# SUPPLEMENTING A COMMERCIAL DIET FOR ATLANTIC SALMON (*Salmo salar L.*) WITH ARGININE, GLUTAMATE OR TETRADECYLTHIOACETIC ACID (TTA). IMPACT ON PRODUCTION EFFICIENCY, SLAUGHTER PARAMETERS AND FLESH QUALITY

Thesis submitted for the degree of Master of Science

by

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May, 2010

# Acknowledgements

This thesis was mainly connected with the project "Integrated and dynamic production of farmed salmon in sea", financed by the Norwegian research Council (NRC, 1799481/I30), and managed by Prof. K-A. Rørvik at Nofima Marin AS. Additionally, some collaboration was also to other projects financed by the Norwegian Research Council and the Fishery and Aquaculture Industry Research Fund, managed by Dr. T. Mørkøre, Nofima Marin AS (NRC/FHF 190479/S40, FHF 900339, FHF 900338). The Fishery and Aquaculture Industry Research Fund also provided direct financial support to the present master thesis.

I would like to thank a number of people who lent their support, expertise, patience, and guidance to me during this study.

Special appreciation is extended to my supervisors, Dr. Turid Mørkøre and Professor Kjell-Arne Rørvik for contributing many hours of guidance, excellent suggestions, kindness, and encouragement throughout the project.

Special thanks to Muhammad Saqib Latif, my classmate, for helping me during this experiment. It was nice working with you, I appreciate you for everything.

Thanks to technical assistance provided by staff at Nofima Marin's research station at Averøy, and the laboratory at Ås, especially Inger Ø. Kristiansen.

Thanks to Skretting As Stavanger for manufacturing the basal diet of this research project.

Finally, I would like to thank all my friends past and present in Ås, especially Sohrab Afsardoost.

I would like to dedicate this thesis to my family: my parents and my brothers who were supporting me during these two years and giving me this great chance.

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# Abstract

The present study was carried out to evaluate the effects of supplementing a commercial diets with 1.5% L-arginine, 1.5% L-glutamate or 0.25% tetradecylthioacetic acid (TTA) on feed intake, feed utilization and growth parameters of Atlantic salmon (*Salmo salar* L.) during the critical periods after sea transfer with regard to seawater adaptation and onwards, from April to September. In the case of TTA, fish were fed until they reached a weight gain of 0.2% of the initial body weight, thereafter the TTA diet was replaced by the control diet. The arginine and glutamate diets were fed throughout the whole experimental period. At the experimental termination, effects of dietary treatments on rigor development, post-mortem energy metabolism and selected quality parameters of fish exposed to normal slaughter handling and stress exposure (crowding stress for 16 hours) were also studied.

6000 smolts of Atlantic salmon with an average weight of 105 grams were transferred to 12 net pens in sea water (500 smolts per each net pen). Three net pens were assigned for each of the four dietary treatments. Fish were sampled initially and once a month (five samplings).

No significant differences in body weight, thermal growth coefficient (TGC), specific growth rate (SGR), feed conversion ratio (FCR) or specific feed ratio (SFR) were observed between dietary treatments, but numerically the performance was the best for the salmon fed the arginine supplemented feed, whereas the numerically lowest performance was observed for the salmon fed the TTA diet. The hepatosomatic index (HSI) of the TTA group was significantly higher compared with the control and arginine groups in June (feeding TTA diet was stopped at this time), whereas in September, the TTA group had the numerically lowest HSI (significant from the glutamate group). Cardio somatic index (CSI) did not differ significantly among the dietary treatments, but the condition factor was lowest of the TTA group, whereas the arginine and glutamate diets tended to enhance muscularity. Pigmentation of fillets did not show any significant differences between dietary treatments, but the fat content of fish fed TTA was significantly lower than other dietary treatments.

Developments in the following parameters were recorded during storage of pre-rigor fillets of non-crowded and crowded salmon in September: rigor contraction, fillet color, gaping, muscle pH, and adenosine triphosphate (ATP). Additionally, cathepsin B, L and B+L activity were analyszd immediately after slaughter. The TTA and arginine groups seemed to have the slowest contraction rate of the non-crowded fish, and after 72 hours storage, the TTA diet was significantly lower compared to the control group. However, in crowded fish, the contraction of the TTA group was significantly highest initially, whereas the numerically lowest contraction was observed for the arginine group. After 12 hours, fillet color of fish fed glutamate was significantly higher than of fish fed control diet in the non-crowded group, but in crowded fish the color of the glutamate group tended to be palest. Muscle pH showed significant variations between dietary treatments for non-crowded fish after 1, 12, 24 and 72 hours storage, where the muscle pH was significantly lower of fish fed TTA diet after 1 and 12 hours and of fish fed

arginine diet after 24 and 72 hours compared to the control diet. In contrast, muscle pH of the TTA group exposed to crowding was significantly highest together with the glutamate diet after 12 hours storage compared to the control group. For crowded fish, cathepsin B activity of the arginine, glutamate and TTA group was significantly lower than of the control group. No significant impact of dietary treatments were observed on fillet gaping or ATP content.

Crowding stress prior to slaughtering significantly accelerated rigor contraction, muscle pH degradation, and ATP depletion. Furthermore, the gaping score was higher in crowded fish, but fillet color and cathepsins activity did not differ significantly.

# Introduction

Increased food production is required because of the world's growing human population. In recent decades, aquaculture has also shown great progress. Fish consumption is considered to be healthier than meat and so its use is being promoted. From a production of below 1 million tonnes in the early 1950s, production in 2006 was reported to have increased to 66.7 million tonnes (51.7 million tones excluding aquatic plants) with a value of US $ 85.9 billion (US $ 78.8 billion excluding aquatic plants) (FAO2008). Increasing consumer demand for aquaculture products is putting more pressure on both producers and exporters. Between the fish species, Atlantic salmon is now firmly established as a farmed species of major commercial importance in Europe especially in the northern part like Norway, Scotland, and the Faeroe Islands. In these countries, the combination of suitable environmental conditions and excellent sites has led to the establishment of a successful industry.

Good nutrition in animal production is essential for an economically sound production of healthy and high quality product for human consumption. In intensive fish farming, 40-50% of the production cost is related to feed, hence the industry and researchers are continuously putting efforts in defining solutions that can reduce the feeding costs. Although the aim of a large proportion of the research achievements related to aquaculture is focusing on optimizing dietary composition for farmed fish, there is only limited and fragmented information available with regard to requirements of essential nutrients for Atlantic salmon.

Cost efficient production of salmon requires that unless attention is paid to the amount of feed consumed by the fish and the amount the feed that is converted into tissue that is edible for humans. Basic understanding of growth and nutrient utilization by the fish are therefore vital areas that should be focused. Amino acids and fatty acids are very important nutrients to be considered when a diet is formulated for fish especially for carnivorous species that have limited ability to digest carbohydrates. Dietary amino acids are required for two purposes, firstly for growth, which mainly consists of protein deposition, and secondly for a number of processes that are described as maintenance. Fatty acids have also different functions where providing energy and serving as structural components are among the most important (Lim & Webster 2002). A trend in salmon feeds has been to use higher levels of lipids, and also to supplementing the diets with increasing amounts of plant ingredients. Reports on nutrient requirements in salmon diets are to a large extent based on feed formulation where the dietary composition was quite different from today. Additionally the growth of the fish was significantly slower.

Until recently, salmon farming was production driven, the fish were exported in the gutted state and the industry had only a limited focus on marked preferences. Today, filleting salmon immediately after slaughtering has accelerated, and the farming industry has become more and more marked driven. Filleting fish in the pre-rigor state requires that the fish are not entering

rigor mortis too soon after harvesting, as it is not recommended to handle fish when they are stiff in rigor. Therefore, it is important to ensure a long pre rigor period in order to enable filleting before the fish enters rigor mortis. It is well known that slaughter handling influences development of rigor mortis, but there is very little information on the impact of dietary composition and development of rigor mortis.

The aim of the present study was to determine the effects of tetradecylthioacetic acid (TTA), arginine and glutamate in separates diets on production efficiency of farmed Atlantic salmon after sea transfer from April to September. Additionally influences of these components on rigor development and post-mortem energy metabolism of fish fillet during normal handling and stress exposure was studied.

# Background

## Production efficiency and product quality

Effective production of salmonids has been obtained through the establishment of breeding programmes, optimization of feed and improved disease treatment. Fish nutrition has improved significantly in recent years with the development of new, balanced commercial diets that promote optimal fish growth and health. The focus on optimizing dietary composition has resulted in reduced production time and less mortality, but negative aspects like low slaughter yield and high fat content are of potential improvement in modern salmonid aquaculture (Gjedrem 1997). There is also an obvious interest for effective feed conversion and high protein utilization for fish farmers because feed constitutes a large fraction of total costs in salmonid aquaculture.

An additional strategy that enhances salmonid value is improvement of the quality. Many studies have considered the effect of feed composition on quality traits in salmonids (Wathne 1995; Bjerkeng et al. 1997; Jobling et al. 1998). Among important quality parameters are the sensory, nutritional, technological, and hygienic properties of fish that are affected by factors such as: feed ingredients, rearing environment, slaughtering techniques and stress in addition to post mortem handling. Slaughtering and handling are major procedures that can cause physiological changes in fish. These changes may lead to increased muscle activity, changes in acid base balance and they may increase blood plasma ion concentrations and decrease water content of tissue of fish in sea water (Mazeaud et al. 1977; Mazeaud & Mazeaud 1981).

## Protein and amino acids

Protein is an essential component of fish feed, required for growth, normal development, reproduction, health and survival of fish. In the fish body, proteins are the primary elements of structural and protective tissues (bones, ligaments, scales, and skin), soft tissues (organs, muscle) and body fluids. They contain carbon (50%), nitrogen (16%), oxygen (21.5%), hydrogen (6.5%) and some of the proteins also contain sulfur, phosphorus and iron. The basic structural component of proteins consists of amino acids. Amino acids provide essential nitrogen for the synthesis of protein and other biological molecules. There are several amino acids in nature whereof ten are considered as essential; i.e. they cannot be synthesized by the fish and must be provided through the diet. The essential amino acids are: methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine and phenylalanine. Optimal synthesis occurs when all the essential amino acids are present in the diet, whereas essential amino acid deficiency limits protein synthesis (Lim & Webster 2002). Amino acids are synthesized from glutamate, which is formed by amination of α-ketoglutarate (Figure 1):

α-ketoglutarate + $NH_4^+$↔ glutamate. Thereafter, alanine and aspartate are formed by transamination of glutamate. All of the remaining amino acids are then constructed from glutamate or aspartate, by transamination of these two amino acids with one α-keto acid.

Because protein is an expensive part of fish feeds, it is important to know the protein requirements of each fish species. Additionally fish need a diet containing either none essential amino acids or the precursors for their synthesis of required amino acids. Protein requirements depend on the size of fish, rearing environment, water temperature, physiological status, genetic composition and dietary energy level. Protein requirements are higher for smaller fish and it usually decreases as fish grow. Protein is consumed for growth of fish if sufficient levels of fats and carbohydrates are present in the diet, otherwise protein may be used as energy source. The requirements are usually lower for herbivorous and omnivorous fish than carnivorous species (Craig & Helfrich 2009).

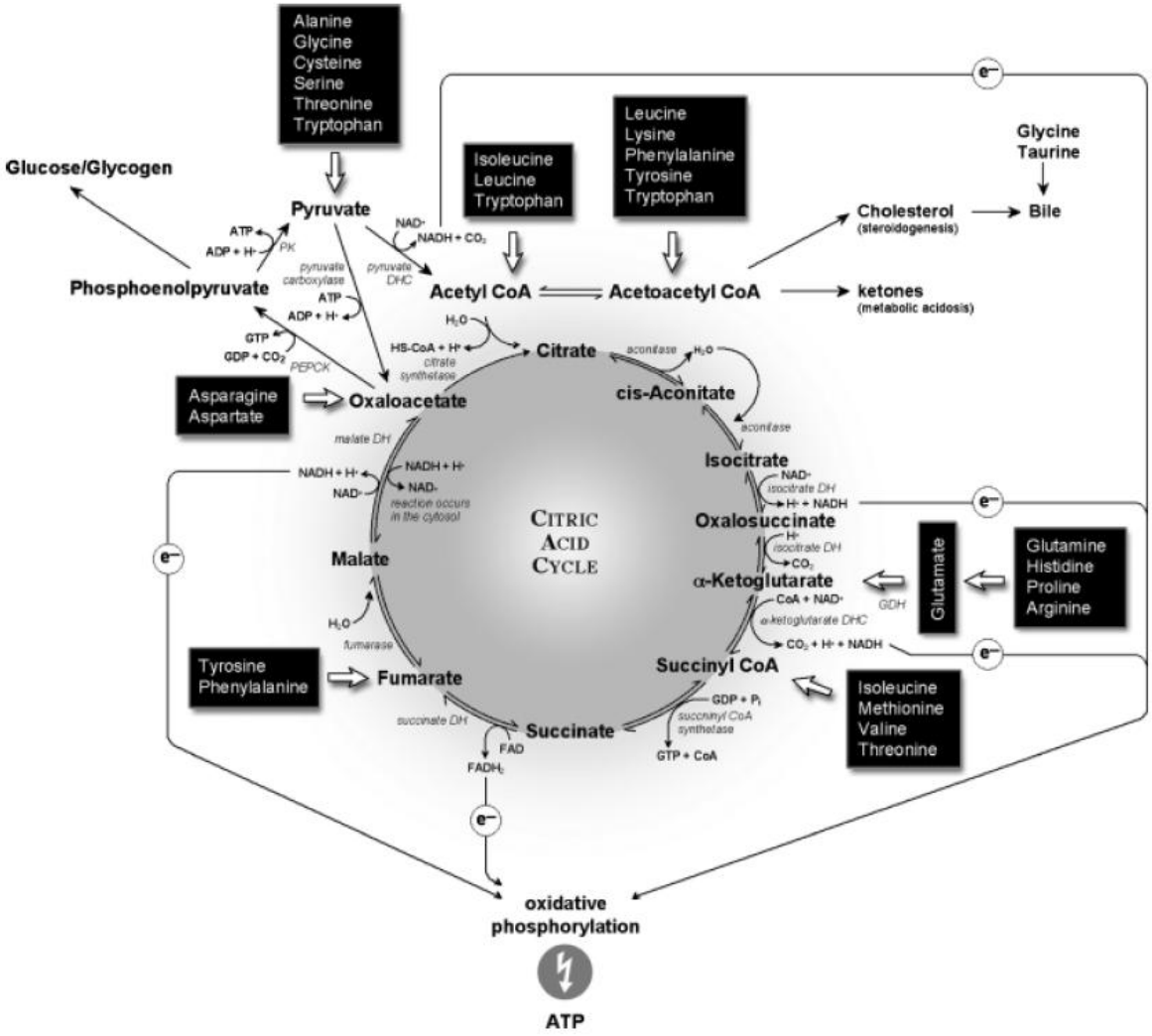


**Figure 1**- Catabolic entry points of amino acid carbon skeletons in the tri-carboxylic acid cycle, showing primary exit points for gluconeogenic, cholesterogenic and ketogenic pathways (scheme: (Finn & Fyhn 2010)).

## Glutamate

The amino acid glutamate (Figure 2) is considered to be the main mediator of excitatory signals in the mammalian central nervous system and is probably involved in most aspects of normal brain function such as: cognition, memory and learning (Fonnum 1984; Kuhar 1984; Collingridge and Lester. 1989). Glutamate also plays major roles in the development of the central nervous system, including synapse induction and elimination, as well as cell migration, differentiation and death. Furthermore, glutamate plays a signaling role in peripheral organs and tissues as well as in endocrine cells (Moriyama et al. 2000).

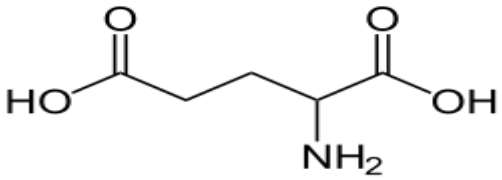


**Figure 2-** The chemical structure of glutamate (Wikipedia 2010a).

Glutamate is an important molecule in cellular metabolism. In the body dietary proteins are broken into amino acids. Transamination is the major process of degradation of amino acids where the amino group of an amino acid is transferred to α-ketoacid, commonly α-ketoglutarate, which after transamination of α-ketoglutarate, gives glutamate.

Approximately 20% of the total amino acids in plant and animal proteins are glutamate and glutamine. These amino acids can be depredated in the gut of fish as well as in terrestrial mammals (Wu & Morris 1998). The substrate for glutamine synthesis is glutamate by ATP-dependent glutamine synthetase, while to generate glutamate, glutamine is hydrolyzed by phosphate-dependent glutaminase (Anderson et al. 2002).

## Arginine

Arginine is an indispensible amino acid, which means that it cannot be synthesized in the body and should be provided in the diet. It is essential for optimal growth of young fish (Wilson 1989) and terrestrial animals (Visek 1984). The chemical structure of arginine is shown in Figure 3.

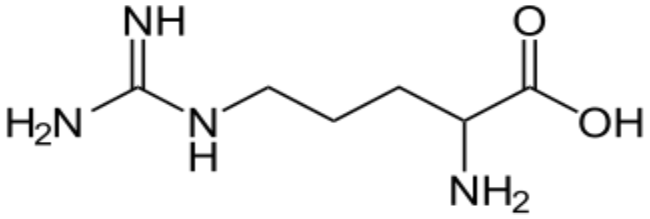


**Figure 3-** The chemical structure of arginine (Wikipedia 2010b).

Arginine is involved in many metabolic pathways such as: protein synthesis and production of urea, nitric oxide (NO), polyamines, proline, glutamate, creatine and agmatine in terrestrial animals (Wu & Morris 1998). Arginine has a very important role in regulating endocrine and reproductive functions and extra-endocrine signaling pathways (Jobgen et al. 2006; Yao et al. 2008). Additionally arginine can activate the release of numerous hormones like insulin, growth hormone and glucagon (Mommsen et al. 2001). Growth and health promoting effects of dietary supplementation of arginine has been reported for some fish species. For example, dietary arginine level has important effect on survival of channel cat fish in response to challenge with *Edwardsiella ictaluri* (Buentello & Gatlin 2001).

Requirements of dietary essential amino acids for animals are determined by different response criteria, whereof growth and feed efficiency are considered as the most important criteria (Santiago & Lovell 1988; Griffin et al. 1994). Additionally, serum amino acid concentrations have been used to clarify certain requirements, but concentrations of essential amino acids in serum of fish are not always responsive to varying concentrations in the diet. Arginine has been reported in this case (Robinson et al. 1981; Kaushik et al. 1988), as urea in plasma or serum has been more responsive than free arginine concentrations (Cho et al. 1992; Tibaldi et al. 1994). Free arginine concentration in muscle and liver, oxidation of L-[$^{14}$C] arginine, urinary excretion of arginine catabolites are other criteria that can be used as guide lines to obtain essential amino acid requirements.

Arginine requirement of many fresh water fish species has been determined, and it varies both within and between fish species. These variations reported in the arginine requirement may be because of variations in the basal diet composition, size physiological status and age of fish, genetic differences, feeding rate and culture conditions that can affect overall growth rate. As an example, the requirements vary from 3.3 g (Tiews & Halver 1979) to 5.4-5.9 g (Ketola 1983) of dietary protein for rainbow trout. Some experiments were also performed to establish the arginine requirement of post smolt Atlantic salmon, although the estimates of arginine requirements differ by more than 25%. Hence, Lall et al. (1994) reported an arginine requirement of 1.6% of dry matter (4.1% of dietary protein) for approximately 100 g Atlantic salmon smolts farmed in sea water, whereas results from an experiment performed with 390g salmon by Berge et al. (1997) indicated an arginine requirement of 5-5.1% of dietary protein based on growth. . In the latter study, the salmon were graded levels of dietary arginine from 2.7% to 6.8% of dietary protein.

## Lipids and fatty acids

Lipids are high energy nutrients that can be partially substituted instead of protein in the fish diet. In this way, protein can be spared for synthesis of new tissue (Pickering & Black 1998; Wilson 1989). The energy density of lipids (9.4 Kcal of $GE^{-1}$) is approximately two times higher than of proteins (5.6 Kcal of $GE^{-1}$) and carbohydrates (4.1 Kcal of $GE^{-1}$). Lipids have several

important roles in the body, besides from being a source of energy, for example they provide the body with essential fatty acids, serve as structural components and they also have several vital regulatory functions(intracellular signaling, local hormonal regulation etc.) (Christie 2010).

Lipids include fatty acid and triacylglycerols. Based on the number of carbon and double bonds, a fatty acid can be saturated (SFA, no double bonds), mono unsaturated (MUFA, one double bond), polyunsaturated (PUFA, >2 double bonds), or highly unsaturated (HUFA, > 4 double bonds). Marine fish oils have generally high amount of omega 3 HUFA and they are considered as the best source of lipid in fish diets (EPA or eicosapentaenoic acid, 20:5n-3 and DHA or docosahexaenoic acid, 22:6n-3 are two major essential fatty acid of this group) (Lim & Webster 2002).

Fish needs dietary lipids to meet essential fatty acid requirements including especially EPA and DHA, to allow normal growth and development of cells and tissues. However, fatty acid requirement differs among fish species. It is clear that cold water fish species need highly unsaturated fatty acids (HUFA) of the n-3 class, whereas warm water fish species require HUFA from either n-3 or n-6 classes or a mixture of them. The major signs of deficiency of essential fatty acids are reduction of growth, increased mortality, decrease in the essential fatty acids in blood and liver phospholipids (Ruyter et al. 2000).

## Tetradecylthioacetic acid (TTA)

Tetradecylthioacetic acid (TTA) is a saturated fatty acid, known as a 3-thia fatty acid that is structurally modified by inserting a sulfur atom at a specific position in the carbon backbone. Figure 4, shows the chemical structure of tetradecylthioacetic acid (TTA).


**Figure 4**- The chemical structure of tetradecylthioacetic acid (TTA) (Kennedy 2007).

Over the past several years, TTA has shown many beneficial properties where its effect on mitochondrial growth and fatty acid oxidation are the most important ones.

Oxidation of fatty acids occurs in the mitochondria of the cell through a process known as beta oxidation. TTA is not processed through beta oxidation due to the position of the sulfur in the carbon chain, but it stimulates the beta oxidation of other fatty acids (Berge & Hvattum 1994). TTA is metabolized in mammals via ω-hydroxylation in the endoplasmic reticulum (Berge et al. 1989; Hvattum et al. 1991; reviewed by Skrede et al. 1997; Berge et al. 2002). Furthermore,

TTA is involved in lipid transport and utilization (Berge et al. 2005). This suggests that TTA may promote fatty acid usage and hence, greater utilization of energy for growth and less accumulation of lipids in the fish body.

It has also been shown that TTA affects mitochondrial growth and gene expression of some important enzymes involved in fatty acid oxidation (Totland et al. 2000). TTA has a significant effect on reducing blood lipids (56% reduction in very low-density lipoprotein VLDL – triacylglycerol) (Asiedu et al. 1996), an effect that might be related to the increase in fatty acid oxidation and increase in gene expression for low density lipoprotein (LDL), receptors that work on removing of LDL cholesterol from circulation (Fredriksen et al. 2004).

TTA is an effective ligand for all three peroxisome proliferators-activated receptors (PPAR) (α, β, γ). The activation of these receptors by TTA is related to the positive effects on gene activation associated with enzymes involved in fatty acid transport and oxidation (Larsen et al. 2005).

Different studies showed that diets containing TTA can be beneficial in aquaculture, but the effects seem to differ between fish species. Studies with Atlantic cod (125-300 grams) and rainbow trout (approximately 800 grams) (Kennedy et al. 2007a; Kennedy et al. 2007b) showed that a diet containing 0.5% TTA had no significant effect on final body weight, growth rate, feed conversion ratio or fat content of these fish species. However, in Atlantic cod, where the liver is the main organ for deposition of excess energy, fish fed TTA had significantly lower hepatosomatic index (HSI). In the cod, TTA also increased the activity of CPT-1 and Acyl Coenzyme-A Oxidase (ACO) in the liver and decreased the ACO activity in white muscle. TTA has been shown to increase the capacity for hepatic proxisomal β-oxidation. In rainbow trout CTP-1 and ACO activity increased in liver and red muscle, and expression of CPT-1 was also increased in white muscle. In a study with Atlantic salmon (86 – 250 grams) (Moya-Falcon et al. 2004) that a basal diet supplemented with either 0%, 0.3% or 0.6% of TTA, many effects of this bioactive fatty acid was considered, fish fed dietary TTAs had significantly lower body weight and SGR compared to control diet, fish fed 0.6% TTA showed the significantly highest HSI and lowest fat content. In this study higher mitochondrial β-oxidation capacity was found in the liver of fish fed TTAs diet and the percentage of n-3 fatty acids especially 22:6n-3 increased, whereas the percentage of saturated fatty acids 14:0 and 16:0 in the fractions of the gills and heart decreased.

Recent studies (Alne 2009) illustrate that TTA can also be beneficial for Atlantic salmon, especially after transferring salmon to sea water, when a reduction in growth performance, condition factor, muscle fat are generally observed. Adding TTA to salmon diets has also shown reduced susceptibility to diseases, i.e. IPN (infectious pancreas necrosis) in $1^+$ salmon, and HSMI (heart and skeletal muscle inflammation) in $0^+$ salmon. The main difference between $1^+$ and $0^+$ smolt is the time of sea transfer. $1^+$ smolt is transferred to sea in spring more than one year after hatching while $0^+$ smolt is transferred to sea in autumn less than a year after hatching. Alne

(2009) also reported that TTA reduced sexual maturation in male post smolt $1^+$ Atlantic salmon. Mortality due to IPN in $1^+$ salmon was decreased from 7.8% to 2.3% by dietary supplementation of 0.5% TTA and reduction in mortality of $0^+$ salmon due to HSMI from 4.7% to 2.5% was also observed by 0.25% TTA in fish diet. Gene expression associated with oxidation of lipid was higher in cardiac ventricles in salmon fed diets supplemented with TTA and periodically reduced levels of plasma urea and increased cardio somatic index and growth were observed.

## Rigor mortis and energy metabolism

Rigor mortis is a notable change in muscles, happening soon after death, when the supply of oxygen to the muscles terminates and blood circulatory system fails. At this time ATP can only be generated by breakdown of glycogen. By degradation of glycogen, lactic acid accumulates coinciding with an acidification of the muscle. As the pH falls, the muscle proteins tend to denature and their ability to retain water declines. Rigor mortis occurs when the ATP level falls and cross bridge cycling between actin and myosin in myofibrils stops and permanent actin and myosin linkages (actomyosin) are created (Pate & Brokaw. 1980). A schematic overview of skeletal muscle contraction is given in Figure 5.

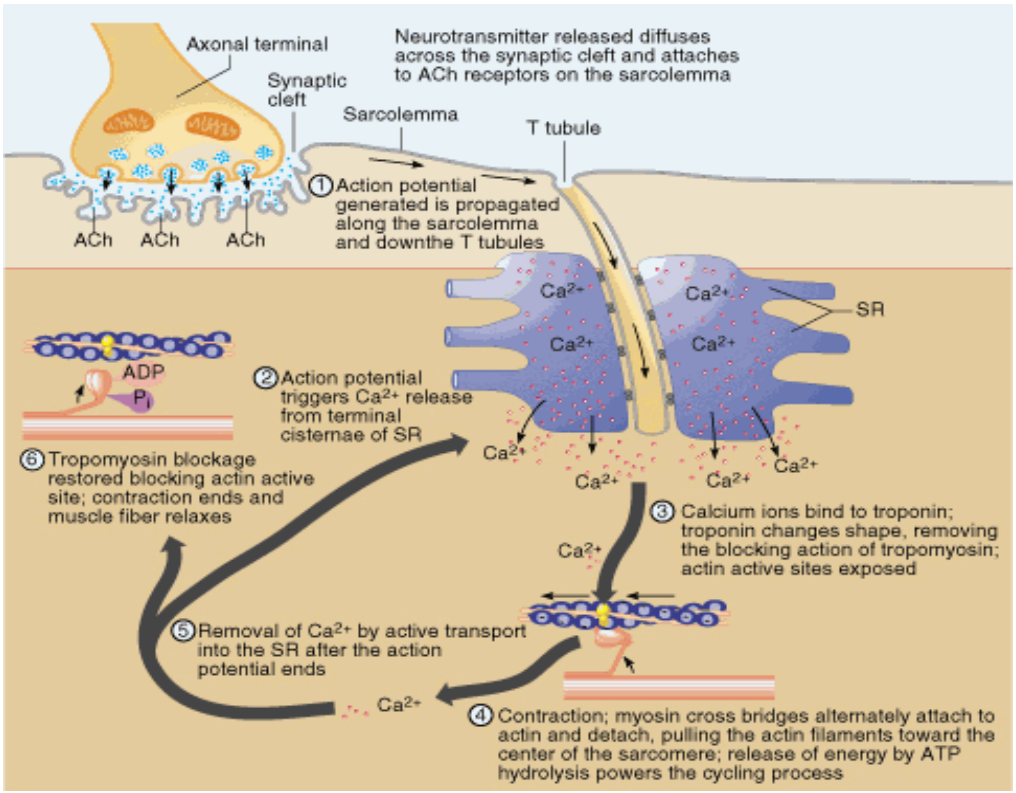


**Figure 5**- A review skeletal muscle contraction (scheme: Cummings 2001).

The initiation of rigor concentration will depend on parameters affecting the level of glycogen, creatine phosphate and rate of muscle metabolism. Stress and high muscle activity during anesthesia and handling of fish can reduce the time to onset of rigor mortis that is provoked by depletion of glycogen and ATP in muscle cells and a rapid drop in muscle pH (Erikson et al. 1997).

Many studies have reported effects of stress on the rate of onset of rigor in fish (Figure 6). For example depletion of muscle ATP and onset of rigor were delayed in un-stressed compared with stressed snapper (*Pagrus auratus*) (Lowe et al. 1993). In exercised Chinook salmon white muscle, the onset of rigor was considered to be quicker than in unexercised fish (Jerrett & Holland 1998). Sigholt et al. (1997) compared muscle metabolite levels in unstressed and stressed Atlantic salmon, and concluded that stressed fish had lower muscle pH, phosphocreatine, ATP level and more rapid onset of rigor. Finally, in another study with Atlantic salmon, the rigor development, post-mortem energy metabolism and quality variations were analyzed during 72 hours cold storage (Morkore et al. 2008). In line the previous studies, the authors reported that pre slaughter stress accelerated rigor development, accelerated lactate formation through post-mortem glycolysis and raised the breakdown of ATP and CP. However, the effect of stress was moderated in fish starved for 35 days prior to harvesting.

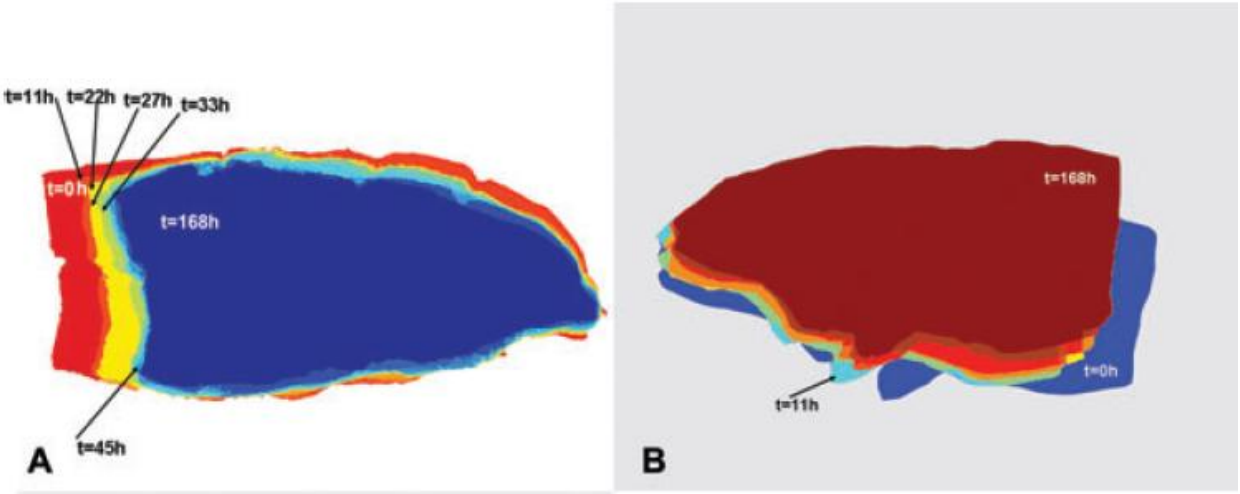


**Figure 6** - Visualization of changes of geometrical features of (A) un-stressed and (B) stressed salmon fillets during rigor at ice storage for seven days. The fillet marked t = 0 h represents initial size and shape measured immediately after slaughter (day 0), and the fillet marked t = 168 h denotes the size and shape of the same fillet at day seven (Scheme: Misimi et al. 2008).

Some studies have evaluated the effect of diet on post-mortem muscle metabolism of fish and other animals. Suontama et al. (2006) evaluated the effect of using three different crustacean meals (*Tysanoessa inermis, Euphausia superb, Themisto libellula)* that were replaced partially instead of fish meal as protein source for Atlantic salmon. The authors found only minor effects

on the flesh quality recorded both by technical and sensory methods. Post-mortem muscle pH was significantly lower in fish fed crustacean diets compared with fish fed fish meal, and increasing the level of non-fish meal protein from *Tysanoessa inermis* significantly reduced the rigor contraction of salmon. In another study with Atlantic cod (Morkore 2006), rigor contraction and quality parameters were analyzed after storage of fillets at 6°C for 48 hours. Fish were fed diets containing 100% fish oil or 60% fish oil and 40% soybean oil. The contraction rate was faster for the fish fed diets with soybean inclusion, but after 48 hours storage the contraction was 21% of the initial fillet length for both fish groups. The ATP content after 1 hour post-mortem was lower in the fish fed soybean oil, but after 16 hours, the ATP content was similar for both fish groups. Muscle pH of fish fed soybean oil was significantly lower than of fish fed fish oil after 1, 8, and 12 hours post mortem.

## Flesh quality parameters

Pre slaughter handling stress also affects fillet quality in other ways. Many studies showed that stress accelerates fish fillet softening, increases gaping and changes the fillet color (Erikson & Misimi 2008; Kiessling et al. 2004; Morzel et al. 2003; Morkore et al. 2008; Roth et al. 2006; Skjervold et al. 2001; Stien et al. 2005) Mammalian and fish post-mortem muscle softening can be associated with cathepsins (Bahuaud et al. 2008; Cheret et al. 2007; Godiksen et al. 2009; Taylor et al. 1995; Yamashita & Konagaya, 1990; Yamashita & Konagaya, 1991). Cathepsins are located in the lysosomes and they are released into cytoplasm and intra cellular spaces after lysosomal disruption due to pH drop (Duston 1983), and they are responsible for myofibrillar and connective tissue degradation (Eggen & Ekholt 1995; Ladrat et al. 2003; Sato et al. 1997; Yamashita, Michiaki & Konagaya, Shiro 1990). Additionally direct mechanical stress of the muscle fibrils or connective tissue, causing the release of proteases, may contribute to the acceleration of muscle structure degradation (Roth et al. 2006). As an example, 24 hours crowding stress of salmon gave significantly lower muscle pH, higher muscle cathepsin L and B gene expression and total activity of cathepsin B tended to increase (Bahuaud et al. 2009). In the same study, a significant correlation was found between muscle pH and cathepsin B+L activity. In another study by the same research group, the influence of dietary n-6 and n-3 fatty acids on lysosomal cathepsins B and L activity was investigated (Bahuaud et al. 2008). In this experiment, fish were fed one of four diets containing 23% crude lipids, with fish oil (FO), rapeseed oil (RO) by low levels of EPA + DHA (10% of total FAs), eicosapentaenoic acid (EPA) enriched-oil (>50% of total FAs) or docosahexaenoic acid (DHA) enriched-oil (>50% of total FAs). The results showed that cathepsin B and cathepsin L total activities in the muscle were lower in the EPA and DHA groups at 0 hour post-mortem and that dietary lipids influenced the level of lysosomal degrading enzyme activity of cathepsin B and cathepsin-L in addition to relative gene expression of cathepsin B.

Gaping is the phenomenon where connective tissues of fish fillets are unable to hold the muscle fibers together (Lovety et al. 1988), and represents one of the major causes of downgrading of Atlantic salmon fillets. Although basal biological mechanisms of gaping are not completely understood, it is considered to be a post mortem phenomenon (Kestin & Warriss 2001). As acute stress can accelerate post-mortem metabolism (Erikson 2001), it may possibly increase the gaping score of fish fillet (Love 1988; Kestin & Warriss 2001) as proposed by (Robb et al. 1999; Einen et al. 2001; Kiessling et al. 2004; Roth at al. 2005). However, little is known about the interaction between dietary composition and susceptibility of salmon fillets to lose tissue organization upon pre-slaughter handling stress.

Fillet color is one of the important product properties of salmon fillets and it depends on astaxanthin and/ or cantaxanthin content of flesh that is related to feed composition and feeding regimes (Kestin & Warriss 2001). Fillet composition is another factor that affects the coloration where the color impression becomes paler in fillet with high fat content (Christiansen et al. 1995). Furthermore, it has also been shown that the fillet color depends on pre-mortem handling stress (Kiessling et al. 2004; Stien et al. 2005), and these variations in color with slaughter is because of changes in muscle structure (Robb et al. 2000), that are also associated with lowered liquid holding capacity (Ofstad et al. 1993). It is reported that fish exposed to high muscle activity prior to slaughter obtained significantly lower color score (corresponding to about one Roche color score unit) compared to un-stressed fish 24 hours post-mortem (Erikson & Misimi 2008).

# Materials and methods

## Fish and experimental design

A feeding trial was carried out at Nofima Marin research station Averoy, Norway, over a period of five months from 15th of April to 15th of September 2009.

The fish used were 6000 smolts of Atlantic salmon (***Salmo salar***, from Salmar ASA) with an average weight of 105 grams. The fish were transferred to 12 net pens of 125 $m^3$ (5m length * 5m width * 5m depth) in sea water April 15th (500 smolt per each net pen). Three net pens were used for each of four dietary treatments.

During the experimental period of five months, five samplings were done, as shown on Table 1.

**Table 1** - Sampling times during the experiment from April to September

| Sampling number | Week 2009 | Dates |
|---|---|---|
| S0 | 16 | April 15th |
| S1 | 22 | May 26-27th |
| S2 | 26 | June 23-24th |
| S3 | 31 | July 29th |
| S4 | 38 | Sep 15-17th |

Sampling S0 was performed in fresh water before transferring the fish to the net pens in sea water. For samplings S1, S2, S3 and S4, which were done in the sea water, the following parameters were recorded: body weight, fork length, liver size, heart size, pigment and fat content of 10 fish per net pen. In addition all fish within each net pen were weighted in order to calculate growth rate and feed utilization within each period. In the last sampling (S4) , fish were exposed to different pre-slaughter handling; normal handling, n=6 per net pen and crowding stress, n = 3 per net pen. At this time point, the following analyses were determined in addition to those previously described: rigor contraction of fillets, colour development, and energy status and ATP and glycogen (pH development) degradation until 72h post-mortem. Additionally activity of cathepsin B and L was analysed.

## Feed and feed composition

The feed used was a commercial extruded dry feed consisting of 3, 4.5, and 7 mm pellets, manufactured by Skretting As Stavanger, Norway. April 15th, June 22nd and August 18th, are the dates when the fish started to feed on the 3, 4.5 and 7 mm pellets, respectively. Before running the experiment, the experimental diets were prepared by coating the control feed with 0.25% Tetradecylthioacetic acid (TTA) (Thiamedica, Bergen Norway), 1.5% L-arginine (Fenchem Biotek Ltd, Nanjing, China) or 1.5% L-glutamate (Meihua Holdings Group Co., Ltd , Hebei,

China). Fish were fed with TTA diet until the body weight increased by 0.2%, and thereafter the TTA diet was replaced by the control diet.

**Table 2** - Composition of the experimental diets

| | pellets size | 3mm | | | | 7mm | | |
|---|---|---|---|---|---|---|---|---|
| | | Diet 1 Control | Diet 2 0.25%TTA | Diet 3 1.5% Glu | Diet 4 1.5% Arg | Diet 1 Control | Diet 3 1.5% Glu | Diet 4 1.5% Arg |
| DM (g kg$^{-1}$) | | 912 | 904 | 912 | 915 | 889 | 897 | 895 |
| In DM: | | | | | | | | |
| | Crude lipid (g kg$^{-1}$) | 275 | 280 | 274 | 269 | 374 | 370 | 369 |
| | Crude protein[1] (g kg$^{-1}$) | 514 | 521 | 518 | 529 | 435 | 438 | 459 |
| | Ash (g kg$^{-1}$) | 72 | 64 | 76 | 75 | 56 | 65 | 52 |
| | Starch (g kg$^{-1}$) | 59 | 59 | 57 | 60 | 82 | 79 | 77 |
| | Energy (MJ kg$^{-1}$) | 25.2 | 25.3 | 25.1 | 25.2 | 27.2 | 27.0 | 27.1 |
| | Astaxanthin (mg kg$^{-1}$) | 63 | 65 | 65 | 66 | 45 | 45 | 46 |
| | Minerals: | | | | | | | |
| | P (g kg$^{-1}$) | 16.2 | 15.6 | 14.9 | 15.4 | 11.5 | 11.0 | 11.4 |
| | Ca (g kg$^{-1}$) | 17.8 | 16.8 | 16.1 | 16.8 | 12.2 | 11.5 | 12.3 |
| | Mg (g kg$^{-1}$) | 2.14 | 2.13 | 2.04 | 2.13 | 1.60 | 1.58 | 1.58 |
| | Na (g kg$^{-1}$) | 7.91 | 7.86 | 10.00 | 8.32 | 8.56 | 8.40 | 6.69 |
| | Fe (mg kg$^{-1}$) | 153 | 234 | 136 | 162 | 136 | 233 | 267 |
| | Ma (mg kg$^{-1}$) | 27.2 | 33.2 | 21.4 | 21.8 | 33.2 | 25.9 | 23.1 |
| | Zn (mg kg$^{-1}$) | 153 | 143 | 145 | 142 | 137 | 135 | 139 |
| | Cu (mg kg$^{-1}$) | 11.8 | 12.3 | 10.4 | 10.7 | 9.1 | 8.6 | 8.2 |
| | Total dispensable amino acids[2] (g kg$^{-1}$) | | | | | | | |
| | Ala | 23.3 | 23.7 | 23.9 | 23.8 | 19.0 | 18.7 | 18.5 |
| | Ammonium* | 29.0 | 29.2 | 29.4 | 29.8 | 25.4 | 24.5 | 25.0 |
| | Asx[3] | 37.7 | 38.3 | 38.3 | 38.3 | 31.5 | 31.1 | 30.8 |
| | Cys | 4.1 | 4.1 | 4.1 | 4.2 | 3.7 | 3.6 | 3.7 |
| | Glx[4] | 64.8 | 65.7 | 75.7 | 65.6 | 57.2 | 66.4 | 55.7 |
| | Gly | 21.8 | 22.2 | 22.3 | 22.2 | 18.5 | 18.1 | 18.2 |
| | Pro | 16.8 | 18.3 | 17.8 | 17.5 | 18.8 | 19.9 | 19.2 |
| | Ser | 17.2 | 17.6 | 17.5 | 17.4 | 15.3 | 15.0 | 14.8 |
| | Tyr | 14.0 | 14.2 | 13.9 | 13.9 | 11.6 | 11.6 | 11.4 |
| | Total indispensable amino acids[2] (g kg$^{-1}$) | | | | | | | |
| | Arg | 28.2 | 28.5 | 28.6 | 39.6 | 24.2 | 23.8 | 34.9 |
| | His | 9.5 | 9.7 | 9.7 | 9.7 | 8.4 | 8.2 | 8.2 |
| | Ile | 18.1 | 18.4 | 18.6 | 18.6 | 16.1 | 15.5 | 15.6 |
| | Leu | 30.4 | 31.1 | 31.0 | 31.2 | 26.9 | 26.3 | 26.3 |
| | Lys | 30.3 | 30.9 | 31.0 | 31.0 | 24.9 | 24.3 | 24.2 |
| | Met | 11.8 | 12.0 | 11.8 | 12.0 | 9.4 | 9.2 | 9.2 |
| | Phe | 18.4 | 18.8 | 18.7 | 18.7 | 17.0 | 16.8 | 16.5 |
| | Thr | 16.9 | 17.2 | 17.1 | 17.0 | 14.2 | 14.0 | 13.8 |
| | Trp | 4.9 | 4.9 | 4.7 | 4.7 | 4.0 | 4.1 | 4.1 |
| | Val | 21.1 | 22.0 | 22.2 | 22.4 | 19.1 | 18.4 | 18.3 |
| | Free dispensable amino acids[2] (g kg$^{-1}$) | | | | | | | |
| | Ala | 1.47 | 1.60 | 1.57 | 1.55 | 2.02 | 2.02 | 2.02 |
| | Ammonia* | 0.04 | 0.05 | 0.04 | 0.04 | 0.05 | 0.05 | 0.05 |
| | Anserine* | 0.40 | 0.42 | 0.38 | 0.38 | 0.00 | 0.00 | 0.00 |
| | Asn | 0.00 | 0.00 | 0.00 | 0.00 | 0.43 | 0.43 | 0.46 |
| | Asp | 0.31 | 0.34 | 0.34 | 0.34 | 0.74 | 0.70 | 0.75 |
| | Glu | 1.24 | 1.38 | 14.78 | 1.50 | 1.77 | 16.34 | 1.70 |
| | Gly | 0.97 | 1.05 | 1.02 | 1.02 | 0.97 | 0.95 | 0.98 |
| | Ornithine* | 0.08 | 0.09 | 0.08 | 0.11 | 0.25 | 0.26 | 0.26 |
| | Phosphoethanolamine* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Phosphoserine* | 0.13 | 0.15 | 0.14 | 0.15 | 0.18 | 0.18 | 0.17 |
| | Pro | 0.38 | 0.33 | 0.31 | 0.42 | 0.32 | 0.34 | 0.35 |
| | Ser | 0.36 | 0.41 | 0.39 | 0.41 | 0.59 | 0.59 | 0.61 |
| | Taurine* | 4.05 | 4.36 | 4.27 | 4.22 | 4.75 | 4.63 | 4.68 |
| | Tyr | 0.37 | 0.38 | 0.37 | 0.37 | 0.78 | 0.74 | 0.76 |
| | Free indispensable amino acids[2] (g kg$^{-1}$) | | | | | | | |
| | Arg | 0.59 | 0.66 | 0.62 | 11.37 | 1.91 | 1.53 | 17.59 |
| | His | 0.64 | 0.71 | 0.70 | 0.70 | 0.63 | 0.64 | 0.69 |
| | Ile | 0.45 | 0.52 | 0.49 | 0.50 | 0.76 | 0.82 | 0.78 |
| | Leu | 1.07 | 1.27 | 1.26 | 1.25 | 2.02 | 1.96 | 1.97 |
| | Lys | 0.96 | 1.05 | 1.02 | 1.01 | 1.59 | 1.50 | 1.55 |
| | Met | 0.45 | 0.51 | 0.51 | 0.55 | 0.67 | 0.64 | 0.65 |
| | Phe | 0.55 | 0.67 | 0.67 | 0.63 | 1.02 | 0.96 | 0.99 |
| | Thr | 0.51 | 0.59 | 0.53 | 0.55 | 0.92 | 0.86 | 0.91 |
| | Val | 0.57 | 0.63 | 0.62 | 0.60 | 1.06 | 1.03 | 1.07 |

[1]Nx6.25
[2]Amino acids given as dehydrated residues
[3]Asx represents Asp and Asn
[4]Glx represents Glu and Gln
*not corrected for water molecule, given as hydrated residues

## Chemical analysis of feed

Feed amino acids were analyzed using a Biochrom 30 amino acid analyzer (Biochrom LTD Cambridge, UK). Free amino acids and nitrogenous compounds were analyzed after deproteinising with sulphosalicylic acid and filtering (0.22 µm Ultrafree CL) (Davis 2002). Prior to total amino acid analysis of the feeds, tryptophan and tyrosine were hydrolyzed with 4.2M NaOH (Hugli & Moore 1972), and the remaining amino acids were hydrolyzed with 6 M HCL (Davies 2002). Analysis of the feeds, both for total amino acids and free amino acids, were done to double check added levels. It became apparent that glutamate quantification was unreliable using only total amino acid analysis. Presumably, glutamate was partially degraded during acid hydrolysis, so the gentler sample treatment involved in the free amino acid analysis resulted in higher glutamate recovery. The feeds were analyzed for dry matter (105°C until constant weight), ash (550°C until constant weight), nitrogen (Kjeltec Auto System, Tecator, Stockholm, Sweden) and energy (Parr 1271 Bomb calorimeter, Parr Instrument Company, Moline, Illinois, USA). Crude fat was determined by the folch method (Folch et al. 1957), and starch in the feeds was analyzed as glucose after enzymatic hydrolysis employing a commercial kit (K-TSTA 05/06, Megazyme, Australia).

## Coating procedure of feed

Coating procedure was performed to make different dietary treatments. It was done according to the following procedure:

Control diet: 25 kg of basis diet (commercial extruded feed) coated with 900 ml distilled water (70°C) in a blender, and then it was dried on a tray for one day (outdoor temperature). Finally it was covered with 500 ml rapeseed oil in the same blender.

TTA diet: 65 gram TTA was dissolved in 900 ml distilled water (70°C) to coat 25 kg of basis diet. After coating, the feed was also dried on a tray for one day and thereafter coated again with 500 ml rapeseed oil. Coating was performed in the same blender.

Arginine diet: 390 gram arginine was dissolved in 900 ml distilled water (70°C) to coat 25 kg of basis diet. After coating, the feed was dried on a tray for a day and thereafter coated again with 500 ml rapeseed oil in the same blender.

Glutamate diet: 25 kg of basis diet was coated with 390 gram glutamate dissolved in 900 ml distilled water (70°C). After coating, the feed was dried on the tray a day and thereafter coated again with 500 ml rapeseed oil in the same blender.

Rapeseed oil was used to prevent the diffusion of TTA, arginine and glutamate in seawater during feeding. The coating procedure was also done for Control diet to make the different treatments the feeds as similar as possible in order to enable extracting the dietary effect per se in the feeding trial.

## Recorded parameters on a daily basis

Sea water temperature and amount of feed consumed were registered every day from April 15th until end of the experiment.

The water temperature at 3 meter depth averaged 12°C during the experiment, with a minimum of 6°C on 17th of April and a maximum of 16.3°C on 11th of August (Figure 7).

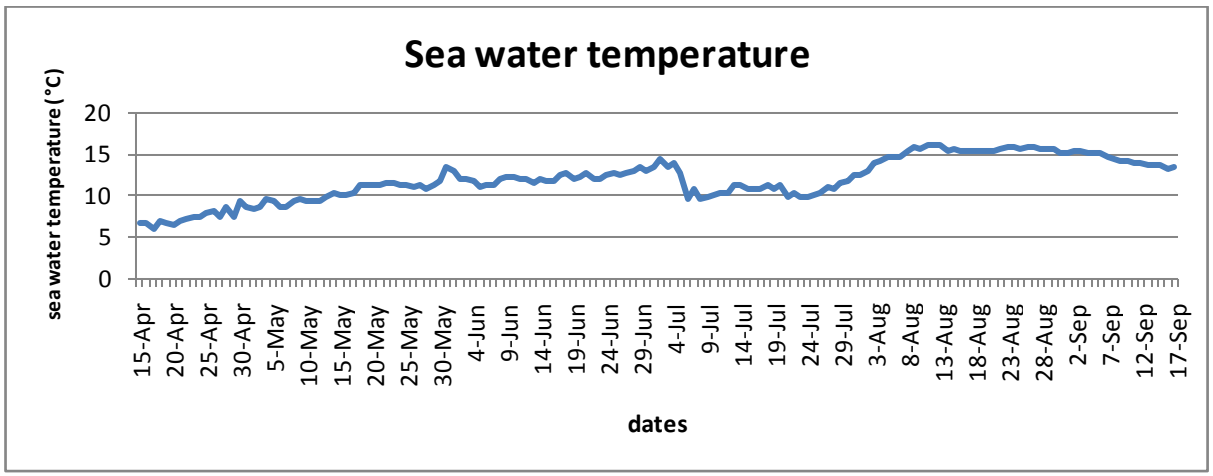


**Figure 7** - Sea water temperatures during the experiment from April 15th to Sep 15th.

The fish were fed by automats in excess of assumed feed intake four times per day, and uneaten feed was collected quickly after each feeding and pumped up into wire mesh strainers as described by Einen et al. (1999) (Figure 8). Feed consumption (g) was recorded to calculate the specific feed ratio (SFR) based on the actual amount of feed eaten. Each diet was examined for recovery of dry matter under the environmental conditions present during the experiment as described by Helland et al. (1996), and the weight of uneaten feed registered was corrected for dry matter losses during feeding and collection.

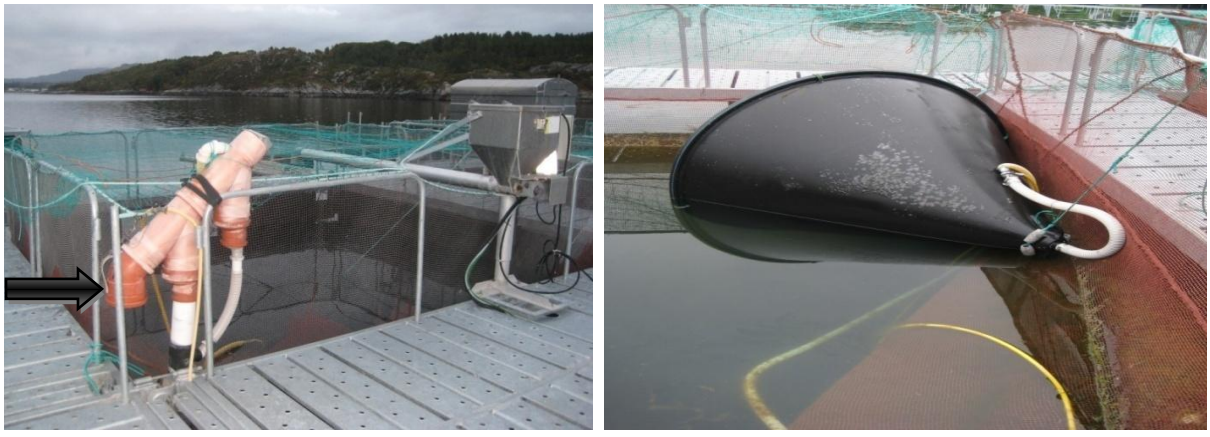

**Figure 8** - Collector system of uneaten feed pellets at Nofima research station, Averoy, Norway (Photo: B.Rahnama)

## Sampling of fish

The fish were transferred in batches to 1000 liter tanks with seawater, where they were anesthetized (MS 222 metacaine, ALPHARMA, Animal Health Ltd., Hampshire, UK, 0.1 g $L^{-1}$), counted and bulk weighted. Thereafter, the average weight of all fish in the net pen was calculated, and ten fish representing the mean weight of the net pen were killed (gill-cut) and sampled for further analysis. The fish were transferred to the land based facilities for washing, gutting and filleting (by hand), and the following slaughter parameters were recorded: whole body weight, fork length, liver weight, heart weight. The Norwegian quality cut (i.e. the cutlet between the posterior end of the dorsal fin and the gut, NQC) were sampled from each left fillet and analyzed for fat and pigment content using image analysis.

## Fat content and pigment analysis

Fat and pigment analyses were performed on the left side of the NQC (Figure 9), using the equipment provided by PhotoFish AS (Ås, Norway). The system consists of closed box with standardized light and color conditions, a digital camera, and a PC for transmitting of the image and software for analyses (Folkestad et al. 2008).

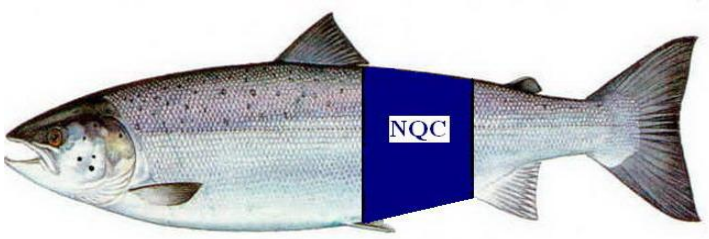


**Figure 9 -** Sampling area for analysis of fat and pigment.

## Slaughtering and filleting procedure of fish for pre rigor quality analysis

At the last sampling (S4, Sep 15-17$^{th}$) a more comprehensive determination of analysis were performed. In addition to normal slaughtering, a number of fish were also exposed to crowding stress prior to slaughtering. First and second days (Sep 15-16$^{th}$) were allocated to Non-crowded fish (normal slaughter handling; in each day fish from 6 net pens). Slaughtering and filleting of Crowded fish were done in the third day (Sep 17$^{th}$).

**Non- crowded fish;** 6 fish were sampled from each net pen, after that they were killed (gill- cut) and transferred to the land based facilities for washing, gutting, filleting (by hand) and further analysis. Filleting was performed within maximum, half an hour after killing. The following

slaughter parameters were recorded for all fish: whole body weight, fork length, gutted body weight, liver weight, and left fillet length. After separating the right and left fillet of each fish they were placed on solid plates to avoid quality deterioration due to handling. The fillets were kept in Styrofoam salmon boxes (3 fillets in each box) filled with ice made of fresh water (fillets were not in contact with the ice). The fillets were stored for up to 72 hours and analyzed in a refrigerated room at $5^0$C.

**Crowded fish;** 3 fish were sampled from each net pen; they were tagged and kept in a 1000L tank. To induce crowding stress, fish were transferred from net pens to the tank one night before slaughtering time, and they were stocked in the tank with approximately 20% running seawater of the total tank volume and with a fixed level of oxygen (7 mg/l). The slaughtering and filleting procedure and storage conditions followed as the same procedure as Non-crowded fish.

## Measurements and data registrations

The length measurements, gaping and color from left fillets and samples for ATP and pH from right fillets for Non-crowded fish (Table 3) and Crowded fish (Table 4) were taken according to the schedule.

**Table 3** - Measurements and sampling times from fillets of non-crowded fish

| Hours after filleting | 0 | 1 | 3 | 6 | 9 | 12 | 24 | 48 | 72 |
|---|---|---|---|---|---|---|---|---|---|
| Length of fillets | * | * | * | * | * | * | * | * | * |
| color | | * | | * | | * | * | * | |
| Gaping | | | | | * | | * | | |
| Muscle samples and pH for group A (first 3 fish of each net pens) | | | | * | | | * | | * |
| Muscle samples and pH for group B (second 3 fish of each net pen) | | * | | | | * | | * | |

**Table 4** - Measurements and sampling times from fillets of crowded fish

| Hours after filleting | 0 | 1 | 3 | 6 | 9 | 12 | 24 | 48 |
|---|---|---|---|---|---|---|---|---|
| Length of fillets | * | * | | * | | * | * | * |
| color | | * | | * | | * | * | * |
| Gaping | | | | | * | | * | |
| Muscle samples and pH (all 3 fish of each net pen) | | * | | | | * | | * |

**Rigor contraction**

Rigor contraction of left fillet was registered through the measurement of fillet length (Figure 10).

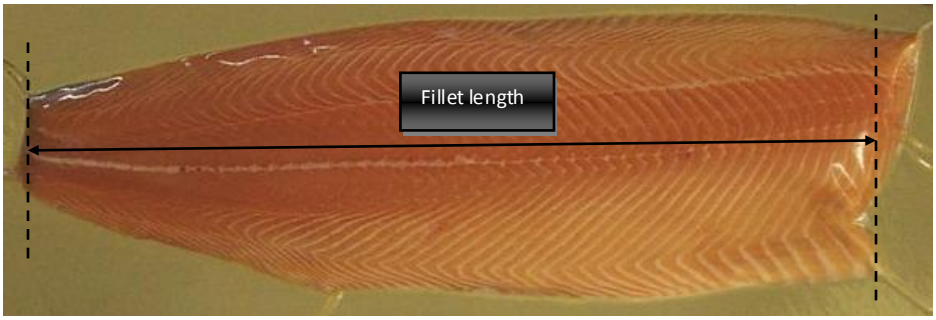


**Figure 10** - Left fillets were used for measurement of fillet contraction during storage.

**pH measurements**

The pH was measured by a pH-meter 330i SET (Wissenschaftlich-Technische-Werkstatten Gmbh & Co. KG, WTW, Weilheim, Germany) with a pH muscle-electrode (Schott pH-electrode, Blueline 21 pH, WTW, Weilheim, Germany) and a temperature probe (TFK325, WTW, Weilheim, Germany) that was directly entered in the fillets are shown on Figure 3.5.

**ATP analysis**

Muscles samples for ATP were taken from the sections as shown on Figure 11. The muscle samples were immediately frozen individually in liquid nitrogen, and stored with dry ice in Styrofoam boxes until they were transferred to a -80$^{o}$C freezer.

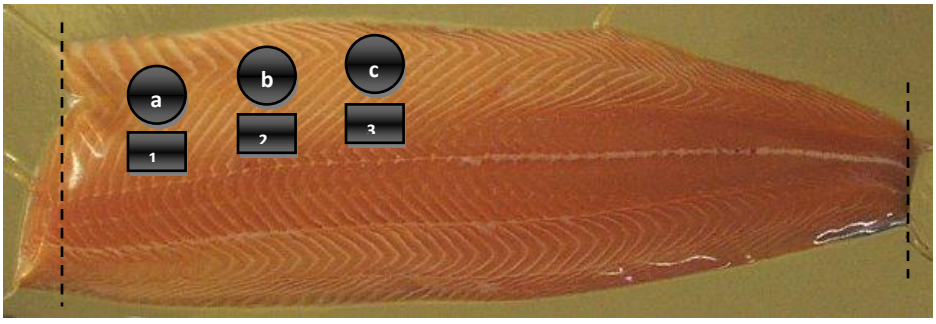


**Figure 11**- Sampling sections for analysis of ATP (a, b and c) and pH (1, 2 and 3)

**Color of fillets**

Fillet color development during storage was registered by using Roche ***Salmo***Fan$^{TM}$ . To ensure consistent color assessment, fluorescent light conditions within a controlled environment are preferred; for this purpose salmon color box was used. The color box was equipped as reported by Bjerkeng et al. (1997) (Figure 12).

**Figure 12** - Registration of fillet color by Roche SalmoFan$^{TM}$

**Fillet gaping**

The fillet gaping was recorded according to a scale ranging from score 0-5, where the score zero represents no gaping and score five is equivalent to extreme gaping (Table 5).

**Table 5** - Scale used to classify salmon fillets according to degree of fillets gaping (Andersen et al. 1994).

| Gaping score | Definition |
|---|---|
| 0 | No gaping |
| 1 | Few small[1] slits (less than 5) |
| 2 | Some small slits (less than 10) |
| 3 | Many slits (more than 10 small or a few large[2]) |
| 4 | Severe gaping (many large slits) |
| 5 | Extreme gaping (the fillet falls apart) |

[1] ‹ 2 cm [2] › 2 cm

## Laboratory analysis

### ATP analysis

ATP was extracted by mixing pooled samples of freeze-dried muscle in 8% $HCLO_4$ (2.5 ml) for 30 minutes before centrifugation (10 min at 11,900g). The supernatant (1 ml) was neutralized with 3 M $K_2CO_3$ (0.3 ml), centrifuged again at 11,900g for 10 min and finally filtered (0.45-µm filter). ATP was analyzed by HPLC in a Waters Alliance liquid chromatograph system (2695) equipped with a photodiode array detector (2996) (Morkore et al. 2010).

### Cathepsins analysis

Samples were taken from the same sections of fillets as the pH measurements were done (Figure 3.5), after that they were quickly frozen in liquid nitrogen before being stored at -80 °C until further analyses. Cathepsin B + L, cathepsin B and cathepsin L total activities were determined by homogenizing 300 mg of muscle in 900 ml extraction buffer (100 mM Na-acetate in 0.2% Triton X-100, pH 5.5) in Precellys tubes, with a Precellys24 homogenizer (Bertin Technologies, France) (2 cycles of 20 s at5500 rpm, separated by a 10 s break). The obtained homogenates were centrifuged at 16,000 G for 30 min and the supernatants were used to determine enzyme activities. Cathepsin B + L activity was measured fluorimetrically, according to the method of Kirschke et al. (1983). The procedure used N-CBZ-Lphenylalanyl-L-arginine-7- amido-4-methylcoumarin (Z-Phe-Arg-Nmec) as a substrate. For cathepsin B + L and N-CBZ-L-arginyl-L-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-Nmec) for cathepsin B. cathepsin L activity was obtained subtracting the result of cathepsin B activity from the result of cathepsin B + L activity. In all cases, the assays were run in triplicates for all cathepsin measurements.

## Data analysis

ANOVA analyses (GLM procedure) were determined by the Statistical Analysis System (SAS), release 8.02 (SAS Institute Inc., Cary, NC, USA) as statistical software and with diet and per-slaughter handling within diet as explanatory variables. Significant differences among means were ranked by Least Squares Means at $p < 0.05$ (Duncan ranking, or pdiff when analyses were missing for some reason).

## Calculations

**Condition factor (CF)**

Condition factor was registered through the measurement of body weight and fork length with the following equation:

CF= Body weight (g) * 100 / Fork length (cm)

**Hepatosomatic and cardio somatic index (HSI & CSI)**

Hepatosomatic Index (HSI) and cardio somatic index (CSI) are defined as the ratio of liver and heart weight to body weight by the following equation:

HSI (%) = Liver weight (g) / Body weight (g) x 100

CSI (%) = Heart weight (g) / Body weight (g) x 100

**Rigor contraction**

Fillet contraction at time T is expressed as percentage of initial length:

$\text{Contraction}_T\,(\%) = 100 - (L_T/L_S)*100$

Where:

$L_T$: Fillet length at time T

$L_S$: Fillet length at filleting (initial fillet length)

# Results

## Growth performance

The growth rate during the whole five month trial showed no significant variation between dietary treatments. Numerically, the specific growth rate (SGR) and thermal growth coefficient (TGC) for the TTA group was the lowest, whereas the arginine group had the highest specific growth rate (SGR) and thermal growth coefficient (TGC), as shown in Table 6.

Comparison of dietary treatments within each sampling period (Figure 13) showed that the body weight of the TTA group was significantly lower compared to the arginine group at S2 (June 23-24$^{th}$) (Figure 14). In other sampling times no significant differences were recorded between dietary treatments.

**Table 6** - Specific growth rate (SGR), thermal growth coefficient (TGC) and feed conversion ratio (FCR) of farmed Atlantic salmon fed different dietary treatments for five months.

| Diets | Initial weight (g) | Final weight (g) | SGR (% day$^{-1}$) | TGC (% day$^{-1}$) | FCR |
|---|---|---|---|---|---|
| Control | 105 | 863.6 | 1.37 | 2.60 | 0.77 |
| Arginine | 105 | 887.3 | 1.39 | 2.65 | 0.77 |
| Glutamate | 105 | 878.0 | 1.38 | 2.63 | 0.77 |
| TTA | 105 | 827.6 | 1.34 | 2.53 | 0.77 |

SGR= (ln Wf−ln Wi) / No. of days×100, where Wf and Wi refer to final and initial mean body weight, respectively.
TGC = [(FBW$^{1/3}$ - IBW$^{1/3}$) / (T*D)]* 1000, where T and D refer to average of temperature and number of days.
FCR = eaten feed (kg)/ biomass increased (kg), where biomass increased calculated as;
final biomass (kg) + mortality biomass (kg) – initial biomass (kg)

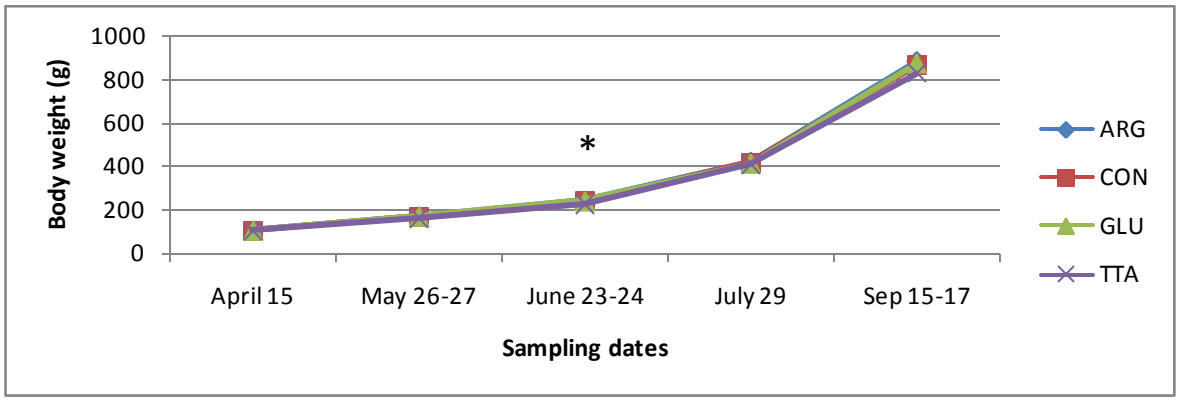


**Figure 13** - Development of body weight during the experiment. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Periods where significant differences were observed between dietary treatment are indicated by *

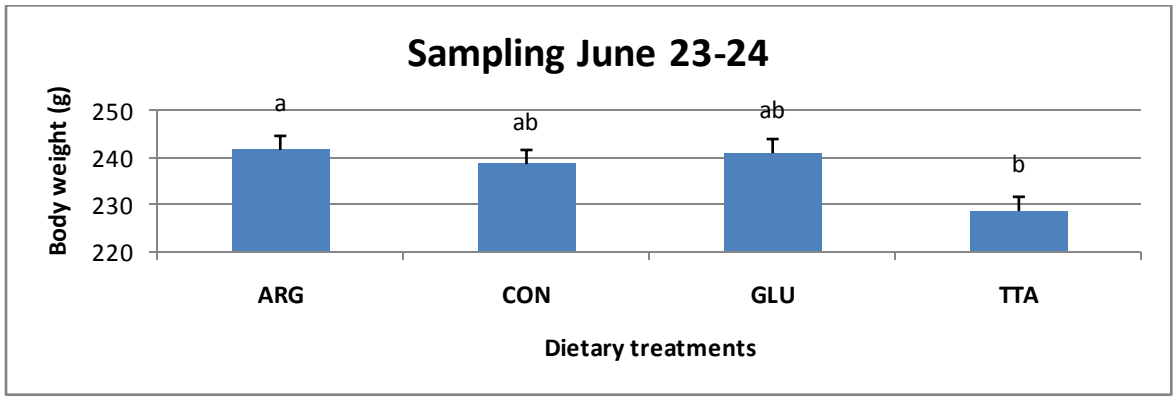


**Figure 14** - Significant differences of body weight between dietary treatments (sampling June 23- 24th).

## Feed conversion ratio (FCR)

Significant differences were observed among the dietary treatments at S1 (May 26-27th) and S4 (Sep 15-17th) (Figure 15). At both sampling times, TTA group showed highest feed conversion ratio. In S1, the TTA group showed significantly higher FCR compared to the control group and in S4 it was significantly higher from the arginine group (Figure 16).

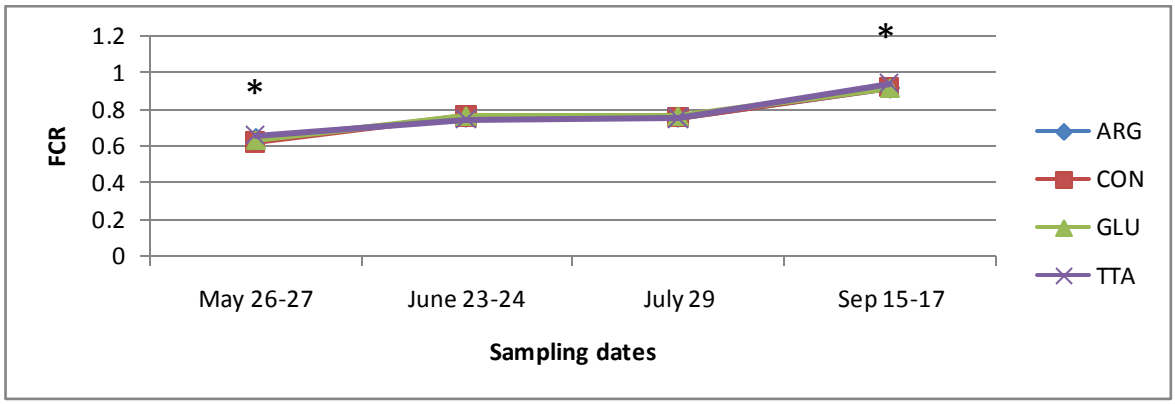


**Figure 15** - Development in feed conversion ratio during the experiment. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Periods were significant differences were observed between dietary treatment are indicated by *.

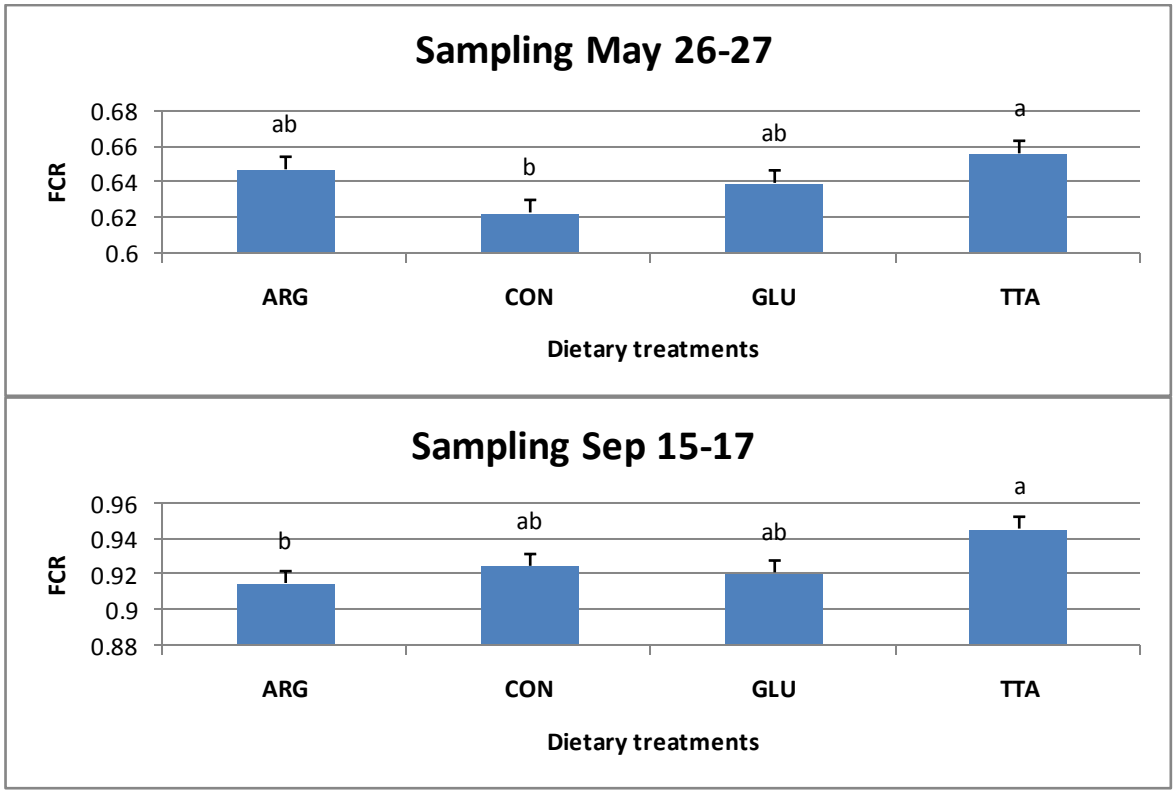


**Figure 16** - Significant differences in feed conversion ratio between dietary treatments at S1, sampling May 26-27$^{th}$ & S4, sampling Sep 15-17$^{th}$. Results are given as mean ± SE, and dietary treatment with no common letters above the error bars are significantly different.

## Specific feed ratio (SFR)

There was no overall significant difference among the dietary treatments, or between dietary treatments within each sampling period (Figure 17).

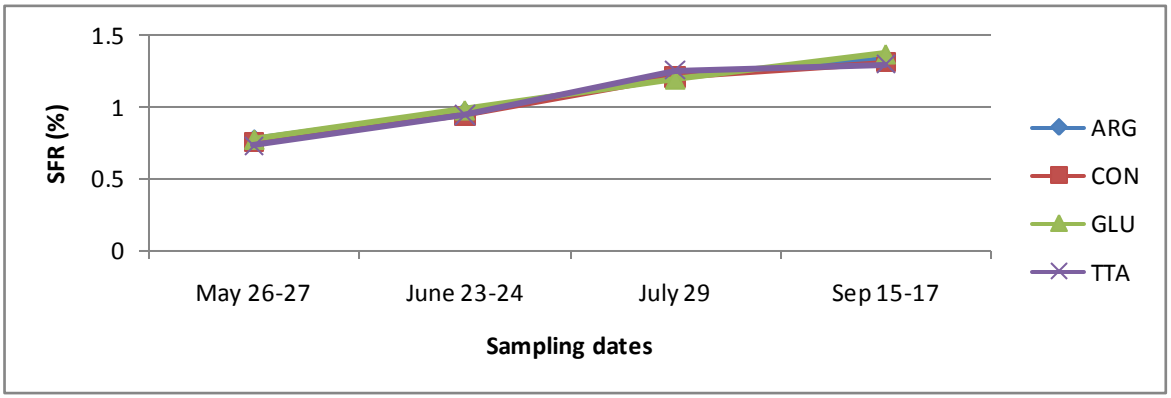


**Figure 17** - Development of specific feed ratio during the experiment. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA.

## Condition factor (CF)

No significant differences were recorded between the diets, except at S2 (June 23-24$^{th}$) and S4 (Sep 15-17$^{th}$) (Figure 18). At S2 the arginine group and at S4 the glutamate group had significantly higher condition factor compared to the TTA group (Figure 19).

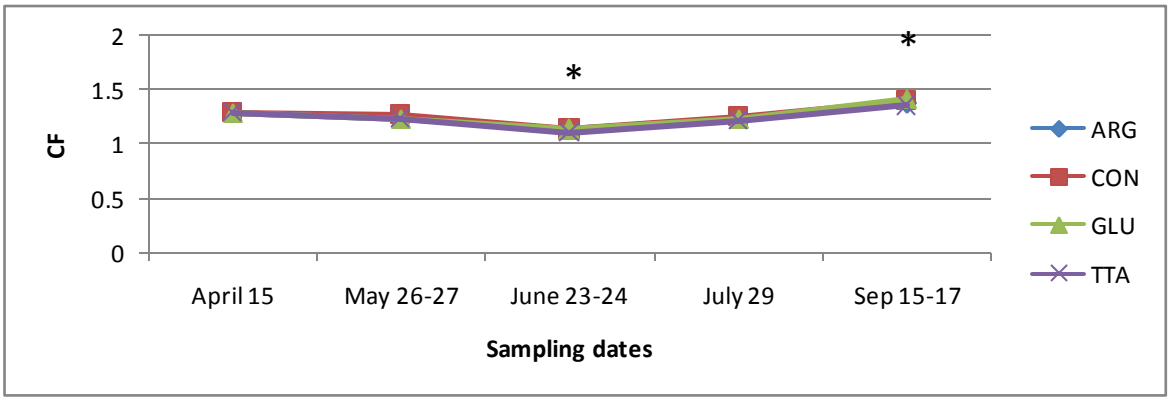


**Figure 18** - Development of condition factor (CF) during the experiment. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Periods where significant differences were observed between dietary treatment are indicated by *.

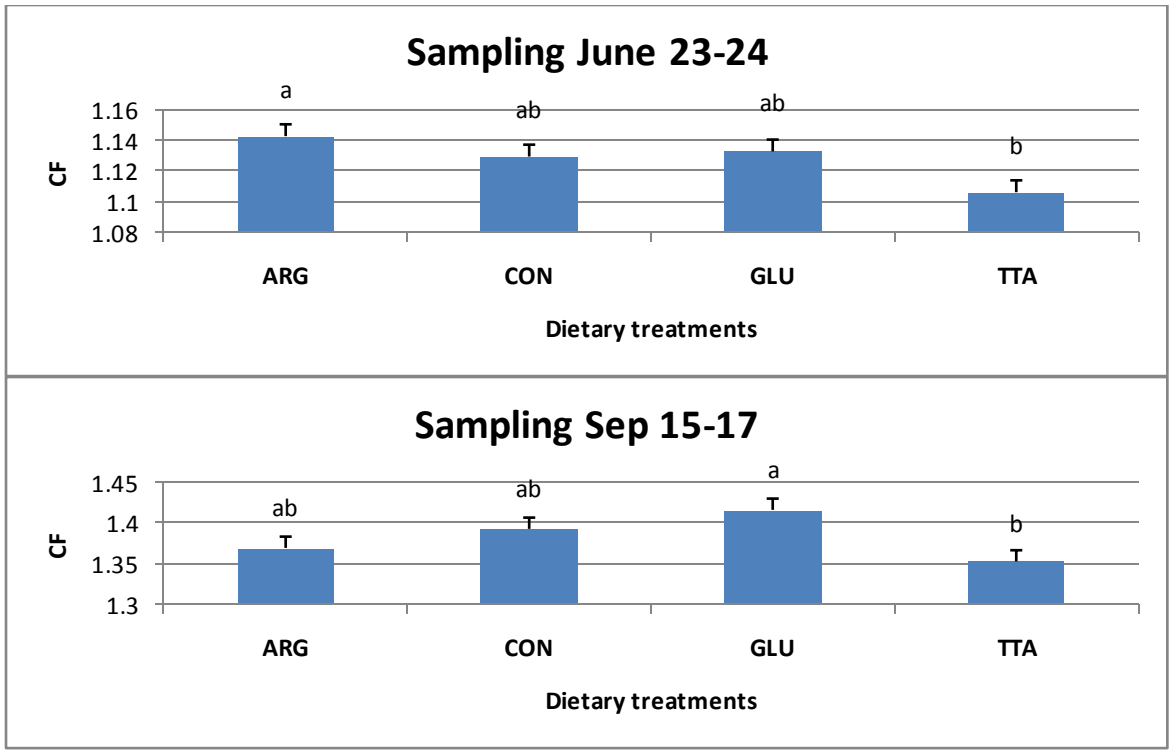


**Figure 19** - Significant differences of the condition factor (CF) between dietary treatments at S2, June 23-24th & S4, Sep 15-17th. Results are given as mean ± SE, and dietary treatment with no common letters above the error bars are significantly different.

## Hepatosomatic Index (HSI)

Significant differences were registered at S2 (June 23-24th) and S4 (Sep 15-17th) (Figure 20). In S2, the TTA group had significantly higher HSI compared to the control and arginine group, and at S4, the glutamate group showed significantly higher HSI compared to the TTA group (Figure 21).

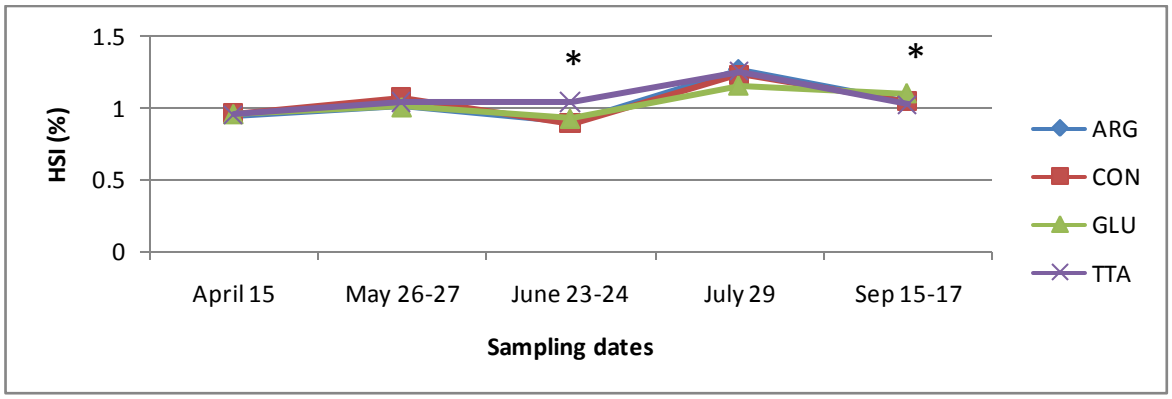


**Figure 20** - Development of hepatosomatic index (HSI %) during the experiment. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Periods were significant differences were observed between dietary treatment are indicated by *

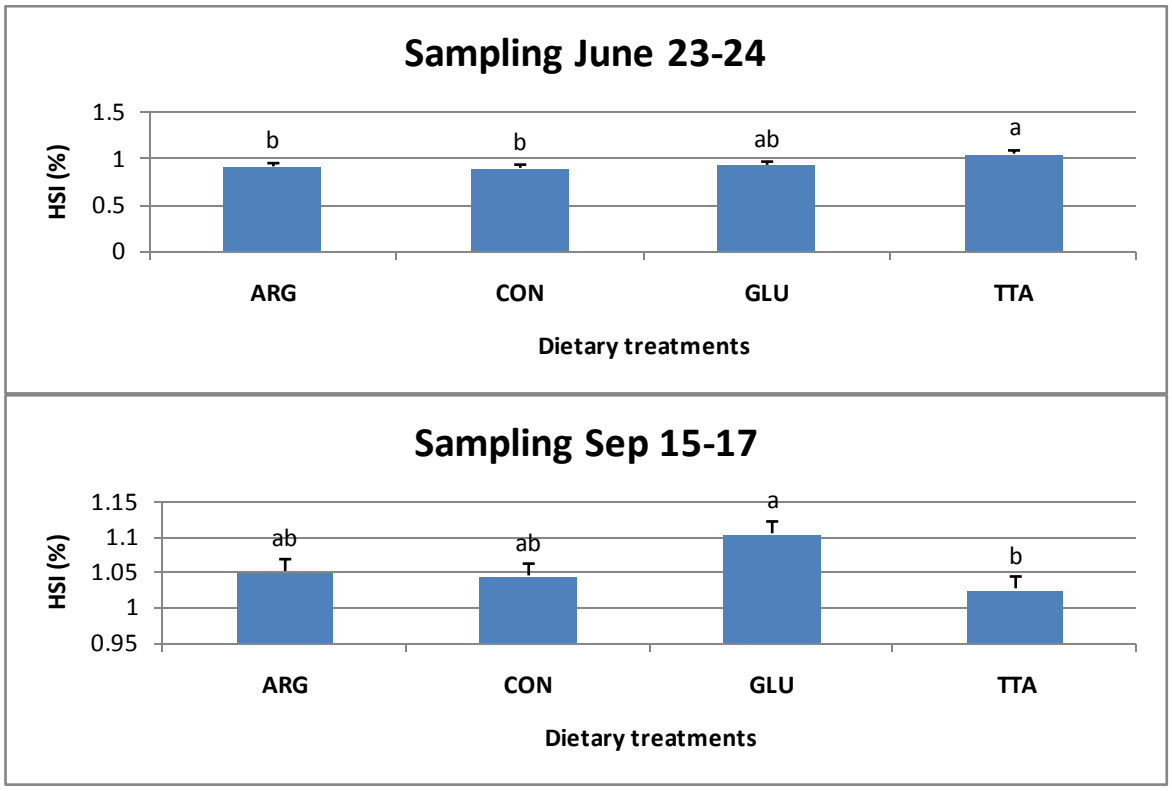


**Figure 21** - Significant differences of hepatosomatic index (HSI, %) between dietary treatments at S2, June 23-24th & S4, Sep 15-17th. Results are given as mean ± SE, and dietary treatment with no common letters above the error bars are significantly different.

## Cardio somatic index (CSI)

There was not overall significant difference between the dietary treatments, or between dietary treatments within each sampling period (Figure 22).

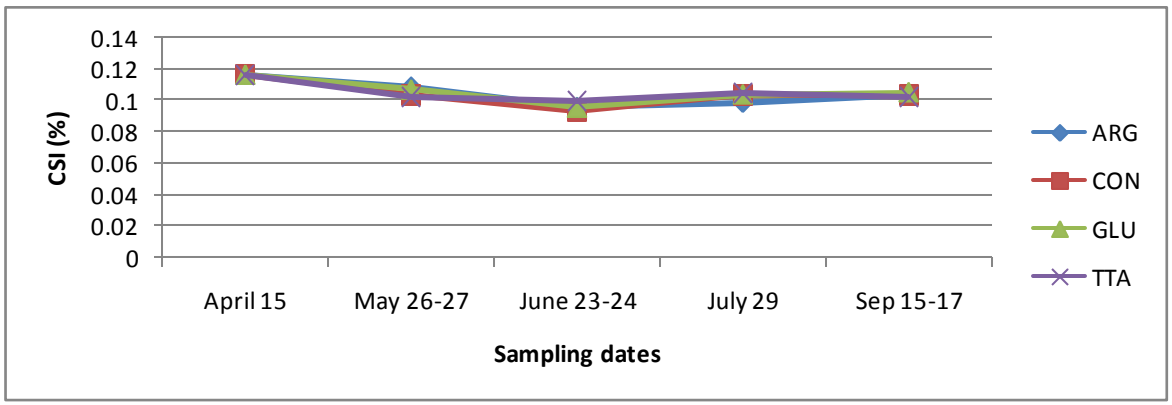


**Figure 22** - development of cardio somatic index (CSI, %) during the experiment. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA.

## Pigmentation

There was not overall significant difference between the dietary treatments, or between dietary treatments within each sampling period. Astaxanthin content decreased from S0 (April 15th) to S1 (May 26-27th), after that it increased gradually until the last sampling (Figure 23).

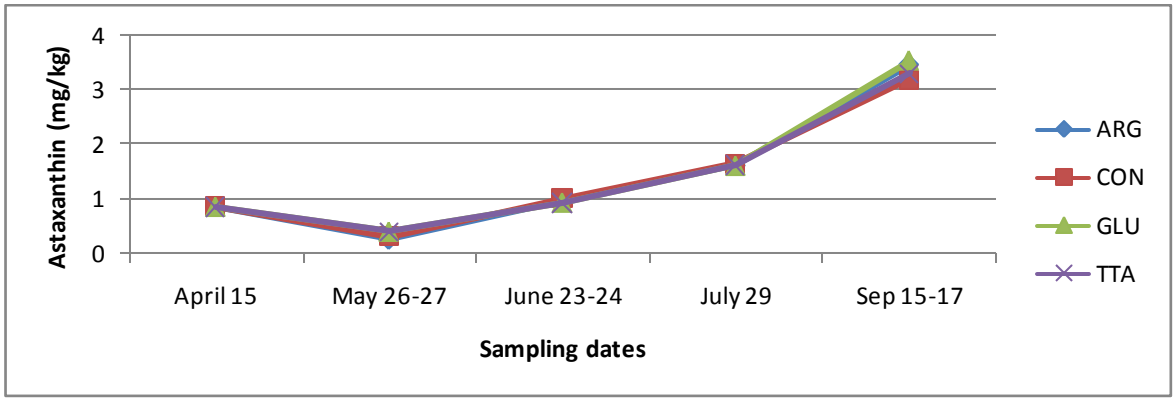


**Figure 23** Developments in astaxanthin content (mg/kg) during the experiment. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA.

## Fat content

In three sampling times, significant differences were observed between the dietary treatments (Figure 24). In S2 (June 23-24th) and S4 (Sep 15-17th) fish fed TTA had the significantly lowest fat content compared to fish fed other diets and in S3 (July 29th) the arginine group showed significantly lower fat content as compared to the control diet (Figure 25).

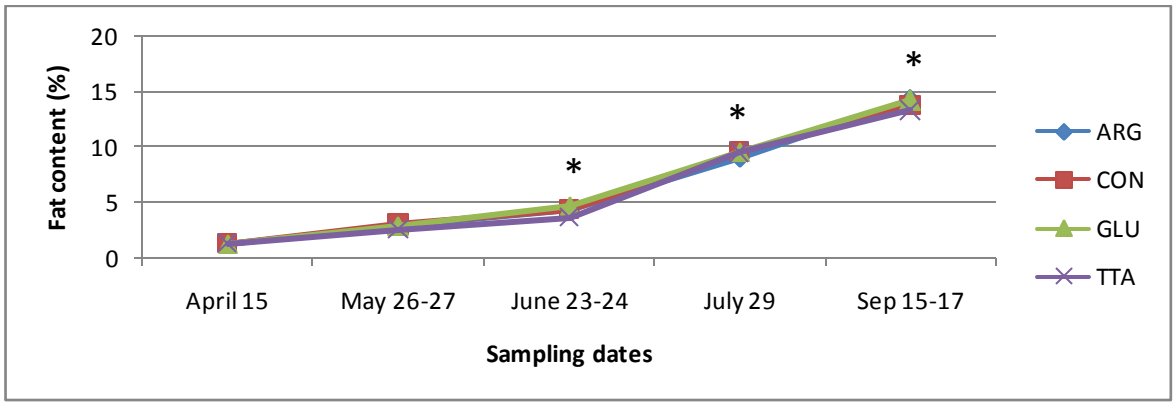


**Figure 24** - development of fat content (%) in muscle during the experiment. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Periods were significant differences were observed between dietary treatment are indicated by *

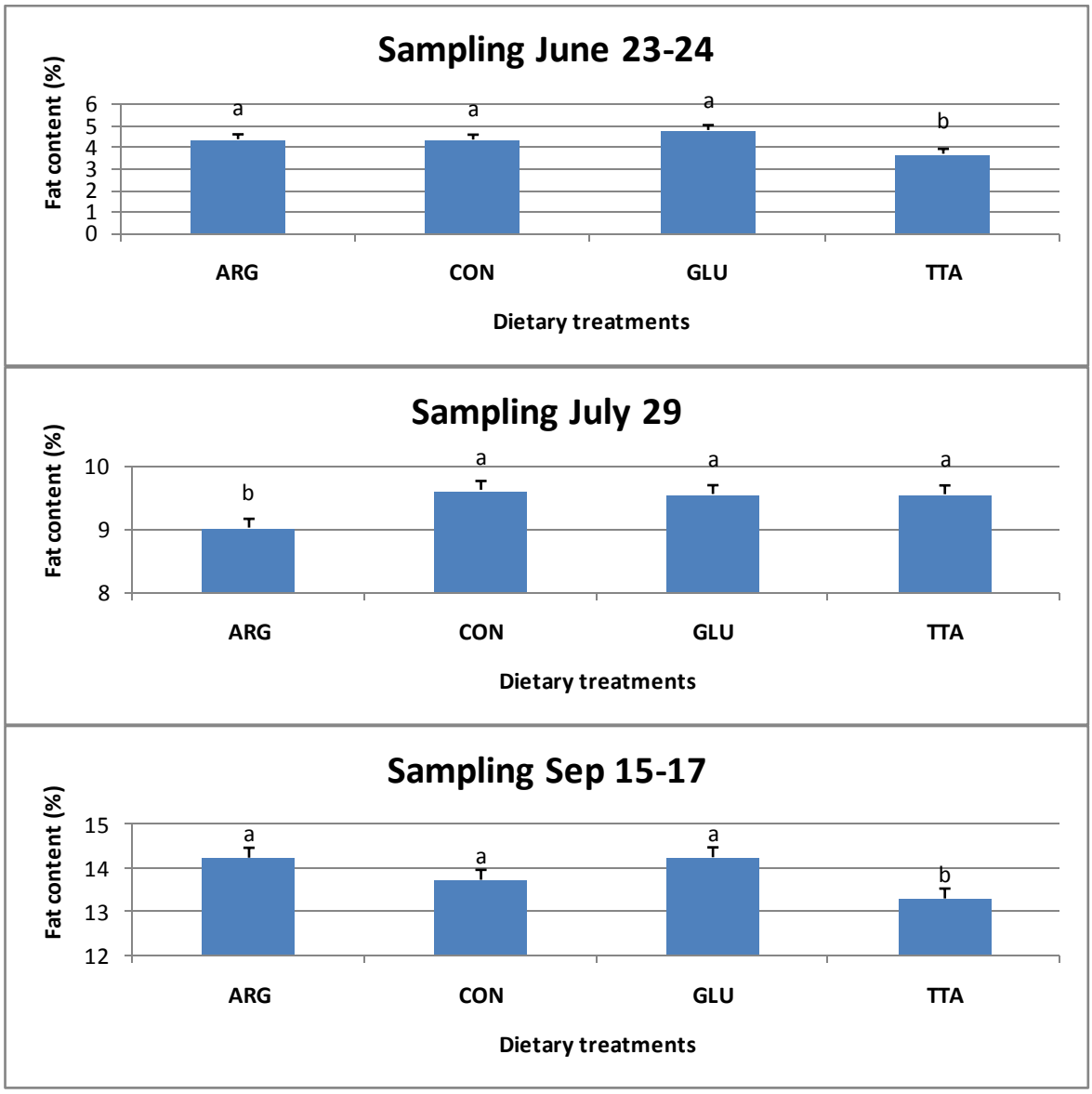


**Figure 25** - Significant differences of muscle fat content between dietary treatments at S2, June 23-24$^{th}$,& S3, July 29$^{th}$ & S4, Sep 15-17$^{th.}$. Results are given as mean ± SE, and dietary treatment with no common letters above the error bars are significantly different.

# Rigor development

## Fillet contraction

**Non-crowded fish:** Rigor contraction of non-crowded salmon increased gradually after slaughter and fillets reached a maximum contraction of approximately 18% after 72 hours (Figure 26). Significant differences between dietary treatments were observed after 9 and 72 hours. After 9 hours there was no significant difference between the dietary treatments compared to control group, but the glutamate group showed the significantly highest contraction compared to arginine and TTA groups. After 72 hours the TTA group had the significantly lowest rigor contraction.

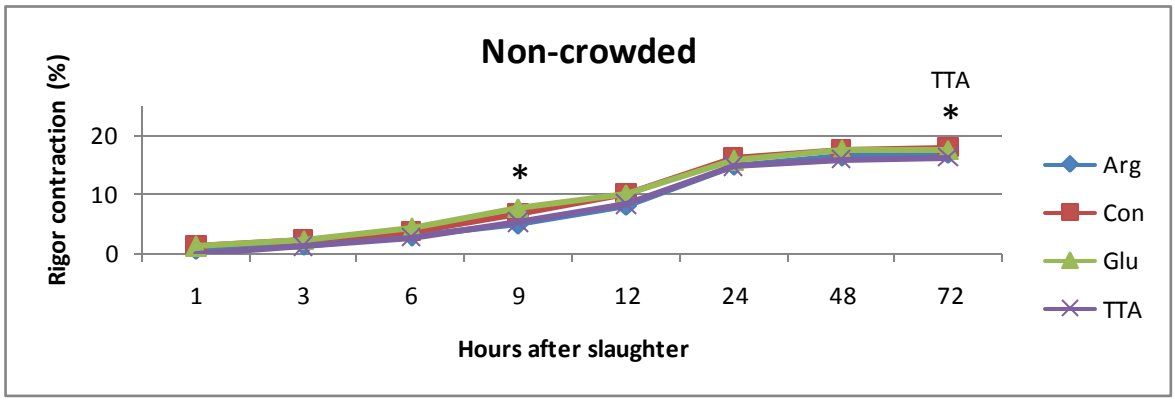


**Figure 26** - Fillet contraction during 72 hours ice storage of Atlantic salmon subjected to normal slaughter handling. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Time points were significant differences were observed between dietary treatment are indicated by * ($P < 0.05$). Dietary treatments that differed significantly from the Con are denoted above the *.

**Crowded fish:** Rigor contraction of crowded fish was measured from 1 to 48 hours after slaughter. After 1 hour the TTA group obtained significantly higher contraction compared to arginine group, but no significant differences were observed between the control group and other dietary treatments (Figure 27). Rigor contraction increased dramatically during the first hour and from 1 to 6 hours storage, at which time the contraction reached approximately 18% (similar to the contraction of non-crowded fish after 72 hours storage).

Crowded fish obtained a significantly higher percentage of contraction at 1 and 48 hours after slaughtering for all dietary treatments (Figure 28).

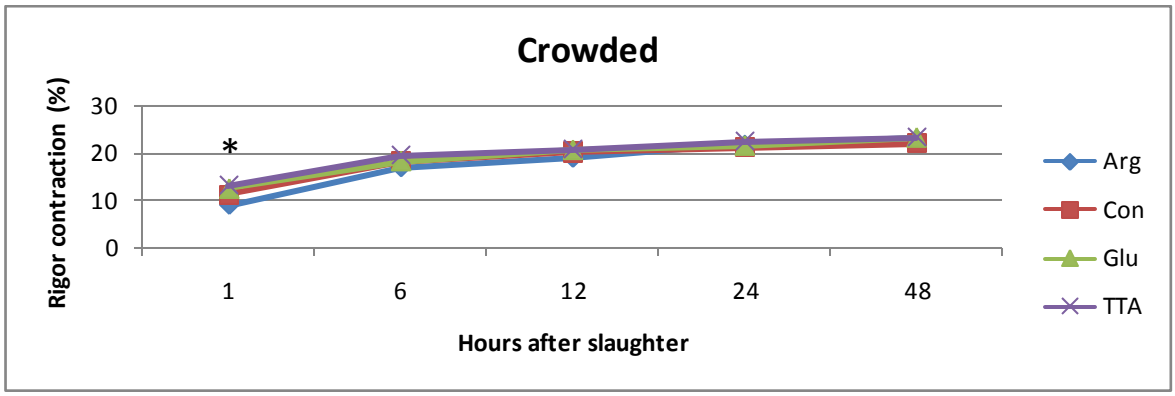


**Figure 27** - Fillet contractions during 48 hours ice storage of Atlantic salmon exposed to crowding stress for 16 hours. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Time points where significant differences were observed between dietary treatment are indicated by * ($P < 0.05$).

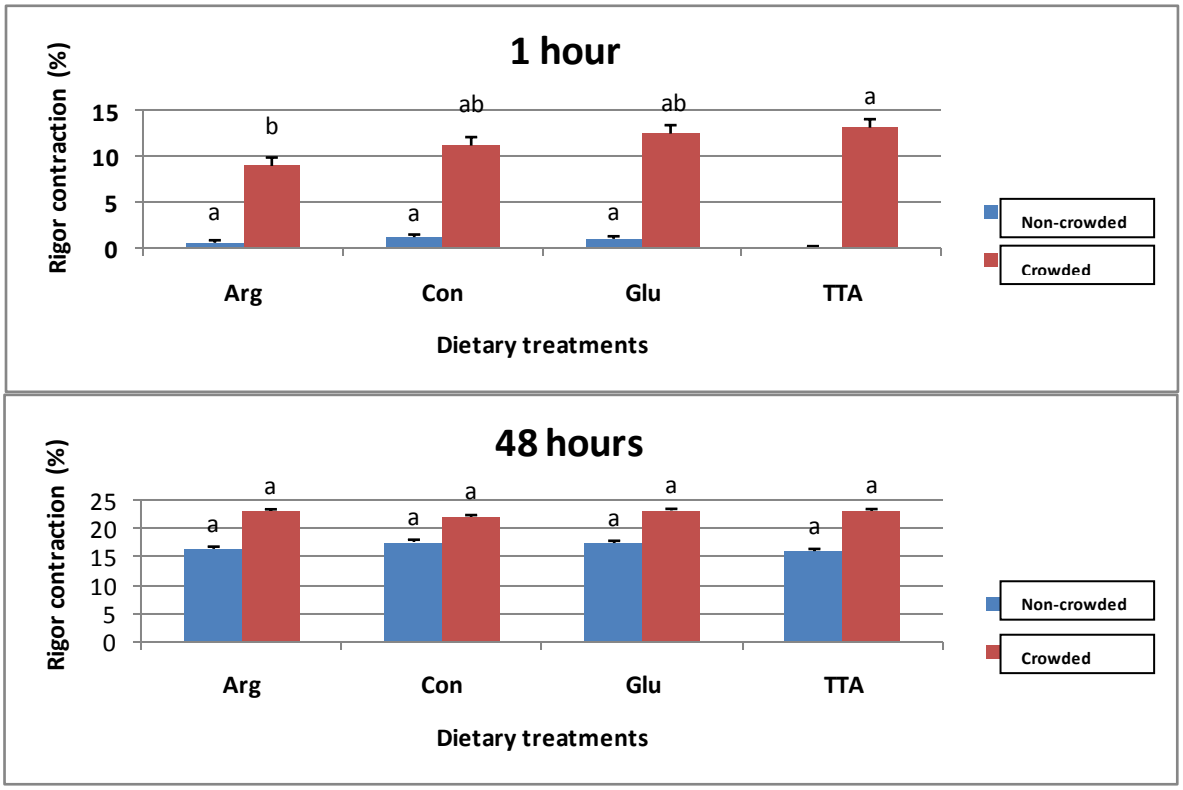


**Figure 28** - Comparison of rigor contraction of non-crowded and crowded fish after 1 and 48 hours ice storage. (★) indicates significant differences between pre slaughter handling. Different letters indicate significant differences between diets within slaughter treatment ($P < 0.05$).

## Fillet color

The fillet color was scored by using *Salmofan*. For both non-crowded and crowded fish, colour was registered from 1 to 48 hours after slaughtering. For non-crowded fish, a significant dietary difference was observed after 12 hours where the glutamate group showed significantly higher fillet color score compared to the control group (Figure 29). The measurements of the crowded fish showed no significant differences among the dietary treatments, although the glutamate group had numerically lowest fillet color score at all sampling points, from 1 to 48 hours (Figure 30).

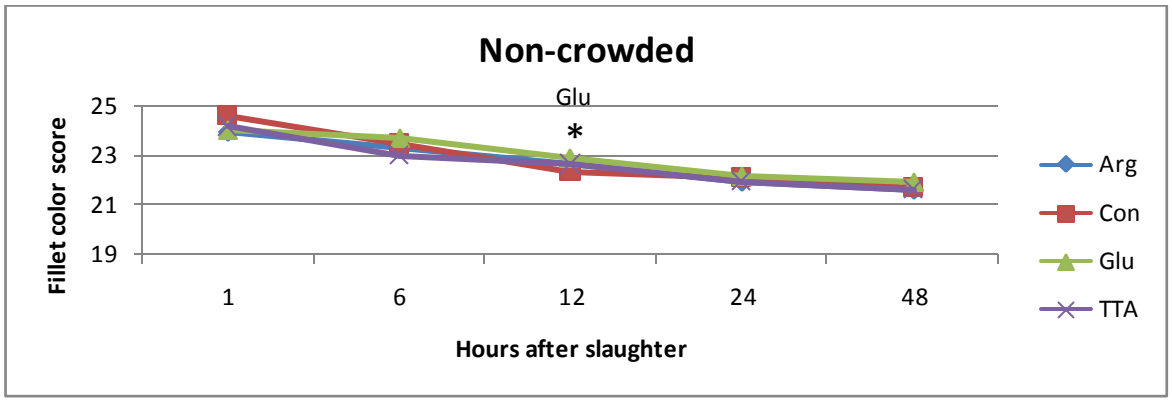


**Figure 29** - Fillet color score during 48 hours ice storage of Atlantic salmon subjected to normal slaughter handling. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Time point were significant difference was observed between dietary treatment is indicated by * ($P < 0.05$). Dietary treatments that differed significantly from the Con are denoted above the *.

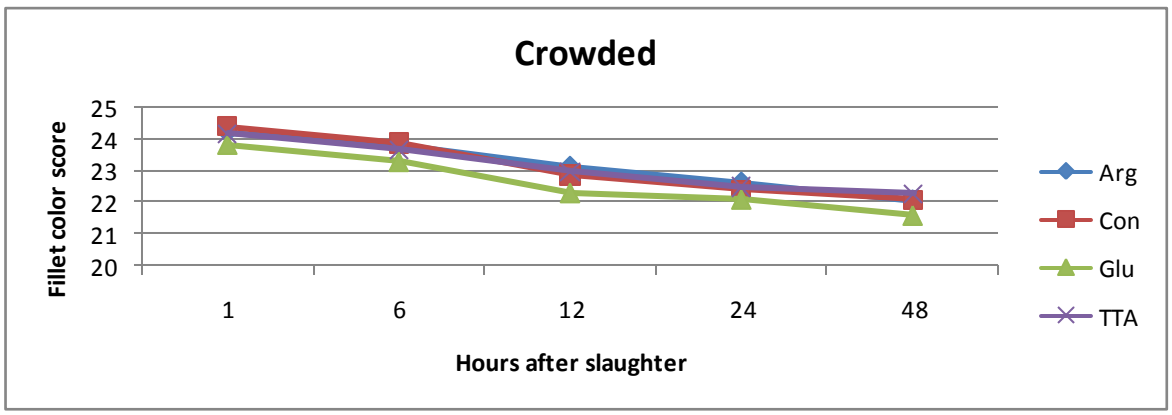


**Figure 30** - Fillet color score during 48 hours ice storage of Atlantic salmon exposed to crowding stress for 16 hours. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA.

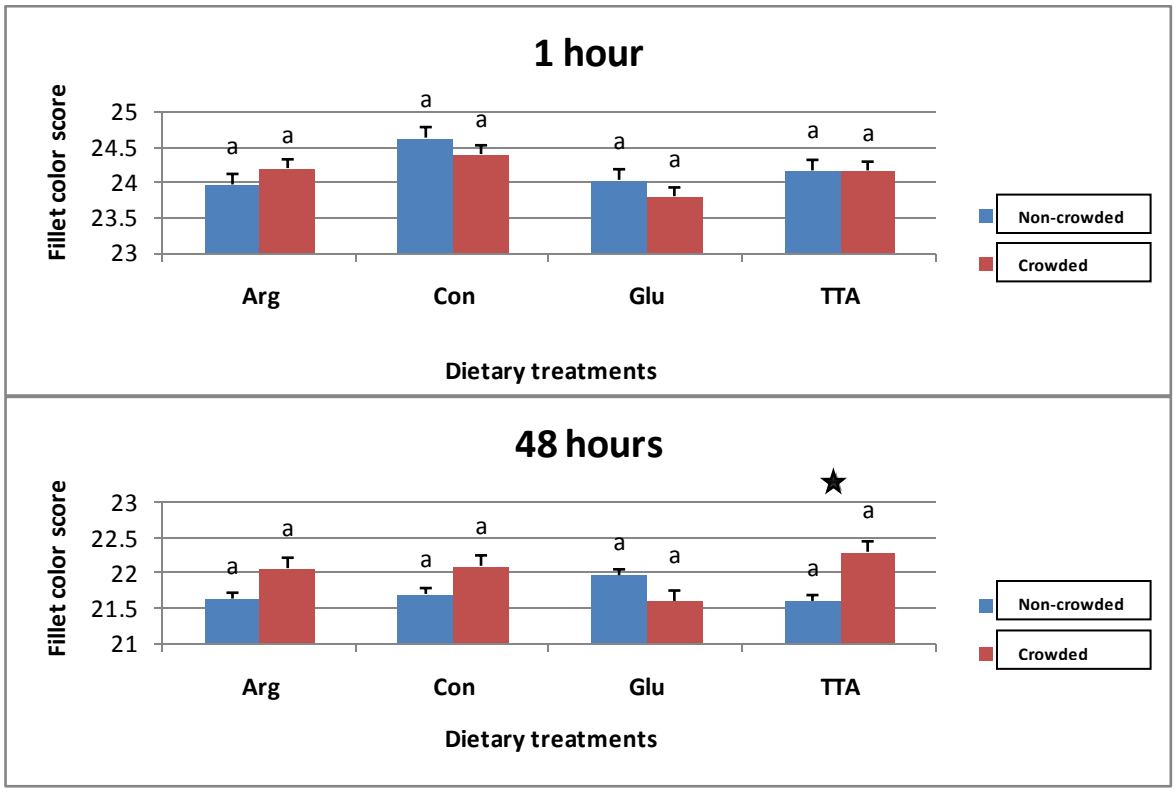


**Figure 31** - Comparison of fillet color of non-crowded and crowded salmon after 1 and 48 hours ice storage. ( ★ ) indicates significant differences between pre slaughter handling. Different letters indicate significant differences between diets within slaughter treatment ($P < 0.05$).

The comparison of non-crowded and crowded fish after 1 and 48 hours showed no significant differences between dietary treatments except for TTA group where crowded fish had significantly higher fillet color score than non-crowded fish after 48 hours (Figure 31).

## Fillet gaping

The fillet gaping was registered according to a scale ranging from score zero to five. It was measured 9 and 24 hours after slaughtering for both non-crowded and crowded fish, and no significant differences were observed between the dietary treatments (Figure 32).

A comparison between the non-crowded and crowded fish showed significantly higher gaping of the crowded arginine and control groups after 9 hours, and for the glutamate and control groups after 24 hours, (Figure 33).

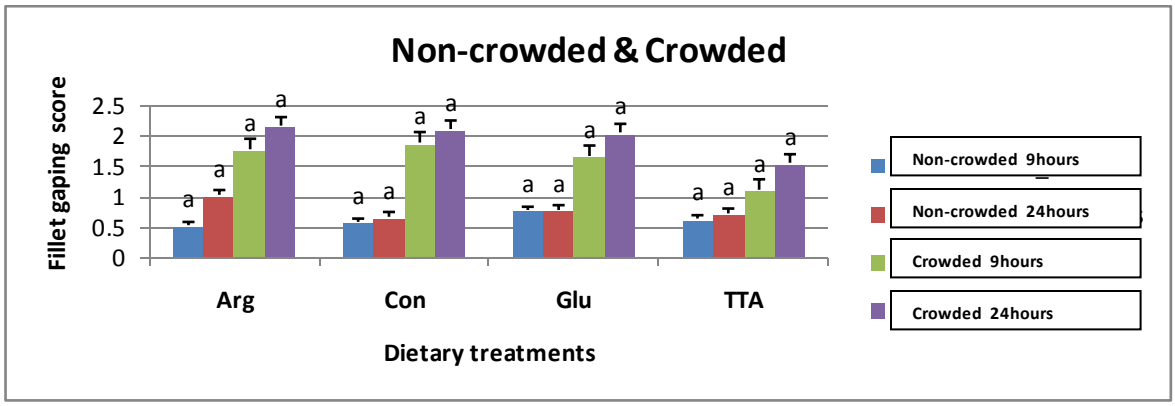


**Figure 32** - Fillet gaping score at 9 and 24 hours ice storage of Atlantic salmon subjected to normal slaughter handling and exposed to crowding stress for 16 hours. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Different letters indicate significant differences between diets within slaughter treatment and time point (P ‹ 0.05).

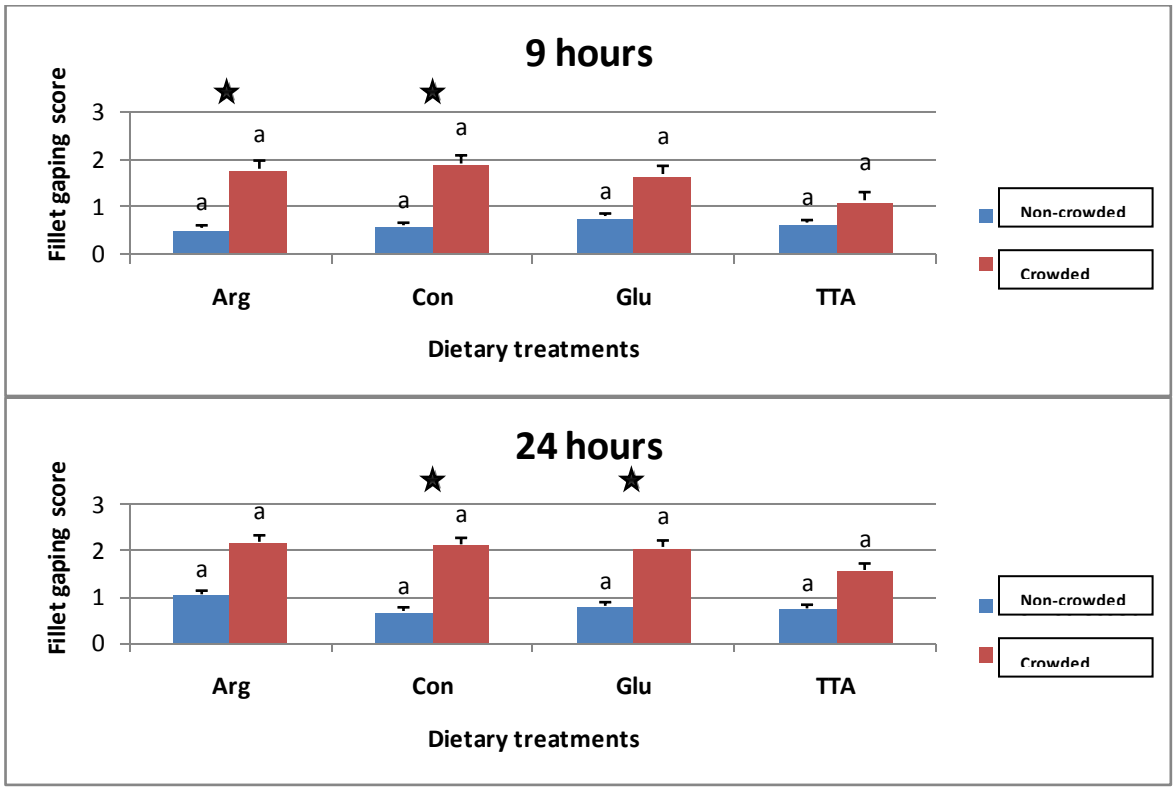


**Figure 33** - Comparison of fillet gaping of non-crowded and crowded salmon after 9 and 24 hours ice storage (★ ) indicates significant differences due to pre slaughter handling within dietary treatment. Different letters indicate significant differences between diets within slaughter treatment (P ‹ 0.05).

## Energy status

Energy status of the salmon fillets was analyzed by determination of pH, Adenosine triphosphate (ATP) and Cathepsins immediately after slaughtering. Post-mortem degradation of ATP and pH development was additionally determined during storage.

## Muscle pH

**Non-crowded fish:** Muscle pH was registered for non-crowded fish from 1 to 72 hours after slaughter. A quick drop in muscle pH occurred between 1 to 6 hours of storage, thereafter it reduced gradually. Significant differences between dietary treatments were obtained after 1, 12, 24 and 72 hours (Figure 34). After 1 and 12 hours, TTA group and after 24 and 72 hours, the arginine group had significantly lower muscle pH compared to the control group.

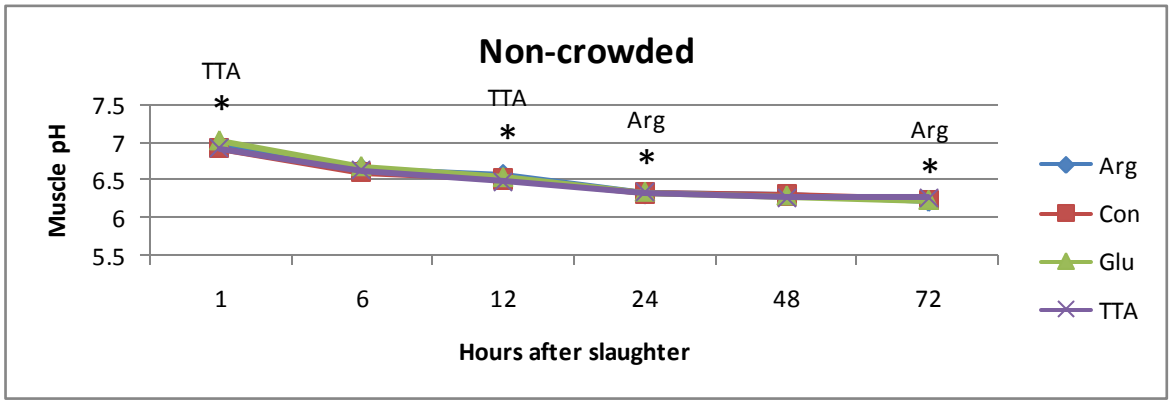


**Figure 34** - Development in muscle pH during 72 hours ice storage of Atlantic salmon subjected to normal slaughter handling. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Time points were significant differences were observed between dietary treatment are indicated by * ($P < 0.05$). Dietary treatments that differed significantly from the Con are denoted above the *.

**Crowded fish:** muscle pH of crowded fish was recorded at 1, 12 and 48 hours after slaughtering. After 1 hour, crowded fish had similar muscle pH as the non-crowded reached after 24 hours. Measurements taken over 12 hours showed that the glutamate and TTA groups had significantly higher muscle pH compared to control (Figure 35).

Comparison of non-crowded and crowded fish showed that after 1 hour they were significantly different, but after 48 hours no significant differences were found between non-crowded and crowded fish from TTA and glutamate groups (Figure 36).

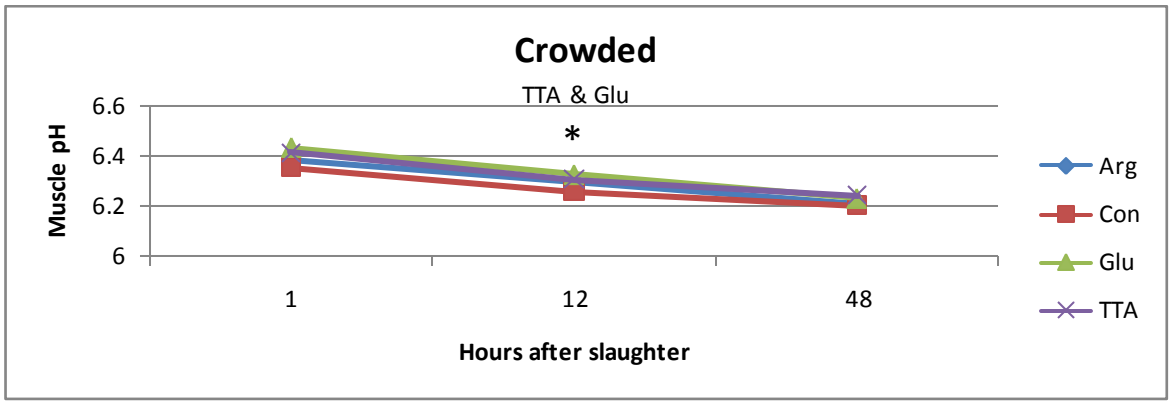


**Figure 35** - Development in muscle pH during 48 hours ice storage of Atlantic salmon exposed to crowding stress for 16 hours. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Time points where significant differences were observed between dietary treatment are indicated by * ($P < 0.05$). Dietary treatments that differed significantly from the Con are indicated above the *.

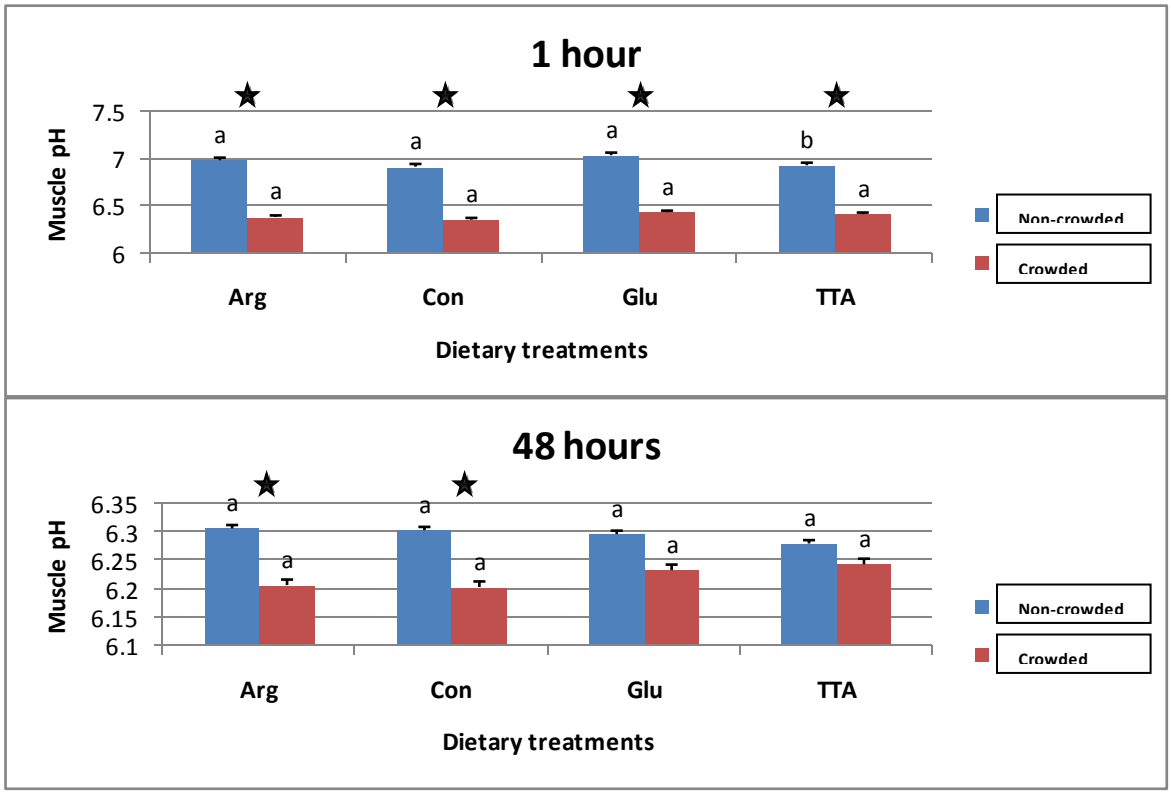


**Figure 36** - Comparison of muscle pH of non-crowded and crowded fish after 1 and 48 hours ice storage ( ★ ) indicates significant differences between pre slaughter handling within dietary treatment. Different letters indicate significant differences between diets within slaughter treatment ($P < 0.05$).

## Adenosine triphosphate (ATP)

The ATP content was analyzed after 1, 12 and 48 hours in both non-crowded (Figure 37) and crowded fish (Figure 38). No significant variation due to dietary treatment was recorded for neither non-crowded nor crowded salmon. The ATP content of non-crowded fish decreased gradually during the storage time, reaching zero level after 48 hours storage. The ATP content in non-crowded fish was higher than of crowded fish at all the registration times. The ATP content of crowded fish dropped quickly from 1 to 12 hours after slaughtering to almost zero level. For both non-crowded and crowded fish, the ATP content was zero at 48 hours.

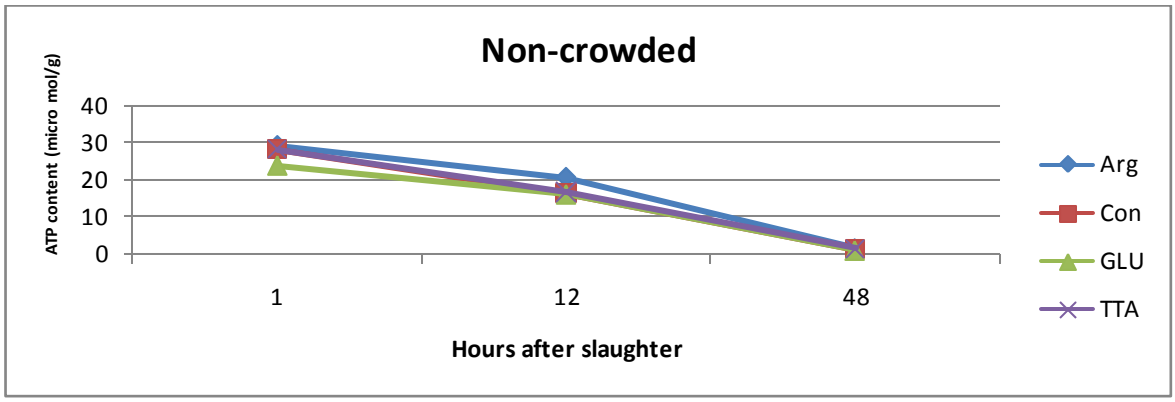


**Figure 37** - Development in ATP content during 48 hours ice storage of Atlantic salmon subjected to normal slaughter handling. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA.


**Figure 38** - Development in ATP content during 48 hours ice storage of Atlantic salmon exposed to crowding stress for 16 hours. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA.

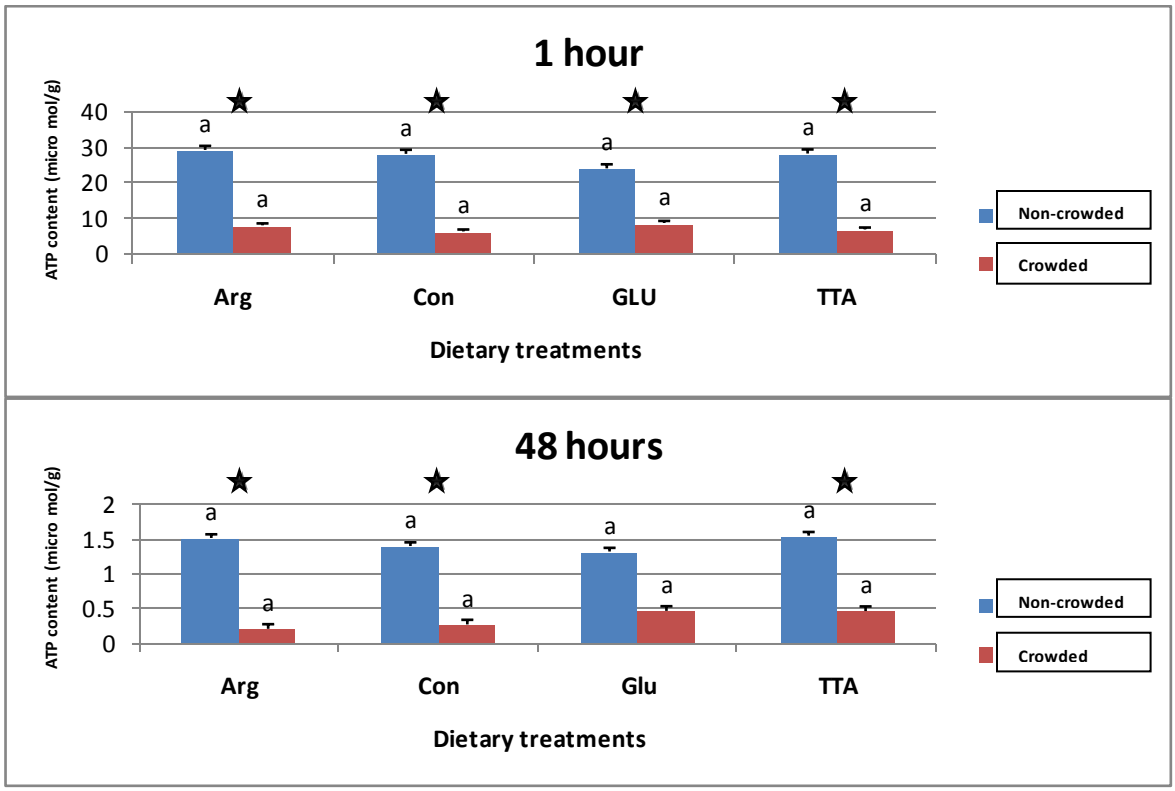


**Figure 39** - Comparison of ATP content of non-crowded and crowded fish after 1 and 48 hours ice storage ( ★ ) indicates significant differences between pre slaughter handling.
Different letters indicate significant differences between diets within slaughter treatment ($P < 0.05$).

Comparison of non-crowded and crowded fish showed that they are significantly different after 1 hour, but after 48 hours no significant differences were observed between non-crowded and crowded fish from glutamate group (Figure 39).

## Cathepsin B, L and B+L activity

The cathepsin B, L and B+L activity of non-crowded fish (Figure 40) didn't differ significantly among the dietary treatments, but for crowded fish cathepsin B activity of arginine, glutamate and TTA group were significantly lower than of the control group. There were no significant variations between the dietary fish groups for cathepsin L and cathepsin B+L activity of the crowded fish (Figure 41).

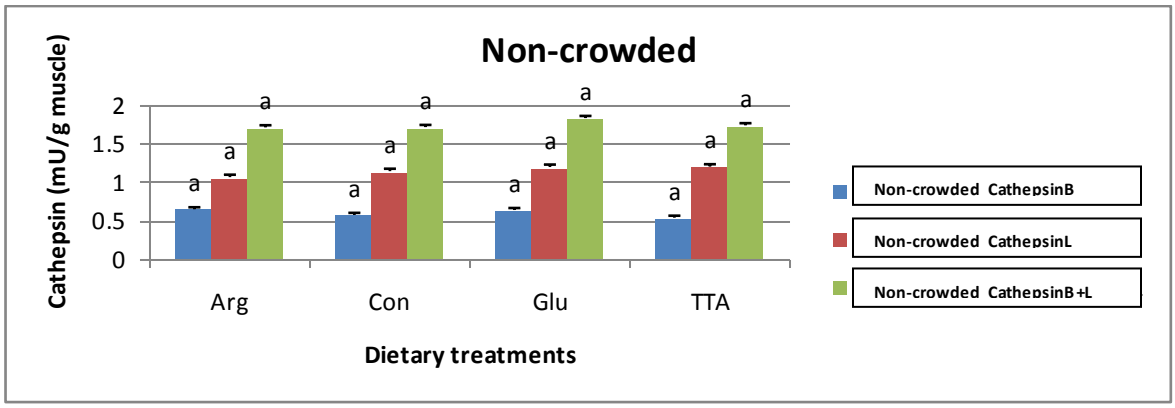


**Figure 40** - Cathepsin B, L and B+L activity of Atlantic salmon subjected to normal slaughter handling. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Different letters indicate significant differences between diets within each parameter, cathepsin B, L and B+L ($P < 0.05$).

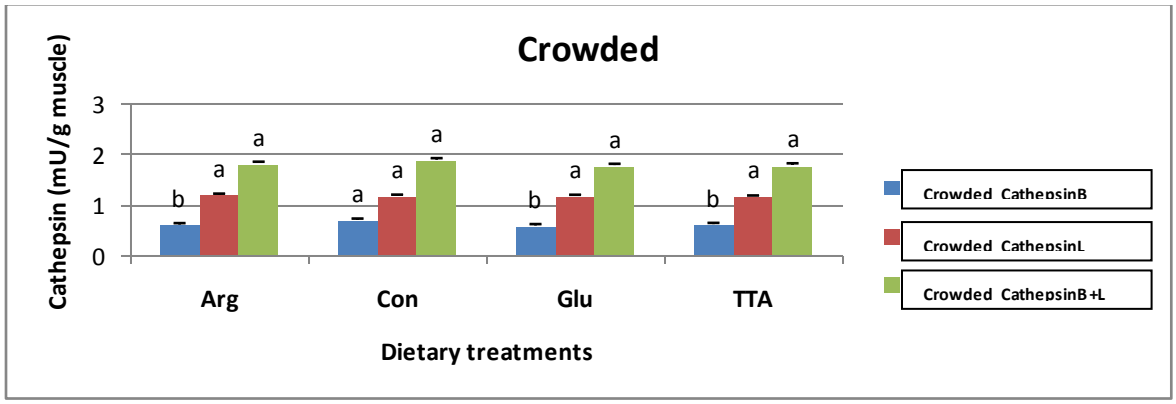


**Figure 41** - Cathepsin B, L and B+L activity of Atlantic salmon exposed to crowding stress for 16 hours. The fish were fed a standard commercial dry feed (Con) or the same feed supplemented with arginine (Arg), glutamate (Glu) or TTA. Different letters indicate significant differences between diets for each of the parameters, cathepsin B, L and B+L ($P < 0.05$).

Comparison of non-crowded and crowded fish showed no significant variations between dietary treatments (Figure 42).

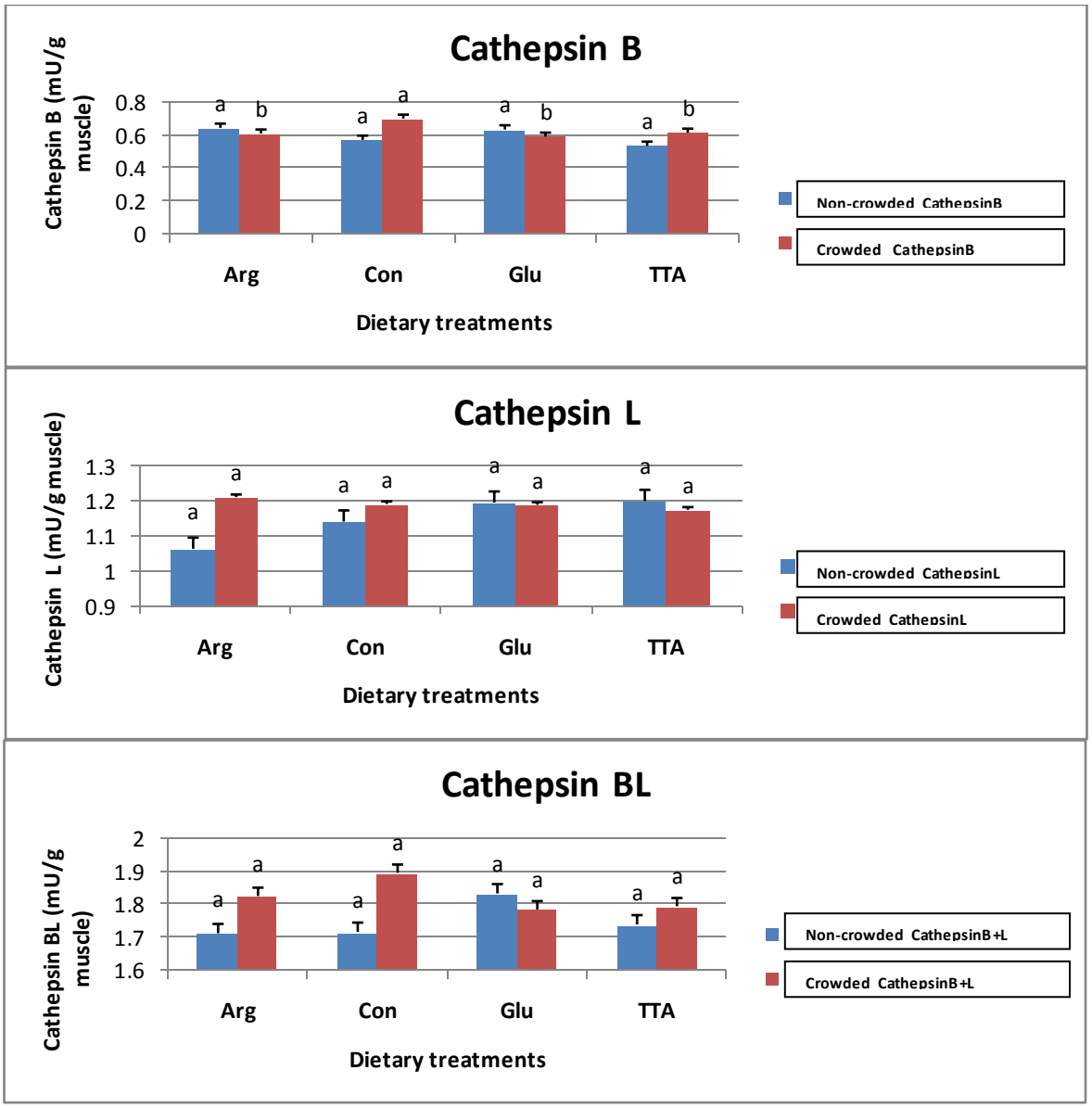


**Figure 42** - Comparison of cathepsin B, L and B+L activity of non-crowded and crowded fish after slaughter. Different letters indicate significant differences between diets within slaughter treatment ($P < 0.05$).

# Discussion

## Growth performance

The present study showed that feeding a diet supplemented with arginine, glutamate or TTA didn't significantly improve the feeding rate, feed utilization or growth of Atlantic salmon reared in sea water during the period April – September (grown from 100 g to approximately 850 g). However, the numerical values showed that the body weight of the salmon fed the arginine diet was 24 g (3%) higher, whereas the salmon fed the TTA diet was 36 g lower (4%), compared with the control. Due to a relatively high variation between net pens within dietary treatment, a higher variation in body weight is required to obtain significant differences between dietary groups. However, in June the body weight of the arginine group was significantly higher compared to TTA group, and in April and May the FCR of the control and arginine group were significantly lower compared to the TTA group. The results therefore indicate that the fish fed the TTA supplemented feed had lower ability to build new tissue of the ingested feed as compared to the other dietary treatments.

Many studies reported the arginine requirement of Atlantic salmon, but most of them were done with small and medium size of fish (up to approximately 400 g), in tanks or under constant environmental conditions. For example Lall et al. (1994) reported an arginine requirement of 17.2 g/kg of dietary dry matter in fish farmed from approximately 100 grams to 300 grams in sea water during eight weeks. In another experiment, Berge et al. (1997) reported an arginine requirement of 21.2 - 21.6 g/kg dietary dry matter based on a growth study where 380 g Atlantic salmon were fed graded levels of dietary arginine from 11.3 to 28.6 g/kg dietary dry matter in tanks for eighteen weeks. In addition, unpublished results from a recent study (Grammes et al. 2010), illustrated that a combination of dietary arginine and glutamate can significantly improve the growth of Atlantic salmon in sea water during periods of high performance and fluctuating environmental conditions. The study carried out under the same conditions and during the same period as the present experiment (from May to September). The arginine content of the control feed and supplemented feed were 22.5 and 30.5 g/kg dietary dry matter, respectively.

It should be mentioned that the level of arginine in the present experiment for control feed was 28.2 and 24.2 g/kg dietary dry matter and for supplemented feed 39.6 and 34.9 g/kg dietary dry matter for pellet size 3.5 mm and 7 mm, respectively. The level of arginine was therefore within the range of requirement reported in earlier studies, also for the control diet. Very little information is available on the requirement of glutamate of salmon diets.

The non significant negative effects of dietary TTA on growth performance in this experiment coincide with studies with Atlantic cod, rainbow trout (Kennedy et al. 2007) and Atlantic salmon (Alne 2009) that showed no significant effect on final body weight or growth rate when the feed was added TTA. However in another study, Moya-Falcon et al. (2004) reported that diet

containing TTA had a negative effect on body weight and the specific growth rate of Atlantic salmon.

## Condition factor (CF)

The CF of all dietary treatments decreased from April to June, for thereafter to increase until September. Significant differences due to dietary treatment were observed in June and September. In June, the arginine group had a significantly higher CF, and in September the glutamate group showed a significantly higher CF compared to the TTA group. Significantly lower CF of the TTA group coincided with reduced growth (June) and high FCR (September).

From previous studies, it is found that the growth of vertebrae and development of muscle mass are two parameters that can control the CF somewhat independently (Einen et al., 1998) and in another study it is documented that CF of salmon show seasonal variation (Morkore and Rorvik., 2001). In the latter study it seemed like the body length increase surpassed body mass increase during late autumn and winter, when the energy demand for maintenance appeared to be high. , Results from the present study likewise indicate impaired ability of the TTA fed fish to synthesize new muscle tissue relative to the growth in body length – when compared to the other experimental diets.

## Hepatosomatic index (HSI) & Cardio somatic index (CSI)

HSI and CSI are defined as the ratio of liver and heart weight to body weight. In this study, significant differences were observed for HSI in June and September. In June, the TTA group had significantly higher HSI compared to the control and arginine group. but in September the TTA group had significantly lower HSI compared to the glutamate group at which time the TTA group had the lowest HSI of all the dietary treatments numerically. The CSI didn't differ significantly between the dietary treatments at any sampling time. It should be noted that the fish in this experiment were fed the TTA diet until they reached a body weight increase 0.2% - approximately until the sampling in June. From June and onwards, the TTA diet was replaced by the control diet.

In the study with Atlantic salmon reported by Moya-Falcon et al. (2004), fish fed 0.6% TTA in the diet likewise reached a significantly higher HSI compared to control diet. Asiedu et al. (1996) showed that "liver mass increased in rats after administration of TTA, and they proposed that the increase was due, at least in part, to an increase in total protein levels and proliferation of mitochondria". Moreover, Demoz et al. (1994) reported that "changes in HSI in rats treated with sulphur-substituted FAs are accompanied by a marked increase in peroxisomal h-oxidation of FAs, as measured by fatty acyl oxidase (FAO) activity".

## Pigmentation

The red flesh color of salmon derives from dietary supplemented carotenoid, which is accumulated as the fish grow (Torrissen et al. 1989; Storebakken & No. 1992). In this experiment the astaxanthin level reduced from April (0.8 mg/kg) to May (0.35 mg/kg), thereafter it increased steadily up to an average level of 3.4 mg/kg in the last sampling (September).

No significant differences were observed between the dietary treatments at any sampling time, but the general variation in astaxanthin level coincides with previous studies, that showed increased pigment retention in spring time with increasing water temperature and reduced feed intake (Ytrestøyl et al. 2006; Rorvik et al. 2009).

## Fat content

The fat content of fillets increased from approximately 1.2% to approximately 14% during the experiment. It rose slowly from April to June where the fillets had approximately 4.5% fat content, thereafter it increased dramatically until the end of the experiment. Significant differences between dietary treatments were recorded in June, July and September. In June and September, the TTA group had the significantly lowest fat content, whereas in July, the arginine group had the significantly lowest fat content among the dietary treatments.

Previous studies have reported the effect of TTA on fat content of fish fillets. A study with Atlantic cod and rainbow trout (Kennedy et al. 2007) showed no significant correlation between TTA and fillet fat content, but in other studies with Atlantic salmon (Moya-falcon et al. 2004; Alne 2009), dietary TTA gave significant reduction of fat in the muscle.

This reduction in fat content can be caused by TTA that is not processed through beta oxidation, but rather stimulates the beta oxidation of other fatty acids in the mitochondria of the cells (Berge and Hvattum. 1994). And it is involved in lipid transport and utilization (Berge et al. 2005). This could suggest that TTA may promote greater fatty acid usage and hence, improved utilization of energy for growth and less accumulation of lipids in the fish body. However, the low CF, growth and FCR of the salmon of the TTA group in June and September is contradicting this hypothesis.

## Fillet contraction

Fillet contraction of non-crowded fish increased after slaughter, reaching approximately 15% after 24 h storage and a maximum contraction of 18% after 72 hours storage. During the whole period, arginine and TTA groups showed less percentage of contraction compared to control and glutamate groups numerically, but no significant differences were observed between the dietary treatments within each time point, except after 9 and 72 hours. After 9 hours, the glutamate

group had significantly higher fillet contraction compared to arginine and TTA groups, but no significant differences were seen between the dietary treatments compared with the control group. After 72 hours, the TTA group showed significantly lower contraction compared to the control group.

The measurements taken from crowded fish during 48 hours after slaughtering showed only significant differences between dietary treatment after 1 hour storage, where the TTA group had significantly higher percentage of contraction compared to arginine group. No significant differences were observed between the control group and other dietary treatments. Furthermore, crowded fish reached a maximum of contraction that was substantially greater than of the non-crowded fish (22%) after 48 hours of slaughter, and high level of contraction (18%) occurred already during the first 6 hours after slaughter. Thus, in the present study, crowded salmon reached a 4% units higher percentage of contraction compared with the non-crowded salmon after 48 hours, and at each time point contraction of crowded salmon was significantly higher from non-crowded fish.

Previous studies have also shown that the onset of rigor for crowded fish is faster than the onset for non-crowded fish (Erikson et al. 1997; Skjervold et al. 1999; Robb et al. 2000; Morkore et al. 2008). In the study reported by Morkore et al. (2008), the percentage of contraction for non-crowded and crowded salmon was approximately 15% and 16%, respectively. Hence, the maximum contraction rate was much greater than previously reported. The reason might be variations in pre-slaughter handling procedures, or the fish size might have affected the results. In the present study the body weight of salmon was 0.9 kg whereas in the study reported by Morkore et al. (2008), the body weight of the fish averaged 3.5 kg.

## Fillet color

Fillet color score of non-crowded and crowded fish were registered from 1 to 48 hours storage after slaughter time. The results showed that the visual color became paler during this period, decreasing slowly from approximately score 24 to score 21 on the *SalmoFan* scale for both non-crowded and crowded fish. Significant differences were observed for non-crowded fish after 12 hours storage, where the glutamate group got significantly higher fillet color score compared to the control group. No significant differences were observed between dietary treatments for crowded fish. In this study, crowded and non-crowded fish showed similar visual color intensity, except for the fillet color of the TTA group where color of the non-crowded fish was significantly paler than of crowded fish after 48 hours storage.

Many studies showed the effect of pre slaughter handling stress on fillet color of fish (Robb & Warriss 1997; Kiessling et al. 2004; Stien et al. 2005), but they found that fillet color became paler for fish exposed to pre-slaughter handling stress compared to fish with normal handling. Thus, the variation pattern in fillet color in the present study did not coincide with earlier results

obtained. However, in previous studies, visual color of the salmon fillets has been recorded in the post-rigor state, while in the present study, fillet color was analyzed during the rigor development. Also the fish in the present study were much smaller than those used in previous studies, thus also the color intensity.

## Fillet gaping

Fillet gaping score was recorded at 9 and 24 hours after slaughter. For both non-crowded and crowded fish, the scores increased numerically from 9 to 24 hours, but significant differences between dietary treatments were observed neither between non-crowded nor crowded fish.

Significant differences were observed after 9 and 24 hours between the non-crowded and crowded fish. Crowded fish had significantly higher gaping score after 9 hours for the arginine and control groups, and after 24 hours for the glutamate and control groups.

Previous studies showed that Gaping is considered to be a post mortem phenomenon (Kestin & Warriss 2001). As acute stress can accelerate post-mortem metabolism (Erikson 2001), it can possibly increase the gaping score of fish fillet (Robb et al. 1999; Einen et al. 2001; Kiessling et al. 2004; Roth at al. 2005).

## Muscle pH

Muscle pH was recorded in non-crowded fish from 1 to 72 hours after slaughter. A quick drop in muscle pH occurred during the first 6 hours after slaughter (from pH 6.9 to 6.6), but after that it reduced slowly, reaching a final level of approximately 6.2. Significant differences between dietary treatments were obtained after 1, 12, 24 and 72 hours storage. After 1 and 12 hours, the TTA group, and after 24 and 72 hours, the arginine group had significantly lower muscle pH compared to the control group.

Muscle pH of crowded fish was recorded at 1, 12 and 48 hours after slaughtering. After 1 hour, the crowded fish had the same level of muscle pH as non-crowded reached after 24 hours (6.3). Significant differences were observed after 12 hours where the glutamate and TTA groups had significantly higher muscle pH compared to the control group.

Comparison of muscle pH of non-crowded and crowded fish showed that after 1 hour, non-crowded fish had significantly higher muscle pH compared to crowded fish. After 48 hours, non-crowded fish from the control and arginine group had significantly higher muscle pH compared to crowded fish, but there were no significant differences between non-crowded and crowded fish fed glutamate or TTA.

In previous studies Einen et al. (2002) and Morkore et al. (2008) also reported that pH for pre rigor fillets decreased from 6.8 to 6.4 after 12 hours. This drop in pH during the early rigor phase is caused by conversion of glycogen to lactate during post-mortem glycolysis in white muscle under anaerobic conditions (Love, 1988). Hence, higher level of lactate due to accelerated glycolysis is causing the lower pH of crowded fish. It is however, interesting to notice that dietary treatment significantly influenced the rate of pH reduction in both non-crowded and crowded fish. It is not possible to give a clear explanation to this variation in pH drop, but it might be that the dietary treatments influenced the buffering capacity of the fish muscle, and/or reflecting higher accumulation of glycogen in the living fish. For example for the salmon fed the arginine feed where the ultimate pH was significantly lower compared with the control in non-crowded fish.

## Changes in Adenosine triphosphate (ATP)

The ATP content in muscle samples of non-crowded and crowded fish during 48 hours storage didn't significantly differ among the dietary treatments. However, the ATP content of non-crowded fish was higher than of crowded fish at all the registration times, and statistical analyses revealed that the ATP content of non-crowded fish was significantly higher compared to crowded salmon after 1 hour and after 48 hours storage in this experiment.

The results of this experiment showed that the ATP content depleted at 48 hours after slaughter. These results do not coincide with earlier results obtained by Thomas et al. (1999), who showed a depletion of ATP after 24 hours, but they coincide with results reported by Morkore et al. (2008) who also found ATP depletion after 48 hours storage. The results obtained in this study are according to previous studies (Thomas et al. 1999; Morkore at al., 2008) that found accelerated ATP depletion in crowded fish compared with non-crowded fish pre-harvesting.

## Cathepsins activity

The cathepsin B, L and B+L activity of non-crowded fish showed no significant variations among the dietary treatments, but for crowded fish cathepsin B activity of arginine, glutamate and TTA groups were significantly lower than control group and there were not any significant variations for cathepsin L and cathepsin B+L activity of crowded fish. Comparison of cathepsins activity of non-crowded and crowded salmon also showed no significant differences.
Previous study with Atlantic salmon (Bahuaud et al. 2009) that analyzed the cathepsin B, L and B+L activity of fish based on pre slaughter handling showed that pre slaughter crowding stress seemed to result in higher cathepsin B + L activity in the muscle, although the cathepsin activity was not significant from non-crowded salmon. The higher activity was observed for cathepsin B activity, but not for the cathepsin L activity.

In salmonids, cathepsin B and cathepsin L activity have been associated with muscle softening, with an optimum pH for activity of 6.0–5.5 and 5.6, respectively (Aoki & Ueno. 1997; Godiksen et al. 2009; Yamashita & Konagaya. 1990). In the same studies, a significant negative correlation was found between muscle pH and level of cathepsin B + L activity. In the present study, crowding stress resulted in lower muscle pH immediately post-mortem compared with fish exposed to non crowding stress. The lower pH could be directly responsible for the indications of an earlier higher activity of cathepsin B+L in the muscle of the stressed salmon.

# Conclusion

The level of dietary supplementations of arginine, glutamate or TTA had no significant positive effects on feeding rate, feed utilization or growth in Atlantic salmon. The present study was not designed to established level of amino acid requirement, but it evaluated the growth of fish with above assumed level of arginine and glutamate during the critical period after sea transfer. However the fish fed arginine had higher body weight and growth compared to other dietary treatments. Additionally, no negative effect of TTA was observed on growth performance although, fish fed TTA had lower body weight, growth and higher FCR compared to other dietary groups. It was demonstrated that hepatosomatic index of fish fed TTA diet was higher than other dietary treatments until it was replaced by control diet, whereas it was the lowest at the end of experiment. The positive effect of TTA has also shown on reducing the fillet fat content.

The positive effects of dietary treatments were also observed during rigor development and post-mortem energy metabolism for both non-crowded and crowded fish, especially on rigor contraction and fillet color of non-crowded and muscle pH and cathepsin B activity of crowded fish after slaughter.

The present experiment demonstrated that the rigor contraction, muscle pH, fillet gaping and ATP content of salmon was significantly altered by crowding stress.

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