Influence of nutritional factors on muscle development and texture of Atlantic salmon (Salmo salar L.). In vivo and in vitro studies.

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INFLUENCE OF NUTRITIONAL FACTORS ON MUSCLE DEVELOPMENT AND TEXTURE OF ATLANTIC SALMON (SALMO SALAR L.).

IN VIVO AND *IN VITRO* STUDIES.

Master Thesis in Aquaculture

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by

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DEDICATION

I would feel pride to dedicate this thesis to my philanthropic family; my affectionate parents, loving and caring sisters and brothers, my sweet bhabi and last but not least, my lovey-dovey fiancée.

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Before all else, I thank Almighty Allah for His infinite mercy to render me alive to see this day. I thank to Allah for all the strengths and abilities He has given to me to come with this write up. I also offer my humblest thanks to our Holy Prophet Muhammad (Peace Be Upon Him), the fountain of knowledge and forever torch of guidance for humanity.

This period of master study has been a real adventure, not only as a professional experience but also as an extremely rich human exposure. Startup of something new is always a challenge and even more challenging in a completely new environment. During this period of my life I have seen the world in a scenario that I have never seen before. Beside this academic experience I have learnt the life in a more critical and practical way. Thanks to all of you who participated in my encouragement, made me laugh, shared your laughs as well as the scientific knowledge with me.

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SUMMARY

The aim of the present study was to examine the effect of amino acids or bioactive fatty acid on the Atlantic salmon flesh quality with main emphasis on the muscle development and fillets texture by *in vivo* and *in vitro* strategies. For the *in vivo* study, the fish were fed a commercial extruded dry feed i.e. control (Con) diet or the same diet supplemented with tetradecylthioacetic acid (TTA), arginine (Arg) or glutamate (Glu) for a period of five months. A total of 12 net-pens with triplicate randomly assigned net pens of each diet were setup. A total of 108 salmon (nine fish per net-pen) were used, whereof two subgroups were made according to pre-slaughter handling: non-crowded, NC (harvested using normal procedure, *n=72*) and crowded, C (exposed to crowding stress for 16 hours before slaughter, *n=36*). Parameters studies included fillet contraction, muscle pH, texture, cathepsin B, L and cathepsin B+L, and histological analysis. For the *in vitro* study, fifty Atlantic salmon parr with an average length of 5-7cm were used for isolation of myosatellite cells. Three experimental treatments, arginine (Arg), glutamine (Gln) and tetradecylthioacetic acid (TTA), in addition to one control (Con) treatment were made and supplemented to the isolated myosatellite cells that were incubated at two different temperatures, either 8°C (10 days) or 16°C (7 days) for PCNA and qPCR analysis. In the PCNA assay, proliferation percentage of blue and brown cells nuclei was performed. In qPCR study, two muscle genes, myogenin (regulatory) and myosin light chain 2 (structural) were selected, and in addition also two genes for the proteases cathepsin B and cathepsin L.

In the *in vivo* study, Arg and TTA diets in NC group and only Arg diet in C group showed a tendency towards lowering the fillet contraction. Muscle pH was significantly increased by Glu diet in C group but unfortunately, pH was significantly lowered by Arg and TTA in NC group. Moreover, Arg and Glu diets showed more firm fillet texture in both NC and C groups whereas TTA diet had only in C group. Data from histology revealed higher tendencies by Arg and Glu diets in increasing the cell numbers of NC and C groups respectively, whereas significantly lower tendency by TTA diet in NC group and vice versa. Further, analysis of cathepsins showed that only the activity of cathepsin B is influenced by the Arg, Glu and TTA diets. Pre-slaughter crowding stress demonstrated significantly negative effect on fillet contraction, muscle pH, as well as the texture. In the *in vitro* study, results from PCNA indicated higher proliferation of muscle cells by Arg and Gln treatments at 8°C and 16°C respectively, whereas a significantly lower proliferation by TTA treatment at both temperatures was observed. Relative gene expression from qPCR analysis

showed an up-regulation of gene expression of myosin light chain2 and myogenin by Arg, Gln and TTA treatments, while no effect of any treatment on gene expression of cathepsin L was found. However, TTA treatment showed a significantly lower expression of cathepsin B at 8°C. In addition, only the expression of cathepsin L was found significantly different between temperatures.

Keywords: *Fish quality, amino acids, bioactive fatty acid, crowding stress, rigor, pH, texture, cathepsins, in vitro myosatellite cells, myogenesis, gene expression.*

TABLE OF CONTENTS

LIST OF ABBREVATIONS

Con	Control
Arg	Arginine
Glu	Glutamate
Gln	Glutamine
TTA	Tetradecylthioacetic acid
PIPES	Piperazinediethanesulfonic acid
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
FBS	Fetal Bovine Serum
BSA	Bovine Serum Albumin
PBS	Phosphate Buffered Saline
HEPES	Hydroxyethylpiperazineethanesulfonic acid
RNA	Ribonucleic acid
cDNA	complimentary Deoxyribonucleic Acid
DEPC	Diethylpyrocarbonate
MRF	Myogenic Regulatory Factors
IGF	Insulin like Growth Factor
C	Crowded
NC	Non-Crowded

LIST OF FIGURES

LIST OF TABLES

1. INTRODUTION

Aquaculture is one of the modern, internationally competitive and perhaps the fastest growing animal production sector in the world, particularly in Norway since 1970s. Atlantic salmon and rainbow trout are the dominating species farmed in Norway. Atlantic salmon is consumed preferably due to its high Omega-3 fatty acids, high protein and high vitamin D content (NIH).

In the recent years consumers have put very high demands on their food. It ought to be healthy, natural and most important should present pleasant appearance, texture, odor and taste (Drobna et al., 2006). Quality is usually evaluated by instrumental or chemical analysis; however, it may be differently perceived and defined depending on the end users (Rødbotten, 2009). Quality in fish can be influenced by several elements, such as breeding (Gjedrem, 1997), feed composition (Thomassen and Røsjø, 1989; Bell et al., 2002; Torstensen et al., 2008), pre-slaughter stress (Sigholt et al., 1997; Roth et al 2009), transport (Erikson et al., 1997), filleting methodology (Roth et al., 2009), storage temperature (Hansen et al., 2007), packaging (Bahuaud et al., 2008) etc.

The muscle is often the main part of the fish, favored by the consumers (Kiessling et al., 2001) and muscle integrity therefore influences the quality characters like sensory quality and texture. Texture is one of the most important quality parameters form consumers point of view as many fish species do not present a strong flavor (Hyldig and Nielsen, 2001). Soft texture and fillet gaping are the major causes of downgrading of Atlantic salmon (Mitchie, 2001). It is investigated that pre-slaughter handling stress greatly disturbs the textural properties of fish (Bagni et al., 2007; Lefevre et al., 2008; Roth et al., 2009). Moreover, the number and size distribution of muscle fibres is an important characteristic of flesh texture (Hatae et al., 1984) as there is a significant correlation between fibre size and the total area (Mørkøre et al., 2009).

Intensively farmed fish exposed to a number of acute and chronic stress. The stressor can be biological, environmental or physiological. Level of stress over time affects growth, reproduction, immunocompetence and meat quality (Erikson et al., 1997; Skjervold et al., 1999). Direct mechanical stress on texture (myofibrils and connective tissue), cause the release of proteases, and these proteases could participate in degrading the muscle structure (Roth et al., 2006). Pre-slaughter stress makes the fillet difficult to process due to earlier onset of rigor (Kiessling et al., 2004; Morkore et al., 2008). An initial low pH after *post-mortem* is the prominent indicator of stress as well as negative element for texture (Poli et al., 2005; Bagni et al., 2007).

Use of amino acids in aquaculture feed is not infancy. Amino acids and their metabolites are necessary for the maintenance, growth, feed intake, nutrient utilization, immunity, behavior, larval metamorphosis, reproduction, as well as resistance to environmental stressors and pathogenic organisms in various fishes (Li et al., 2009). It has been ascertained too that, beside the cell signaling molecules, amino acids are regulators of gene expression and protein phosphorylation cascade (Wu, 2009). Amino acids may be beneficial in improving fillet taste and texture (Li et al., 2009).

Due to general shortage of marine sources for aquaculture feed particularly salmon, researches on alternative fatty acids (vegetables) are in focus (Madsen et al., 2002; Moya-Falcon et al., 2004; Kennedy et al., 2007). Tetradecylthioacetic acid (TTA) is a bioactive fatty acid which belongs to the family of 3-thia fatty acids and contain sulphur atom at third position from carboxyl terminus (Kennedy et al., 2007). TTA has been reported to modulate the expression of several important genes (Kleveland et al 2006; Gjøen et al., 2007; Kennedy et al., 2007), reduce body fat (Madsen et al., 2002; Wensaas et al., 2009) has great influence on inflammatory processes (Fredriksen et al., 2004; Bivol et al., 2008; Alne et al., 2009). Increased use of the vegetable fatty acids in fish feed cause fat deposition in fish (Roselund et al., 2001). Due to the marvelous effect of TTA on lipid metabolism (Kennedy et al., 2007), TTA could be used as a beneficial tool in increasing the product quality.

The present study was taken into consideration in order to investigate the effect of amino acids (arginine (Arg), glutamine (Gln), and glutamate (Glu)) and bioactive fatty acid, tetradecylthioacetic acid (TTA) on the muscle development as well as the fillets texture of Atlantic salmon. The main goals of the study were based on two different approaches,

- Firstly, to observe the *in vivo* effects by dietary supplementation of the TTA, Arg and Glu on mechanical properties and muscle cells morphology of Atlantic salmon fillets.
- Secondly, to observe the *in vitro* effects by supplementing the TTA, Arg and Gln on growth pattern of isolated myosatellite cells morphology and gene expression of selective genes related to muscle development of Atlantic salmon.

The overall objective was to test the hypothesis that dietary supplementation of Arg, Glu, Gln and TTA will have beneficial effects on muscle cell development, hence texture on Atlantic salmon fillets.

2. THEORETICAL BACKGROUND

The background consists of four main sections. The first section gives a general introduction and discussion of fish quality and flesh texture. The second section focuses mainly on the muscle structure, muscle contraction, pH, and lysosomal enzymes (cathepsins). The third section include the myogenesis and important genetic regulatory pathways involved in the paradigms of muscle development, whereas in the final section, physiological aspects of different bioactive components used in the present study and a brief overview of *in vitro* cell culturing is discussed.

2.1. Fish quality

The term quality means different things to different people. Hence, quality is a complex terminology and can be defined in a number of ways based on who and for what is being defined, such as consumer-based *"Quality is the degree to which a specific product satisfies the wants of a specific consumer"* (Gilmore, 1974), manufacturing-based *"Quality is the degree to which a specific product conforms to a design or specification"* (Gilmore, 1974), product-based *"Quality refers to the amounts of the unpriced attributes contained in each unit of the priced attribute"* (Leffler, 1982), value-based *"Quality is the degree of excellence at an acceptable price and the control of variability at an acceptable cost"* (Broh, 1982). According to the standard ISO 8402 Quality is defined as:

"The totality of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs" (http://www.fao.org/fishery/topic/1521/en).

Considering fish products, quality actually relates to gastronomic delights, purity, nutrition, safety, consistency, fairness, value and product excellence. This means “Quality” have a multifaceted definition. The term quality is generally categorized into 5 sub-categories:

- Sensory quality; the quality presents the properties of what our sense organs perceive like odor, color, taste, appearance, texture etc.
- Nutritional quality; the quality presents the health promising values like protein content, lipid content, lipid composition, vitamins, minerals etc.
- Hygienic quality; the quality presents the contamination status of the product like microorganisms, heavy metals, antibiotics etc.

- Technological quality; the quality presents the ability of product to satisfy the processing test like water holding capacity, pH, fats (saturated or non-saturated), content of connective tissue etc.
- Ethical quality; the quality presents consumers expectations and standards in accordance with the fish products like food security, animal welfare, handling of animal before slaughtering etc.

During the last decades, focus on food safety is highlighted tremendously due to more knowledge in general among the consumers. Knowledge of how consumers evaluate the quality of fish is thus necessary in order to identify and describe valid quality indicators. Several studies have been focused on consumer preferences of fish (Wandel & Bugge, 1997; Verbeke et al., 2007; Rødbotten et al., 2009). According to Peri (2006), quality can be defined as the requirements necessary to satisfy the needs and expectations of the consumers. Consumers are interested more towards taste, freshness, physical appearance, nutritional value and food safety of the product. However, the focus of interest among consumers may vary in different societies and at different times (Wandel & Bugge, 1997).

The main characteristic of fish quality is often associated with freshness (Sveinsdottir, 2003). Quality can be measured by various means like chemical analysis (Kent et al; 2004), instrumental analysis (Macagano, 2005; Casas et al., 2006; Morkore et al., 2009) and or sensory descriptive analysis (Gonzalez-Fandos, 2005). Numerous kind of factors are involved in influencing the product quality of fish both by *pre-mortem* and *post-mortem*; such as breeding (Gjedrem, 1997), feed composition (Thomassen and Røsjø, 1989; Bell et al., 2002; Bransden et al., 2003; Rora et al., 2005; Drobna et al., 2006; Torstensen et al., 2008; Pratoomyot et al., 2008), feeding strategies (Einen, 1999), handling and pre-slaughter stress (Sigholt et al., 1997; Thomas et al., 1999; Poli et al., 2005; Bagni et al., 2007; Roth et al 2009), transport (Erikson et al., 1997), slaughtering method (Roth et al., 2009), storage temperature (Sigholt et al., 1997; Hansen et al., 2007) and seasonal variations (Johnston et al., 2004).

2.2. Flesh texture

Fillet texture is one of the most important quality parameters of fish for producers, processors and consumers. Many fish species do not bear a sound flavor and therefore texture becomes most important for consumers acceptability (Hyldig & Nielsen, 2001). Important flesh

quality attributes are nutritional value, safety, flavor, color, preservation and processing characteristics of fillets (Haard, 1992). Soft texture and fillet gaping are the major causes of downgrading of Atlantic salmon (Mitchie, 2001).

Flesh quality in fish is becoming more and more meaningful for the industry for processing purposes (Haard, 1992; Gjedrem, 1997) as well as a valued sensory characteristic for consumers (Haard, 1992). Texture is a very complex sensory phenomenon and there is no agreement on how to define it, however, a variety of definitions are available in the literature. For instance, Szczesniak (1963) defined texture as "combination of the physical structure of the food and the characteristics of the food during mechanical treatment". Guinard and Mazzucchelli (1996) summarized texture and mouthfeel of foods and beverages as multi-parameter qualities "evaluation in the mouth is a highly dynamic process in which physicochemical properties of the food are continuously altered by chewing, salivation, potentionally, body temperature. A variety of mechanoreceptors embedded in the tongue, palate, gums, periodontal membrane, and muscles and tendons of the jaws is involved in the perception of texture and mouthfeel".

Texture is often expressed in terms of flesh firmness and can be measured either by instrumental or by sensory analysis. The main instrumental techniques used to measure the texture in fish are puncture, compression, shear and tensile techniques. Instrumental measurements are favored over sensory evaluations since instruments may minimize variation among measurements due to human factor and are more precise (Abbott, 1997 cited by Casas et al., 2006). Moreover, firmness is irregular along the whole length of fillet (Casas et al., 2006).

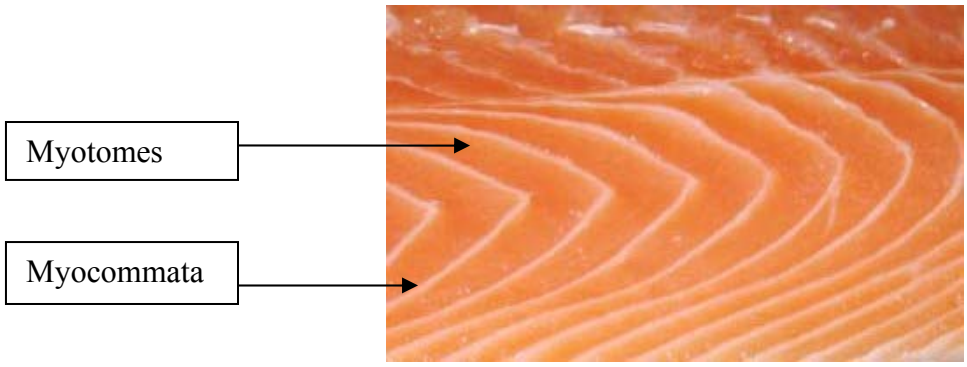

Fig. 2.1. Myotomes (muscle blocks) and myocommata (connective tissue) in Atlantic salmon flesh. (Accessed form http://www.mstevensandson.co.uk/shop/product_info.php?products_id=127).

Flesh texture can be influenced by several factors for instance, starvation before slaughtering (Mørkøre et al., 2008), fillet processing methods and storage temperature (Sigholt et al., 1997; Skjervold et al., 2001; Roth et al., 2009), *post-mortem* processing techniques (Veiseth-Kent et al., 2010), pre-slaughter handling stress (Bagni et al., 2007; Lefevre et al., 2008; Roth et al., 2009) and fish species, harvesting season, photoperiod regimes (Johnston et al., 2004; Espe et al., 2004; Hagen et al., 2007). *Post-mortem* factors influencing texture include glycolysis, pH and rigor mortis.

The number and size distribution of fibres is referred to as muscle cellularity and thought to be an important characteristic of flesh texture (Hurling et al., 1996). In fish, flesh is constituted of adjacent muscle blocks of myotomes which are separated by sheets of collagenous tissue called myocommata (Hyldig & Nielsen, 2001; fig. 2.1). The connective tissue forms a supporting network through the whole fish fillet and textural properties seem to be dependent of chemical composition and structural properties, in particular to the myofibril and connective tissue proteins. These two components, thus predict the overall picture of the texture. However, texture varies by muscle region, species and fibre distribution of the muscle (Hatae et al., 1984 and 1990). The strength of raw salmon muscle is higher when the fibre diameter is smaller as there is a positive correlation between fibre diameter and sensory firmness of fish (Hatae et al., 1990; Hurling et al., 1996). Lower number of muscle fibres decreases the sensory score for firmness, chewiness and mouthfeel (Johnston et al., 2000). Flesh texture, thus seems to be a multifactorial and complex sensory property rather than a physical structure based only on the muscle fibre.

2.3. Atlantic salmon muscle structure

Fish muscle structure holds water, protein and other nitrogenous compounds, lipids, carbohydrates, vitamins and minerals. However, the chemical composition varies from species to species and even among fish of same specie depending on the age, sex season and environment. Generally, the fish muscle contains 66-81% water, 16-21% protein, 0.2-25% lipid, <0.5% carbohydrates and 1.2-1.5% ash (FAO, 2005). The majority of fish skeletal muscle comprises more than 50% of the whole body mass.

The skeletal muscle of fish differs from those of mammals and birds due to short bundles of myotome (muscle blocks) and thin layers of myocommata (connective tissue), and this unique structure in turn gives the fish meat a soft flaky texture (Britannica.com). The skeletal muscle of

fish can be divided into two main fibre types, red and white. These two fibre types differ greatly in physiologically, biochemically and in organization (Kilarski, 1967; Johnston et al., 1972). The red aerobic fibres are responsible for the slow locomotion while white anaerobic fibres are for the agility.

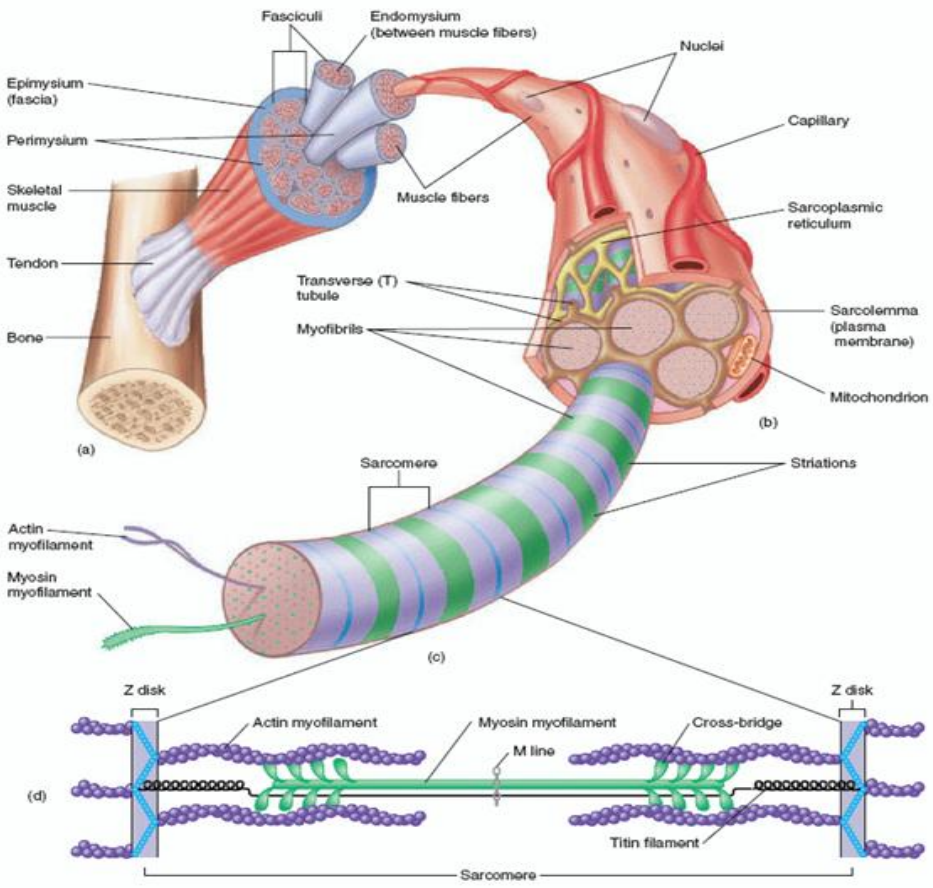

Fig. 2.2. A top to down organization of skeletal muscle in vertebrates. (a) Whole skeletal muscle (b) Single muscle fibre (c) Single myofibril (d) Contractile filaments actin and myosin. (Accessed form http://www.shoppingtrolley.net/skeletal%20muscle.shtml

An ultrastructure of skeletal muscle reveals a complex pattern of organization (fig. 2.2). Whole muscle when seen in cut shows that they are covered by a layer of connective tissue called epimysium. Looking further at a cross section view, it becomes visible that skeletal muscle consisits of bundles of muscle fibres called fasciculi which are surrounded by another connective tissue called perimysium. Each fascicule contains several numbers of muscle fibres. A detailed view on muscle fibres reveals that they too are covered by a layer of fibrous connective tissue called endomysium. Beneath this muscle fibre is the plasma membrane called sarcolemma (cytoplasm of cell, sarcoplasm, sarcoplasmic reticulum and smooth endoplasmic reticulum). In each muscle fibre

there are several myofibrils. Finally, these myofibrils hold several bundles of myofilamets called actin (thin filament) and myosin (thick filament), which are the fibres responsible for the contraction and relaxation process.

Considering the fish quality, *post-mortem* tenderization of the fillet is an important parameter needs to understand. It has been determined that deterioration of muscle is due to the proteolytic degradation of minor components linking the structural units together (Olafsdottir et al., 1997). It has been investigated that breakages in the muscle cell cytoskeleton and connective tissue i.e. myofibre-myofibre and myofibre-mycommata detachments, are the main causes of *post-mortem* fillet tenderization (Bahuaud et al., 2008). Degradation of the extracellular matrix also contributes to the tenderization phenomenon (Taylor et al., 2002). In addition, mechanical stress on the muscle fibre or connective tissue is another participant in the softening of the muscle structure, thereby causing the release of proteases (Roth et al., 2006). Low post-mortem pH during pre-slaughter stress also has an indirect effect (by accelerating the activity of proteases) on muscle structure shape (Bahuaud et al., 2010). Further, low muscle pH or mechanical stress causes the provoked shrinkage of myofibrils by increasing the tensions in connective tissue and resulting in the acceleration of overall muscle structure (texture) degradation (Bahuaud et al., 2010).

2.3.1. Muscle contraction and pre-slaughter stress

Rigor mortis means the stiffening of the muscle of animals shortly after death. The most dramatic change immediately post-mortem is onset of rigor mortis. Right after death muscle is totally relaxed and soon after it becomes stiff and inflexible which indicates the rigor mortis condition (Huss, 1995). Rigor mortis starts immediately or shortly after death if the fish is starved and the glycogen reserves are depleted or if the fish is stressed (Huss, 1995). Rigor development is generally dependent of adenosine tri-phosphate (ATP) level in the muscle, the species, storage and water temperature, handling and biological status (Huss, 1995; Elvevoll et al., 1996) and stocking densities of the fish (Skjervold et al., 1999). Moreover, the onset of rigor is dependent of the red and white muscle, as rigor development is slower in white muscle compared to red muscle (Kobayashi et al., 2004).

It is known that handling stress prior to slaughtering (Sigholt et al., 1997) and pre-slaughter crowding stress (Bahuaud et al., 2010) affect the fish fillet quality. Moreover, crowding the fish is a stressor and this has been proved by the study of Einarsdottir and Nilssen (1996). A schematic

diagram indicating the relationship between stress and other parameters is illustrated in figure 2.3. Onset of rigor mortis is generally used as an indicator of *pre-mortem* stress (Nakayama et al., 1992) and meat quality (Stroud 1969). After *post-mortem* fish usually derives energy anaerobically, and this results in a lowered post-mortem muscle pH (Thomas et al., 1999; Poli et al., 2005; Bagni et al., 2007) and rigor development (Erikson et al., 1997). This low pH ultimately deteriorates the muscle quality (Nakayama et al., 1996; Sigholt et al., 1997). Pre-slaughter stress cause an earlier rigor onset that makes difficult to process pre rigor fillets (Skjervold et al., 1999; Kiessling et al., 2004; Morkore et al., 2008). Furthermore, increased fillet gaping, fillet softening, change in skin and fillet color and increased drip loss are the principal indicators caused by stress (Skjervold et al., 2001; Kiessling et al., 2004; Roth et al., 2006; Morkore et al., 2008).

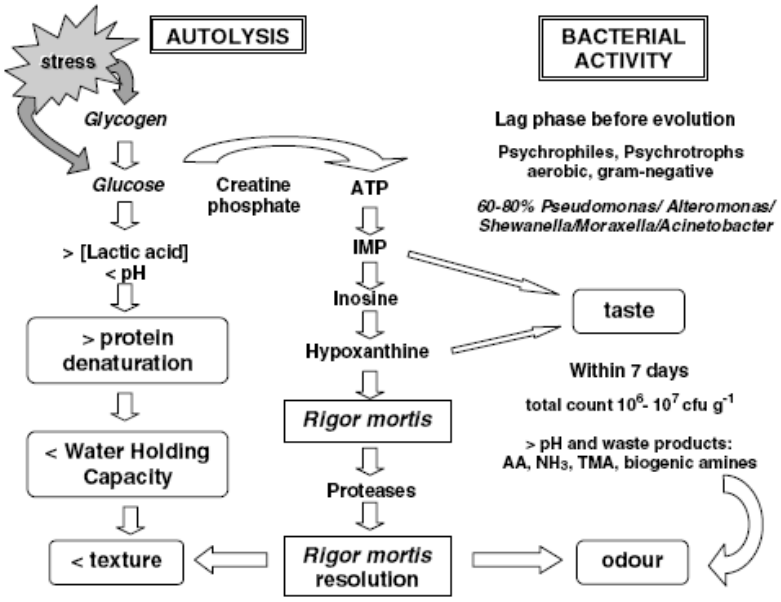

Fig. 2.3. Relationship of stress and the quality parameters. Adapted from Poli et al (2005).

2.3.2. Muscle pH

After harvesting, muscle passes through several changes such as rigor mortis, dissolution of rigor mortis, autolysis and bacterial spoilage. The occurrence of these changes is mainly due to breakdown of cellular structures. Within these *post-mortem* changes, protein degradation, ATP degradation, drop of pH, lipid oxidation, production of undesirable compounds like trimethylamine have strong impact on product quality (Ocana-Higuera et al., 2009). Under stressed or exhausting conditions, white muscle mainly derives energy from glycogen reserves anaerobically and this in turn results in the production of lactic acid. This production of lactic acid, therefore, depends on the

nutritional status and amount of stored glycogen in living tissue (Huss, 1995). A brief overview of the energy production post-mortem is illustrated in figure 2.4.

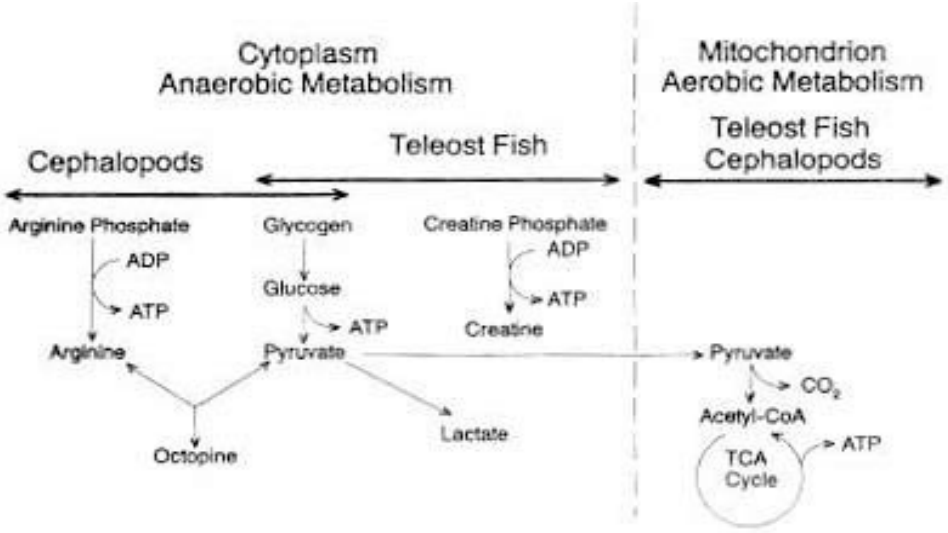

Fig. 2.4. Aerobic and anaerobic breakdown of glycogen in cephalopod and fish muscle. Adapted from (Huss, 1995)

Muscle pH of the unstressed Atlantic salmon immediately *post-mortem* fall in the range of 7 (Hansen et al., 2007). The reduction rate in the *post-mortem* pH has profound effect on physical properties of muscle, such as water holding capacity, texture etc (Huss, 1995). It is estimated that greater muscle activity and pre slaughter stress results in ultimate low *post-mortem* muscle pH (Erikson et al., 1997; Thomas et al., 1999; Poli et al., 2005; Bagni et al., 2007). Temperature is one of the main factors that causes changes in *post-mortem* pH, as a moderate temperature in early *post-mortem* produce a slow decline in pH (Bruce & Ball 1990). But, these results presents a contradiction as no effect of temperature on muscle pH was recorded by Sigholt et al. (1997).

2.3.3. Muscle lysosomal cathepsins

Lysosomes are organelles that contain a variety of enzymes, where main class of lysosomes is proteases such as cathepsins. Cathepsins are divided into three main groups based on the amino acid of their active site that confers the catalytic activity, namely cysteine (cathepsins B, C, F, H, K, L, N, O, S, T, U, Wand X), aspartyl (cathepsins D and E) and serine (cathepsins A and G) (Tardy et al., 2006). Out of all of these, cathepsin B and L are of great interest as they are suspected to cause post-mortem softening of muscle (Yamashita & Konagaya, 1991). Both cathepsin B and L seem to degrade the muscle proteins. For instance, Yamashita & Konagaya (1991) proposed that cathepsin

B mainly hydrolyzes the connectin, nebulin and myosin, whereas cathepsin L together with connectin, nebulin and myosin also degrade the α-actinin and troponin T and I.

Recently, it was suggested that cathepsin B and L cause the major degradation of the extracellular matrix and breakage in muscle cell cytoskeleton and connective tissue (Bahuaud et al., 2009). The degradation activity of cathepsin B and L is associated with the pre-slaughter condition such as super chilling, short and long term crowding stress (Bahuaud et al., 2008 and 2010) and the feed fed to the fish (Bahuaud et al., 2009). Further, there is a positive correlation between muscle pH and cathepsin B and L activity (Bahuaud et al., 2010). Relative gene expression of cathepsin B and L is also linked to pre-slaughter stress and feed. Pre-slaughter crowding stress significantly boosts the expression of cathepsin L (Bahuaud et al., 2010), whereas feed has a significant impact on expression of cathepsin B (Bahuaud et al., 2009).

2.4. Myogenesis

The basic and earliest events of myogenesis in all vertebrates and fish in particular, are the specification of stem cells to myoblasts, proliferation, cell cycle exit, differentiation, migration and fusion (Johnston, 2006). The final event of myogenesis is the formation of myofibrils, which is accompanied by expression of the structural genes and synthesis of specific proteins of the contractile system (Ozernyuk et al., 2004). Two main phases of muscle development can be discerned based on the life cycle of the fish. First phase happens in yolk sac larval stage, when inner white and outer red muscle zones develop, and second phase happens in free swimming larval stage when yolk is resolved and uptake of food is from new limited sources (Koumans & Akster, 1995). The embryonic phase of myogenesis, however, associates with myofibre hyperplasia and hypertrophy. It is well known that embryonic hyperplasia in teleosts is greatly affected by environmental conditions (Stickland et al., 1988; Koumans & Akster, 1995; Rescan, 2001), specifically temperature and oxygen concentrations (Johnston, 2006).

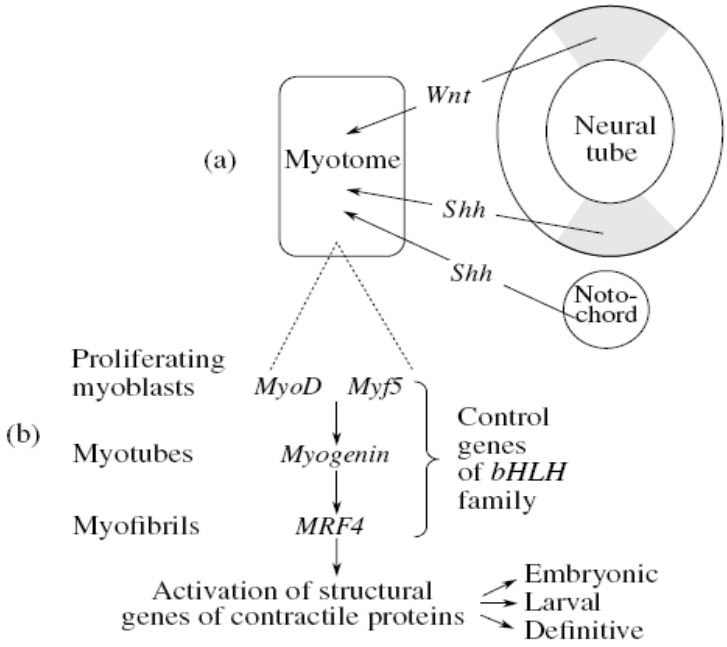

Fig. 2.5. (a) Diagrammatic illustration of the notochord and neural tube influencing events on determination of muscle precursors in myotomes and (b) The regulatory effect of *bHLH* gene family. Adapted from Ozernyuk et al., (2004).

A variety of genes are involved in the development of myotomes (muscle blocks). As demonstrated by Ozernyuk et al. (2004), the formation of myotomes is controlled by the activity of Shh (Sonic Hedgehog) gene family in the notochord and basal neural tube as well as by the Wnt gene family activity in the dorsal neural tube (fig. 2.5). Beside this, there are several other important genetic regulatory pathways that take part in the muscle development. These pathways are discussed in the next section in detail.

2.5. Important genetic regulatory pathways

The myogenic regulatory pathways are crucial for understanding the differentiation mechanism of muscle development. Several significant regulatory pathways have been elucidated in the past two to three decades. A brief description of these is as follows.

2.5.1. The MyoD gene family

MyoD belongs to a larger class of DNA binding proteins containing a basic helix loop helix (bHLH) domain. MyoD was the first myogenic regulatory gene identified and is expressed only in myoblasts and skeletal muscle tissue, instead of cardiac or smooth muscle (Olson, 1990). MyoD gene family consists of four transcription factors namely, Myod, myogenin, Myf5 and

MRF4/herculin/Myf6 in vertebrates, and have shown to carry distinct but overlapping functions (Rescan, 2001). These transcription factors of MyoD family are highly conserved between mammals and fish and are required for myogenic lineage determination and muscle differentiation (Olson, 1990; Rescan, 2001; Berkes & Tapscott, 2005; Johnston et al., 2008).

It has been investigated that Wnt, Shh and other signaling pathways contribute to muscle determination and differentiation by inducing expression of Myf5 and MyoD (Ozernyuk et al., 2004) as illustrated in figure (2.5). Furthermore, differentiation of myotubes is dependent of myogenin. Accordingly it becomes visible that MyoD and Myf5 are important for terminal myoblasts proliferation, while myogenin is important for terminal myoblasts differentiation and MRF4 has aspects of both functions (Berkes & Tapscott, 2005), thereby activating muscle specific transcription through binding to a DNA consensus sequence known as E-box present in the promoter of several genes. MyoD family is antagonized by many other growth factors; one major factor in this regard is HLH protein which is an inhibitor of DNA binding (Olson, 1990).

2.5.2. Myostatin

Myostatin also known as the GDF-8 is a member of the transforming growth factor-β gene family which was explored as a first negative regulator of muscle growth in mammals (McPherron et al., 1997). Elucidated studies on myostatin in teleost fish revealed the existence of two distinct myostatin genes (Ostbye et al., 2001; Rescan, 2001). In mammalian, cell culture myostatin down-regulate the expression of key transcriptional factors of muscle development such as MyoD and Myf5 (Amthor et al., 2004) and arrest the transition of myoblasts form G1 to S-phase of cell cycle (Thomas et al., 2000).

In addition to being a potential negative regulator of muscle deposition, it is suggested that myostatin also perform an immmunomodulatory role (Helterline et al., 2007) and possess some other kind of functions in a wide variety of tissues (Kocabas et al., 2002). Inactivation of myostatin leads to the significant deposition of muscle mass. This has been demonstrated by a study on gilthead sea bream (Rebhan & Funkenstein, 2008) and a study on myostatin-null mice (McPherron et al., 1997; fig. 2.6). Further, it is shown that myostatin inhibits the proliferation and differentiation of satellite cells (McFarland et al., 2006; Thomas et al., 2000).

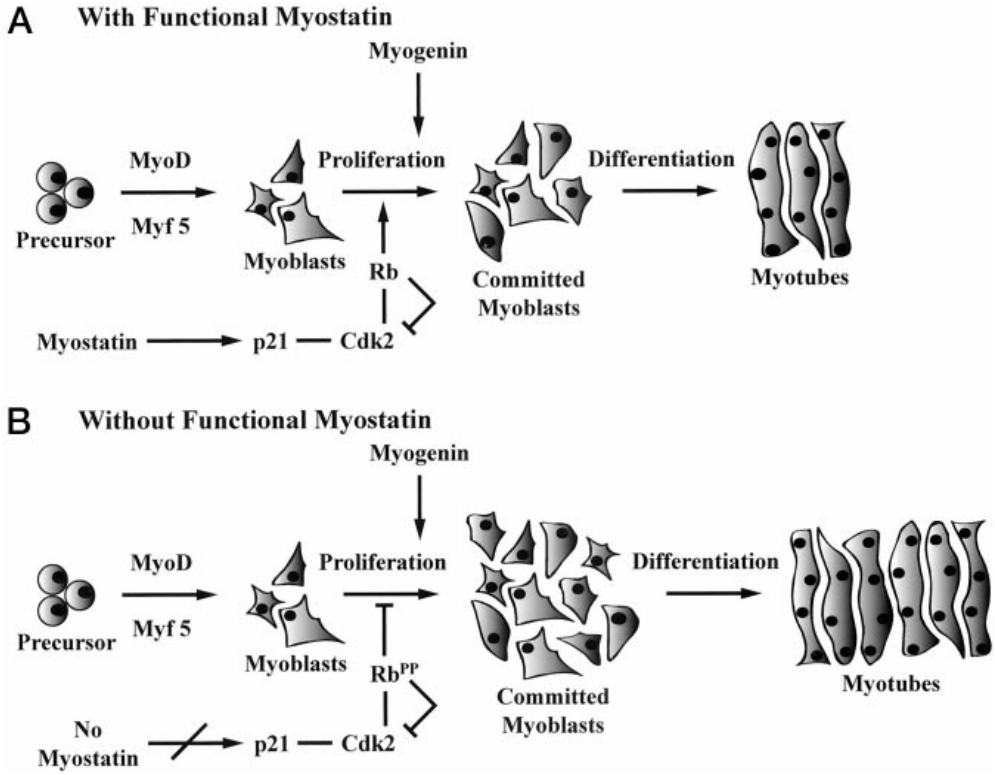

Fig. 2.6. A model illustrating the functional role of myostatin in muscle growth. (A) Muscle growth with functional myostatin. (B) Muscle growth with nonfunctional myostatin. Adapted from Thomas et al (2000).

2.5.3. Follistatin

Follistatin is a secreted glycoprotein which is expressed in wide variety of mammalian tissues including gonads, pituitary gland, pregnancy membranes, vasculature and liver etc (Philips & de Krestor, 1998). Follistatin was first identified as a strong inhibitor of follicle stimulating hormone (Philips & de Krestor, 1998) and subsequently in other regions of the adult body associated with reproductive functions (Patel, 1998). Moreover, a later discovery revealed that follistatin is an inhibitor of many members of transforming growth factor-β gene family including myostatin (Patel, 1998), and is a regulator of amniote myogenesis (Amthor et al., 2004; Macqueen & Johnston, 2008).

To date follistatin is only known to antagonize the function of myostatin which is a powerful inhibitor of muscle growth (Amthor et al., 2004). For example, the inhibitory effect of recombinant follistatin and myostatin prodomian on fish myostatin activity has been figured out by Rebhan & Funkenstein (2008). The authors proposed that enhanced muscle growth could be achieved by this approach. In addition, follistatin is an essential component for normal development as follistatin

knock-out mice died soon after birth with a number of defects in skeletal muscle development (Patel, 1998). Macqueen & Johnston (2008) demonstrated that follistatin is expressed in multiple tissues, including fast and slow muscles, in different fish species.

2.5.4. Insulin like growth factor (IGF) system

Insulin like growth factor-I (IGF-I) and IGF-II represents the main endocrine and autocrine regulators of skeletal muscle (Johnston et al., 2008). Liver is the main endocrine source of IGFs therefore paracrine activity is observed in several tissues (O'Dell & Day, 1998; Moriyama et al., 2000; Johnston et al., 2008). The IGFs play a critical role in preadolescent growth by mediating rapid metabolic changes and have long term growth promoting effects as regulators of cell proliferation (O'Dell & Day, 1998). It is well known that biological functions of IGF-I are highly conserved in vertebrates (Moriyama et al., 2000) and contributes to the compensatory growth in fish (Montserrat et al., 2007).

In mammals pituitary gland produces a growth hormone which is monitored by hypothalamic hormones. This growth hormone later binds to its target organ mainly in liver and cause the synthesis and release of insulin like growth factor-I (Moriyama et al., 2000). This IGF-I later binding to a IGF-I receptors regulate the protein, lipid, carbohydrate and mineral metabolism in cells, differentiation and proliferation in cells and ultimately the body growth (Moriyama et al., 2000). The mode of action of IGF-II as growth promoting agent is carried out by binding to two distinct IGF receptors, type 1 and type 2 (O'Dell & Day, 1998). Type 1 receptor cause the stimulation of RNA and DNA synthesis, cell proliferation and differentiation and cell survival whereas type 2 receptor involved in targeting of lysosomal enzymes to lysosomes and also the degradation of IGF-II (O'Dell & Day, 1998).

It was known that IGF-I play its role by mediating growth hormone actions but a recent study on vertebral and muscle tissue of Atlantic salmon indicated that IGF-I can act independently while regulating growth (Nordgarden et al., 2006). Regeneration is a coordinate process in which stem cells maintain the structure and cellular basis of muscle regeneration. Based on the results of Musaro et al. (2007), IGF-I is proved to be a powerful enhancer of stem cell mediated regeneration and could be a innovative tool to develop strategies to improve muscle regeneration in muscle diseases.

2.5.5. Clapain and Calpastatin system

The two ubiquitous calpains, μ-calpain and m-calpain, and calpastatin are the Ca^{+2} dependent cysteien proteinases that constitute a large and diverse family. Calpastatin is a multiheaded protein, which is expressed in different isoforms having one, three or four inhibitory domains and different N-terminal sequences and is a specific inhibitor of calpain proteases (Goll et al., 1998; Salem et al., 2005a). The two forms of calpain and calpastatin have been cloned and sequenced for a number of species including human, mouse rat, monkey etc and sufficient data is available on calpain system from these organisms (Goll et al., 2003). Calpains are involved in the many physiological functions primarily the muscle proteolysis both *ante* and *post-mortem* (Salem et al., 2005b). Calpastatin plays a central role in muscle growth and meat quality (Salem et al., 2005a). Molecular characterization of calpains and calpastatin and their relationship to muscle growth have been investigated in many studies (Goll et al., 1992, 1998; Salem et al., 2005a, b; Saito et al., 2007), but the mechanism of their action still need to be elucidated in fish particularly (Saito et al., 2007).

2.6. Biological aspects of Amino acids

Amino acids are crucial part of life and perform significant functions in metabolism. An important function in this regard is the building blocks of proteins. Amino acids are classified into two classes on the basis of their biological requirements i.e. dispensable and indispensable. Conditionally dispensable amino acids are those that an organism synthesize in adequate amounts while indispensable are those whose carbon skeleton cannot be synthesized de novo by the body sufficiently to meet the necessary needs. The indispensable amino acids in mammals and fish are Arg, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and alanine (Wu, 2009).

In recent years, it is ascertained that amino acids are regulators of gene expression and the protein phosphorylation cascade (Jousse et al., 2004; Bruhat et al., 2009; Brasse-Lagnel et al., 2009). The transcription, translation and post-translational modifications are the biochemical events involved in gene expression (Jousse et al., 2004; fig. 2.7). The regulation of gene expression by amino acids usually involves the transfer of information encoded in a gene into either RNA or protein (Wu, 2009).

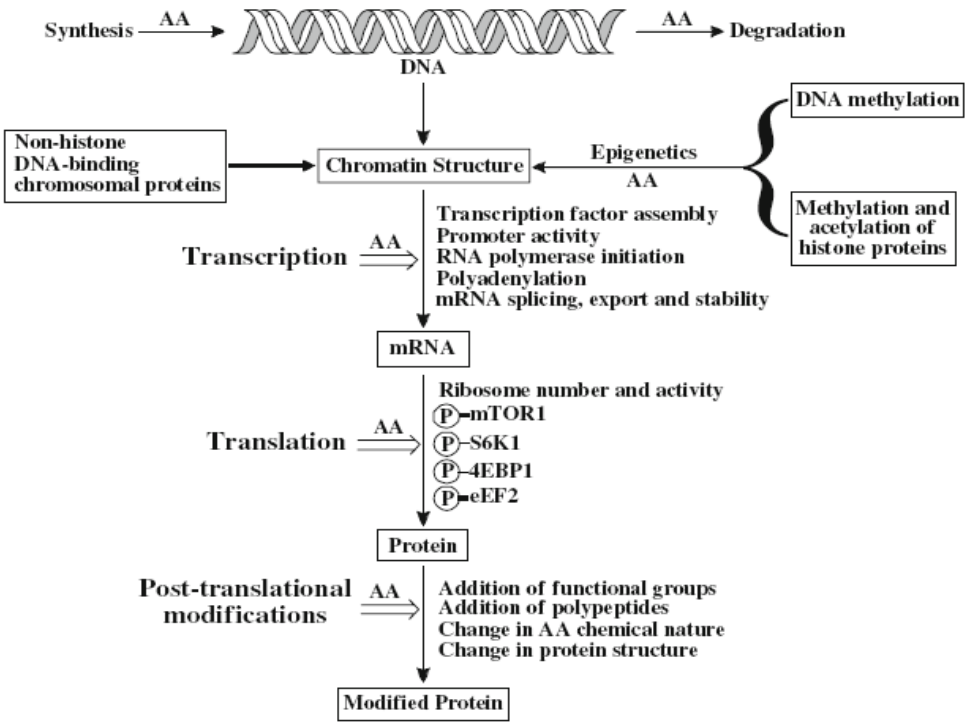

Fig. 2.7. Possible mechanisms involved in the regulation of gene expression in cells by amino acids. Adapted from Wu (2009)

2.6.1. Arginine (Arg)

Arg is one of the 20 most common naturally occurring amino acids. Arg is classified as an essential amino acid for birds, carnivores and young mammals, and a semi-essential or conditionally essential for adults (Tapiero et al., 2002a; fig. 2.8). Arg was first investigated in crystalline form by Schulze & Steiger (1886) and the presence of Arg as a component of animal protein was identified by Hedin (1895). Arg is an amino acid of versatile functions and involved in many metabolic pathways in animal cells such as synthesis of proteins, nitric oxide, urea, polyamine, proline, glutamate, creatine and agmatine (Wu & Morris, 1998).

Arg is identified as potential immunomodulator and is useful in severe sepsis and postoperative stress by two immunomodulatory actions; firstly, the arginase pathway by which polyamines are synthesized that may lead to the lymphocyte mitogenesis and secondly, the production of nitric oxide which has a strong role in the maintenance of vascular tone, immune system gastrointestinal tract and coagulation (Evoy et al., 1998). Beside these functions, Arg also plays a crucial role in lowering the blood pressure (Gokce, 2004).

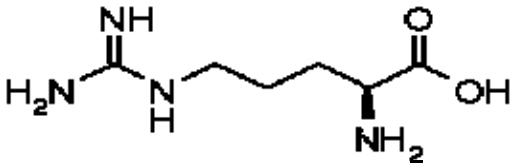

Fig. 2.8. Chemical structure of argrinine (Arg). (Accessed from http://www.chemie.fuberlin.de/chemistry/bio/aminoacid/arginin_en.html).

It is documented that infusion of amino acids in fish nutrition stimulates muscle growth (Brown & Cameron, 1991), but little information is available on the mechanism involved. Although, it is known that Arg metabolism lead to the production of ornithine, the precursor for putrescine, which is important for the synthesis of polyamines. And this polyamine is highly responsible for muscle growth (Mommsen, 2001). Furthermore, Arg activates the release of glucagon, glucagon-like peptide-I and somatostatins which are the growth regulating molecules. Metabolic pathway of Arg and its products which play a significant role in muscle growth are illustrated in figure 2.9.

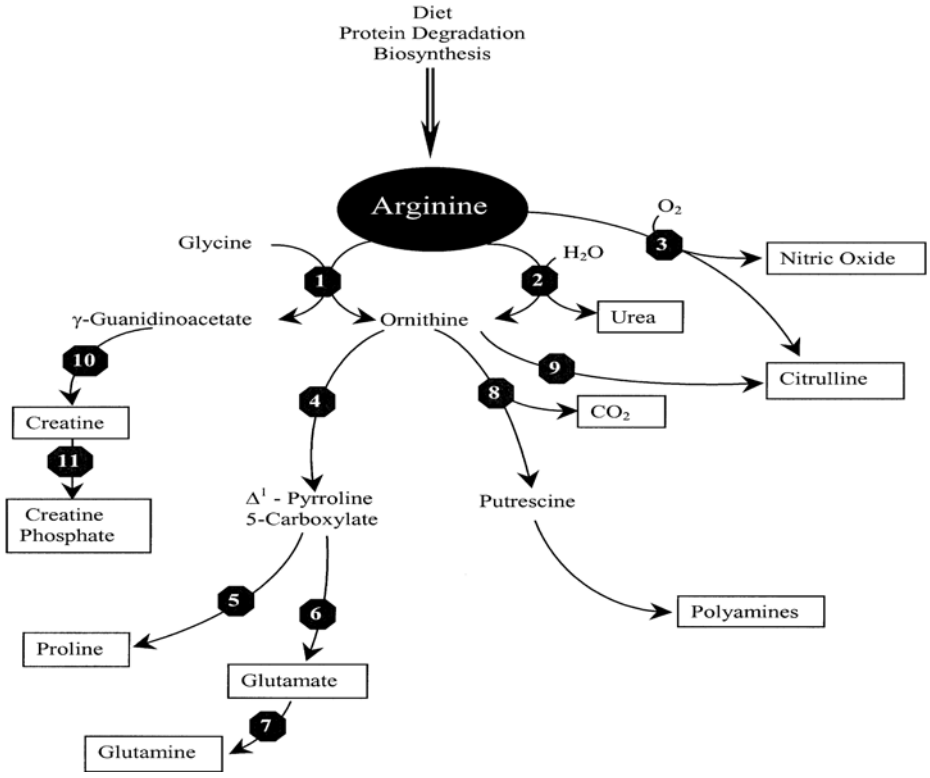

Fig. 2.9. Metabolic products of arginine (Arg). The products inside the boxes are responsible for muscle growth. Adapted from Mommsen, (2001).

Several studies have dealt with the dietary Arg requirement and its metabolic functions in fish (Kim et al., 1983 and 1992; Ketola, 1983; Walton et al., 1986; Kaushik et al., 1988; Cynober et al., 1995; Twibell and Brown, 1997; Buentello & Gatlin 2000; Park et al., 2005; Saavedra et al., 2008). It is known that deficiency of indispensible amino acids leads to the decrease in food intake, reduced growth, and negative nitrogen balance (Evoy et al., 1998).

2.6.2. Glutamate (Glu) and Glutamine (Gln)

Glu and Gln together with ornithine, histidine, Arg and proline comprise approximately 25% of the dietary amino acids intake and constitute the "Glutamate family" of amino acids (Tapiero et al., 2002b). Glu and Gln are interrelated to each other and are essential amino acids for brain metabolism and function (Struzynska & Sulkowski, 2004). Chemical structures of Glu and Gln are presented in figure 2.10.

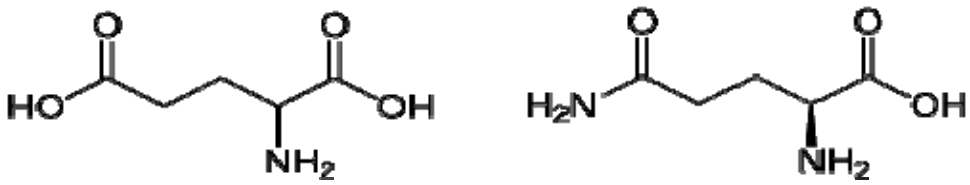

Fig. 2.10. Differences in the chemical structures of L-Glutamate (Glu, left side) and L-Glutamine (Gln, right side) and. (Accessed from http://en.wikipedia.org/wiki/Glutamine).

Glutamate: Glu is the dispensable amino acid, meaning that body can sufficiently synthesize its required amounts. The salts and carboxylate anions of glutamic acid are known as glutamates. Glu is the main constituent of dietary proteins and is present from 11 to 22% by weight of glutamic acid in animal protein and around 40% Glu by weight in plants protein (Tapiero et al., 2002b). The most abundant form of Glu is present as monosodium Glu and is used for flavor enhancement in daily life (Wu, 2009). Glu was discovered in 1866 by Karl Heinrich Leopold Ritthausen and later in 1907 identified by Kikunae Ikeda. In addition to the taste enhancer, Glu exhibit its own taste named umami (Ikeda, 2002) which means savory taste.

Glu like other amino acids is absorbed and metabolized in small intestine (Burrin & Stoll, 2009). A variety of pathways are involved in Glu metabolism, however, major proportion of Glu is metabolized during its transformation through enterocytes (Blachier et al., 2009). Glu first transforms to alanine in intestinal mucosal cells and to glucose afterwards and finally to lactate in liver (Stegink et al., 1979). Glu serves an important role in bridging the urea cycle with the Krebs

cycle (Wu, 2009). It is also well known that Glu serves as precursor for several other amino acids including alanine, aspartate, ornithine, Arg and proline (Reeds et al., 2000 Tapiero et al., 2002b; Blachier et al., 2009; fig. 2.11) and bioactive molecules such as glutathione (Burrin & Stoll, 2009). Out of all of these, proline is the most important in muscular point of view as it synthesizes the collagen and connective tissue (Tapiero et al., 2002b).

In addition to serve as a precursor, Glu has a variety of other functions in the living cells. One of the most exciting functions of Glu is to perform as a major excitatory neurotransmitter in the vertebrate central nervous system (Meldrum, 2000; Tapiero et al., 2002b) and this process is assisted by two main groups of Glu receptors, i.e. ionotropic and metabotropic (Tapiero et al., 2002b). Recent findings suggested that Glu is the major oxidative fuel in the intestinal mucosa and is responsible for maintenance and protection of mucosa (Burrin & Stoll, 2009; Blachier et al., 2009). Furthermore, Glu performs an important role in synaptic maintenance and plasticity and is also involved in the learning and memory process (Tapiero et al., 2002b). Very limited information is available about Glu and its effects on fish biology, however, a few studies has been discussed in this regard. For instance, Glu has proved to exert effects on the steroidogenesis in rainbow trout (Leatherland & Renaud 2004). Further, several studies have shown that Glu is greatly involved in the contraction and depolarization in crayfish, lobster and crustacean muscle (Robbins, 1959; Takeuchi & Takeuchi, 1964; Shinozak & Shibuya, 1974; Frank, 1974; Colton & Freeman, 1975).

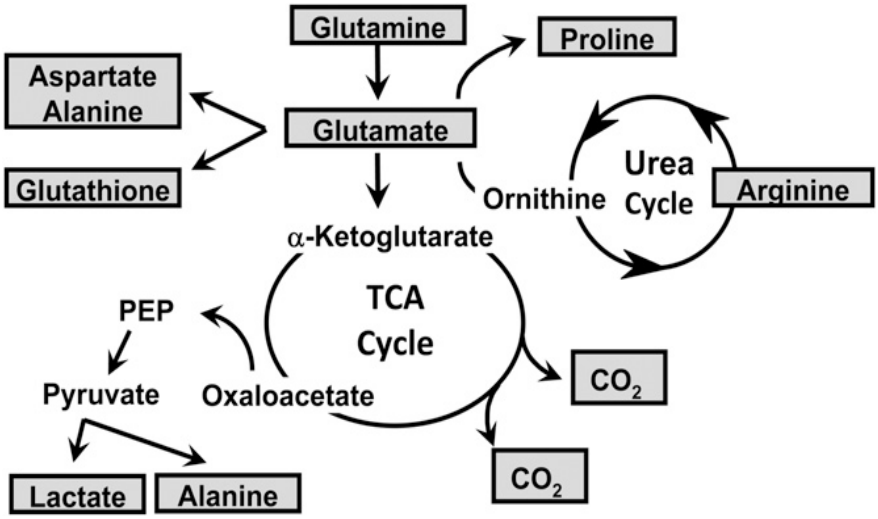

Fig. 2.11. Metabolic products of glutamate (Glu). Adapted from Burrin & Stoll (2009).

Glutamine: Gln is the most abundant type of α-amino acid in the blood and represents roughly 20% of free amino acids in plasma (Hall et al., 1996; Watford, 2008). Gln like Glu has

traditionally been categorized as a dispensable amino acid, though; recent findings suggested that Gln is a conditionally indispensable amino acid (Wu, 2009; Mates et al., 2009). Metabolism of glutamine gives rise to a number of significant products important for body (fig. 2.12). Small intestine is often the major site for dietary glutamine uptake; therefore, skeletal muscle and lung are the major export sites (Tapiero et al., 2002b; Newsholme et al., 2003; Watford, 2008).

Gln is a multifaceted amino acid which performs a variety of physiological functions in living cells. Most exciting function of Gln as nutrient for cell survival and proliferation *in vitro* (Ehrensvard et al., 1949) has been known since 60 years back, therefore the confirmation regarding this fact was later supported by the study of Eagle et al (1956). Nowadays, Gln is considered as a necessary nutrient for cell growth and proliferation, especially lymphocytes, fibroblasts, enterocytes and tumor cells (Wilmore & Shabert, 1998; Abcouwer, 2000; Tapiero et al., 2002b; Mates et al., 2002 and 2009; Newsholme et al., 2003; Matheson et al., 2008; Wu, 2009). Beside this, Gln performs several other functions such as, regulation of gene expression (Newsholme et al., 2003; Wu, 2009), immune function (Wilmore & Shabert, 1998; Abcouwer, 2000; Wu, 2009), inhibition of apoptosis (Mates et al., 2002; Wu, 2009), major fuel for proliferating cells (Newsholme et al., 2003; Watford, 2008; Wu, 2009), nitrogen reservoir and synthesis of nucleotides (Newsholme et al., 2003; Watford, 2008; Wu, 2009).

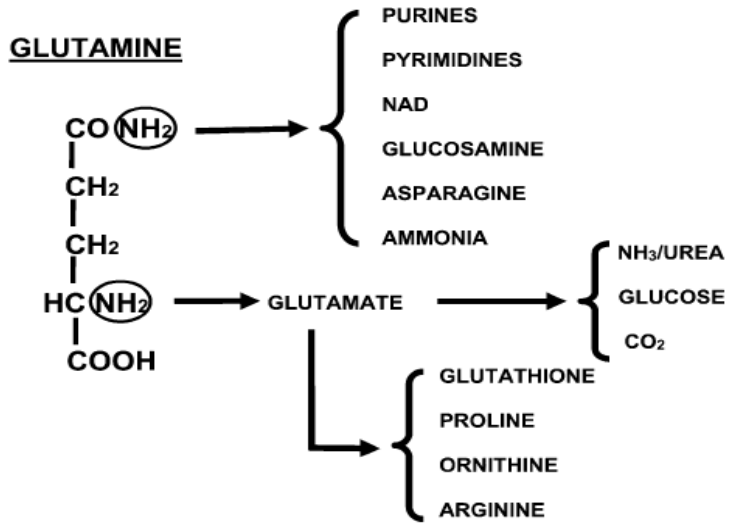

Fig. 2.12. Metabolic products of glutamine (Gln). Adapted from Watford (2008).

Gln demands under stressed conditions increased drastically, as discussed earlier, it is involved in a number of physiological processes. It is found that Gln oxidation in bony fish and

teleost fish is variable and it set high demands for the former fish species (Chamberlin et al., 1991). Gln has been proved to be effective in protecting the intestinal epithelial cells of jian carp under oxidative stress situations (Chen et al., 2009). Gln play a central role in the detoxification of ammonia in many fish species (Randall & Tusi, 2002) such as rainbow trout (Wicks & Randall, 2002). Out of all the amino acids, Gln-responsive genes and transcription factors modulate number of processes such as inflammatory response, proliferation, metabolism, apoptosis and survival (Brasse-Lagnel et al., 2009; fig. 2.13).

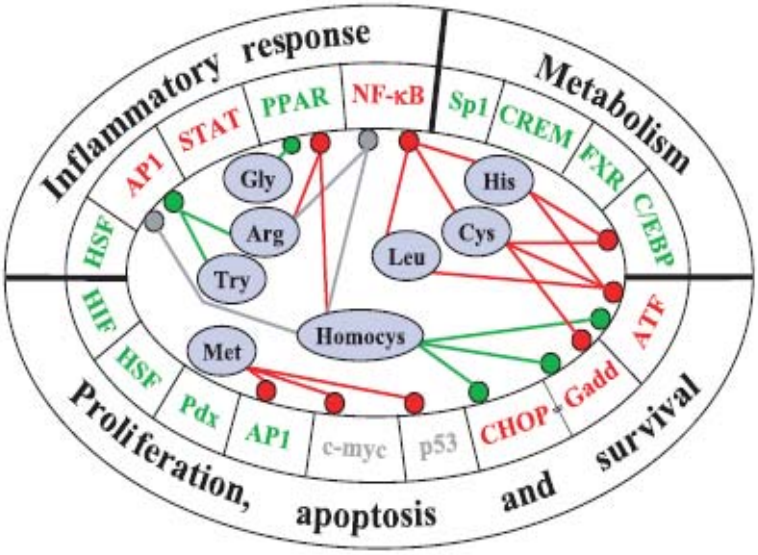

Fig. 2.13. Glutamine (Gln) modulated families of transcriptional factors to regulate physiological processes. Different colors indicate the families of transcription factors modulated by Gln depending on the effect; red (inhibition), green (activation), grey (inhibition or activation). Adapted from Brasse-Lagnel et al (2009).

2.7. Biological aspects of 3-thia fatty acids

3-thia fatty acids are a group of fatty acids in which a sulfur atom replaces the β-methylene group in the alkyl chain (Berge et al., 1989). Like amino acids, 3- thia fatty acids also perform a critical role in many physiological processes in the living cells. For instance, they play significant roles in gene expression (Kleveland et al., 2006; Kennedy et al., 2007), oxidation of other fatty acids (Moya-Falcon et al., 2004 and 2006), changed lipid composition (Gjøen et al., 2007), increased survival during inflammation (Alne et al., 2009) etc.

2.7.1. Tetradecylthioacetic acid (TTA)

TTA is a bioactive fatty acid and belongs to a family of 3-thia fatty acids that exhibit a sulphur atom at the third position from the carboxyl terminus. The chemical structure of TTA is presented in figure 2.14. There is a resemblance in the chemical properties of thia fatty acids and ordinary fatty acids, but their metabolism and metabolic effects are far different from each other. This difference mainly depends on the position of the sulfur atom (Skrede et al., 1997). The origin of preparation of long chain thia fatty acids is from 1920 (Spydevold & Bremer, 1989). Catabolism of TTA cannot occur through β-oxidation pathway and is instead processed through ω-oxidation (Skrede et al., 1997).

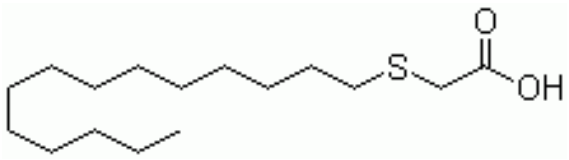

Fig. 2.14. Chemical structure of tetradecylthioacetic acid (TTA). (Accessed from http://www.chemblink.com/products/2921-20-2.htm).

TTA influences many physiological and biochemical processes, and has a great effect on inflammatory processes. For example, TTA has been shown as a potent anti inflammatory agent in two kidney one clip hypertension in Wister rats (Bivol et al., 2008). TTA also reduces the inflammation in circumstances which are not related to hypertension (Dyroy et al., 2005). For instance, Fredriksen et al. (2004) concluded that TTA can act as an anti-inflammatory agent in patients infected with HIV. In addition, TTA has shown the impact on the survival rate on S0 Atlantic salmon infected with heart and skeletal muscle inflammation (Alne et al., 2009). Long term feeding of the TTA leads to increased liver weight due to the stimulatory effect on the up-regulation of many hepatic enzymes genes (Skorve et al., 1990).

Changes in the tissue fatty acids composition is highly influenced by TTA. For instance, Moya-Falcon et al (2004) investigated the changed fatty acid composition in several tissues of Atlantic salmon. Similar results were documented in rainbow trout by Kennedy et al. (2007) and in Atlantic salmon by Gjøen et al. (2007). Studies regarding the effect of TTA on lipid related genes in fish have been performed by many authors (Kennedy et al., 2007). Another major function of TTA is inhibition of the fat deposition through oxidation of fatty acids (Madsen et al., 2002; Wensaas et al., 2009). However, high doses of TTA in order to increase the fatty acids oxidation and reduction

in fat deposition may cause poor growth, inhibition of inflammatory response and increased mortality (Gjøen et al., 2007).

2.8. *In vitro* cell culturing

In vitro cell culturing means the culturing of cells outside the body of an organism in a controlled environment, such as in a test tube or Petri dish (Kail & Cavanaugh, 2006). Cell culture has become a fruitful tool in cell and molecular biology today. The term cell culture is generally used for the removal of cells, tissues or organs form an animal or plant and then after their subsequent placement into a congenial environment for growth. Satellite cells with high proliferative potential have been isolated and cultured in turkey and fish (Koumans et al., 1990; Matschak & Stickland, 1995; Vegusdal et al., 2003; Mcfarland et al., 2006). Cell culturing is used for a variety of purposes such as, model systems, toxicity testing, cancer research, virology, genetic engineering, gene therapy etc. Out of these model systems provide good model for studying nutritional studies. One of the major advantages of *in vitro* system is the increased control over physiochemical (ph, temperature, osmotic pressure, oxygen) and physiological environments which is not possible in *in vivo* systems (Mothersill & Austin, 2003). In addition *in vitro* system is often more cheaper and provide the results much rapid than *in vivo* system (Mothersill & Austin, 2003).

3. MATERIALS AND METHODS

This section is divided into two parts. The first part describes the *in vivo* study while the second part describes the *in vitro* study.

3.1. IN VIVO STUDY

3.2. Fish and Experimental Design

The feeding trial was carried out at Nofima Research Station (Averøy), on the west coast of Norway, for a period of five months starting from 15th of April to 15th of September 2009. The water temperature at three 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. Day before commencing the experiment, 6000 smolts of Atlantic salmon (*Salmo salar* L, obtained from the farming company Salmar ASA) with an average weight of 105 grams were transferred to 12 net-pens with a volume of 125 m^3 (5m length, width, and depth) in 33 % sea water (500 smolts per net-pen).

The fish were fed a commercial extruded dry feed i.e. control (Con) or the same diet supplemented with tetradecylthioacetic acid (TTA), arginine (Arg) or gltamate (Glu) manufactured by Skretting (Stavanger, Norway). A total of 12 net-pens with triplicate randomly assigned net pens of each diet were setup. The TTA diet was fed to the fish for 60 days; thereafter it was replaced by the Con diet. For the present study a total of 108 salmon (nine fish per net-pen) were used for studying muscle pH, fillet contraction, cathepsin B, L and cathepsin B+L, texture and histological analysis. The fish were randomly selected from each net-pen and divided into two groups, where the fish in the first group were harvested as carefully as possible (non-crowded (NC) group; *n=72,* six per net-pen) whereas the fish in the second group were harvested after exposing the fish to crowding stress (crowded (C) group; *n=36,* three per net-pen). The NC group was sub-divided into two batches, batch A (first three fish of each net pen) and batch B (last three fish of each net pen). Fish in the C group were tagged and crowded in a 1000L tank for sixteen hours before slaughtering, in order to induce stress. Oxygen level was monitored at a constant level at 7mg/l. An overview of the whole experimental design is given in the attachments section.

3.3. Slaughtering of fish

Slaughtering and filleting procedure were accomplished within three days. The first two days were designated for the NC group i.e. six net-pens each day, while on the last day, all the fish in the C group were slaughtered and filleted. The fish were killed with a blow to the head, gill-cut,

and bled in a tank with running sea water. The fish were hand-filleted within half an hour *post-mortem* by an experienced worker. The fillets were placed on solid smooth plastic trays, (to allow the fillets to contract freely) in closed Styrofoam boxes (three fillets in each box) with ice, and stored in a cold room (5°C) for further analysis. The right fillets were used for muscle pH, texture measurement, cathepsin B, L and cathepsin B+L and histological analysis, whereas the left fillets were used for length measurements in order to calculate rigor contraction.

3.4. Fillet contraction

The fillet length was measured using a standard measuring tool in centimeters (cm) at various time points (table 3.1). The length measurements were taken from the lowest point in the curve at the anterior end, to the highest point on the curve at posterior end. The relative decline in the length of fillet indicates the fillet contraction. The contraction in percentage was calculated as follows:

Fillet length (cm) at time t / initial fillet length x 100

Where t = 1, 6, 12, 24, 48 and 72 hours post harvesting. The fillets were cut even at the anterior and posterior end after filleting to ensure more accurate length measurements.

3.5. Muscle pH

A pH meter 330i SET (Wissenschaftlich-Technische-Werkstätten GmbH & Co. KG WTW, Weilheim, Germany) connected to a muscle electrode was used for the muscle pH measurement at different time points (table 3.1). The measurements were carried out by inserting the electrode into the white muscle at the same point as the instrumental texture analyses (fig. 3.1).

3.6. Instrumental texture measurement

Texture is normally measured as the force required inflicting tissue breakage or cleavage of muscle filaments (Kiessling et al., 2004). Each right fillet from each group of salmon was used for texture measurement at three different time points (table, 3.1). Measurements were done on the front part of the fillets at three different positions (fig. 3.1), in the dorsal section, above the mid line, by keeping a distance of 3cm between each measurement as described by (Morkore & Einen, 2003). A texture analyzer TA-XT2 (Stable Micro Systems Ltd, Surrey, UK) equipped with flat-ended cylindrical probe (12.5 mm diameter, type p/0.5) and 25kg load cell was used for the measurements

(fig. 3.2). The test speed was 1 mms^{-1} and penetration depth of 90% of the fillet height. The parameter recorded was the area under the force-time graphs denoted as total work i.e. the maximum resisting force and total force required to cut the sample.

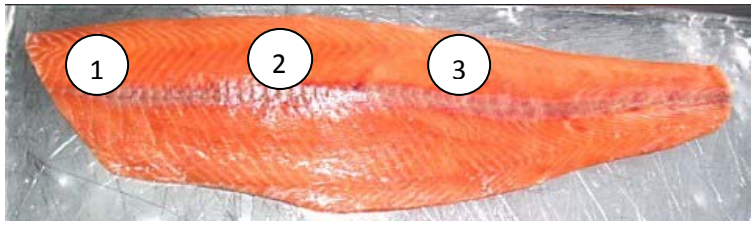

Fig. 3.1. Texture measurement from the right fillet at three different locations (1, 2, and 3) at different time points (1, 6, 12, 24, 48, and 72).

Fig. 3.2. Texture analyzer TA-XT2 (Stable Micro Systems Ltd, Surrey, UK) used for the texture measurements.

3.7. Histology procedure

Sampling for microscopic observations was performed on both NC and C groups of fish (table, 3.1). Muscle samples were taken directly after texture measurements one hour after harvesting, right next to the place of instrumental texture measurement. The samples were stored in small glass vials containing 5ml of 2.5% glutaraldehyde in Piperazinediethanesulfonic acid (PIPES) buffer at 5°C at IHA laboratory UMB (Ås). The samples were re-cut to obtain cross-sections and the embedding preparation in plastic blocks. Small glass-vials containing PIPES buffer without glutaraldehyde, pre-marked with the sample identity were used. For each fish, two plastic blocks (A

and B) were prepared for microscopic analyses. In each block A or B, 1 to 2 samples (sample 1 and sample 2) of fish muscle were fixed. The direction of the muscle fibres was oriented in the plastic blocks in order to obtain cross-sections only.

Samples collected in small glass vials were re-cut (under a fume hood) to fit the embedding moulds, transferred to the new glass vials, filled up with 5 ml of PIPES buffer (without glutaraldehyde) and kept overnight in the refrigerator (4°C) for rinsing purposes. The next day, PIPES buffer was taken out with the help of a Pasteur-pipette from each glass vial and were treated through a series of steps as follows;

- PIPES buffer (without glutaraldehyde) was added again 2x15min
- PIPES buffer was removed and 70 % Ethanol was added 2x20 min
- 70% Ethanol was removed and 96 % Ethanol was added 2x20 min
- 96% Ethanol was removed and 100 % Ethanol was added 1x20 min
- 100% Ethanol was removed and Solution A* 1:2 was added 1x120 min
- Solution A* 1:2 was removed and Solution A** 1:1 was added 1x60-120min
- Solution A** 1:1 was finally removed and Solution A was added and kept overnight in the refrigerator (4°C)

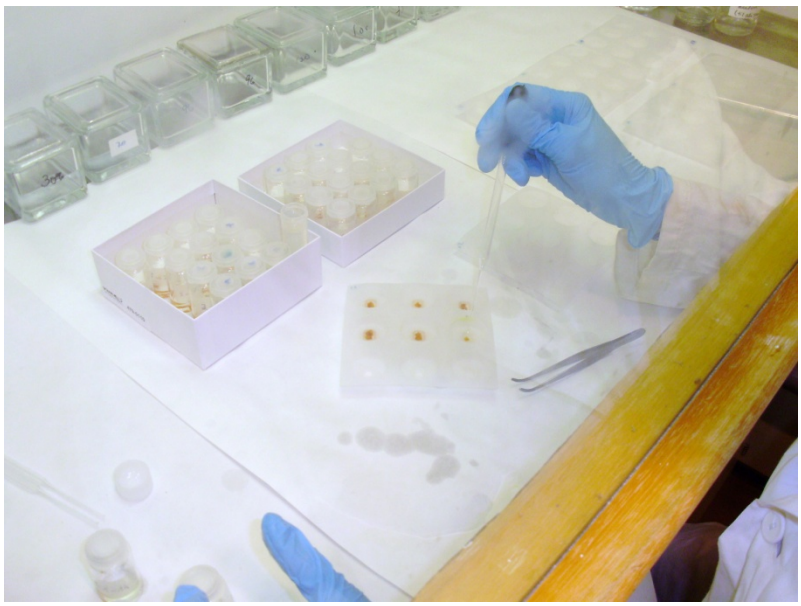

Fig. 3.3. Placement of the samples into histo-mould with a small volume of solution B for polymerization process.

Next day each sample was placed in the histo-moulds (fig. 3.3). A little volume of Solution B was poured in histo-mould hole before the samples were placed, and the hole was then filled up to the top after placement of the samples, in order to sufficiently cover the mould area. All samples were then left overnight at room temperature under the fume hood for polymerization in the mould.

After the polymerization process all the polymerized samples were covered with plastic adapters on histo-mould. The plastic adapters were then filled up with a Glue Solution to sufficiently cover the adapter's area. Samples were left again overnight at room temperature, under the fume hood, in histo-moulds for drying purpose. The samples were marked on each adapter before taking them out from the histo-mould.

Sectioning of the embedded samples was performed with the help of a semi-automatic Microtome (Leica RM 2165, Nusscloch, Germany). Briefly, after adjustments of each histo-block into the Microtome as well as speed of rotator and thickness of the sections from the computer attached to the Microtome, sectioning procedure was carried out. Tissue sectioning was done in two steps; first the samples were trimmed at 8µm and then sectioned for 3µm to be placed on glass-slides for microscopic observations. Tissue sections were then picked up with the help of a pincer and transferred into a Water Bath (Leica HI 1210, Nusscloch, Germany) at 50°C. Afterwards, they were placed carefully on 2 glass-slides/3-4sections/histo-block avoiding wrinkles. The glass-slides were dried on a slide dryer (Leica SW 85, Nusscloch, Germany) at 50°C.

After few hours of drying, the slides were stained with Toluidine blue solution. Briefly, after adjusting slides on a special slide-stand (ten-slides/stand) they were submerged in Toluidine solution for 4 min and later rinsed in a bowl with cold water for 4 min. The stained slides were dehydrated through different gradients of ethanol and xylene. Starting with 70% ethanol, then 96% ethanol, then 2x100% ethanol and eventually two times with xylene (10-15 times up and down at every step). Later, the stained tissues were covered precisely with cover-slips, avoiding air-bubbles, and with a drop of Eukitt glue. Slides were then led horizontally in fume hood for a few hours to later be able to observe them microscopically.

Following buffers and solutions were used for the preparation of the histology samples.

- 0.1M PIPES buffer with 2.5% glutaraldehyde, pH 7.2 for the fixation of the samples in the glass vials.

- 0.1 M PIPES buffer without glutaraldehyde, pH 7.2 for rinsing of the samples after fixation and just before embedding preparation
- Solution A (100ml Technovit® 7100 "Heraeus Kulzer, Denmark" in 1g of Hardener I) was used in the embedding preparation process.
- Solution A* 1:2 (Solution A: Ethanol 100%) was used in the embedding preparation process.
- Solution A** 1:1 (Solution A: Ethanol 100%) was used for embedding preparation process.
- Solution B (15g of Solution A and 1.13g of Hardener II) was used for polymerization of the samples
- Glue Solution (10g Technovit® 3040 "Heraeus Kulzer, Denmark" and 5g Universal Solution) was used to glue histo-blocks to the polymerized samples in the histo-moulds.
- Toluidine solution (T3260, Sigma Aldrich®), 0.05% Toluidine in 0.1 M of Sodium-Acetate was used for staining of microscopic slides following the method of Ofstad et al (2006) with slight modification.

3.7.1. Microscopy

Micrographs for counting the relative number of fibres were taken using the light microscope Leica CTR 6000B (Leica Microsystems, Nusscloch, Germany). Additionally, a digital camera (Evolution MP Color, Media Cybernetics Inc., Silver Spring, MD) attached to the microscope was used to capture images of the stained muscle samples. All image acquisitions were controlled by Image Pro Plus 4.0 software from Media Cybernetics (Silver Spring, MD). The magnification of each area was set 20x and relative number of myofibres from each diet (Con, Arg, Glu and TTA) were counted in both non-crowded and crowded groups (n = 16 per treatment/group; fig. 3.4).

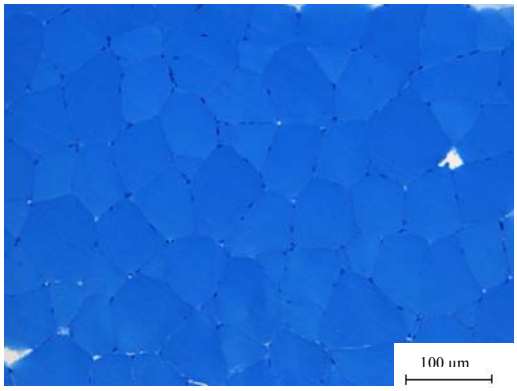

Fig. 3.4. Micrograph as an illustration for counting the number of myofibres.

3.8. Cathepsins analysis

For cathepsins analysis samples were taken from the sections of the fillets where pH and texture measurements were done (fig. 3.1). The muscle pieces were quickly frozen in liquid nitrogen before being stored at -80°C until further analyses. Cathepsin B and cathepsin L and total activities of both were determined by homogenizing 300mg of muscle in 900ml extraction buffer (100mM 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 10s break). The obtained homogenates were centrifuged at 16,000G for 30min 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.

3.9. Data recording procedure

Data recording of the NC and C groups were performed as shown in table 3.1. The rigor development was recorded using the left fillet sides while pH, texture measurement and muscle sampling for histological and cathepsins analysis were recorded on the right fillets.

Table 3.1. Summary depicting the recording of data from right fillets for pH, texture measurement and sampling for histological and cathepsins analysis at different time points.

Non-crowded group								
List of parameters recorded	**Time *post-mortem***							
	1	**3**	**6**	**9**	**12**	**24**	**48**	**72**
pH and texture measurement of batch A (first 3 fish of each net pen; right fillets)			*			*		*
pH and texture measurement of batch B (last 3 fish of each net pen; right fillets)	*				*		*	
Fillet length (left fillets)	*	*	*	*	*	*	*	*
Muscle sampling for histology and cathepsin B, L and cathepsin B+L (right fillets)	*							
Crowded group								
pH and texture measurement (all 3 fish of each net pen; right fillets)	*				*		*	
Fillet length (left fillets)	*		*		*	*	*	
Muscle sampling for histology and cathepsin B and cathepsin B+L (right fillets)	*							

3.10. IN VITRO STUDY

3.11. Fish and experimental design

Atlantic salmon for the *in vitro* study were reared on commercial diet at Nofima Marin's research station at Sunndalsøra (Norway). The salmon was transferred to and kept at Norwegian Institute for Water Research at Solbergstrand (Norway) until isolation of the myosatellite cells. Fifty Atlantic salmon parr with an average length of 5-7cm (fig. 3.5) were transported to Nofima Marine, Ås in polythene bag (20 liter) containing aerated water. The fish were kept alive in a tank in the laboratory and oxygen was added to the water by the use of an aquarium pump. The experimental design consisted of three treatment groups, arginine (Arg), glutamine (Gln) and tetradecylthioacteic acid (TTA), in addition to one control (Con) group. Muscle cells for both Proliferating Cell Nuclear Antigen (PCNA) assay and quantitative Polymerase Chain Reaction (qPCR) analysis were incubated at either 8°C or 16°C. PCNA assay and qPCR analysis were performed on fixed and harvested cells, from all experimental groups. In the qPCR study, two muscle genes, myogenin (regulatory) and myosin light chain2 (structural), in addition two genes for the proteases cathepsin B and cathepsin L were selected to evaluate the effects on muscle development and degradation.

3.12. Isolation of myosatellite cells

Myosatellite cells were isolated essentially as described by Koumans et al. (1990), with the modifications developed by Matschak and Stickland, (1995) and Vegusdal et al. (2003). The position of the skeletal muscle tissue isolation is illustrated in figure 3.5.

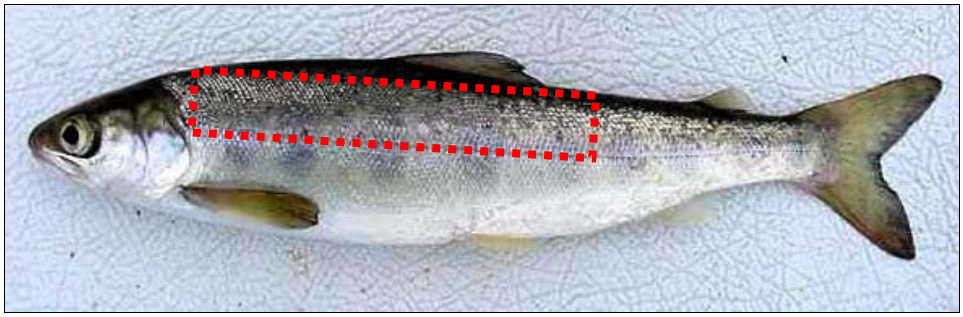

Fig. 3.5. Position (red mark) of isolation of skeletal muscle tissues from Atlantic salmon parr. The fish were stunned by a blow to the head and killed by decapitation. Mucus was scraped off and fish was dipped in ethanol thrice. Skin was cut across the fish right in front of the gut up to the ridge (red dotted line indicating the respective area) following the lateral line. The skin was removed with tweezers and tissue was cut down and collected in a tube contained 90% L-15 together with Antibiotic-Antimycotic solution placed on an ice box.

3.13. Cell culturing and splitting

Isolated myosatellite cells were resuspended in muscle growth media (90% L-15, 10% FBS (fetal bovine serum), 2mM L-glutamine, 0.01M HEPES (hydroxyethyl-piperazineethane sulfonic acid) buffer, 10ml/l Antibiotic-Antimycotic solution) seeded on laminin (L2020) coated culture flasks (25 cm^2), and incubated for three hours at 13°C without CO_2. Attached cells were extensively washed with L-15 medium before adding of fresh growth media. The suspension containing non-attached cells were centrifuged (15min at 300rpm and 4°C), resuspended in growth media and reseeded in laminin coated flasks (Nunc™, Denmark) (fig. 3.6). This was repeated four times after three hours incubation at 13°C.

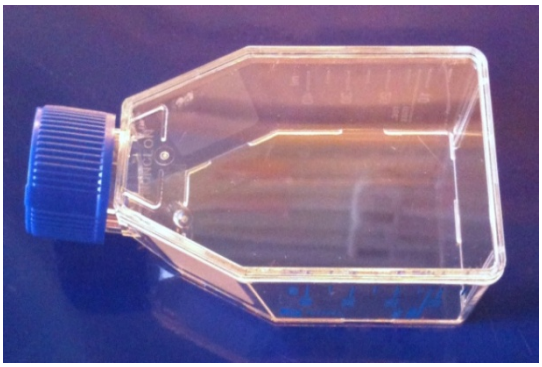

Fig. 3.6. Cell culture flasks (Nunc™, Denmark) (25cm^2) used for seeding of isolated myosatellite cells

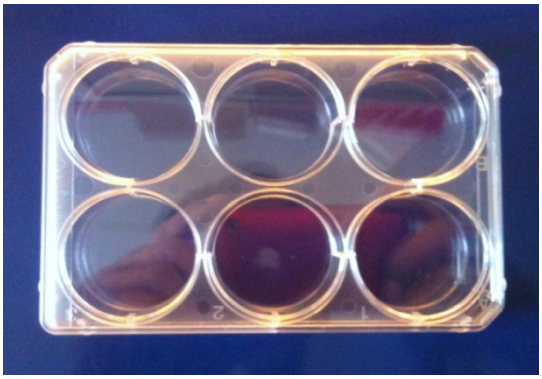

Fig. 3.7. Six-well plates (Nunc™, Denmark) (9.6 cm^2/well) for qPCR study, three plates for 8°C and three for 16°C containing quadruplicate wells of each treatment (Arg, Gln, TTA, Con).

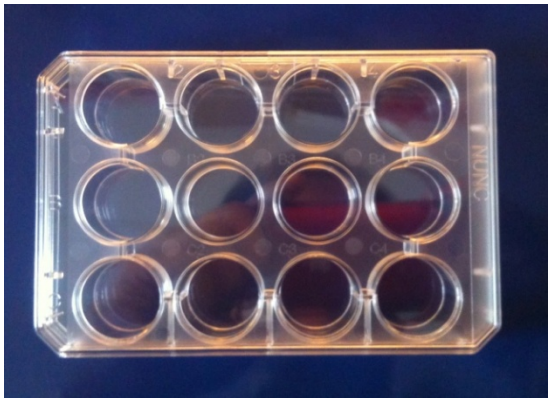

Fig. 3.8. Twelve-well plates (Nunc™, Denmark) (3.5 cm^2/well) equipped with Thermanox coverslips (18mm) for PCNA study, one plate for 8°C and one for 16°C containing triplicate wells of each treatment (Arg, Gln, TTA, Con).

On the fourth day muscle cells were spilt by trypsination and seeded on six-wells (Nunc™, Denmark) (9.6 cm^2/well) laminin coated plates for qPCR study (fig. 3.7) and twelve-wells (Nunc™, Denmark) (3.5 cm^2/well) plates with laminin coated coverslips (18mm) for the PCNA study (fig. 3.8).

Trypsination was performed by first removing the medium in the culture flasks. The cells were washed twice with phosphate buffered saline (PBS) to remove all the traces of fetal bovine serum (FBS), (A15-501, PAA Laboratories, Austria). A 500µl of 0.1% trypsin (T4549) was added to the cell flask and immediately decanted off. The cells were detached by adding 500µl of 0.1% trypsin, incubation for 2-3 minutes, and gentle shaking of the flasks. The process was carefully followed in the microscope to be able to stop the process when most of the cells were loosen. Trypsin treatment was terminated by adding growth medium containing 10% FBS. To get a homogenous culture, trypsinated cells from all culture flasks were pooled and seeded on laminin coated plates (day 0).

3.14. Procedure for TTA solubilization

TTA, provided by TiaMedica (Bergen, Norway), was made water soluble by dissolving it with bovine serum albumin (BSA). The procedure for dissolution and concentration formulation was as follow. BSA and TTA were initially separately dissolved before the fatty acids were complexed to BSA in the ratio TTA: BSA (2.5:1).

Solution 1; 25mM TTA (MW 288.6) in 0.1M NaOH

- 0.0072g TTA7ml

- TTA was dissolved with strong heating in a sterile tube.

Solution 2; 3.16mM BSA (MW 67000) in PBS

- 0.212g BSA/ml
- Solution was carefully stirred avoiding foam formation.
- Solution was kept under 50°C to avoid denaturation of BSA

For making 10ml of 6mM TTA concentration, 7.6ml of Solution 2 was carefully added in a 2.4ml of Solution 1 and pH (7) was adjusted.

3.15. Preparation and addition of substrates

Together with Con, three experimental treatments Arg, Gln and TTA were tested on the muscle cells. Arg and Gln were obtained in commercial form, while TTA was provided by TiaMedica and water-solubilized with the procedure mentioned previously. Arginine and glutamine were added in a double concentration as compared to the concentration in L-15. Arg concentration in L-15 is (0.5 g/l, 2.87mM) and Gln concentration in L-15 is (0.3 g/l, 2.05mM).

Finally, the following concentrations were made and used for Arg, Gln and TTA treatments.

- Arg concentration after twofold = 1.0 g/l (5.74mM)
  Molecular Weight (MW) L-Arginine; 174.2
- Gln concentration after twofold = 0.6 g/l (4.1mM)
  Molecular Weight (MW) L-Glutamine; 146.15
- TTA concentration in full growth medium = 0.0072 g/ml (0.6mM)
  Molecular Weight (MW) TTA; 288.6
- Composition of Con growth media = 90% L-15, 10% FBS, 2mM L-glutamine, 0.01M HEPES buffer, 10ml/l Antibiotic-Antimycotic solution

In order to make the experimental concentrations, 15mg of each substrate (Arg, Gln and TTA) was added in three separate tubes, containing a mixture of antibiotic (3ml), 0.01M HEPES, (H0887) buffer (0.3ml), FBS (3ml) and 90% L-15 (26.4ml) and mixed well. In case of Con treatment, normal cell growth media was used. Growth medium already present in the wells of

plates was removed and washed with 90% L-15 for the Arg, Gln and Con treatments, whereas 100% L-15 was used for TTA.

After 8 days of cultivation, the cells were confluent and the incubation with the different treatments was started. The cells were transferred to either 8°C or 16°C and acclimatized for 3-4 hours before the substrates were added. Approximately 3ml of different treatments were added to the six-well plates and 1ml to twelve-well (equipped with Thermnaox coverslips) plates. The end structure was quadruplicate-wells/treatment in case of qPCR study whereas triplicate-wells/treatment for PCNA study. The plates were then incubated at either 8°C (10 days) or 16°C (7 days) temperature with renewal of treatment concentrations only once after four days of treatments addition. After 10 and 7 days of treatments addition, cells were harvested for qPCR analysis as well as fixed for PCNA assay.

3.16. Proliferating cell nuclear antigen (PCNA) assay

Assessment of cell proliferation was assessed by immunocytochemical detection of PCNA (ZYMED® Laboratories Inc, USA). PCNA staining was performed essentially according to the protocol provided by ZYMED® Laboratories (fig. 3.9) with two additional steps, i.e. after blocking the endogenous peroxidase activity followed by PBS washing, cells were treated with 0.1% Triton X-100 for 10 min and rinsed off, in order to disrupt the cell membrane.

Fig. 3.9. Photo shoot for staining of the cultured cells for PCNA assay.

Later the cells were washed with PBS (3x2min) and then after the protocol supplied in the kit was followed. PCNA positive nuclei were stained dark brown while non-stained cells became blue.

3.16.1. Microscopy

A Carl Zeiss Axio Observer-A1 (Carl Zeiss Microimaging GmbH Gottingen, Germany) light microscope was used to view the stained cells. Additionally digital Carl Zeiss Axiocam MRc5 (Carl Zeiss Microimaging GmbH Gottingen, Germany) integrated with the microscope was used to capture digitized cell images. All image acquisitions were controlled by Image Pro Plus 4.0 software from Media Cybernetics (Silver Spring, MD). Blue and brown nuclei were counted within four different areas of each slide. Percentage of the blue and brown nuclei relative to the total number of nuclei was calculated. The magnification of each area was set 25x and the average number of nuclei was in the range of 152-311 (min and max respectively). The number of blue and brown nuclei, and percentage of blue and brown nuclei within each of the four areas were used as repeated measurements for the statistical analysis (n = 5 per treatment).

3.17. Gene expression analysis

3.17.1. RNA extraction

RNA isolation form cells, cultured at two different temperatures (8°C and16°C), was achieved by using RNeasy® Mini kit (Qiagen, USA) according to the manufacturer's protocol. The cells (in the six-well plates) were washed twice with PBS. Then the cells were harvested with the help of a cell scraper (TPP®, Switzerland) in 350µl buffer RLT with 0.014M β-merkaptoethanol. RNase-Free DNase Set (Qiagen, USA) was used to eradicate genomic DNA. DNase I treatment was performed on column during the RNA isolation procedure according to the manufacture's protocol. The integrity of the RNA was verified spectrophotometrically (NanoDrop® ND-1000 Spectrophotometer Wilmington, Delaware USA) by the ratio of absorbance at 260/280nm (indicates purity ratio between DNA and RNA) and 260/230nm (indicates purity of nucleic acids). The RNA solution was then stored at -80°C for further analysis.

3.17.2. First strand cDNA synthesis

cDNA was synthesized from 250ng RNA in a total volume of 25µl by using TaqMan®Gold RT-PCR Kit (Applied Biosystems, USA). The components of the cDNA synthesis reaction were mixed on ice according to table 3.2.

Table 3.2. Quantity of the solutions used for the synthesis of cDNA master-mix.

cDNA master-mix	
10x TaqMan® RT Buffer	2.5 µl
25mM $MgCl_2$	5.5 µl
10mM dNTP mixture	5 µl
Oligo $d(T)_{16}$	1.25 µl
RNase Inhibitor	0.5 µl
Reverse Transcriptase (50 U/µl)	0.625 µl
RNA 250ng	x µl
DEPC-H_2O	9.625 – x µl
Total	15.375 µl

The cDNA synthesis was run on PTC-200 Peltier Thermal Cycler (MJ Research, USA) with the following program.

- 25°C for 10min (primer incubation)
- 48°C for 60min (Reverse Transcriptase step)
- 95°C for 5min (Reverse Transcriptase inactivation)
- 4°C for ∞

The reverse transcription product (cDNA) was stored at -20°C until use.

3.17.3. Quantitative polymerase chain reaction (qPCR) analysis

PCR master mix consisted of 1µl forward and reverse primer (final concentration of 0.5µM), 4µl 1:10 dilution of cDNA and 5µl LightCycler 480 SYBR Green-I Master (Roche Applied Science, Germany). All samples were analyzed in parallels with a non-template control for each gene. qPCR was performed in 96 wells optical plates on LightCycler® 480 (Roche Diagnostics GmbH Mannheim, Germany). The running conditions were as follow

- 95°C for 5min (Pre-incubation)
- 50 cycles of 95°C for 15sec, 60°C for 1min (Amplification)
- 95°C for 10sec, 65°C for 1min, heating until 97°C (Melting curve)

- 40°C for 10sec (Cooling step)

The real time PCR was performed in a LightCycler® 480 device (Roche Diagnostics, Mannheim, Germany) with gene specific primers (table, 3.3). Two reference genes, Elongation factor 1a and RNA polymerase2 were evaluated using the geNorm software (Vandesomplele et al., 2002). RNA polymerase was found to be more stabile. A melting curve analysis (95ºC for 5seconds and 65ºC for 1minute, 97ºC) was run to confirm presence of a single PCR product. Also the primer efficiency of each primer pair was calculated. The relative gene expression level was calculated according to the ΔΔCt method and adjusted for differences in primer efficiency (Pfaffl, 2001).

Table. 3.3. Real time PCR primer sequences for relative gene expression of elongation factor 1a, RNA polmerase2, myogenin, myosin light chain2, cathepsin B and cathepsin L.

Target genes	**Forward primer (5´-3´)**	**Reverse primer (5´-3´)**	**GenBank accession no**
EF1a*	CACCACCGGCCATCTGATCTACAA	TCAGCAGCCTCCTTCTCGAACTTC	AF321836
RNA poly2*	TAACGCCTGCCTCTTCACGTTGA	ATGAGGGACCTTGTAGCCAGCAA	CA049789
Myo*	ATTGAGAGGCTGCAGGCACTTG	GTGCGGTAGTGTAAGCCCTGTGTT	DQ294029
MLC2*	CCATCAACTTCACCGTCTTCCTCAC	CAGCCCACAGGTTCTTCATCTCC	NM001123716
cat B*	AGGGGGGAACTCCTTACTGGCT	CGATGCCACAGTGGTCCTTACCT	DR696159
cat L*	GTATAGTGAAATGTGTGACC	AACCAGAGCAATAATTCAAG	CB502996

(*): Elongation factor 1a (EF1a), RNA polymerase2 (RNA poly2), myogenin (Myo), myosin light chain2 (MLC2), cathepsin B (cat B), cathepsin L (cat L).

3.18. Statistical Analysis

Data were analysed by ANOVA using the SAS program (Version 9.2; SAS Institute Inc., Cary, USA). The alpha level was set to 5% ($P < 0.05$).

4. RESULTS

This section is also divided into two parts. The first part describes the *in vivo* study while the second part describes the *in vitro* study.

4.1. IN VIVO STUDY

4.2. Fillet contraction

4.2.1. Non-crowded (NC) group

The progress of rigor development in the NC group is presented in figure 4.1. During the whole storage period, a maximum contraction of 18% was observed. The fillets contracted rapidly during the initial 24h of storage among all the diets (15-16% contraction). Thereafter the contraction rate decreased during the remaining storage period until 72h (further 1.4-2.1% units contraction). Numerically, the TTA and Arg diets showed 0.6-1.8% units lower contraction at each time point in comparison to the Con diet, whereas the Glu diet showed a similar contraction patters as the Con diet, deviating with only 0.5-1% units compared with the Con diet. Despite the numerical differences, no significant variations were observed between dietary treatments compared to Con diet, except at 72h when the contraction of the TTA diet was significantly lower ($p = 0.02$), and TTA diet also tended to be lower than the Glu diet ($p = 0.09$). Further, at 9h *post-mortem* the contraction of the Glu diet was significantly higher compared with the Arg diet ($p = 0.01$) and the TTA diet ($p = 0.02$).

4.2.2. Crowded (C) group

In case of C group, an initial higher contraction (≈12%) was observed for all the diets compared with NC group. The contraction rate was rapid from 1h to 6h of storage (18.5%) and then continued to increase at a slower rate until the final storage of 48h (fig. 4.2). Maximum contraction was 22-23% of initial fillet length. Numerically the contraction of the Arg diet was 1-2% units lower compared with the Con diet up to 24h storage, and in contrast to NC group, the TTA diet and the Glu diet showed higher numerical contraction compared to the Con diet at each time point. Although the numerical differences were consistent, there were no significant differences in contraction over the whole storage period between the experimental diets compared to the Con diet. However, at 1h *post-mortem* a significant difference ($p = 0.02$) was observed between the Arg and

TTA diets and the Arg diet also tended to be lower compared with the Glu diet at this time point (p = 0.08).

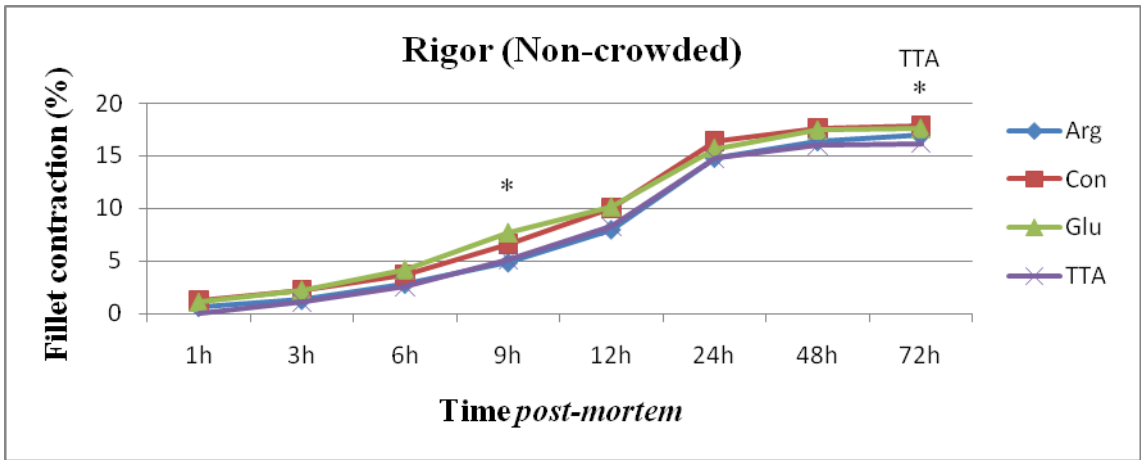

Fig. 4.1. Fillet contraction (mean) of the NC group during the storage period of 72h *post-mortem* of *pre-rigor* fillets of Atlantic salmon. The sign (*) represents significant differences between the dietary treatments (Con, Arg, Glu and TTA) whereas TTA on top of the sign (*) indicates significant difference ($p < 0.05$) form the Con diet.

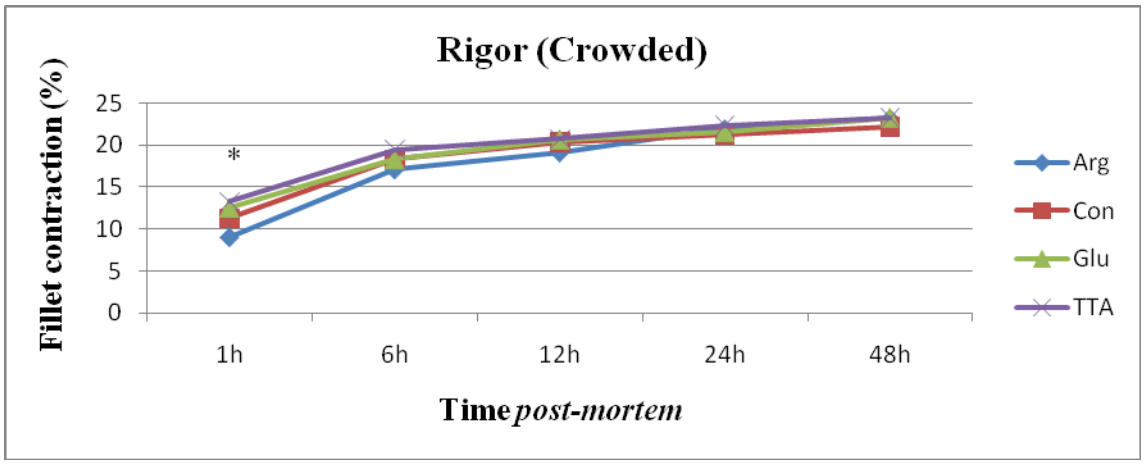

Fig. 4.2. Fillet contraction (mean) in the C group (16h crowding stress) during the storage period of 48h *post-mortem* of *pre-rigor* fillets of Atlantic salmon. The sign (*) represents significant differences ($p < 0.05$) between the dietary treatments (Con, Arg, Glu and TTA).

4.2.3. Comparison of NC and C groups

A comparison of fillets contraction within the dietary treatment was performed between NC and C groups at 1h and 48h of storage (fig. 4.3). Results from the comparison showed that crowding stress caused higher percentage of contraction both at 1h and 48h *post-mortem* compared to NC group.

Significant differences were observed between NC and C groups within all dietary groups (Con, Arg, Glu and TTA), both at 1h and 48h *post-mortem*.

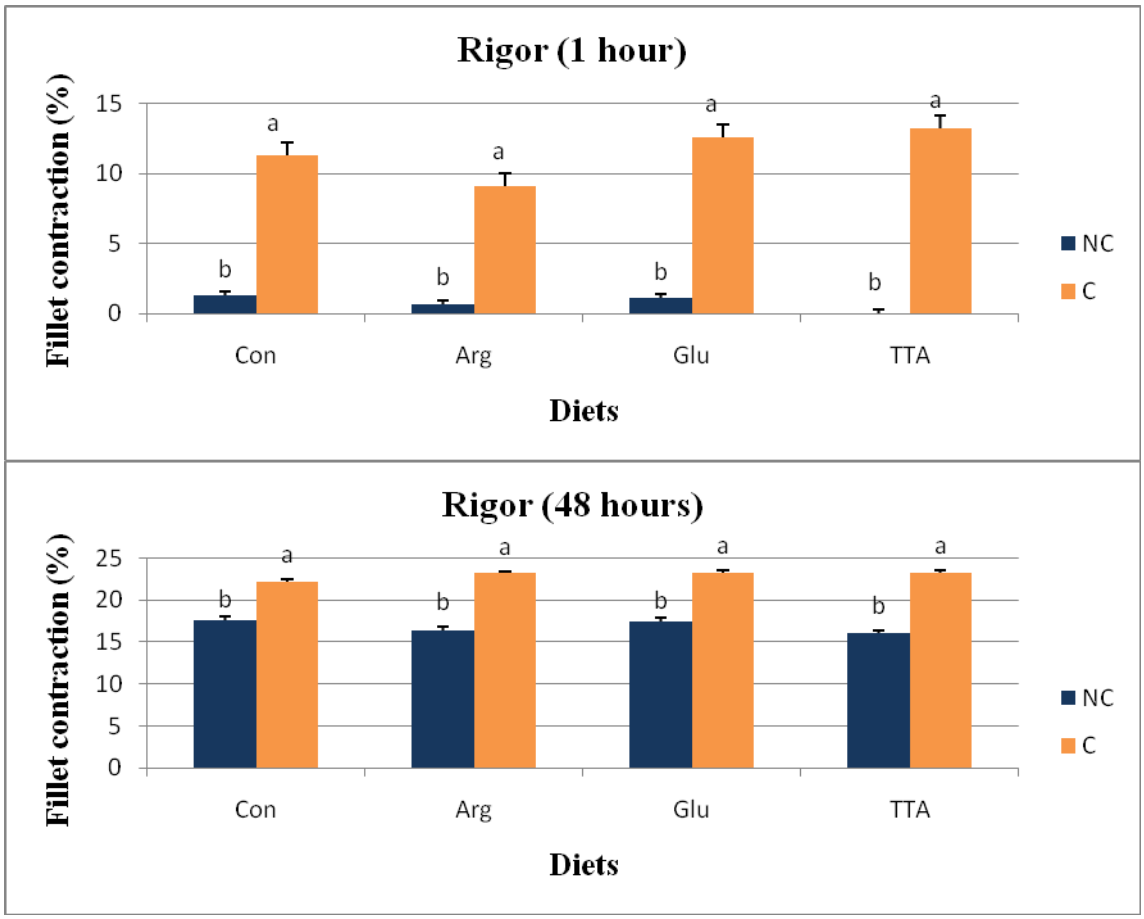

Fig. 4.3. Fillet contraction (mean ± SE) of *pre-rigor* fillets of NC and C groups of Atlantic salmon, after 1h and 48h *post-mortem*. Different letters denote significant differences ($p < 0.05$) between NC and C groups within different dietary treatments.

4.3. Muscle pH

Change in muscle pH was significantly influenced by all the diets (Arg, Glu and TTA) in both NC and C groups compared to Con diet. In addition, crowding stress also caused significant influence on muscle pH between NC and C groups within all the diets (Arg, Glu, TTA and Con).

4.3.1. Non-crowded (NC) group

The muscle pH of the NC group declined gradually with time, from an initial pH of 7.0 (1h) to a final pH of 6.25 (72h) on average (fig. 4.4). Muscle pH varied significantly between dietary treatments. The numerically lowest muscle pH was found in TTA diet compared to Con diet

throughout the whole storage period. In addition, muscle pH was higher for the Arg diet at 12h and 48h *post-mortem* while the pH of the Glu diet was higher at 6h *post-mortem* in comparison to the Con diet.

Significant differences were found only for the TTA and Arg diets at 1h, 12h, 24h and 72h *post-mortem* compared to the Con diet. At 1h and 12h *post-mortem,* muscle pH was significantly lower for the TTA diet than of the Con diet. Furthermore, the pH of the TTA diet was significantly lower compared with the Arg diet ($p = 0.005$) and Glu diet ($p = 0.01$) at 1h *post-mortem,* and compared with the Arg diet ($p = 0.003$) at 12h *post-mortem*. At 24h and 72h *post-mortem,* significantly lower muscle pH was found for the Arg compared to Con diet. A non-significant difference of $p = 0.08$ between Con diet and TTA diet at 24h *post-mortem* and between Con diet and Glu diet at 72h *post-mortem* was also found. No significant difference between Con diet and Glu diet was found.

4.3.2. Crowded (C) group

A comparatively lower initial pH (6.4) was found in of the C group than of the NC group although the ultimate pH (6.2) was similar in both groups (fig. 4.5). Also in contrast to NC group, there was a smooth decline in the muscle pH of the C group. During the whole storage period, the numerical muscle pH was higher in Glu diet than Con diet. Lowest final muscle pH (6.2) was found in Con diet. In case of the C group, muscle pH was significantly higher ($p = 0.03$) in Glu diet than Con diet at 12h storage, otherwise no significant variation was observed due to dietary treatment. However, there tended the pH of the TTA diet tended to be higher compared with Con diet at 12h ($p = 0.06$) and 48h ($p = 0.08$) *post-mortem* respectively.

4.3.3. Comparison of NC and C groups

Likewise fillet contraction, comparison of muscle pH within the diets was also performed between NC and C groups at 1h and 48h of storage period (fig. 4.6). Results showed a comparatively higher muscle pH in NC group than C group at both 1h and 48h storage. Significant differences were observed between NC and C groups within all the diets including Con diet at 1h *post-mortem.* However, at 48h *post-mortem* significant differences between NC and C groups were found only within Con diet and Arg diet. No significant differences were found within Glu ($p = 0.08$) and TTA ($p = 0.29$) diets among the both groups.

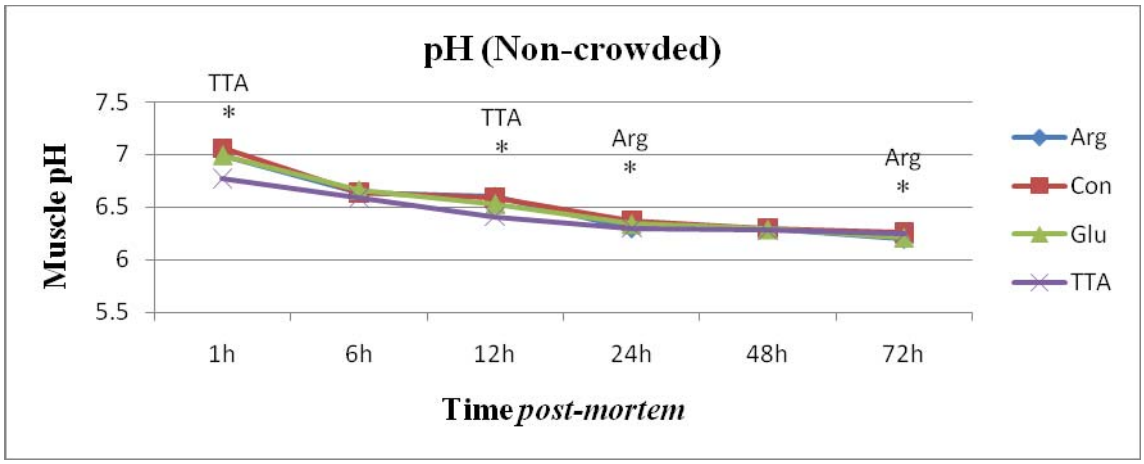

Fig. 4.4. Development in pH (mean) of *pre-rigor* fillets of Atlantic salmon during the storage period of 72h *post-mortem* of the NC group. The sign (*) indicate significant differences between dietary treatments, whereas diets denoted on top of the sign (*) indicate significant differences ($p < 0.05$) form Con diet.

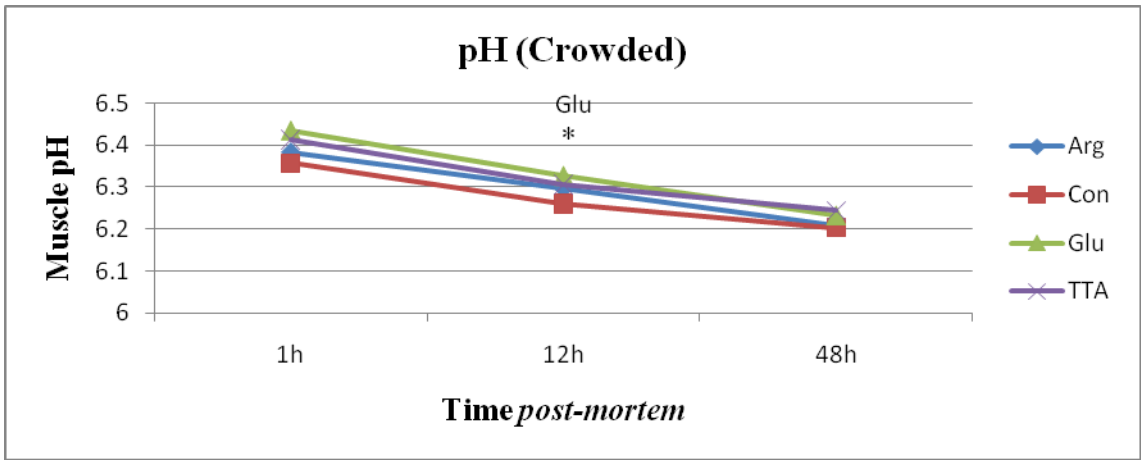

Fig. 4.5. Development in pH (mean) of *pre-rigor* fillets of Atlantic salmon during the storage period of 48h *post-mortem* of C group (16h crowding stress). The sign (*) indicate significant differences between dietary treatments, whereas diets denoted on top of the sign (*) indicate significant differences ($p < 0.05$) form Con diet.

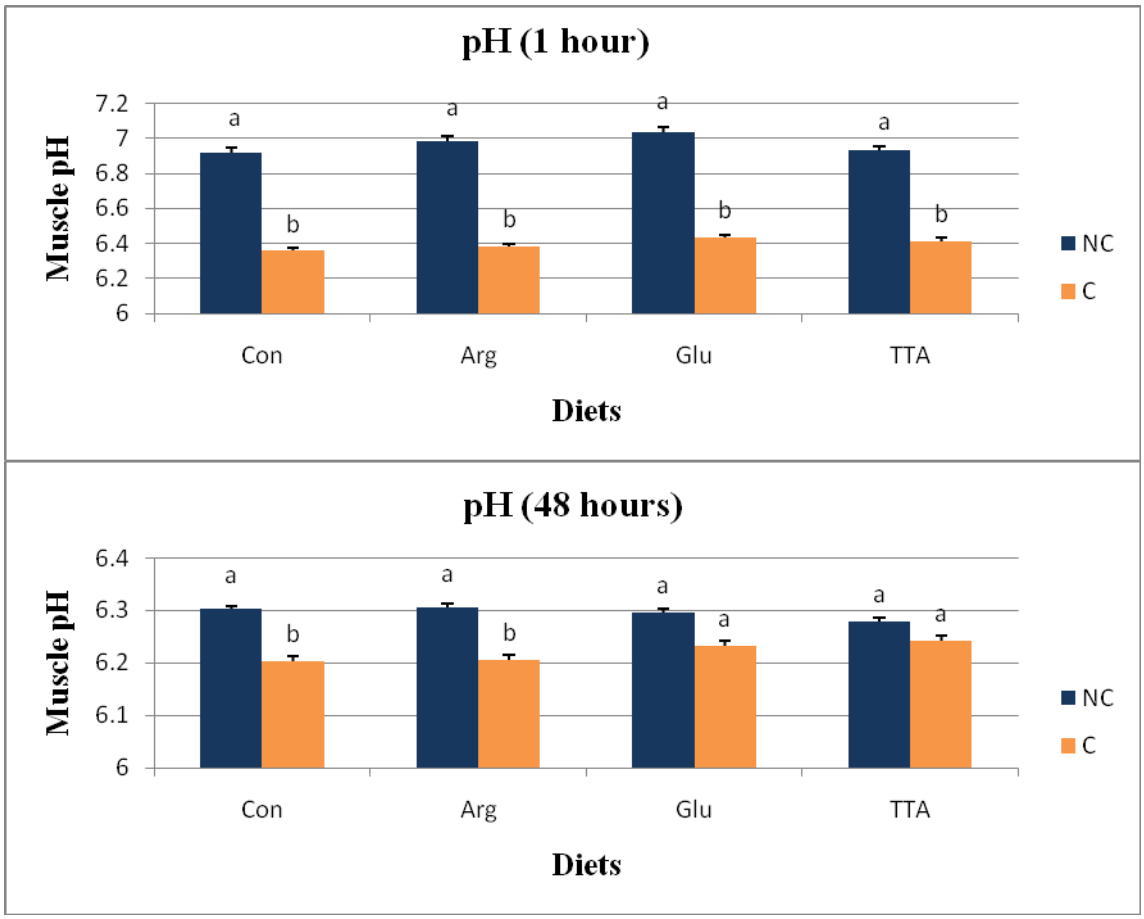

Fig. 4.6. Development in pH (mean) of *pre-rigor* fillets of NC and C groups of Atlantic salmon, after 1h and 48h *post-mortem*. Different letters denote significant differences ($p < 0.05$) between NC and C groups within different dietary treatments.

4.4. Texture analysis

Fillet firmness, determined as the total area under force-time graphs during instrumental analyses, showed significant influence of all the experimental diets (Arg, Glu and TTA) in both NC and C groups when compared to Con diet.

4.4.1. Non-crowded (NC) group

Results from the instrumental texture measurements showed a great variation in the total area between different diets (fig. 4.7). The average value was ranging from an initial total area of 230 (N*sec) to a final total area of 91 (N*sec). A rapid decline was found in total area under the graphs in all the diets from 1h to 6h *post-mortem*. Afterwards, the area remained stable in Arg and Glu diets until 24h *post-mortem* whereas it was highly decreased in TTA diet. The decreasing pattern was linear in Con diet during the whole storage period. Total area became similar in all the

diets from 48h *post-mortem* until the 72h *post-mortem.* At 72h storage, the total area was numerically higher of the Arg and Glu diets (114 N*s) and lowest for the Con diet (92 N*s), but the variation was not significant according to ANOVA. Statistical results showed significant differences in all the diets compared to Con diet. Arg diet was significantly higher at 1h and 24 *post-mortem*, Glu diet at 12h and 24h *post-mortem* whereas TTA diet at 12h *post-mortem*.

4.4.2. Crowded (C) group

In contrast to NC group, instrumental texture measurement results in C group showed a more linear decrease for all the diets (fig. 4.8). The total area in C group showed an average lower initial value of 180 (N*sec) to an average final lower value of 80 (N*sec). Arg diet showed the numerically highest initial (186.85 N*sec) and also final (90.4 N*sec) total area. The total area did not decrease significantly for any dietary treatment from 1h *post-mortem* to 12h *post-mortem.* Significant differences were only in Arg and Glu diets at 48h *post-mortem* compared to Con diet (higher values for the experimental diets; 90 vs. 78 N*s).

4.4.3. Comparison of NC and C groups

Comparison of total area under force-time graphs within the diets was performed between NC and C groups at 1h and 48h *post-mortem* (fig. 4.9). Results from the comparison revealed comparatively higher total area in NC group than C group at both 1h and 48h *post-mortem* within all the diets (Arg, Glu, TTA and Con). Significant differences were found within Arg, Glu and TTA diets at 1h *post-mortem*, while at 48h *post-mortem,* significant differences were found within all the diets (Arg, Glu, TTA and Con) between NC and C groups.

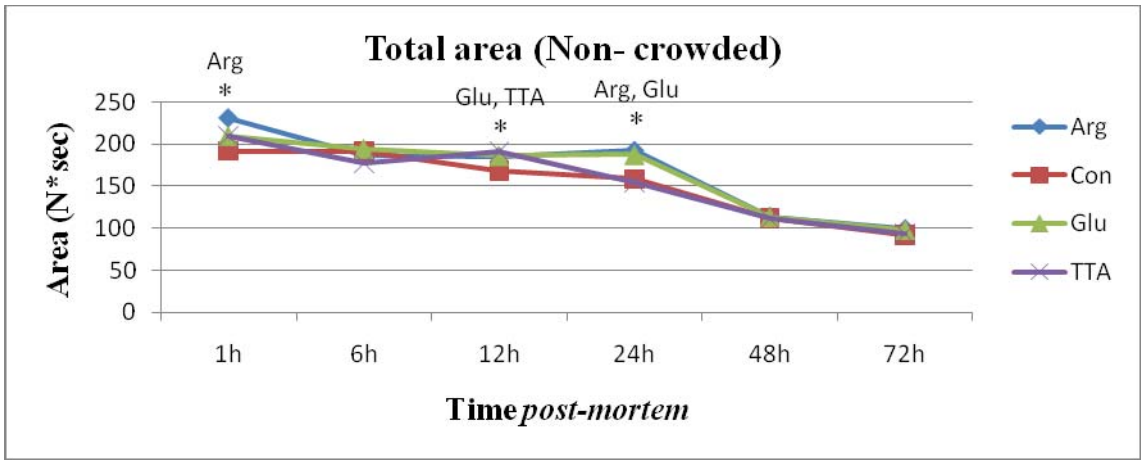

Fig. 4.7. Change in total area under the force-time graphs (mean) of *pre-rigor* fillets of Atlantic salmon during the storage period of 72h *post-mortem* in NC group. The sign (*) indicate significant differences between dietary treatments, whereas diets denoted on top of the sign (*) indicate significant differences ($p < 0.05$) form Con diet.

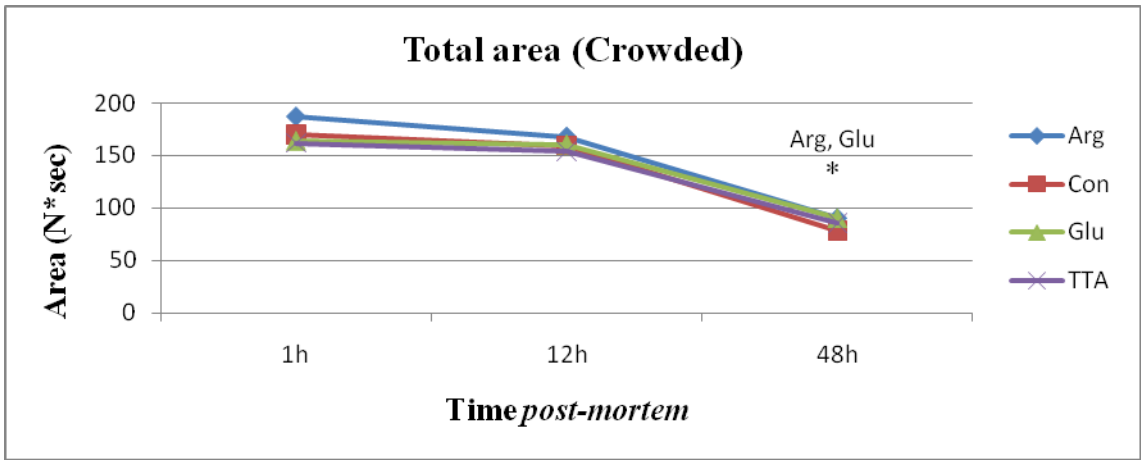

Fig. 4.8. Change in total area under the force-time graphs (mean) of *pre-rigor* fillets of Atlantic salmon during the storage period of 48h *post-mortem* in C group (16h crowding stress). The sign (*) indicate significant differences between dietary treatments, whereas diets denoted on top of the sign (*) indicate significant differences ($p < 0.05$) form Con diet.

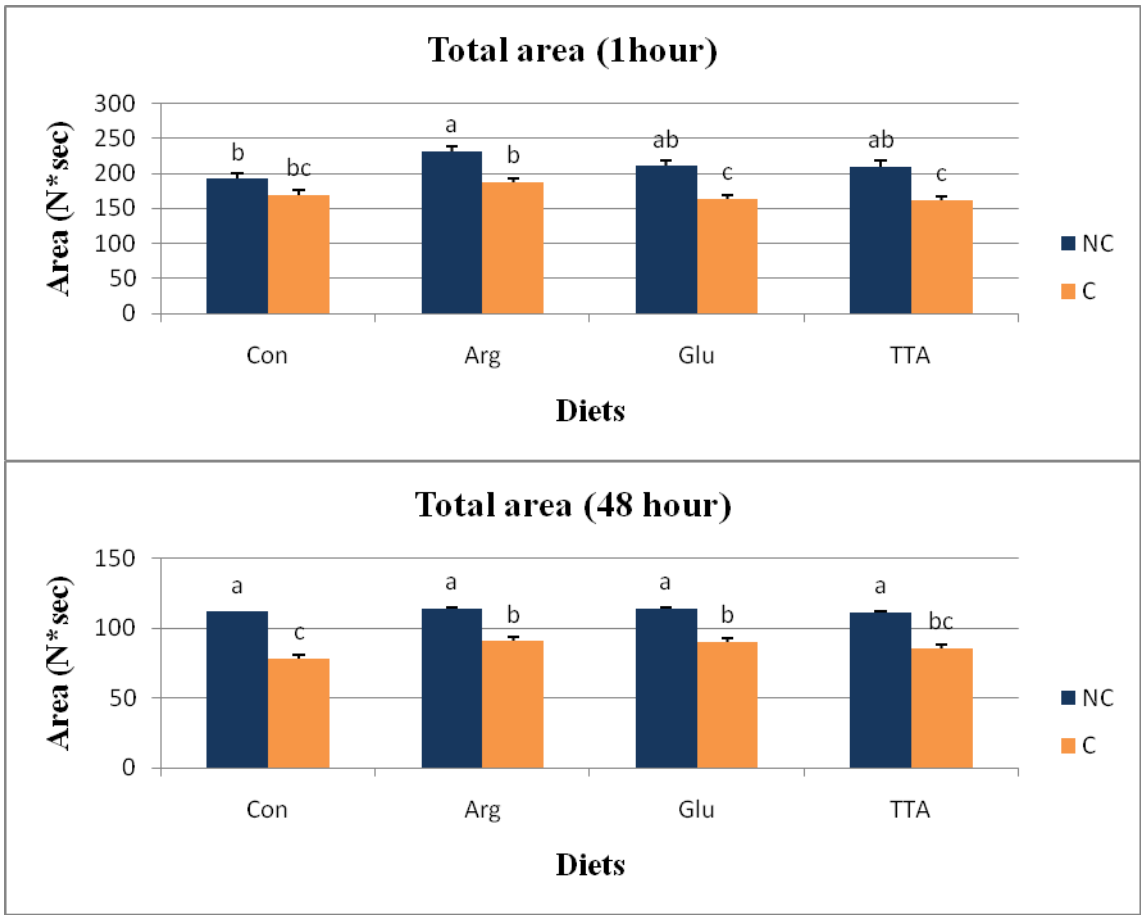

Fig. 4.9. Change in total area (mean ± SE) under the force-time graphs of *pre-rigor* fillets of NC and C groups of Atlantic salmon, after 1h and 48h *post-mortem*. Different letters denote the significant differences ($p < 0.05$) between NC and C groups within different dietary treatments.

4.5. Histological analysis

4.5.1. Non-crowded (NC) group

The relative number of muscle fibres of the NC group showed significant effect of dietary treatment, although no significant differences were found between experimental diets (Arg, Glu and TTA) compared to Con diet (fig. 4.10). However, the relative number of muscle fibres of the Arg diet (n=88.9) was significantly higher compared to the TTA diet (n=80.6), while the Glu diet (n=84.4) and Con diet (83.9) had intermediate number of muscle fibres. Results thus indicate that the Arg group had smaller muscle fibres whereas the TTA group had larger muscle fibres.

4.5.2. Crowded (C) group

In contrast to the NC group, the relative number of muscle fibres was significantly higher in TTA diet compared to Con diet of the crowded group (fig. 4.11). The number of muscle fibres in

Arg and Glu diets were not significantly different from Con diet (n=82.8) but numerically the number of muscle fibres were higher in Arg and Glu diets (n= 84.8 and 88.6, respectively).

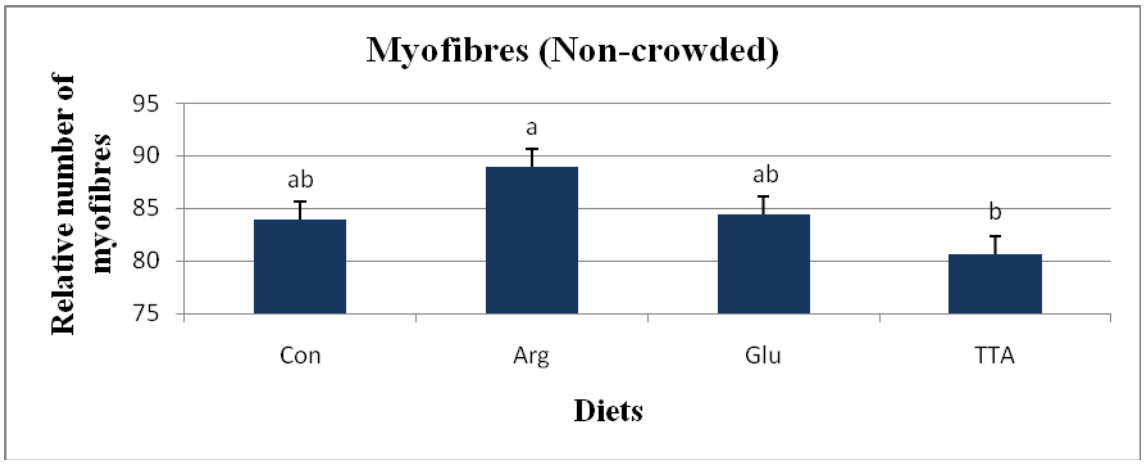

Fig. 4.10. Relative number of myofibres (mean ± SE) in NC group of *pre-rigor* fillets of Atlantic salmon after 1h *post-mortem,* determined by histological examination. Different letters denote significant differences ($p < 0.05$) between dietary treatments (Con, Arg, Glu and TTA).

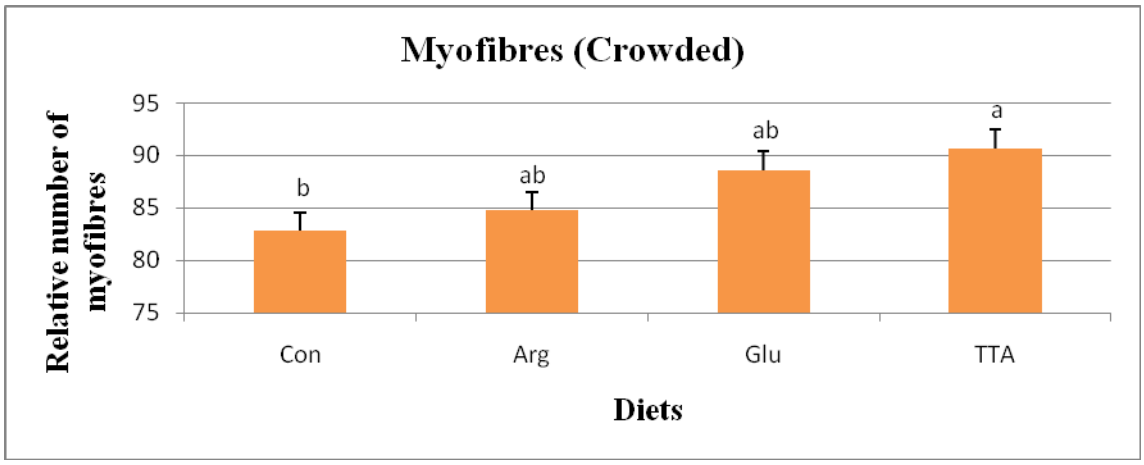

Fig. 4.11. Relative number of myofibres (mean ± SE) in C group of *pre-rigor* fillets of Atlantic salmon after 1h *post-mortem,* determined by histological examination. Different letters denote the significant differences ($p < 0.05$) between dietary treatments (Con, Arg, Glu and TTA).

4.5.3. Comparison of NC and C groups

Comparison of NC and C groups within different diets showed no significant differences, except for the TTA diet (fig. 4.12). Micrographs of the fish fed TTA diet are presented in figure 4.13 to illustrate general morphological differences between the NC and C group. Micrographs from TTA diet were chosen, as there was a significant difference between NC and C group.

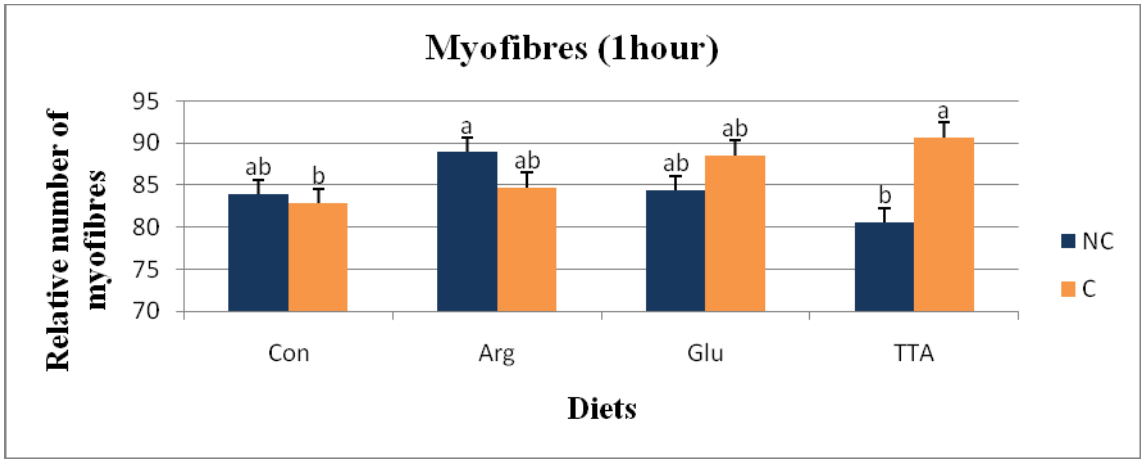

Fig. 4.12. Relative number of myofibres (mean ± SE) in NC and C group of *pre-rigor* fillets of Atlantic salmon after 1h *post-mortem* determined by histological examination.. Different letters denote significant differences ($p < 0.05$) between dietary treatment within pre-slaughter handling procedure (NC and C).

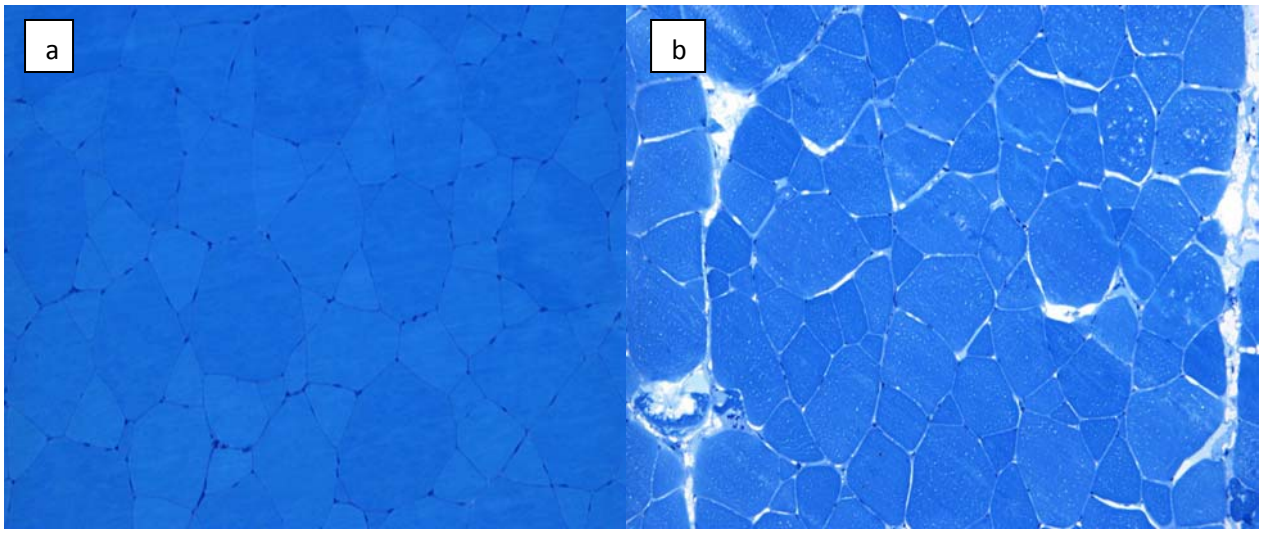

Fig. 4.13. An example of micrograph of NC group and C group. (a) Micrograph from the fish fed TTA diet in NC group and (b) Micrograph from the fish fed TTA diet in C group. Light microscope images; Magnification = 20x

4.6. Cathepsins analysis

No effect of all the experimental diets (Arg, Glu and TTA) on the activities of cathepsins (cathepsin B, cathepsin L and cathepsin B+L) was found in NC when compared to Con diet. Additionally, crowding stress only showed significant variation between the Glu and Con diet for activity of cathepsin B+L

4.6.1. Non-crowded (NC) group

Activities of cathepsin B, cathepsin L and cathepsin B+L in NC group are presented in figure 4.14. Results from statistical analysis showed that, none of the diet significantly influenced the activity of cathepsin B, cathepsin L and or cathepsin B+L compared to Con diet. Cathepsin B activity, in Arg and Glu diets (0.64mU/g muscle) and cathepsin B+L activity in Glu diet was however slightly higher than of the Con diet (0.57mU/g muscle), although not significant.

4.6.2. Crowded (C) group

The numerical cathepsin L activity in Arg and Glu diets was slightly higher than Con diet whereas cathepsin B and cathepsin B+L activities were slightly lower in all the experimental diets compared with the Con diet (fig. 4.15). However, no significant differences in the activities of cathepsin L and cathepsin B+L were found in any of the diets compared to the Con diet, but the activity of cathepsin B was significantly lower in all the diets (Arg, Glu, and TTA) compared with the Con diet.

4.6.3. Comparison of NC and C groups

The activity of cathepsin B was similar in the NC and C group, whereas cathepsin L and cathepsin B+L activities were irregular in different diets between the NC and C groups (fig. 4.16). Results from the statistical analysis showed no significant difference in any of the activity of the cathepsins.

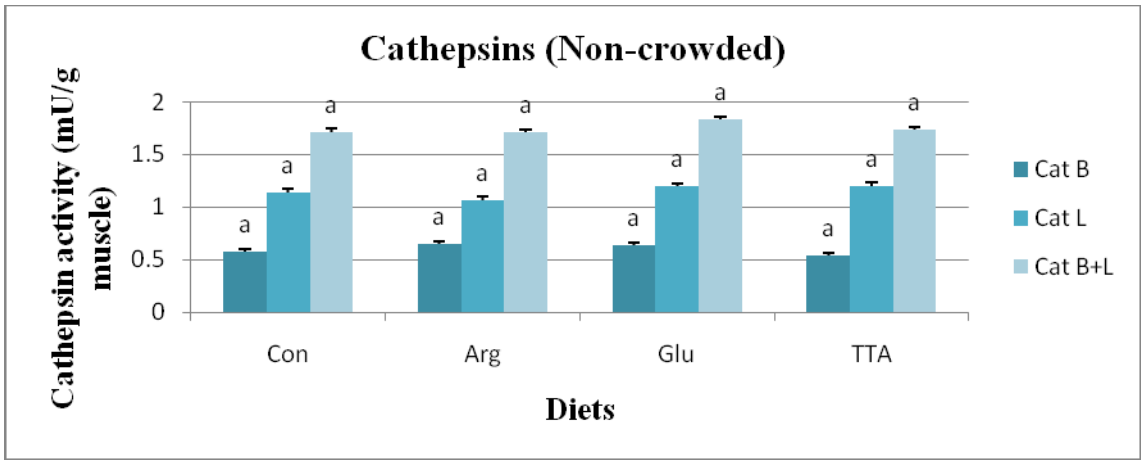

Fig. 4.14. Activity of cathepsin B, L and B+L (mean ± SE) in muscle of NC group of Atlantic salmon fed different diets (Arg, Glu and TTA) including Con diet at 1h *post-mortem*. Different letters denote the significant differences ($p < 0.05$) between different diets from Con diet in cathepsin B, L, and B+L activity.

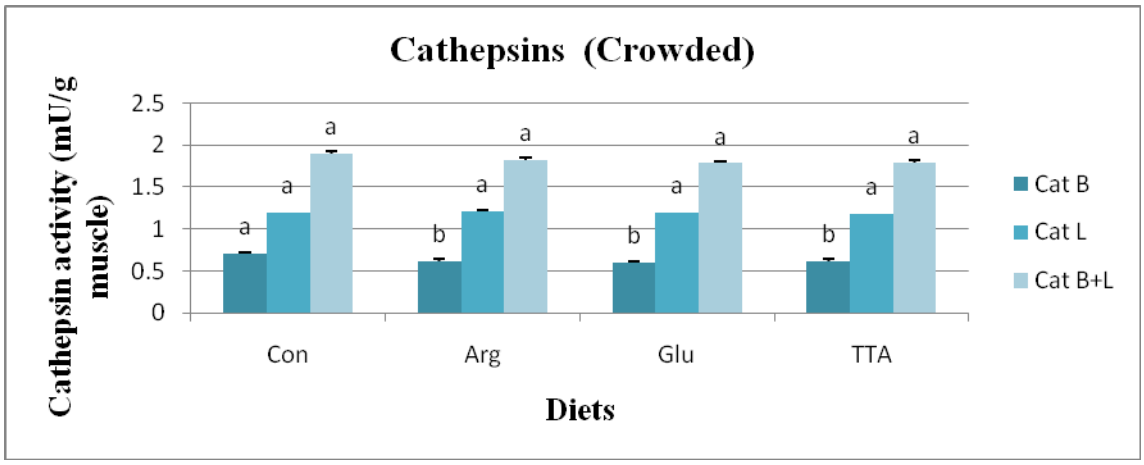

Fig. 4.15. Activity of cathepsin B, L and B+L (mean ± SE) in muscle of C group (16h) of Atlantic salmon fed different diets (Con, Arg, Glu or TTA). Different letters denote significant differences ($p < 0.05$) between diets in cathepsin B, L, and B+L activity, respectively. Analyses were performed in muscle sampled 1h *post-mortem.*

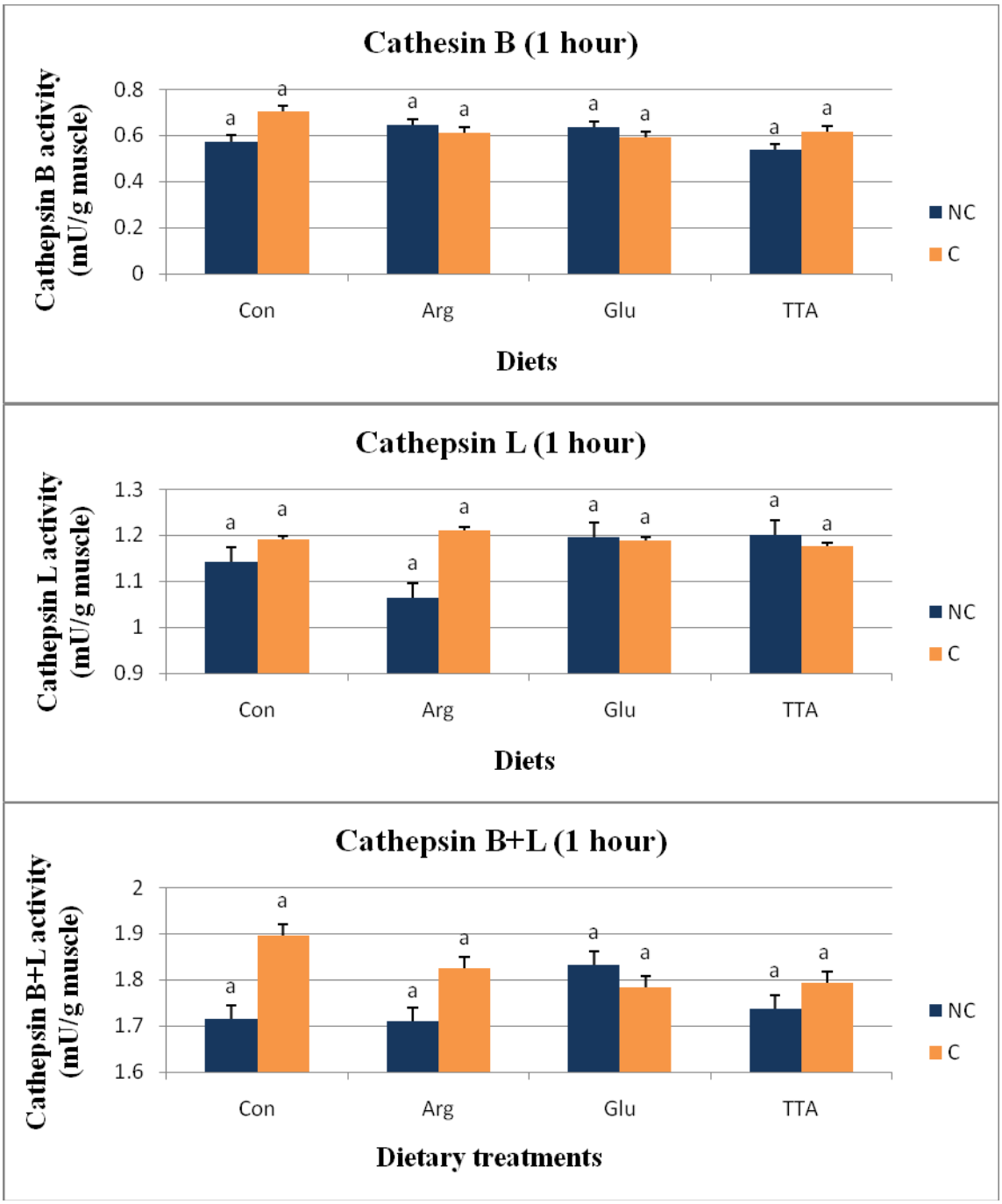

Fig. 4.16. Activity of cathepsin B, L and B+L (mean ± SE) in muscle of NC and C groups of Atlantic salmon fed different diets (Con, Arg, Glu and TTA) at 1h *post-mortem*. Different letters denote the significant differences ($p < 0.05$) between NC and C groups within different dietary treatments.

4.7. IN VITRO STUDY

4.8. Proliferating cell nuclear antigen (PCNA) assay

4.8.1. Numbering of cells nuclei

The number of blue cells nuclei, brown cells nuclei and total cells nuclei were determined. Results from the proliferating cell nuclear antigen (PCNA) analysis revealed a great variation between the numbers of PCNA negatively stained nuclei (blue cells nuclei), PCNA positively stained nuclei (brown cells nuclei) and total cells nuclei, between both treatments and temperatures and within treatment (table, 4.1 ; fig. 4.17, 4.18). However, the variation was inconsistent with regard to treatment.

At 8°C, the numbers of blue, brown and total cells nuclei were lower in TTA treatment compared to Con, but the Arg and Glu treatments did not differ significantly from Con. Similarly, the number of blue and total cells nuclei was lower in the TTA treatment compared to Con at 16°C. The number of blue cells nuclei was numerically highest of the Arg treatment at 8°C, but at 16°C the number was significantly lower compared with the Con and similar with the TTA. The number of brown cells nuclei of the Arg group was numerically highest at 16°C; hence the total number of cells nuclei did not differ between the Arg and Con treatment. The Gln treatment did not differ significantly from the Con, but at 16°C, the number of blue cells nuclei was higher compared with the Arg and TTA treatment.

Table 4.1. Summary of the counting of blue, brown and total number of cells (mean ± SE). Different letters denote the significant differences ($p < 0.05$) between the treatments (Arg, Gln and TTA) and temperatures (8 C°, 16C) from Con.

Treatments	Blue cells nuclei	Brown cells nuclei	Total cells nuclei
Con 8	$187.4^{ab} \pm 15.2$	$63.0^{c} \pm 3.5$	$250.4^{b} \pm 17.5$
Arg 8	$207.0^{a} \pm 19.7$	$48.2^{cd} \pm 7.6$	$256.2^{b} \pm 24.0$
Gln 8	$158.2^{bc} \pm 2.1$	$46.4^{cde} \pm 2.0$	$204.6^{bc} \pm 1.6$
TTA 8	$138.2^{c} \pm 9.7$	$13.6^{e} \pm 1.5$	$152.0^{d} \pm 10.8$
Con16	$208.8^{a} \pm 27.7$	$99.4^{ab} \pm 7.8$	$308.2^{a} \pm 25.9$
Arg 16	$149.6^{bc} \pm 17.1$	$125.0^{a} \pm 30.5$	$274.6^{ab} \pm 18.1$
Gln 16	$204.4^{a} \pm 2.1$	$106.8^{ab} \pm 10.1$	$311.2^{a} \pm 9.9$
TTA 16	$152.0^{c} \pm 17.1$	$76.6^{bcd} \pm 4.1$	$228.6^{bc} \pm 18.8$

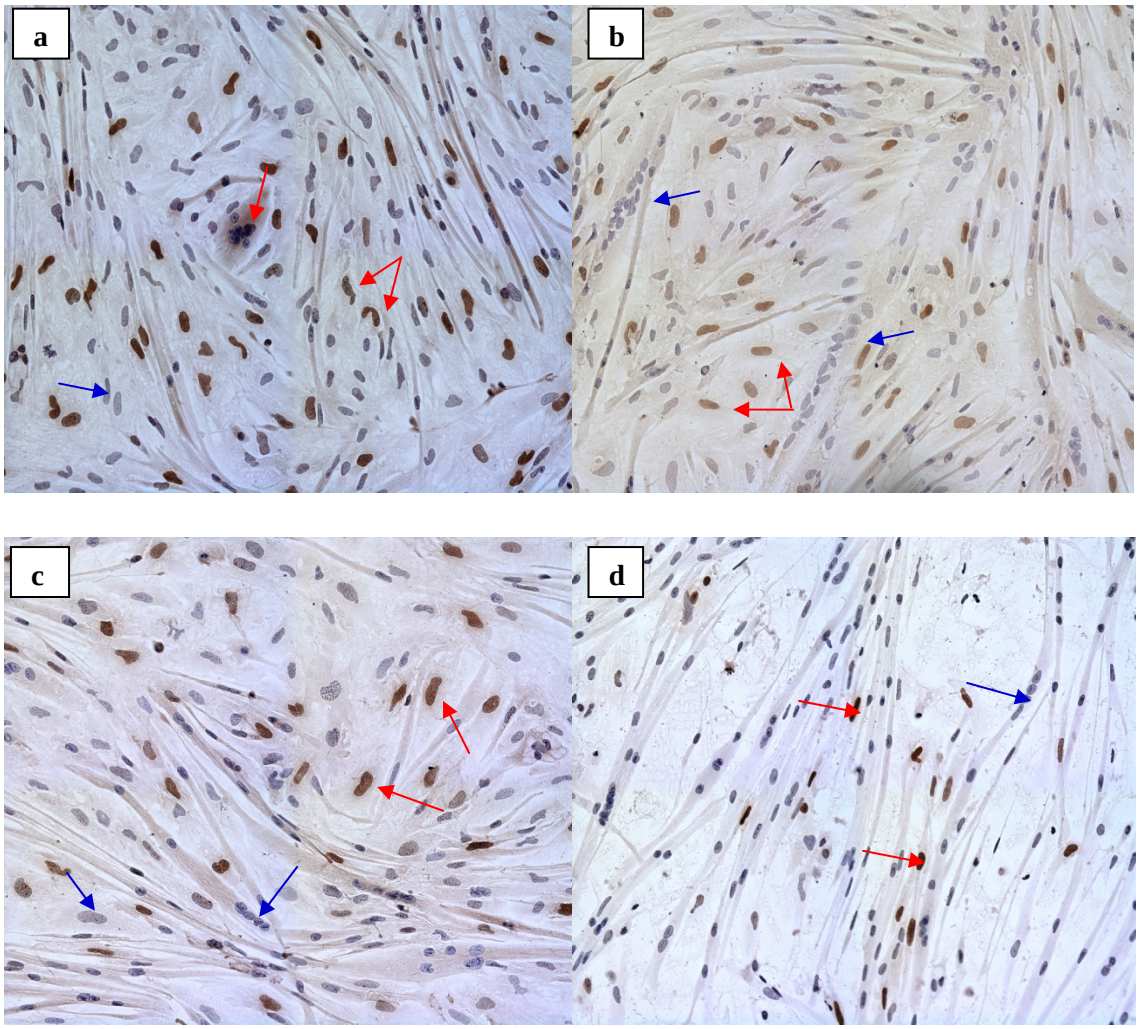

Fig. 4.17. Light microscope images (magnification = 25x) illustrating the muscle precursor cell morphology and proliferating versus non-proliferating cells nuclei at 8°C. Blue arrows indicate the non-proliferating cells nuclei, whereas red arrows indicate the proliferating cells nuclei. (a) Proliferation in Con treatment (b) Proliferation in Arg treatment. (c) Proliferation in Gln treatment. (d) Proliferation in TTA treatment.

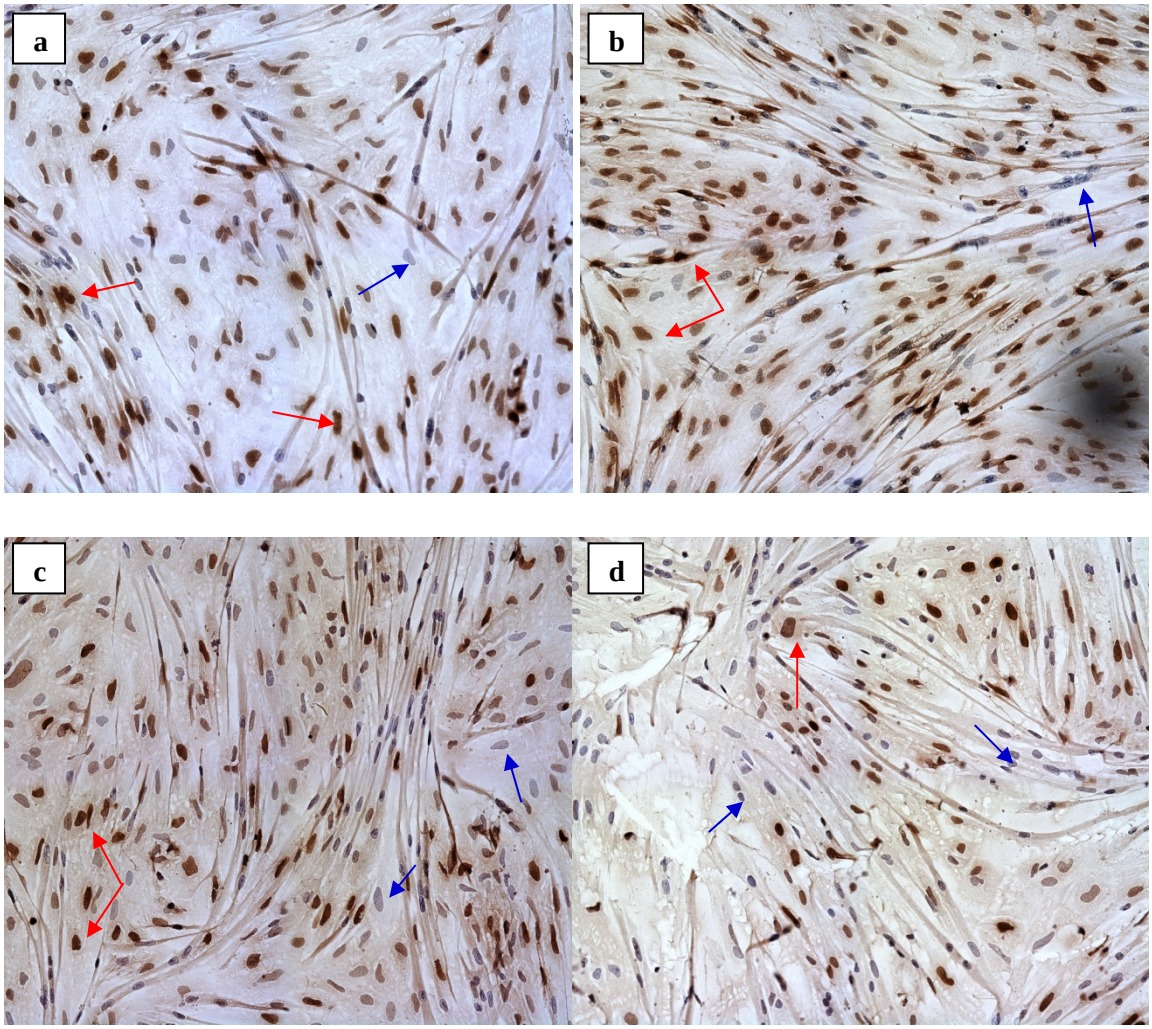

Fig. 4.18. Light microscope images (magnification = 25x) illustrating the muscle precursor cell morphology and proliferating versus non-proliferating cells nuclei at 16°C. Blue arrows indicate the non-proliferating cells nuclei, whereas red arrows indicate the proliferating cells nuclei. (a) Proliferation in Con treatment (b) Proliferation in Arg treatment (c) Proliferation in Gln treatment (d) Proliferation in TTA treatment.

4.8.2. Proliferation percentage (blue cells nuclei)

Percentage of PCNA negatively stained nuclei (blue cells nuclei) is presented in figure 4.19. Results from PCNA assay analyses revealed higher percentage at 8°C than 16°C. Percentage of blue cells nuclei was higher in TTA treatment (90.9%) at 8°C compared to Con and the percentage was higher at 8°C than at 16°C. At 16°C percentage of blue cells nuclei of Gln and TTA treatments (66%) were similar as for the Con (65%), while a lower percentage was found for the Arg treatment (56%). With the exception of higher percentage of blue cells nuclei for the TTA treatment at 8°C ($p = 0.0004$) and lower for the Arg treatment at 16°C ($p = 0.051$), no significant differences between were found treatments at either temperatures compared to Con. Furthermore, a significant

difference was seen between TTA and Gln treatments (p = 0.01) at 8°C (lower percentage in Gln). The percentage of blue cells nuclei was significantly lower at 16°C vs. 8°C for all treatments.

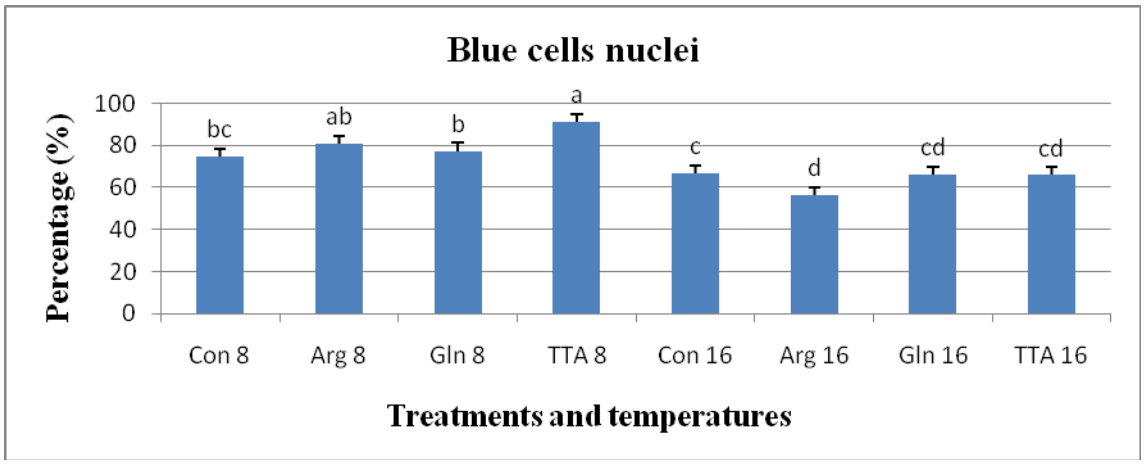

Fig. 4.19. Percentage of PCNA negatively stained nuclei (mean ± SE) at two different temperatures (8°C, 16°C) and treatments (Con, Arg, Gln and TTA). Different letters denote the significant differences (p< 0.05) between treatments.

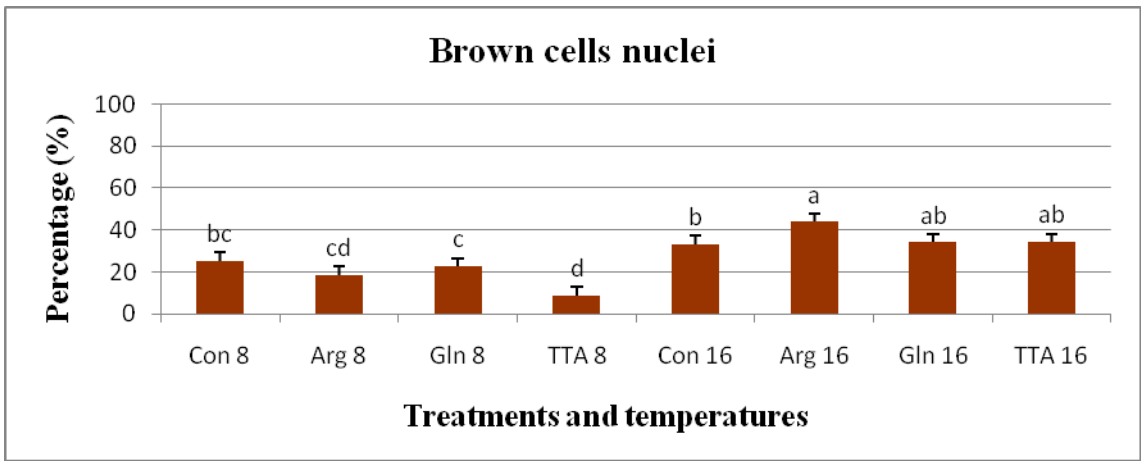

Fig. 4.20. Percentage of PCNA positively stained nuclei (mean ± SE) at two different temperatures (8°C, 16°C) and treatments (Con, Arg, Gln and TTA). Different letters denote the significant differences (p< 0.05) between treatments.

4.8.3. Proliferation percentage (brown cells nuclei)

A percentage analysis of PCNA positively stained nuclei (brown cells nuclei) is presented in figure 4.20. Results of the PCNA assay revealed higher percentage at 16°C than 8°C. At 16°C, the highest percentage brown cells nuclei was found in Arg treatment (43%) (p = 0.051). At 8°C, percentage was lower for the TTA treatment compared to Con (p <0.0001), but otherwise no significant variation was found.

4.9. Gene expression analysis

Dietary treatments (Con, Arg, Gln, and TTA) showed significant impact on gene expression of myosin light chain2 and myogenin at both temperatures (8°C and16°C) compared to Con. Dietary treatments (Arg, Gln, and TTA) showed no impact on gene expression of cathepsin B and cathepsin L at both temperatures (8°C and16°C) compared to Con. A summary of the *P* values of treatment, temperature, interaction between treatment and temperature, and model is given in addition to the R^2 in table 4.2.

Table. 4.2. Summary of *P* values of treatment (Arg, Gln, TTA), temperature (8°C and16°C), interaction between treatment and temperature, and model. Furthermore R^2 is given.

Parameter	*P* value treatment	*P* value temperature	*P* value interaction	*P* value model	R^2
Myosin light chain2	<.0001	0.8789	<.0001	<.0001	0.88
Myogenin	0.0014	0.2609	0.0002	<.0001	0.86
Cathepsin B	0.0434	0.0947	0.0441	0.0263	0.45
Cathepsin L	0.7849	<.0001	0.5120	<.0001	0.72

4.9.1. Relative gene expression of Myosin light chain2

Results from PCR analysis showed that expression of myosin light chain2 was higher at 16°C than 8°C (fig. 4.21). Highest expression was found in TTA treatment (1.48) at 8°C and in Gln treatment (1.04) at 16°C. The expression was similar in Arg (0.67) and Gln (0.66) treatments at 8°C, and in Arg (0.83) and TTA (0.81) treatments at 16°C. Myosin light chain2 expression was significantly higher in all the experimental treatments compared to Con at both 8°C and 16°C. Further, when different experimental treatments were compared, a significant difference between TTA and Arg treatments ($p < .0001$), and TTA and Gln treatments ($p < .0001$) was found at 8°C. There was no significant difference between Arg and Gln treatments at 8°C. At 16°C, significant differences between Gln and Arg treatments ($p = 0.02$), and Gln and TTA treatments ($p = 0.01$) were also found.

A comparison of the same treatments at two different temperatures (8°C and16°C) revealed significant variation only between Gln treatments and TTA treatments. Myosin light chain2

expression in case of TTA treatment was significantly higher (p <.0001) at 8°C compared to TTA treatment at 16°C. In contrast, Gln treatment was significantly higher (p = 0.0002) at 16°C than that of 8°C. Though there was no significant difference between Arg treatments between the two temperatures, a non-significant difference of p = 0.07 was found.

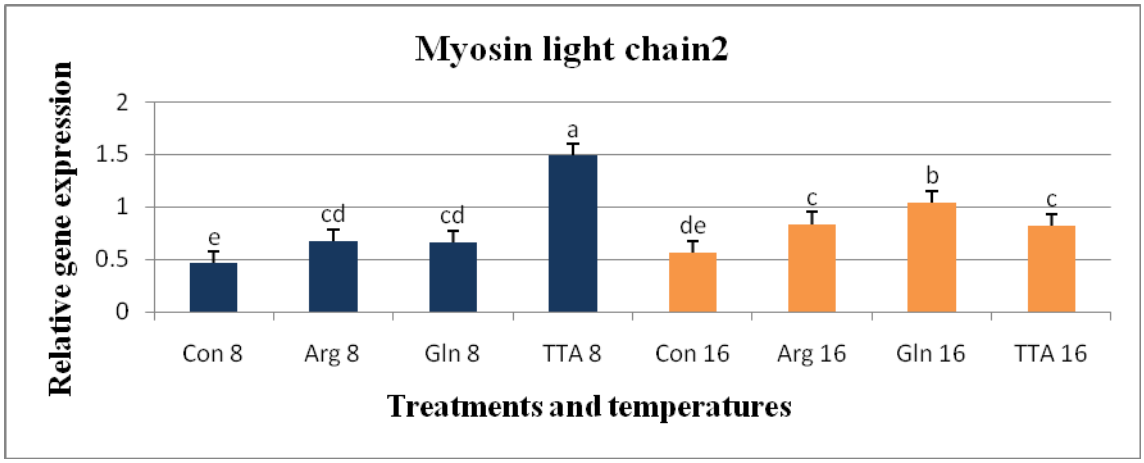

Fig. 4.21. Relative gene expression (mean ± SE) of myosin light chain2 in muscle precursor cells of Atlantic salmon, supplemented with three different substrates (Arg, Gln and TTA) together with Con, when kept at two different temperatures (8C°, 16C°). Different letters denote significant differences (p< 0.05) between treatments and temperatures.

4.9.2. Relative gene expression of Myogenin

Results from the PCR analysis revealed that relative gene expression of myogenin was also influenced by treatment (fig. 4.22), although the expression was not so different between the temperatures, except for the TTA treatment. Arg and Gln treatments had strong influence on the up-regulation of myogenin at both temperatures, and also the TTA treatment at 8°C. Gln treatment (1.35) at 16°C and Arg treatment (1.21) at 8°C presented the highest myogenin expression compared to Con. There was a slight difference between the expression of Arg (1.21) and Gln (1.20) treatments at 8°C, and at 16°C a significant difference was observed between these treatments (p = 0.0008),. Relative gene expression of myogenin in case of TTA treatment (0.70) was lower than Con (0.89) at 16°C (p = 0.009). In addition, comparison between different treatments showed significant differences between TTA and Arg treatments (p = 0.01), and TTA and Gln treatments (p = 0.02) at 8°C.. Moreover, variation between Arg and TTA treatments (p <.0001), and Gln and TTA treatments (p <.0001) were also found.

Significant differences between 8°C and 16°C were found only between Gln and TTA treatments. Relative gene expression in case of TTA treatment was significantly higher ($p < .0001$) at 8°C compared to 16°C. In contrast, Gln treatment was significantly higher ($p = 0.02$) at 16°C than that of 8°C.

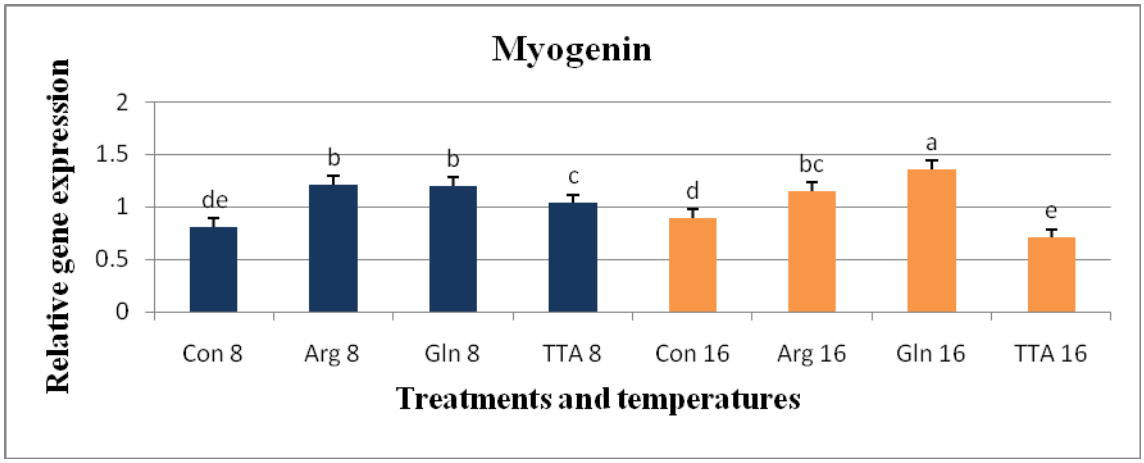

Fig. 4.22. Relative gene expression (mean ± SE) of myogenin in muscle precursor cells of Atlantic salmon, supplemented with three different substrates (Arg, Gln and TTA) together with Con, when kept at two different temperatures (8C°, 16C°). Different letters denote significant differences ($p < 0.05$) between treatments and temperatures.

4.9.3. Relative gene expression of cathepsin B

Results from the PCR analysis showed that expression of cathepsin B was only influenced by TTA treatment at 8°C ($p = 0.04$) (fig. 4.23), when compared with the Con. Moreover, a significant difference between TTA and Gln treatments ($p = 0.009$) was found at 8°C. At 16°C, none of the treatment was significantly different from Con, but comparison between different treatments showed significant differences between Arg and Gln treatments ($p = 0.04$) and also between Arg and TTA treatments ($p = 0.03$). Expression was similar between the two temperatures, except for the TTA treatment, where relative gene expression in TTA treatment was significantly higher ($p = 0.0021$) at 16°C compared to that at 8°C.

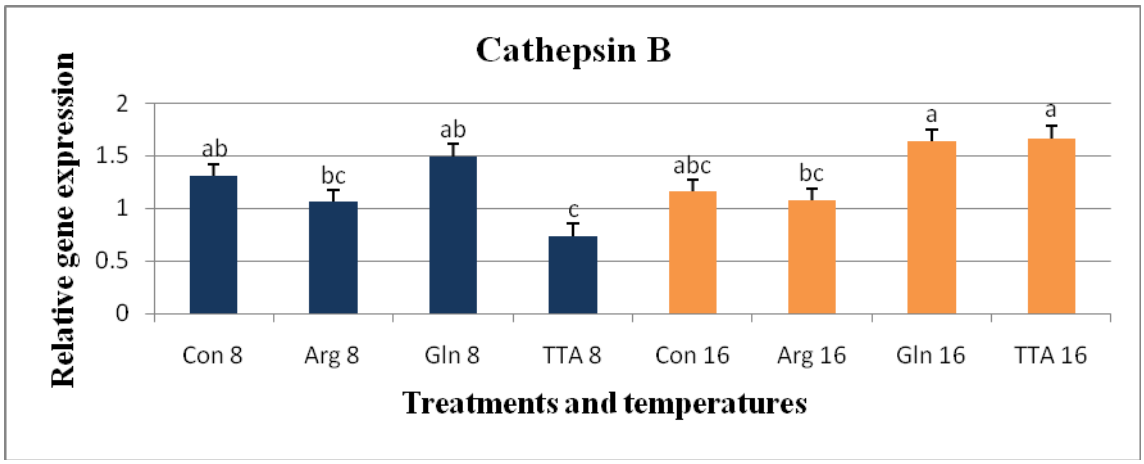

Fig. 4.23. Relative gene expression (mean ± SE) of cathepsin B in muscle precursor cells of Atlantic salmon, supplemented with three different substrates (Arg, Gln and TTA) together with Con, when kept at two different temperatures (8C°, 16C°). Different letters denote significant differences (p< 0.05) between treatments and temperatures.

4.9.4. Relative gene expression of cathepsin L

Results from PCR analysis revealed that none of the treatments influenced the relative gene expression of cathepsin L, but the expression was almost twice as high at 16°C than 8°C for all treatments (fig. 4.24).

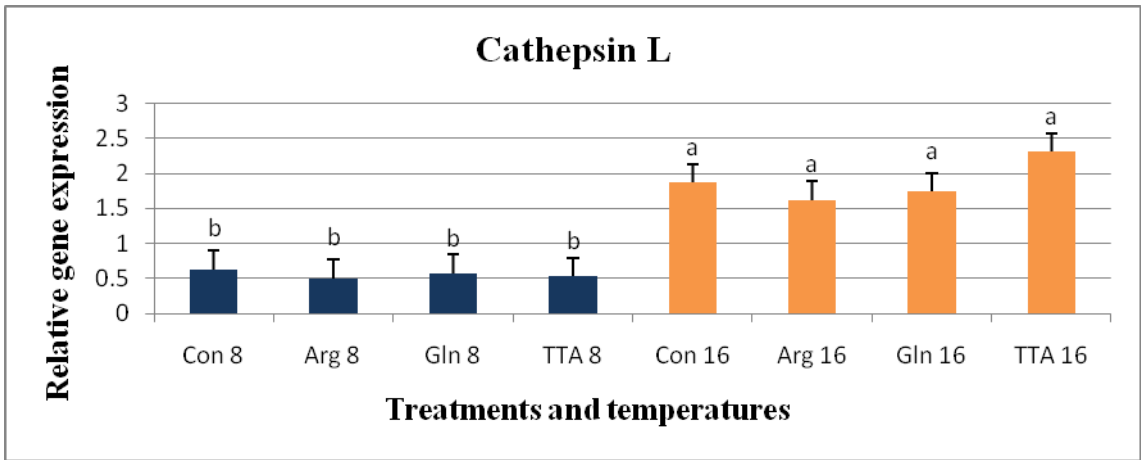

Fig. 4.24. Relative gene expression (mean ± SE) of cathepsin L in muscle precursor cells of Atlantic salmon, supplemented with three different substrates (Arg, Gln and TTA) together with Con, when kept at two different temperatures (8C°, 16C°). Different letters denote significant differences (p< 0.05) between treatments and temperatures.

5. DISCUSSION

In the present thesis two different approaches were used, i.e. *in vivo* (Arg, Glu and TTA) and *in vitro* (Arg, Gln and TTA) studies. In the *in vivo* study, fillet contraction, muscle pH, texture, histology and cathepsins analysis were performed while for the *in vitro* study, PCNA assay and gene expression analysis were performed.

Rigor development in the present study is defined as shortening in the length of the fillets original length. Feeding Atlantic salmon diet, supplemented with Arg, Glu or TTA did not exert any significant impact on contraction percentage of either non-crowded (NC) or crowded (C) group. The final contraction percentage was in the order of TTA < Arg < Glu < Con diets in NC group while Con < TTA < Glu < Arg diets in C group. In both the NC and C group, the contraction rate seemed to be slowest for the Arg diet (similar development for Arg and TTA diet in the NC group). Very few studies have been conducted which shows the impact of bioactive components on rigor development. The lower contraction showed by amino acids (Arg and Glu) in NC group was, however, in line with the work of Suontama et al. (2006) who found significant lower contraction in Atlantic salmon fed krill protein. High percentage of contractions (NC = 15-16%; C = 20-21%) were measured during the first 24h *post-mortem* and thereafter the contraction rate became slower (NC = further 1.4-2.1% units; C = further 1.1-2.1% units) until the final storages. Thus, a maximum contraction of 18% and 22-23% were observed for the NC and C group, respectively. The results in NC group were slightly higher than previously reported (Skjervold et al., 2001; Sorensen et al. 1997; Morkore et al. 2008), where the final contraction percentage in rested salmon was in the range of 15-16%. The significantly faster contraction rate and higher final contraction in the C group (average 11.4% at 1h post-mortem compared to near 0% for the NC group at 1h) is in agreement with the earlier studies showing higher contraction due to pre-slaughter handling stress (Nakayama et al., 1992; Huss, 1995; Elvevoll et al., 1996; Erikson et al., 1997; Skjervold et al., 1999; Thomas et al., 1999; Kiessling et al., 2004 and 2006; Roth et al., 2006; Morkore et al., 2008), probably reflecting fast and continuous shortening of the sarcomeres in the myofibrils (actin and myosin).

Change in muscle pH was significantly influenced by dietary treatment in both NC and C groups. An initial muscle pH of 7.0 and final pH of 6.25 after 72h storage was observed in the NC group, which is in line with the pH suggested by previous studies (Hansen et al., 2007; Morkore et al., 2008; Bahuaud et al., 2010). Significant lower muscle pH in NC group was observed in Arg and

TTA diets compared to Con diet at 1h, 12h, 24h, and 72h *post-mortem*, whereas the muscle pH was similar in all the diets (Arg, Glu, TTA and Con) at 6h *post-mortem*. Muscle pH related to dietary treatment was investigated by Suontama et al. (2006), but our results showed significant differences at 72h *post-mortem* which the previous study could not find due to dietary effects. Rapid decline in muscle pH during the first 6h *post-mortem* (pH 7- 6.6) of the NC group suggests that most of the biochemical changes occur immediately after death, as was also proposed by Hansen et al. (2007). As expected, an initial lower muscle pH (6.4) was found in the C group (Erikson et al., 1997; Thomas et al., 1999; Poli et al., 2005; Bagni et al., 2007; Lefever et al., 2008), although the fish in these former studies were not subjected to the same amount of stress and type of nutrition. The ultimate production of lactic acid, which to a large extent determines the final muscle pH *post-mortem*, depends on the pre-slaughter nutritional status and stored glycogen (Huss, 1995; Skjervold et al., 2001). Similar ultimate muscle pH as that determined in the C group was reported by Lefevre et al. (2008), who found no impact of stress on final pH of rainbow trout. Enhanced glycolysis in the salmon exposed to pre-slaughter crowding stress is expected to be the cause of elevated production of lactic acid, and concomitant reduction of muscle pH in the C group compared to NC group at both 1h and 48h post-mortem.

One of the main aspects of this thesis was to study the impact of different diets (Arg, Glu and TTA) on the flesh firmness (texture). For the present study, total area under the force-time graph determined by instrumental puncture analyses was selected as a firmness determinant. It was hypothesized that Arg, Glu and TTA diets would give firmer texture and this was confirmed, as each of the diets presented significant higher firmness compared with the Con diet in both NC and C groups. Compared with the Con diet, a significantly firmer texture was found in Arg, Glu and TTA diets at 1h, 12h, and 24h *post-mortem* of NC group, and in Arg and Glu diets at 48h *post-mortem* of C group. The firmer texture of the Arg and Glu diets evidenced their effects on muscle fibres synthesis as Glu is a precursor of important amino acids, alanine, proline, arginine (Reeds et al., 2000 Tapiero et al., 2002b; Blachier et al., 2009). In particular proline and arginine (arginine is also precursor for proline) that are involved in the synthesis of collagen and connective tissue (Tapiero et al., 2002b). In addition, firmer texture in Arg and Glu diets of NC group might reflect increased recruitment of new fibres. The firmer texture in TTA diet is difficult to explain. Comparison of NC and C groups at 1h and 48h *post-mortem* revealed significantly softer texture (lower total area) in C group. Softer fillet texture due to pre-slaughter stress observed in this study are in line with the reports of Sigholt et al. (1997), Roth et al. (2006), and Lefevre et al. (2008). The

softer texture in the C group suggests weakening of muscle fibres due to breakage as a direct effect of stress on muscle cell cytoskeleton and connective tissue (myofibre-myofibre and myofibre-mycommata detachments) (Roth et al., 2006; Bahuaud et al., 2009), and/or degradation of the extracellular matrix (Taylor et al., 2002). Increased breakage of the muscle fibres might also be attributed directly or indirectly to the lower *post-mortem* muscle pH (Bahuaud et al., 2010), which is ultimately associated with loss of fillet firmness (Taylor et al., 2002).

Muscle growth is a dynamic process in fish, and recruitment of new fibres (hyperplasia) and enlargement of existing fibres (hypertrophy) within a muscle mass often continues well past the age of sexual maturity. Because hyperplasia is associated with small fibres and hypertrophy is correlated with fibres of greater dimensions, the size of individual fibres can be used to assess muscle growth (Zimmermann & Lowery, 1999). In the present study, salmon fed the diet supplemented with Arg diet tended to have smaller fibre cross-sectional areas (higher number of fibres per unit area). These results fit well to the obtained texture results showing higher firmness of the Arg group, as several studies have documented an inverse relationship between firmness and fibre cross-sectional area (Hatae et al., 1990; Hurling et al., 1996). Muscle cross sections examined in salmon exposed to crowding stress showed no significant change in the Con, Arg and Glu group, but significantly higher amount of fibres were recorded in the TTA group. The results therefore indicate that muscle cells of the fish with the TTA diet schrinked upon crowding stress, probably due to osmotic stress. Osmotic stress occurs when the concentration of molecules in solution outside of the cell is different than that inside the cell. When this happens, water flows either into or out of the cell by osmosis, thereby altering the intracellular environment. Hyperosmotic stress causes water to diffuse out of the cell, resulting in cell shrinkage (Go et al., 2004).

Cathepsins could be used as a useful tool for examining the post-mortem muscle structure as they play a major role in the degradation of muscle proteins (Yamashita & Konagaya, 1991), thus texture deterioration. Although dietary effects were observed with regard to texture properties, no pronounced variation was found in activity of cathepsin B, cathepsin L and cathepsin B+L in experimental diets compared to the Con diet of NC group whereas the activity of cathepsin B was found significantly lower in C group. The cathepsin B activity in the Arg and Glu diets was however slightly higher than in the Con diet of the NC group. In addition, cathepsin B+L activity in Glu diet was higher than Con diet. These higher activities of cathepsin B and cathepsin B+L of NC group tally the results of Bahuaud et al. (2009), who found higher activities of cathepsin B and

cathepsin B+L by feeding salmon with fish oil and or rapeseed oil. However, the authors found lower activities with eicosapentanoic acid-enriched oil and and docosahexanoic acid-enriched oil. Comparison of the NC and C groups reveled no significant differences in the activities of cathepsin B, cathepsin L and or cathepsin B+L within each of the diets Arg, Glu, TTA, and Con. These non-significant activities of all the cathepsins are not consistent with the reports of Bahuaud et al. (2010), who observed significant effect of crowding stress on each of cathepsin B and cathepsin L activity, but in their study they subjected the salmon for a long term stress of 24h as compared with 16h in the present study.

Gln was used in the *in vitro* study because it is an essential nutrient for cell survival and growth (Ehrensvard et al., 1949; Eagle et al., 1956). PCNA assay was used as a marker for cell proliferation as it has been widely used in many studies for assessing cell proliferation (Koumans et al., 1990; Matschak & Stickland, 1995; Vegusdal et al., 2003 and 2004). Percentage of blue and brown cells nuclei, and counting of the blue, brown and total cells nuclei revealed a variable picture between treatments (Arg, Gln and TTA) compared to Con, and between temperatures (8°C and 16°C). Number of total cells nuclei in Arg treatment (256.2) at 8°C and Gln treatment (311.2) at 16°C were found higher than of the Con treatment at the same temperatures (250.4, 308.2 respectively), although the differences were not significant. These indicated higher numbers of cells in Gln treatment evidenced its effect on *in vitro* cell proliferation (Eagle et al., 1956; Newsholme et al., 2003; Watford, 2008; Wu, 2009). The effect of Arg with regard to muscle growth stimulation still need to be elucidated (Brown & Cameron, 1991), but it is known that cyclic process of Arg results in the formation of polyamines which are highly responsible for muscle growth (Mommsen, 2001). TTA treatment seemed to be deleterious to the muscle cells (Gjøen et al., 2007) at both temperatures (8°C and 16°C) as evaluated by number of total cells nuclei and percentage of blue and brown cells nuclei. TTA treatment presented significantly lower numbers of total cells nuclei (152.0 = 8°C and 228.6 = 16°C) compared with the Con treatment (250.4, 308.2 respectively). Temperature has a great influence on growth rate of fish muscle (Mathers et al., 1993; Johnston et al., 2006; de Assis et al., 2004), and muscle growth is associated with the increased nuclear numbers (Koumans et al., 1993), as also observed in this study. Significantly higher number of total cells and brown cells nuclei were found within all the treatments at 16°C than 8°C. These findings are not in consistent with the results of Matschak & Stickland, (1995), who found no effect of temperature on the number of proliferating myosatellite cells and brown cells nuclei of salmon. Although, they speculated that no difference in the nuclear number was due to the fact that

unstained cultures were compared and this led to an underestimate of the actual number present in the cultures. The deviation between the present study and the study of Matschak & Stickland, (1995) might also be attributed to different temperature ranges used (5°C and 11°C vs. 8°C and 16°C in the present study). The higher proliferation of cells at 16°C than 8°C, reflects the temperature dependent behavior of muscle cells as well as interaction with the treatments.

Gene-expression analysis is frequently used in biological research for better understanding of the different mechanisms at cellular level. Reverse transcription (RT) followed by a polymerase chain reaction (PCR) is the most effective technology to amplify and detect trace amounts of mRNA (Heid et al., 1996). In this study expression of four genes were selected to evaluate the effects on muscle development and degradation respectively. These included two muscle genes, myogenin (regulatory) and myosin light chain 2 (structural), and two genes for the proteases cathepsin B and cathepsin L. Relative gene expression of myosin light chain and myogenin was significantly higher in all the experimental treatments (Arg, Gln and TTA) at both temperatures compared to the Con treatment. Gene expression of myosin light chain at 8°C in TTA treatment (1.48) was more than twofold as compared to Con (0.46). The higher gene expression by Arg and Gln treatments confirmed the fact that amino acids are involved in the regulation of gene expression (Skorve et al., 1990; Newsholme et al., 2003; Kennedy et al., 2007; Wu, 2009; Li et al., 2009). The up-regulation mechanism of muscle gene expression by Arg and Gln and TTA is difficult to explain. However, it is believed that muscle growth due to Arg and Gln supplementation is because they serve as precursors for several other amino acids (Mommsen, 2001; Watford, 2008). For instance, Gln is the precursor for Glu, that in turn is the precursor for Arg and the metabolic products of Arg are greatly responsible for muscle growth (Mommsen, 2001; Tapiero et al., 2002b; Blachier et al., 2009), particularly proline and polyamines (Tapiero et al., 2002b). Arg also activates the release of glucagon, glucagon-like peptide-I and somatostatins which are the growth regulating molecules (Mommsen et al., 2001). There was no significant difference in gene expression (myosin light chain2 and myogenin) between temperatures (8°C and 16°C), except of the Gln treatment, although the ratios were slightly higher at 16°C.

Results from the relative gene expression of cathepsin B revealed significantly lower expression in TTA treatment than in Con at 8°C, and higher but non-significant expression at 16°C. The higher expression of TTA at 16°C is in line with the work of Bahuaud et al. (2009), who suggested that dietary lipids increase the gene expression of cathepsin B. However the lower

expression of cathepsin B at low temperature suggests that the activity of thia fatty acids differ with the change in temperature. Furthermore, gene expression of cathepsin L was significantly higher at 16°C compared to 8°C. This higher expression of cathepsin L at higher temperature indicates a temperature dependency of this enzyme.

6. CONCLUSIONS

Data from the *in vivo* study suggest that inclusion of bioactive components (Arg, Glu and TTA) in feed to Atlantic salmon put positive effect in minimizing the fillet contraction, increasing the fillet firmness (texture) as well as the number of myofibres. However, these components embed a negative effect on muscle pH too. In addition, pre-slaughter crowding stress is a negative factor for the fillet contraction, pH and texture of the fish.

Results from *in vitro* study suggest that supplementation of the Arg, Gln and TTA substrates up-regulate the gene expression of myosin light chain2 and myogenin at both temperatures (8°C, 16°C). Based upon light microscopy pictures, Arg and Gln also seem to improve the proliferation rate of muscle cells whereas TTA seems to be lethal for muscle cells.

In conclusions, Arg, Glu, Gln and TTA supplementation have significant impact on muscle development and texture of Atlantic salmon as observed by *in vivo* and *in vitro* studies. Therefore, extra care should be paid on inclusion of TTA concentrations while feed formulations as higher doses can be deleterious as observed in muscle cell proliferation.

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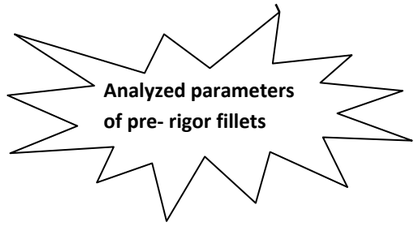
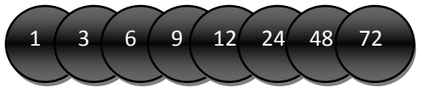
Starting point of project (Sampling 0)
15. Apr.2009

Sampling 1
15-17. Sep. 2009

Time scale: 22, 25 26 June

Time *post-mortem*





Con

TTA

Arg

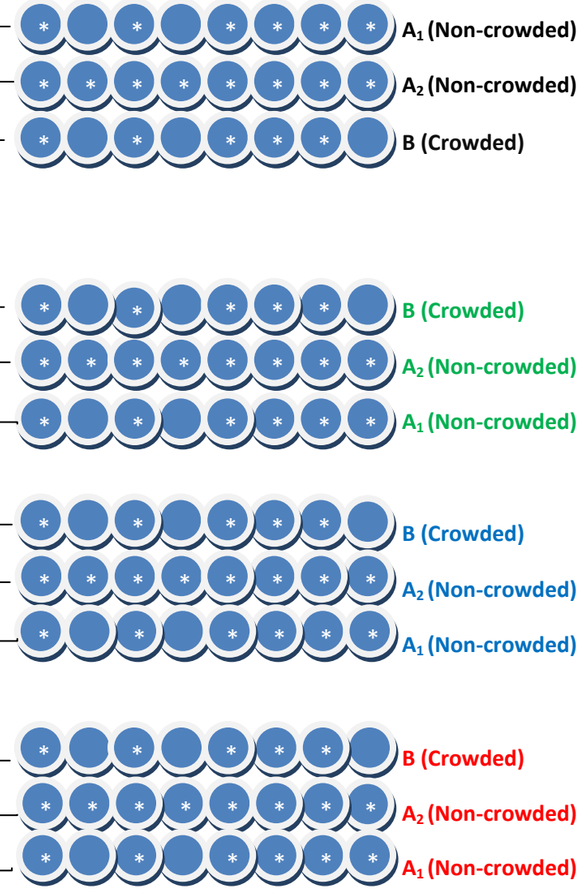
Glu

Fillet length (1, 3, 6, 9, 12, 24, 48 and 72 hours for Non-crowded groups and 1, 6, 12, 24 and 48 hours for crowded group)

Texture measurement (1, 6, 12, 24, 48 and 72 hours for Non-crowded group and 1, 12 and 48 hours for crowded group)

Muscle pH (1, 6, 12, 24, 48 and 72 hours for Non-crowded group and 1, 12 and 48 hours for crowded group)

Muscle samples (1 hour for Non-crowded group and 1 hour for crowded group)



An overview of the experimental design of the study

Date (22, 25 and 26) of June is the date for shifting of TTA diet to Control for net pen number 9, 6 and 1 respectively. TTA diet was fed to the fish when reached to 0.2% of body weight.

Pre-rigor fillets analysis: analysis of 6 fish (non-crowded) and 3 fish (crowded) from each net pen in the sampling time from 15-17th of September.

A_1= Measurements of muscle pH, texture and muscle samples collection for histology and cathepsins from right fillet of non-crowded fish.

A_2= Measurements of fillet length from left fillet of non-crowded fish.

B = Measurements of muscle pH, texture and muscle samples collection for histology and cathepsins from right fillet as well as measurements of fillet length from left fillet of crowded fish.