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Dietary Impact of Feed on Performance, Health and Tissue Melanization of Atlantic Salmon (Salmo salar L.)

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Abstract:

Good performance, robustness to diseases and stress, and flesh quality according to consumer expectations are important success factor in farming of Atlantic salmon (*Salmo salar* L). In recent years, superficial hyper-pigmented "black spots" are the major fillet quality problem that causes severe economic losses to the salmon industry. The aim of current research thesis was to investigate the effect of diet on black spots, robustness to stress, health parameters and product yield. Diets offered during the research trail were either a standard commercial salmon feed or the same feed supplemented with antioxidants (vitamin-E and selenium), copper or zinc. The experiment was conducted in seawater in triplicate net pens per dietary treatment from of June to September 2014. Harvesting was done either by a standard method or after crowding stress. The zinc group showed significantly higher body weight (4067 ± 60 g vs 3797 ± 53 g), body length (69 ± 0.3 cm vs 67.7 ± 0.3 cm), fillet weight (2607 ± 40 g vs 2394 ± 35 g) and fillet yield (64.1 ± 0.1 % vs 63.2 ± 0.2 %), and lower melanization of the peritoneum. The antioxidant group showed lower melanization of skeletal muscle and the zinc group also tended to have lower degree of melanization. The copper supplementation showed no significant effect, but a tendency to higher incidence of dark pigmented spots. Light microscopy of the melanized tissue samples revealed aggregation of mononuclear, pigmented cells or darkly stained particles inside the cells surrounding the tissues. It is concluded from the current study trail that supplementation of zinc increases growth performance and improves fillet yield. The antioxidant supplementation improves health status with lowest incidence of pigmented spots.

Key words: Dark pigmented spots, dietary treatments, histopathology, fillet quality, stress resistance, Atlantic salmon.

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1 INTRODUCTION

Aquaculture is the second largest industry in Norway with cultivation of Atlantic salmon *(Salmo salar L.*) as the dominating specie and contributing significantly to the economic development of the country (FAO, 2005). Salmon has high nutritional value with high quality proteins, omega-3 fatty acids, vitamins and minerals. These nutritional components are contributing positively in reducing diseases like atherosclerosis and cardiovascular diseases in human (Porter, 2005, Børresen, 2008).

Salmon farmers aim to produce fast growth and healthy fish with attractive visual appearance, flavor and texture (FAO, 2005; Paterson *et al*., 1997; Kiessling *et al*., 2006). Generally the customer preferences are variable (Greenhoff & MacFie, 1994). In 1992 Koteng reported that 5% of the salmon buyers were not satisfied with dark hyper-pigmentation of the fillet (black spots) (Koteng, 1992). Nowadays the main problem of salmon fillet quality is melanin hyper-pigmentation which imparts undesired black color (Berg *et al*., 2012). A recent study has showed that the probability of melanization in salmon fillet has been recorded up to 19% (Mørkøre *et al*., 2015).

Abnormal pigmentation may be associated with various pathological changes in organs and tissues. The origin of these pigments may be endogenous or exogenous. The pigment of endogenous origin includes hemoglobin, porphyrin, derivatives of lipids and melanin. The term melanosis refers to the accumulation of melanin in abnormal sites (Thomson, 1984). Pigment producing cells (melanocytes) are responsible for the production of melanin which imparts dark color to the fish fillet (Hearing *et al*., 1991). The production of melanin in the muscles is not yet

clearly understood. A close connection has been reported between pathogens and melanin spot in fish. Moreover melano-macrophages were observed at the site of affected area after bacterial infection (Ribelin & Migaki, 1975). In salmon the pigment producing granules were identified as an inflammatory response that relates the immune system to the pigmentary system (Larsen *et al*., 2012). Melanin pigmentation is often observed in organs as a result of vaccination, and stress due to vaccination was suggested to be the reason for dark pigmentation in salmon fillets (Koppang *et al*., 2005).

During stress, the oxidation process tends to influence the ratio of reactive oxygen species (ROS) and antioxidant molecules (Finkel & Holbrook, 2000). Antioxidants like α-tocopherol has been documented to minimize the process of melanogenesis (Yamamura *et al*., 2002) and glutathione (GSH) which is a biologically important reducing agent, also play a determining role in melanogenesis (Marmol *et al*., 1993). Carotenoids are oxidation-sensitive molecules and hence can act as antioxidant (McGraw, 2006; van Schantz *et al*., 1999: Hartley & Kennedy, 2004). Similarly melanin could also act as an antioxidant which is sensitive to oxidation stress (McGraw, 2006: Moreno & Moller, 2006).

Zinc acts as a co-factor in enzymes which carry out nucleic acid and protein metabolism (Tacon, 1990). Copper exhibit pro-oxidant activity it also acts as a co-factor for the enzyme tyrosinase and stimulate the expression of melanin based signals on exposure to free radicals formed during oxidation (Galvan & Alonso-Alvarez, 2008). The antioxidants occupy the binding site of the free radicals which protect the membranes from oxidation (Knox *et al*., 1984).

The main aim of the current study was to determine the effect of dietary supplementation of antioxidants (Se, vit-E), zinc (Zn) or copper (Cu) on salmon performance, health, robustness to

stress and flesh quality with special emphasis on black spots. To achieve this goal, the following parameters were analyzed: biometric traits (body weight, body length, fillet yield, carcass yield and organ indices), melanization of tissues (fillet, organ and abdominal wall,) histopathology of the black spots and blood chemistry (total protein, cortisol and glucose).

2 THEORATICAL BACKGROUND

2.1 Fish Health

The presence of melanin in fish fillets decreases the product quality (Koteng, 1992). In vertebrates the melanin synthesis takes place in melanocytes and further proceed in melanosomes which are intracellular organelle related to lysosomes (Orlow, 1995; Raposo *et al.*, 2002). In mammals the origin of melanocytes is the embryonic neural tube (Sulaimon & Kitchell 2003). These melanocytes have affinity to transforme into inflamed tissue (Thomson, 1984). The tissue regeneration and inflammatory reaction response in salmonids have close similarity with mammals (Finn & Nielson, 1971). Additionally these melanocytes also trigger the involvement of melano-macrophages (Roberts 1975; Agius & Roberts 2003). The origin of melanosomes in visceral organs and muscle tissue in fish is not exactly known (Agius & Roberts 2003).

In poikilothermic vertebrates the phenomenon of melanogenesis take place in cells derived from mesenchyma of the haematopoitic lineage (Sichel, Scalia, Mondio & Corsaro, 1997). In teleost the melano-macrophages have shown macrophage like properties but the exact mechanism is not known (Agius & Roberts 2003). It has been proposed that the role and formation of melanin in various tissue and organ level are related with non-specific immune mechanism. This mechanism also describe the presence of melanin around the peritoneum and visceral organs of poikilothermic vertebrates (Mackintosh, 2001).

It has been documented that melanin spots developed from non-specific inflammatory response induced by physical stress like vaccination. The most vaccines available in markets are in injectable form which contain non-matabolizable mineral oil. This non-metabolizable mineral oil triggers intense inflammatory response as compared to the metabolizable mineral oil (Spickler

& Roth 2003). Mineral oils consist of long chains of inert hydrocarbons. These oils are not activated by biochemical reactions. They are usually obtained as a byproduct during petroleum distillation (Murray *et al*., 1972). The side effect of mineral oil based adjuvant vaccines has been reported in lab and domesticated animals. Due to intense inflammatory response made by oil based vaccines it is not recommended for human application (Gupta *et al*., 2003). Recent studies also revealed that oil based vaccines may induce immune complex mediated glomerulonephritis and auto-immune response glomerulonephritis (Shaheen *et al*., 1999; Satoh *et al*., 2003; Kuroda *et al*., 2004). The side effects of oil based vaccines was not documented in fish but specific inflammatory response at the site of injection and area around peritoneum was observed (Lillehaug *et al*., 1992; Midtlyng, 1996; Poppe & Breck 1997; Mutoloki *et al*., 2004). The observed consequences of vaccination are granulomatous peritonitis, adhesions of peritoneum and visceral organs, stunted growth and reduced fillet quality (Poppe *et al*., 2002).

Usually in salmonids the injectable site for vaccination is posterior part of abdominal cavity but the occurrence of melanin spots is random and more abundant in the anterior part of the fillet (Mathiassen *et al.,* 2007). Most recent studies revealed the similar pattern of melanin formation in unvaccinated salmon (Norwegian School of Veterinary Science, 2013). In Atlantic salmon melanin formation takes place in melanosomes of the muscle tissue which make strong connection between immune response and pigment producing mechanism (Larsen *et al*., 2012).

The Norwegian salmon industry have succeeded in controlling the problems regarding the fish health and minimize the administration of antibiotics by introducing vaccinations (Poppe, 2006). But vaccination may induce some side effects in the fish like decrease in feed intake and growth rate. However, the fish injected with normal saline do not show decrease in feed intake and growth rate. Other factor may affect vaccination like fish size, temperature, photoperiod, feeding

time, fish density, handling and management. In some cases the vaccinated fish also attain the same length and body weight as compared to unvaccinated fish when transfer to sea water. During normal conditions, vaccinated and unvaccinated fish may show similar growth rate. Other factors like breeding, low bioavailability of phosphorus in feed, rapid growth, environmental contaminants and environmental temperature may also play key roles in decreased feed intake and growth rate. So vaccination alone is not the only detrimental factor which induces developmental anomalies in salmon. Moreover Norwegian slaughterhouses recorded variable amount of melanin pigments from the fish that had been reared, vaccinated and slaughtered under the same conditions. Also the defense mechanism of all the fishes are not exactly the same. These findings conclude that different factors are dependent on each other. For many years vaccine was suggested as the principle cause of melanin spots in the fillets and organs, but experimental trails have shown that most probably the melanin spots in fillets and organs can have different origin and it is also unlikely that physical stress induced by handling during vaccination is the main factor in inducing melanin spots. These statements are validated by the recorded data from in-depth evaluation of different associated determinants (Berg *et al.,* 2007 and Mørkøre, 2012).

Pancreas disease (PD) caused by salmon alpha virus (SAV) can lead to the formation of melanin spot in salmon fish fillets. The exact relationship between level of infection and melanin formation is not clearly established by the industry (Mørkøre, 2008). PD is a viral infection and leads to significant losses to the Norwegian aquaculture, especially Atlantic salmon and rainbow trout infecting at an average of 90 localities every year since 2006 (Jansen *et al*., 2010). In chronic infected muscles the desired bright red color is not obtained even though high levels of astaxanthin are administered. The incidence of dark spots and discoloration of the fillet can occur at the same time (Mørkøre, 2012).

Stress is also a contributing factor to the development of melanin in fillets. Stress is defined as collective response of biological reactions in response to abnormal stimuli that tends to disturb the organisms homeostasis. The stimulus could be physical, mental or emotional, internal, nutritional and external to the environment. Nutritional stress may correspond to foreign particles in the feed that stimulate body defense mechanism and macrophages starts producing e.g. lipofuscin and hemosiderin (Mørkøre, 2012; Thorsen, 2006). Hemosiderin is a golden brown pigment formed by the breakdown hemoglobin by macrophages and lipofuscin occur in different cells. The presence of lipofuscin indicates the formation of free radicals and composed of protein-phospholipid complex (Krause, 2005). If the haemorrhage areas was occupied by macrophages of the anterior kidney then it would result in increased deposition of pigments (blood/melanin). The experimental trails conducted on carp has revealed that melanin deposition can occur due to the presence of foreign particles that tend to induce increased level of melano-macrophages, lipofuscin and hemosiderin at the anterior lobe of the kidney. One possible assumption is that the macrophages from anterior lobe of kidney migrate to the haemorrhagic site that ultimately leads to the melanin deposition in the surrounding area. The other possibility is that foreign particle presence in the feed will tend to elevate the formation of the melanization in the surrounding area and the foreign particles present in feed that tend to increase the elasticity of the walls of the blood vessel and increase the internal immunity, thus decreasing the melanin spot formation.

2.2 Fillet Quality

The incidence of melanin spots on salmon fillets is increasing and they do not vanish while baking or smoking and imparts bad impression on the product quality (Mørkøre, 2008). In 2006, the processing industry reported 30% degradation of salmon fillet due to melanin pigmention

(Thorsen, 2006). In 2007, an industry reported that approximately 8-20% of the total fillets had melanin spot and 4% of them were discarded (Mathiassen *et al.,* 2007).

Recently in 2013, the incidence of melanin spots in fillet with light grey spots with diameter ≥3 cm was estimated around 12% while 2% of the fillet had darkly stained melanin spot with a diameter ≤ 3cm (FAO, 2013). Geographically, the incidence of melanin was not randomly distributed. The southern part of Norway had a prevalence of 22%, northern part of Norway 12% while the prevalence of melanin spots in the central area of Norway was 15% (Mørkøre, 2012). In many food items, melanin was a natural component and no side effect, allergic reaction or toxicity was reported (FAO, 2013). The customer do not prefer any discoloration or dark pigmentation of the fillet as they associate discoloration with inferior quality. The melanin spot decreases market price and fillets with dark spots are downgraded in the production line as the fillet containing melanin can't be marketed as a whole fillet (Mathiassen *et al.,* 2007). Melanin spot can have a variety of shades. Darkly stained fillet spots were due to melanin while red to greyish black spots could be due to blood pigment as a result of haemorrhages or scar tissue formation or combination of both (FAO, 2013).

It is an established fact that dark fillet spots were an inflammatory response or tissue damage by immune system of the fish (FAO, 2013). The melanin was found not only around the peritoneal membrane of the abdominal wall but also anywhere on surface or deep in fillets and measuring length around 1-4 cm (Mørkøre, 2008). The incidence of melanin spot increases with the age of the fish which also led to the hypothesis that the phenomenon of dark spot formation can't be related to the vaccination only (Mørkøre, 2012).

Vitamin E also known as α-tocopherol is an important lipid soluble antioxidant. In human the Vit-E together with other vitamins has a role to stop bleeding. In 2011 it was reported that the inclusion of Vit-E prior to harvesting improved the robustness to stress during slaughtering as indicated by biomarkers in the blood. Vit-E inclusion in feed also improved gut health and muscle texture (Mørkøre, 2012). Vit-E also increases the product shelf life as it has a role of antioxidant and prevent the formation of free radicals during oxidation of lipids (Baker, 2001).

2.3 Melanin

Melanin is a high molecular weight, complex biopolymer of indole quinone, insoluble and stable pigment (Jacobson 2000). Naturally it is found in most animals and plants and responsible for skin, eye and hair color in human (Mørkøre & Prytz 2013). The melanin simply means black pigment which is originated from animals (Swan, 1974).

Melanin is synthesized by specialized dendritic cells, melanocytes derived from ectoderm of the skin. At the cellular level the amount of melanization is monitored by specific organelles known as melanosomes that are produced in different size and densities. The amount of production of melanosomes is genetically controlled. After production, the melanosomes are transferred to keratinocytes and hair shaft, in the skin and hair bulb respectively from where final distribution take place in response to certain stimuli (Hearing *et al.* 1991).

The process of melanin synthesis (melanogenesis) is regulated by a variety of extracellular factors like ultraviolet radiation (Bolognia *et al.,* 1989), interferon (Kameyama *et al.,* 1989) and substrates (Slominski *et al.,* 1987). These factors hyper-sensitize the melanocyte stimulating hormone (MSH) receptors. MSH is a peptide hormone secreted from the posterior lobe of pituitary gland. The MSH increases the production of eumelanin. The melanosomes work together with

neighboring cells were engulfed and distributed by the skin keratinocytes and distribute themselves around the nucleus and protecting the inner cell organelles from harmful effects (Hearing *et al.* 1991). There are three different structures of melanin, i.e. eumelanin, pheomelanin and neuromelanin, but the most prevalent melanin found in teleosts is eumelanin (Bagnara & Matsumoto 1998; Agius & Roberts, 2003).

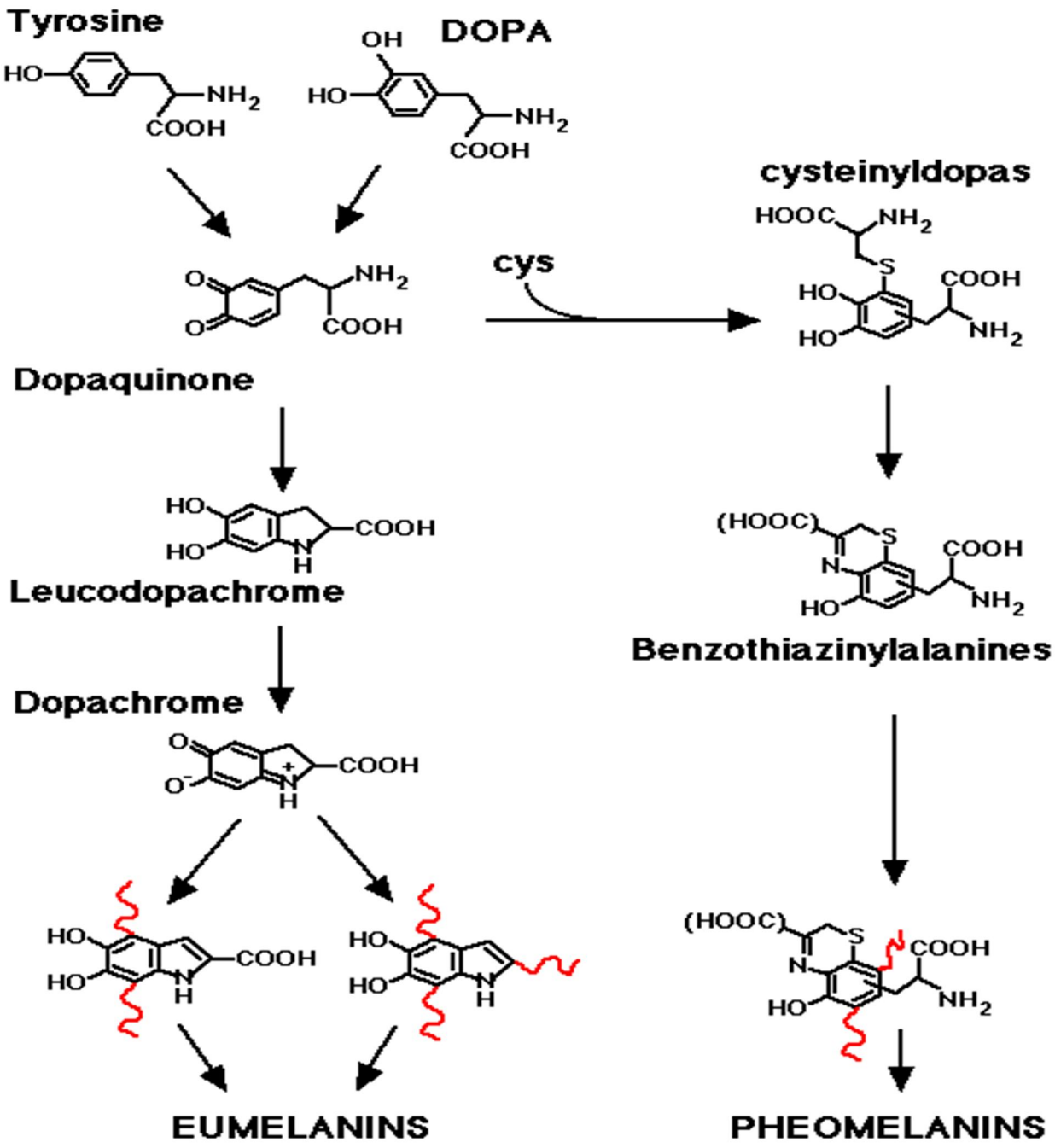

Figure 2-1: Structural representation of eumelanin and pheomelanin.

Melanin has been used for enhancing taste and flavor in many dishes in Venice since 14th century. Today Italian black macaroni also has melanin as principle ingredient to enhance flavor. Nowadays many natural herbs containing melanin are consumed to facilitate digestion and other health benefits (FAO, 2013).

People in western world urge to increase their skin tone by melanin production. Certain food items are now available in the market that stimulate melanin production like soy, beans, apricots, eggs, oyster containing copper, liver, shell fish, turkey, chicken, fish and certain dairy products like yogurt and cheese (Mørkøre, 2012).

Melanin is a photosensitive pigment as it has tendency to absorb and dissipate radiation like UVR (Meredith & Sarna, 2006). Melanin is a photo-protective agent and used in sunscreen lotions. UVR causes rancidity of fats, oils and dairy products by producing free radicals, thus degrading the product quality. The melanin has capability to absorb these free radicals and they are used as preservation of various products (NPS, 2013). In Avians, melanin gave more strength to the feathers and protect them against bacterial degradation.

2.4 Vitamin E

Vitamin E is a fat soluble vitamin discovered during studies conducted on rats in 1922 (Thorsen J. 2006). It protects the plasma membrane from free radicals produced during oxidation (Hamre *et al.,* 1998). The two important components of vitamin E are tocopherols and tocotrienols. Among these α-tocopherol and γ-tocopherol are widely studied and commercially available being exclusively used in feed industry. Natural sources of vitamin E are eggs, liver, vegetable oils, green vegetables and plants (McDowell, 1989). It is well documented that vitamin E plays a key role in fish immune mechanism. It increases fish health, aids the specific and non-specific immune

mechanisms against certain diseases, increases fillet quality and reduces mortality (Ispir *et al.,* 2011; Halver & Hardy *et al*., 2002). Certain abnormalities have been documented due dietary deficiency of vitamin E like anemia, muscular dystrophy, erythrocytic fragility and pale coloration of the fillet (NRC, 1993). The requirements of vitamin E does not remain constant during different stages of life. The daily requirement of Atlantic salmon is 120mg/kg of dry feed. Various factors which determine the digestion and absorption of vitamin E are water temperature, level of selenium in the diet and ratio of antioxidant level and dietary fat (Hamre *et al*.,, 1995).

2.5 Minerals

Minerals play important roles in the daily cell activities of fish and other animals. Fish fulfill their requirements by dietary intake or uptake from the water. The body requirements are controlled by homeostatic mechanism, which determine the amount required for cell metabolism. Minerals play important roles in maintaining acid-base balance, formation of skeleton and as a co-factor in enzymes and hormones. Mineral deficiency may result in stunted growth and development of pathological conditions while excess of minerals may results in toxicity (Watanabe *et al.,* 1997).

Zinc acts as a co-factor in enzyme function which carry out metabolism of carbohydrates, lipids and proteins. It is an integral part of nucleic acid and protein metabolism. It also play important roles in the function of hormones like corticosteroids, insulin, glucagon, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Tacon, 1990).

In view of importance of zinc, it should be included in fish feed. Normally it is absorbed through digestive tract and to some extent via gills (Peterson *et al.,* 1997). The daily requirement of zinc by Atlantic salmon varies from 37-67 mg/kg (Maage & Julshamn, 1993). The inclusion

level of Zinc for salmon feed ranges from 80-118mg/kg (Tacon and De Silva, 1983). Commercial feed industry marketing feed with 150 mg/kg of Zinc (Nutra Olympic, 2014) and 200mg/kg (EFSA, 2014). In rainbow trout its deficiency causes immunosuppression and eye lens cataract and also affects mineral deposition and function of gonads in common carp (Kiron *et al.,* 1993). Higher doses of zinc may cause cross reactions with copper and iron which play important roles in red blood cells (RBC) synthesis (Knox *et al.,* 1984).

2.6 Stress Physiology

The physiological response to stress stimulus has unique mechanism in the survival of the organism. This physiological response pattern is highly specific in vertebrates. Random and repeated exposure to stressors has deleterious effects on the physiology of organisms like alteration and incoordination in function of nervous system, growth and development, metabolism, reproductive system and immune system function. Fish exhibits unique pattern of physiological and biochemical changes in response to handling and crowding stress. The stress response in fish has been divided into different categories like primary response, secondary response and tertiary response (Mazeaud *et al.,* 1977).

Primary response include active release of stress hormones like cortisol and catecholamine into the blood circulation. Secondary response includes immediate effect of these hormones at tissue and cellular level such as effect on acid-base balance, cellular function, immune response, mineral ions balance, respiration, growth and metabolism. The tertiary response is comprised of detrimental adaptations at the individual level and even at the population level. These feedback mechanisms include variation in performance factors like disease resistance, growth and behavior.

The stress hormones like cortisol and catecholamine regulates various physiological and biochemical processes such as activation of metabolic pathways that amend hematology and blood chemistry. For example in carbohydrate metabolism these hormones stimulates glucose synthesis by gluconeogenesis, which results in the escalation of blood plasma glucose concentration (Barton and Iwama, 1991; Nakano et al., 2014). The fish may encounter deleterious effects if it was not accustomed to these stressors. The response to these stressors induced high energy demanding mechanisms, which deprive energy away from basic life processes like reproduction and metabolic processes like growth and development.

Secondary response includes immediate effect of these hormones at tissue and cellular level such as effect on acid-base balance, cellular function, immune response, mineral ions balance, respiration, growth and metabolism. The tertiary response is comprises of detrimental adaptations at the individual level and even at the population level. These feedback mechanisms include variation in performance factors like disease resistance, growth and behavior.

In a normal production system, fish were exposed to a variety of artificial and natural stressors such as handling, holding, collecting, sorting, transportation and vaccination. These normal routine procedures influence fish health and physiology. In intensive aquaculture systems, water quality and fish crowding were important parameters contributing to fish health and stress (Barton *et al*., 2000). Temperature, pH, ammonia, nitrite, nitrate, dissolved oxygen, carbondioxide, salinity, hardness and alkalinity were common water quality parameter that influence the physiological stress (Portz *et al.,* 2006). To evaluate the fish health status, blood parameters were used as indicators of fish physiology in recent studies. Variation in glucose and cortisol level in blood are most widely used to determine the stress status in fish (Pickering & Pottinger, 1989; Barton & Iwama, 1991; Mazeaud *et al.,* 1977, Silbergeld, 1974).

2.7 Handling stress by vaccination

The history of vaccination was first documented by Polish scientists in 1938 under the supervision of Snieszko, where vaccination of common carp with bacterial culture Aeromonas punctate was performed to develop the immunity. In 1942 another scientist Duff, worked on vaccination against Aeromonas salmonida (Gudding & Muiswinkel). The vaccination against the viral infection was conducted for the first time by the Russian pathologist Goncharov in 1951 (Goncharov & Mikriakov, 1968). The most advancement about vaccination in fish at molecular level started between 1950 and 1960. Fish immunity by vaccination gained similar importance as for other vertebrates. Aquaculture practices are still needed to be addressed in many developing countries when compared with other animal husbandry practices (Muiswinkel, 2008). The knowledge and development of vaccination in aquaculture is increasing with the passage of time. The use of antibiotics, which is a big issue these days, can be controlled by better knowledge of vaccination and farming techniques.

Vaccines are suspensions containing live, killed, attenuated or modified microorganisms/ toxins which act as antigens which on administration into the body stimulate the body immune system to produce specific antibodies and prevent the individual against future outbreak of a disease. The vaccine preparation can be oil based or water based. The oil or water adjuvant enhances the efficacy and life span of the vaccine. In fish the vaccine can be orally or through injection. In oral route the vaccine can be mixed / coated with feed or bio-encapsulated. The vaccine is absorb through mucosal surface of the gastrointestinal tract and stimulate the body immune system. For injectable vaccine anesthesia is needed in order to minimize the stress and mechanical injuries. The route of injection can be intramuscular or intraperitoneal, but the most

widely used route of injection with minimum side effect is intraperitoneal (Komar *et al.,* 2004). The desired site is located on the midline of the ventral side of the abdominal wall just in front of the caudal fin. The accuracy of injection minimize the mortality and other complications (Intervet International B.V, 2005). Injectable vaccine is a preferred method and provide better immunity as each and every fish receive the desired amount of vaccine that is required to initiate the body's immune system and this method is done under trained staff (Komar *et al.,* 2004).

In salmonids, threshold level of maternal antibodies in young fish fry was too low to provide immunity against infections (Lillehaug *et. al.,* 1996). This deficiency was compensated by early development of immune system (Johnson *et. al.,* 1982). Vaccination results made it a demanding phenomenon. The fish health organizations consider it against fish welfare as it activates the immune system of fish against infections. Studies have shown that the immune response mechanism was similar in fish, birds and mammals and considered vaccination as a good immune-prophylaxis tool. In Norway the use of vaccination has been successfully monitored by qualified scientists in government and private sector. A good communication is important among the industry and the governing authorities. This mutual understanding makes possibility for the scientists to develop and market the vaccine at better speed (Gudding & Muiswinkel, 2013).

3 MATERIALS AND METHODS

The study was conducted at Marine Harvest Research Station Averøy, Kristiansund, Norway from June to September 2014 as a part of research project "Dark spots in salmon fillet" funded by the Norwegian Fishery and Aquaculture Industry Research Fund (FHF). In total 1680 1+ smolt of *Atlantic salmon* were kept in 12 net pens with 140 fishes each. The 12 net pens were divided into three randomized block design. Each block consisted of four groups zinc group (Zn), copper group (Cu), antioxidant group (Selenium and vit-E) or control group. The feed used during the research trail was a standard commercial diet (9mm) manufactured by Skretting AS, Averøy, Kristiansund, Norway. The volume of each seawater net pen was 125m^3. The temperature was recorded every day at the depth of 3m and the average value during the entire period of study was 9°C.

Table 3-1: Feed trail design showing the distribution of dietary treatments fishes among different net pen.

BLOCK 1		*BLOCK 2*		*BLOCK 3*	
F1	**F3**	**F5**	**F7**	**F9**	**F11**
Cu	**AntiOx**	**K**	**Zn**	**Zn**	**Cu**
number 140	number 140	number 140	number 140	number 140	number 140
biomass 346	biomass 349	biomass 347	biomass 342	biomass 346	biomass 344
weight (kg) 2.47	weight (kg) 2.49	weight (kg) 2.48	weight (kg) 2.44	weight (kg) 2.47	weight (kg) 2.46
F2	**F4**	**F6**	**F8**	**F10**	**F12**
K	**Zn**	**Cu**	**AntiOx**	**K**	**AntiOx**
number 140	number 140	number 140	number 140	number 140	number 140
biomass 348	biomass 343	biomass 351	biomass 351	biomass 350	biomass 348
weight (kg) 2.48	weight (kg) 2.45	weight (kg) 2.51	weight (kg) 2.51	weight (kg) 2.50	weight (kg) 2.49

A standard commercial diet (Ottoline premium, 9mm, Skretting, Averøy, Norway) was used as control diet while the diets for the other groups were modified. The gross composition of the control feed was: protein 35.6 %, fat 36.3 %, water 6.9 %, and ash 4.6 %. The content of free astaxanthin was 50 mg/kg. For Zn group the standard commercial diet was supplemented with

100mg Zn/kg by mixing the feed with Zinc Sulphate Hepta-hydrate (VWR Z4750 Sigma Aldrich International) ($ZnSO_4.7H_2O$). The calculated amount of $ZnSO_4.7H_2O$ was poured in known volume of water and then mixed with feed in 25 kg batches. Specialized drum was used for mixing to ensure homogenous concentration on each feed batch. The feed was then coated with rapeseed oil to prevent the leeching and taste effect of nutrients. The feed was then air dried before fed to the fish. For Cu group the standard commercial diet was supplemented with 12 mg/kg $CuSO_4.5H_2O$ (VWR Chemicals) using the same procedure as for the Zn feed. The Cu content in the control feed was 11 mg/kg. For Antioxidant group 0.2 mg/kg organic selenium (Sel-Plex, Alltech) & 500 mg/kg vitamin-E (dl-α-tocopherol acetate DSM Nutritional products, Basel Switzerland) was mixed in rapeseed oil as they are fat soluble and coated on the feed. Each group was offered feed 5 times a day. The uneaten feed was collected at the bottom and pumped into a wired mesh stainer (Einen *et al.* 1999).

The salmon were vaccinated on 4 April, 2013 against Vibriosis (O-1, O-2), furunkulosis, cold water Vibriosis, winter ulcers (Moritella viscosa) and Infectious Pancreas Necrosis (MSD Animal Health (Norvax Minova 6). The unvaccinated fish group were injected with salt water (1% NaCl) in order to standardize the protocol.

3.1 Analysis

The fish group was sampled on August 2014. The fish were desensitized using anaesthesia followed by percussive stunning. The blood was collected aseptically in a heparinized syringe from caudal vein. After blood sampling, the sample fish were allowed to bleed at ambient water temperature by cutting gill arches. The removal of visceral organs and filleting was done manually by trained staff. Fish parameters recorded were: the appearance of heart and liver, body weight,

gutted weight, fillet yield, abdominal fat deposition, body confirmation, frequency of melanin spots in fillets, blood parameters and histological manifestation of melanized tissues. Among the different parameters analyzed in blood plasma glucose, total protein and cortisol level will be focused for results while the detailed blood chemistry parameters are given as attachment in the appendix

Figure 3-1: Analysis at Marine Harvest research station

3.2 Histology

The pigmented spot in fillet were recorded and inspected separately. Tissue dissection and fixation of sample taken from the affected area and the normal tissue were done in 10% natural buffer formalin for histology sampling. At least 4 fishes from each group containing melanin spot

were analyzed. The processing of melanin samples for histology was done at Norwegian School of Veterinary Science, Oslo, Norway according to the routine standard operating procedures for haematoxylin and eosin staining. The prepared slides were analyzed at microscopic lab Nofima, Ås, Norway. Each slide was visualized and recorded from three different areas randomly at 10x resolution and then focus on melanin spot at higher resolutions (20x, 40x, 63x and 100x).

4 BIOMETRIC TRAITS

4.1 Melanin in Fillet

The scoring of the melanin spots was done manually by visual analysis from scale 0-3. The position of the melanin spot was also recorded (Mørkøre, T., 2012).

4.2 Melanization of Peritoneum

The characterization of melanin around visceral organs and abdominal wall was done macroscopically and graded from 0-3 VAS scales (Taksdal *et al*. 2012). The description of the scale was as under:

0 = no melanin

1 = melanin present as tiny spots or dots.

2 = observable amount of melanin.

3 = melanin present in large amount around abdominal wall/ visceral organs.

4.3 Data Analysis

The random block design data was analyzed by the programme Statistical Analysis System 9.1 (SAS Institute Inc.). The interpretation of results was done by LS mean (± SEM) and the level of significance was $P \leq 0.05$.

4.4 Calculations

Feed conversion ratio, FCR = (feed intake, g) / (net weight gain, g)

Condition factor, CF = Weight (g) / (fish length, cm) × 100

Weight gain, WG = Initial wt.W_1 (g) – final wt.W_0 (g)

Hepato-somatic index, HSI = Liver weight (g) / Body weight (g) × 100

Cardio somatic index, CSI = Heart weight (g) / Body weight (g) × 100

Carcass yield, CY = Gutted weight (g) / Body weight (g) × 100

Fillet yield, FY = Fillet weight (g) / Body weight (g) × 100

5 RESULTS

The results will be described into two parts. The first part describes macroscopic evaluation of tissues including biometric traits and melanization of tissues. The second part describes blood chemistry and microscopic evaluation of the melanized tissue samples. The results will be presented graphically as LS means ±Standard error, SE.

5.1 Biometric traits

The Zn group showed significantly higher body weight (4067.8g) and gutted weight (3653.5g) as compared to the antioxidant, Cu and control groups (Figure 5.1).

The Zn group showed significantly higher body length (69.1cm) as compared to the antioxidant, Cu and control groups (Figure 5.2).

The Zn group showed significantly higher fillet weight (average 2606.8) as compared to the antioxidant, Cu and control groups (Figure 5.3).

The liver weight showed no significant differences among the different dietary treatments while the Zn group had significantly higher heart weight (average 4.57) as compared to the antioxidant, Cu and control (Figure 5.4).

The condition factor (average 1.22) showed no significant differences among the different dietary treatment groups (Figure 5.5).

The hepatosomatic index (average 0.88) showed no significant differences among the different dietary treatment groups (Figure 5.6).

The carcass yield (average 89.78) showed no significant differences among the different dietary group but the antioxidant and Zn groups showed significantly higher fillet yield (average 64.1 and 63.7 respectively) as compared to the Cu and control groups (Figure 5.7).

The average melanin score of the abdominal wall varied from 1.3 for the Zinc to 1.7 for copper. The Zinc group showed significantly lower score for melanin in the abdominal wall as compared to the Antioxidant, Copper and control groups (Figure 5.8).

The average melanin score in organs varied from 0.75 for the copper group to 0.84 for the control and copper. The dietary treatments showed no significant differences among the different groups (Figure 5.8).

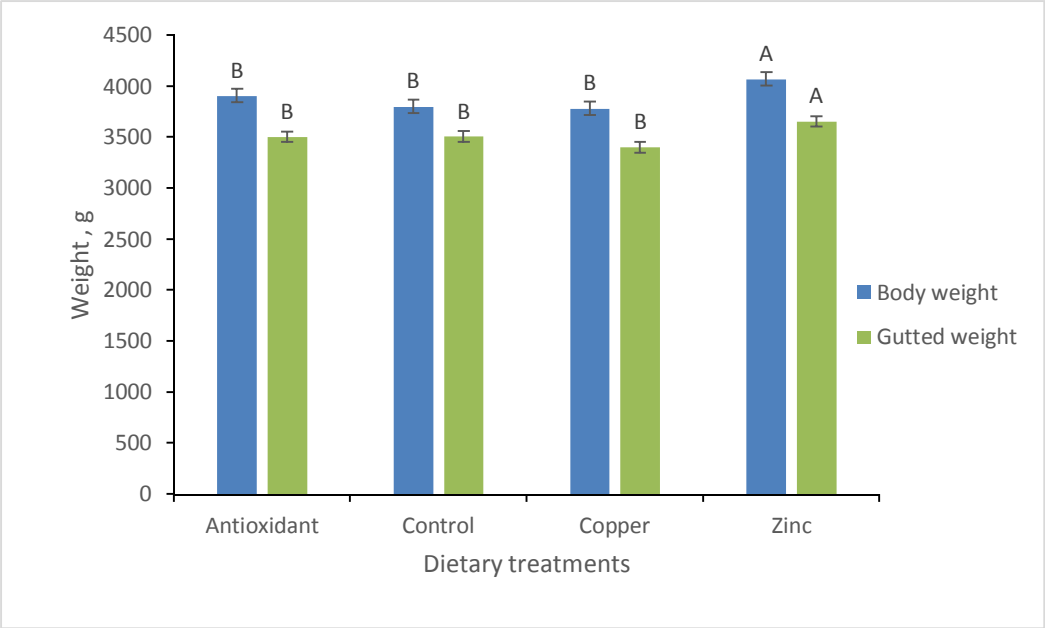

Figure 5-1: Body weight (g) and gutted weight (g) of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05)

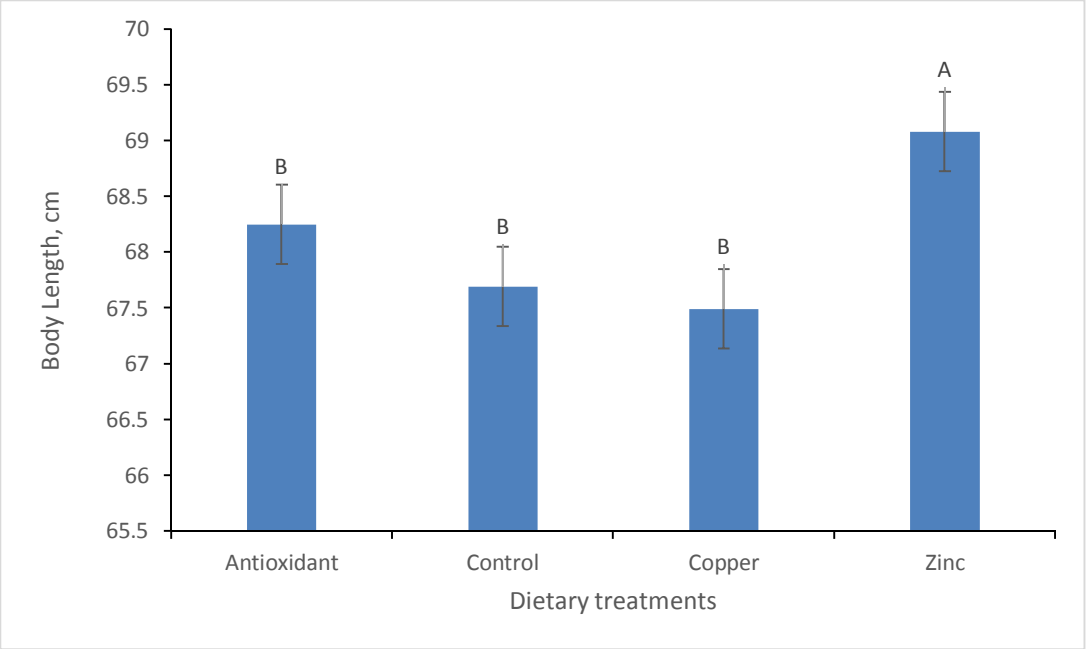

Figure 5-2: Body length (cm) of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05)

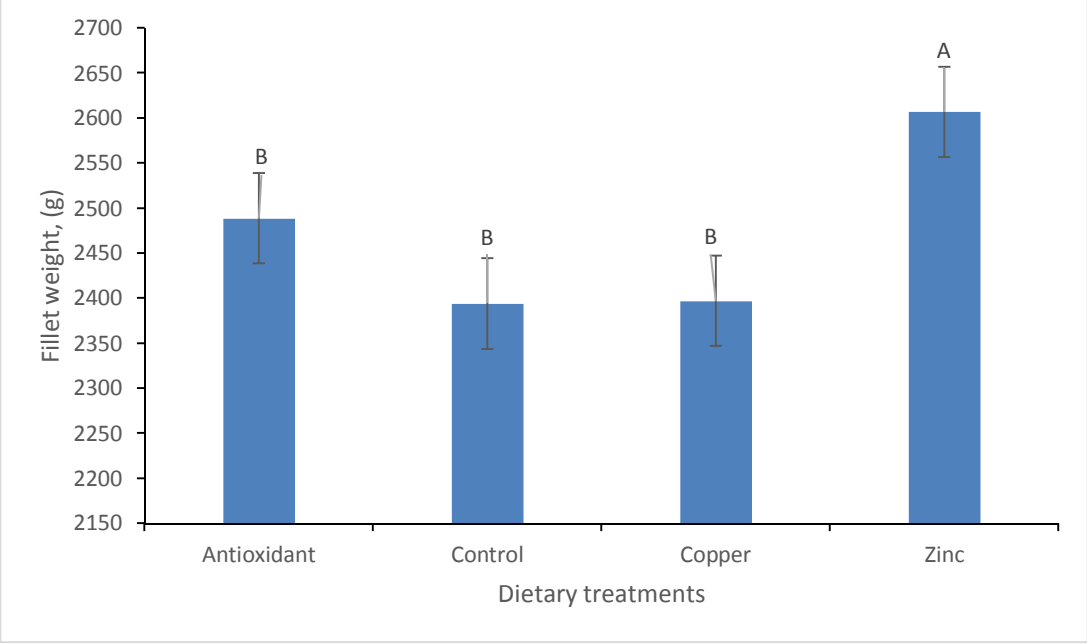

Figure 5-3: Fillet weight (g) of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05)

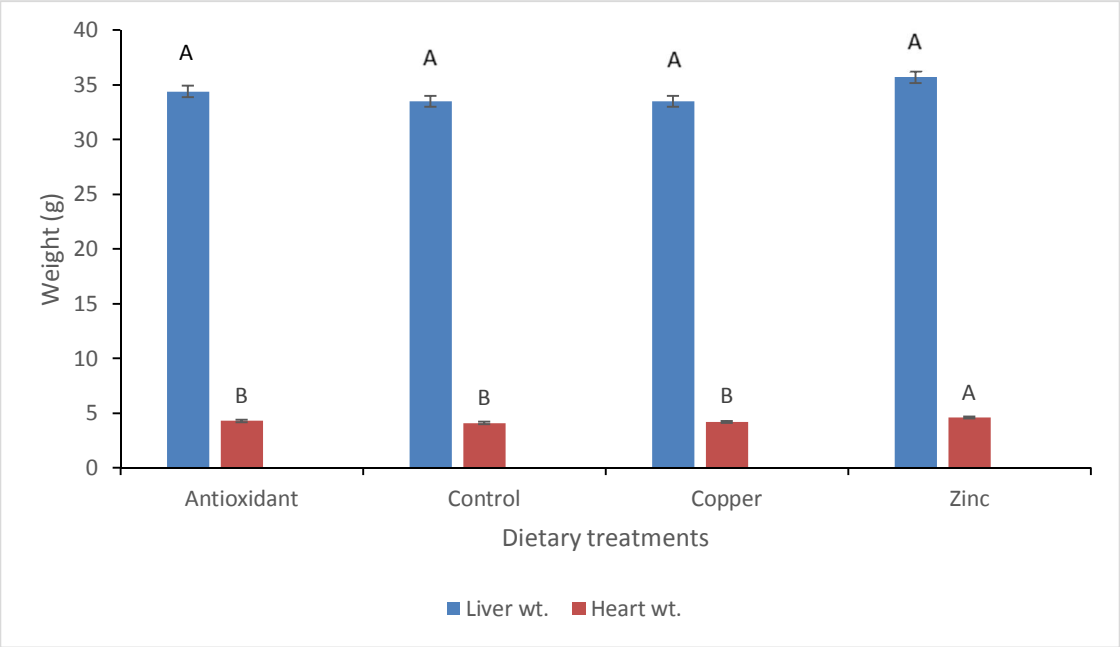

Figure 5-4: Liver and heart weight (g) of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05)

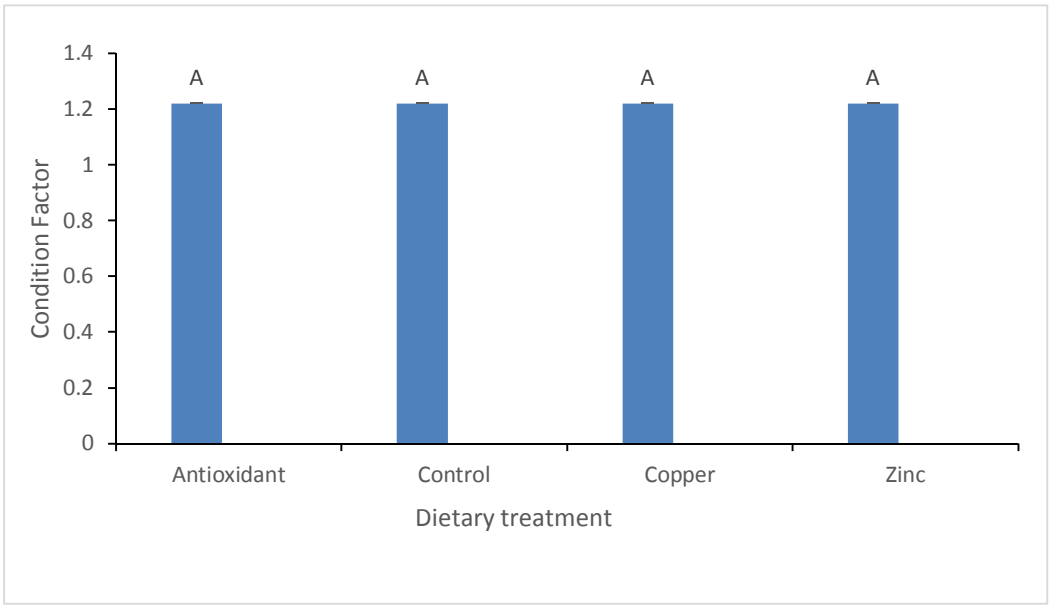

Figure 5-5: Condition factor of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE. The same superscripts indicate no significant differences between dietary treatments (P<0.05)

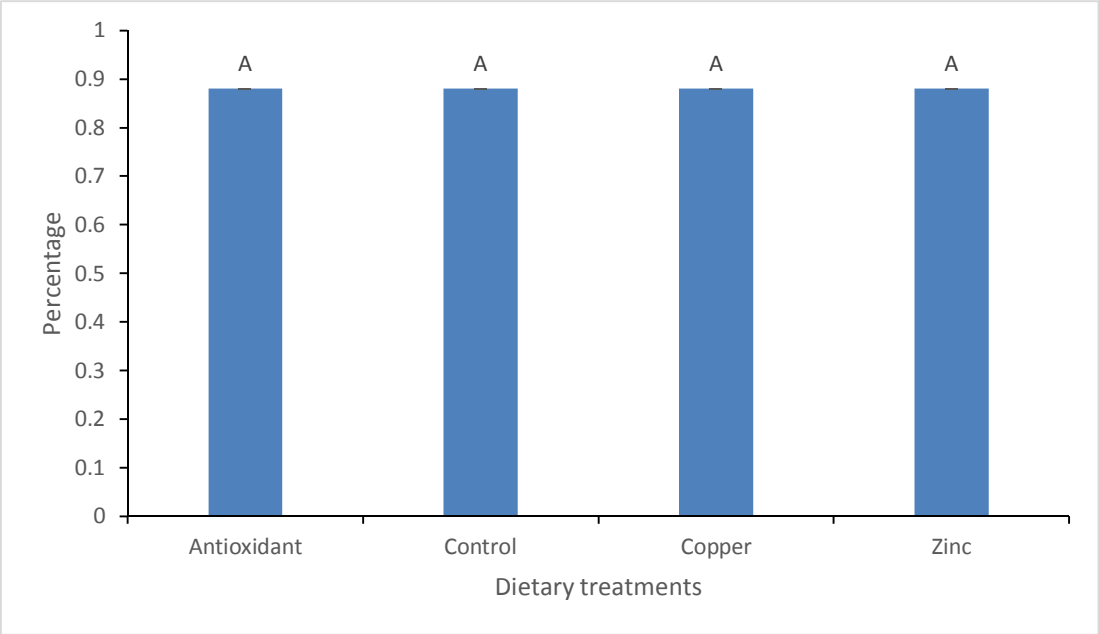

Figure 5-6: Hepatosomatic index values of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE. the same superscripts indicate no significant differences between dietary treatments (P<0.05)

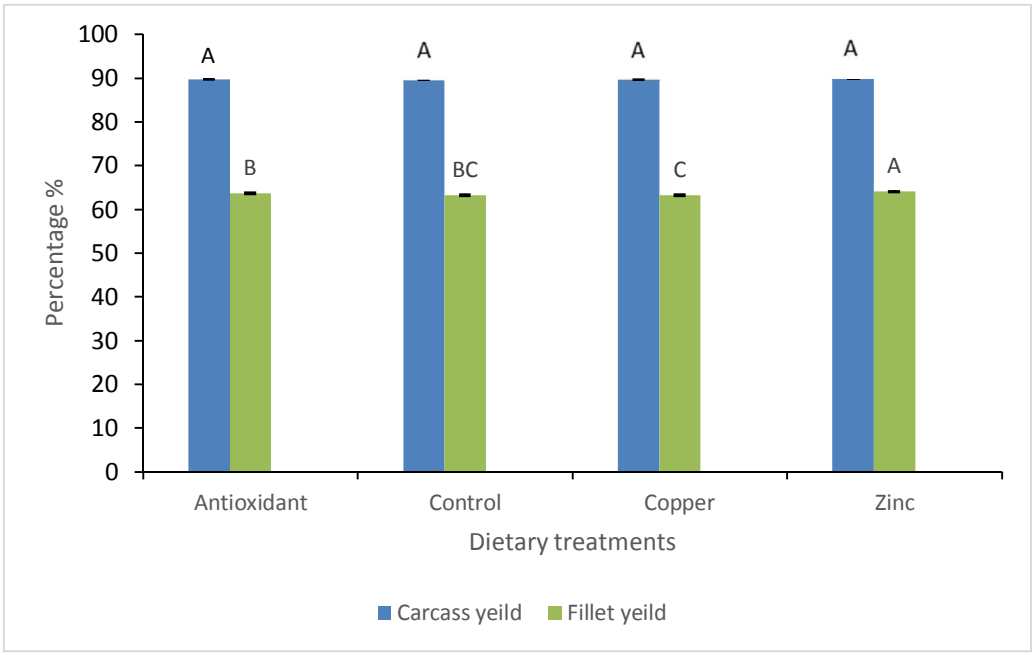

Figure 5-7: Carcass yield and fillet yield (%) of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05)

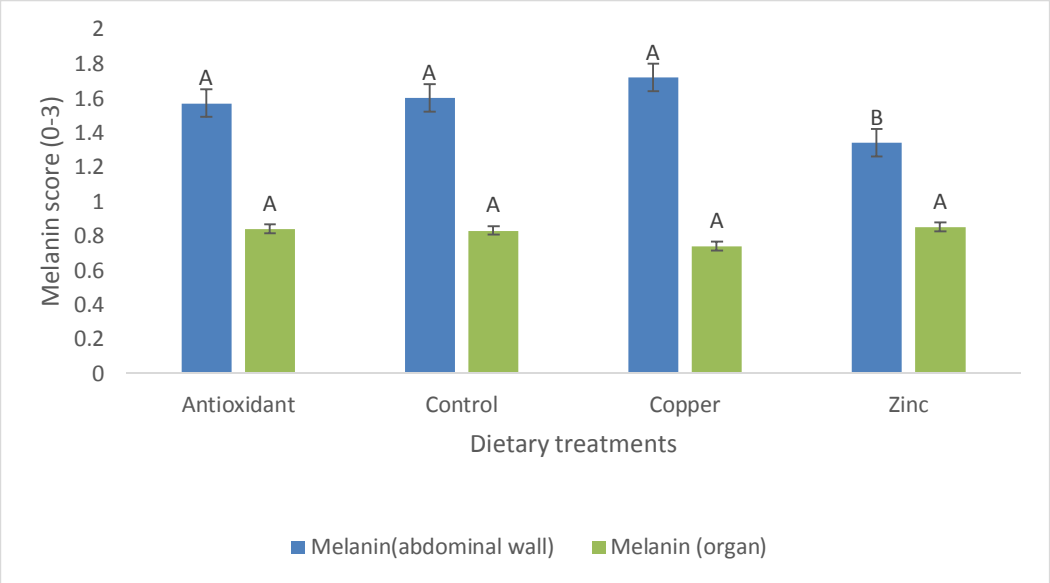

Figure 5-8: Melanin score (0-3) in abdominal wall and organs (0-3) of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts you choose indicate significant differences between dietary treatments (P<0.05)

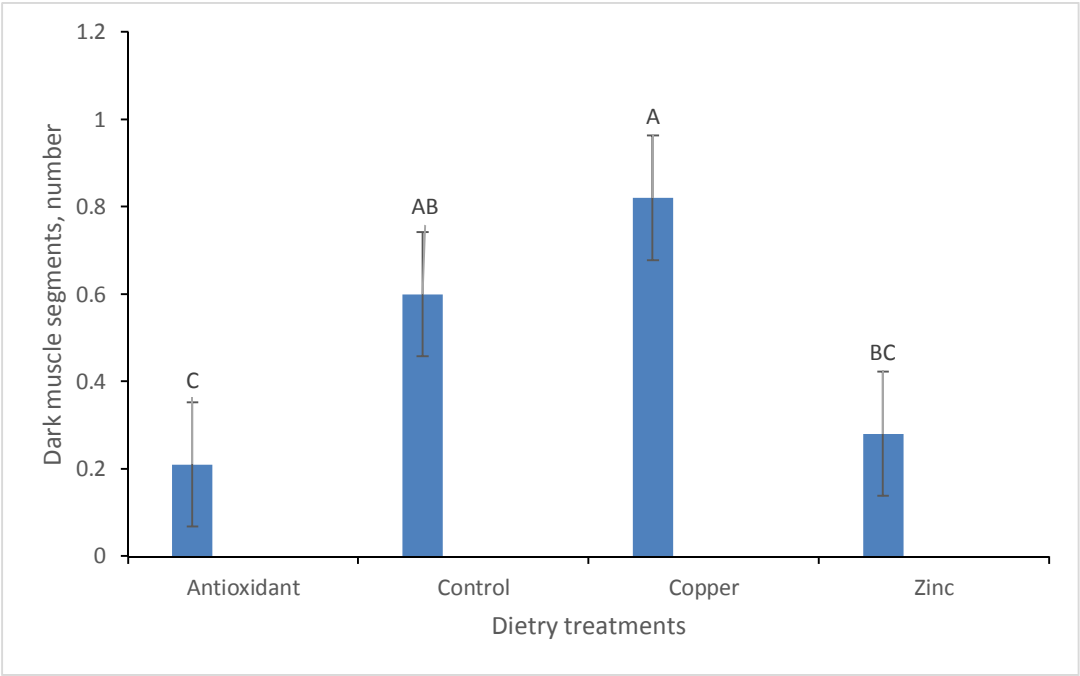

Figure 5-9: Dark muscle segment of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts you choose indicate significant differences between dietary treatments (P<0.05)

Table 5-1: Data from biometric traits of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05)

Biometric traits	Antioxidant	Control	Cu	Zn	P-value		
					Diet	Sex	Model
Body weight, g	3906.05^{B} ±66.93	3797.90^{B} ±53.61	3780.20^{B} ±46.97	4067.75^{A} ±60.52	0.0013	0.0007	0.0001
Gutted weight, g	3502.70^{B} ±58.31	3405.50^{B} ±47.82	3398.60^{B} ±42.12	3653.50^{A} ±54.36	0.0011	0.0005	0.0001
Body length, cm	68.25^{B} ± 0.40	67.69^{B} ±0.30	67.49^{B} ±0.26	69.08^{A} ±0.33	0.0031	0.0031	0.0001
Condition factor	1.22^{A} ±0.01	1.22^{A} ±0.01	1.22^{A} ±0.01	1.22^{A} ±0.01	0.9818	0.0731	0.4931
Fillet weight, g	2488.60^{B} ±42.76	2393.85^{B} ±34.73	2396.85^{B} ±31.47	2606.80^{A} ±39.91	0.0001	0.0044	0.0001
Fillet yield, %	63.70^{B} ±0.16	63.22^{BC} ±0.11	63.20^{C} ±0.08	64.07^{A} ±0.14	0.0001	0.0026	0.0001
Carcass yield, %	89.72^{A} ±0.0.16	89.68^{A} ±0.16	89.91^{A} ±0.16	89.82^{A} ±0.16	0.6027	0.3302	0.5508
Liver weight, g	34.45^{A} ±0.66	33.51^{A} ±0.52	33.50^{A} ±0.59	35.77^{A} ±0.59	0.0223	0.0061	0.0006
Hepato somatic index, HSI, %	0.88^{A} ±0.009	0.88^{A} ±0.009	0.88^{A} ±0.009	0.88^{A} ±0.009	0.9897	0.8857	0.9970
Heart weight, g	4.26^{B} ±0.07	4.12^{B} ±0.07	4.19^{B} ±0.07	4.57^{A} ±0.07	0.0002	0.001	0.001
Cardio somatic index, CSI, %	0.11^{A} ±0.001	0.11^{A} ±0.001	0.11^{A} ±0.001	0.11^{A} ±0.001	0.3081	0.0314	0.0435
Melanin abdominal wall, score	1.57^{A} ±0.06	1.6^{A} ±0.08	1.72^{A} ±0.09	1.34^{B} ±0.08	0.0001	0.0095	0.0017
Melanin organs, score	0.84^{A} ±0.08	0.83^{A} ±0.08	0.74^{A} ±0.07	0.85^{A} ±0.09	0.8909	0.8159	0.6433

5.2 Blood chemistry:

The blood samples taken from all fishes were analyzed for total protein including albumin and globulin which are taken as stress indicator in fish. Under stress slaughter condition the total protein value showed significantly lower level of total protein as compared to the standard slaughter method (Figure 5.9).

Cortisol was also used as an indicator of stress. The Antioxidant, control and copper groups slaughter under stress condition showed significant higher level of cortisol in blood circulation when compared to the standard slaughter method (Figure 5.10).

The glucose level was also used as an indicator of stress. The stress slaughter treatment of Antioxidant, Control, Copper and Zinc group showed significantly higher level of glucose in blood at the time of slaughter when compared with standard slaughter treatment of the respective group (Figure 5.11)

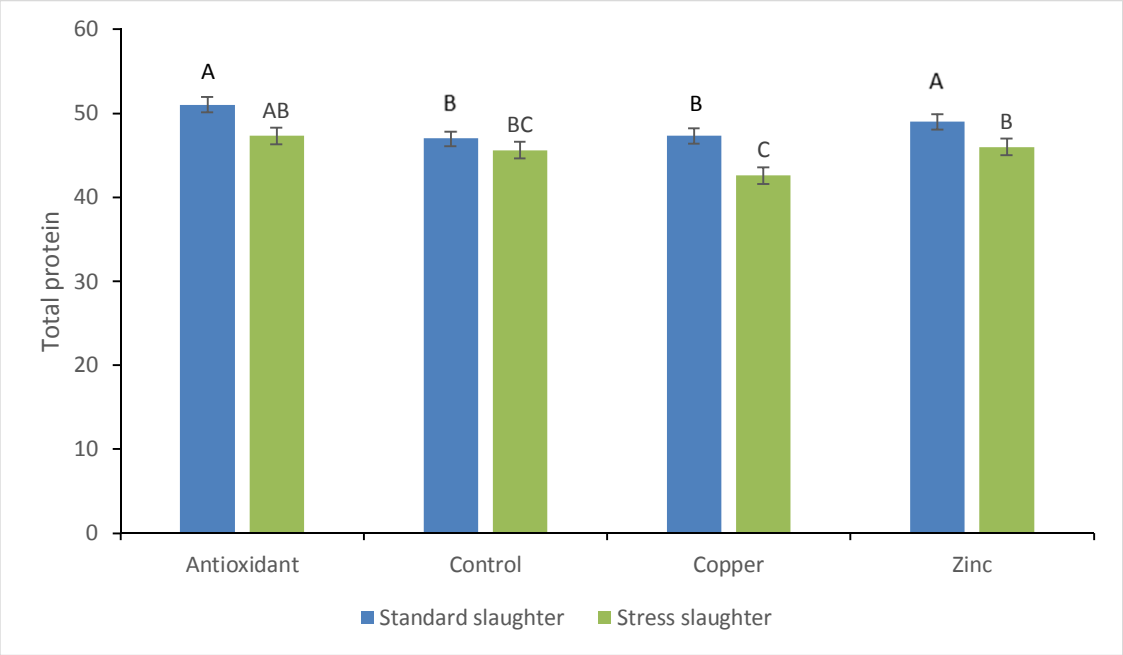

Figure 5-10: Total protein (g/L) of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. The salmon were slaughtered according to standard procedure or upon crowding stress. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05).

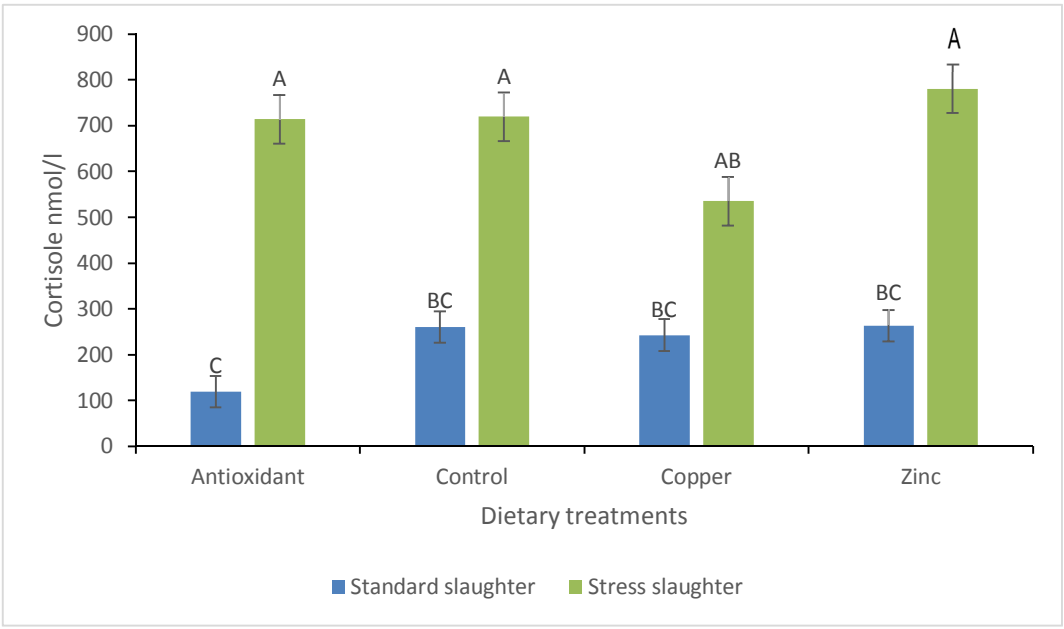

Figure 5-11: Cortisol (nmol/L) of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. The salmon were slaughtered according to standard procedure or upon crowding stress. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05).

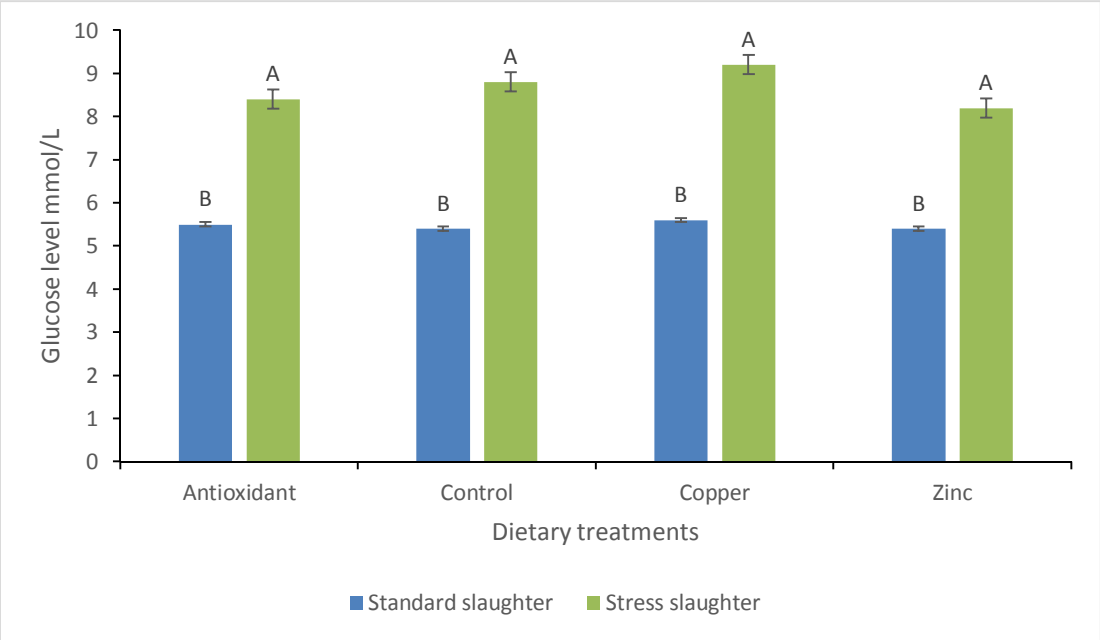

Figure 5-12: Glucose level (mmol/L) in blood of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. The salmon were slaughtered according to standard procedure or upon crowding stress. Results are presented as LS mean ± SE and different superscripts indicate significant differences between dietary treatments (P<0.05).

5.3 Histology of Melanized Tissue

The body weight, gutted weight, fillet weight, fillet yield and body length of the fish containing melanin showed slightly decreased score through the entire sampling. Focal discoloration from grey to brownish-black was prominent after filleting. Mostly the abnormal pigmentation was seen under the peritoneum in the abdominal wall while some of the pigmented spots showed no relationship with the abdominal wall with occurrence of dark pigmentation in the deep musculature. The number of pigmented spots was mostly one per fillet but in some cases up

to three spots was identified. The size of the pigmented spots varied from few millimeters to several centimeters.

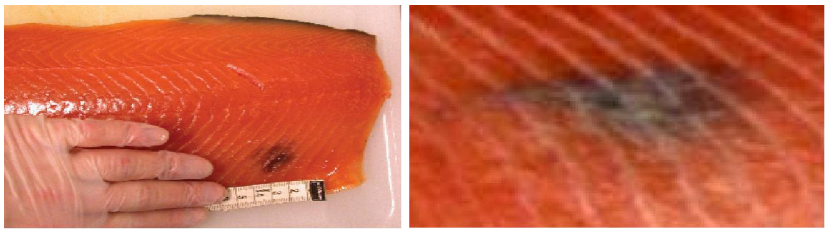

Figure 5-9: Typical melanin spot in salmon fillet.

The changes in well-defined pigmented spot indicated by arrow reflect chronic polyphasic necrotizing myopathy in the vicinity of the pigmentation while degeneration of muscle fiber was also prominent.

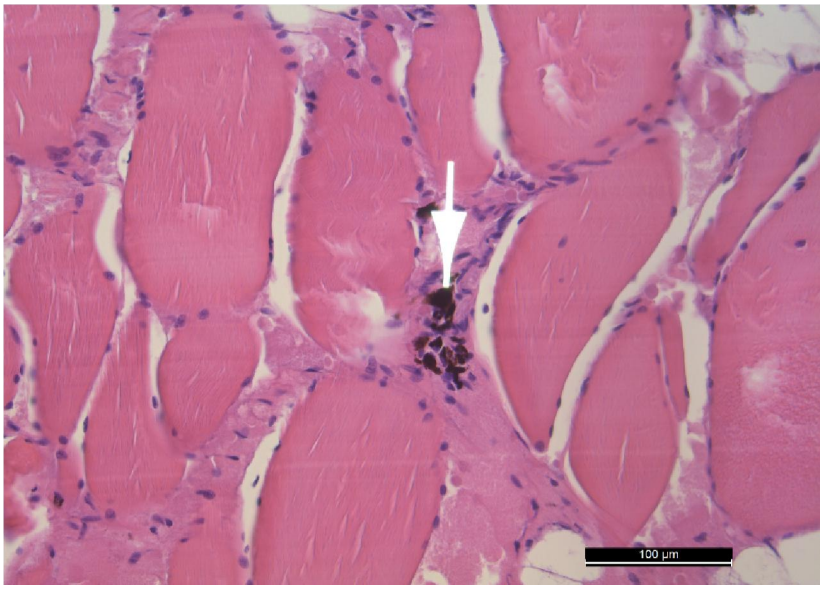

Figure 5-10: A polyphasic necrotizing myopathy containing pigmented cells indicated by arrow head (H&E staining, scale bar = 100µm)

The pigmented spots contain abundant melano-macrophage like cells which appeared as granulomas and are considered as an indication of chronic inflammation followed by regeneration. These changes frequently contain vacuoles. These vacuoles appeared empty and surrounded by

numerous melano-macrophages forming a border of pigmentation separating them from surrounding tissue.

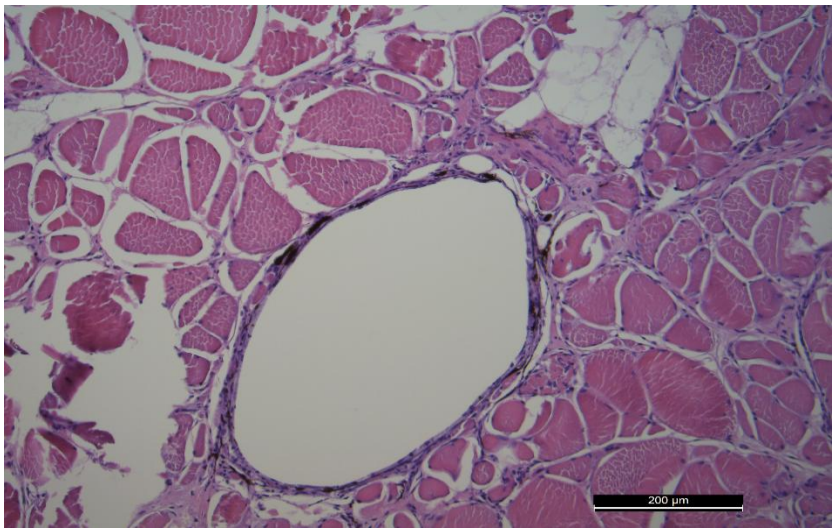

Figure 5-11: Pigmented spots surrounding by melano-macrophages forming a vacuole (H&E staining, scale bar = 200µm)

The melano-macrophage like cells surrounded by the pigmented cells and destruction of muscle fiber forming granuloma showed an indication of chronic inflammation followed by fibrosis. Various vacuoles and degenerative fibrous tissue (indicated by arrow) were also present in the surrounding area.

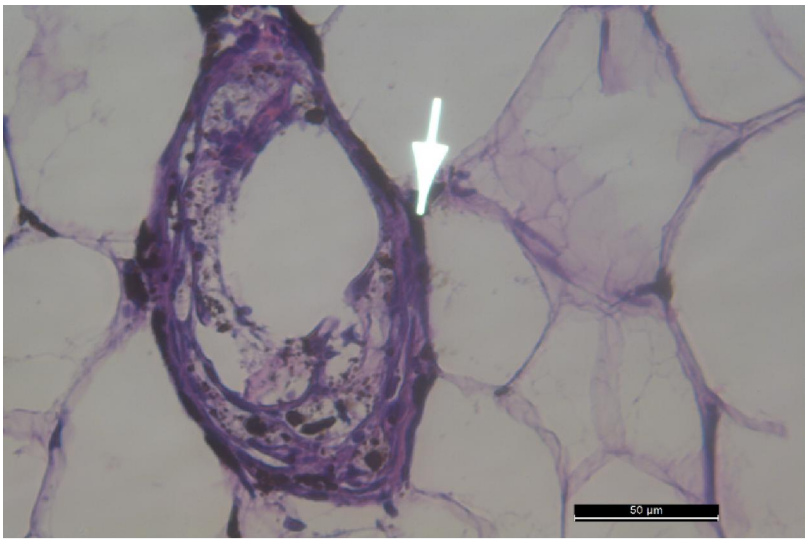

Figure 5-12: Degenerative fibrous tissue with pigmented cells indicating chronic inflammation (H&E staining, scale bar = 200µm)

Petechial haemorrhage with red blood cells containing darkly stained nuclear granules in addition to melano-macrophage like cells (indicated by arrow) infiltrating the surrounding tissues indicating the possibility of acute inflammation.

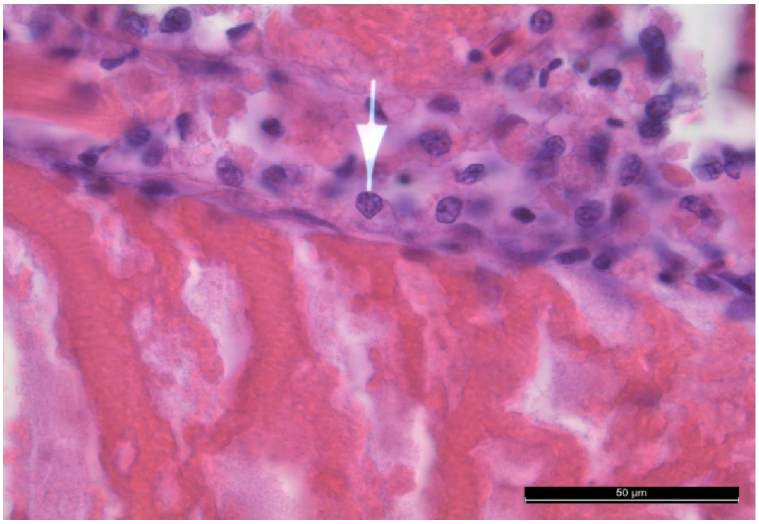

Figure 5-13: Petechial haemorrhage with darkly stained nuclear granules indicating acute inflammation (H&E staining, scale bar = 50µm)

Aggregation of darkly stained cells containing nuclear granules (indicated by arrow) and mono-nuclear cells in the surrounding tissue containing pigmented cells indicating the possibility of inflammation.

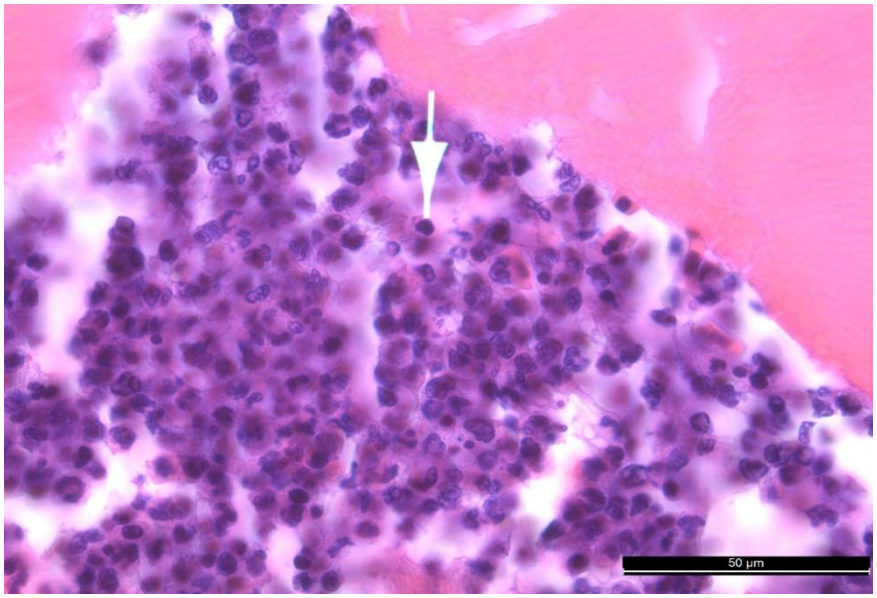

Figure 5-14: Tissue containing nuclear granules in area surrounding inflammation (H&E staining, scale bar = 50µm)

Granulomatous mass containing large number of melano-macrophages like cells (indicated by arrow) in the affected area followed by fibrosis and mass containing degenerative tissue showing abnormal cell morphology.

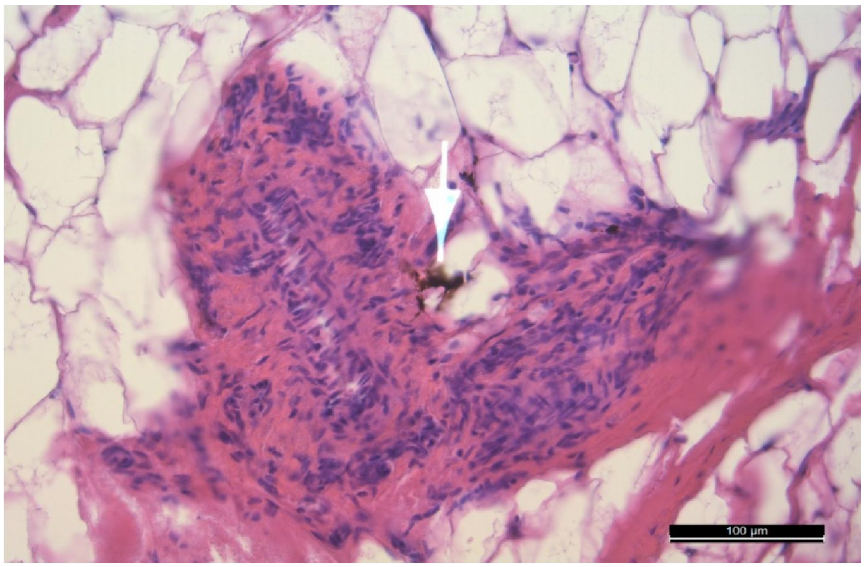

Figure 5-15: Tissue with abnormal morphology (H&E staining, scale bar = 100µm)

The irregularly arranged melano-macrophages found in the degenerative muscle fiber containing abundant amounts of melanin granules (indicated by arrow).

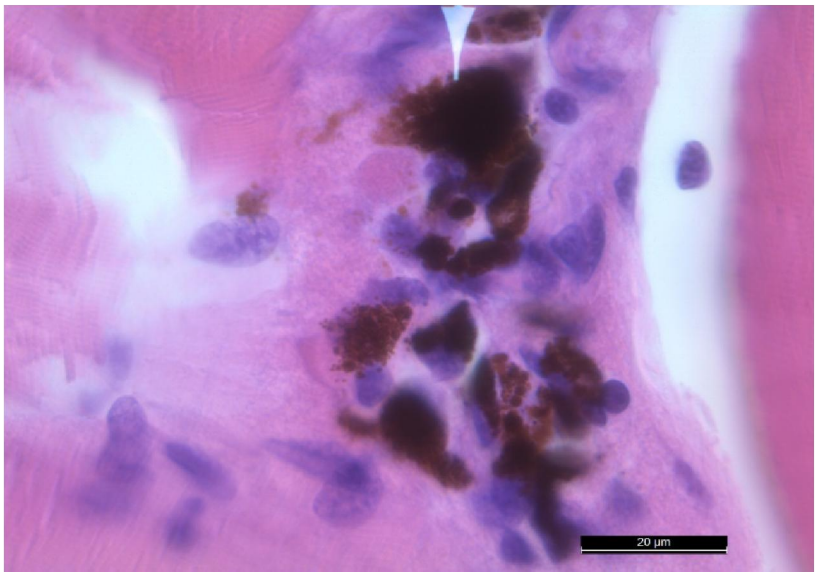

Figure 5-16: Degenerative muscle fiber containing melanin granules (H&E staining, scale bar = 20µm)

The irregularly shaped variable amount of pigmented melano-macrophages like cells abundantly found in the granulomatous tissue giving an appearance of dendritic shaped cells

(indicated by arrow) with melanin. This structural difference in various melano-macrophages like cells was evident in unorganized inflamed tissue.

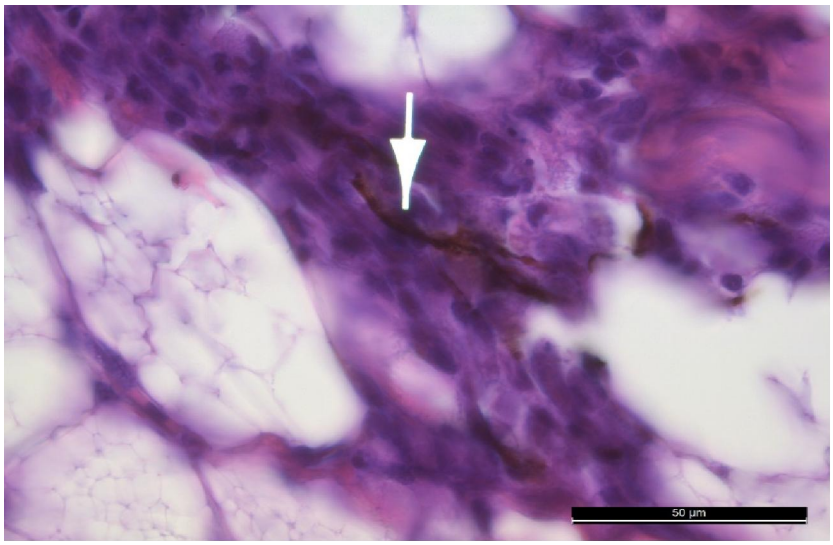

Figure 5-17: Dendritic shaped cells with melanin (H&E staining, scale bar = 50µm)

Amorphous mass containing proteineous material in space between myocytes forming a vacuole with macrophage like cells indicating partial degeneration.

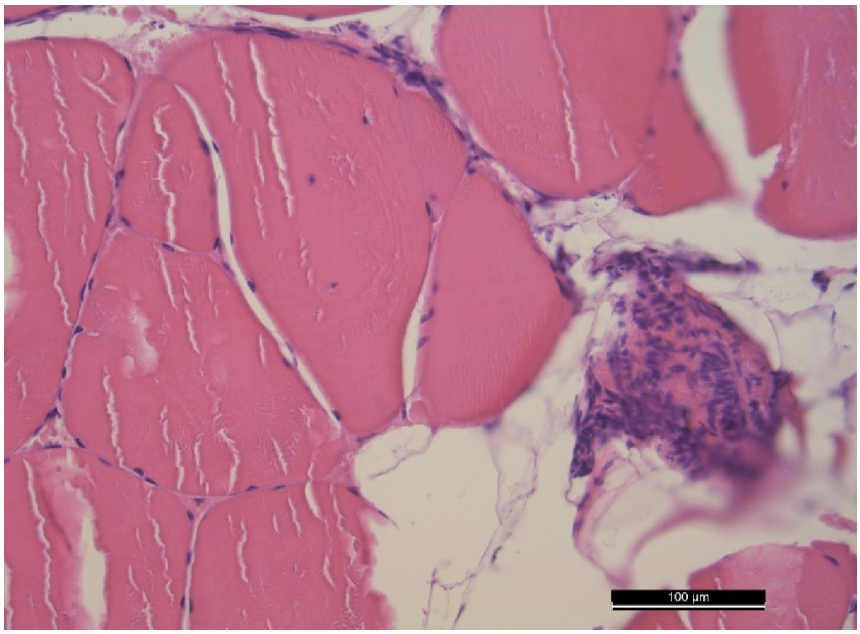

Figure 5-18: Partial degeneration of myocytes (H&E staining, scale bar = 100µm)

The muscle fiber invaded by melano-macrophages and mononuclear cells containing pigmented and non-pigmented granules indicating acute inflammation.

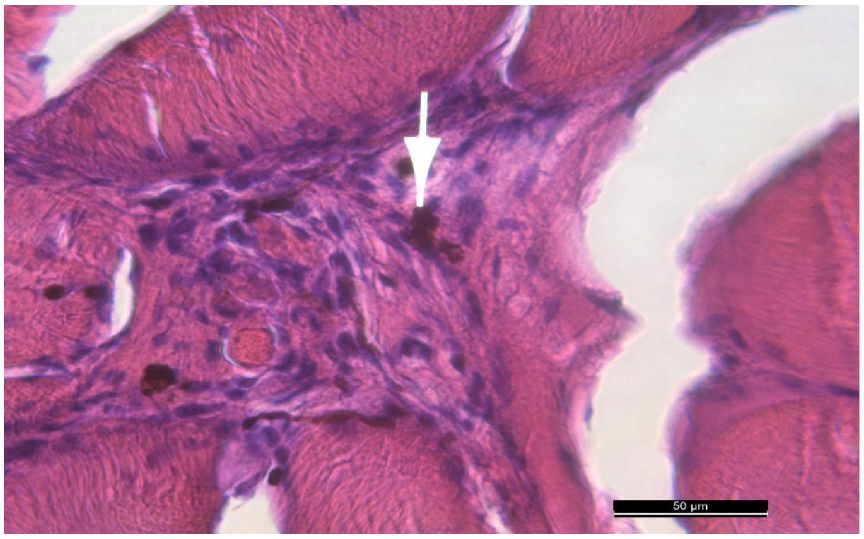

Figure 5-19: Acute inflammation containing pigmented and non-pigmented granules (H&E staining, scale bar = 50µm)

Cross section of normal muscle tissue from reference sample.

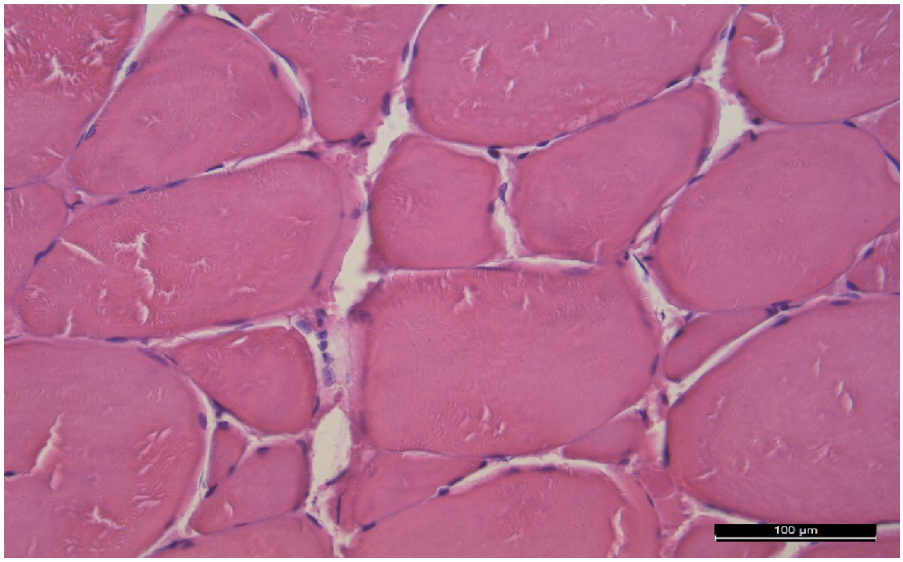

Figure 5-20: Normal muscle tissue (H&E staining, scale bar = 100µm)

A normal reference slide showing blood capillary.

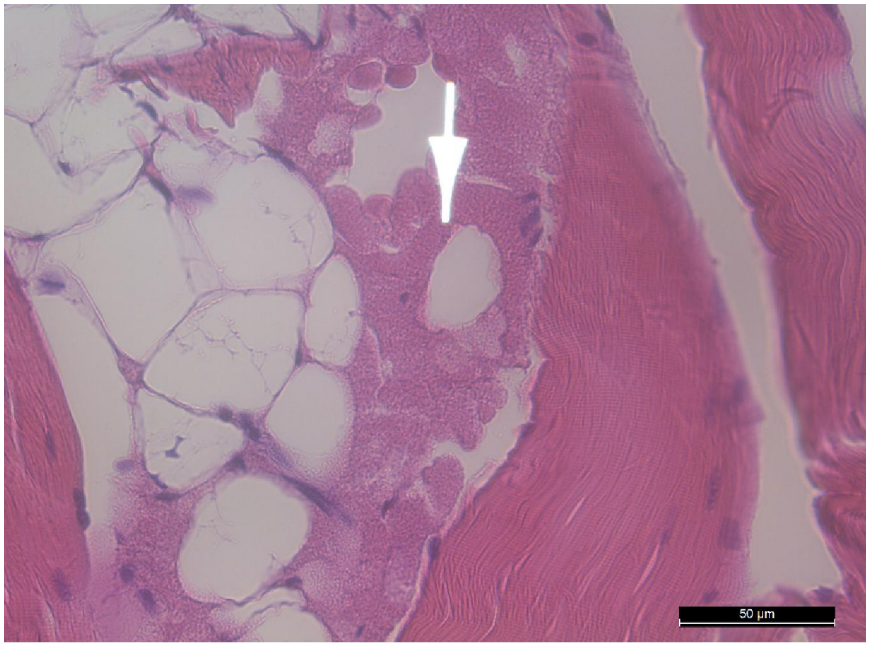

Figure 5-21: Reference slide (H&E staining, scale bar = 50µm

Longitudinal section of normal muscle fiber showing organized pattern of muscle fibers indicated by arrow head from reference sample.

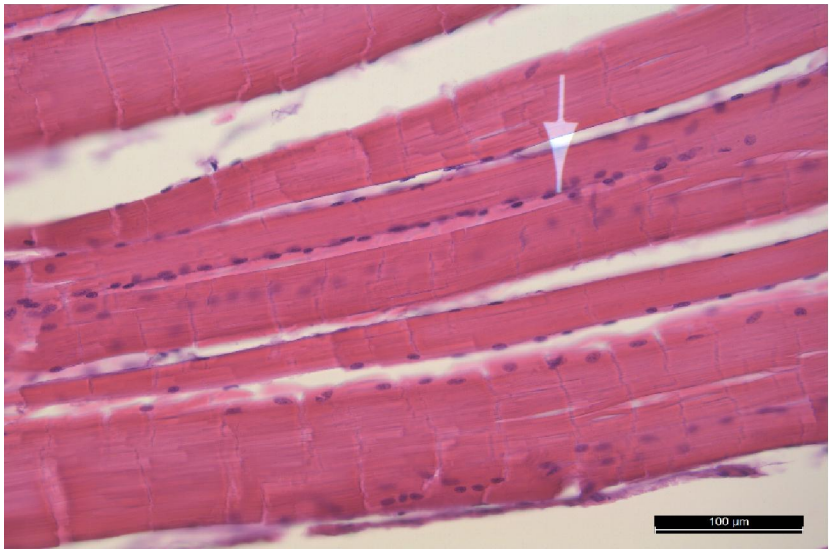

Figure 5-22: Longitudinal section of muscle fiber (H&E staining, scale bar = 100µm)

6 DISCUSSION

The discussion will also be described into two parts. The first part describes macroscopic evaluation of tissues including biometric traits and melanization of tissues. The second part describes blood chemistry and microscopic evaluation of the melanized tissue samples.

6.1 Biometric Traits

The condition factor (CF) that was evaluated by the ratio of fish weight and length. Higher values of CF indicate optimum growth that could gained under current trail. When we discuss the results among the different dietary treatments there was no significant difference in relation to the CF. The lower CF values could be due to different factors like light duration, temperature, disease outbreak, handling and management practices Folmar & Dickhoff (1980). The higher values reflect increased growth of muscles and fat storage around visceral organs. The different diets contributed equally in growth and development and it eventually increases with the passage of time and this was a good indication from economic point of view.

The results from fillet weight (FW) indicated that the Zn group showed higher fillet growth while Antioxidant group and Zn group indicated increased fillet yield when compared to other dietary treatments. The Zn was essential micronutrient and integral part of many enzymes as cofactor which carries number of metabolic reactions while antioxidant is essential to prevent denaturation of fats. Hence both groups showed optimum fillet yield.

The hepatosomatic index (HSI) was an indicator of fish health metabolism. The general appearance of liver was very important to determine the overall health status as it was the metabolic center and played key role in number of different pathways like hydroxylation of fats bile

secretions. Light brown to dark brown color and abnormal appearance indicates metabolic disorders Larsson *et al*., (2014).

The organ adhesion remained unaffected by different dietary treatments but vaccination handling induces stress which resulted in greater organ adhesions. The organ adhesion adversely affects the fish health status. So there is also a need to develop more efficient methods of vaccination which minimize the handling stress. Studies carried out by Poppe (1997); Haugarvoll *et al*., (2010) and Drangsholt, (2011) also indicates that oil based vaccines induces inflammation response which ultimately resulted in organ adhesion between the visceral organs itself and the body wall.

Koppang *et al.,* (2005) indicated that deposition of abnormal pigment in tissues and organs were due to some pathological conditions. Inflammation and granulomas could be formed at the injection site or adjoining tissues as a result of mineral oil based vaccines. These foreign molecules integrate from site of injection to various body tissues and organs as a result of autoimmune response by fish defense mechanism. Ultimately formation of dark pigments around visceral organs in the current study could be due to vaccination side effect. Kiron *et al.,* 1993. Ispir *et al.,* 2011 and Halver, 2002 documented that Zn and Vitamin E inclusion in diet can trigger fish immune response which resulted in increased melanin formation while the results from our current study did not validate this findings. It was also indicated that Zn group contributed to minimize the chance of melanin deposition in fillet as evident from the research trail. It could be assumed the Zn being a part of co-factor, occupied the reaction site during melanin formation pathway but further studies needed to investigate the real mechanism of how it contribute to prevent such melanin deposition. The Zn group also showed dark brown color which could be due to

hyperactivity of hepatocytes involved in detoxification of harmful components and immune system.

Melanin formation around peritoneum showed that Zn group tends to minimize the occurrence of melanin when compared to other dietary treatments while the Cu group showed the highest numerical value. Study conducted in 2010 explained the melanin formation around peritoneum as an inflammatory response of vaccination. The mineral oil component present in vaccine could be the potential source of intraperitoneal granulomas (Koppang *et al*., 2010).

Melanin pigment formation in salmon fillet is currently an important product quality issue. Any pigmentation or discoloration in salmon fillet was assumed to be inferior product quality, which ultimately led to decrease price and sometimes even resulted in rejection of the fillet containing such melanin pigmentation (Mathiassen *et al*., 2007). Studies conducted by Koppang in 2010 demonstrated that pigmentation or discoloration of salmon fillet has been documented in fish from British Colombia, Chile, Canada and Scotland as post vaccination effect while countries like Australia and Tasmania did not showed such pigmentation where intraperitoneal vaccination was not practiced. He also documented that no such pigmentation was documented from salmon fish of wild origin. The pigmentation of white muscle in vaccinated and farmed Atlantic salmon could be explained as granulomatous or secondary inflammatory response much identical to the foreign body type inflammatory response and the absence of pathogens at the particular site were the contributing factors for such pigmentation. (Koppang *et al*., 2005). Another study was conducted by Larsen and co-workers in 2012 also demonstrated that the abnormal pigmentation of skeletal muscle of salmon was possibly due to inflammatory response by pigment producing granuloma cells of fish immune system (Larsen *et al*., 2012). One possible factor that elicits such inflammatory immune response was intra-peritoneal vaccination technique and resulted in the

formation of pigmented (melanin) spots around peritoneum (Mørkøre, 2008). In 2012, Mørkøre demonstrated that the occurrence of pigmented (melanin) spots also tend to increase with age and size of the fish. Vaccination could not be the only contributing factor for the development of melanin spots but a phenomenon that could be related to the aging of fish. One possible reason could be that with aging of the fish which render weak immune system due to other infections or stress mechanisms. (Mørkøre *et al*., 2012). The Zn group (0.34) showed decrease trend in organ adhesion and melanin in organ while the Cu group (0.72) showed highest tendency for these traits. So diet supplementation with Zn had contributed positive effect on minimizing the occurrence of melanin. The location of melanin spot also showed no significant difference among different dietary treatments. Most of the melanin spots were found in the anterior part of the abdomen in salmon. These results were similar as presented by Mørkøre, 2012 who documented that mostly the melanin spot were found to be present at the anterior part of the abdomen in salmon fillet (Mørkøre *et al,*. 2012).

6.2 Microscopic Evaluation and Blood Chemistry

In our current study, histopathology mostly revealed pigmented and granular changes in the white muscle of the skeletal tissue of the Atlantic salmon. This can be regarded as granulomatous inflammatory conditions much similar to the foreign body type occupied by epithelioid cells, macrophage-like cells and melano-macrophages. The lack of advance techniques used for identification of foreign particles (like bacteria and viruses) and other possible justification tend to postulate that stress due to injecting intraperitoneal vaccination triggered immune mediated inflammatory response which could be the most possible etiological factor responsible for these mechanisms. The majority if the pigmented granules were present in the adjoining tissues of the peritoneal cavity (Gupta *et al,*. 1993). The variation in color from grey to blackish appearance was

an index of the aggregation of melano-macrophages. Tissue embedding during processing samples for histology imparts lipid molecules into the tissue stained sections were regarded as oil adjuvant. The debris containing vesicles were regarded as organizing structure in the process of formation. The toxic side effect of oil adjuvant based vaccines is the current subject of interest. In food producing animals' oil adjuvant based vaccines are recommended only when it did not stimulate inflammation response (Spickler & Roth 2003).

In teleosts there are no scientific reports published on pharmacokinetics and drug distribution of injected mineral oil adjuvants. In poultry a group of scientists elaborated the rapid diffusion of the injected vaccine containing mineral oil components to the surrounding tissues especially the tissues with high vascularization. The hydrocarbons contained in mineral oil based vaccines were excreted as conjugated antigen-antibody complex in the egg yolk of the hen. However the excretion of these hydrocarbons from the body in non-laying hen was much slower (Franchini *et al*,. 1984). In another experiment the mineral oil adjuvants injected in one leg could not identified in the other leg of the same poultry bird (Piretti *et al*,. 1982). In rats it was documented that radioactive hydrocarbons when injected in intraperitoneal cavity distributed eventually to the liver and more specifically to the fat cells. Keeping in view these findings it can be concluded that salmonids store such lipids derived from mineral oil based adjuvants in their red and white musculature (Bollinger 1970). But the exposure these adjuvants present in food could not document any injurious effect to human health (Nash *et al*., 1996).

A well-documented metabolic response to stress stimuli is elevated level of glucose in the plasma concentration of the blood. Glucose is a basic energy generating currency of the cell required for the oxidation of basic metabolic processes and increased demand of energy in relation to stress in fish. One of the key roles of liver metabolism was synthesis of new glucose

molecules, gluconeogenesis, in order to ensure the uninterrupted energy supply in the form of glucose to the extra-hepatic tissue, gills, brain and heart the period of stress (Mommsen *et al*., 1999). The rapid depletion of glucose in response to the stress from the liver activate adrenergic receptors and stimulate the glycogenolytic pathway which mobilize and maintain the plasma level of glucose and restoration of used glycogen molecules of the liver (Mommsen *et al.,* 1999; Aluru and Vijayan, 2007). The standard slaughter treated group showed elevated level of blood glucose level that is highest for copper (9.2 mmol/L) and lowest for zinc (8.2 mmol/L), while the standard slaughter treated group showed much similar results within the different dietary treatments but significantly different level of blood glucose when compared with stress slaughter treated group. These results validate the studies that have documented hyper-activity of liver glucose oxidation enzymes involved in the glycolysis immediately after acute stress exposure. This mechanism is important to meet the higher energy requirement of the liver in order to maintain the internal homeostatic environment (Wiseman *et al*., 2007; Mommsen *et al*., 1999; Iwama *et al*., 2006). The other enzyme involved in glycolysis like kinases, and dehydrogenases also fluctuate in the same manner during acute stress. The availability of these enzymes are the rate limiting step for glycolysis. Meanwhile the gluconeogenesis is also activated and these results are supported by the hyper-activity of enzymes like phosphatases and synthetases soon after stress. In liver the most appropriate substrate for gluconeogenesis are lactate, albumin, globulin and glycerol. These substrates were upregulated soon after exposure to stress indicating the activation of proteolytic pathways (Wiseman *et al*., 2007). In the current study the total protein obtained by cumulative sum of albumin and globulin level, also indicates such decreased level in stress treated group. The enzymes play critical role in substrate pharmacokinetics and adaptation to specific stress response in fish.

Another metabolite related to stress response is indicated by the level of cortisol which is usually elevated on exposure to stress. The higher level of cortisol stimulates energy demanding mechanisms such as synthesis of protein to retain homeostasis (Wendelaar Bonga, 1997; Mommsen *et al*., 1999). The higher level of cortisol also resulted in elevation of blood glucose level in fish (Mommsen et al., 1999; Iwama et al., 2006). There is very little information available for the signaling role of cortisol in proteolytic pathway at molecular level in fish. Although lysosomal utilization and degradation has shown to be the basic mechanism regulating the catalysis of muscle protein during migration, spawning and starvation (Mommsen, 2004). The role of cortisol in the breakdown of muscle protein is well established fact in mammals. This proteolytic pathway is activated by the lysosomal enzyme especially cathepsin D which act as promotor and mainly responsible for the mechanism involved in the breakdown of muscle protein (Tsukuba *et al*., 2000). In mammalian model system, the degradation mechanism carried by the liver lysosomes has been well documented (Roberts *et al*., 1997; Mommsen, 2004). Further studies are needed to investigate the exact mechanism of cortisol activating liver proteolysis. There are various mechanisms involved in the stimulation of proteolytic pathways like external environmental factors contributing stress to the fish and internal hormonal regulation which play important role in the metabolism to make adjustments necessary to minimize stress. The utilization of lysosomal enzyme cathepsin D as well as cortisol in trout validates the role of lysosomal mechanism in response to stress (Wiseman *et al*., 2007; Aluru & Vijayan, 2007). Cortisol also play key role in other basic physiological behavior like immune function, osmoregulation and neuroendocrine functions. Further studies needed to investigate the cortisol-mediated response in different organs like brain, gills, pancreas, intestine and gonads on exposure to stress. Evaluation and identification of cortisol-mediated response to stress will be a revolutionary step in fish genomics.

7 Conclusions

The current research project exhibited the significant importance and variation of the tissue evaluation of the dietary supplementations of antioxidant, copper and zinc when compared to control group on melanization, fish health, fillet quality and stress resistance of Atlantic salmon. It can be summarized as follows:

- Supplementation of zinc: improved growth, fillet weight, total protein and blood glucose. It also showed minimum incidence of melanin formation in darkly stained muscles, organ and peritoneum.
- Supplementation of antioxidant: improved fillet yield, total protein and blood glucose. It also showed improved health parameters. Melanization of organ and peritoneum showed no significant differences while less number of darkly stained muscles in salmon.
- Supplementation of copper: increased number of melanin in darkly stained muscle, organ and peritoneum. It showed decreased fillet yield.

Histopathological findings also showed darkly stained particles inside the cell surrounding the melanized tissue. Nature and origin of such particles is the hour of need for further investigation possibly with electron microscope or molecular techniques.

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9 Appendices

9.1 Appendix A

Data from hematology of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts you choose indicate significant differences between dietary treatments (P<0.05)

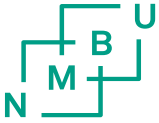
Blood parameters	Standard Slaughter				Stress Slaughter			
	Antioxidant	Control	Copper	Zinc	Antioxidant	Control	Copper	Zinc
Total protein	51.0 ^{A} ±0.3	47.3 ^{B} ±0.4	47.0 ^{B} ±0.3	49.0 ^{A} ±0.3	47.3 ^{AB} ±0.3	45.7 ^{BC} ±0.4	42.7 ^{C} ±0.4	46.0 ^{B} ±0.2
Albumin	25.0 ^{A} ±0.6	23.7 ^{A} ±0.7	23.7 ^{A} ±0.3	24.0 ^{A} ±0.6	23.7 ^{A} ±0.3	23.0 ^{AB} ±1.0	21.3 ^{B} ±0.9	23.3 ^{B} ±0.4
Globulin	26.0 ^{A} ±1.0	23.7 ^{B} ±0.3	23.3 ^{BC} ±0.7	25.0 ^{A} ±0.2	23.7 ^{B} ±0.3	22.7 ^{BC} ±0.0	21.3 ^{C} ±0.3	22.7 ^{BC} ±0.3
Aspartate aminotransferase	537.7 ^{AB} ±103.2	426.7 ^{B} ±36.6	416.0 ^{B} ±55.1	438.3 ^{B} ±31.5	395.0 ^{AB} ±25.0	411.0 ^{A} ±25.0	378.3 ^{AB} ±21.9	484.7 ^{A} ±21.7
Alkaline phosphatase	171.7 ^{A} ±7.3	158.3 ^{A} ±10.2	147.0 ^{B} ±9.9	168.3 ^{A} ±2.6	178.3 ^{A} ±7.9	157.0 ^{A} ±8.1	131.7 ^{B} ±4.3	171.0 ^{A} ±2.8
Glutamate dehydrogenase	52.7 ^{A} ±20.7	30.0 ^{C} ±1.2	42.3 ^{B} ±15.3	44.7 ^{BC} ±12.4	43.0 ^{B} ±8.3	45.0 ^{B} ±10.4	29.0 ^{C} ±1.2	36.0 ^{BC} ±7.2
Lactate dehydrogenase	544.7 ^{AB} ±31.3	311.7 ^{C} ±50.9	381.3 ^{C} ±49.0	444.7 ^{BC} ±29.7	474.3 ^{ABC} ±40.1	612.0 ^{A} ±115.9	321. ^{C} ±23.7	404.3 ^{B} ±42.3
Total bilirubin	1.7 ^{AB} ±0.3	2.3 ^{A} ±0.3	1.7 ^{AB} ±0.3	2.0 ^{A} ±0.3	1.0 ^{B} ±0.0	1.0 ^{B} ±0.0	1.3 ^{B} ±0.3	1.0 ^{B} ±0.0

Blood parameters	Standard Slaughter				Stress Slaughter			
	Antioxidant	Control	Copper	Zinc	Antioxidant	Control	Copper	Zinc
Cortisol	119.3 ^{C} ±16.4	260.3^{BC} ±133.7	242.7 ^{BC} ±163.8	263.3^{BC} ±94.5	714.3 ^{A} ±96.1	720 ^{A} ±104.2	535 ^{AB} ±24.1	780.7^{A} ±189.1
Cholesterol	10.1^{A} ±0.2	9.3 ^{ABC} ±0.3	9.2 ^{ABC} ±0.3	9.8 ^{AB} ±0.2	9.6 ^{AB} ±0.2	9.0 ^{BC} ±0.5	8.6 ^{C} 0.2	9.2 ^{ABC} ±0.3
Glucose	5.5 ^{B} ±0.3	5.4 ^{B} ±0.4	5.6 ^{B} ±0.1	5.4 ^{B} ±0.3	8.4 ^{A} ±0.6	8.8 ^{A} ±0.3	9.2 ^{A} 0.2	8.2 ^{A} ±0.2
Urea	1.26 ^{B} ±0.1	1.2 ^{B} ±0.1	1.4 ^{B} ±0.1	1.4 ^{B} ±0.1	1.56 ^{A} ±0.2	1.4 ^{B} ±0.1	1.26 ^{B} ±0.2	1.53 ^{A} ±0.2
Phosphorous, P	5.7 ^{BC} ±0.3	5.9 ^{BC} ±0.3	5.1 ^{C} ±0.2	5.6 ^{BC} ±0.3	6.1 ^{AB} ±0.2	6.9 ^{A} ±0.3	5.6 ^{BC} ±0.5	6.1 ^{AB} ±0.4
Calcium, Ca	3.7 ^{BC} ±0.0	3.2 ^{C} ±0.0	3.3 ^{C} ±0.0	3.3 ^{C} ±0.0	3.6 ^{AB} ±0.1	3.7 ^{A} ±0.2	3.5 ^{ABC} ±0.2	3.5 ^{ABC} ±0.1
Potassium, K	0.7 ^{A} ±0.0	1.0 ^{A} ±0.3	1.1 ^{A} ±0.3	1.1 ^{A} ±0.3	1.1 ^{A} ±0.3	1.3 ^{A} ±0.3	1.0 ^{A} ±0.2	1.2 ^{A} ±0.2
Chlorine, Cl	138 ^{B} ±1.5	139 ^{B} ±0.6	141 ^{B} ±1.5	138 ^{B} ±0.8	148.3 ^{A} ±1.2	149.3 ^{A} ±1.2	149.7 ^{A} ±2.7	151.7^{A} ±1.6
Iron, Fe	8.7 ^{A} ±0.3	7.7 ^{A} ±1.3	7.3 ^{A} ±0.9	6.3 ^{A} ±0.7	8.7 ^{A} ±0.3	6.3 ^{A} ±0.9	7.3 ^{A} ±0.9	7.0 ^{A} ±0.4

9.2 Appendix B

Data from hematology of Atlantic salmon (Salmo salar L.) fed a standard diet (Control) or the same diet added Vitamin E and selenium (Antioxidant), copper (Copper) or zinc (Zinc) for 12 weeks. Results are presented as LS mean ± SE and different superscripts you choose indicate significant differences between dietary treatments (P<0.05)

Blood parameters	P-Value			
	Model	Stress	Diet	Stress & Diet
Total protein	0.0187	0.0036	0.0305	0.7737
Albumin	0.0888	0.022	0.1068	0.6014
Globulin	0.0084	0.0015	0.0184	0.7562
Aspartate aminotransferase	0.484	0.3296	0.5058	0.3714
Alkaline phosphatase	0.484	0.3296	0.5058	0.3714
Glutamate dehydrogenase	0.8422	0.6242	0.7464	0.6235
Lactate dehydrogenase	0.0531	0.49	0.1383	0.0311
Creatinine	0.0013	0.0001	0.1491	0.9845
Total bilirubin	0.0265	0.0010	0.5472	0.3193
Cholesterol	0.1968	0.0692	0.0987	0.9753
Glucose	< 0.001	< 0.001	0.6792	0.8037
Urea organ	0.0159	0.0122	0.0108	0.6397
Calcium, Ca	0.0156	0.003	0.6419	0.4512
Potassium, K	0.8181	0.2529	0.7116	0.8645
Chlorine, Cl	< 0.001	< 0.001	0.6170	0.5441
Iron, Fe	0.2836	0.7643	0.0902	0.6309
Cortisol	0.0004	0.0001	0.481	0.4526



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