Effect of tryptophan enriched diets on aggression in hierarchical groups of juvenile Atlantic salmon (*Salmo salar*)

Master Thesis in Aquaculture Biology

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June 2010

Front page illustration: www.flickr.com

Acknowledgments

I would like to thank my supervisor Ian Mayer who gave me this great opportunity, for his valuable comments on this manuscript and for his friendship. During this project I had the opportunity to use the laboratory facilities at Uppsala University, were Svante Winberg was kind enough to guide me through the process of how an HPLC works. I also enjoyed some time at Denmark's Technical Institute research facilities in Hirtshals, were Erik Höglund helped me to analyze the data in all possible manners; taking it from just numbers to results!

During the experiments period, Frank Midtøy was constantly available for my very frequent questions and always had smart ideas as to how to improve the design, for this I sincerely thank you. In the creation of the experimental design I would also like to thank for their comments and help Anders Fernö, Justin Meager, Olav Moberg and Jan Egil Skjaraasen, who was also kind enough to guide me along the statistics analysis. Thank you for your patience and time.

My thanks to Kristine Wilson for ALL the times that she came to my rescue and to Cindy Pedrosa for her help during the experiments. I also would like to thank the students at DTU in Hirtshals, for all the brain storming over the results and support.

I would like to thank my family, for always supporting me in everything I have done, even though I may be half a world away, muchísimas gracias! To my wife's family, Stein and Inger, for their never ending encouragement and support.

I would most of all like to thank my wife, Marion, for her patience, help, encouragement and PATIENCE, during all my frustrated moments and less than happy situations that I spent in front of the computer and in labs. Thank you for standing by me, for your love and well, for your patience!

I would also like to thank my dog, Kaisa, for taking me out on walks when I needed to get some fresh air!!

Abstract

Aggression in fish hierarchies often leads to one dominant individual monopolizing resources (food/shelter), thus increasing their fitness. Meanwhile individuals in subordinate ranks, endure high stressful conditions, which often lead to disease and higher mortality rates. Levels of serotonin have been linked to agonistic behavior. Enriching fish diets with tryptophan (TRP), the natural precursor for serotonin, has been used in several vertebrate species in order to control aggression levels.

Twelve hierarchies consisting of 4 fish in each were studied. Six groups were given a TRP enriched diet and six groups a control diet. Aggressive acts performed and those received were observed during 7 days, 15 min per day, in order to establish hierarchies. Whole brains were excised and immediately frozen from each fish at the end of the trial, in order to analyze the monoamine levels by means of HPLC chromatography.

TRP groups were found to have a higher overall aggression compared to control groups. Dividing the groups into their hierarchical ranks, middle rank individuals were overall more aggressive, displaying also more aggressive attacks towards dominants in TRP groups, compared with control groups. These results were opposite to what was expected. Serotonergic activity, measured as the [5-HIAA]/ [5HT] ratio, was higher in more aggressive individuals, which is also opposite from what has been found before. High amounts of received aggression were found to be positively correlated with high [5-HIAA]/ [5HT] ratios.

This is the first time (as far as we know) that the effect of a TRP enriched diet on fish hierarchical groups has been studied. Our contradictory results show how complicated the social interactions and mechanisms in fish hierarchies are, since previous studies have only looked at fish interacting in pair-wise contests. We propose that under true hierarchical conditions, other factors are involved in the overall control of aggression; therefore, attempting to control agonistic behavior in groups of fish with TRP may not be sufficient. This could be an important finding that needs to be studied further, especially in the context of fish welfare in intensive aquaculture.

Sammendrag (Norwegian)

Aggressivitet I fiskehierarkier fører ofte til at et individ blir dominant og monopoliserer fór/ ly. Disse styrker dermed sine fysiske egenskaper. Samtidig finner vi individer som må underordne seg og som dermed lever under høyt stress, noe som ofte fører til sykdom og høyere dødlighet. Nivåer av serotonin har blitt knyttet til aggressiv atferd. Hjernen bruker tryptofan (TRP) til å produsere serotonin. Derfor har en tryptofanrik diett blitt brukt til flere ryggvirvel-arter for å kontrollere aggressivitetsnivå.

Tolv hierarkier med 4 fisker i hver, ble studert. 6 grupper ble behandlet med en TRP-rik diett og 6 grupper med en kontrolldiett. For å observere etablering av hierarkier, ble antall aggressiv handlinger som ble utført og påført observert over 7 dager i 15 minutter per dag. Dereter, ble hver enkelt fiskehjerne tatt ut og umiddelbart frosset ned for å analysere monoamine nivåer ved hjelp av en HPLC kromtografimaskin.

Det ble avdekket at TRP-gruppene påførte mer aggression en kontrollgruppene. Etter at gruppene ble fordelt i forhold til hierarki, ble de funnet at individer tilhørende middelgruppe, var generelt mer aggressive. Disse individene viste også mer aggressiv atferd mot dominante individer. Dette resultatet var motsatt av det forventede. Serotoninaktivitet, målt som konsentrasjonen [5-HIAA]/ [5HT], var høyere hos de mer aggressiv individene, noe som også er det motsatte av tidligere funn. Hos individer som ble påført mye aggresjon ble det fastslått en positiv korrelasjon med høy [5-HIAA]/ [5HT].

Det er første gangen (så vidt kjent) en har studert effekten av en TRP-rik diett i fiskehierarkigrupper. At resultatene skiller seg fra tidligere funn, viser at mekanismer og verkselvirkninger i fiskehierkier er mer komplisert enn antatt. Dette kan skyldes at tidligere studier er gjort på grupper bestående av bare 2 fisker (pair-wise contests). En antakelse er at også andre faktorer kan påvirke den generelle aggresjon i et hierarki med mer enn 2 nivåer. Videre vil det derfor ikke være tilstrekkelig å benytte TRP-rik diett til å påvirke aggressiv oppførsel. Dette kan være et viktig funn, hvor det trengs dypere studier, spesielt med henblikk på fiskevelferd i intensive akvakulturer.

Resumen (Spanish)

La agresión en jerarquías de peces genera un individuo dominante que monopoliza los recursos (alimento y/o albergue) e individuos subordinados que viven en condiciones elevadas de estrés, lo cual con lleva a enfermedades y alta mortalidad. Niveles de serotonina han sido vinculados con comportamiento agresivo. El uso de una dieta rica en triptofano (TRP), el precursor natural de serotonina, ha sido utilizada en varias especies de vertebrados para controlar la agresión.

Doce jerarquías (de 4 peces c/u) fueron analizadas. 6 grupos fueron tratados con una dieta rica en TRP y 6 con una dieta control. La agresión ejecutada y recibida fue observada por un periodo de 7 días, durante 15 min. c/día, para establecer las jerarquías. Al final, los cerebros completos de cada pez fueron removidos y congelados, para ser luego analizados en relación al contenido de niveles de monoaminas, utilizando una máquina de cromatografía HPLC.

En general, los grupos TRP mostraron un nivel de agresión más elevado. Al dividir los grupos en sus rangos jerárquicos, encontramos que los individuos de rango medio (en grupos TRP), mostraron en general niveles de agresión elevados y mayores ataques contra individuos dominantes. La actividad serotonérgica, medido como la concentración de [5-HIAA]/ [5HT], fue más alta en los individuos más agresivos. Estos resultados fueron contradictorios a lo obtenido en estudios previos. Niveles altos de agresión recibida fueron correlacionados positivamente con niveles altos de la fracción [5-HIAA]/ [5HT].

Esta es la primera vez (hasta donde conocemos) que los efectos en agresión en grupos jerárquicos de peces con una dieta rica en TRP son estudiados. Los resultados contradictorios que hemos obtenido, demuestran como las interacciones sociales y los mecanismos en jerarquías de peces son más complicados de lo previsto; ya que estudios previos fueron realizados en peces interactuando en parejas (“pair-wise contests”). Proponemos que en condiciones verdaderas de jerarquía, otros factores están involucrados en el control del comportamiento agresivo. Por lo tanto el uso de una dieta rica en TRP para controlar los niveles de agresión, no es suficiente. Esto podría ser un resultado importante para próximos estudios, especialmente en el contexto del bienestar de los peces en sistemas intensivos de acuacultura.

Index

1. **Introduction** 1

1.1 Study species 1
1.2 Domestication/aquaculture 1
1.3 Aggression, dominance and territories/hierarchies 2
1.4 Monoamines, serotonin and tryptophan 4

2. **Materials and methods** 7

2.1 Experimental facilities 7
2.2 Experimental fish 7
2.3 Experimental design 7
2.4 Dietary tryptophan 12
2.5 Video recordings 12
2.6 Agonistic behavior 12
2.7 Hierarchies 13
2.8 Brain dissections 14
2.9 Measurement of brain monoamines 15
2.10 Statistical analysis 16

3. **Results** 17

3.1 Hierarchical ranks 17
3.2 Aggression 17
Performed acts of aggression 17
Received aggression 21
Attacks performed 22
3.3 Serotonergic activity ([5-HIAA]/[5-HT]) 24
3.4 Serotonergic activity and hierarchy ranks 26
3.5 Serotonergic activity and aggression 28
Performed acts of aggression 28
Dominants 30
Middle 31
Subordinates 32
Received aggression 33
Dominants 35
Middle 36
Subordinates 37
3.6 Summary of results 38

4. **Discussion** 40

4.1 Aggression 40
4.2 Serotonergic activity 42
4.3 Hierarchies and serotonergic activity 44
Dominants 45
Middle 47
Subordinates 48
4.4 Methods and sources of error 49

5. **Conclusions** 53

6. **References** 54

7. **Appendix I** 60

8. **Appendix II** 63

9. **Appendix III** 64

1. Introduction

1.1 Study species

The family Salmonidae has been the topic of many studies over the last 50 years, especially the members of the subfamily Salmoninae which comprises 30 species in seven genera (Klemetsen, Amundsen et al. 2003). In this group we find, our study species, the Atlantic salmon, *Salmo salar*, which has gained an increasing economical importance in the last 30 to 40 years due to its commercial exploitation by the aquaculture industry (Jonsson and Jonsson 2006).

The Atlantic salmon is an anadromous species that spawns in fresh water, from late summer to early winter. The females dig their eggs down into the gravel and several months later well-developed alevins hatch. The alevins remain in the gravel while its yolk reserves hold. Following their emergence in spring, when the juvenile salmon start feeding, the fish, now called parr, maintain territories by aggressive interactions. After one, or more (usually two years) in fresh water, the parr undergo a metamorphosis, termed parr-smolt transformation (smoltification), in which the freshwater parr change into seawater adapted smolts. Parr-smolt transformation involves a number of distinct morphological, physiological and behavioral changes which pre-adapts the smolts for seawater life. As smolts their territorial behaviour is lost In favor of schooling and as a group they leave for the ocean waters. They stay several years in the ocean feeding on small fish and crustaceans in small pelagic groups. After several years they swim back in large schools, normally to their native rivers, in order to reproduce (Keenleyside and Yamamoto 1962; Debelius 1997; Hutchison and Iwata 1997).

1.2 Domestication/Aquaculture

The process of domestication, that includes an evolutionary and environmental adaptation of an organism to an environment provided by man (Mork, Bjerkeng et al. 1999), promotes not only the selection of desirable traits in salmonid fish, as for example rapid growth, but also unintentionally selects for some traits that may be counterproductive to the industry; as for example, higher aggression and dominance (Swain and Riddell 1990; Mork, Bjerkeng et al. 1999; Huntingford 2004; Huntingford and Adams 2005; Pearsons, Fritts et al. 2007).

There is substantial evidence showing that animals utilize different strategies, or coping styles, in order to obtain access to limited resources, such as bold risk takers as opposed to shy individuals. In natural settings these strategies perform best under different environmental conditions. The aquaculture environment provides in contrast only one type of environment for the fish, which amongst other things, provides fish with a predictable highly localized food source, high densities and a predator free environment. Under these conditions, the risk taking aggressive individuals are favored, as they are able to monopolize the food sources and dominate, thus creating favorable conditions for hierarchies to be formed (Huntingford 2004; Sundström, Petersson et al. 2004; Huntingford and Adams 2005; Pike, Samanta et al. 2008). In contrast, the submissive, shy individuals tend to avoid aggression by keeping close to the bottom or sides, not fighting for the food resources and in many cases displaying a dark coloration to signal submission, and in this way reducing the cost associated with aggressive conflicts (Johnsson and ÅKerman 1998; O'Connor, Metcalfe et al. 1999; Höglund, Balm et al. 2000; O'Connor, Metcalfe et al. 2000; Suter and Huntingford 2002).

Aquaculture environments can in turn expose individuals to higher levels of stress, as social interactions are increased under conditions of high densities in which there is no possibility of escaping. It is common practice to mix different age groups, which also promotes aggressive interactions. In the case of subordinate fish, the constant pressure from dominant fish may lead to diminished food intake and impaired growth, and potentially immunosuppression as a result of chronic stress (Iwama 1998; Conte 2004; Estevez, Andersen et al. 2007).

1.3 Aggression, dominance and territories/hierarchies

A territory is defined as a fixed space from which an individual or group of mutually tolerant individuals actively excludes competitors from a specific resource or resources. For the vast majority of animal groups, food has been shown to be the most common resource that promotes the formation of territories (Maher and Lott 2000).

Aggression in fish can be a very important facet of their social behavior used in order to maintain territories, gain access to mates and/or survival against predators (Mork, Bjerkeng et al. 1999). In the case of Atlantic salmon in the wild, the young parr stay close to the bottom of the river bed facing upstream. As food particles or intruders are encountered, the territorial fish dashes forwards in order to catch the food or attack and chase away the intruder. After this has been accomplished, the parr quickly return to the same resting point to guard their territory (Keenleyside and Yamamoto 1962). Territories are thus maintained by agonistic behaviors, defined as all activities directly associated with fighting, such as nips, butts, chases, and threats or displays (Keenleyside and Yamamoto 1962; Pearsons, Fritts et al. 2007).

Dominant individuals quickly establish territories and subordinates are sometimes excluded to more open areas, where they swim in small groups, since it is not possible to defend any sort of territory (Keenleyside and Yamamoto 1962). In this case, dominant individuals actively exclude others from favorable feeding areas, even suppressing their foraging activity (Adams, Huntingford et al. 1998; Skajaa 2006). It has been argued that in the case of aquaculture systems due to the dimensions of the tanks/nets, it is not possible for the fish to establish territories. In these circumstances the establishment of hierarchies is then favored (Fernö and Holm 1986; MacLean, Metcalfe et al. 2000; Sundström, Lõhmus et al. 2003).

A hierarchy is characterized as a pecking order in which dominant individuals obtain preferential access to the food resources. The less dominant subordinate individuals are only able to obtain food when the more dominant individual(s) allows them. In extreme cases, the more subordinate individuals may be completely restrained from obtaining food in order to avoid the aggression from the dominant fish (Jobling 1995; Kadri, Huntingford et al. 1996; Moutou, McCarthy et al. 1998; Sæther and Jobling 1999; Hollis, Langworthy-Lam et al. 2004).

The unequal distribution of resources promoted by the formation of dominant held territories and hierarchies, has economical implications in aquaculture systems due to loses (death of fish) and poor growth by subordinate individuals (MacLean, Metcalfe et al. 2000; Vøllestad and

Quinn 2003; Huntingford 2004; Huntingford and Adams 2005). Fin damage is one of the first signs of aggression in fish, and although it may be due to other factors, it is one of the main predictors for aggression in Atlantic salmon culture systems (MacLean, Metcalfe et al. 2000; Jonsson and Jonsson 2006). On many occasions, the hierarchies and dominance status may lead to large size differences within a sibling population. The development of a large size variation within a population can lead to increase rates of cannibalism, which is a common problem with many cultured species. While cannibalism is not a major problem in salmon farming, it can be a major production problem in other farmed species, such as Atlantic cod, *Gadus morhua* (Baras and Jobling 2002).

1.4 Monoamines, serotonin and tryptophan

Monoamine neurotransmitters across different groups of animals, are involved in the control of different behavioral patterns: aggression, mating, feeding, stress reactions and regulation of neuroendocrine and autonomic functions (Jacobs and Azmitia 1992; Winberg and Nilsson 1993). The major monoamines are; the catecholamines, dopamine (DA), norepineprine (NE) and epinephrine (E), plus the indoleamine serotonin (5-hydroxytryptamine, 5-HT) (Winberg and Nilsson 1993).

These different monoamine neurotransmitters are stimulated according to social interactions between individuals. For example, in subordinates behavioral inhibition (suppressed aggression, reduced feeding, decrease in loco-motor activity), is promoted by increased levels of serotonin, which in turn stimulates the hypothalamus-pituitary-interrenal (HPI) axis (Winberg and Nilsson 1993; Øverli, Harris et al. 1999). Meanwhile, increased levels of dopamine have been linked to dominant individuals (Winberg and Nilsson 1993).

The role of serotonin in controlling aggressive behavior has been extensively studied in different groups of animals. Some examples are: lizards, *Anolis carolinensis* (Deckel and Fuqua 1998), roosters, *Gallus domesticus* (Shea, Douglass et al. 1990), humans (Young 1996), crustaceans (although higher levels of serotonin have been correlated with dominant individuals as opposed to vertebrates) (Edwards and Kravitz 1997) and several species of fish;

including rainbow trout, *Oncorhyncus mykiss* (Winberg, Carter et al. 1993; Øverli, Harris et al. 1999; Winberg, Øverli et al. 2001), grouper, *Epinephelus coioides* (Hseu, Lu et al. 2003), Atlantic cod (Höglund, Bakke et al. 2005) and Atlantic salmon (Cubitt, Winberg et al. 2008) amongst others. Across the different vertebrate groups, it has been consistently found that increased levels of serotonin are associated with decreased levels of aggression.

While the levels of serotonin have been linked to social status in hierarchies and territorial disputes (Winberg, Carter et al. 1993; Cubitt, Winberg et al. 2008), it has been found that it is possible to externally alter the levels of serotonin by increasing the availability of its main precursor; L-tryptophan or L-5-hydroxitryptophan (TRP) (Jacobs and Azmitia 1992; Winberg and Nilsson 1993; Aldegunde, Garcia et al. 1998; Aldegunde, Soengas et al. 2000; Schjolden, Pulman et al. 2006). The serotonergic pathway is shown in figure 1.

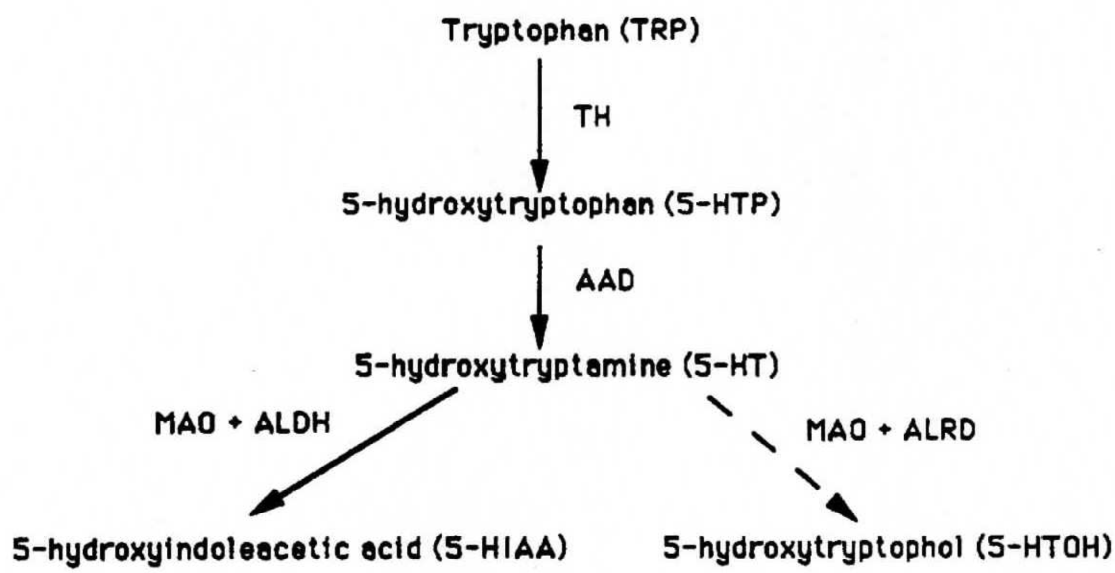

Figure 1: Pathway of serotonin metabolism. TH: tryptophan hydrolase; AAD: aromatic L-amino acid decarboxylase; MAO: monoamine oxidase; ALDH: aldehyde dehydrogenase; ALRD: aldehyde reductase (Winberg and Nilsson 1993).

Winberg and Nilsson (1993), explain how serotonin (5-HT) is synthesized from the amino acid tryptophan, which is transported from the blood via non-specific carriers and through the blood-brain barrier. They point out that the limiting factor for this transport appears to be the availability of TRP, especially in fish. Thus by feeding fish a TRP enriched diet, leading to elevated 5-HT levels, the potential exists to suppress aggression, especially in dominant individuals. This decrease in aggression by dominant individuals would facilitate the breakdown of the hierarchy structure, which in turn would lead to a decrease in the unequal distribution of food resources that is created by hierarchies, as well as a reduction in cannibalism in culture systems (Winberg, Øverli et al. 2001; Hseu, Lu et al. 2003; Lepage 2004; Höglund, Bakke et al. 2005).

The ratio between the catabolite 5-hydroindolacetic acid (5-HIAA) to the transmitter 5-HT is often used to estimate serotonergic activity. This is especially true in the case of stress and behavior studies; since the available 5-HT is greater than the immediate demand. In other words, the 5-HT activity in this case is expressed as changes in the [5-HIAA]/[5HT] ratios (Winberg and Nilsson 1993).

The purpose of this study is to test the hypothesis that elevated dietary levels of L-tryptophan will diminish aggressive acts and in turn, diminish the strength of the established hierarchy in a group of juvenile Atlantic salmon. In order to achieve this goal there were 2 criteria analyzed:

- Aggressive interactions between groups of fish with elevated dietary tryptophan compared to control groups.
- Analysis of brain monoamine levels (with emphasis on serotonergic activity levels), between control and experimental groups.

2. Materials and methods

2.1 Experimental facilities

All experiments were conducted in Bergen, Norway at the High Technology Center which is part of the Mathematics and Biology Faculty. Two experiments were conducted, the first from the 29th of May until the 10th of July, 2009 and the second from the 29th of October until the 11th of November, 2009. The analysis of the brain monoamine levels were conducted at the University of Uppsala, Sweden at the Department of Neuroscience, part of the Bio-Medical Center and at the Technical Institute of Denmark research facilities, located in Hirtshals, Denmark.

2.2 Experimental fish

All fish were obtained from The Industrial and Aquatic Laboratory (ILAB) at The High Technology Center at the University of Bergen (UIB). The fertilized eggs were originally obtained from Akva Gen which rears the eggs until the eyed stage, approximately 6 to 12 weeks after fertilization. At this point the eggs are moved to the Urke Fiskeoppdrett AS facilities located in Urke, Norangsfjorden. The fish were reared under conditions of natural temperature and photoperiod regime of light:dark (LD) 24:0, until they reached a weight of 20-25 grams, after which they were moved to the ILAB facilities. At ILAB, the fish are kept at a temperature between 4 and 6 ^{o}C and under a LD 12:12 photoperiod. The fish were fed commercial food pellets constantly during light hours, by an automated system.

2.3 Experimental design

This thesis was based on 2 replicate experiments, using the same experimental facilities, and fish from the same stock of Atlantic salmon. Details for the 2 experiments are as follows:

Experiment 1

Fifty three 1 year old parr were collected from ILAB, having a mean length and weight of 13.3 ± 0.68 cm and 23.4 ± 3 gm respectively. For individual recognition, the fish were tagged at random with T-bar anchor tags, consisting of a 2 cm long filament in 4 different color combinations, completely black, completely white, top half white, lower black or top half black and lower white. The tags were attached using an Avery Dennison 10312 fine fabric tag applicator for fine anchor or TBF tags. The tags were applied on the flesh next to the dorsal fin after the fish had been anaesthetized with Metacaine (MS-222) at a concentration of 0.5 g per 5 L of water. At the same time as tagging, the anesthetized fish were also measured and weighed. Immediately after tagging, fish were placed in well aerated containers where they were monitored until normal swimming activity had resumed. After complete recovery they were moved into a large aquarium, measuring 300 cm length, 70 cm height and 51.5 cm width, for a 23 day acclimation period. During the acclimation period the fish were monitored in order to check for possible infections resulting from the tagging procedure. The water level was kept at 40 cm height, giving a tank volume of approximately 1000 L, with a 9 L/min flow rate of fresh Bergen tap water. The density in this tank was 1 kg/m^3. The fish were kept under an LD 12:12 photoperiod, while the temperature was gradually raised from 6 to 12 ^{o}C in order to promote fish activity. Light was provided by 2 lamps consisting of two 36 Watt halogen light bulbs controlled by a timer to maintain the specified light regime.

Four black plastic circles (25 cm diameter), were placed 30 cm apart on the bottom of the aquarium. These black circles were provided in order to stimulate the defense of a resource in a form of a territory, as previous experiments have shown that salmon prefer a dark region over the white substrate (Metcalfe, Valdimarsson et al. 2003). In addition, 2 small boulders were also provided on top of the black circles since it has been observed that Atlantic salmon respond to the presence of boulders. Dominant individuals may aggressively protect boulder areas if they are deemed good sheltering and/or feeding areas (Kemp, Armstrong et al. 2005). The fish were hand fed once a day an equivalent to 2% body mass per fish, with the feed being distributed above the black circles. This was done in order to enforce territorial behavior around the black circles.

Following the acclimation period, 4 fish were transferred to each of 6 small glass aquaria in order to conduct the observational studies. The aquarium measured 70 cm in length, 30 cm width and 35 cm height. The water level was kept at 30 cm height, giving a tank volume of approximately 70 L, with a 1 L/min flow rate of fresh Bergen tap water. The density in this tank was 0.5 kg/m³. The fish were kept under the same LD 12:12 photoperiod and at the same temperature as provided during the acclimation period. Light was provided by 1 lamp consisting of one 18 Watt halogen light bulb, situated 20 cm from the water, directly above each aquarium, controlled by a timer to maintain the specified light regime. At the centre of each aquarium was placed one black plastic circle with two stones (Fig. 2). The fish were hand fed once a day a quantity of food equivalent to 2% body mass per fish, with the pellets being distributed above the black circles. In such a way, this area represented a defendable resource, over which the fish could become territorial over. Following transfer, the fish were allowed to acclimatize for 3 days in the small aquaria before behavioral observations were conducted.

Experiment 2

Twenty four fish were collected from ILAB, having a mean length and weight of 14.5 ± 0.25 cm and 32.3 ± 2.4 gm respectively. The fish were anesthetized with Metacaine at a concentration of 0.5 g per 5 L of water, in order to weight and measure them. Immediately after this, fish were placed in well aerated containers where they were monitored until normal swimming activity had resumed. After complete recovery the fish were randomly separated into 6 different aquaria with 4 fish per aquarium. The acclimating period during this experiment was 7 days and not longer since the fish were not tagged. In order to individually identify the fish, dorsal fin marks were individually noted and utilized (Fig. 3). The same aquaria and experimental design was used in Experiment 2 as in Experiment 1 (Fig. 2). The fish were similarly hand-fed once a day, a quantity of food equivalent to 2% body mass per fish, with the feed being presented directly above the black plastic circle containing the two small stones.

During both experiments the oxygen and temperature were monitored each day using a micro processor Oximeter, Oxi 320 with an oxygen sensor CellOx 325. The temperature and percentage oxygen saturation during the experiments, was maintained at 12.6 ± 0.1 ^{o}C and 89.6 ± 3.1% respectively during Exp. 1 and 10.1 ± 0.1 ^{o}C and 93.6 ± 1.4% during Exp. 2.

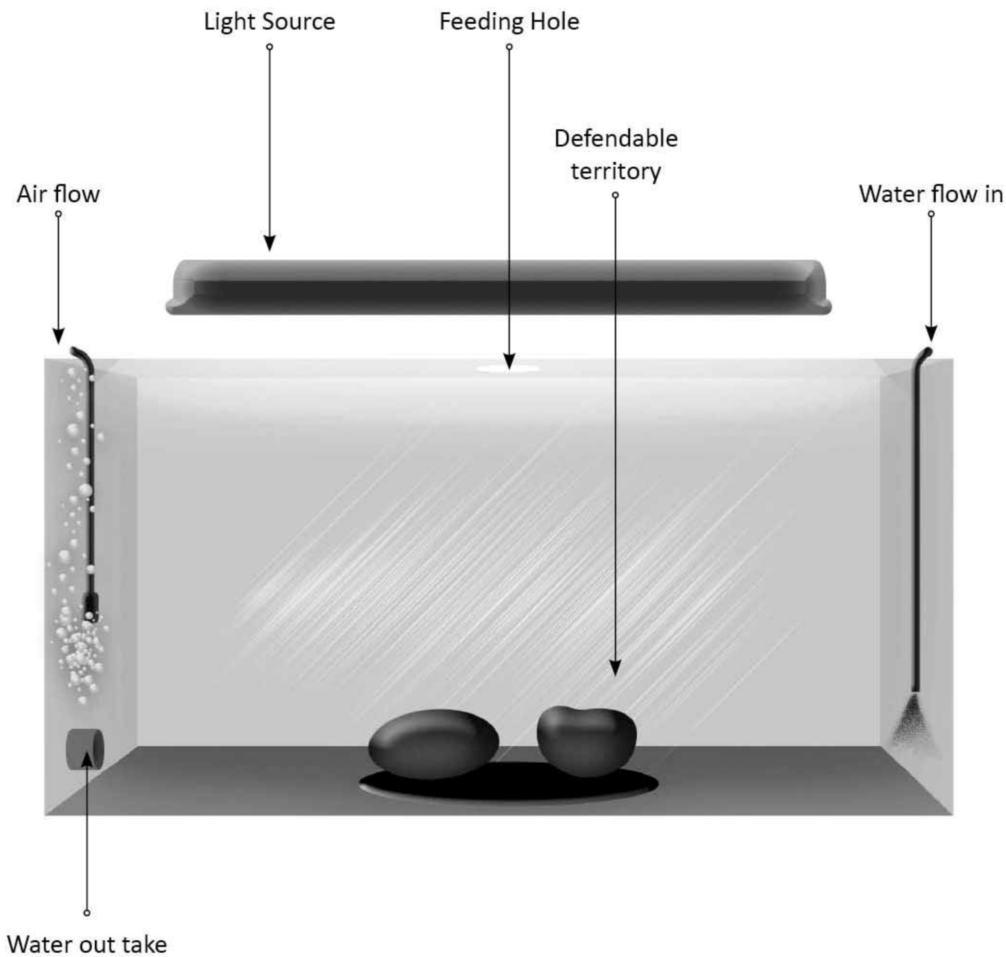

Figure 2: Schematic representation of experimental setup, showing the water flow coming into the system and leaving through an out take at the bottom. The tank was gently aerated by means of an air-stone. A lid was provided in order to hinder fish jumping out of the tanks and in the center of this lid a hole was made in order to feed the fish. On top of this lid, we find the light source. The dark circle with rocks in the middle represents a possible defendable territory situated right underneath the food source.

For both experiments, the 6 aquaria (described above) were used to house 4 fish, which were individually recognized by either external tags (Experiment 1), or individual marks on their dorsal fins (Experiment 2, as described above). For experiment 1, 24 fish were randomly selected out of the 53 and moved to the experimental tanks. The start of the experiment was on the 30th of June and lasted until the 10th of July. In the case of second experiment, the starting date was the 5th of November, ending on the 11th of November.

In both studies there were 2 experimental groups (in triplicate): TRP-fed and control-fed fish. As described above, the fish were provided with 1 black plastic circle with two small boulders from which food (TRP-supplemented or control feed) was presented directly above once a day.

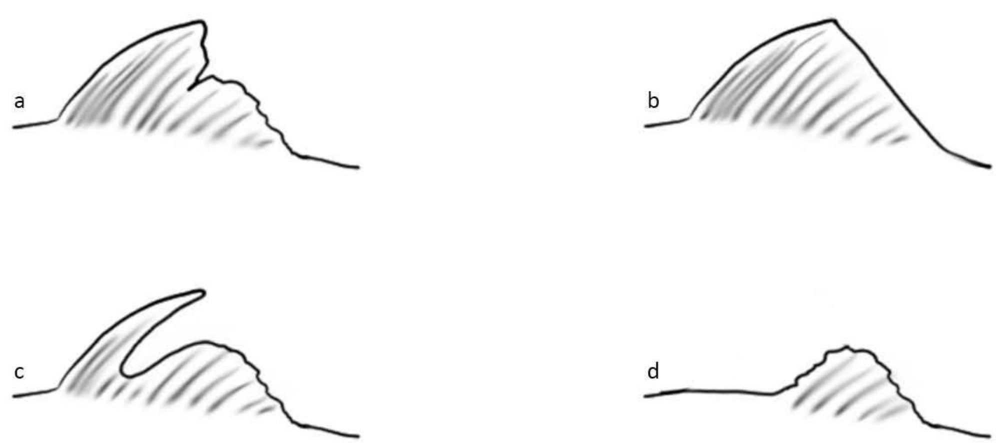

Figure 3: Dorsal fin diagrams exemplifying individual marks criteria used in order to recognize experimental fish in each aquarium during experiment 2.

2.4 Dietary tryptophan

The food pellets were provided by BIOMAR AS (Trondheim). The tryptophan feed contained 4 times as much tryptophan than the control feed, which had the same concentration of tryptophan that commercial feeds utilize. During a previous studies, it was found that this percentage of tryptophan in the feed was optimal in order to suppress aggression in cultivated fish (Basic, von Krogh et al. 2009). The TRP-feed was maintained in a freezer at a temperature of – 80 ^{o}C during the acclimation period. During the experiment, after feeding the fish, the feed was kept refrigerated.

2.5 Video recordings

Video recordings were taken every day for each of the 6 aquaria. Three Panasonic super dynamic CCTV cameras were used in order to record behavioral activity for the experimental fish. In order to capture all tanks, the recordings were taken for the first 3 tanks and immediately after that, the remaining 3. The video cameras were connected to 3 video recorders; a JVC HRS6950, a Phillips VR550 and a Samsung DVD-V6600 (DVD/VCR). All video was recorded on Sony E240CDG video tapes.

For each aquarium, the fish were video recorded for 15 minutes each day, consisting of 5 minutes before feeding and the remaining 10 minutes while feeding and immediately after. This method has been proven effective in other agonistic behavioral studies as it includes the most significant part of the day in which aggressive interactions occur, which is the period immediately after feeding (Ryer and Olla 1991; Skajaa 2006; Noble, Kadri et al. 2007). Also, all observations were recorded during the day since it has been observed that there is a substantial reduction in aggressive interactions at night, when fish appear to ignore each other (Kadri, Metcalfe et al. 1997).

2.6 Agonistic behavior

Keenleyside and Yamamoto (1962) provided a detailed description of territorial behavior in juvenile Atlantic salmon and it is their descriptions of agonistic behavior that were used in order to analyze and quantify aggressive interactions during this study. The behaviors are described as follows:

- **Charging**: the simplest and most direct form of attack in which the resident (fish that is holding a territory or is higher in the hierarchy), fixes the intruder (subdominant) optically, raises its dorsal fin slightly and swims directly and quickly towards the other fish.
- **Nipping**: it consists of one fish biting another and it is usually the culmination of a charge. Nips are directed at the tail region in most cases, although dorsal, pelvic and/or pectoral fins may also be targeted.
- **Chasing**: repeated charges with attempts to nip at the tail of a retreating fish. In aquaria were space is limited, these chases are often exaggerated since the fish is not able to swim away. The chase usually ends when the subordinate fish remains immobile at the surface or bottom.
- **Fleeing**: when the subordinate fish is been attacked, it retracts all its fins and swims directly and quickly away from its opponent. Again, in aquaria were space is limited, this may lead to the fish freezing at the bottom or going into a head-up position near the water surface.

Keenleyside and Yamamoto (1962) described one more agonistic behavior which they call displaying; which is when 2 individuals asses each other and try to intimidate the opponent by displaying their fins in different body postures. For the purpose of this study though, this behavioral act was not included. From other behavioral studies it has been found that displays may be fairly ambiguous and therefore difficult to establish as a measure of aggressive behavior for the observed fish (Cutts, Brembs et al. 1999).

2.7 Hierarchies

In order to establish the hierarchies, the aggressive interactions between individuals were totaled and arranged in a matrix in order to be then analyzed by using the David's scores method of ranking individuals in small groups. In order to obtain David's scores, first we have to calculate the proportion of wins by individual (*i*) in his interactions with another individual *j*. P_{ij} is the number of times that *i* defeats *j* (α_{ij}) divided by the total number of interactions between *i* and *j* (n_{ij}), i.e. $P_{ij} = \alpha_{ij}/n_{ij}$.

The following equation is then used:

$$DS = w + w_2 - l - l_2$$

Where w = the sum of *i*'s P_{ij} values, w2 = the summed *w* values (weighted by the appropriate P_{ij} values) of those individuals with which *i* interacted with, *l* = the sum of *i*'s P_{ji} values and l_2 = the summed *l* values (weighted by the appropriate P_{ji} values) of those individuals with which *i* interacted with (Gammell, De Vries et al. 2003). After obtaining each individuals DS, they were arranged in a hierarchy from dominant (rank 1) to subordinate (rank 4). An example is given on figure 4.

Table 1: Example of aggressive acts matrix and David's scores method, displaying the final ranked hierarchy.

		Loses						
		Bk	W	BW	WB	W1	W2	DS
Victories	Bk	----	19 (1)	14 (0.47)	29 (0.37)	1.838462	1.76631	1.353846
	W	0 (0)	----	0 (0)	2 (0.087)	0.086957	0.203587	-5.65217
	BW	16 (0.53)	4 (1)	----	12 (0.2)	1.733333	1.535719	0.933333
	WB	49 (0.63)	21 (0.91)	48 (0.8)	----	2.341249	2.620993	3.364994
	l1	1.161538	2.913043	1.266667	0.658751			
	l2	1.089387	3.029674	1.069052	0.938496			

Rank: **WB (1) -> Bk (2) -> BW (3) -> W (4)**

2.8 Brain Dissections

At the end of each of the 2 experiments, all fish were anaesthetized with an overdose of Metacaine, at a concentration of 3 g per 5 L of water. The fish were weighed, measured, and then quickly humanely killed by decapitation. Following decapitation, the whole brain (Fig. 4) was quickly excised from the fish and rapidly frozen for preservation and further analysis.

- **Experiment 1**: Whole brains were placed in individually labeled 1.5 ml cryo-tubes which were then placed in a Styrofoam box containing dry ice. Immediately following the last dissected brain, the frozen samples were then transferred for storage at -80 ^{0}C.
- **Experiment 2**: Whole brains were placed in individually labeled 1.5 ml cryo-tubes and quickly snap-frozen in liquid nitrogen. The frozen samples were then placed in a Styrofoam box containing dry ice and following the last dissected sample, were transferred for storage at -80^{0}C.

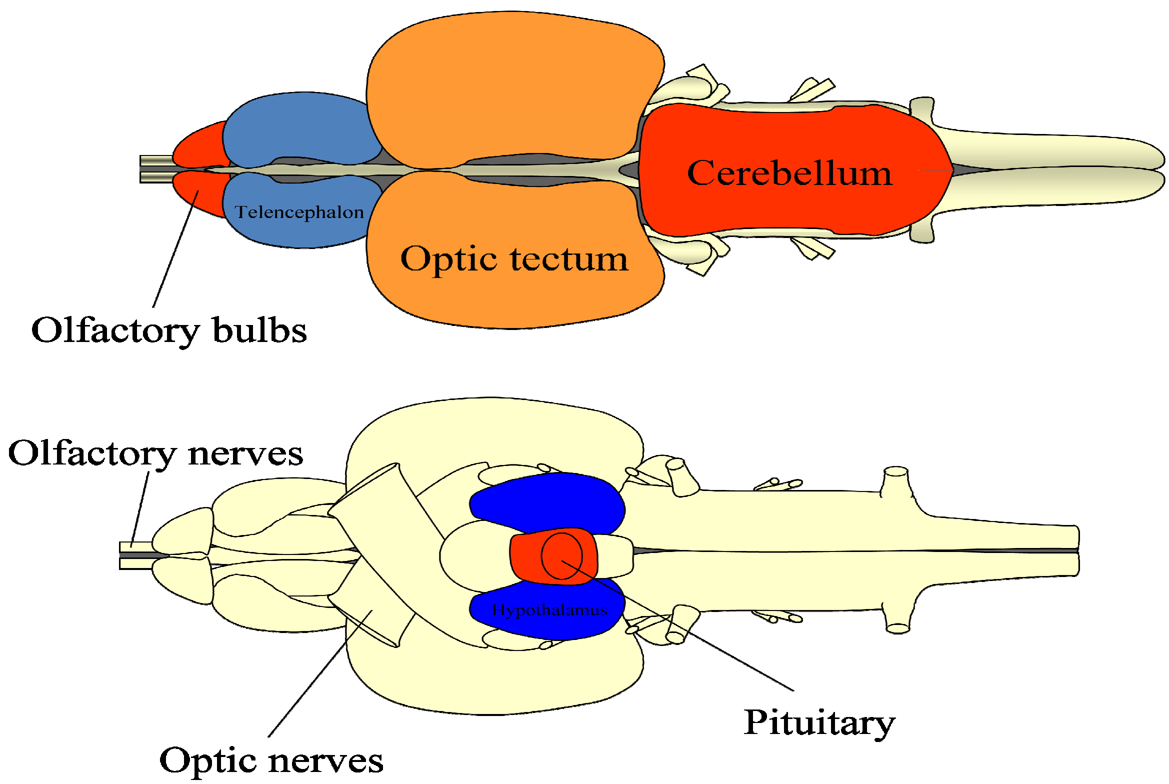

Figure 4: Schematic diagrams of the dorsal and ventral view of a teleost brain (Øverli 1999).

2.9 Measurement of brain monoamines

Brain monoamine levels were measured according to the method described by Øverli, Harris et al. (1999). In brief, the frozen brain samples were homogenized in 4% (wt/vol) ice-cold perchloric acid (PCA) containing 0.2% EDTA and 40 ng/ml epinine (deoxyepinephrine, the internal standard), using a Potter–Elvehjem homogenizer. Brain [5-HT] and [5-HIAA] were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection (EC).

2.10 Statistics analysis

Values presented are means ± standard error. The data analyzed were monoamine concentrations, serotonergic activity ratios and aggressive interactions. We determined aggression to be a function of monoamine activity, more specific a function of the serotonergic activity. Thus the monoamine concentrations and serotonergic activity would be the independent variables and aggression the dependent variable. Since the independent variables were not statistically different between experiments, all data was pooled together. The data were subjected to a linear mixed effects model; applied by using the statistical software package R (version 2.11). This model was chosen since we have clustered data, fish tanks with several fish within each aquarium. In which the categorical predictor representing the clusters (aquaria) was determined to be a random factor (we were not interested in these specific aquaria, but only in the fixed effects variable, fish been fed TRP-feed or control feed and the possible effect that this would have on monoamine levels which in turn would alter aggression levels). In this model independent observations are assumed but blocks formed by the subject variables (tanks), are supposed to be independent.

The level of statistical significance was set at $p < 0.05$. To fulfill the assumption of normal distribution, monoamine concentrations were log transformed and the [5-HIAA]/ [5-HT] ratios were subjected to arcsine transformation as has been done in similar studies before (Øverli, Harris et al. 1999; Winberg, Øverli et al. 2001; Höglund, Bakke et al. 2005).

3. Results

A total number of 12 tanks were analyzed each containing 4 individuals for a total of 48 fish. Out of these individuals a total of 1012 behavioral observations were made. Statistical analysis of this data showed no significant differences between the control group and the tryptophan fed groups in terms of brain monoamine levels and levels of aggressive behavior (see Appendix I for raw data and Appendix II for statistical results).

3.1 Hierarchical ranks

Fish were separated into their respective ranks following their David' scores number. In this way, for each aquarium, the fish obtaining the highest and the lowest score were classified as being dominant and subordinate respectively, while the remaining two intermediate fish (2 &3) were classified as being in the middle (Table 2).

3.2 Aggression

Performed acts of aggression

Treatment with TRP supplemented or control feed seems to have affected the overall acts of aggression performed by the study individuals (Table 3), although the difference appears to be very small. These difference are found to be opposite from what was expected, since it shows the tryptophan group were slightly more aggressive than control groups (Fig. 5).

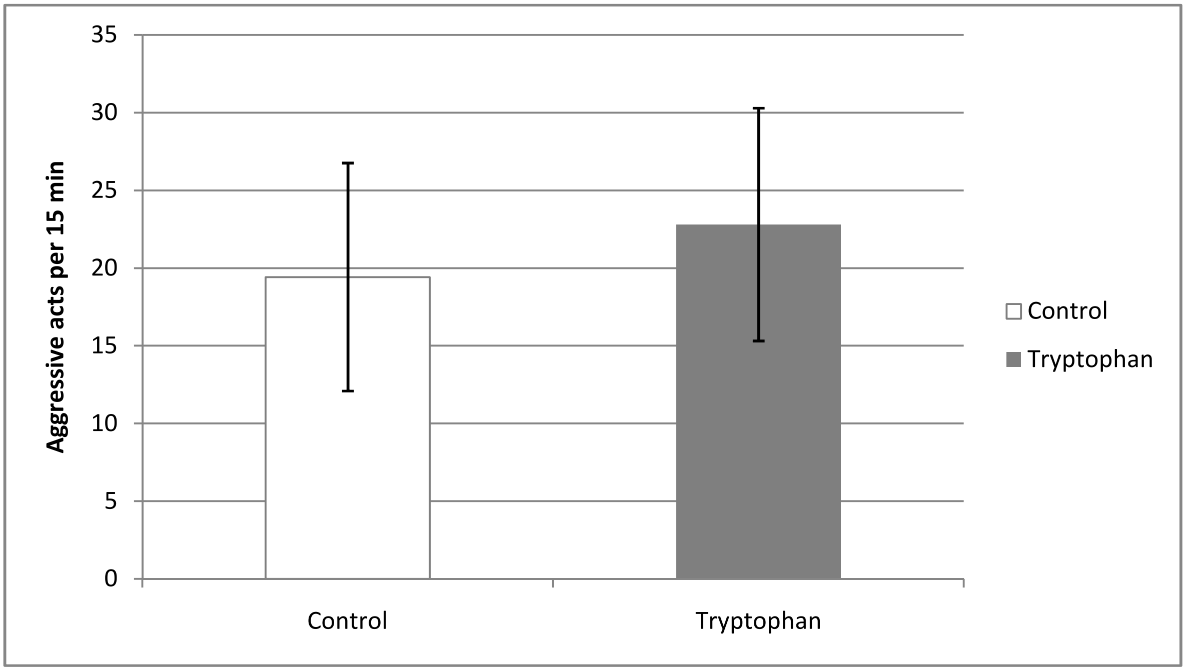

Figure 5: Mean number of aggressive acts with standard deviation per 15 min performed by control fed groups and tryptophan fed groups.

Furthermore, when we take into account the overall aggression performed by individuals per hierarchical position, we find that dominants and subordinates in control groups were, as expected, more aggressive than dominants in tryptophan (TRP) groups. Individuals in the middle (fishes 2 & 3 in the hierarchy) were unexpectedly found to be considerably more aggressive in the TRP groups than those in control groups (Table 3, Figure 6).

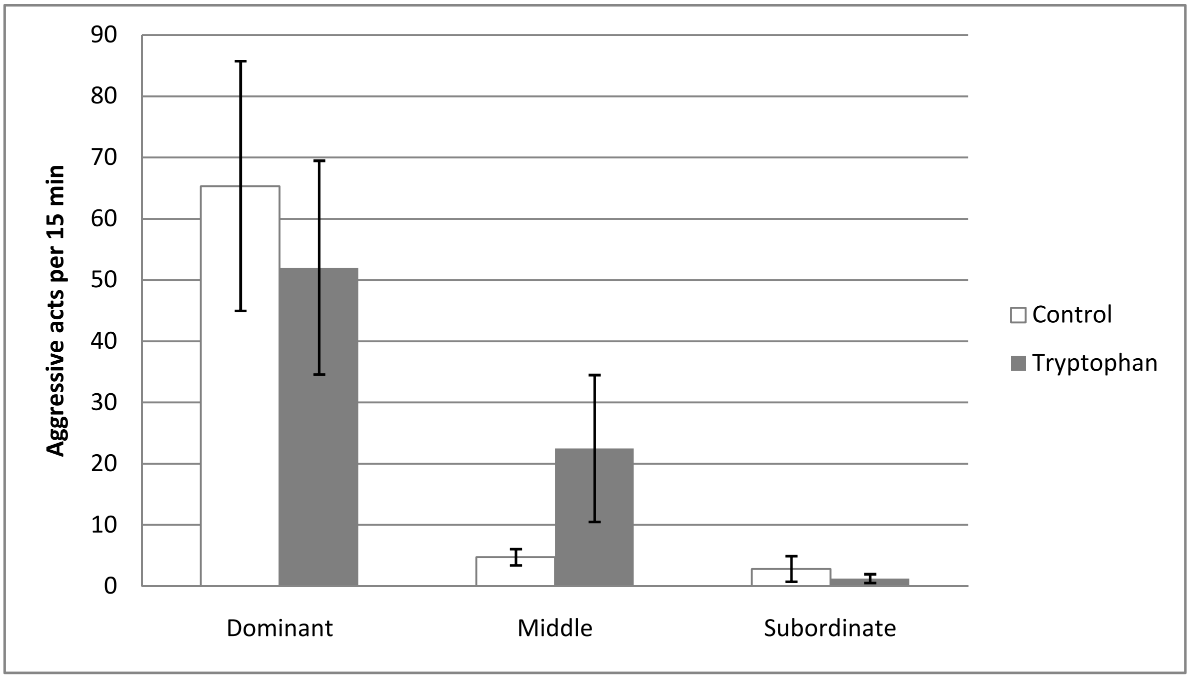

Figure 6: Mean number of aggressive acts with standard deviation per 15 min performed by control fed groups and tryptophan fed groups, divided by their corresponding hierarchical status.

Table 3: Mean number of aggressive acts with standard deviation per 15 min performed by the whole group and by individuals divided according to their hierarchical rank on control fed groups and TRP fed groups.

Behavior	Control	Tryptophan
Average aggressive acts	19.42 ± 6.6	22.8 ± 6.8
Dominant's average aggression	65.34 ± 20.38	52 ± 17.44
Middle's average aggression	4.75 ± 1.32	24.78 ± 13.15
Subordinate's average aggression	2.84 ± 2.09	1.32 ± 0.64

Table 2: Mean number of aggressive acts with standard deviation performed per 15 min, David's scores (DS) obtained, with corresponding hierarchical rank and concentration of 5-HIAA/5-HT ratios.

Tank	Treatment	Aggressive acts /15 min	David's scores	Rank	5-HIAA/5-HT
1	T	15.25 ± 5.392	3.364994426	I	0.552841593
1	T	11.625 ± 4.11	1.353846154	II	0.770283453
1	T	11.75 ± 4.154	0.933333333	III	0.397925974
1	T	0.125 ± 0.044	-5.652173913	IV	0.490090422
2	C	15.125 ± 5.347	6.846560847	I	0.557462178
2	C	0.25 ± 0.088	2.857142857	II	0.352526988
2	C	0.375 ± 0.133	-1.851851852	III	0.436009703
2	C	0	-6	IV	0.408224676
3	T	10.75 ± 3.801	5.644189383	I	0.41919116
3	T	2.5 ± 0.883	0.235294118	II	0.333076479
3	T	0.125 ± 0.044	-1.131994261	III	0.455380826
3	T	0.25 ± 0.088	-2.707317073	IV	0.377858692
4	T	3.25 ± 1.145	5	I	0.521338616
4	T	0	0	III	0.282270445
4	T	0.5 ± 0.177	0	III	0.375089464
4	T	0.75 ± 0.265	-5	IV	0.477692437
5	C	10.5 ± 3.712	5.692307692	I	0.39283507
5	C	0.25 ± 0.088	2.8	II	0.246406173
5	C	0.5 ± 0.177	-1.2	III	0.35347548
5	C	1.625 0.574	-6	IV	0.334680199
6	C	12.75 ± 4.508	3.591911765	I	0.522042499
6	C	1.125 ± 0.398	0.5	II	0.385063406
6	C	0	-1.154411765	III	0.38272038
6	C	0.375 ± 0.133	-2.882352941	IV	0.540991014
7	T	3 ± 1.5	4	I	0.537913572
7	T	1 ± 0.707	0	II	0.370145594
7	T	0	-1	III	0.312325517
7	T	0	-3	IV	0.259908076
8	C	9.625 ± 3.403	5	I	0.529994888
8	C	1 ± 0.707	1	II	0.322207137
8	C	2.5 ± 1.768	-1	III	0.286148432
8	C	0.000	-4	IV	0.399162023
9	C	1 ± 0.5	6	I	0.281145003
9	C	1.5 ± 0.75	0.3125	II	0.287026734
9	C	3.4 ± 1.521	0.25	III	0.325222624
9	C	1 ± 1	-5	IV	0.363003183
10	T	13 ± 7.506	1	I	0.368953304
10	T	0	-1	IV	0.712520287
10	T	0	0	IV	0.492792253
10	T	0	0	IV	0.519865381
11	T	3.375 ± 1.193	5.666666667	I	0.285956321
11	T	1.8 ± 0.805	0.666666667	II	0.416457508
11	T	1 ± 0.707	-3	III	0.495387569
11	T	0.000	-2.666666667	IV	0.329606161
12	C	1 ± 0.707	4	I	0.741370513
12	C	1 ± 0.707	1.733333333	II	0.256107493
12	C	1 ± 1	-2.2	III	0.499907325
12	C	0	-3.2	IV	0.433830156

Received aggression

It is expected that by feeding fish with elevated levels of dietary tryptophan, aggression received would diminish compared to individuals been fed a normal commercial diet. This was not the result found during these experiments, in which TRP groups averaged a higher amount of aggressive acts received (Table 4) as shown on Figure 7.

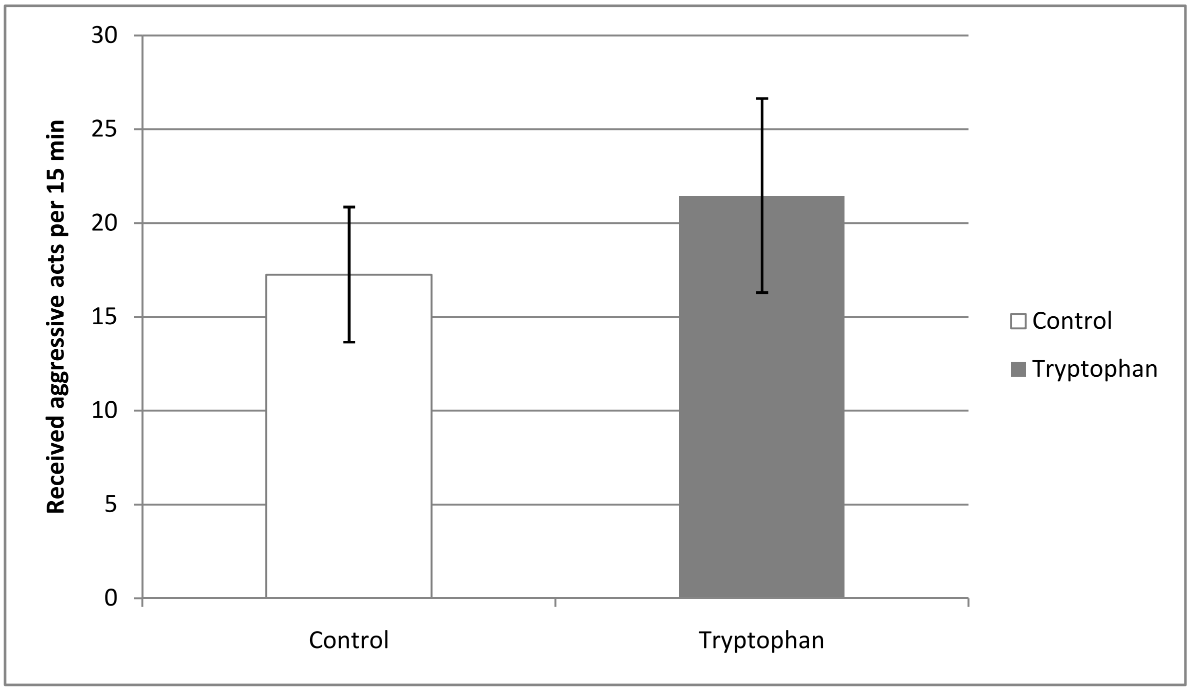

Figure 7: Mean number of aggressive acts received per 15 min with standard deviation in control fed groups and tryptophan fed groups.

In order to further investigate these results, we analyzed the received aggression in terms of the different hierarchical groups and as seen in Figure 8. Dominant and middle individuals in TRP groups received on average higher amounts of aggression compared to control groups, while subordinate groups showed less received acts of aggression in the TRP groups, as it was expected (Table 4).

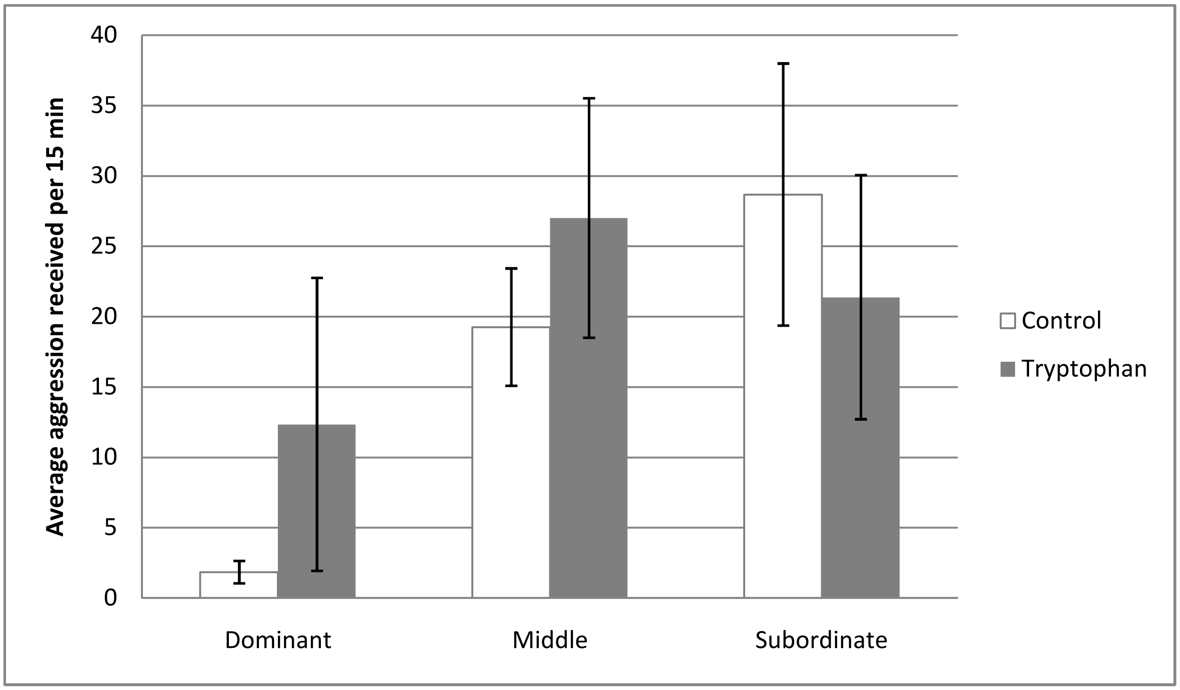

Figure 8: Mean number of aggressive acts received per 15 min with standard deviation by control fed groups and tryptophan fed groups, divided by their corresponding hierarchical status.

Attacks performed

We find that there seems to be a tendency for individuals in the middle of the hierarchy to increase their aggression in the TRP groups, this increment in aggression appears to be directed towards the dominant individuals, especially if we compared the amount of aggression performed by the middle groups in control groups towards the dominants (Fig. 9). On the other hand individuals with dominants status in TRP groups display a slight increase in attacks towards middle and subordinates (Fig. 10).

Table 4: Mean number of aggressive acts with standard deviation per 15 min in whole groups and by individuals divided according to their hierarchical rank on control fed groups and TRP fed groups.

Behavior	Control	Tryptophan
Average received aggression	17.25 ± 3.6	21.46 ± 5.17
Dominant's average received aggression	1.83 ± 0.79	12. 33 ± 10.41
Middle's average received aggression	19.25 ± 4.16	27 ± 8.51
Subordinate's average received aggression	28.67 ± 9.31	21.37 ± 8.67

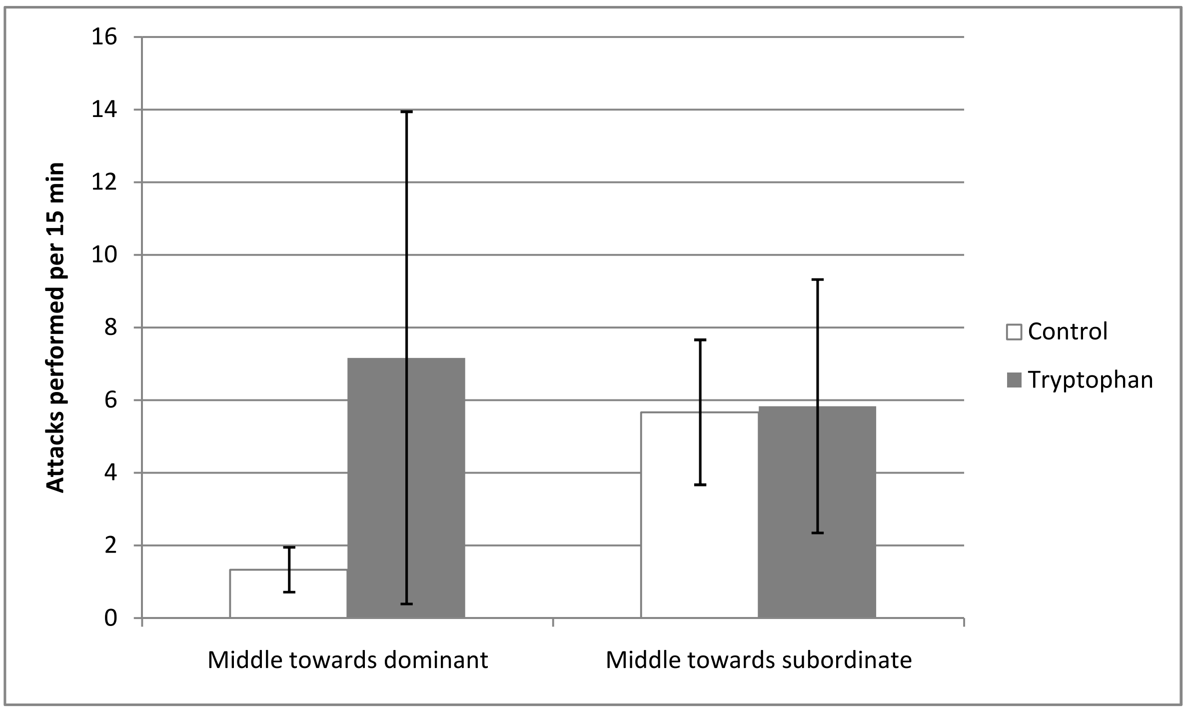

Figure 9: Mean number of attacks performed per 15 min with standard deviation by individuals in the middle groups towards dominants and subordinates in control fed groups and TRP fed groups.

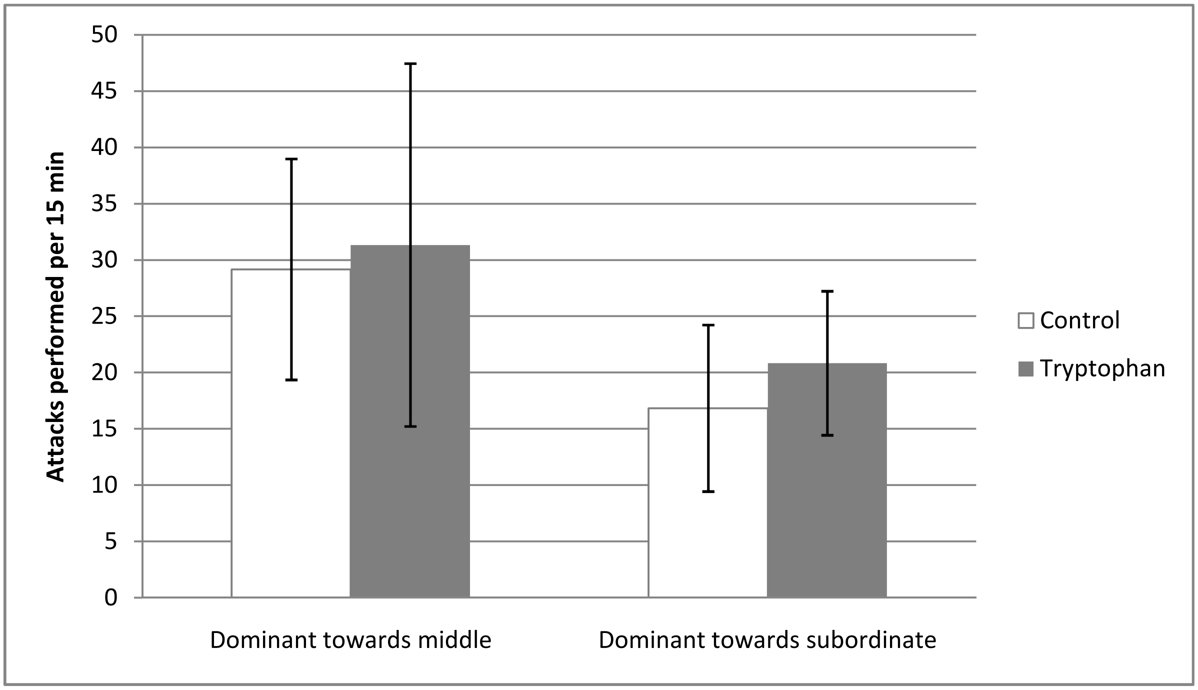

Figure 10: Mean number of attacks performed per 15 min with standard deviation by individuals with dominant status towards middle ranking individuals and subordinates in control fed groups and TRP fed groups.

We propose that individuals in a hierarchy, which are affected by social interactions within the group, may be further affected by the introduction of tryptophan in the food and the possible change in serotonergic activity in ways that have not been contemplated before in groups of fish. This will be analyzed further during the discussion.

3.3 Serotonergic activity ([5-HIAA]/ [5-HT])

The average brain concentration of serotonin [5-HT] in tryptophan fed groups (131.49 ± 10.27) was higher than in control fed groups (119.11 ± 10.07). The concentration of serotonin's main metabolite (5-HIAA) (53.56 ± 4.55), was also found to be higher than control fed groups (46.23 ± 4.22). These differences did not achieve statistical significance, but are in accordance with what is expected when feeding fish an enriched tryptophan diet.

The serotonergic activity was measured as the fraction of the concentration of the tryptophan's main metabolite over the concentration of available tryptophan. Individuals fed with an incremented amount of tryptophan feed show in average elevated ratios of [5-HIAA]/ [5-HT], compared with control fed groups (Table 5 and Fig. 11).

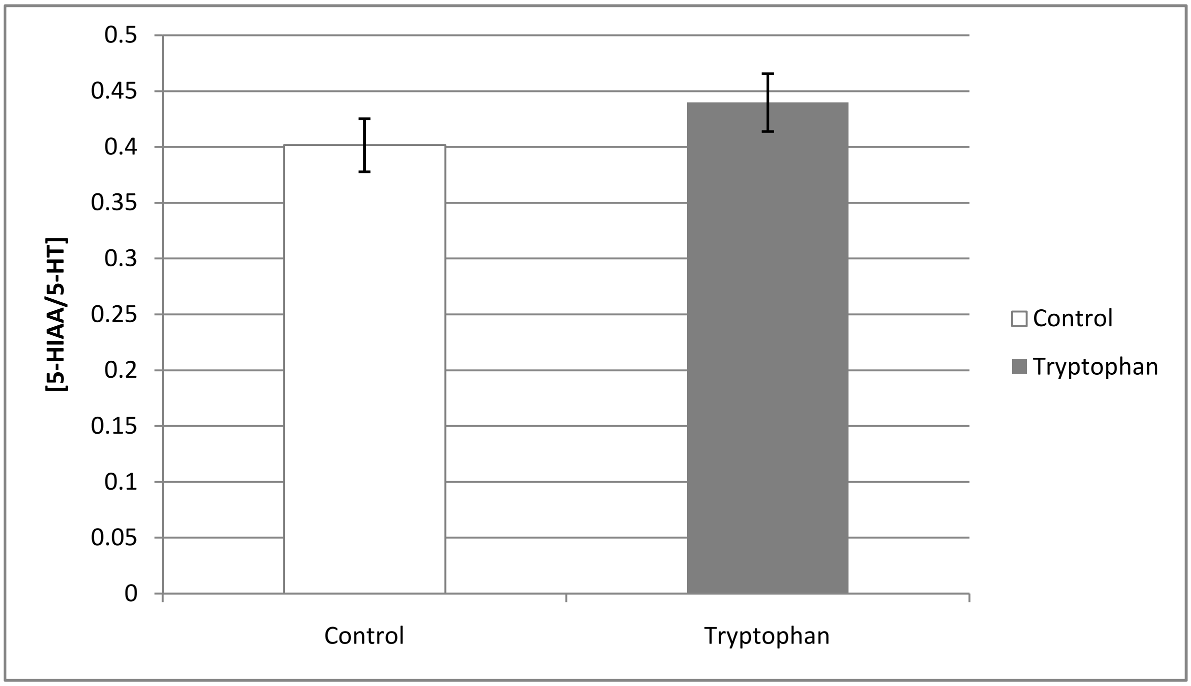

Figure11: Mean concentration of 5-HIAA/5HT ratios with standard deviation in control fed groups and TRP fed groups.

Table 5: with Mean concentration of 5-HIAA/5HT ratios with standard deviation by the whole groups and by individuals divided into their respective hierarchical groups.

Groups	Control	Tryptophan
Average [5-HIAA/5HT] ratio	0.4 ± 0.02	0.44 ± 0.03
Dominant's average [5-HIAA/5HT] ratio	0.5 ± 0.06	0.45 ± 0.04
Middle's average [5-HIAA/5HT] ratio	0.34 ± 0.02	0.42 ± 0.04
Subordinate's average [5-HIAA/5HT] ratio	0.41 ± 0.03	0.48 ± 0.05

Furthermore, separating the groups by ranks, we find that individuals in the middle and subordinates, show higher ratios in the tryptophan fed groups (Table 5), than control groups (Fig. 12). Individuals with dominant status were expected to have the lowest 5-HIAA/5-HT concentration ratios, which we did not find in these experiments.

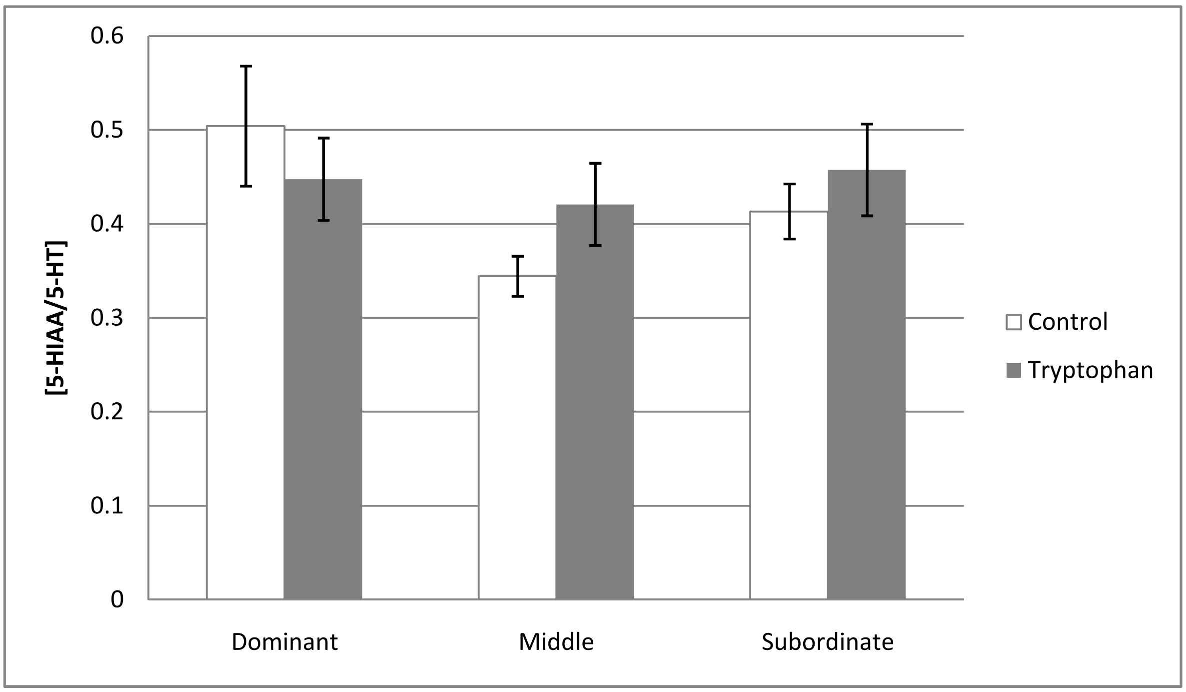

Figure12: Mean concentration of 5-HIAA/5HT ratios with standard deviation in control fed groups and TRP fed groups by individuals divided into their corresponding hierarchical ranks.

3.4 Serotonergic activity and hierarchy ranks

It is expected that within a hierarchy, the most aggressive and therefore dominant fish will have a high [5-HIAA]/[5-HT] ratio, at the same time the fish presenting the highest [5-HIAA]/[5-HT] ratio would be the most subordinate. Data from this experiment has shown that this was not the case here. The control groups show in average the highest ratio belonging to rank 1 and the rest follow the expected pattern of most dominant been lowest (Fig. 13).

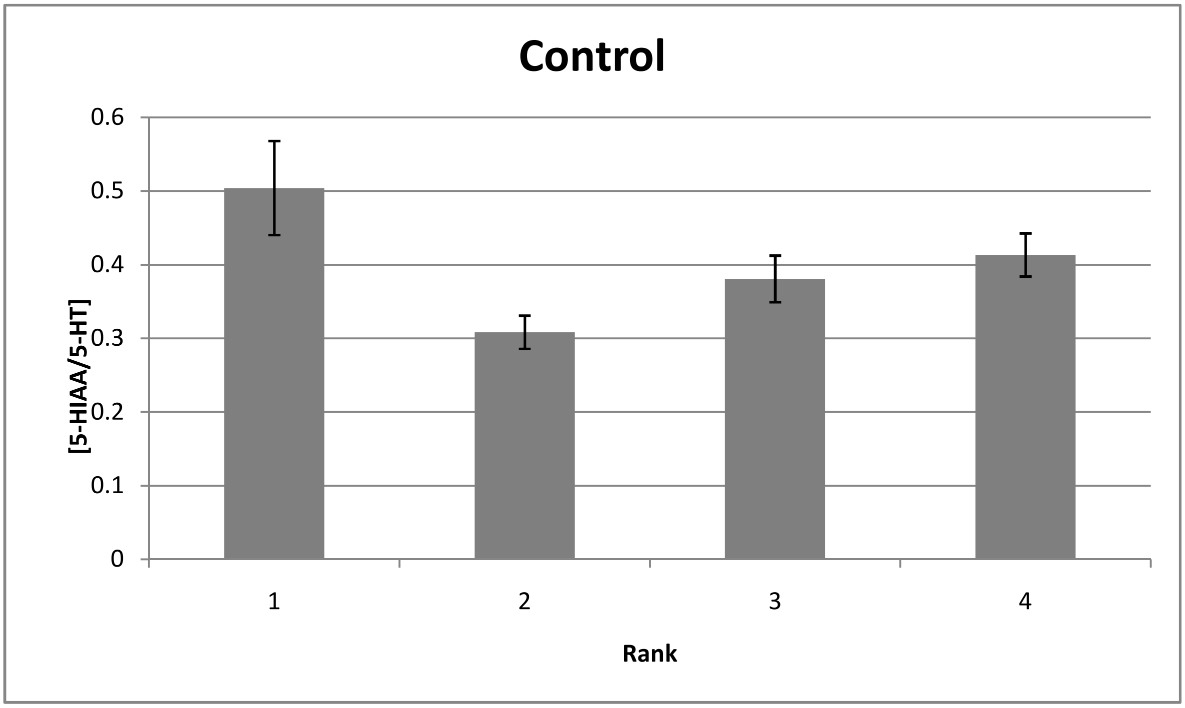

Figure13: Mean concentration of 5-HIAA/5HT ratios with standard deviation in control fed groups by individuals divided into their corresponding hierarchical ranks.

In the TRP groups we found the highest ratio belonging to rank 2 followed by rank 4, then 1 and finally 3. Although this order seems to differ very much from what was expected, we have to keep in mind that this ratio is been highly affected by not only social interactions, but by the enriched tryptophan diet as well (Fig. 14).

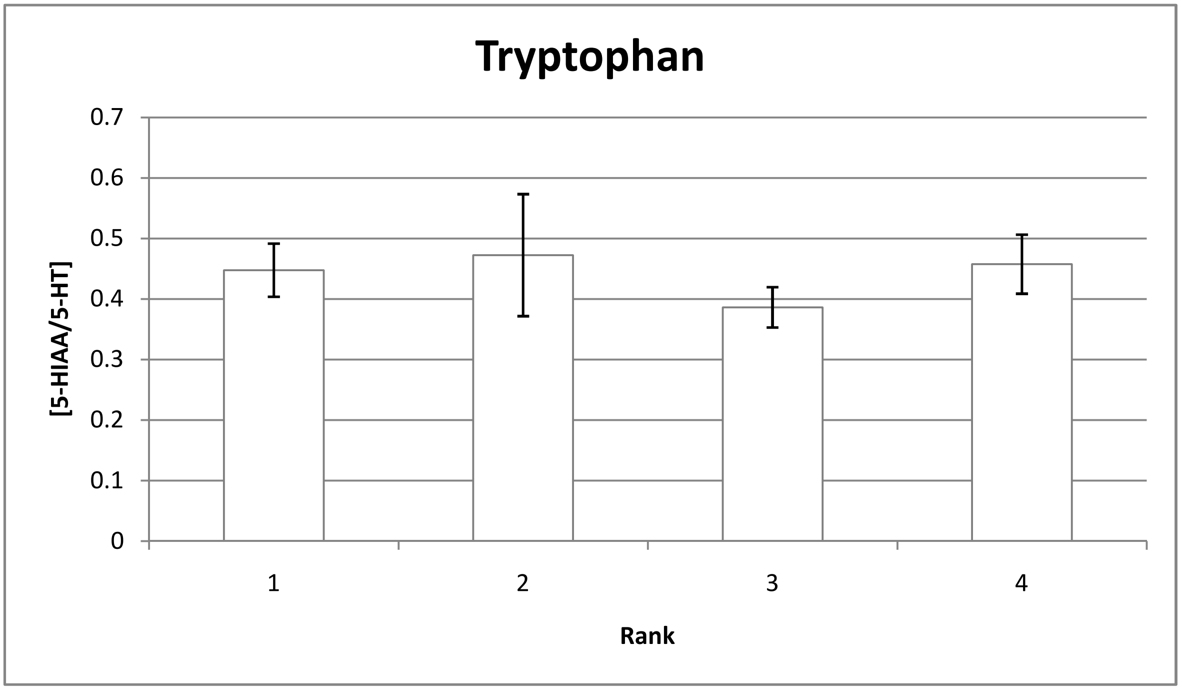

Figure14: Mean concentration of 5-HIAA/5HT ratios with standard deviation in TRP fed groups by individuals divided into their corresponding hierarchical ranks.

3.5 Serotonergic activity and aggression

Performed acts of aggression

It is expected that the amount of aggression is correlated to the [5-HIAA]/ [5-HT] ratios. Data from these experiments shows that as the amount of aggressive acts increase, we find also higher concentration ratios (Fig. 15). This was not expected since it is believed that the [5-HIAA]/ [5-HT] ratios are inversely correlated with the amount of aggression.

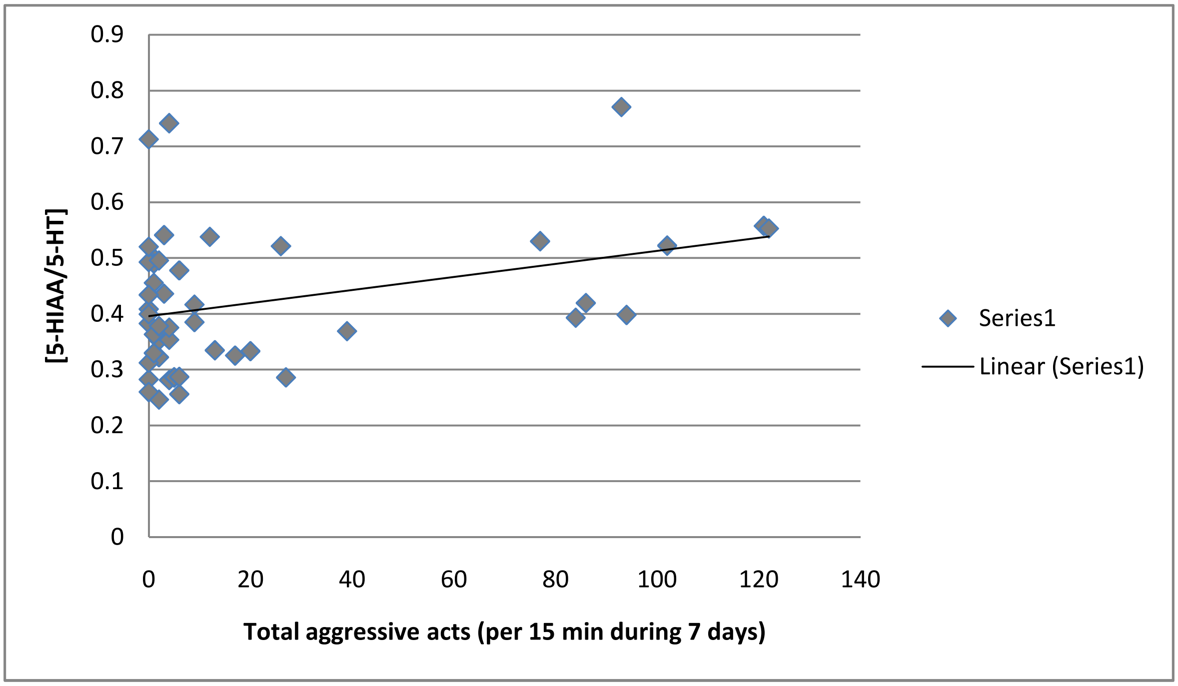

Figure15: Serotonergic activity ratios (5-HIAA/5HT) displayed by individuals in relation to their total amount of aggressive acts performed per 15 min, during 7 days, in all groups.

Performing a liner mixed model effect on the relationship between aggression and [5-HIAA]/ [5-HT] showed significant results ($p= 0.0220$, $t= 2.396198$, 35df), so we find that aggressive acts performed are affected by the brain serotonergic activity.

Analyzing the data by diving the groups into TRP fed and control fed (Fig. 16), we find again the same tendency for [5-HIAA]/[5-HT] ratios to be higher in individuals performing the most amount of aggressive acts. This seems to be true for both experimental groups. Statistical analysis showed that there were no significant differences between [5-HIAA]/ [5-HT] ratios in tryptophan and control fed groups ($p= 0.389$, $t= 0.900596$, 10df).

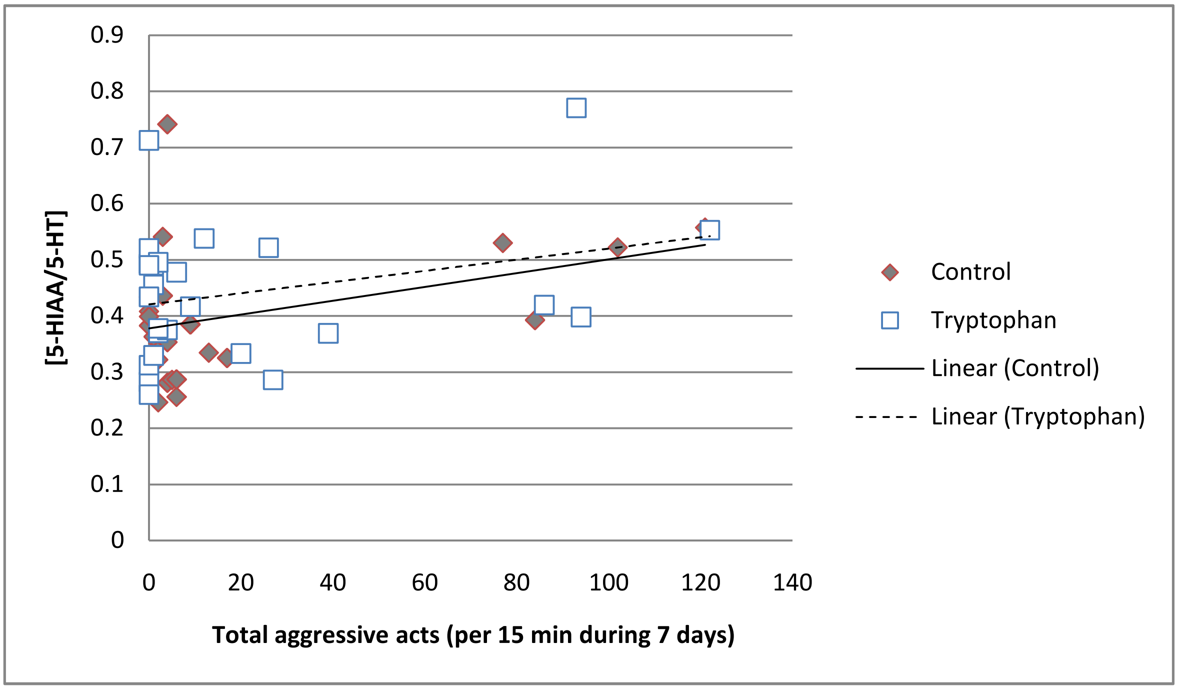

Figure16: Serotonergic activity ratios (5-HIAA/5HT) displayed by individuals in relation to total amount of aggressive acts performed per 15 min, during 7 days. In control fed groups and TRP fed groups.

When groups were analyzed by hierarchical status (Dominant, middle and subordinate groups), we start to see some differences between both groups.

- **Dominants**

Dominants in TRP groups (Fig. 17), exhibit the same tendency of higher ratios in more aggressive fish, while in control groups no real tendency seems to be displayed. However, no statistical significance was found between performed aggression and [5-HIAA]/ [5-HT] in dominant groups ($p = 0.6676$, $t = 0.4423887$, 10df).

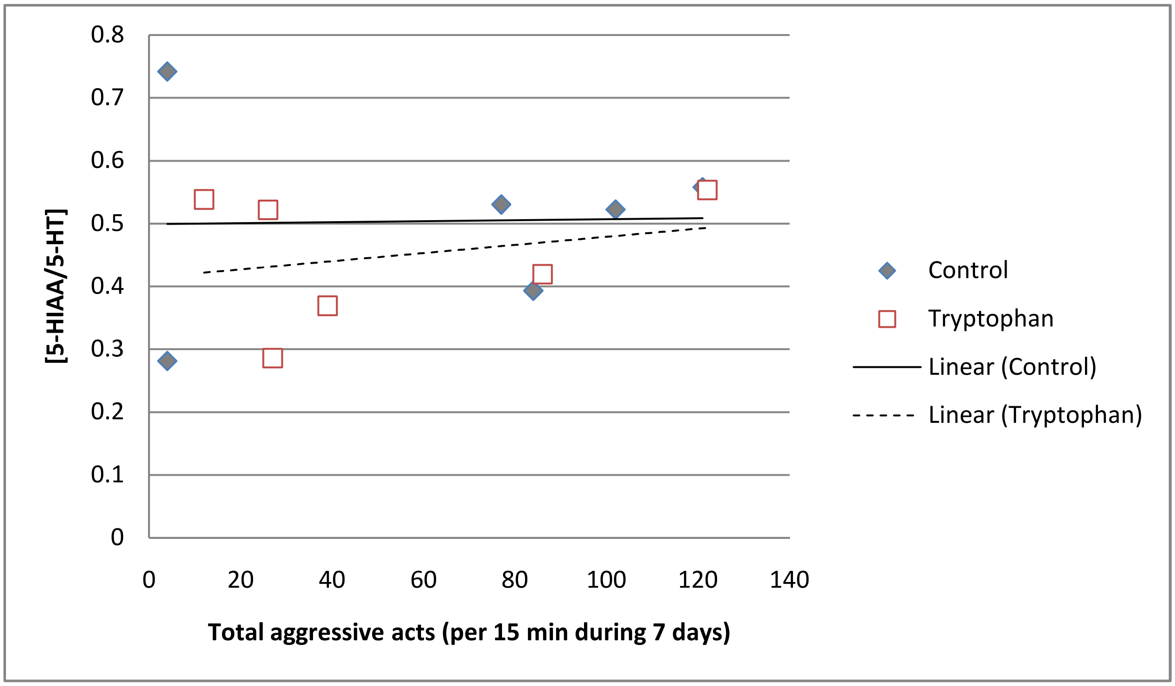

Figure17: Serotonergic activity ratios (5-HIAA/5HT) displayed by dominant individuals in control fed groups and TRP fed groups in relation to total amount of aggressive acts performed per 15 min during 7 days.

➢ Middle groups

Individuals in tryptophan fed groups situated in the middle showed a higher tendency for aggressive individuals displaying higher ratios of [5-HIAA]/ [5-HT] (Fig. 18). Control fed groups seem however to display opposite tendencies, which is what would be expected. These results were not statistically significant, having a p=0.6844, t= -0.4185191, 9df, for the [5-HIAA]/ [5-HT] ratios to be correlated to amount of aggression.

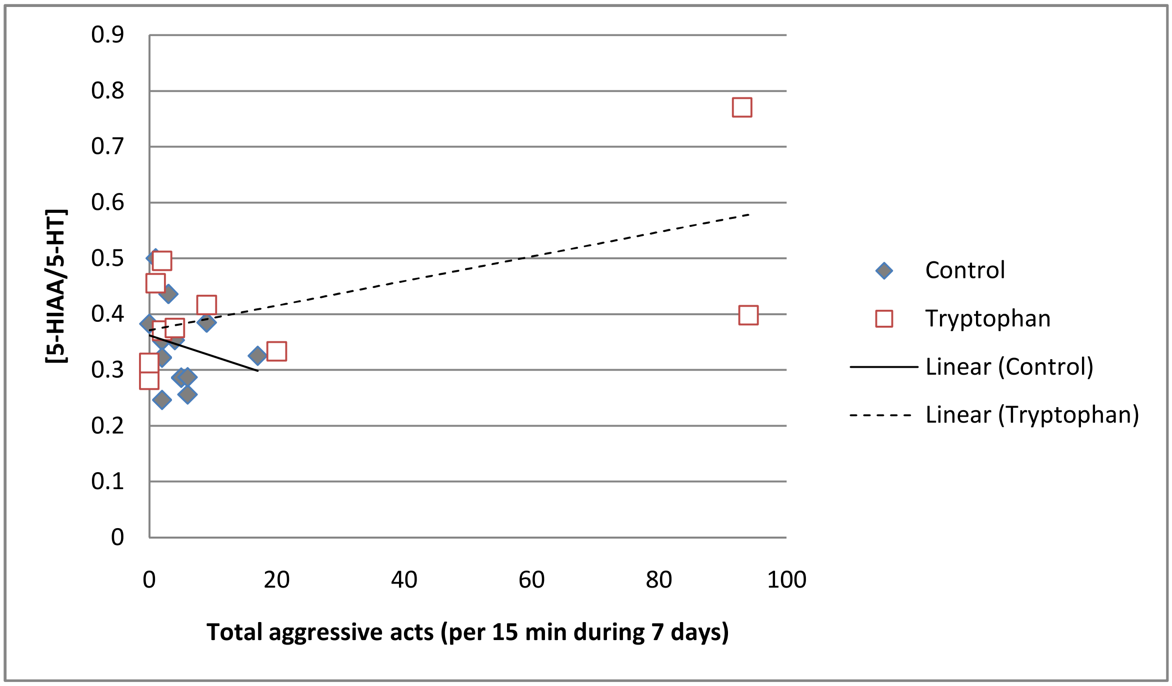

Figure18: Serotonergic activity ratios (5-HIAA/5HT) displayed by individuals situated in the middle ranks on control fed groups and TRP fed groups in relation to total amount of aggressive acts performed per 15 min, during 7 days.

- **Subordinates**

Subordinate groups in contrast, showed tendencies in both groups for the inverse relationship between [5-HIAA]/ [5-HT] ratios and amount of aggression. Which is what is expected (Fig. 19). Statistically, however the relationship between performed aggression with [5-HIAA]/ [5-HT] was found to be not significant (p=0.6382, t= -0.484924, 10df)

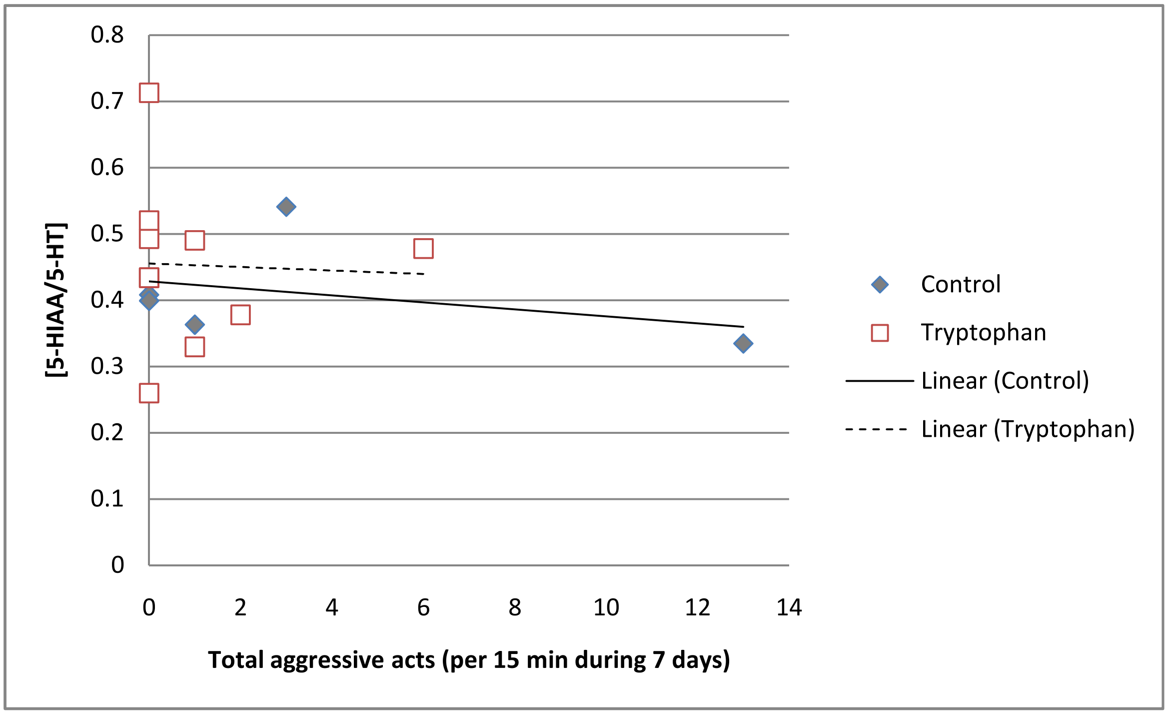

Figure19: Serotonergic activity ratios (5-HIAA/5HT) displayed by subordinate individuals in control fed groups and TRP fed groups in relation to total amount of aggressive acts performed per 15 min, during 7 days.

Received aggression

The results of these experiments show that individuals which received higher amounts of aggression, display higher ratios of [5-HIAA]/ [5-HT] (Fig. 20). Although this relationship is what is expected, it was found to be statistically not significant (p= 0.1442, t= 0.900596, 35df).

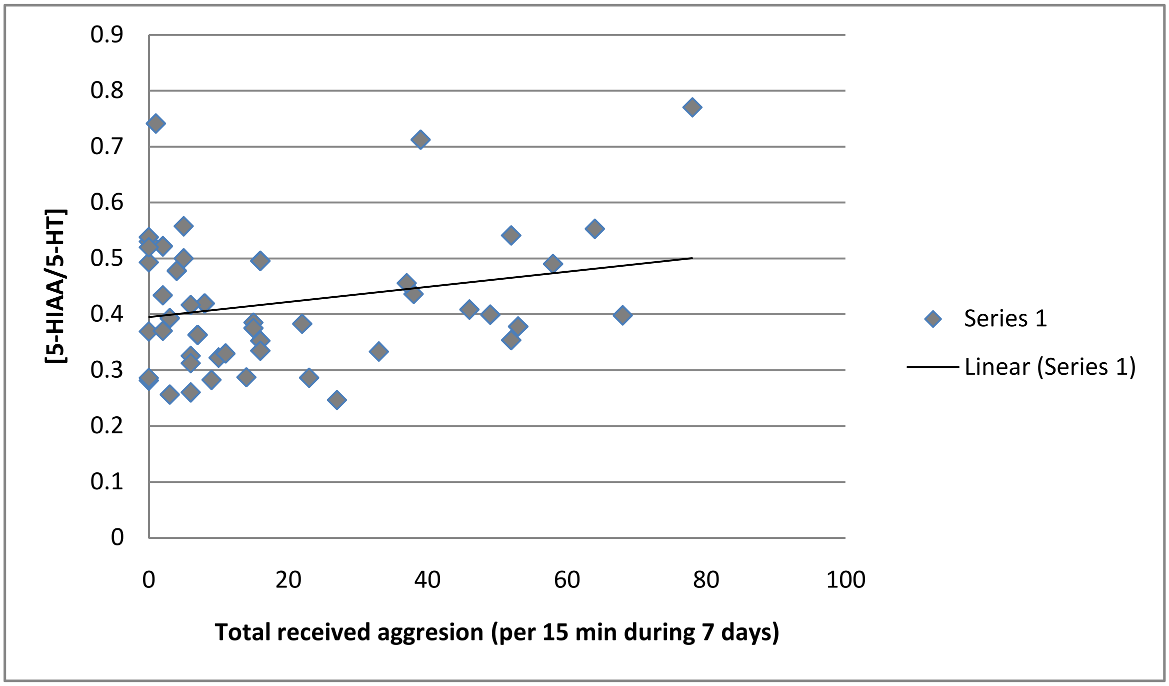

Figure20: Serotonergic activity ratios (5-HIAA/5HT) displayed by individuals in relation to total amount of aggression received per 15 min during 7 days. In both experimental groups.

It was found that dividing the groups by experimental treatment, TRP groups displayed a strong tendency for higher [5-HIAA]/ [5-HT] ratios in individuals receiving more aggression. While in control groups, it appears to be opposite (Fig. 21). Although the statistical analysis showed that there were no significant differences between [5-HIAA]/ [5-HT] ratios in tryptophan and control fed groups (as mentioned previously). Furthermore, no statistical differences were found between [5-HIAA]/ [5-HT] and received aggression (p= 0.1442, t=1.493805, 35df).

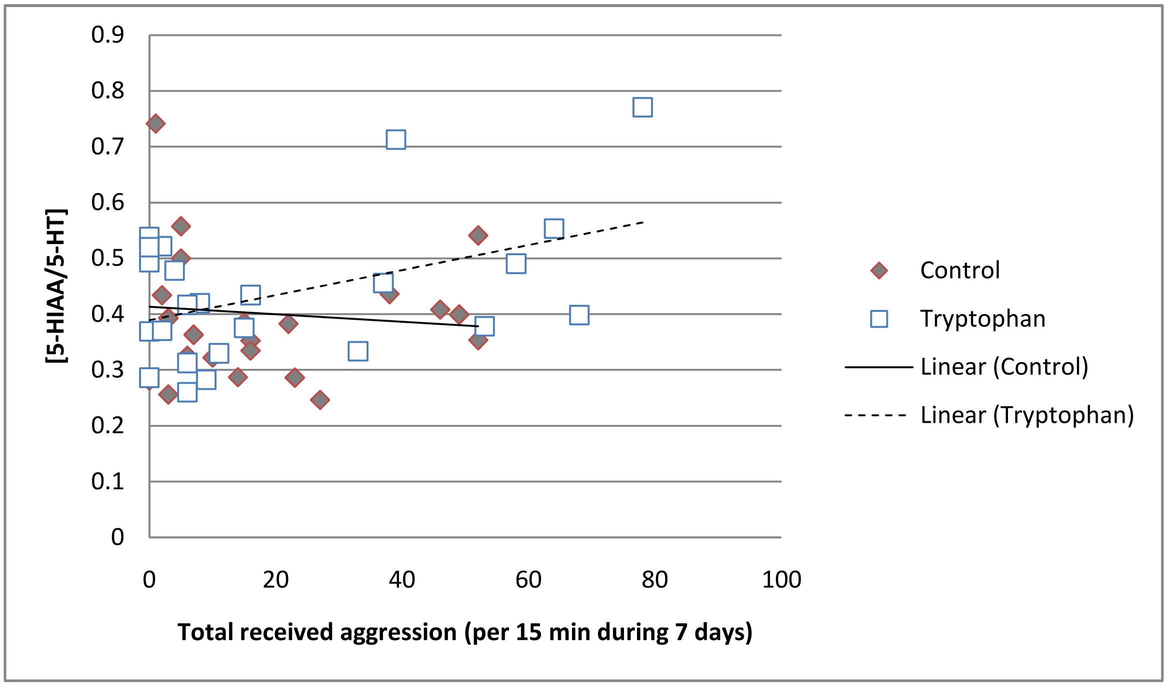

Figure21: Serotonergic activity ratios (5-HIAA/5HT) displayed by individuals in control fed groups and TRP fed groups in relation to total amount of aggression received per 15 min during 7 days.

Again, when the fish are divided into the three hierarchical groups (Dominants, middle and subordinate fish) some tendencies are observed regarding received aggression.

- **Dominants**

As expected, dominant individuals in both control and TRP, groups showed tendencies in which individuals receiving the most amount of aggression also displayed higher amount of [5-HIAA]/[5-HT] ratios (Fig. 22). Statistically the relationship between received aggression with [5-HIAA]/ [5-HT] was found to be not significant (p=0.5529, t= 0.614004, 10df).

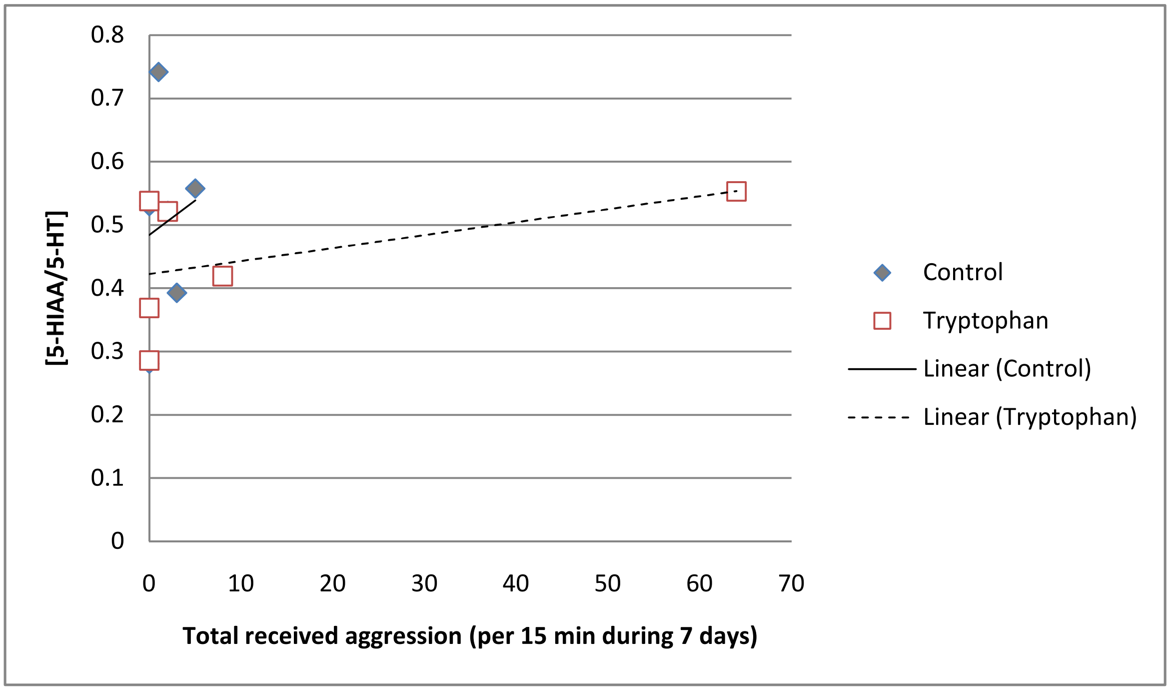

Figure22: Serotonergic activity ratios (5-HIAA/5HT) displayed by dominant individuals in control fed groups and TRP fed groups in relation to total amount of received aggression per 15 min during 7 days.

- **Middle groups**

Individuals in the middle of the hierarchy show a strong tendency in the TRP group in which fish receiving higher amounts of aggression displayed higher [5-HIAA]/[5-HT] ratios. Control groups on the other hand show no real tendency. Statistically the relationship between received aggression with [5-HIAA]/ [5-HT] was found to be significant (p=0.0157, t= 2.903247, 9df) (Fig. 23).

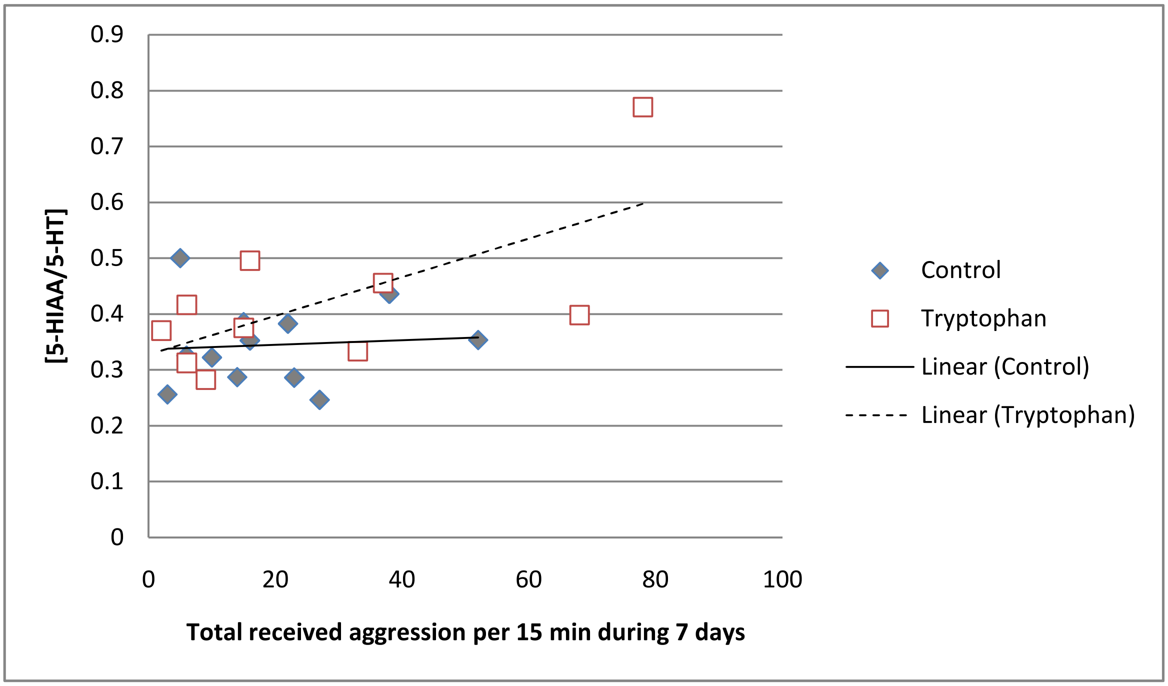

Figure23: Serotonergic activity ratios (5-HIAA/5HT) displayed by individuals situated in the middle ranks on control fed groups and TRP fed groups in relation to total amount of received aggression per 15 min during 7 days.

- **Subordinates**

Subordinate groups show as well tendencies in agreement with the other groups (Fig. 24). Although, statistically the relationship between received aggression with [5-HIAA]/ [5-HT] was found to be not significant (p=0.2429, t= 2.492486, 10df).

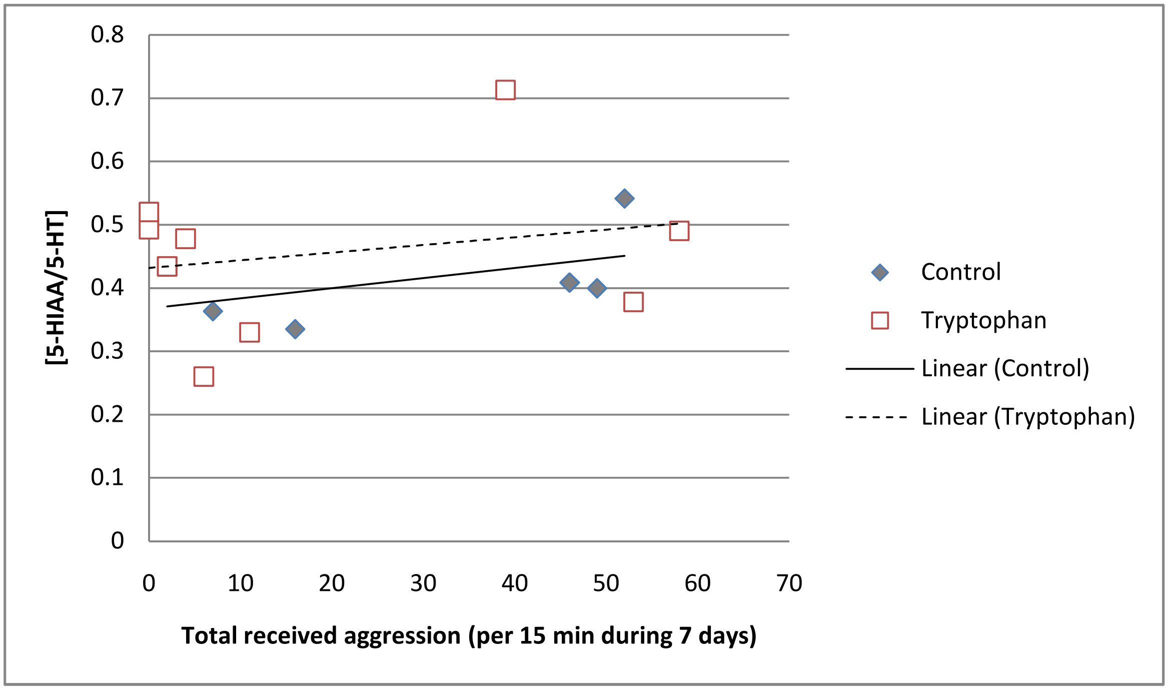

Figure 24: Serotonergic activity ratios (5-HIAA/5HT) displayed by subordinate individuals in control fed groups and TRP fed groups in relation to total amount of received aggression per 15 min during 7 days.

Overall we found that there is a strong relationship between [5-HIAA]/ [5-HT] and aggression and although we found no statistical significance between TRP groups and Control groups in terms of [5-HIAA]/ [5-HT] ratios, we found a tendency for TRP groups displaying higher [5-HIAA]/ [5-HT] ratios than control groups, which is in accordance with other studies.

3.6 Summary of results

The main results obtained in these studies are:

- Overall, tryptophan groups appear to be more aggressive than control groups.
- Specifically, the middle groups in the hierarchy appear to increase their aggression when fed increased dietary levels of tryptophan.
- High levels of [5-HIAA]/ [5-HT] were present in highly aggressive individuals, especially in tryptophan treated groups.

- High levels of [5-HIAA]/ [5-HT] were present in individuals receiving high levels of aggression also.

Most of these results are unexpected and indeed opposite to results obtained on previous studies, although most of these studies have been based primarily on pair-tests. These unexpected results therefore, bring the following questions:

- Why do tryptophan fed groups display an overall increased aggression compared to control groups. Specifically, why are middle groups more aggressive?
- It is expected that fish with high levels of [5-HIAA]/ [5-HT] are less aggressive. Why have we obtained the opposite?

4. Discussion

Our results show that aggression is clearly linked with levels of brain monoamines and this in turn affects social interactions within hierarchies. This is to our knowledge, the first study undertaken to investigate the possible alteration of hierarchies by means of diminished aggression using a tryptophan enriched diet. As such our results may represent an important step into understanding possible mechanisms that may shape social interactions in fish.

4.1 Aggression

In these experiments there were no statistically significant differences found between groups of fish fed either a TRP enriched diet or control diet, when tested for aggression acts displayed for a period of 1 week. Although not significant, we did find a difference between groups, but the results, which are contradictory to what was expected, showed that TRP groups increased their aggression during the course of the experiments. A number of studies have been conducted on different vertebrate species, correlating the levels of aggressive behavior with dietary levels of tryptophan (Shea, Douglass et al. 1990; Young 1996; Winberg, Øverli et al. 2001; Hseu, Lu et al. 2003; Lepage 2004; Höglund, Bakke et al. 2005). All these studies demonstrated a reduction of aggression in those individuals been fed a tryptophan enriched diet. The rationale behind these observations is that tryptophan is the natural precursor for serotonin and feeding tryptophan enriched-diets results in elevated levels of endogenous serotonin (Johnston, Atkinson et al. 1990; Aldegunde, Garcia et al. 1998; Aldegunde, Soengas et al. 2000). Serotonin has been found to be a major regulatory factor in the control of agonistic behavior, as well as been involved in mediating the stress response in teleost fish (Winberg and Nilsson 1993; Edwards and Kravitz 1997; Deckel and Fuqua 1998; Lepage, Tottmar et al. 2002; Lepage, Molina Vílchez et al. 2003). Taking into consideration these previous studies, clearly indicating that elevated serotonin levels, through the feeding of tryptophan-enriched diets, results in suppressed aggression, it was surprising to find the opposite effects in these present experiments. Therefore, we analyzed the groups further by their hierarchical status.

When comparing the 3 hierarchical groups (dominant, middle and subordinate) there was a tendency for both the dominant and subordinate individuals in the TRP groups to show a reduction in aggression, compared to controls. In contrast, the opposite was found for the middle fish (2 out of the 4), with the TRP-fed fish being more aggressive than the controls. The middle groups are in fact responsible for the overall increase of aggression by TRP groups, when analyzed as a whole, since it is so pronounced. We know that salmonid fish socially interact with each other in order to form hierarchies and that these social interactions create a great deal of stress upon all individuals, but especially in subordinate individuals (Winberg, Carter et al. 1993; Moutou, McCarthy et al. 1998; Øverli, Harris et al. 1999; Øverli, Korzan et al. 2004; Summers and Winberg 2006).

When looking at received aggression in TRP groups, we noticed that there was an increase in received aggression specifically for dominants and individuals in the middle compared to control groups. When Atlantic salmon are first introduced to a new environment, they usually increase their aggression until hierarchies are established (Cutts, Metcalfe et al. 2002).It is also known that aggression in salmonids increases in captivity, especially when fish are kept in small tanks and/or cages and have no means of escaping or signaling submission to dominant individuals (Keenleyside and Yamamoto 1962; Sundström, Lõhmus et al. 2003; Estevez, Andersen et al. 2007). Both control and TRP groups experienced this situation, the main difference was the amount of dietary tryptophan, which as mentioned before, should theoretically reduce the level of agonistic behavior. It has been proposed that when environmental situations change, the fish may adapt their agonistic behavior to maximize their success, for example during the change from fresh water into salt water, when juvenile salmonids change from displaying territorial to schooling behavior (Ryer and Olla 1991; Damsgård and Arnesen 1998). We propose that since incremented tryptophan in the diet may reduce the aggression in dominants, it may also lead to individuals in subsequent ranks increasing their levels of aggression in order to gain the position of dominance. This could explain why middle-order individuals in the TRP groups became more aggressive and subsequently attacked the dominants more than in the control groups, but at the same time creating a vicious circle of aggression in which they would also receive more aggression from defending individuals in dominant positions. It is hard to try to explain all the possible factors that may be in operation during these social interactions and we believe that more experiments need to be done in this area in order to understand better all factors that may be at play.

These studies serve to indicate the complexity of social interactions in fish hierarchies. It is immediately apparent that the picture, in terms of aggression-monoamine levels, is far more complex when studied in larger groups of fish, as compared to only a pair of competing fish. In the latter, there will clearly be a winner (dominant) and a loser (subordinate). In contrast, in larger groups, most individuals in the hierarchy will be both dominant over some fish, while at the same time subordinate to others.

4.2 Serotonergic activity

Tryptophan is the main precursor for the production of serotonin and the amount of free tryptophan seems to be the only limiting factor in this process. Therefore it would be expected to find increased levels of serotonin in groups which have been fed a TRP enriched diet, as it was found in our groups, although this was not statistically significant. Serotonin's main metabolite, 5-HIAA also was found to be higher in TRP groups (not significant) as expected; furthermore, their values are in accordance with values obtained with Atlantic cod fed on an enriched TRP diet (Höglund, Bakke et al. 2005) These results were not further considered in the analysis, since as explained before, changes in the concentration of [5-HIAA]/ [5-HT] are a better indicator for serotonergic acivity, since the available 5-HT is greater than the immediate demand required when stress or aggressive interactions are been experienced (Winberg and Nilsson 1993)

We found that there was a strong correlation between the serotonergic activity and the amount of aggression performed. Although, we found no statistically significant differences between TRP and control groups, the correlation between [5-HIAA]/ [5-HT] ratios (as a measure for serotonergic activity) and aggression, was found in both groups. Serotonergic activity has been correlated with agonistic and stress tackling behavior in several fish species (including rainbow trout, grouper and Atlantic cod). It has been found that high [5-HIAA]/[5-HT] ratios are found in less aggressive individuals, therefore by feeding TRP and increasing the [5-HIAA]/[5-HT] ratio you would be affecting aggression as well, in an inverse manner corresponding to the amount of TRP in the feed (Winberg, Øverli et al. 2001; Lepage, Tottmar et al. 2002; Hseu, Lu et al. 2003; Lepage, Molina Vílchez et al. 2003; Höglund, Bakke et al. 2005).

We found elevated [5-HIAA]/ [5-HT] ratios in TRP groups consistent with other studies, although in our case, there seems to be a trend in which individuals with high [5-HIAA]/ [5-HT] ratios, also displayed the highest amount of aggression. The relationship between agonistic behavior and neurotransmitters is complicated, since this behavior is in fact a combination of several different behaviors and they in turn may be controlled by different neurotransmitters. Also, other factors that we may not be aware of at this time, may be regulating agonistic behavior (Winberg and Nilsson 1993). It is now generally accepted that low [5-HIAA]/ [5-HT] ratios are found in more aggressive individuals. In light of this it is difficult to understand our results, but we have to keep in mind that it has also been found that incremented TRP may increase or decrease aggression in animals dependent on their social situation (if they are in groups or by themselves) (Edwards and Kravitz 1997), and [5-HIAA]/[5-HT] ratios in fish may also be affected by individual factors such as perceived social threat, variation in threat levels assessments and motivation to convey aggressiveness (Summers and Winberg 2006). Finally [5-HIAA]/ [5-HT] ratios in salmonids are also affected by stress (Winberg and Nilsson 1993; Øverli, Harris et al. 1999; Höglund, Balm et al. 2000; Lepage 2004).

The results for received aggression did not show any significant differences in our study, although we found that individuals who had received the most amount of aggression, had higher [5-HIAA]/ [5-HT] ratios. Received aggression is a constant source of social stress and as mentioned before, high stress levels are linked to high [5-HIAA]/ [5-HT] ratios (Winberg and Nilsson 1993; Øverli, Harris et al. 1999; Höglund, Balm et al. 2000; Lepage 2004). Therefore, our results for received aggression and serotonergic activity are in agreement with previous studies and show how important stress is in influencing serotonergic activity levels.

4.3 Hierarchies and serotonergic activity

In these studies we found some unexpected results regarding hierarchies and [5-HIAA]/ [5-HT] levels. High levels of aggression were linked to high levels of [5-HIAA]/ [5-HT] ratios, contradictory to what other studies have found.

Most studies conducted on salmonids and Atlantic cod, studying the relationships between dominants and subordinates, have used dyadic contests (Hutchison and Iwata 1998; Øverli, Harris et al. 1999; Winberg, Øverli et al. 2001; Lepage, Tottmar et al. 2002; Metcalfe, Valdimarsson et al. 2003; Sundström, Lõhmus et al. 2003; Lepage 2004; Höglund, Bakke et al. 2005; Sverdrup 2008). This method is often chosen because of its simplicity, but it only measures the ability of an individual to compete against only 1 opponent and often only for a specific period of time, generally until the outcome of an aggressive encounter, when 1 fish clearly becomes dominant over the other (Johnsson, Winberg et al. 2006).

Some earlier studies in Artic charr (*Salvelinus alpinus*), examined monoamine levels in groups of fish and their hierarchies (Winberg, Nilsson et al. 1991; Winberg, Nilsson et al. 1992). In these studies a strong correlation was found between hierarchical rank and [5-HIAA]/ [5-HT] ratios, with the lowest ratios being found in dominant individuals. Meanwhile, to our knowledge, no studies have been conducted exploring the relationship between an elevated TRP diet and possible changes in hierarchical aggression and their corresponding monoamines levels. Previous studies in teleost fish linking the reduced aggression experienced in dominant individuals after been fed increased ratios of TRP food, have only looked at pair-wise interactions between individuals (Winberg, Øverli et al. 2001; Hseu, Lu et al. 2003; Höglund, Bakke et al. 2005).

Hierarchical interactions can be very complicated and there are several factors that may affect aggression levels, social stress and consequent [5-HIAA/5HT] (Fig. 25). In some instances it may be maladaptive to reduce aggression, even when high [5-HIAA]/ [5-HT] levels are present in areas of the brain involved in the control of aggression (Summers and Winberg 2006).Therefore to obtain contradictory results in this study, only illustrates the fact that we still lack some knowledge regarding fish social relationships and their physiological regulation.

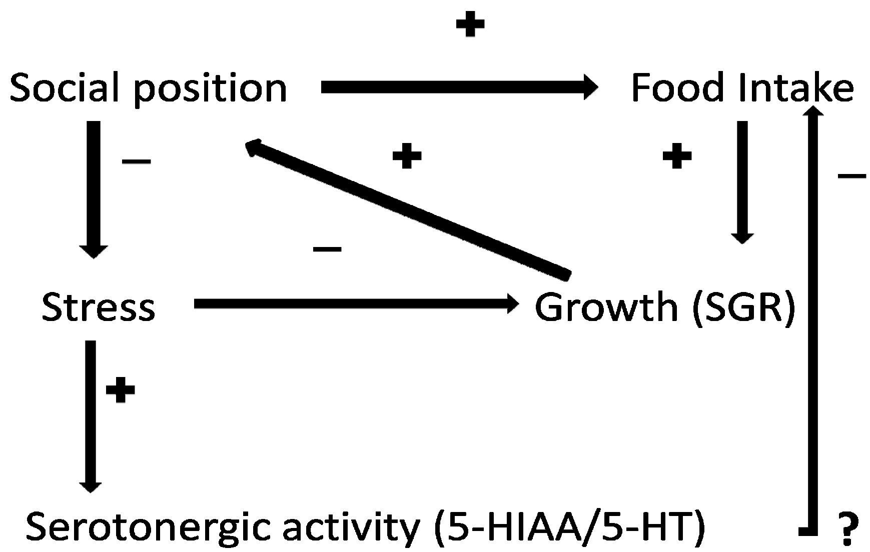

Figure 25: Schematic representation of the possible relationships between social position, food intake, stress, growth and serotonergic activity that may be affecting individuals within a hierarchy. The arrows indicate either a positive (+) or negative (-) effect of these inter-related factors. The question mark indicates an uncertain connection (Winberg, Carter et al. 1993).

In our experiments, involving four fish, the hierarchical positions were referred to as being dominant, middle (the two intermediate fish) and subordinate.

Dominants

The individuals classified as dominants in our experiments were the ones that were most aggressive and we found that they had high levels of [5-HIAA]/ [5-HT] ratios. It was also found a tendency in which the most aggressive also had the higher [5-HIAA]/ [5-HT] ratio values.

Winberg et al. (1991 and 1992) studied groups of four Arctic charr, and the hierarchies they form, correlating them to their monoamine levels, specifically to their [5-HIAA]/ [5-HT] ratios. They found a strong correlation for individuals in dominant positions exhibiting low [5-HIAA]/ [5-HT] ratio values. In a later article the same author states *that* although [5-HIAA]/[5-HT] ratios probably reflect the hierarchical position individuals occupy, the relationship between these variables is complicated by other factors, such as stress (Winberg, Carter et al. 1993).

The dominant position is established initially at the expense of increased aggression which increases stress levels. Higher [5-HIAA]/[5-HT] ratios have also been reported during this initial phase (Øverli, Harris et al. 1999). Later the hierarchy is maintained by prior experience and recognition between individuals, so as to reduce aggressive interaction. This generally leads to dominants monopolizing food sources, increasing their growth rate and in general achieving bigger sizes than subordinates, as has been observed in both salmonids and Atlantic cod (Jobling 1995; Tupper and Boutilier 1995; Rhodes and Quinn 1998; Salvanes and Braithwaite 2005). After this initial period of increased aggression, higher stress and high [5-HIAA]/ [5-HT] levels, it has been reported that dominant fish experience a reduction in [5-HIAA]/ [5-HT] ratios, since the same levels of aggression are not required to maintain their place in the hierarchy (which in turn reduces their stress levels), as explained above. (Øverli, Harris et al. 1999). This means that it could be possible that the low levels of [5-HIAA]/ [5-HT] found in dominant fish may be due to decrease stress levels experienced once the hierarchy has been established.

Once hierarchies in Atlantic salmon are established, they remain quite stable. Challengers may arise and some times, are able to displace or share the dominant positions with previous dominants (Fernö and Holm 1986). It would be possible then to imagine that in a situation where dominant individuals are eating more of the TRP food than the others, which would lead to elevated [5-HIAA]/ [5-HT] ratios and consequently, a diminution in their aggressive behavior. Other fish may arise as challengers for the dominant positions and this may provoke dominants to try and hold their dominant position by maintaining their aggression levels and in turn increase their stress levels. This could be an explanation as to why dominant individuals in our experiments were displaying high [5-HIAA]/ [5-HT] ratios and yet still remain quite aggressive, especially towards middle individuals (possible challengers for the dominant position).

When looking at received aggression, we also found that there was a tendency for individuals who received the most amount of aggression, displaying higher [5-HIAA]/ [5-HT] ratios. This would be in accordance with previous studies (conducted on Atlantic salmon, rainbow trout and Arctic charr), were an increase in aggression would represent an increase in social stress and this in turn would lead to increase the levels of serotonergic activity (Winberg, Carter et al. 1993; Øverli, Harris et al. 1999; Höglund, Balm et al. 2000; Lepage, Tottmar et al. 2002; Cubitt, Winberg et al. 2008). This strengthens our possible explanation for the increased serotonergic activity levels found for dominant fish in our experiments.

Middle groups

The middle ranks 2 and 3 in our experiments showed an increase in aggression within TRP groups compared to control groups. Individuals in the middle ranks experience a situation that may be very variable: they are dominated by one or several others, but at the same time they dominate over others (Keenleyside and Yamamoto 1962; Moutou, McCarthy et al. 1998). This gives them the opportunity to sometimes challenge the dominant individuals and take their positions as mentioned in the previous section (Fernö and Holm 1986).

Moutou et al. (1998) found that in rainbow trout hierarchies, individuals in the middle ranks showed extensive fin damage, a sign of increased aggression, and that this could be related to having the lowest inter-renal activity, a physiological indicator of high levels of stress. Under conditions of increased stress, we expect the [5-HIAA]/ [5-HT] levels to increase (see previous references).

In previous hierarchy studies, it was found that individuals in the middle ranks had an inverse relationship between rank and [5-HIAA]/ [5-HT] ratios. This would indicate that it is expected for middle ranks to have higher ratios than dominants, but at the same time, they should suppress their aggression because of these higher ratios (Winberg, Nilsson et al. 1991; Winberg, Nilsson et al. 1992).

Having found high [5-HIAA]/ [5-HT] ratio levels in individuals that are highly aggressive therefore is very contradictory to what has been found before. We believe that, as mentioned before, there might be other unknown factors controlling aggression and that introducing higher levels of dietary TRP may destabilize the hierarchy in unforeseen ways that in turn may provoke the increase of aggression in middle rank individuals. In order to test this hypothesis, more studies are needed focusing on the interaction between tryptophan, aggression and middle ranks in the hierarchy and possible increase in stress levels.

On the other hand, when analyzing the amount of received aggression by middle ranked fish, it was found that as the levels of received aggression increased, so did the [5-HIAA]/ [5-HT] levels. This is in accordance to previous studies in which received aggression is related with high stress levels and in turn associated with high serotonergic activity ratios (see previous references).

To summarize, we believe that the conditions experienced by middle rank individuals in a hierarchy, may make the use of TRP as a controlling agent for levels of aggression unpredictable. In these circumstances, high levels of [5-HIAA]/ [5-HT] may not be the controlling factor in agonistic behavior.

Subordinates

In our studies, subordinates displayed a tendency in which the most aggressive individuals had the lowest [5-HIAA]/ [5-HT] ratios. Even though this relationship was not statistically significant, it is found to be in accordance with other studies.

The subordinates in salmonid hierarchies have to deal with a great deal of social stress and this is reflected by high levels of [5-HIAA]/ [5-HT] (Winberg, Nilsson et al. 1991; Winberg, Nilsson et al. 1992; Winberg and Nilsson 1993; Winberg, Carter et al. 1993; Øverli, Harris et al. 1999; Höglund, Balm et al. 2000; Cubitt, Winberg et al. 2008). The increase in [5-HIAA]/ [5-HT] ratios may lead to behavioral inhibition by subordinates as a survival technique in order to reduce the number of attacks. But at the same time, the increased serotonergic activity can cause a reduction in the food intake and high levels of cortisol that in turn, affect the immune response (amongst other physiological factors). All these factors reduce the fitness in subordinate individuals (Winberg and Nilsson 1993; Winberg, Carter et al. 1993).

It was also found that individuals which received the highest levels of aggression, also displayed higher levels of serotonergic activity (did not achieve statistical significance), which is in accordance with the increment in social stress that we have discussed in the previous paragraph.

Our results are in accordance with others and add to the belief that stress is a major factor affecting the subordinate ranks. This issue of subordinate fish being exposed to chronic stress is a welfare issue that needs to be studied further, especially in context to the aquaculture industry.

4.4 Methods and sources of error

Understanding the mechanisms that control the establishment of social structures in fish is a challenging task. It is known that, in fish social groups, individuals are able to recognize each other and react upon individual social signals from others (Höglund, Balm et al. 2000; Suter and Huntingford 2002; Magnhagen, Braithwaite et al. 2008). It is more complex, though, understanding how these mechanisms and their interactions affect individuals in larger groups and/or in different environments, as for example fish in the wild or in captivity (such as in aquaculture). While some believe that as the group size grows it is less likely for individuals to recognize one another (Croft, James et al. 2005), others believe that inter-individual interactions are still possible and important in large groups (Pike, Samanta et al. 2008). For the purpose of this experiment it was not practical to study large groups of fish, since it becomes very challenging to recognize individuals and their interactions with one another. This limits the amount of information that we may be able to gather from fish interactions in groups. It is not correct to assume that what happens in a group of 4 fish in an aquarium is the same that happens in the wild or in commercial aquaculture systems. On the other hand, in order to understand what are the possible mechanisms that are at play during fish social interactions, many have opted for conducting intruder-resident experiments (pair-wise interactions) (Øverli, Harris et al. 1999; Winberg, Øverli et al. 2001; Lepage, Tottmar et al. 2002; Metcalfe, Valdimarsson et al. 2003; Sundström, Lõhmus et al. 2003; Lepage 2004; Höglund, Bakke et al. 2005; Sverdrup 2008) and the information obtained from these experiments have been crucial in understanding the physiological mechanisms controlling these interactions. Our results clearly demonstrate that the underlying mechanisms controlling the establishment and maintenance of hierarchies in larger groups of fish are far more complex. Most likely, this reflects the fact that most fish in a hierarchy are not solely “dominant” or “subordinate”, but are dominant over some while subordinate to others.

A number of studies have been undertaken on groups of salmonid fish, correlating hierarchy status based on aggression (Keenleyside and Yamamoto 1962; Fernö and Holm 1986; Holm and Fernö 1986; Winberg, Nilsson et al. 1991; Winberg and Nilsson 1993; Moutou, McCarthy et al. 1998; MacLean, Metcalfe et al. 2000) and/or feed consumption (Winberg, Carter et al. 1993; Kadri, Huntingford et al. 1996; Cubitt, Winberg et al. 2008). These studies have paved the way for understanding hierarchies, aggression and possible physiological effects that may incur in fish. My experiment followed this line of experimentation utilizing small groups of fish and analyzing their aggressive interactions in relation to monoamine levels as a physiological parameter.

Our study fish were one year old Atlantic salmon parr that according to their average size (14.91 ± 0.12 cm), were capable of parr-smolt transformation if given the right environmental signals (Taranger, Carrillo et al. 2009). Many agree that fish close to the smoltification process or during this process considerably reduce their aggression and abandon hierarchies in order to form shoals (Ryer and Olla 1991; Hutchison and Iwata 1997). However, some believe that this change of behavior may be a consequence of habitat shift (fresh water to salt water) rather than a consequence of the physiological changes associated with smoltification. It has been stated rather, that when parr and smolts are given the same habitat conditions, they exhibit the similar agonistic behavior (Damsgård and Arnesen 1998). In our experiments, individuals often preformed acts of aggression, where sometimes the aggression seemed to escalate considerably by the aggressor chasing the other around the tank, while the recipient tried to get away but could not because of space restrictions. On the other hand, typical parr defending territorial behavior was not observed. This diminished the amount of information that could be obtained, especially since the hierarchy was not obvious to the eye, until aggressive acts were counted. Even though these factors may have limited our experiments, it was still possible to assess hierarchical status by amount of aggression observed.

The number of replicates in these studies was compromised due to the available resources; a small room which could only house a maximum of 6 aquaria at a time, plus amount of video cameras which were used for the study. It would have been ideal to analyze 12 tanks during 1 trial and then complement this with 12 more tanks immediately following. Increasing the number of test individuals would provide more information that can be then analyzed in terms of trends a population may be following (Imsland, Folkestad et al. 1994). But as we were restricted by space and available recording equipment, we maximized the replicates by following the explained design.

In order to study brain monoamine levels and the changes in their concentrations due to social interactions and stress in fish, it is common practice to divide the brain into three main areas: brain stem, telencephalon and hypothalamus (see Fig 4). In this way differences in monoamine concentrations can be specifically studied in those brain areas that are known to control certain functions in fish (Johnston, Atkinson et al. 1990; Winberg, Nilsson et al. 1991; Winberg, Nilsson et al. 1992; Winberg and Nilsson 1993; Winberg, Carter et al. 1993; Aldegunde, Garcia et al. 1998; Øverli, Harris et al. 1999; Aldegunde, Soengas et al. 2000; Winberg, Øverli et al. 2001; Lepage, Tottmar et al. 2002; Lepage, Molina Vílchez et al. 2003; Lepage 2004). In this study brain analysis was conducted in whole brains. This limits the amount of information that can be obtained from physiological pathways, but it still provides accurate information on monoamine concentration levels in fish brains and their possible links to agonistic behavior, as has been conducted by at least one other experiment in Atlantic cod (Höglund, Bakke et al. 2005).

It has been discussed in this paper how aggression leads to stress and the effects this has on fish behavior. To date, the most common method used to assess the level of stress in fish, is by measuring plasma cortisol levels (Øverli, Harris et al. 1999; Höglund, Balm et al. 2000; Lepage, Tottmar et al. 2002; Lepage, Molina Vílchez et al. 2003; Øverli, Korzan et al. 2004; Cubitt, Winberg et al. 2008). This method has proven to be very reliable and when used in combination with the measurement of brain monoamine levels, can give a better picture of how social interactions may be affecting fish behavioral interactions. In our experiments, plasma samples for cortisol analysis were not taken, although this could have helped assessing the level of stress in individuals, helping to explain monoamine levels as well. In future experiments, this factor should be considered in order to obtain more information.

5. Conclusions

Understanding social interactions between fish, especially in larger groups is not an easy task. Behavioral studies involving larger groups of fish are limited by the ability to recognize individuals in these large groups and their interactions with each other. During these experiments, we have encountered unexpected and contradictory results from previous studies, although the vast majority of these earlier studies have involved only pair-wise contests. However, this is the first time (as far as we know), that the effect of feeding tryptophan enriched diets on aggression and its consequences in a hierarchy, has been tested in a larger group of fish. We found that even though tryptophan feed may increase levels of serotonergic activity, individuals seem not to diminish their aggression levels. This was especially noticeable in the middle ranked fish, which displayed a significant increase in aggression. We propose that under true hierarchical conditions, other factors are involved in the overall control of aggression. This could be an important finding that needs to be studied further, especially in the context of fish welfare in intensive aquaculture, where high levels of aggression and stress are less than ideal for good fish welfare.

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7. Appendix I

Table 6: Raw data for experiments 1 and 2. A total of 12 tanks treated with either tryptophan enriched or control diets with individually recognized individuals, either by tags (black, white, black and white or white and black variations) or by individual dorsal fin markings (A, B, C or D were assigned to each fish). Monoamine concentrations for serotonin and 5-HIAA, serotonergic activity ratios (5-HIAA/5-HT), total aggressive acts performed and total aggressive acts received are given for each individual along with their hierarchical rank level.

exp	tank	treat	indiv	rank	ser	hiaa	hiht	tagg	ragg
1	2	C	W	I	48.28815	26.91882	0.557462	121	5
1	5	C	BW	I	106.1134	41.68505	0.392835	84	3
1	6	C	W	I	83.17267	43.41967	0.522042	102	2
2	8	C	A	I	204.57	106.7999	0.529995	77	0
2	9	C	A	I	145.64	39.81975	0.281145	4	0
2	12	C	B	I	89.662	66.3124	0.741371	4	1
1	1	T	WB	I	87.395	48.31559	0.552842	122	64
1	3	T	W	I	105.3372	44.15643	0.419191	86	8
1	4	T	BW	I	49.74691	25.93498	0.521339	26	2
2	7	T	A	I	244.02	130.2754	0.537914	12	0
2	10	T	A	I	192.71	68.67708	0.368953	39	0
2	11	T	B	I	176.24	50.23063	0.285956	27	0
1	2	C	Bk	II	92.74347	32.69457	0.352527	2	16
1	5	C	W	II	75.13844	18.51458	0.246406	2	27
1	6	C	Bk	II	61.45135	23.66267	0.385063	9	15
2	8	C	D	II	117.63	37.43351	0.322207	2	10
2	9	C	B	II	167.81	47.26137	0.287027	6	14
2	12	C	C	II	177.8	44.12391	0.256107	6	3
1	1	T	Bk	II	67.02948	51.6317	0.770283	93	78
1	3	T	WB	II	127.1238	42.34195	0.333076	20	33
2	7	T	D	II	171.89	63.91347	0.370146	2	2
2	11	T	C	II	153.39	61.87697	0.416458	9	6
1	2	C	WB	III	92.78991	40.4573	0.43601	3	38
1	5	C	WB	III	83.53145	29.52632	0.353475	4	52
1	6	C	BW	III	81.57046	31.21868	0.38272	0	22
2	8	C	B	III	215.08	60.97987	0.286148	5	23
2	9	C	D	III	132.32	42.46776	0.325223	17	6
2	12	C	D	III	147.97	72.31998	0.499907	1	5
1	1	T	BW	III	150.6523	59.94845	0.397926	94	68
1	3	T	BW	III	61.35882	27.94163	0.455381	1	37
1	4	T	Bk	III	103.6633	29.26108	0.28227	0	9
1	4	T	WB	III	116.4378	43.67457	0.375089	4	15
2	7	T	B	III	163.88	51.1447	0.312326	0	6

2	11	T	A	III	124.13	59.52444	0.495388	2	16
1	2	C	BW	IV	139.4171	56.9135	0.408225	0	46
1	5	C	Bk	IV	58.23188	19.48906	0.33468	13	16
1	6	C	WB	IV	81.42092	44.04799	0.540991	3	52
2	8	C	C	IV	217.2	84.5423	0.399162	0	49
2	9	C	C	IV	128.45	46.17748	0.363003	1	7
2	12	C	A	IV	110.66	48.06669	0.43383	0	2
1	1	T	W	IV	80.63421	39.51805	0.49009	1	58
1	3	T	Bk	IV	144.1826	54.48063	0.377859	2	53
1	4	T	W	IV	38.17071	18.23386	0.477692	6	4
2	7	T	C	IV	170.15	43.56645	0.259908	0	6
2	10	T	B	IV	175.78	74.58245	0.71252	0	39
2	10	T	C	IV	130.30	63.91585	0.492792	0	0
2	10	T	D	IV	152.08	78.05612	0.519865	0	0
2	11	T	D	IV	169.53	54.33479	0.329606	1	11

Table 7: Raw data for experiments 1 and 2. A total of 12 tanks treated with either tryptophan enriched or control diets with individually recognized individuals, either by tags (black, white, black and white or white and black variations) or by individual dorsal fin markings (A, B, C or D were assigned to each fish). Logarithmic transformed monoamine concentrations for serotonin and 5-HIAA, arcsine transformed serotonergic activity ratios (5-HIAA/5-HT), total aggressive acts performed and total aggressive acts received are given for each individual along with their hierarchical rank level.

exp	tank	treat	indiv	rank	lser	lhiaa	ahiht	tagg	ragg
1	2	C	W	I	1.683841	1.430056	48.29963	121	5
1	5	C	BW	I	2.02577	1.61998	38.81189	84	3
1	6	C	W	I	1.919981	1.637687	46.26335	102	2
2	8	C	A	I	2.310842	2.028571	46.71961	77	0
2	9	C	A	I	2.163281	1.600098	32.02107	4	0
2	12	C	B	I	1.952608	1.821595	59.43229	4	1
1	1	T	WB	I	1.941487	1.684087	48.03326	122	64
1	3	T	W	I	2.022582	1.644994	40.3496	86	8
1	4	T	BW	I	1.696766	1.413886	46.22298	26	2
2	7	T	A	I	2.387425	2.114862	47.17437	12	0
2	10	T	A	I	2.284913	1.836812	37.40284	39	0
2	11	T	B	I	2.246115	1.700969	32.32688	27	0
1	2	C	Bk	II	1.967283	1.514476	36.42285	2	16
1	5	C	W	II	1.875862	1.267514	29.76166	2	27

1	6	C	Bk	II	1.788531	1.374064	38.3552	9	15
2	8	C	D	II	2.070518	1.573261	34.58533	2	10
2	9	C	B	II	2.224818	1.674506	32.3947	6	14
2	12	C	C	II	2.249932	1.644674	30.40245	6	3
1	1	T	Bk	II	1.826266	1.712916	61.36112	93	78
1	3	T	WB	II	2.104227	1.626771	35.24878	20	33
2	7	T	D	II	2.235251	1.805592	37.47361	2	2
2	11	T	C	II	2.185787	1.791529	40.19081	9	6
1	2	C	WB	III	1.967501	1.606997	41.32354	3	38
1	5	C	WB	III	1.92185	1.470209	36.47971	4	52
1	6	C	BW	III	1.911533	1.494415	38.21718	0	22
2	8	C	B	III	2.3326	1.785187	32.33906	5	23
2	9	C	D	III	2.121621	1.628059	34.76996	17	6
2	12	C	D	III	2.170174	1.859258	44.99469	1	5
1	1	T	BW	III	2.177976	1.777778	39.11018	94	68
1	3	T	BW	III	1.787877	1.446252	42.4401	1	37
1	4	T	Bk	III	2.015625	1.46629	32.09274	0	9
1	4	T	WB	III	2.066094	1.640229	37.76654	4	15
2	7	T	B	III	2.214526	1.708801	33.97706	0	6
2	11	T	A	III	2.093863	1.774695	44.73572	2	16
1	2	C	BW	IV	2.144316	1.755215	39.71168	0	46
1	5	C	Bk	IV	1.765161	1.289791	35.3462	13	16
1	6	C	WB	IV	1.910736	1.643926	47.35125	3	52
2	8	C	C	IV	2.33686	1.927074	39.18251	0	49
2	9	C	C	IV	2.108732	1.66443	37.04897	1	7
2	12	C	A	IV	2.043998	1.681844	41.19759	0	2
1	1	T	W	IV	1.906519	1.596796	44.43219	1	58
1	3	T	Bk	IV	2.158913	1.736242	37.93028	2	53
1	4	T	W	IV	1.58173	1.260879	43.72145	6	4
2	7	T	C	IV	2.230832	1.639152	30.65129	0	6
2	10	T	B	IV	2.244969	1.872637	57.57662	0	39
2	10	T	C	IV	2.11496	1.805609	44.58701	0	0
2	10	T	D	IV	2.182072	1.892407	46.1385	0	0
2	11	T	D	IV	2.229254	1.735078	35.03756	1	11

8. Appendix II

Table 8: Statistical data. Results from mixed linear effect models performed on variables used to assess differences in aggression and monoamine levels between TRP and control groups. Analysis was carried either on all groups pooled together or by hierarchical ranks (dominants, middle rank individuals or subordinates). Degrees of freedom, t values and p values are given (** represents a statistically significant value).

Variables analyzed	Groups	Degrees of freedom	*t* value	*p* value
Total performed aggression dependent on treatment	All groups	10	0.2720091	0.7911
Received aggression dependent on treatment	All groups	10	0.3882152	0.706
Total performed aggression and 5-HIAA/5-HT	All groups	35	2.396198	0.0220**
5-HIAA/5-HT dependent on received aggression	All groups	35	1.493805	0.1442
5-HIAA/5-HT dependent on treatment	All groups	10	0.900596	0.389
Serotonin dependent on treatment	All Groups	10	0.433312	0.674
5-HIAA dependent on treatment	All Groups	10	0.814643	0.4342
Total performed aggression dependent on treatment	Dominants	10	-0.497165	0.6298
Received aggression dependent on treatment	Dominants	10	1.005692	0.3383
Total performed aggression and 5-HIAA/5-HT	Dominants	10	0.4423887	0.6676
5-HIAA/5-HT dependent on received aggression	Dominants	10	0.614004	0.5529
5-HIAA/5-HT dependent on treatment	Dominants	10	-0.729656	0.4823
Total performed aggression dependent on treatment	Middle	9	0.980977	0.3007
Received aggression dependent on treatment	Middle	9	0.2626363	0.5587
Total performed aggression and 5-HIAA/5-HT	Middle	9	-0.4185191	0.6844
5-HIAA/5-HT dependent on received aggression	Middle	9	2.903247	0.0157**
5-HIAA/5-HT dependent on treatment	Middle	9	1.645734	0.1342
Total performed aggression dependent on treatment	Subordinates	10	-1.828732	0.0974
Received aggression dependent on treatment	Subordinates	10	-0.5667114	0.5834
Total performed aggression and 5-HIAA/5-HT	Subordinates	10	-0.484924	0.6382
5-HIAA/5-HT dependent on received aggression	Subordinates	10	2.492486	0.2429
5-HIAA/5-HT dependent on treatment	Subordinates	10	0.338839	0.7417

9. Appendix III

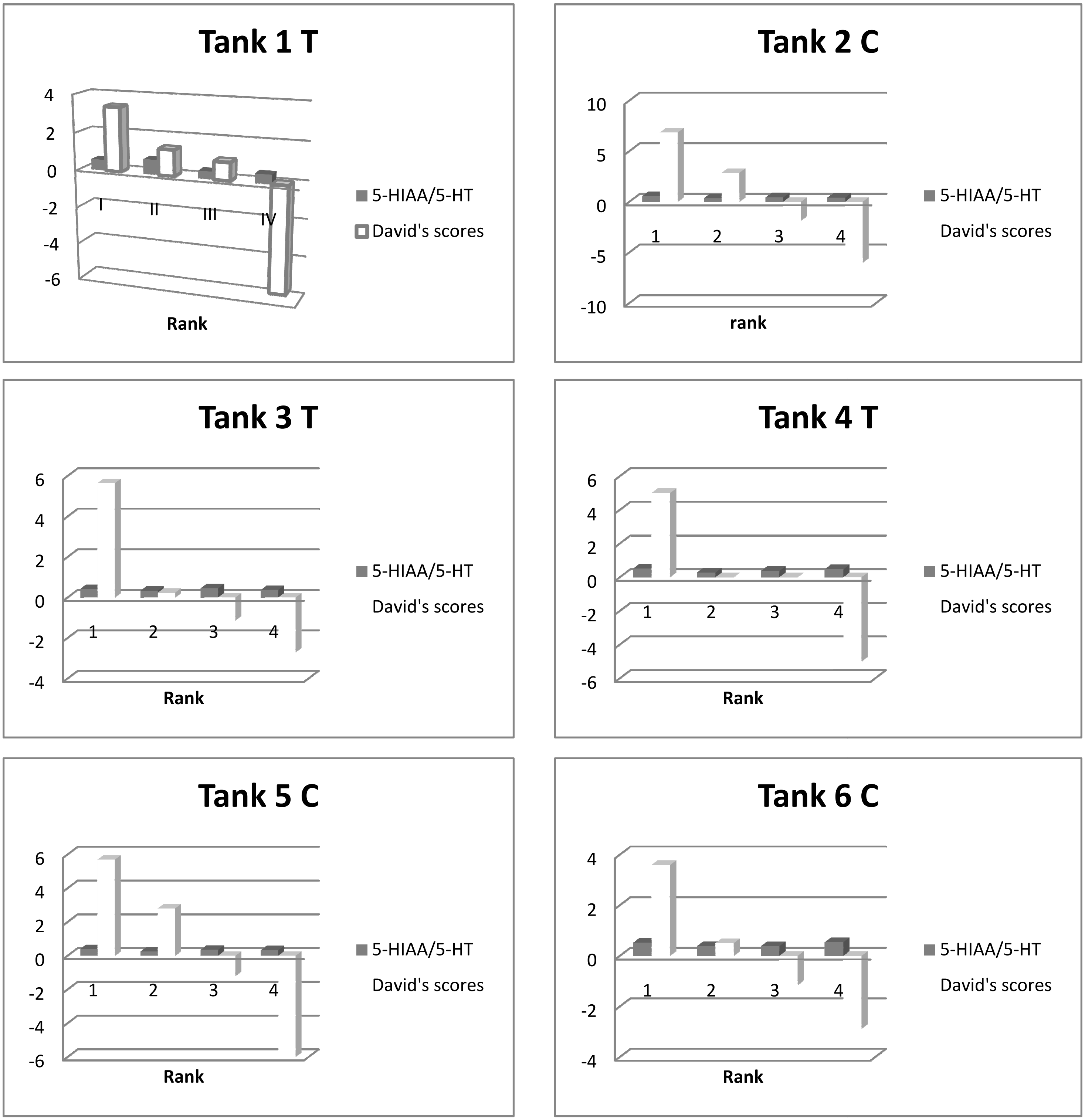

Figure 26: David's hierarchical ranks and the corresponding serotonergic activity ratios for each fish per aquarium

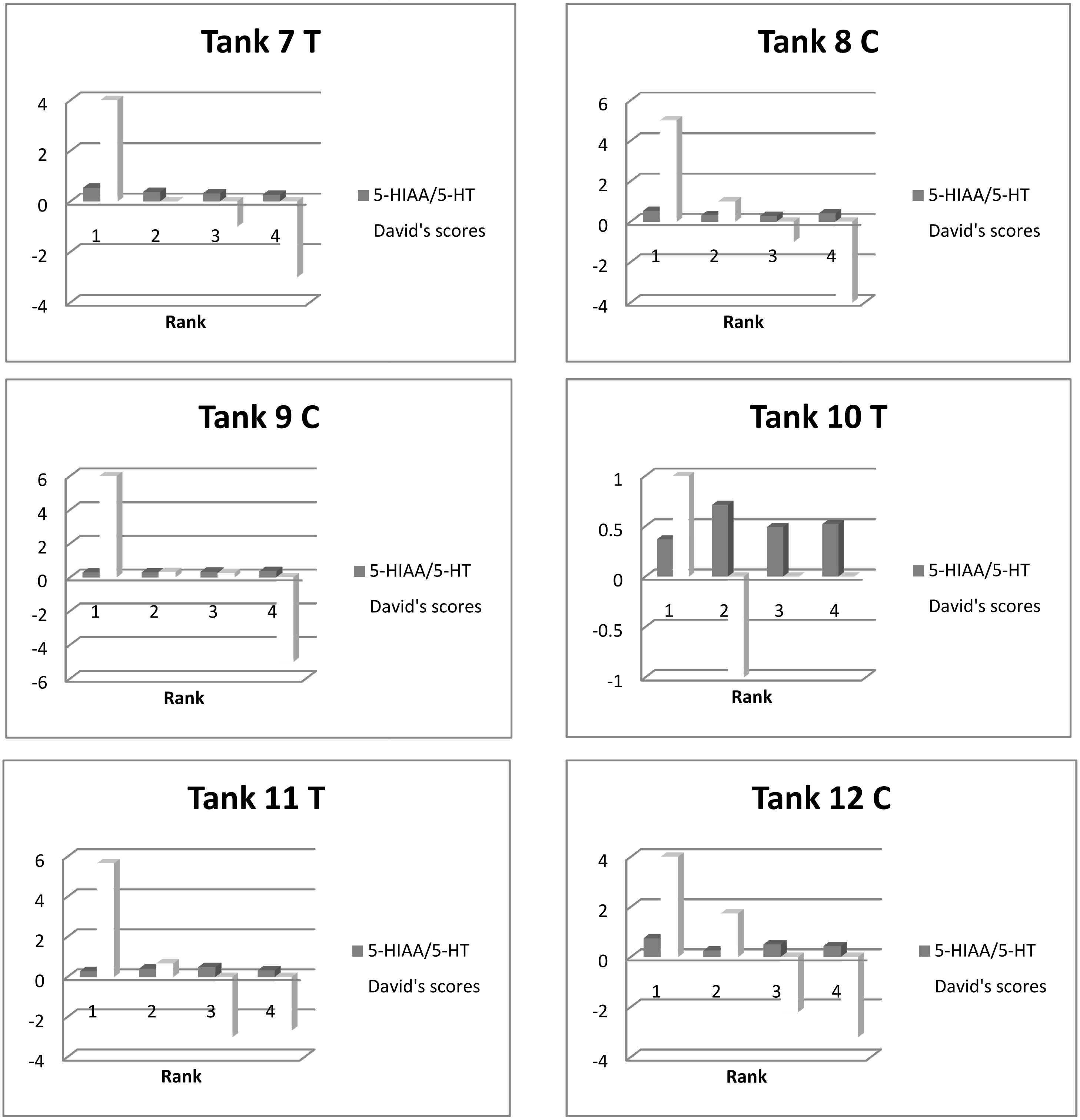

Figure 27: David’s hierarchical ranks and the corresponding serotonergic activity ratios for each fish per aquarium