcohol or social instigation in mice: reduction by the 5-HT1B receptor agonist CP-94,253. *Psychopharmacology (Berl.)* **146,** 391-399.

Folkvord, A., Otterå, H. (1993). Effects of initial size distribution, day length, and feeding frequency on growth, survival, and cannibalism in juvenile Atlantic cod (*Gadus morhua* L.). *Aquaculture* **114**, 243-260.

Fuller, R. W., Roush, B. W. (1973). Binding of tryptophan to plasma proteins in several species. *Comp. Biochem. Physiol.* **46B,** 273-276.

Green, H., Sawyer, J. L. (1966). Demonstration characterization and assay procedure of tryptophan hydroxylase in rat brain. *Anal. Biochem.* **15 (1),** 53-&.

Hecht, T., Pinienaar, A. G. (1993). A review of cannibalism and its implications in fish larviculture. *J. World Aquacult. Soc.* **24**, 246-261.

Hen, R. (1996). Mean genes. *Neuron* **16 (1),** 17-21.

Hoffman, B. J., Mezey, E., Brownstein, M. J. (1991). Cloning of a serotonin transporter affected by antidepressants. *Science* **254 (5031),** 579-580.

Höglund, E., Balm, P. H. M., Winberg, S. (2000). Skin darkening, a potential social signal in subordinate arctic charr (*Salvelinus alpinus*): the regulatory role of brain monoamines and pro-opiomelanocortin-derived peptides. *J. Exp. Biol.* **203 (11)**, 1711-21.

Höglund, E., Balm, P. H. M., Winberg, S. (2002). Stimulatory and inhibitory effects of 5-HT_{1A} receptors on ACTH and cortisol secretion in a teleost fish, the Arctic charr (*Salvelinus alpinus*). *Neurosci. Lett.* **324,** 193-196.

Höglund E, **Kolm N., Winberg S.** (2001). Stress-induced changes in brain serotonergic activity, plasma cortisol and aggressive behavior in Arctic charr (*Salvelinus alpinus*) is counteracted by L-DOPA. *Physiol. Behav.* **74 (3),** 381-389.

Hseu, J. R., Lu, F. I., Su, H. M., Wang, L. S., Tsai, C. L., Hwang, P. P. (2003). Effect of exogenous tryptophan on cannibalism, survival and growth in juvenile grouper, *Epinephelus coioides*. *Aquaculture* **218**, 251-263.

Huntingford, F., Turner, A. (1987). *Animal conflict*, pp 158-159 and 167-189, Chapman and Hall, London New York.

Jacobs, B. L., Azmitia, E. C. (1992). Structure and function of the brain serotonin system. *Physiol. Rev.* **43,** 563-578.

Jequier, E., Lovenber., W., Sjoerdsm., A. (1967). Tryptophan hydroxylase inhibition-mechanism by which P-chlorophenylalanine depletes rat brain serotonin. *Mol. Pharm.* **3 (3),** 274-&.

Jequier, E., Robinson, D. S., Lovenber., W., Sjoerdsm., A. (1969). Further studies on tryptophan hydroxylase in rat brainstem and beef pineal. *Biochem. Pharm.* **18 (5),** 1071-&.

Jobling, M. and Wandsvik, A. (1983). Effect of Social Interactions on Growth-Rates and Conversion Efficiency of Arctic Charr, Salvelinus-Alpinus L. *J. Fish Biol.* **22**, 577-584.

Kah, O. and Chambolle, P. (1983). Serotonin in the brain of the goldfish, *Carrasius auratus*. *Cell Tissue Res.* **234,** 319-333.

Kestemont, P., Jourdan, S., Houbart, M., Melard, C., Paspatis, M., Fontaine, P., Cuvier, A., Kentouri, M. and Baras, E. (2003). Size heterogeneity, cannibalism and competition in cultured predatory fish larvae: biotic and abiotic influences. *Aquaculture* **227**, 333-356.

Korzan, W. J., Summers, T. R., Ronan, P. J., Renner, K. J., Summers, C. H. (2001). The role of monoaminergic nuclei during aggression and sympathetic social signaling. *Brain Behav. Evol.* **57,** 317-327.

Larson, E. T. and Summers, C. H. (2001). Serotonin reverses dominant social status. *Behav. Brain Res.* **121,** 95-102.

Lepage, O., Tottmar, O. and Winberg, S. (2002). Elevated dietary intake of L-tryptophan counteracts the stress-induced elevation of plasma cortisol in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **205**, 3679-87.

Lepage, O., Vilchez, I. M., Pottinger, T. G. and Winberg, S. (2003). Time-course of the effect of dietary L-tryptophan on plasma cortisol levels in rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* **206**, 3589-99.

Long, J. B., Youngblood, W. W., Kizer, J. S. (1982). A microassay for simultaneous measurements of *in vivo* rates of tryptophan hydroxylation and levels of serotonin in descrete brain nuclei. *J. Neurosci. Meth.* **6,** 45-58.

Lookingland, K. J., Shannon, N. J., Chapin, D. S., Moore, K. E. (1986). Exogenous tryptophan increases synthesis, storage and intraneuronal metabolism of 5-hydroxytryptamine in the rat hypothalamus. *J. Neurochem.* **47,** 205-212.

Lyons, W. E., Mamounas, L. A., Ricaurte, G. A., Coppola, V., Reid, S. W., Bora, S. H., Wihler, C., Koliatsos, V. E., Tessarollo, L. (1999). Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 15239-15244.

McGeer, E. G., Peters, D. A. V., McGeer, P. L. (1968). Inhibition of rat brain tryptophan hydroxylase by 6-halotryptophans. In *Life Science part 1; Physiology and Pharmacology, and part 2; Biochemestry General and Molecular Biology* **7 (12P2),** 605-&.

McMenamy, R. H. (1977). Albimin binding sites. In: *Albumin structure, function and uses* (eds)Rosenoer, V. M., Oratz, M., Rothschild, M. A. Pergamon Press, Oxford.

Metcalfe, N. B. (1986). Intraspecific variation in competitive ability and food-intake in salmonids - consequences for energy budgets and growth-rates. *J. of Fish Biol.* **28**, 525-531.

Miczek, K. A., Hussain, S., Faccidomo, S. (1998). Alcohol-heightened aggression in mice: attenuation by 5-HT1A receptor agonists. *Psychopharmacology (Berl.)* **139,** 160-168.

Mongeau, R., Blier, P., Montigny, C. (1997). The serotonergic and noradrenergic systems of the hippocampus: their interactions and the effects of antidepressent treatments. *Brain Res. Rev.* **23,** 145-195.

Nelson, R. J. and Chiavegatto, S. (2000). Aggression in knockout mice. *Inst. Lab. Anim. Res. J.* **41,** 153-162.

Nelson, R. J. and Chiavegatto, S. (2001). Molecular basis of aggression. *Trends in Neurosci.* **24**, 713-719.

Noakes, D. L. G., Letherland, J.F (1977). Social dominance and interrenal activity in Rainbow trout, *Salmo gairdneri* (Pisces, Salmonidae). *Env. Biol. Fish.* **2,** 131-136

Nutt, D., Forshall, S., Bell, C., Rich, A. Sandford, J., Nash, J., Argyopoulos, S. (1999). Mechanisms of action of selective serotonin reuptake inhibitors in the treatment of psychiatric disorders. *Eur. Neuropsycopharmacol.* **3,** S81-S86.

Otterå, H., Folkvord, A. (1993). Allometric growth in juvenile cod (*Gadus morhua*) and possible effects on cannibalism. *J. of Fish Biol.* **43,** 643-645.

Parent, A., Poitras, D. and Dubé, L. (1984). Comparative neuroanatomy of central monoaminergic systems. In *Handbook of Chemical Neuroanatomy* vol 2, pp 409-439 (ed. A. Björklund and T. Hökfelt), Amsterdam, New York, Oxford: Elsevier.

Perreault, H. A. N., Semsar K., Godwin J. (2003). Fluoxetine treatment decreases territorial aggression in a coral reef fish. *Physiol. Behav.* **79 (4-5),** 719-724.

Pozefsky, T., Felig, P., Tobin, J. D., Soeldner, J. S., Cahill, G. F. (1969). Amino acid balance across tissues of the forearm in postabsorptive man. Effects of insulin at two dose levls. *J. Clin. Invest.* **48,** 2273-2282.

Raleigh, M. J. and McGuire, M. T. (1991). Bidirectional relationship between tryptophan and social behavior in vervet monkeys. In *Kynurenine and Serotonin Pathways* (eds Schwartcz, R. *et al.*) New York: Plenum Press; 289-298.

Ruibal, C., Soengas, J. L., Aldegunde, M. (2002). Brain serotonin and the control of food intake in rainbow trout (*Oncorhyncus mykiss*): effects of changes in plasma glucose levels. *J. Comp. Physiol. A.* **188,** 479-484.

Saudou, F., Amara, D. A., Dierich, A., Lemeur, M., Ramboz, S., Segu, L., Buhot, M. C., Hen, R. (1994). Enhanced aggressive behaviour in mice lacking 5-HT1B receptor. *Science* **265,** 1875-1878.

Scott, D. B. C., Currie, C. E. (1980). Social hierarchy in relation to adrenocortical activity in *Xiphophorus helleri* Heckel. *J. Fish. Biol.* **16,** 265-277.

Shannon, N. J., Gunnet, J., Moore, K. E. (1986). A comparison of biochemical indices of 5-hudroxytryptaminergic neuronal activity following electrical stimulation of the dorsal raphe nucleus. J. Neurochem. **47,** 958-965.

Shea, M. M., Douglass, L. W., Mench, J. A. (1990). The interaction of dominance status and supplemental tryptophan on aggression in *Gallus domesticus* males. *Pharm. Biochem. Behav.* **38,** 587-591.

Summers, C. H., Summers, T. R., Moore, M. C., Korzan, W. J., Woodley, S. K., Ronan, P. J., Hoglund, E., Watt, M. J. and Greenberg, N. (2003). Temporal patterns of limbic monoamine and plasma corticosterone response during social stress. *Neuroscience* **116**, 553-563.

Tagliamonte, A., Biggio, B., Vargiu, L., Gessa, G. L. (1973). Free tryptophan in serum controls brain tryptophan level and serotonin synthesis. *Life sci.* **12,** 277-287.

Takeuchi, Y. (1988). Distributin of serotonin neurons in the mammalian brain. In *Neuronal Serotonin* (Edited by N. N. Osborne and M. Hamon). John Wiley & Sons, Chichester, New York, Bribane, Toronto, Singapore.

Tamir, H., Gershon, M. D. (1990). Serotonin-storing secretory vesicles. *Ann. N. Y. Acad. Sci.* **600,** 53-66.

Törk, I. (1990). Anatomy of the serotonergic system. In *The Neuropharmacology of Serotonin* (Edited by P. M. Whitaker-Azmitia, S. J. Peroutka), pp 9-36. Ann. New York Aced. Sci. Vol 600., New York.

Van der Meeren, T. (2002). Larver og tidlig yngel in: Havbruks rapport 2002. Fisken og Havet, særnummer 3-2002 (ed. By Johan Glette et al.): 70-73.

Van der Vegt, B. J., Lieuwes, N., Cremers, T. I. F. H., de Boer, S. F., Koolhaas, J. M. (2003). Cerebrospinal fluid monoamine and metabolite concentrations and aggression in rats. *Horm. Behav.* **44,** 199-208.

Wilson, R. P. (1994). Utilization of dietary carbohydrate by fish. *Aquaculture* **124,** 67-80.

Winberg, S., Carter, C. G., McCarthy, I. D., He, Z., Nilsson, G. E., Houlihan, D. F. (1993). Feeding rank and brain serotonergic activity in rainbow trout, *Oncorhyncus mykiss* (Walbaum). *J. Exp. Biol.* **179,** 197-211.

Winberg, S., Lepage, O. (1998). Elevation of brain 5-HT activity, POMC expression, and plasma cortisol in socially subordinate rainbow trout. *Am. J. Physiol.* **274**, 645-654.

Winberg, S. and Nilsson, G. E. (1993). Roles of brain monoamine neurotransmitters in agonistic behavior and stress reactions, with particular reference to fish. *Comp. Biochem. and Physiol. C-Pharmac Toxicol. Endocrinol.* **106**, 597-614.

Winberg, S., **Nilsson G. E.** (1996). Multiple high-affinity binding sites for [H-3]serotonin in the brain of a teleost fish, the Arctic charr (*Salvelinus alpinus*). *J. Exp. Biol.* **199 (11),** 2429-2435.

Winberg, S., Øverli, Ø., Lepage, O. (2001). Suppression of aggression in rainbow trout (*Oncorhynchus mykiss*) by dietary L-tryptophan. *J. Exp. Biol.* **204**, 3867-3876

Yoshida, M., Nagatsu, I., Kawakami-Kondo, Y., Karasawa, M., Spatz, M., Nagatsu, T. (1983). Monoaminergic neurons in the brain of goldfish as observed by immunohistochemical techniques. *Experienta* **39,** 1171-1174.

Zimmerman, A. E., Kells, D. I. C., Yip, C. C. (1972). Physical and biological properties of guinea pig insulin. *Biochem. Biophys. Res. Commun.* **46,** 2127–2133.

Øverli, Ø., Harris, C. A. and Winberg, S. (1999). Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout. *Brain. Behav. Evol.* **54**, 263-275.

Øverli Ø, **Korzan, W. J., Höglund, E., Winberg, S., Bollig, H., Watt, M., Forster, G. L., Barton, B. A., Øverli, E., Renner, K.J., Summers, C. H.** (2004). Stress coping style predicts aggression and social dominance in rainbow trout. *Horm. Behav.* **45 (4),** 235-241.

Øverli, Ø., Winberg, S., Damsgard, B. and Jobling, M. (1998). Food intake and spontaneous swimming activity in Arctic char (*Salvelinus alpinus*): role of brain serotonergic activity and social interactions. *Can. J. of Zool.* **76**, 1366-1370.